INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

DISSERTATION

STUDIES ON FATTY ACID AND ACYLATED HOMOSERINE LACTONE BIOSYNTHESIS IN *Pseudomonas aeruginosa*

Submitted by

Tung T. Hoang

Department of Microbiology

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

COLORADO STATE UNIVERSITY

FORT COLLINS, COLORADO

SPRING 2000

COLORADO STATE UNIVERSITY

December 14, 1999

WE HEREBY RECOMMEND THAT THIS DISSERTATION PREPARED UNDER OUR SUPERVISION BY TUNG T. HOANG ENTITLED "STUDIES ON FATTY ACID AND ACYLATED HOMOSERINE LACTONE BIOSYNTHESIS IN *Pseudomonas aeruginosa*" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Herbert P. Schweizer, Ph.D. (Supervisor)
Department of Microbiology

John T. Belisle, Ph.D.
Department of Microbiology

Claudia Gentry-Weeks, Ph.D.
Department of Microbiology

Julia M. Inamine, Ph.D.
Department of Microbiology

Carol A. Ishimaru, Ph.D.
Department of Bioagricultural Sciences and Pest Management

Ralph E. Smith, Ph.D. (Interim Department Head)
Department of Microbiology

ABSTRACT OF DISSERTATION

STUDIES ON FATTY ACID AND ACYLATED HOMOSERINE LACTONE BIOSYNTHESIS IN *Pseudomonas aeruginosa*

This study was initiated to define the pathways for fatty acid (FA) and acylated homoserine biosynthesis in *Pseudomonas aeruginosa*. To date, a complete FA biosynthetic pathway has only been defined in *Escherichia coli*. This pathway is essential and is central to the biosynthesis of membrane phospholipids, LPS, lipoproteins, lipoic acid, and biotin, and therefore offers attractive targets for various antimicrobial agents. At the onset of this study, there were conflicting results in the literature concerning the role of FA synthesis in acylated homoserine lactone (HSL or autoinducers) synthesis and thus provided additional incentives to define both pathways. Some investigators believed that the acyl groups of HSLs were derived from the FA biosynthetic pathway, while others argued that the FA degradative pathway may be the source of acyl groups. HSLs in *P. aeruginosa* are responsible for regulation of the majority of the virulence factors. Hence, the goals of the present study were to define the FA biosynthetic pathway in conjunction with HSL biosynthesis to not only reveal potential targets for inhibition of growth, but also inhibition of virulence gene expression in this bacterium.

Some unique genetic technologies were developed to facilitate the process of achieving the above goals. First, the development of a phage CTX-based chromosomal integration system allows the integration of gene cassettes at a defined location, *attB*, in the *P. aeruginosa* chromosome without disrupting any cellular functions. The mini-CTX integration plasmids are equipped with *FRT* sites that were configured to allow *in vivo* removal of unwanted plasmid DNA sequences with *Saccharomyces cerevisiae* Flp-recombinase. In two steps, the newly described system allows stable, single-copy, chromosomal integration of any

DNA segment to be studied, e.g. autoinducer regulated transcriptional *lacZ* gene fusions. Deletion of plasmid-borne promoters by Flp recombinase-mediated excision and flanking of the integrated gene cassette by strong phage T4 transcriptional terminators minimizes transcriptional interferences from external promoters. This system will facilitate studies involving various reporter gene fusion constructs in *P. aeruginosa* (and potentially other *Pseudomonads*) from a single chromosomal copy. It will also allow for the genetic engineering of many useful laboratory strains, as well as strains for use in industrial settings where gene expression from plasmids may not be desirable. Second, a broad-host-range system for isolation of marked and unmarked chromosomal mutations was developed utilizing an engineered Flp-*FRT* system. A series of new gene-replacement vectors was constructed which facilitate mutant construction in *P. aeruginosa* and other bacteria. Together, these two newly developed systems allow easy *P. aeruginosa* chromosome manipulation by integration and/or deletion of genes or sequences.

Novel genetically engineered strains and vectors were created to allow the purification of problem proteins. This was in part necessitated since the present study involved the purification of 14 different proteins for complete assembly of the FA and HSL biosynthetic pathways *in vitro*. During this project, three proteins (RhlI, LasI, and ACP) were especially problematic and could only be expressed and purified after the development of low-copy number expression vectors and appropriate host strains. RhlI and LasI (the two autoinducer synthases) were extremely insoluble when overexpressed from high copy number plasmids, and ACP (acyl carrier protein) requires post-translational modification for function. So far, this system provides the best way to purify ACP, in terms of yield and percentage of holo-ACP, as well as ease and expense of the procedures involved.

A connection of the FA biosynthetic (Fab) pathway to HSL biosynthesis was made possible by the combination of the technologies developed above, followed by *in vitro* assembly of the complete pathway. Purified RhlI could efficiently synthesize biologically active *N*-butyryl-L-homoserine lactone (BHL) and *N*-hexanoyl-L-homoserine lactone (HHL) only from the respective acyl-ACP substrates but was very inefficient when the respective acyl-CoA substrates were present. This corroborated the notion that the Fab pathway provided

the acyl groups for HSL synthesis. Mutant analysis demonstrated that FabI (enoyl-ACP reductase) was required for efficient synthesis of both autoinducers (BHL and HHL) in *P. aeruginosa*. In a RhlI/FabI coupled reaction, the synthesis of BHL was inhibited by triclosan, an inhibitor of FabI. The studies also demonstrated that *P. aeruginosa* contains another FabI homologue. Utilizing purified proteins, the complete Fab pathway was assembled *in vitro* and coupled to purified LasI for the synthesis of *N*-[3-oxo-dodecanoyl]-L-homoserine lactone (3-oxo-C_{12}-HSL). Fab pathway inhibitors inhibited 3-oxo-C_{12}-HSL synthesis, indicating that this *in vitro* system may be useful for drug screening. Finally, the *E. coli* acyl-ACP synthase membrane-associated protein was purified by affinity chromatography and used to label ACP with various fatty acids. This enzyme will be useful in future experiments because it allows enzymatic synthesis of substrates for various stages of FA biosynthesis, as well as substrates for the synthesis of HSL.

Tung T. Hoang
Department of Microbiology
Colorado State University
Fort Collins, Colorado 80523
Spring 2000

ACKNOWLEDGMENTS

I am very grateful to my supervisor Dr. Herbert P. Schweizer for his relentless supervision, encouragement, and support from the first experiment done to the last paragraph written in this dissertation. I am thankful for receiving an opportunity to work in Dr. Schweizer's laboratory, and he has allowed me to fully develop as a scientist with my own thoughts and ideas. I almost gave up several times during the program, due to family reasons. Thanks to Dr. Schweizer's opinion and advice, I was able to keep things in perspective. Our many conversations and the knowledge that I have learned will help me with the next phase of life and will stay with me throughout my academic career.

I am very thankful to Drs. John T. Belisle, Claudia Gentry-Weeks, Julia M. Inamine, and Carol A. Ishimaru for their help and support as committee members, and their attitudes and personalities made it very comfortable for me to approach them for help.

I would like to thank everyone in Dr. Schweizer's lab (Dr. RoxAnn Karkhoff-Schweizer, Anna Becher, Kerry Beinlich, Rungtip Chuanchuen, and Sarah Sullivan) and Marcus-leVell Lambert for their technical help and for their friendship which made things much easier and fun.

Last but not least, I acknowledge the receipt of a Student Fellowship from the American Cystic Fibrosis Foundation and the Dr. William K. Riddell Memorial Graduate Scholarship from the College of Veterinary Medicine and Biomedical Science at Colorado State University.

DEDICATION

I dedicate this work to my family and friends because of their patience, kindness, and understanding. I think quite often of them and they are my motivational force to complete and achieve so much. I also dedicate this work to both Drs. Herbert and RoxAnn Schweizer. They have been my extended family for the past six years. To everyone who comes through the Schweizer laboratory I leave the following thoughts to hopefully help you through the tough times, like they did for me:

To have lived well,
laughed often and loved much;
to have gain the respect of intelligent people
and the love of children;
to have filled a niche
and accomplished a task;
to have left the world better-
whether by an improved poppy,
a perfect poem or a rescued soul;
to have appreciated earth's beauty
and not failed to express it;
to have looked for the best in others
and have given the best of yourself.
That is achievement.

by R.L. Stevenson

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A.	*Agrobacterium*
A. t.	*Agrobacterium tumefaciens*
A_{540nm}	absorbance at 540 nm
A_{600nm}	absorbance at 600 nm
Aas	acyl-ACP synthase
AccABCD	acetyl-CoA carboxylase
AcCoA	acetyl-coenzyme A
ACP	acyl carrier protein
acpP	ACP encoding gene
AcpS	ACP synthase
acpS	AcpS encoding gene
ADP	adenosine diphosphate
aGM_1	asialoganglioside 1
aGM_2	asialoganglioside 2
AHL	acylated homoserine lactone(s)
AI	autoinducer
AIDS	acquired immune deficiency syndrome
AMP	adenosine monophosphate
Ap	ampicillin
apr	alkaline protease encoding gene
asd	aspartate-β-semialdehyde dehydrogenase structural gene
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
att	locus for phage attachment

Az	azithromycin
β–Gal	β-galactosidase
BHL	*N*-(butyryl)-L-homoserine lactone
bla	β-lactamase encoding gene
bp	base pair(s)
bhr	broad-host-range
C_4	acyl chain length four carbons long
C_6	acyl chain length six carbons long
C_8	acyl chain length eight carbons long
C_{10}	acyl chain length ten carbons long
C_{12}	acyl chain length twelve carbons long
C_{18}	acyl chain length eighteen carbons long
Cb	carbenicillin
°C	degree celcius
C.	*Chromobacterium*
CDB	chitin-binding domain
Cer	cerulenin
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
Cl^-	chloride ion
CNS	central nervous system
cm	centimeter
Cm	chloramphenicol
CoA	coenzyme A
CSF	cerebrospinal fluid
CTX	cytotoxin
Cv	crystal violet
CV	*Chromobacterium violaceum*
Da	Dalton(s)

DAP	diaminopimelic acid
Dia(z)	diazaborine
DKP	diketopiperazine
DNA	deoxyribonucleic acid(s)
dNTP	deoxyribonucleoside triphosphate(s)
dTDP	deoxythymidine diphosphate
DTT	dithiothreitol
E	enzyme
E.	*Escherichia* or *Erwinia*
EDTA	ethylenediamine tetraacetic acid
Em	erythromycin
ER	endoplasmic reticulum
FA	fatty acid(s)
Fab	fatty acid biosynthesis
FabA	β-hydroxyacyl-ACP dehydratase
FabB	β-ketoacyl-ACP synthase I
FabD	malonyl-CoA:ACP transacylase
FabF	β-ketoacyl-ACP synthase II
FabG	β-ketoacyl-ACP reductase
FabH	β-ketoacyl-ACP synthase III
FabH2	a second FabH homologue
FabI	enoyl-ACP reductase
FabJ	a second putative enoyl-ACP reductase
FabZ	β-hydroxyacyl-ACP dehydratase
Fad	fatty acid degradation
FadR	*E. coli* fatty acid metabolism regulatory protein
FRT	Flp recombinase target
g	gram(s) or acceleration of gravity
GC	gas chromatography

ΔG	free energy
GFP	green fluorescent protein
GI	gastrointestinal
Gm	gentamicin
H.	*Haemophilus*
HHL	*N*-(hexanoyl)-L-homoserine lactone
His6 or H6	hexahistidine-containing peptide
H(S)L	homoserine lactone(s)
HPLC	high-pressure liquid chromatography
h	hour(s)
IC_{50}	50% inhibitory concentration
IgA	immunoglobulin alpha
IgG	immunoglobulin gamma
int	phage CTX integrase gene
IPTG	isopropyl-β-D-thiogalactopyranoside
K.	*Klebsiella*
kb	kilobase(s) or 1000 bp
kDa	kilodalton(s)
kg	kilogram(s)
Km	kanamycin
l	liter(s)
lacI	*E. coli lac* repressor structural gene
lacZ	β-galactosidase encoding gene
lacY	lactose permease
lasA	elastase encoding gene
lasB	elastase encoding gene
LasI	*P. aeruginosa N*-[3-oxododecanoyl]-L-HSL synthase
LasR	*N*-3-oxo-C_{12}-HSL-binding regulatory protein
LB	Luria-Bertani medium

LD_{50}	lethal dose killing 50 percent of specimens
log	logarithic growth phase
LPS	lipopolysaccharide(s)
LuxI	*V. fischeri N*-(3-oxo-hexanoyl)-L-HSL synthase
M.	*Mycobacterium*
M	molar
mM	millimolar
MaCoA	malonyl-coenzyme A
MALDI	matrix assisted laser desorption ionization
MBC	minimal bactericidal concentration
MCAC	metal chelation affinity chromatography
MCS	multiple cloning site(s)
mg	milligram(s)
MIC_{50}	minimal inhibitory concentrations inhibiting 50% of strains tested
MIC_{90}	minimal inhibitory concentrations inhibiting 90% of strains tested
min	minute(s)
ml	milliliter(s)
M_r	molecular weight
MS	mass spectrometry
MTA	methylthioadenosine
NAD^+	oxidized β-nicotinamide adenine dinucleotide
NADH	β-nicotinamide adenine dinucleotide reduced form
$NADP^+$	oxidized β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate reduced form
NEC	necrotizing entercolitis
ng	nanogram(s)
nm	nanometer(s);
NNIS	National Nosocomial Infections Surveillance

Nov	novobiocin
NTA	nitilotriacetic acid
nt	nucleotide(s)
ori	origin of replication
oriT	origin of transfer
P.	*Pseudomonas*
PABA	*p*-aminobenzoic acid
PAGE	polyacrylamide gel electrophoresis
P_{lac}	*E. coli lac* operon promoter
P_{lacUV5}	*E. coli lac* operon UV5 promoter
P_{trc}	*E. coli trp-lac* hybrid promoter
pabC	gene encoding 4-amino-4-deoxychorismate lyase
PAGE	polyacrylamide gel electrophoresis
PAI	*Pseudomonas* autoinducer
PBS	phosphate-buffered saline
PBP	penicillin binding protein(s)
PCR	polymerase chain reaction
ϕ	phage
pI	isoelectric point
PIA	*Pseudomonas* isolation agar
PMSF	phenylmethylsulfonyl fluoride
PMN	polymorphonuclear leukocytes
pol	polymerase structural gene
Pol	polymerase
PPi	pyrophosphate
PQS	*Pseudomonas* quinolone signal
psi	pounds per square inch
PTSB	peptone-trypticase soy broth
Ql	quinolone(s)

r	resistance/ resistant
RBS	ribosome-binding site
Rep	replication protein
RhlAB	rhamnosyltransferase
RhlI	*P. aeruginosa* BHL synthase
RhlR	C_4-HSL binding regulatory protein
Rif	rifampin
RmlD	*M. tuberculosis* dTDP-L-rhamnosyl synthase
rpm	revolutions per minute
rpoS	stationary phase sigma factor
RT	room temperature
S.	*Staphylococcus* or *Salmonella*
s	sensitive/susceptible
sacB	*Bacillus subtilis* gene encoding levansucrase
SAM	*S*-adenosylmethionine
SDS	sodium dodecyl sulfate
Sm	streptomycin
Std	standard(s)
TBE	tris-borate-EDTA
TIGR	The Institute for Genomic Research
TLC	thin layer chromatograph(y)
TLM	thiolactomycin
TOF	time of flight
TraI	*A. tumefaciens N*-(3-oxo-octanoyl)-L-HSL synthase
TraR	*A. tumefaciens N*-(3-oxo-octanoyl)-L-HSL binding regulatory protein
Tc	tetracycline
tet	tetracycline-resistance encoding gene
toxA	exotoxin A encoding gene

Tri	triclosan
Ts	temperature sensitive
u	unit(s)
µl	microliter
µM	micromolar
µg	microgram(s)
UTP	uridine triphosphate
V.	*Vibrio*
v/v	volume by volume
wt	wild-type
XGal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
xcpP	gene encoding a protein of the general secretory pathway
xcpR	gene encoding a protein of the general secretory pathway

LIST OF PUBLICATIONS

Parts of this dissertation have been published in the following articles or via database deposition:

Articles

Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer. 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene 212:77-86.

Hoang, T. T., Y. Ma, R. J. Stern, M. R. McNeil, and H. P. Schweizer. 1999. Construction and use of low-copy number T7 expression vectors for purification of problem proteins: purification of *Mycobacterium tuberculosis* RmlD and *Pseudomonas aeruginosa* LasI and RhlI proteins, and functional analysis of RhlI. Gene 237:361-371.

Hoang, T. T., and H. P. Schweizer. 1999. Characterization of the Pseudomonas aeruginosa enoyl-acyl carrier protein reductase: a traget for triclosan and its role in acylated homoserine lactone synthesis. J. Bacteriol. 181:5489-5497.

Kutchma, A. J., T. T. Hoang, and H. P. Schweizer. 1999. Characterization of a *Pseudomonas aeruginosa* fatty acid biosynthetic gene cluster: purification of acyl carrier protein (ACP) and malonyl-coenzyme A:ACP transacylase (FabD). J. Bacteriol. 181:5498-5504.

Hoang, T. T., A. J. Kutchma, A. Becher, and H. P. Schweizer. 2000. Integration proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. Plasmid 43:59-72.

GenBank Depositions

Sequence	**Accession Number**
fab gene cluster	U91631
fabI	AF104262
mini-CTX1	AF140576
min-CTX2	AF140577
mini-CTX-*GFP*	AF140578
mini-CTX-*lacZ*	AF140579
pEX18Ap	AF004910
pEX18Gm	AF047518
pEX18Tc	AF047519
pFLP2	AF048702
pNam	AF147463
pViet	AF147464

CHAPTER 1

INTRODUCTION

1.1 MICROBIOLOGY OF *P. aeruginosa*

A *Pseudomonas aeruginosa* pure culture was first isolated in 1882 from a patient with blue green discolored cutaneous wounds (135). The coloration is due to *P. aeruginosa* production of at least two pigments, a fluorescent yellow pigment and a blue pigment called pyocyanin (135, 140). It is a Gram-negative, aerobic, rod-shaped bacterium which commonly inhabits soil, water, sewage, plant surfaces, and many other oligotrophic environments. In addition, *P. aeruginosa* can grow at temperatures ranging from 5° to 42°C (21). This rod shape bacterium ranges from 1 to 3 μm in length and from 0.5 to 1.0 μm in width (12, 26). The organism can be motile, having one polar flagella (3). Survival in very diverse environments or in oligotrophic environments requires many metabolic capabilities (14), which is reflected by the large *P. aeruginosa* genome size as compared to other bacteria (*P. aeruginosa*, 6.3 megabases; *E. coli*, 4.6 megabases; and *H. influenzae*, 1.8 megabases) (9, 15, 32, 56) and its genome is currently the largest bacterial genome sequenced thus far. Although lacking the Embden-Meyerhof pathway, *P. aeruginosa* has the ability to metabolize various compounds (mannose, fructose, glucose, ribose, xylose, glycerol, acetate and C_4 to C_{10} fatty acids) for carbon and energy sources, utilizing the Entner-Doudoroff pathway, the pentose phosphate cycle, the tricarboxylic acid cycle and β-oxidation (14). *P. aeruginosa* has a dimorphic lifestyle and can exist as planktonic (individual free-swimming) cells or as microcolonies (aggregation of cells) enclosed in slime-protected biofilms (16, 17). Planktonic cells are derived from microcolonies when nutrients are in abundance. Biofilm microcolonies allow the capturing of nutrients in oligotrophic environments such as streams and rivers. Planktonic

cells produce numerous virulence factors and are susceptible to antimicrobial factors such as antibiotics, complements, and phagocytosis. However, biofilm (alginate) enclosed microcolonies are extremely resistant to these antimicrobial factors and are nearly impossible to eradicate in chronic infections. Thus, the remarkable ability to readily interconvert between the dimorphic phases not only allows survival in various environments, but can also lead to different scopes of *P. aeruginosa* pathogenesis from acute to chronic infections. Being an opportunistic pathogen, *P. aeruginosa* is different from other human pathogens, in that it is able to colonize and cause disease in warm- and cold-blooded vertebrates, insects, aquatic animals and plants (3, 26, 104).

1.2 *Pseudomonas aeruginosa* INFECTIONS

While *P. aeruginosa* can be transiently present in diverse areas of the body (for examples, the skin, the respiratory tract, and nasal cavity), it is normally unable to cause disease in healthy individuals. However, there is great morbidity and mortality associated with *P. aeruginosa* infections, and the organism is extremely difficult to eradicate with available antimicrobial agents. This is best exemplified in cystic fibrosis patients, where chronic infection with the same clone of *P. aeruginosa* occurs for 30 to 40 years, although they routinely undergo anti-*Pseudomonas*-drug therapy. Being an opportunistic pathogen, *P. aeruginosa* causes disease in patients who are immunocompromised (patients on chemotherapy for cancer and patients with humeral or cellular defects, including AIDS patients) (3). Neutropenic patients are very susceptible to *P. aeruginosa* infection and subsequent septicemia (3). Alternatively, the defense of the host can be breached (in immunocompetent individuals) as in corneal ulcerations or skin burns, or can be artificially overcome as with assisted ventilation or by an indwelling catheter. Many instances of *P. aeruginosa* infections to immunocompetent individuals are due to direct inoculation of a sufficient bacterial load into otherwise sterile sites, thus causing bone and joint infections, endocarditis, meningitis, urinary tract infections, ocular infections, septicemia (occurring in severe burns) and malignant external otitis (26).

1.2.1 *P. aeruginosa* Nosocomial Infections

Nosocomial infections, including infections of the urinary tract, wound, lung and bloodstream, by *P. aeruginosa* are alarming due to high levels of antimicrobial resistance (6, 10, 111). Virtually all *P. aeruginosa* isolates causing infections in National Nosocomial Infections Surveillance (NNIS) hospitals from 1985 to 1991 were resistant to cephalothin (98.4%), cefazolin (96.9%), cefuroxime (94.6%), cefixitin (94.2%), and cefamandole (92.6%). Fortunately, resistance to other β-lactams such as cefotaxime (33.7%), moxalactam (30.1%), and ceftizoxime (45.9%) was less common. Among the cephalosporins, resistance was much less with ceftazidime (8.6%) and cefoperazone (10.3%). Aminoglycosides resistance was reported at 12.9% for gentamycin, 6.3% for tobramycin and 5.5% for amikacin. Nearly any type of hospital equipment or utensil can serve as a reservoir for *P. aeruginosa*, and other reservoirs include food, environmental sources, medications, fluids, prostheses, and disinfectants (6). Thus, patient colonization most likely results from exposure to these sources or transiently colonized hands of health-care workers. *P. aeruginosa* is infrequently associated with contaminated blood during blood culture collection or processing, but rather can be traced to contaminated vacuum tubes, skin antiseptics, or blood culture processing errors (6). *P. aeruginosa* infections in hospitals are serious enough to require contact isolation (private room), depending on the instances. Major skin, wound, and burn infections that are draining or cannot be covered by a dressing, as well as patients with pneumonia due to multiply resistant strains of *P. aeruginosa* should be isolated (6). Patients infected with strains that are resistant to all aminoglycosides (gentamycin, tobramycin, and amikacin) must also be isolated.

Among clinical *P. aeruginosa* infections, lung infections are associated with the highest mortality rate, and the mortality rates associated with *P. aeruginosa* pneumonia range from 50 to 80% (95). Clinical conditions placing certain populations at high risk for *P. aeruginosa* pneumonia are patients requiring respiratory assistance in intensive care units, neutropenic patients, patients with both primary or acquired hypogammaglobulinemia (lymphocytic leukemia), and cystic fibrosis patients. *P. aeruginosa* bacteremia in conjunction with pneumonia is associated with highest mortality, with many studies indicating 100% mortality (95). There has been an increase in *P. aeruginosa* pneumonia from 1980 to 1987,

with 25,000 to 30,000 reported cases of *P. aeruginosa* pneumonia in the United States during 1987 alone (95). According to the National Nosocomial Infections Surveillance (NNIS) System, *P. aeruginosa* was the leading cause of hospital-acquired pneumonia from 1985 to 1991 among several microbes, including *E. coli*, *Enterococci*, *S. aureus*, coagulase-negative staphylococci, *Enterobacter* species, and *K. pneumoniae*. In other studies, mechanically ventilated patients had incidences of nosocomial pneumonia ranging from 9 to 68%, and mortality rates ranged from 33 to 71% (10). The action of *P. aeruginosa* toxins, proteases and ciliostatic compounds (rhamnolipid and pyocyanin) lead to lung damage and decrease mucociliary action (see chapter 1.3 and table 1.1). The reduced normal bronchopulmonary clearance enhanced airway colonization and persistence then lead to invasion of tissue in high risk patients, as well as people with advanced age, surgery, and malnutrition (95, and references therein).

Although *P. aeruginosa* almost never produces urinary tract infections in patients with hydrodynamically normal urinary tracts, infections can occur when there are important structural or functional abnormalities of the urinary tract (68). Cases have been reported originating from contaminated fluid during urological procedures and bathing in contaminated water (whirlpool), but the majority of cases are from long-term use of urinary catheters and external drainage devices (68). The infections are not benign, can involve all structures of the urinary tract and are extremely difficult to eradicate. Until the predisposing factors are removed, antimicrobials therapies are not very effective against *P. aeruginosa* urinary tract infections (68). Since most patients with *P. aeruginosa* urinary tract infections are immunocompent, methods to improve immune status of the host will probably not be helpful (68).

1.2.2 *P. aeruginosa* Skin and Soft Tissue Infections

P. aeruginosa skin and soft tissue infections are more than just nosocomial wound infections or burn infections, and involve less severe, but annoying infections such as folliculitis, cellulitis, toe web infections, and green nail infections. *P. aeruginosa* occurred in 42% of wounds sustained during the Vietnam war (118). From 1985 to 1991 the NNIS reported *P. aeruginosa* was found in 7.1% of surgical site (wound) infections in ward areas.

However, in intensive care units, *P. aeruginosa* was found in 10.9% of surgical site infections (118). Thermal, chemical, and electrical injury to tissue has been most commonly associated with severe and life-threatening *P. aeruginosa* infections (118). Studies have shown that burns indeed cause immunosuppression and defects in chemotaxis of leukocytes, and the degree of injury correlates with the level of impairment of opsonic capability of serum (118, and references therein). The burn allows release of ready available nutrients and sites of attachment; and the combination of systemic disability of the host, large mass infection and bacterial replication, and the toxigenic potential of *P. aeruginosa* allows vascular invasion leading to thrombosis, necrosis, and, occasionally, sepsis (118). Studies of burn wound sepsis have shown a range of 25 to 75% of deaths that are due to *P. aeruginosa* (118). The immunosuppressive state can often promptly be reversed by surgical removal of the thermally injured tissue mass and followed by skin graphs. Other studies indicate that isolation of patients allow delays in colonization, and time for improvement of immune system function of the host reduces rates of bacteremia (118). In the burnt mouse model, *P. aeruginosa* protease-deficient strains were less virulent than wild-type, and overproducer strains grew more rapidly on burnt tissue (54, 55). *P. aeruginosa* proteases are able to degrade collagen and other connective tissue, complement and IgG, leading to invasion (see chapter 1.3 and table 1.1). The combination of *P. aeruginosa* degradation of complement and IgG and the loss of leukocytes chemotaxis in burns allows escape from the host defense. Although *P. aeruginosa* can be isolated from many transiently wet body areas, e.g. the feet, groins, digital, perineum and axillae (armpits), it is only able to cause cutaneous infections under conditions of high inoculum, hyperhydration, or injury of the outermost layer of the epidemis. The bacteria also attach readily to hydrated, traumatized or thermally injured skin (118). Folliculitis due to *P. aeruginosa* can be obtained from underchlorinated (or brominated) hot tubs, whirlpools, recreational waterslides, and swimming pools (118). *P. aeruginosa* folliculitis is usually not fatal and is characterized by rash followed by papular and papulopustular lesions (can be >1 cm), but is usually resolves itself in healthy individuals. *P. aeruginosa* cellulitis can occur in immunocompromised patients with surgical wounds or cutaneous ulcers (118). Other instances are due to breaks in the dermal barrier, causing nonfatal but

unpleasant and painful toe web infections or green nails characterized by a bluish-green discoloration of the nail plate (118).

1.2.3 *P. aeruginosa* Infections of The Gastrointestinal Tract

P. aeruginosa is also recognized as a colonizer of the gastrointestinal (GI) tract in immunocompromised and immunocompetent hospitalized adults, and children who have undergone surgery. In the GI tract, *P. aeruginosa* can cause severe necrotizing localized disease by means of toxin production, or disseminate into the bloodstream to cause sepsis, shock, or metastatic infection (71). Disease resulting from gastrointestinal tract colonization can be local, such as toxin-mediated severe enteritis or necrotizing entercolitis of infants (71). Invasive perianal or perirectal disease can also be seen in neutropenic adults (71). In neonates, *P. aeruginosa* can cause the uncommon, but often severe and fatal disease of necrotizing entercolitis (NEC). During the first week(s) of feeding when the infant lacks passive immunity to enteric organisms, *P. aeruginosa* can be acquired and cause NEC, in which the bacteria may overgrow, produce cytotoxins and damage mucosal cells (71). In another study, during a period of 19 months, 6% of 363 liver transplants were followed by *P. aeruginosa* bacteremia and the port of entry was found to be the abdomen in 35% and biliary tract in 13% of the patients. Therefore, although *P. aeruginosa* is not part of the normal flora of the intestine, it can survive in the GI tract and can be isolated in stool, and contamination from food, water, or other environmental sources can cause severe infections in predisposed patients via the GI tract.

1.2.4 *P. aeruginosa* Bacteremia

Because of the continuing high mortality rate, *P. aeruginosa* bacteremia is among the most feared types of infections treated by clinicians (3). The mortality rate ranges from 30 to more than 60%, and mortality rates as high as 80 to 100% have been documented (3). Patients at risk for *P. aeruginosa* bacteremia in the hospital include those with neutropenia, thermal injuries, intravascular monitoring devices, organ transplantation, peritoneal dialysis, AIDS, those in premature neonatal nurseries, and those requiring instrumental monitoring of the genitourinary or respiratory tracts. Thousands of cases of *P. aeruginosa* bacteremia have

been documented and reviewed by Baltch (3), including war casualties, malnourished children, patients undergoing chemotherapy for cancer, and AIDS patients. Because *P. aeruginosa* planktonic cells can produce numerous virulence factors and LPS, they can be invasive and can cause systemic manifestations once found in the bloodstream. In contrast to cystic fibrosis (chapter 1.2.9) where high serum antibody concentrations usually indicate poor prognosis, high serum antibody titers to LPS and exotoxin A in *P. aeruginosa* bacteremia are associated with greater survival rate (3). Besides hospital-acquired bacteremia, many cases of *P. aeruginosa* community-acquired bacteremia with high mortality rates have been documented. In one study involving children, the mortality rate of community-acquired bacteremia was 83% versus 59% for hospital-acquired cases; while the reverse was true for adults with 44% versus 71%, respectively (3). In community-acquired bacteremia, *P. aeruginosa* enters mostly through the skin, respiratory tract, and genitourinary tract, but neutropenic patients can also get *P. aeruginosa* bacteremia from the gastrointestinal tract. Thus, ingestion of contaminated food and water by these patients must be prevented, since *P. aeruginosa* can survive and colonize in the gastrointestinal tract (3). Among cancer patients, patients with leukemia are five times more vulnerable to *P. aeruginosa* bacteremia than patients with solid tumors, and the prognosis was poorest when the source for bacteremia was from the lung or anorectal region (4, 114). To prevent colonization by *P. aeruginosa* in these patients, antibiotics that are not absorbed orally and laminar flow rooms with reverse isolation were introduced (112, 113).

Because of the seriousness of *P. aeruginosa* bacteremia, treatment must be immediate and specific with an antipseudomonal β-lactam (penicillin or cephalosporin) and an aminoglycoside (tobramycin or amikacin), as soon as the infectious agent is identified as *P. aeruginosa* (3). Synergy of β-lactam plus aminoglycoside combinations occurs more frequently with *P. aeruginosa* than with *Enterobacteriaceae*, and delay in treatment of 4-8 h significantly decreases the chances for patient survival (3). It is well known that *P. aeruginosa* can cause acute, rapidly progressing systemic bacteremic infections with or without shock. Thus, besides antibiotics, therapy should also include immunoglobulins, vaccines, and modulation of cytokines.

1.2.5 *P. aeruginosa* Endocarditis

P. aeruginosa endocarditis (infections of heart valves or the endovascular lining of the heart) is primarily related to predisposing host conditions (prosthetic heart valve, intracardiac tubing, wire or patch, leukemia, cancer, anticancer chemotherapy, and prolonged neutropenia) or host behavioral practices, for example intravenous drug abuse (60). Among cases of Gram-negative bacillary endocarditis, *P. aeruginosa* is the causative agent in the majority of cases among drug abusers, more than one-half of cases involving rheumatic/congenital valves, and one-quarter of cases of prosthetic valve endocarditis (60). Among drug abusers, however, *P. aeruginosa* endocarditis has increased in frequency and in some localities has periodically displaced staphylococcal endocarditis (60). Unsterile practices of drug abusers, as well as non-sterile drugs and diluents provide a direct route for aquaphilic, aerophilic bacteria such as *P. aeruginosa* into the bloodstream.

Strains causing endocarditis were found to be mostly of serotypes 01, 04, 06 and 011, and the last two serotypes were predominant. They also had a particular phenotype when bacteremia was also present, being resistant to neutralization and lysis by antibodies and complements (60). Biofilm formation (see chapter 1.3) may allow the organism to acquire this resistance and also to adhere to surfaces, even in the fast-moving and forceful stream of blood in the heart. Among Gram-negative bacteria, *P. aeruginosa* was observed to adhere to human and pig heart valves better than *E. coli* (40). Because of its ability to live in a biofilm state, *P. aeruginosa* endocarditis, resulting from colonization of pacemaker wires or other foreign appliances, is nearly impossible to eradicate. Blood cultures are usually positive, and medical treatment may quench the bacteremia and temporarily eliminate symptoms, but it almost never cures the infection (60).

P. aeruginosa endocarditis of the tricuspid valve is the least serious when compared to mitral or aortic valve infections. If detected and treated early and vigorously with effective antibiotics, tricuspid valve infection is the only form of *P. aeruginosa* endocarditis with high probability of survival; even so, the rates of septic pulmonary embolism and heart failure are 75% and 25%, respectively (60). Mitral and aortic valve endocarditis caused by *P. aeruginosa* is very serious, requiring both urgent valvulectomy and valve replacement, as well as anti-infective therapy. Once the infection has been established in these valves, continuous

bacteremia and embolization occur unless the afore mentioned measures are taken (60). As with pacemaker infections described above, the bacteremia caused by susceptible strains can be eliminated with aminoglycoside and antipseudomonal β-lactam combination therapy, but this is ineffective in sterilizing the infected valves, except for the tricuspid valve (60). Thus, in all cases of *P. aeruginosa* endocarditis, surgical removal of the infected valve, prosthesis or foreign body might be an absolute requirement for a cure. Even when both medical and surgical interventions are taken, many cases of mitral or aortic valve infection only lead to 75% or 38% cure rates, respectively. Since blood from the left regions of the heart is highly oxygenated and destined for delivery to the rest of the body, mitral and aortic valve endocarditis can also lead to septic emboli and lesions in multiple organs (brain, kidney, spleen, and others) and the skin (cause purple annular skin lesions and sometimes classical ecthyma gangrenosum).

1.2.6 *P. aeruginosa* Infections of The Central Nervous System

P. aeruginosa rarely caused diseases of the central nervous system (CNS), except in neonates, neurosurgical patients, patients with penetrating head injuries, granulocytopenic patients, alcoholics and intravenous drug users. *P. aeruginosa* causes approximately 2% of cases of neonatal bacterial meningitis and 16% of all cases of Gram-negative meningitis in neurosurgical patients, due to lack of a normal flora and fully functional immune system in neonates or due to direct wound contamination during neurosurgery and trauma, respectively (106). Granulocytopenic patients are at high risk of *P. aeruginosa* meningitis, resulting from hematogenous dissemination of the infection from colonization of the urinary tract, upper respiratory tract and/or gastrointestinal tract (106). The result of meningitis is inflammation of the meninges (the three membranes dura mater, pia mater, and arachnoid) that envelop the brain and spinal cord. Multiplication and lysis of bacteria generates bacterial cell wall components (for example LPS) in the subarachnoid space that induce the release of inflammatory cytokines (TNFα and IL-1) produced by macrophage and monocytes, brain astrocytes and microglial cells. TNFα and IL-1 are strong chemoattractants for leukocytes, resulting in pleocytosis or an accumulation of cells in the cerebrospinal fluid (CSF) (106). In addition to the increase in blood-brain barrier permeability and intracranial pressure, there is

an increase in CSF proteins in the serum, decrease in glucose (bacteria and leukocytes utilization), and change in lactate concentrations typical of bacterial meningitis (106). The LPS also plays an important role in systemic complications of these infections, including fever, hypotension, oliguria, adult respiratory distress syndrome, and disseminated intravascular coagulation (3, 106).

1.2.7 *P. aeruginosa* Infections of The Ear, Bone and Joint

P. aeruginosa ear infections can manifest themselves in the form of malignant external otitis, simple external otitis and chronic otitis media. *P. aeruginosa* is the cause of 99% of adult malignant external otitis (41). Malignant external otitis, a disease caused specifically by *P. aeruginosa*, is commonly seen in elderly patients with diabetes mellitus and can lead to osteomyelitis of the base of the skull or temporal bone, and also to cranial nerve palsy (paralysis) (41, 42). Although it is a typical disease of elderly diabetics, there are increasing reports of nondiabetics and younger people developing this disease (107, 119). Children tend to develop facial nerve paralysis earlier than adults who develop cranial nerve dysfunction as early as 1 week after onset of symptoms, but usually 2 months into the illness (41). The onset of unrelenting otalgia (earache) is usually nocturnal and may be accompanied by otorrhea (purulent discharge) in 50-80% of patients (108). Pain is remarkably severe and often remains unrelieved by narcotic analgesics (41). The drug of choice for treatment of *P. aeruginosa* malignant external otitis is ciprofloxacin (42). Adjunctive treatment includes hyperbaric oxygen treatment, which is believed to enhance phagocytotic killing, microangiogenesis (small blood vessel development) and killing by aminoglycosides (41). Simple external otitis (external canal infection) is very common with frequent isolation of *P. aeruginosa* from the drainage and is associated with humid atmospheric conditions, aural water exposure and ear canal trauma (41). Although quite painful, pain is not as severe as with malignant external otitis and can be treated with an ear wick soaked in anti-inflammatory agents (hydrocortisone) and an antimicrobial (neomycin sulfate) (41). *P. aeruginosa* can cause a third type of ear infection known as chronic otitis media with otorrhea resulting from a tympanic membrane perforation; this often occurs in children, most likely due to a relatively immature Eustachian tube (41). *P. aeruginosa* has

been recovered from as many as 62% of cases of chronic otitis media (63). Most patients usually have Eustachian tube dysfunction and middle ear problems and tympanic membrane perforation resulting from the inability of the Eustachian tube to ventilate the middle ear cleft properly (41). Over time, the draining middle ear fluid will macerate the skin of the external auditory canal and canal infection ensues (41). Treatment is the same as for simple external otitis, but if the middle ear is inflamed, most clinicians would recommend systemic agents.

P. aeruginosa community-acquired bone and joint infections are uncommon, except in cases of hemetogenous osteomyelitis (inflammation of bone) found in intravenous drug abusers (IVDA), osteochondritis (inflammation of bone and cartilage) as a complication of a puncture wound of the foot, or in diabetic patients with foot infections (76). *P. aeruginosa* alone or in combination with other microbes (*Staphylococcus* or *Streptococcus* species) was the causative agent in more than 80% of skeletal infections found in IVDA from 1970 to 1980 (76). In acute hematogenous osteomeylitis, if initial medical treatment fails, medullary (bone marrow) and/or soft tissue removal in conjunction to antibiotic treatment may be necessary (76). In chronic osteomeylitis, the infection will not regress until the causes of persistent contamination, including infected necrotic bone, hardware and poorly perfused soft tissue envelope surrounding the bone are removed, and adequate drainage, wound protection, and antibiotics may be necessary (76). Most patients with *P. aeruginosa* joint infections have predisposing conditions, including immunosuppression, chronic renal failure, diabetes mellitus, rheumatoid arthritis, trauma or surgery to the joint, or are elderly (76). Thus, the source of infection does not have to be direct inoculation to the joint, but can be by a hematogenous route via seeding from urinary tract infections. *P. aeruginosa* was found to be the causative agent in 11% of prosthetic joint infections in the elderly (76). Cases of community-acquired osteochrondritis due to *P. aeruginosa* are from people with puncture wounds, for example by stepping on a nail (76), and some studies indicate that *P. aeruginosa* may account for more than 90% of osteochondritis of the foot due to puncture wounds (2).

1.2.8 *P. aeruginosa* Ocular Infections

P. aeruginosa ocular infections often lead to a poor outcome, because the organism is able to destroy ocular tissue leading to corneal ulceration, permanent scarring, corneal

perforation and extension of the corneal purulence into the surrounding sclera resulting in loss of the eye (142). The frequencies of *P. aeruginosa* corneal infections have increased dramatically with the widespread use of cosmetic and soft contact lenses. On the surface of the eyeball, the ocular drainage system along with important host proteins (secretory IgA, complement, albumin, lactoferrin, lysozyme, and beta lysin) in the tear fluid is required to prevent bacterial colonization (142). However, corneal ulcers may develop following even slight abrasions of the corneal surface epithelium by objects contaminated with *P. aeruginosa*, such as vegetation (tree branches or corn stalks), mascara brushes and other cosmetic applicators, fingernails, contact lenses, or imbedded corneal foreign bodies (142). If inflammation occurs, there can be localized cellular migration in addition to edema, and corneal infiltrate occurs, a hallmark of bacterial corneal infection (142). Although *P. aeruginosa* is only responsible for about 2% of bacterial conjunctivitis, it is now the most common cause of Gram-negative corneal ulceration and corneal melting in contact lens wearers. It is also the most common cause of corneal ulceration in uncompromised hosts, particularly after corneal injury, where the cornea can perforate leading to anterior chamber collapse (142). Contact lens wearers frequently have minor corneal surface disruptions because of frequent lens insertion and removal, and extended wear of contact lenses allows adherence of *P. aeruginosa*. Extended wear of contact lenses is associated with a six-fold higher incidence of corneal infections than daily wear because protein build-up on both side of the lens allows adherence of *P. aeruginosa* in biofilms (142). The rare, but very serious infections, *P. aeruginosa*-caused sclerokeratitis and endophthalmitis can occur as a result of injuries to the sclera and vitreous cavity, respectively (142). The former infection may extent into the meninges, and the latter is a true emergency with high risk of loss of ocular function and irreparable damage to the integrity of the globe itself (142).

1.2.9 Cystic Fibrosis and *P. aeruginosa* Infections

The disease cystic fibrosis (CF) is an autosomal-recessive inherited disorder that occurs in 1 of 1000 live births in mostly people of Caucasian descent, and currently 1 in 2000 people in the United States and Canada has CF (66). The disease is characterized by lung infections, pancreatic insufficiency, increased sweat Cl^- concentration, and obstruction of

exocrine glands of the gastrointestinal, genitourinary, and respiratory tracts, all resulting from a defect in and translocation of a chloride channel to the apical plasma membrane of epithelial cells (66, 120). Individuals with severe cases frequently die before the age of 30 (66). The defect was found to be caused by mutations of a gene encoding a plasma membrane protein designated the cystic fibrosis transmembrane conductance regulator (CFTR). To date, researchers have found more than 720 different mutations in CFTR giving rise to CF, and mutant CFTR alleles have been found in as many as 5% of Caucasians of European descent (100). The *CFTR* gene was cloned in 1989 and mapped to the long arm of chromosome 7 (120). The most frequently occurring mutation (~70%) leading to CF was found to be a deletion of a single phenylalanine codon at position 508 (ΔF508), although disease causing mutations can be found in any of the five domains of CFTR (see below). CFTR is a single polypeptide integral membrane protein of 1480 amino acids consisting of five major domains, including two nucleotide (ATP) binding folds, a regulatory domain and two transmembrane spanning regions (66). The latter act as an anion channel gated by the two ATP binding and hydrolysis domains, which are in turn regulated by phosphorylation of the regulatory domain. The ΔF508 deletion of CFTR is localized within one of the two putative ATP binding/ATP hydrolysis domains, resulting in an improperly folded CFTR and retention in the endoplasmic reticulum (ER) due to underglycosylation (66). It has been shown that ΔF508 CFTR when retained in the ER is functional but more readily targeted for degradation (66, and references therein). The prevalence and reason for the existence and maintenance of defective CFTR in the human population has been discussed, and may be advantageous because other bacteria utilize wild-type CFTR as receptor for invasion into the host (101). *Salmonella typhi* translocation across the gastrointestinal tract into the submucosa, across the epithelium, was reduced by 86% in heterozygous ΔF508 *CFTR* mice as compared to wild-type mice (101). Thus, the mutation may act to prevent or reduce the occurrences of these or other infectious diseases, much like sickle-cell anemia does for the malaria parasite.

The defective chloride channel impairs epithelial ion transport, leading to viscous secretions, reduced mucociliary clearance and decline of pulmonary function resulting from chronic *P. aeruginosa* infections in the endobronchial space (27). Recently, some investigators found that airway epithelial cells utilize CFTR as the receptor for specific

endocytosis of *P. aeruginosa* via *P. aeruginosa* LPS outer core. This may help to clear the bacterium from healthy lungs through desquamation of epithelial cells (100, and references therein). Also, the defective Cl^- channel is believed to prevent release of antibacterial factors (e.g. human β-defensin-1) or may cause inactivation of these factors by the high salt content in the CF airways (66). For these reasons, in early adulthood CF patients eventually succumb to chronic infections by various microbes, including *Staphylococcus aureus, Haemophilus influenzae, Burkholderia cepacia* and, especially, *Pseudomonas aeruginosa* (120, 124). Mucoid (biofilm) *P. aeruginosa* is by far the predominant organism in the lung of most CF patients, and is the cause of high morbidity and mortality associated with CF and one ml of sputum from these patients can contain up to 10^7 to 10^8 *P. aeruginosa* colony forming units (124). Over 93% of CF patients between the age of 18 and 24 are infected with *P. aeruginosa* (27). Infection by mucoid *P. aeruginosa* may start early in life and 20% of infants with CF are colonized/infected with *P. aeruginosa* during the first two years of life; earlier acquisition is associated with worse pulmonary function and earlier mortality (110). CF patients who harbor nonmucoid *P. aeruginosa* and/or *S. aureus* maintain more than 90% of their expected lung function for many years (100). Nonmucoid *P. aeruginosa* phenotypes can be seen early in the infection and often displace other pathogens such as *S. aureus*, but it is the conversion of nonmucoid *P. aeruginosa* (planktonic) to the mucoid form (biofilm enclosed microcolonies) during the infection that eventually causes deterioration of the host in chronic infections. The mucoid variant is LPS rough, serum sensitive and exhibits low toxigenicity. The conversion from nonmucoid to mucoid forms *in vivo* during the chronic infection can be reversed *in vitro* after several passages of the mucoid variants on laboratory media (120, 121). The mucoid exopolysaccharide producing forms display enhance ability to bind to tracheal epithelial cells, interfere with binding to macrophage and other phagocytes, scavenge reactive bacterial oxygen intermediates, impede chemotaxis of polymorphonuclear leukocytes and impede diffusion of antibiotics to bacteria (17, 120). Once colonization occurs, the same clone of *P. aeruginosa* can persist for decades from initial colonization to death of the patient, and host deterioration is a result of vicious cycles of bacterial growth and inflammation (100). Since the immune system is functional in CF patients and the immunogenic exopolysaccharide alginate is a potent polyclonal B cell activator, antibodies

are produced that opsonize *P. aeruginosa* nonmucoid (planktonic) cells, but these antibodies do not opsonize mucoid or biofilm enclosed microcolonies (100). Antibodies are produced against numerous bacterial antigens which cause a rise in immune complexes, resulting in stimulation of neutrophils to release more proteases in classic type III hypersensitivity reactions (27). Immune complexes provoke the influx of large numbers of inflammatory cells, particularly polymorphonuclear leukocytes (PMN) into the endobronchial space, and the break down of these cells releases viscous DNA, further impairing lung function. The recruited PMNs also ingest and kill *P. aeruginosa* cells, and release toxic oxygen intermediates that further damage lung tissues (120). Neutrophils are the predominant inflammatory cells in infected bronchi of CF patients, and there is an elevated level of proinflammatory cytokines (TNFα and IL-1) in bronchial secretions; further inflammation ensues with recruitment of more PMNs and causes further tissue damage (120). Thus, nonsteroidal anti-inflammatory agents (e.g. ibuprofen) can be used to ease CF respiratory infections. *P. aeruginosa* infections in CF patients are different than other types of infections due to *P. aeruginosa* discussed above, in that higher serum antibodies are inversely correlated with pulmonary status. Growth as biofilm in CF lungs also protects *P. aeruginosa* from phagocytosis and complement mediated killing (120). The combination of *P. aeruginosa* toxins and proteases, as well as failed phagocytosis which releases numerous hydrolytic compounds (oxygen radicals and lysosomal enzymes) and neutrophil-derived elastase, all contribute to significant chronic lung damage. *P. aeruginosa* proteases (see chapter 1.3) and neutrophil elastase cleave various protease inhibitors, such as human α_1-proteinase inhibitor and secretory leukocyte proteinase inhibitor, allowing uncontrolled protease action in the lungs of CF patients resulting in lung damage (27). Although the role of T-lymphocytes in the lumen of the infected CF airways is not known, it is believed that cleavage of T-cell CD2, CD4 and CD8 by neutrophil uncontrolled elastase release can suppress antigen recognition and amplification (27). The combination of defective mucociliary clearance, enhanced host mucin secretion, bacterial production of mucoid causing exopolysaccharide alginate and DNA release by lysis of bacterial and host cells, all contribute to a viscous sputum that eventually deteriorates lung functions of CF patients leading to suffocation (120). Thus, respiratory tract disease in CF patients is characterized by chronic progressive deterioration in

pulmonary function with intermittent exacerbations and remissions of disease (120). Since *P. aeruginosa* is almost impossible to eradicate from CF patients, the most optimistic goal is long-term suppression rather than eradication of the infection, and other agents can be used to alleviate the suffering of CF patients. Protease inhibitors, as well as DNase, hold promise in prolonging lung function in CF patients (27). In addition, nucleotides (UTP and ATP) can be used to induce chloride secretion via other chloride channels besides CFTR, and amiloride, which blocks sodium channels, both have been shown to be effective and improve mucociliary clearance in CF patients (120).

1.3 *Pseudomonas aeruginosa* VIRULENCE AND PATHOGENESIS

Although *P. aeruginosa* is an opportunistic pathogen, the disease outcome is often fatal. Even when the immune system is functional, for example in CF patients, these bacteria persist for years and always pose the potential threat of phase conversion to the planktonic phase, during which they secrete various harmful extracellular hydrolytic compounds that cause tissue damage. The ability of *P. aeruginosa* to persist and infect various tissues of the body is due to many virulence determinants. Mekalanos (79) defined virulence determinants as those factors that contribute to infection, as well as to disease, excluding those that are required for multiplication on "non-living" media. *P. aeruginosa* virulence determinants, and their mechanisms and actions are summarized in table 1.1. Loss of any of these determinants leads to decreased or lack of virulence. Acting in concert and expressed in a timely manner, these virulence determinants allow colonization, persistent survival, invasion and damage to host tissues; and each virulence determinant contributes differently to the wide scope of diseases caused by *P. aeruginosa*.

Several virulence components are responsible for colonization, damage to host cells, and inactivation of host defense (complement, antibodies, immune cells), allowing the organism to persists during acute or chronic infections. All *P. aeruginosa* virulence factors are chromosomally encoded, with the exception of cytotoxin (or leukocidin) which is carried on a temperate phage. One μg of this toxin is sufficient to be lethal to a 20 g Swiss white

Table 1.1. ***P. aeruginosa*** **virulence determinants.**

Virulence factors	Mechanism of action	Potential role in virulence
Mucoid exopolysaccharide alginate (polyuronic acid)	Physical barrier	Barrier to antibiotics, phagocytosis, bactericidal oxygen radicals, scavenging and binding of complement and antibody; allows establishment of chronic infections in CF
Proteases		
LasA	Proteolytic nicking of elastin cross-linking peptides	Destruction of host defenses, and tissue invasion and damage; epithelial cell tight junction separation; degradation of fibrinogen and fibronectin; cleavage of antibodies creating non-functional blocking antibodies; inactivation of α_1-antiproteinase, complement components, and cytokines; cleaves C3b receptors from neutrophils; stimulates mucus secretion (detrimental in CF patients)
LasB Alkaline protease	Proteolytic degradation of LasA-nicked elastin	
Lysine-specific protease	(?)	
Exotoxin A	ADP-ribosylation of eukaryotic elongation factor-2, inhibition of protein synthesis	Tissue destruction; toxic to macrophages; T-cell mitogen (superantigen); inhibits granulocyte and macrophage progenitor cell proliferation
Exoenzyme S	ADP-ribosylation of eukaryotic proteins vimentin (cytoskeletal protein) and $P21^{c\text{-}H\text{-}ras}$ (oncogene)	Dissemination of organism; tissue destruction; adherence to epithelium
Siderophores Pyochelin (pyocyanin) Pyoverdin	High-affinity binding of iron; scavenging of iron from host transferrin and lactoferrin	Inhibits ciliary beating; toxic to other bacterial species and human cells; enhances oxidative metabolism of neutrophils; inhibits lymphocyte proliferation

Table 1.1. *P. aeruginosa* virulence determinants (continued).

Virulence factors	Mechanism of action	Potential role in virulence
Pili	Distant attachment to host cells	Initiation of colonization
Lipase	Cleaves phospholipids of eukoryotic cells and lung surfactants(?)	Tissue damage
Cytotoxin (leukocidin)	Pore formation in cell membranes	Cytotoxic to neutrophils and lymphocytes; tissue inflammation and destruction; decreased granulocyte function
Phospholipase C (heat-labile hemolysin)	Degrades phospatidylcholine and sphingomyelin; formation of diacylglycerol or ceramide and phosphorylcholine	Destroys lung surfactant; tissue damage; generation of inflammatory mediators
Rhamnolipid (heat-stable hemolysin)	Detergent like properties	Lethal to leukocytes, stimulates mucus secretion; inhibits ciliary beating; affects ion transport across epithelium; enhances phospholipase C hemolytic activity
Flagellum	Motility and adherence	Chemotaxis; invasion; and initial adherence to host cells

Compiled from references (135, 140).

mouse (140). Cytotoxin has also been shown to decrease granulocyte function (phagocytosis, killing, and chemotaxis) (5). Because the LD_{50} of *P. aeruginosa* LPS in animal studies varies from 20 to 450 μg, *P. aeruginosa* lipopolysaccharides (LPS) has been suggested by some to be less toxic as compared to LPS of *Enterobacteriaceae* (57, 75) and it is therefore not listed in table 1.1. However, others have reported the potency of *P. aeruginosa* LPS to be similar LPS from other Gram-negative bacteria, and anti-LPS antibodies can lead to protection (3). Indeed, IgG antibodies to LPS and cytotoxin can lead to better survival in *P. aeruginosa* bacteremia (3, 140). Intravenous infusion of *P. aeruginosa* into experimental animals can cause shock and administration of LPS (1 μg/g) in burned mice can be lethal (136). It has been demonstrated that exotoxin A potentiates LPS action in animal models, causing an increase in serum TNFα concentration and mortality rates (92). Thus, even if *P. aeruginosa* LPS is less toxic as compared to enterobacterial LPS, its combination with *P. aeruginosa* toxins can cause comparable, if not greater harm. *P. aeruginosa* LPS activates mucin secretion by epithelial cells, which is detrimental in CF patients, since mucin provides additional receptors for *P. aeruginosa* adhesion, and also obstructs airways and impedes bacterial clearance (66, and reference therein).

Damage to host cells caused by several virulence factors may destroy the ciliary beating mechanisms which would otherwise protect the epithelium, and also expose new receptors for bacterial adherence on damaged cells (135). Several bacterial adhesins, including flagellar protein, exoenzyme S and alginate, all contribute to colonization of the host (135). Since fibronectin coats the surface of the mucosa, increase in adherence to epithelial cells correlates with loss of cell surface fibronectin, which is cleaved by *P. aeruginosa* proteases (139, 140). *P. aeruginosa* adheres to host asialoganglioside 1 and 2 (aGM_1 and aGM_2), but not to the sialylated (acidic sugar) homologues (100); however, no specific aGM_1 and aGM_2 adhesins have been identified. *P. aeruginosa* proteases cause hemorrhagic and necrotic effects when injected under the skin, and in animal lungs the hemorrhage and necrosis resembles that seen in *P. aeruginosa* pneumonia (140). *P. aeruginosa* elastase has extremely broad *in vitro* substrate specificity including: human lung elastin; immunoglobulin complement factors C1, C3, C5, C8, and C9; human type III and IV collagen; human α_1-proteinase inhibitor; laminin of basement membrane; mucins; human

transferrin; and bronchial proteinase inhibitor (140). Degradation of human α_1-proteinase inhibitor and laminin allows endogenous serine proteases to cause tissue damage and *P. aeruginosa* invasion, respectively. It has been suggested that *Pseudomonas* proteases supply the bacterium with nutrients by breaking down proteins (e.g. in burnt skin). *P. aeruginosa* has two hemolysins, rhamnolipid and phospholipase C. The former is a glycolipid acting as a detergent to emulsify eukaryotic cell membranes and it is a ciliostatic factor. Thus, rhamnolipids damage host cells and prevent ciliary clearance of the organism by lung epithelial cells. Besides its hemolytic action, destruction of lung surfactant (phosphatidylcholine) by phospholipase C can lead to alteration in host cell response and metabolism via diacylglycerol, and the generated phosphorylcholine can further induce alginate and more phospholipase C production (67, 117, 126). Purified phospholipase C from *P. aeruginosa* causes dermonecrosis, paralysis, increased vascular permeability and death in mice (LD_{50} = 6 μg) (7). In addition, non-hemolytic phospholipases have been discovered (133), but they are currently not well characterized. *P. aeruginosa* produces two ADP-ribosyltransferases, exotoxin A and exoenzyme S. In the burnt mouse model, exoenzyme S deficient mutants are 2000-fold less virulent than the parental strain (85). Clinically, exoenzyme S is produced at high levels in isolates from acute pneumonia patients, but it is at low levels in bacterial cells recovered from burn wound infections (138). The significance of this virulence determinant in *P. aeruginosa* pneumonia was further demonstrated when purified exoenzyme S was administered intratracheally into rat lungs, where extensive nectrotizing and lung damage occurred. Purified exoenzyme S also caused vacuolization and membrane damage to monolayer cultures of bronchial fibroblasts (137). The most toxic substance produced by *P. aeruginosa* is exotoxin A and purified exotoxin A is lethal to various animals (140). Similar in its action to diphtheria toxin, exotoxin A of *P. aeruginosa* ADP-ribosylates eukaryotic elongation factor 2 and inhibits protein synthesis in different organs in animals (59, 91).

1.4 *Pseudomonas aeruginosa* TREATMENT AND RESISTANCE TO ANTIMICROBIALS

There are few classes of antipseudomonal drugs that are available for clinical use, including β-lactams, aminoglycosides, quinolones, and sometimes polymyxin and rifampin. Polymyxin B was released for clinical use against *P. aeruginosa* infections in 1947 and was the only antibiotic available through the 1950s for this bacterium (19). Polymyxin E (colistin) soon followed in 1961, but both polymyxins are relatively ineffective in immunocompromised, bacteremic, and patients with deep-tissue infection and was associated with a high degree of clinical toxicity. Less toxic drugs that are more active against *P. aeruginosa* soon followed with the release of gentamycin in 1963 and carbenicillin in 1967. Still, the mortality due to *P. aeruginosa* bacteremias continued to be higher than those associated with other Gram-negative bacteria and increased used of these drugs was followed by resistant organisms (19). Serum levels of free drugs (unbound by serum proteins) have been good predictors of the active drugs concentration at most extracellular tissue sites within the body. However, penetration of antimicrobials into special sites (e.g. cerebrospinal fluid, humors of the eye, the prostate, and respiratory secretions such as sputum and tracheal aspirate) are not predictable by serum levels and sometimes inhibitory concentrations by available drugs are not achievable. Thus, the introduction of a large variety of antipseudomonal antibiotics in the past 17 years with enhanced pharmacodynamic and pharmacokinetic properties have controlled *P. aeruginosa* infections significantly. However, antimicrobial resistance continues to emerge with their continued use, especially when used in monotherapy. Hence, the continual research for drugs with high antipseudomonal activities, low selection of resistance, and good pharmacokinetic properties is mandatory.

A large variety of β-lactam antibiotics are active against *P. aeruginosa*. These include carboxypenicillins (carbenicillin and ticarcillin), ureidopenicillins (piperacillin and azlocillin), many third generation cephalosporins (ceftazidime, cefempime, cefsulodin, and cefpirome), the carbapenems (imipenem and meropenem) and aztreonam (monobactam) (19, 49, 141). Inhibition of growth by β-lactam antibiotics is by interfering with the final step in the synthesis of peptidoglycan, specifically by inhibiting the transpeptidase that catalyzes the

cross-linking reaction (19). This is only one of several enzymes inhibited by β-lactams, collectively known as penicillin-binding proteins (PBPs), which are numbered according to decreasing molecular weights. In addition to transpeptidase, PBPs also include carboxypeptidase and endopeptidase (141). Inhibition of PBP2 results in loss of rod shape and formation of large ovoid cells; while inhibition of PBP3 results in filament formation without septa (19). Thus, this class of antimicrobial agents inhibits cell wall synthesis and separation, and the bactericidal activity depends directly on the rate of growth. In addition, β-lactams have been found to activate the endogenous autolytic system of bacteria, a process that initiates cell lysis and death (128). The difference in permeability of β-lactams between *P. aeruginosa* and *E. coli* (12 to 100 fold difference) has been explained by the small diameter outer membrane major porins (C, D2, and E) of *P. aeruginosa*. Most strains of *P. aeruginosa* resistant to carbapenems lost the D2 porin protein (19). The primary mechanism for resistance to penicillins, cephalosporins and aztreonam is enzymatic hydrolysis of the β-lactam ring by β-lactamases. Plasmid encoded β-lactamases in *P. aeruginosa* can render the organism resistant to carboxy- and ureidopenicillins, but this type of resistance can sometime (7%) be irreversibly inhibited by β-lactamase inhibitors, such as clavulinic acid, sulbactam, and tazobactam (19, 141). However, chromosomally-encoded β-lactamase, which is resistant to β-lactamase inhibitors, can occur in as many as 40% of cases where monotherapy treatment with antipseudomonal penicillins and cephalosporins is used (19) and some β-lactamase inhibitors can by extruded by MexAB-OprM multidrug efflux pump (74; see below). The MIC_{50} and MIC_{90} (concentrations that inhibit 50% or 90% of the strains tested) for β-lactams range from 2 to 32 mg/l and 50 to 256 mg/l, respectively; and the activities of these agents are affected by inoculum size, and the minimal bactericidal concentrations (MBCs) tend to be two times higher than the MICs (19). The bactericidal activity of β-lactams against *P. aeruginosa* is characterized by a rise in the killing rate with increasing concentrations up to a point of maximal effect (19). This point of maximal effect is about four to five times the MIC, and beyond this point, increasing concentrations do not enhance the rate or extent of killing. Similar observations have been made in animal infection models (37, 134). Hence, dosing frequencies should be designed to provide serum concentrations above the MIC throughout the dosing interval and continuous infusion of β-lactams would ensure that serum levels

exceed the MIC virtually all the time (19). There is no postantibiotic effect (term used to describe the inhibition of regrowth after serum concentration is below the MIC) of β-lactams on *P. aeruginosa*, except for carbapenems. Thus, high drug concentrations beyond four to five times the MIC do not kill *P. aeruginosa* faster, and bacterial regrowth occurs very soon after serum or tissue levels fall below the MICs (19). Unlike other β-lactams (e.g. amoxicillin and phenoxymethyl penicillin), antipseudomonal penicillins are not orally absorbed and are destroyed by gastric acid. Hence, to combat *P. aeruginosa* infections, intravenous administration of 4 g (piperacillin, azlocillin, and mezlocillin) to 5 g (carbenicillin) of β-lactams are required to achieve clinically beneficial serum concentrations of 330 to 450 mg/l (19). In addition, *in vitro* studies demonstrated that piperacillin has activity against *P. aeruginosa* that is twice that of azlocillin, four times that of mezlocillin and ticarcillin, and about 8 times that of carbenicillin (141). The antipseudomonal penicillins exhibit good distribution into extravascular tissues that requires only passive diffusion, but the extent of penetration into certain fluids or tissues (cerebrospinal fluid, aqueous humor, prostate tissue, and respiratory secretions) is poor (19). The levels of antipseudomonal β-lactams for aqueous humor are reported to average 2 to 7% of serum concentration, 4 to 30% for cerebrospinal fluid, 25 to 30% for prostate tissue and 4 to 20% for respiratory secretions. The cephalosporins appear to exhibit a slightly better respiratory concentration profile than penicillins and concentrations of ceftazidime obtained in respiratory secretions are usually above the MIC_{50} (19). The average half-life of β-lactams in serum is short (1 to 1.5 h), and the majority is eliminated by the kidneys and to a lesser extent by the liver. Carbenicillin and ticarcillin exhibit the greatest extent of renal elimination, with approximately 90% of a dose of each recovered unchanged in the urine (19). Besides acting as a hapten to create allergic reactions (3 to 10% frequency), the β-lactams can exhibit a variety of adverse effects, including diarrhea, rash, sodium overload and hypokalemia (carboxypenicillins), neutropenia (ureidopenicllins and carbapenems), thrombocytopenia (cephalosporins and aztreonam), and seizure and nausea are associated with carbapenems (19). The frequencies of these adverse effects range from 2 to 5 % of the cases.

The aminoglycosides antibiotics have been major agents used in the treatment of *P. aeruginosa* infections. This group contains natural antibiotics (e.g. gentamycin, tobramycin,

sisomicin) and semisynthetic derivatives (e.g. amikacin, netilmicin, isepamicin) (18, 19). Monotherapy with aminoglycosides give better therapeutic results against *P. aeruginosa* than monotherapy with β-lactams. The aminoglycosides exert activity by binding irreversibly to the bacterial 30S ribosomal submit, thus interfering with protein synthesis and bacterial growth (28). Lysis of bacteria following exposure to aminoglycosides usually occurs hours after the lethal event. However, the requirements for aminoglycosides to enter the cytoplasm include an energy- and oxygen-dependent transport mechanism that is inhibited by anaerobiosis and low pH (28). The binding of aminoglycosides to the outer membrane is initiated by ionic interaction which is linearly related to aminoglycoside concentration. This binding disrupts the outer membrane and increases further permeability to aminoglycosides (19). Transport of aminoglycosides across the cytoplasmic membrane occurs in two energy-dependent phases i) the slow initial phase is dependent on the electrical potential across the cytoplasmic membrane and ii) the second accelerated phase is triggered by binding of aminoglycoside to its ribosomal target (19, and references therein). At least 14 enzymes found in *P. aeruginosa* can inactivate various aminoglycosides. The majority are acetyltransferases that inactivate gentamycin, tobramycin, sisomicin and netilmicin (109). Amikacin and isepamicin are inactivated by a subset of the 6'-N-acetyltransferases encoded by transposons located on plasmids (109, 116). In the United States, the percentage of strains containing a 2''-O-adenyltransferase, a 3-N-acetyltransferase or a 6'-N-acetyltransferase varied from 26 to 31%, while in Japan 88% of strains had 6'-N-acetyltransferase activity and in Chile 94% of strains contained 3-N-acetyltransferases (19). The MIC_{50} of aminoglycosides is low, ranging from 1 mg/l for tobramycin and 4 mg/l for gentamycin, and the MIC_{90} is 4 mg/l for tobramycin and 16 mg/l for gentamycin. The MIC_{50} and MIC_{90} for netilmicin, amikacin, and isepamicin are double that of gentamicin (19). Due to prolonged postantibiotic effects of aminoglycosides, once-daily single high doses have demonstrated equal or better efficacy than those observed with more frequently divided dosing regimen. This regimen has been associated with less drug accumulation in the kidney cortex and lower incidence of nephro- and ototoxicity (19). Thus, these results support the practice of giving high dosages of aminoglycosides at extended intervals. Emergence of resistance can be prevented *in vitro* by obtaining peak concentrations at least 8 to 10 times the MIC, and in some serious

infections peak aminoglycoside levels at 8 to 10 times the MIC are required for recovery of 85% of patients (19). Due to the hydrophilic nature of aminoglycosides, they are not absorbed orally, and 2 mg/kg (for tobramycin, gentamycin, and netilmicin) or 15 mg/kg (for amikacin and isepamicin) are infused intravenously over a period of 30 minutes. This amount is much lower than that required for β-lactams, because serum protein binding of aminoglycosides is low, averaging around 10%; hence, virtually all of the aminoglycoside present in serum is in the form of free active drug (19). Aminoglycosides enter extravascular and extracellular sites easily, and are found in prostate and respiratory secretions in levels as high as 96% of serum levels. However, they do not enter aqueous humor, noninflamed CSF or respiratory secretions to any appreciable degree (19). The average serum half-life for aminoglycosides is about 1.5 to 2 h, and over 90% can eventually be cleared by the kidneys (19). Thus, nephrotoxicity can be as high as 8 to 14% of the cases, with gentamycin and tobramycin being responsible for the more severe cases (19). Ototoxicity (hearing loss) frequencies can be quite high with amikacin (12%) and gentamycin (8%); and less frequently other type of toxicities can occur, such as vestibular ototoxicity and neuromuscular blockade.

Other commonly used antipseudomonal antibiotics are the quinolones, which inhibit the A subunit of bacterial DNA gyrase (an enzyme that forms supercoils in bacterial DNA) preventing the sealing of nicks in DNA, and mammalian DNA gyrase is unaffected at therapeutic concentrations (1, 19, 58, 77). The two DNA strands wind around each other in a right handed helix and DNA gyrase forms supercoil twists in the opposite direction to the double helix (e.g. negative supercoil) (77). This is extremely important to fit the chromosomal DNA (>1300 μm in length) into a cell that is 1 to 2 μm in size. In addition, the negative supercoiling allows the DNA to unwind during replication and transcription (77). Unlike the self-promoted uptake pathway of aminoglycosides, crossing of the outer membrane by quinolones is mediated by nonporin or porin pathways and crossing of the inner membrane occurs by simple diffusion (19). Among the quinolones, ciprofloxacin is the most active drug against *P. aeruginosa* with an MIC_{50} and MIC_{90} of 0.25 and 1 mg/l, respectively; followed by norfloxacin with an MIC_{50} and MIC_{90} of 1 and 2 mg/l, respectively (19). Other quinolones (fleroxacin, ofloxacin, temafloxacin, sparfloxacin, and enoxacin) exhibited MIC_{50} and MIC_{90} of approximately 1 and 4 mg/l. As with aminoglycosides,

quinolones exhibit concentration-dependent bactericidal activities and demonstrate postantibiotic effects. Unlike the β-lactams, the activities of quinolone drugs are not altered by inoculum size or the presence of human serum, and quinolones are capable of killing nongrowing *P. aeruginosa* (19). These drugs have benefits over the aminoglycosides and β-lactams in that they are absorbed quite efficiently when administered orally. For example, when giving a 500 mg oral dose, 70% of the ciprofloxacin is absorbed, reaching peak serum concentrations averaging 2.3 mg/l, which is above the MIC_{50} and MIC_{90} (19). Quinolones also have advantages over aminoglycosides and β-lactams in that they reach high concentrations in intracellular and extracellular fluids, and in less accessible sites such as prostate and respiratory secretions, exceeding the serum concentration by as much as 300 to 400%. The half-life in serum is superior ranging from 4 to 17 h (19). All quinolones exhibit low serum protein binding, and range widely in their degrees of renal and hepatic elimination (58). Ofloxacin and lomefloxacin are recovered in the urine unchanged at 75-80%, while the recovery rates are quite low with ciprofloxacin at 35%, norfloxacin at 30% and sparfloxacin at 9% (19). Like other antipseudomonal antibiotics, quinolones have several side effects. The major concern is that quinolones impair joint cartilage formation in immature animals; hence their use in children and pregnant females is not recommended (19). Other common adverse effects include nausea with frequencies of 2 to 10%, while headache, dizziness, irritability, and rash occur in 1-4% of cases (19). In addition, resistance of *P. aeruginosa* to quinolones can be found in as many as 10-20% of patients. Primary mechanisms of resistance to quinolones is via mutations in DNA gyrase or mutations in the MexR regulator that regulates the *mexE-mexB-oprM* efflux operon (58). However, unlike β-lactams and aminoglycosides, drug modification of quinolones as mechanism of resistance has not yet been found.

Other antipseudomonal drugs are sometimes used, including polymyxin B and E and rifampin, but these drugs exhibit high toxicities and are only used in special cases and in combination with other antipseudomonal drugs. Polymyxins exert antibacterial activity by binding to the LPS and phospholipids of cell membranes, resulting in leakage of intracellular material (19). Polymyxins are eliminated unchanged by the kidneys, and are associated with high incidences of nephrotoxicity (15 to 25%) and neurotoxicity (19). Rifampin efficiently penetrates into cerebrospinal fluid (uninflamed) and aqueous humor (50-100% of serum

concentration), but 80% of it binds to serum proteins (19). Clinical trials with rifampin demonstrated improvements when the drug was added to therapies in four patients in whom therapy with a carbenicillin-aminoglycoside combination was failing. In another study involving 123 patients, rifampin in addition to β-lactam and aminoglycoside therapy did not significantly improve mortality (24% for all three drugs and 17% for two drugs), but it increased the frequency of bacteriological cure (2% failure for all three drugs and 14% failure for two drugs) (19). However, side effects of rifampin include hepatoxicity, flu-like illness and interstitial nephritis; it also imparts an orange color to saliva, sweat, urine, and tears (19). Rifampin resistance can be due to mutations in the bacterial DNA-dependent RNA polymerase.

Since resistance often occurs with monotherapy and leads to eventual failure of therapy, combination therapy is often utilized to give synergy and prevent emergence of resistant *P. aeruginosa*. Synergy implies that the combined effect of co-administered drugs is significantly greater than the sum of their separate effects. For example, one method of detecting synergy is that the MIC for each drug needs to be reduced at least four-fold when used in combination (19). Synergy between β-lactams and aminoglycosides occurs at frequencies ranging from 37 to 65% for strains tested, and aminoglycosides have been shown to enhance the penetration of β-lactams (19). Antagonism is rarely observed with β-lactam and aminoglycoside combinations. Double β-lactam combinations have very low frequencies of synergy, ranging from 4-22%, but antagonism between some β-lactams has been observed and is attributed to induction of β-lactamase by certain β-lactams. Synergy between β-lactams and quinolones occurs at frequencies ranging from 10-36%, and often additive effects are observed in the absence of synergy (19). Lastly, synergistic combinations of aminoglycosides and quinolones are rarely observed. Resistance can be reduced by combinations of an antipseudomonal β-lactam with an aminoglycoside or a quinolone, but not with two β-lactam combinations (19).

The benefits of synergy can be demonstrated *in vivo* in animal models, where endpoints of success include survival, decreased organism counts and prevention of the emergence of resistance (19). For β-lactam and aminoglycoside combinations, therapy was superior to monotherapy in 50-60% of studies using survival as endpoint, 80% of studies

using organism counts, and in about 50% of studies using prevention of emergence of resistance (19, and references therein). However, combination therapies of β-lactams and aminoglycosides proved not to be better than single-drug therapy in pneumonia models when the aminoglycoside was the single drug rather than β-lactam (19). Thus, any comparison must include susceptibility data, pharmacodynamic and pharmacokinetic properties, and efficacy in animal models of infection.

Antimicrobial pharmacokinetics are altered dramatically in CF patients, and strains of *P. aeruginosa* dissociate into multiple different phenotypic forms often with different antimicrobial susceptibility patterns (127). Since the infection in CF is chronic and *P. aeruginosa* is rarely ever eradicated, resistance to multiple antimicrobial agents develops frequently (25, 39, 127). Mucoid strains are resistant to penetration of some antibiotics. Patients with CF inactivate or eliminate antimicrobials (e.g. aminoglycosides) more rapidly than normal patients and require higher dosages, because of expanded venous plasma volume and increased urine flow rate (102). Parenteral doses of antimicrobials in CF patients in Denmark showed substantially increased survival rates, but parenteral administered drugs penetrated poorly into sputum (19). Therefore, investigators have administered antibiotics by the aerosol route, which appears to be safe and effective at delivering a high dose of antibiotics to the site of infection. However, because of the ubiquitous nature of *P. aeruginosa*, aerosol generating equipment must be sterile designed to and create small enough particles able to penetrate deep in the lungs.

The combination of low permeability of the outer membrane of *P. aeruginosa* and potential of activation of several tripartite multidrug efflux pumps has produced new threats and has been treated with great concerns in recent years (43, 74, 84, 86, 87, 122, 123). *P. aeruginosa* has an outer-membrane permeability that is 12-100 fold lower than that of *E. coli* (43, and references therein). However, upon exposure to drugs, the periplasmic concentrations of many antibiotics reach 50% of their external concentrations within 10 to 30 s in *P. aeruginosa*, a rate that is much slower than in *E. coli* (86, 87). Hence, the decreased outer membrane permeability for drugs is expected to result in a slower accumulation, but will nevertheless reach equilibrium concentrations across the outer-membrane (86, 87). Thus, other drug elimination mechanisms are needed to explain the high intrinsic resistance of this

bacterium. In recent years, it was discovered that *P. aeruginosa* possesses several three-component multidrug efflux systems that are made up of an inner-membrane proton-dependent antiporter (MexB, D, or F), a periplasmic membrane fusion protein (Mex A, C, or E), and an outer membrane channel (OprM, J, or N) (86, 87). These pumps, arranged in three separate chromosomally-located operons, are normally regulated by their corresponding regulator, and their constitutive expression is caused by mutations in the genes encoding these regulatory proteins. The MexAB-OprM complex can pump tetracycline (Tc), chloramphenicol (Cm), quinolones (Qls), erythromycin (Em), azithromycin (Az), sodium dodecyl sulfate (SDS), crystal violet (Cv), novobiocin (Nov), rifampin (Rif) and several β-lactams (ampicillin, carbenicillin, cefoperazone, cefepime, cefpirome, cefotaxime, ceftazadime, penicillin, and ticarcillin) (122, 123). this efflux pump is constitutively expressed in the commonly used laboratory strain PAO1, which was originally a clinical isolate. This pump can be further overexpressed by exposure to and selection of repressor mutations on nalidixic acid containing medium (*nalB* mutants) (84, 122, 123). Expression of two other well characterized multidrug efflux pumps MexCD-OprJ and MexEF-OprN can be achieved by exposure to norfloxacin, and the resulting strains contain mutations in *nfxB* and *nfxC*, respectively (84, 86, 87). MexCD-OprJ can extrude Tc, Cm, Qls, Nov, Em, Az, SDS, Cv, Rif, and forth generation cephem but not conventional β-lactams or carbapenems. However, the MexEF-OprN system can extrude carbapenems (imipenem and meropenem), as well as Cm and Qls (86, 87, 122, 123). More recently, multidrug aminoglycoside efflux pumps have been discovered in a related bacterium *Burkholderia pseudomallei* (81) and a similar pump (MexXY) has recently been described in *P. aeruginosa* (15). Thus, there exist even more threatening mechanisms of resistance than traditional single drug resistance mechanism for all classes of currently available antipseudomonal drugs, namely β-lactams, aminoglycosides, quinolones and rifampin. In addition, multidrug resistance can increase the MIC of certain drugs to an extent that renders the drugs absolutely useless. For example, the carbenicillin MICs of 80% of clinical isolates from the British Isles can be 2000 times higher than that of pump-deficient mutant strains (86). Therefore, future combinational therapy must be reconsidered and should reflect drugs that are effluxed by different pumps. Combinational therapies with drugs that are effluxed by the same pump have the potential to select for

multidrug resistance by a single mutation in one of the regulatory proteins. However, the chances of getting resistance by combinational therapy with drugs that are extruded by different pumps are significantly reduced, because such resistance in *P. aeruginosa* would require multiple mutations to cause expression of more than one pump.

1.5 CELL-TO-CELL COMMUNICATION IN BACTERIA

Among Gram-positive bacteria, including *Staphylococcus aureus*, *Bacillus subtilis*, *Lactococcus lactus* and *Streptococcus pneumoniae*, the common cell-density-dependent autoinducer molecules released for intercellular communication are peptide pheromones (115). These peptides do not diffuse into the cell, but are sensed by two-component regulatory systems which involve a sensor and a response-regulator (65). Once extracellular pheromones are detected by the sensor, the signal is passed to and activates the cytoplasmic response regulator via phosphorylation of specific amino acid residues. In *S. aureus*, peptide pheromones are involved in expression of virulence exoproteins such as δ-lysin; while *S. pneumoniae* and *B. subtilis* peptide pheromones activate natural competence. Antimicrobial peptides in *L. lactus*, *B. subtilis*, and *S. epidermidis* are also activated by other similar peptide pheromones (65). Many Gram-positive bacteria can produce multiple pheromone signals, thus highlighting the importance of intercellular communication (65, 115).

Other types of pheromone molecules have been identified in a variety of different organisms. *Myxococcus xanthus* utilizes amino acids and proteins to communicate intercellularly, and this process is required for fruiting body development (62). *Streptomyces* species produce γ-butyrolactone as an intercellular signaling molecule for antibiotic production (115). *Micrococcus luteus* produces a protein for promoting growth and resuscitation of cells from a dormant state (83). Hence, there are a variety of different molecules produced in different microbes, but the common theme between all signifies the importance for intercellular communication.

In Gram-negative species, intercellular communication involves cell density-dependent production of *N*-acylated-L-homoserine lactones (22, 34, 82, 99). Numerous Gram-negative bacteria have been shown to produce such intercellular communication

molecules, including *Pseudomonas* sp., *Vibrio* sp., *Agrobacterium* sp., *Erwinia* sp., *Rhizobium* sp., *Aeromonas* sp., *Yersinia* sp., *Seratia* sp. and *Burkholderia* sp. (34, 73, 82, 99). These autoinducers (AI) are diffusable molecules synthesized from acyl-acyl carrier protein (acyl-ACP) and *S*-adenosylmethionine by an AI synthase (see chapter 1.8) and accumulate in late-log to stationary-phase of growth. Low basal levels of AIs are synthesized in early-log phase grown cells, and accumulate as cell density increases to reach a threshold level. These AIs are generally thought to be freely diffusable in and out of the cell. However, *P. aeruginosa* *N*-[3-oxo]-dodecanoyl-L-homoserine lactone is as substrate for the MexAB-OprM multidrug efflux pump (30). The binding of these intercellular-diffusable autoinducers to the amino-terminus of the corresponding LuxR (e.g. RhlR and LasR in *P. aeruginosa*; figure 1.2) family of regulators allows activation of the helix-turn-helix at the carboxy-terminus of these regulators, which leads to activation of transcription of the synthase gene and therefore further increased AI production (36). As well, the AI-regulator complex activates various behaviors in different bacteria. *Vibrio fischeri* and *Vibrio harveyi* AIs, both being symbionts of marine animals, can activate an operon of luminescence genes (33, 35). *Erwinia* species AIs activate exoenzyme and carbapenem synthesis, while *P. aeruginosa* AIs activate various virulence determinants. This similarity between Gram-negatives, as well as multiple AIs in a single bacterial species (*P. aeruginosa* and *V. fischeri*) and its multi-gene regulatory role again highlight the significance of intercellular communication as a quorum to synchronize a specific behavioral response.

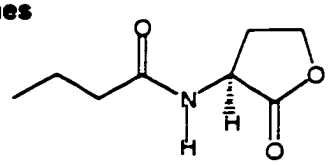
1.6 QUORUM REGULATION OF VIRULENCE FACTORS IN *P. aeruginosa*

P. aeruginosa virulence regulation by a cell density-dependent mechanism is the best understood among Gram-negative bacteria. Almost all virulence factors studied in *P. aeruginosa* so far are regulated via *N*-butyryl-L-homoserine lactone (C_4-HSL) and/or *N*-[3-oxo]-dodecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) autoinducer-dependent mechanisms (figure 1.1A). These include LasA and LasB (elastases), ToxA (exotoxin A), AprA (alkaline protease), RhlAB (rhamnosyltransferase), pyocyanin and biofilm maturation

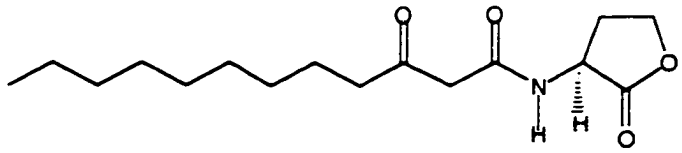
(11, 22, 93, 94, 96, 99, 131). The homoserine lactones (HSLs) C_4-HSL and 3-oxo-C_{12}-HSL bind to their corresponding regulatory proteins RhlR and LasR, respectively, to activate the transcription of various virulence genes (figure 1.2)(97). Furanones produced by the macroalga *Delisea pulchra* have been found to inhibit HSL-dependent regulatory systems by mimicking acylated-HSLs of bacteria (figure 1.4C) (38) and thus there is promise for the potential inhibition of AI-induced pathways.

Recently, two other classes of compounds isolated by organic extractions of *P. aeruginosa* clarified culture supernatants have been described, 2-heptyl-3-hydroxy-4-quinolone and cyclic peptides (diketopiperazines) the structures of which are depicted in figures 1.1B and C (53, 98). These compound apparently play a role in *P. aeruginosa* intercellular communication. The diketopiperazines (DKPs) can induce bioluminescence in an *E. coli* HSL detection strain that contains *luxR* and the *lux* operon promoter of *Vibrio fischeri* coupled to the bioluminescence operon (*luxCDABE*) of *Photorhabdus luminescens*, and it was suggested that DKPs can interact with LuxR to activate the bioluminescence operon (53). However, no direct interaction of DKPs with LuxR was demonstrated and the activation threshold of DKPs (~1 mM) was several order of magnitudes greater than the 1 nM concentration of the natural HSL autoinducer (3-oxo-hexanoyl-HSL) needed for activation of bioluminescence. The authors (53) did not estimate the actual concentrations of DKPs in the extracted culture supernatants and it is conceivable that the concentrations needed for activation may not be achievable in an overnight culture of *P. aeruginosa*. Although these compounds were extracted from *P. aeruginosa*, these authors reported no studies of the effects of DKPs on the expression of any *P. aeruginosa* HSL regulated genes. In addition, no evidence was presented on the growth phase-dependent synthesis or regulation of DKPs, and such molecules could be constitutively synthesized and accumulate in *P. aeruginosa* cultures. Clearly, more studies are needed before such molecules can be assigned a role in intercellular communication as a quorum. Pesci et al. (98) have demonstrated yet another cell-to-cell communication molecule in *P. aeruginosa*, 2-heptyl-3-hydroxy-4-quinolone, and it is the first *Pseudomonas* quinolone signal (PQS; figure 1.1B). By utilizing *lasI* and *lasR* mutants, the synthesis of this molecule was determined to be dependent on 3-oxo-C_{12}-HSL and LasR (figure 1.2). PQS could be isolated from strain PAO-JP2 (Δ*lasI*, Δ*rhlI*) containing a truncated

A) Homoserine lactones

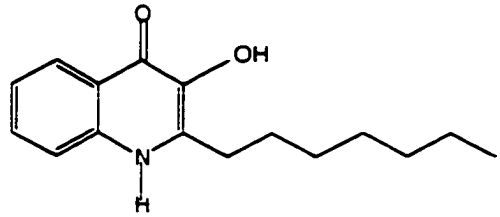


N-butanoyl homoserine lactone



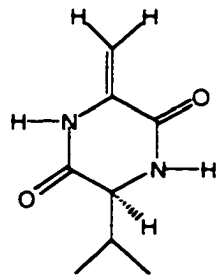
N-(3-oxo)-dodecanoyl homoserine lactone

B) Quinolone

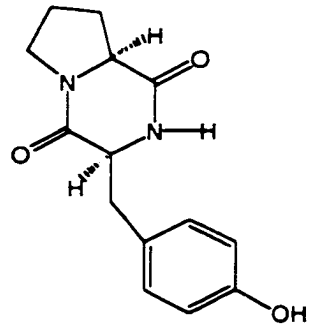


2-heptyl-3-hydroxy-4-quinolone

C) Cyclic Peptides (diketopiperazines)



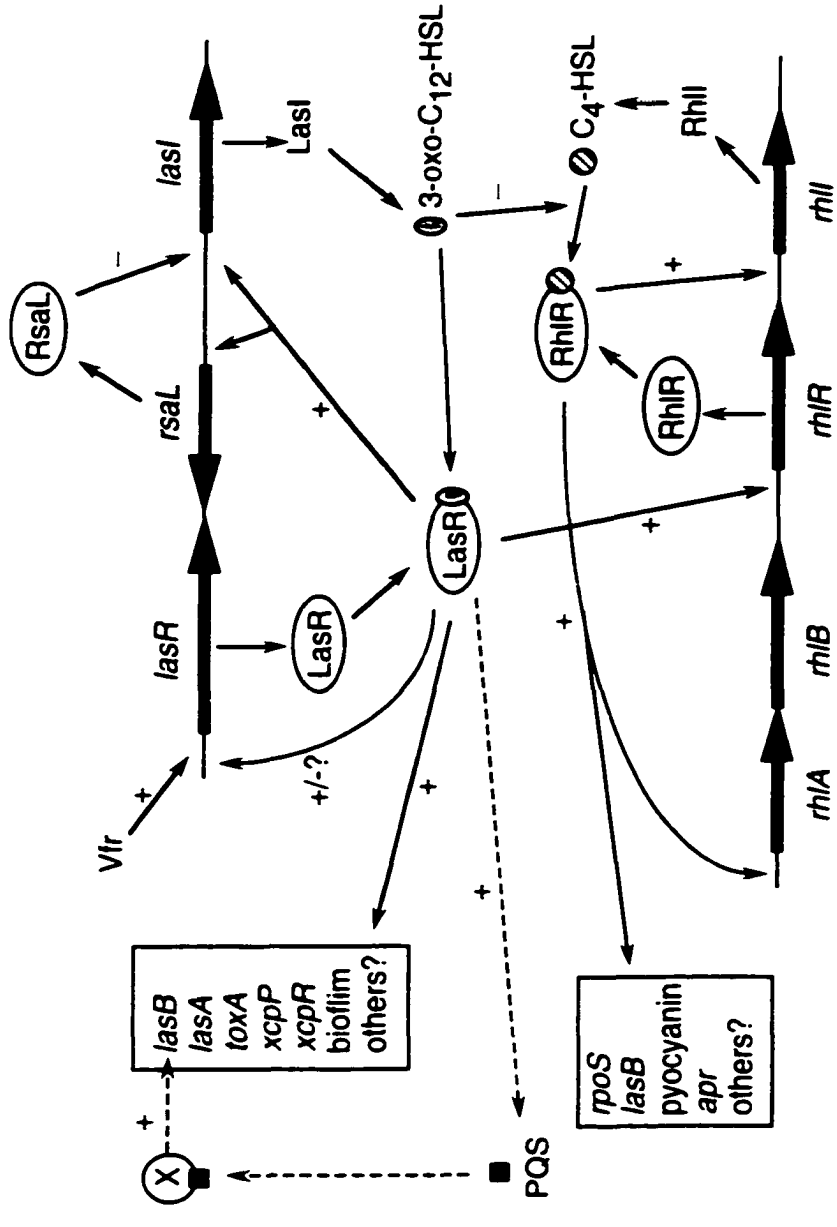
cyclo(Δ-Ala-L-Val)



cyclo(L-Pro-L-Tyr)

Figure 1.1 Cell-cell communication molecules of *P. aeruginosa*.

Figure 1.2. Model of quorum-sensing-dependent gene regulation in *P. aeruginosa*, showing the interaction of the two homoserine-lactone (HSL) autoinducer (AI) systems in virulence gene regulation. Plus (+) indicates activation and minus (-) indicates repression. Basal levels of 3-oxo-C_{12}-HSLs produced in log-phase culture bind to LasR, and upon reaching stationary-phase, Vfr (a cyclic AMP receptor protein) activates further *lasR* expression. Activation of *lasI* and *lasR* by LasR-3-oxo-C_{12}-HSL and Vfr, respectively, increases the concentration of LasR-3-oxo-C_{12}-HSL complexes to a threshold level that activates numerous genes indicated by (+) and also activates *rhlR*. Fine tuning control occurs via 3-oxo-C_{12}-HSL which inhibits binding of C_4-HSL to RhlR indicated by (-), until RhlR levels or C_4-HSL concentrations increase significantly enough to alleviate this inhibition; this allows controlled timing of virulence gene expression of the Rhl system and a mechanism of control for this autoinduction cycle. A similar control of the Las system occurs via the negative regulator RsaL. The potential role of the recently discovered *Pseudomonas* quinolone signal (PQS)for activation of *lasB* is also indicated. Abbreviations: *xcpP* and *xcpR*, genes that encode proteins of the general secretory pathway; *rpoS*, stationary phase sigma factor; other abbreviations are explained in text. Diagram modified from Pesci et al. (97).



LasI protein that allows for HSL-independent constitutive activation of LasI-dependent genes. Extracted PQS was found to activate one of the virulence factors of *P. aeruginosa*, *lasB* (elastase), as indicated in figure 1.2. However, the activation was weak at the concentration of PQS (6 μM) estimated to be present in stationary-phase cultures of *P. aeruginosa*. The concentration of PQS required to give half maximal activation of *lasB* was over 100-fold greater than individual 3-oxo-C_{12}-HSL and C_4-HSL concentrations required to give half maximal activity in their respective assays (~100 μM of natural PQS versus 1 μM of HSLs). Thus, this compound is more potent than DKPs but is still much less potent than HSL autoinducers. Pesci et al. (98) justify the importance of PQS in intercellular communication by suggesting that although the concentration required to activate the *P. aeruginosa* virulence gene *lasB* was much higher than HSLs, the estimated concentrations of PQS in stationary-phase of 6 μM was an underestimation due to incomplete extraction of PQS in clarified culture supernatant. However, this may not be the case, since PQS is even more hydrophobic than C_4-HSL and should be efficiently extracted by the same organic solvent (ethyl acetate)(90). Hence, it remains to be seen how important PQS is for virulence gene activation in *P. aeruginosa* and whether the concentrations needed for this activation will be achievable in stationary-phase culture. The two HSLs of *P. aeruginosa* (C_4-HSL and 3-oxo-C_{12}-HSL) are much more potent than DKPs or PQS in activation of virulence genes and their concentrations in stationary-phase culture exceed the concentrations required for maximal activation of virulence genes by several fold (93, 94, 98).

P. aeruginosa virulence factors are every toxic and are normally only expressed under conditions of high cell density (96, 97, 140). *P. aeruginosa* high cell densities can be demonstrated in CF lungs where 10^7-10^8 colony forming units per ml of sputum are commonly found (124). In this setting, many virulence genes (e.g. *toxA*, *lasA*, and *lasB*) are expressed, presumably in a cell-density dependent manner (figure 3.4) (125). In the burnt mouse model, burn wound sepsis became irreversible after reaching a certain *P. aeruginosa* cell density ($\geq 10^5$/g) (118). Fortunately, in healthy individuals *P. aeruginosa* acts as an opportunistic pathogen not because its virulence factors are not potent enough to infect a healthy host but because these extremely toxic factors (140) are tightly controlled when cells are present at low densities. This tight regulation is required since expression of some of these

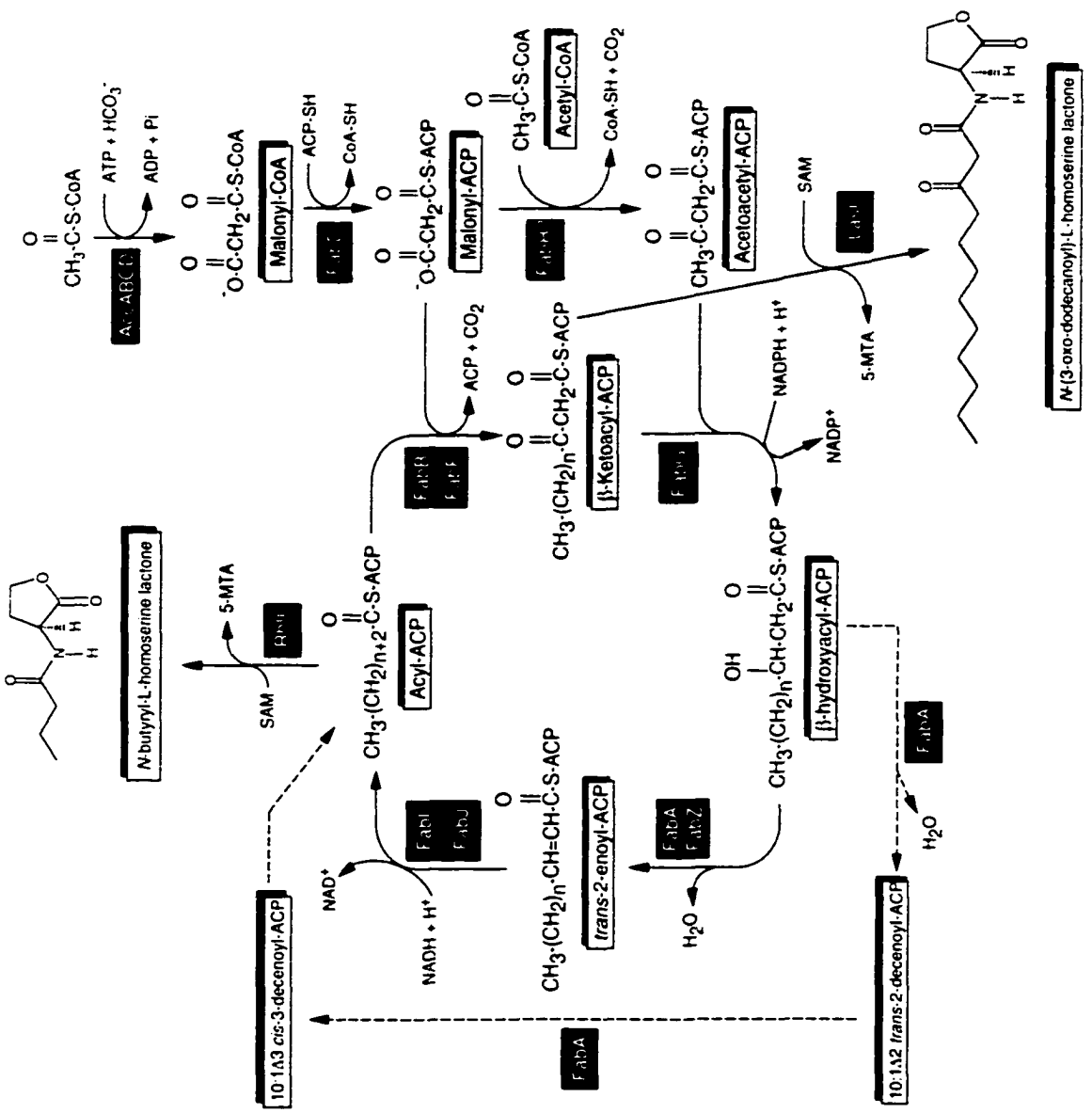
virulence factors at low cell density could stimulate the host immune response and thus would allow detection and development of an effective host immune response for clearance of the bacteria. Conversely, the expression of virulence genes at high cell density allows a synchronized overwhelming assault on the host and therefore allows circumvention of the host defense response.

1.7 BIOSYNTHESIS OF FATTY ACIDS AND ACYLATED HOMOSERINE LACTONES IN *Pseudomonas aeruginosa*

Unlike type I fatty acid biosynthesis (Fab) in eukaryotes where acyl chain extension and modification occurs on a large multifunctional protein, bacteria in general contain type II dissociated fatty acid synthase systems in which several individual proteins catalyze acyl chain elongation and modification (20). However, *Mycobacterium tuberculosis* is one known exception, containing both type I and type II systems (31, 64). The Fab proteins in bacteria (type II) are believed to be dissociated and no evidence exists as to whether formation of a multienyme complex occurs within cells. However, such multienzyme complexes may exists inside bacterial cells and may be disrupted upon cell lysis. Central to all steps in Fab is the acyl carrier protein (ACP) which carries various acyl chain lengths with different modifications and delivers the acyl chains to various Fab proteins (105). Thus, if Fab multienzyme complexes do not exist, these proteins must either have very high activity or be present in large numbers inside the cell to allow recognition of the various mixtures of fatty acids carried on ACP. Regardless, a high output of the pathway is essential considering the fast growth rates of bacteria and the requirements of the acyl-intermediates for synthesis of numerous cellular compounds, including lipid A, phospholipids, lipoproteins, protein-bound biotin and lipoic, acid and acylated HSLs (20). The Fab pathway is well established in *E. coli* and offers a starting point for its study in other bacteria. Using the *E. coli* pathway as a starting point, we were able to clone various *fab* genes and show functions for various Fab enzymes in *P. aeruginosa* (chapters 5 and 6) (51, 52, 70).

We have been able to quickly establish the *P. aeruginosa* Fab pathway (figure 1.3). The first committed step in Fab involves the synthesis of malonyl-CoA from acetyl-CoA,

Figure 1.3. Saturated and unsaturated fatty acid and acylated homoserine lactone biosyntheses in *P. aeruginosa*. Solid arrows indicate catalyzed reactions involved in the elongation and modification cycles of fatty acid synthesis and the shunting of acyl-ACP intermediates into the synthesis of *N*-butyryl-L-homoserine lactone and *N*-(3-oxo-dodecanoyl)-L-homoserine lactone. Dashed arrows indicate committed steps leading to the synthesis of unsaturated fatty acids. The function of FabH is still uncertain in the *P. aeruginosa* Fab pathway as described in the text. FabJ reflects a possible difference in the *P. aeruginosa* pathway with respect to the *E. coli* pathway as described in text. Abbreviations: ACP, acyl carrier protein; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CoA, coenzyme A; FabA (and FabZ), β-hydroxyacyl-ACP dehydratase; FabB, β-ketoacyl synthase I; FabD; malonyl-CoA:ACP transacylase; FabF, β-ketoacyl synthase II; FabG, β-ketoacyl-ACP reductase; FabH, β-ketoacyl synthase III; FabI; enoyl-ACP reductase; 5-MTA, methylthioadenosine; NAD^+ and NADH, oxidize and reduce form of nicotinamide adenine dinucleotide; $NADP^+$ and NADPH, oxidized and reduced form of nicotinamide adenine dinucleotide phosphate; SAM, *S*-adenosylmethionine; LasI, *N*-(3-oxo)-dodecanoyl homoserine lactone synthase; RhlI, *N*-butyryl homoserine lactone synthase.



requiring one molecule of ATP. This reaction is catalyzed by acetyl-CoA carboxylase (AccABCD), which is a multiprotein complex consisting of the α– and β–subunit of carboxyltransferase (AccA and AccD), the biotin carboxyl carrier protein (AccB), and biotin carboxylase (AccC) (8, 20). The activated intermediate is then transferred to ACP by malonyl-CoA:ACP transacylase (FabD). The initiation of Fab occurs by condensation of malonyl-ACP with acetyl-CoA, which in *E. coli* is catalyzed by β-ketoacyl-ACP synthase III. Unlike *E. coli*, the *P. aeruginosa* FabH homologue is not found in the fatty acid gene cluster with *acpP* (encoding ACP) to date and the *P. aeruginosa* FabH homologue has not yet been positively identified. Only one FabG (β-ketoacyl-ACP reductase) exists in *P. aeruginosa* as in *E. coli*, as demonstrated by the inability to isolate a *P. aeruginosa fabG* knockout mutant. This enzyme catalyzes the reduction of 3-oxo-fatty acids utilizing NADPH as the reductant (see chapter 8). However, another FabG homolog of *P. aeruginosa* termed RhlG has been identified and it is involved in rhamnolipid synthesis (13). FabA and FabZ are β-hydroxyacyl-ACP dehydratases catalyzing the conversion of β-hydroxyacyl-ACP to *trans*-2-enoyl-ACP for different chain lengths of fatty acids. However, FabZ, as in *E. coli* (47), seems to have specificity for shorter chain length fatty acids (C_4 to C_8), while FabA has a higher specificity for longer chain length fatty acids (C_{10} to C_{14}) (see chapter 8). The importance of FabZ in short chain fatty acid biosynthesis may lie in that short chain fatty acids are essential in lipid A synthesis, and hence the *fabZ* genes in *E. coli* and *P. aeruginosa* are located in the same cluster as LPS biosynthetic genes (103). The last enzyme in the fatty acid cycle, an enoyl-ACP reductase (FabI), reduces the double bond of *trans*-2-enoyl-ACP to a fully saturated acyl-ACP and utilizes NADH. Genetic evidence indicates that there may yet exist another enoyl-ACP reductase, termed FabJ, in *P. aeruginosa* (51) (see chapter 6). This is the first evidence in any bacterium for separate enzymes catalyzing the enoyl-ACP reductase reaction. Subsequent condensation steps in fatty acid elongation utilize FabB and FabF, β-ketoacyl-ACP synthase I and II, respectively. Each round of the Fab cycle adds two carbons to the growing acyl chain. Thus, to synthesize butyryl-ACP and 3-oxo-dodecanoyl-ACP for HSL biosyntheses, one round and five rounds of the Fab cycle are required, respectively.

Unsaturated fatty acid biosynthesis in *E. coli* is essential, since phospholipids contain C_{16} and C_{18} monounsaturated fatty acids (*cis*-9-hexadecenoic acid and *cis*-11-octadecenoic

acid) attached to the second carbon of glycerol and varying amount of *cis*-11-octadecenoic acid (*cis*-vaccenic) acid attached to the first position of glycerol, depending on growth temperature (20, 69). Unsaturated fatty acid biosynthesis in *E. coli* is initiated at the level of β-hydroxy-decanoyl-ACP (figure 1.3). It is dehydrated to *trans*-2-decenoyl-ACP and isomerized to *cis*-3-decenoyl-ACP. This product then bypasses the reduction step catalyzed by FabI to conserve the double bond, and is further elongated to *cis*-9-hexadecenoic acid and *cis*-11-octadecenoic acid by the action of FabB (not FabF) and the rest of the Fab enzymes. Thus, *E. coli* mutants of *fabA* or *fabB* are viable, as long as unsaturated fatty acids such as oleic acid (*cis*-9-octadecenoic acid) are supplied in the growth medium. Hoang and Schweizer (52) have demonstrated that *P. aeruginosa fabA* and *fabB* mutants survive in media supplemented with oleic acid. This demonstrated that FabA and FabB in *P. aeruginosa* are not necessary for saturated fatty acid synthesis but rather are more important for unsaturated fatty acid biosynthesis. Our ability to isolate a *P. aeruginosa fabF* mutant indicated that this enzyme is not required for unsaturated fatty acid biosynthesis and that FabB can replace the missing FabF synthase function (69). As with *E. coli*, *P. aeruginosa fabF* mutants contained reduced levels of *cis*-11-octadecenoic acid and grew at 42°C but not at 28°C, a temperature at which membrane fluidity is essential (23, 24, 69).

Because of the essential nature of the Fab pathway, it offers several attractive targets for various antimicrobial agents. Some natural fungal derived antimicrobial agents (cerulenin and thiolactomycin) have been described that inhibit the Fab pathway, i.e. the reactions catalyzed by β-ketoacyl-ACP synthases (KAS; figure 1.4A) (45, 46, 80, 88, 132). Cerulenin irreversibly inhibits FabB and FabF (KAS I and II), but this antimicrobial agent is of no therapeutic value since it also inhibits eukaryotic fatty acid synthase systems. However, thiolactomycin, first isolated by a Japanese group in the early 1980s from a *Nocardia* species, has activity towards type I Fab systems but does not inhibit eukaryotic type II Fab systems (45, 80, 88). Subsequently, thiolactomycin was found to inhibit the Fab system of *E. coli* and *P. aeruginosa* (46) and overexpression of KAS I (*fabB*) of *E. coli* imparted resistance to thiolactomycin (129). Thiolactomycin has been shown both *in vitro* and *in vivo* to inhibit all three KAS enzymes of *E. coli* but overexpression of *fabH* (encoding KAS III) did not confer resistance. Since, FabF (KAS II) is not essential FabB (KASI) therefore seems to be the major

target for thiolactomycin (20). FabA can be irreversibly inhibited *in vitro* by the acetylenic substrate analog 3-decenoyl-*N*-acetylcysteamine but *in vivo* saturated fatty acid synthesis continues normally in the presence of this compound (29, 50), presumably since FabZ can replace FabA in saturated fatty acid synthesis. Inhibitors of FabI have also been described, namely diazaborine and triclosan; but these synthetic compounds are quite toxic to eukaryotic cells (figure 1.4) (78, 130). The crystal structure of the *E. coli* FabI along with the inhibitor triclosan has recently been elucidated (48, 72). Although the Fab pathway offers several potential targets for Fab inhibitors, more research is needed to find compounds that not only inhibit the bacterial Fab cycle but are also relatively non-toxic to eukaryotic systems.

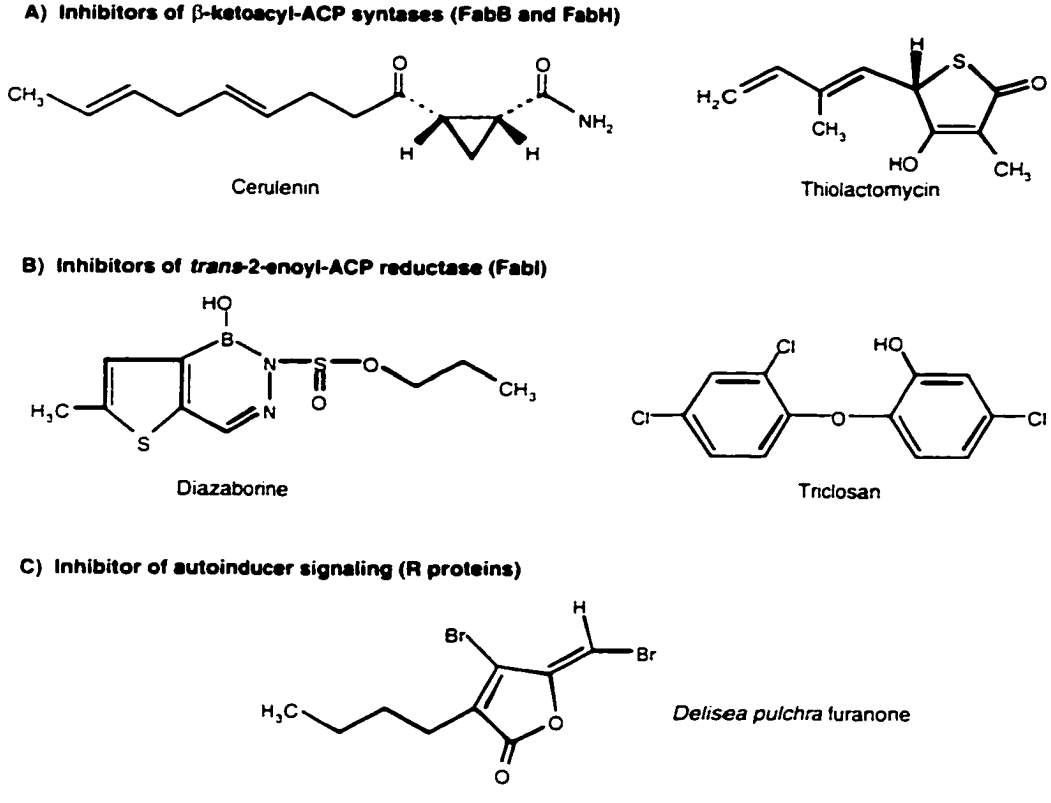
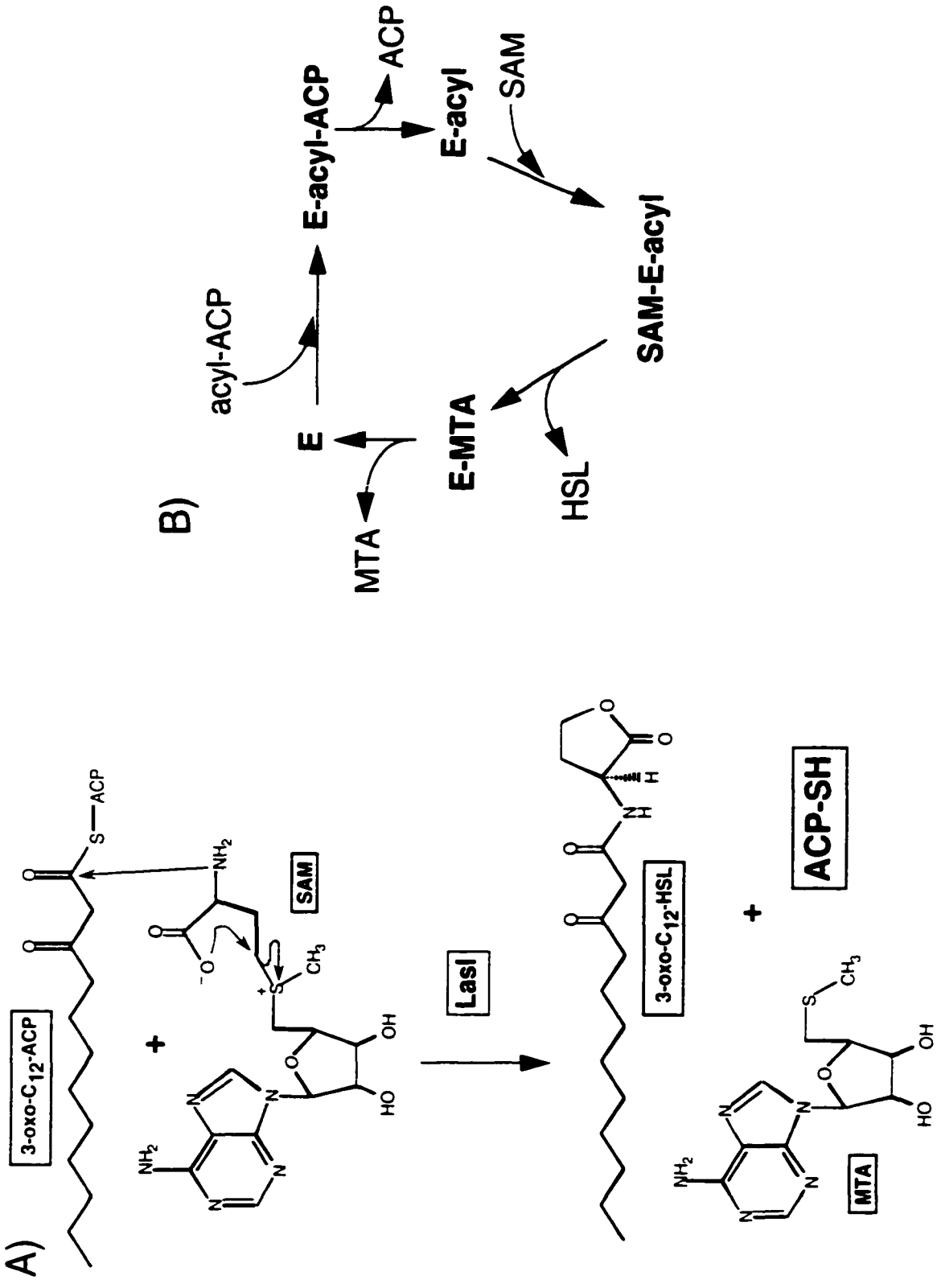


Figure 1.4. Inhibitors of the fatty acid biosynthetic pathway and autoinducer signaling.

At various stages the butyryl-ACP and 3-oxo-dodecanoyl-ACP acyl intermediates shunt off the Fab cycle and react individually with *S*-adenosylmethionine (SAM) in reactions catalyzed by RhlI and LasI to produce C_4-HSL and 3-oxo-C_{12}-HSL, respectively (figure 1.3 and 1.5A) (see chapters 6 and 8). Jiang et al. (61) presented data suggesting that the acyl chain of butyryl-HSL in RhlI reactions may be derived from butyryl-CoA (obtained during fatty acid degradation) rather than butyryl-ACP, and indeed RhlI could synthesize C_4-HSL from butyryl-CoA and SAM. However, these studies were done using high substrate concentrations (0.5 mM) and large quantities (8 mg) of relatively unpure RhlI protein per reaction. Thus, contaminating proteins possibly contributed significantly to the reactions and observed products. *Agrobacterium tumefaciens* HSL [*N*-(3-oxo-octanoyl)-L-HSL] was the first HSL to be entirely synthesized *in vitro* utilizing purified TraI (3-oxo-C_8-HSL synthase), SAM and an *E. coli* clarified cell lysate (82). These authors proposed a ping-pong mechanism for the synthesis of HSL catalyzed by TraI (figure 1.5B), where the acyl chain of acyl-ACP is first transferred to TraI via covalent bond formation to a cysteine residue and then ACP leaves before SAM binding. However, site-directed mutagenesis studies with autoinducer synthases (RhlI and LuxI) indicated that cysteine residues on these proteins are not required for activity (44, 89). Recent results by Parsek et al. (90) indicated that the mechanism of HSL synthesis rather occurs via a bi ter mechanism, where two substrates (acyl-ACP and SAM) bind to into the enzyme before the three products leave (ACP, Acyl-HSL and MTA). However, there are conflicting results within this publication that suggest the mechanism may in fact could also be a ping-pong mechanism. Hence, the scheme presented in figure 1.5B reflects my current hypothesis. In this model, the ACP leaves the enzyme-acyl complex before SAM enters, although cysteine may not be involved in forming a thioester with the acyl chain. Unpublished data from our laboratory, where ACP release is measured in a RhlI reaction (figure 1.5A) using Ellman's reagent seems to support this hypothesis. These unpublished data also showed that RhlI may be able to cleave acyl-ACP in the absence of SAM, indicating yet another potential function for RhlI. However, more research is needed to confirm this observation. Hence, the elusive mechanisms by which autoinducer synthases function await structural studies.

Figure 1.5. Proposed mechanism of acylated homoserine lactone biosynthesis. Autoinducer synthases catalyze the cyclization of the lactone ring from *S*-adenosylmethionine (SAM) and transferring of acyl groups to produce acylated-HSLs and 5'-methylthioadenosine (MTA). (A) Steps involved in synthesis of 3-oxo-C_{12}-HSL from 3-oxo-C_{12}-ACP and SAM by 3-oxo-C_{12}-HSL synthase (LasI). (B) Proposed ping-pong mechanism for autoinducer synthases where acyl-ACP binds to the synthase. ACP leaves the acyl-enzyme (E-acyl) intermediate before SAM binding to form a SAM-E-acyl complex. Subsequent lactonization and transacylation will form and release HSL as indicated in (A), and MTA is the last product to leave the enzyme. Abbreviations: ACP, acyl carrier protein; E, enzyme; HSL, homoserine lactone; LasI, 3-oxo-C_{12}-HSL synthase; MTA, methylthioadenosine; SAM, *S*-adenosylmethionine.



1.8 GOALS OF RESEARCH AND PREVIEWS OF CHAPTERS

At the onset of the studies presented in this dissertation, very little was known about the biosynthesis of fatty acids in *P. aeruginosa*. Only the *fabAB* operon of *P. aeruginosa* was cloned and characterized, and its role in unsaturated Fab was demonstrated (52). In addition, the committed step in Fab involving AccABCD in *P. aeruginosa* was partially characterized (8). Since the origins of the acyl-chains of HSLs were still disputed, I hypothesized that the acyl chains of HSLs are derived from Fab. The following specific aims were pursued to test this hypothesis:

I) To purify the histidine-tagged fatty acid biosynthetic (Fab) enzymes and the two homoserine lactone (HSL) synthases of *P. aeruginosa* (chapters 4, 5, and 8).

II) To assemble the Fab pathway *in vitro* and couple the Fab enzyme(s) to the HSL synthases leading to the production of *N*-butyryl-L-homoserine lactone and *N*-(3-oxo)-dodecanoyl-L-homoserine lactone (chapter 8); thus, defining the *P. aeruginosa* Fab pathway and HSL biosynthetic pathways.

III) To develop a promoter-reporter gene fusion technology for studying autoinducer regulated genes in *P. aeruginosa* (chapter 3).

In the process of achieving these specific aims, several technologies were developed including:

VI) Development of a gene-replacement system for isolation of unmarked *fab* mutations and HSL synthase mutants (chapter 2).

V) Engineering of low-copy number expression vectors and host strains for expression and purification of insoluble proteins (chapter 4).

IV) Development of enzymatic systems for synthesis of defined acyl-ACP substrates representing various stages of the Fab cycle for studies of certain Fab enzymes (Chapter 7).

The research presented in this dissertation for the first time biochemically defines the Fab and HSL pathways of *P. aeruginosa*. This research also will facilitate future screens for novel inhibitors of Fab enzymes and HSL synthases, aimed at inhibiting growth and virulence gene expression of *P. aeruginosa*.

1.9 REFERENCES

1. **Andes, D. R., and W. A. Craig.** 1998. Pharmacodynamics of Fluoroquinolones in Experimental Models of Endocarditis. Clin. Infect. Dis. **27:**47-50.

2. **Baldwin, G., and M. Colbourne.** 1999. Puncture Wounds. Pediactrics in Review. **20:**21-22.

3. **Baltch, A. L.** 1994. *Pseudomonas aeruginosa* Bacteremia, p. 73-127. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

4. **Baltch, A. L., and P. E. Griffin.** 1977. *Pseudomonas aeruginosa* bacteremia: clinical study of 75 patients. Am. J. Med. Sci. **274:**119-129.

5. **Baltch, A. L., M. C. Hammer, R. P. Smith, T. G. Obrig, J. V. Conroy, M. B. Bishop, M. A. Egy, and F. Lutz.** 1985. Effect of *Pseudomonas aeruginosa* cytotoxin on human serum and granulocytes and their microbicidal, phagocytic and chemotactic functions. Infect. Immun. **48:**498-506.

6. **Beck-Sague, C. M., S. N. Banerjee, and W. R. Jarvis.** 1994. Epidemiology and Control of *Pseudomonas aeruginosa* in U.S. Hospitals, p. 51-71. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

7. **Berk, R. S., D. Brown, I. Coutbiho, and D. Myers.** 1987. In vivo studies with two phospholipase C fractions from *Pseudomonas aeruginosa*. Infect. Immun. **55:**1728-1730.

8. **Best, E., and V. C. Knauf.** 1993. Organization and nucleotide sequence of the genes encoding the biotin carboxyl carrier protein and biotin carboxylase protein of *Pseudomonas aeruginosa* acetyl coenzyme A carboxylase. J. Bacteriol. **175:**6881-6889.

9. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, and B. M. Ying Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453-1462.

10. **Bowton, D. L.** 1994. Nosocomial Pseumonia in the ICU-Year 2000 and Beyond. Chest **115:**28S-33S.

11. **Brint, J. M., and D. E. Ohman.** 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. J. Bacteriol. **177:**7155-7163.

12. **Burrows, W.** 1973. Textbook of Microbiology. W.B. Saunders Company, Philadelphia, PA.

13. **Campos-Garcia, J., A. D. Caro, R. Najera, R. M. Miller-Maier, R. A. Al-Tahhan, and G. Soberon-Chavez.** 1998. The *Pseudomonas aeruginosa rhlG gene* encodes an NADPH-dependent β-ketoacyl reductase which is specifically involved in rhamnolipid synthesis. J. Bacteriol. **180:**4442-4451.

14. **Clarke, P. H., and L. N. Ornston.** 1975. Metabolic pathways and regulation - Part I and II. p. 191-341. *In* P. H. Clarke and M. H. Richmond (ed.), Genetics and Biochemistry of *Pseudomonas*, John Wiley & Sons Ltd., NY.

15. **Pathogenesis Corporation.** 1999. Pers. Commun.

16. **Costerton, J. W., and H. Anwar.** 1994. *Pseudomonas aeruginosa*: the microbe and pathogen, p. 1-20. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

17. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318-1322.

18. **Craig, W. A., and D. Andes.** 1997. Aminoglycosides Are Useful for Severe Respiratory Tract Infections. Seminars in Respiratory Infections. **12:**271-277.

19. **Craig, W. A., and S. C. Ebert.** 1994. Antimicrobial Therapy in *Pseudomonas aeruginosa* Infections, p. 441-516. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

20. **Cronan, J. E., and C. O. Rock.** 1996. Biosynthesis of membrane lipids, p. 612-636. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. Brooks Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*. Am. Soc. Microbiol., Washington, DC.

21. **Cruickshank, R., J. P. Duguid, B. P. Marmion, and R. H. A. Awain.** 1975. Medical Microbiology, 12 ed, vol. 2. Churchill Livingstone, NY.

22. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295-298.

23. **DeMendoza, D., and J. E. Cronan.** 1983. Thermal regulation of membrane lipid fluidity in bacteria. TIBS(February):49-52.

24. **DeMendoza, D., A. L. Klages-Ulrich, and J. E. Cronan.** 1983. Thermal regulation of membrane fluidity in *Escherichia coli*. J. Biol. Chem. **258:**2098-2101.

25. **Diver, J. M., T. Schollaardt, H. R. Rabin, C. Thorson, and L. E. Bryan.** 1991. Persistence mechanism in *Pseudomonas aeruginosa* from cystic fibrosis patients undergoing ciprofloxacin therapy. Antimicrob. Agents Chemother. **35:**1538-1546.

26. **Doggett, R. G.** 1979. *Pseudomonas aeruginosa*: Clinical Manifestations of infection and Current Therapy. Academic Press Inc., NY.

27. **Doring, G.** 1997. Cystic fibrosis respiratory infections: interactions between bacteria and host defence. Monaldi Arch. Chest Dis. **52:**363-366.

28. **Edson, R. S., and C. L. Terrell.** 1999. The Aminoglycosides. Mayo Clin. Proc. **74:**519-528.

29. **Endo, K., G. M. J. Helmkamp, and K. Block.** 1970. Mode of inhibition of β-hydroxydecanoyl thioester dehydrase. Specificity of substrates and acetylenic inhibitors. J. Biol. Chem. **245:**4293-4296.

30. **Evans, K., L. Passador, R. Srikumar, E. Tsang, J. Nezezon, and K. Poole.** 1998. Influence of the MexAB-OprM efflux system on quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. **180**:5443-5447.

31. **Fernandes, N. D., and P. E. Kolattukudy.** 1996. Cloning, squencing and characterization of a fatty acid synthase-encoding gene from *Mycobacterium tuberculosis* var. bovis BCG. Gene **170:**95-99.

32. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J.-F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudeck, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. Science **269:**496-512.

33. **Fuqua, C., and E. P. Greenberg.** 1998. Cell-to-cell communication in *Escherichia coli* and *Salmonella typhimurium*: they may be talking but who is listening? Proc. Natl. Acad. Sci. USA **95:**6571-6572.

34. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. **50:**727-751.

35. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. **176:**269-275.

36. **Gambello, M. J., and B. H. Iglewski.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J. Bacteriol. **173:**3000-3009.

37. **Gerber, A. U., C. Feller, and H. P. Brugger.** 1984. Time course of the pharmacological response to beta-lactam antibiotics in vitro and in vivo. Eur. J. Clin. Microbiol. **3:**592-597.

38. **Givskov, M., R. De Nys, M. Manefield, L. Gram, R. Maximilien, L. Eberl, S. Molin, P. D. Steinberg, and S. Kjelleberg.** 1996. Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. J. Bacteriol. **178:**6618-6622.

39. **Godfrey, A. J., L. E. Bryan, and H. R. Rabin.** 1981. Beta-lactam-resitant *Pseudomonas aeruginosa* with modified penicillin-binding proteins emerging during cystic fibrosis treatment. Antimicrob. Agents Cheomother. **19:**705-711.

40. **Gould, K., C. H. Ramirez-Ronda, R. K. Holmes, and J. P. Sandford.** 1975. Adherence of bacteria to heart valves in vitro. J. Clin. Invest. **56:**1364-1370.

41. **Grandis, J. R., and V. L. Yu.** 1994. *Pseudomonas aeruginosa* Infections of the Ear, p. 401-419. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker. NY.

42. **Grossman.** 1997. The role of fluoroquinolones in respiratory tract infections. J. Antimicrobial Chemotherapy. **40**(Suppl. A): S59-S62.

43. **Hancock, R. E.** 1998. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative Gram-negative bacteria. Clin. Infect. Dis. **27**(Suppl. 1):S93-S99.

44. **Hanzelka, B. L., A. M. Stevens, M. R. Parsek, T. J. Crone, and E. P. Greenberg.** 1997. Mutational analysis of the *Vibrio fischeri* LuxI polypeptide: critical regions of an autoinducer synthase. J. Bacteriol. **179:**4882-4887.

45. **Hayashi, T., O. Yamamoto, H. Sasaki, A. Kawaguchi, and A. Okazaki.** 1983. Mechanism of action of the antibiotic thiolactomycin inhibition of fatty acid synthesis of *Escherichia coli*. Biochem. Biophys. Res. Comm. **115:**1108-113.

46. **Hayashi, T., O. Yamamoto, H. Sasaki, and H. Okazaki.** 1984. Inhibition of fatty acid synthesis by the antibiotic thiolactomycin. J. Antibiot. **37:**1456-1461.

47. **Heath, R. J., and C. O. Rock.** 1996. Roles of the FabA and FabZ β-hydroxyacyl-acyl carrier protein dehydratase in *Escherichia coli* fatty acid biosynthesis. J. Biol. Chem. **271:**27795-27801.

48. **Heath, R. J., J. R. Rubin, D. R. Holland, E. Zhang, M. E. Snow, and C. O. Rock.** 1999. Mechanism of triclosan inhibition of bacterial fatty acid synthesis. J. Biol. Chem. **274:**11110-11114.

49. **Hellinger, W. C., and N. S. Brewster.** 1999. Carbapenems and Monobactams: Imipenem, Meropenem, and Aztreonam. Mayo Clin. Proc. **74:**420-434.

50. **Helmkamp, G. M. J., D. J. Brock, and K. Bloch.** 1968. β-hydroxydecanoyl-ACP thioester dehydrase. Specificity of substrates and acetylenic inhibitors. J. Biol. Chem. **243:**3229-3231.

51. **Hoang, T. T., and H. P. Schweizer.** 1999. Characterization of the *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase (FabI): a target for triclosan and its role in acylated homoserine lactone synthesis. J. Bacteriol. **181:**5489-5497.

52. **Hoang, T. T., and H. P. Schweizer.** 1997. Fatty acid biosynthesis in *Pseudomonas aeruginosa*: Cloning and characterization of the *fabAB* operon encoding β-hydroxyacyl-acyl

carrier protein dehydratase (*fabA*) and β-ketoacyl-acyl carrier protein synthase I (*fabB*). J. Bacteriol. **179:**5326-5332.

53. **Holden, M. T. G., S. R. Chhabra, R. Nys, P. Stead, N. J. Bainton, P. J. Hill. M. Manefield, N. Kumar, M. Labatte, D. England, S. Rice, M. Givskov, G. P. C. Salmond, G. S. A. B. Stewart, B. W. Bycroft, S. Kjelleberg, and P. Williams.** 1999. Quorum-sensing cross talk: isolation and chemical characterization of cylcic dipeptides from *Pseudomonas aeruginosa* and other Gram-negative bacteria. Mol. Microbiol. **33**(6)**:**1254-1266.

54. **Holder, I. A., and C. G. Haidaris.** 1979. Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: extra-cellular protease and elastase as *in vivo* virulence factors. Can. J. Microbiol. **45:**593-599.

55. **Holder, I. A., and A. N. Neely.** 1989. Combined host and specific anti-pseudomonas directed therapy for *Pseudomonas aeruginosa* infections in burn mice. J. Burn Care Rehab. **10**:131-137.

56. **Holloway, B. W., U. Roemling, and B. Tuemmler.** 1994. Genomic mapping of *Pseudomonas aeruginosa* PAO. Microbiol. **140:**2907-2929.

57. **Homma, J. Y., H. Tanimoto, I. A. Holder, N. Hoiby, and G. Doering.** 1991. *Pseudomonas aeruginosa* in human diseases. Antibiot. Chemother. **44:**48-53.

58. **Hooper, D. C.** 1998. Clinical application of quinolones. Biochim. Biophys. Acta **1400:**45-61.

59. **Iglewski, B. H., and D. Kabat.** 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc. Natl. Acad. Sci. USA. **72:**2284-2288.

60. **Jackson, G. G.** 1994. Infective Endocarditis Caused by *Pseudomonas aeruginosa*. p. 129-157. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

61. **Jiang, Y., M. Camara, S. R. Chhabra, K. R. Hardie, B. W. Bycroft, A. Lazdunski, G. P. C. Salmond, G. S. A. B. Stewart, and P. Williams.** 1998. In vitro biosynthesis of the *Pseudomonas aeruginosa* quorum-sensing signal molecule *N*-butanoyl-L-homoserine lactone. Mol. Microbiol. **28:**193-203.

62. **Kaplan, H. B., and L. Plamann.** 1996. A *Myxococcus xanthus* cell density-sensing system required for multicellular development. FEMS Microbiol. Lett. **139:**89-95.

63. **Kenna, M. A., C. D. Bluestone, J. S. Reilly, and R. P. Lusk.** 1986. Medical management of chronic suppurative otitis media without cholesteatoma in children. Laryngoscope **96:**146-151.

64. **Khisimuzi, M., R. A. Slayden, Y. Zhu, S. Ramaswamy, X. Pan, D. Mead, D. D. Crane, J. M. Musser, and C. E. Barry III.** 1998. Inhibition of a *Mycobacterium tuberculosis* β-ketoacyl ACP synthase by isoniazid. Science **280:**1607-1610.

65. **Kleerebezem, M., L. E. N. Quadri, O. P. Kuipers, and W. M. de Vos.** 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol. Microbiol. **24:**895-904.

66. **Ko, Y. H., and P. L. Pedersen.** 1997. Frontier in Research on Cystic Fibrosis: Understanding Its Molecular and Chemical Basis and Relationship to the Pathogenesis of the Disease. J. Bioen. and Biomem. **29:**417-427.

67. **Krieg, D. P., J. A. Bass, and S. J. Mattingly.** 1988. Phosphorylcholine stimulates capsule formation of phosphate-limited mucoid *Pseudomoas aeruginosa*. Infect. Immun. **56:**864-873.

68. **Kunin, C. M.** 1994. Infections of the Urinary Tract Due to *Pseudomonas aeruginosa*, p. 237-255. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

69. **Kutchma, A. J.** 1998. M.S. Thesis. Colorado State University, Fort Collins.

70. **Kutchma, A. J., T. T. Hoang, and H. P. Schweizer.** 1999. Characterization of a *Pseudomonas aeruginosa* fatty acid biosynthetic gene cluster: purification of acyl carrier protein (ACP) and malonyl-coenzyme A:ACP transacylase (FabD). J. Bacteriol. **181:**5498-5504.

71. **Lepow, M. L.** 1994. *Pseudomonas aeruginosa* Colonization and Infection of the Gastrointestinal Tract, p. 421-491. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

72. **Levy, C. W., A. Roujeinikova, S. Sedelnikova, P. J. Baker, A. R. Stuitje, A. R. Slabas, D. W. Rice, and J. B. Rafferty.** 1999. Molecular basis of triclosan activity. Nature **398:**383-384.

73. **Lewenza, S., B. Conway, E. P. Greenberg, and P. Sokol.** 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. J. Bacteriol. **181:**748-756.

74. **Li, X.-Z., R. Srikumar, and K. Poole.** 1998. β-lactamase inhibitors are substrates for the multidrug efflux pumps of *Pseudmonas aeruginosa*. Antimicrob. Agents. Chemother. **42:**399-403.

75. **Lui, P. V.** 1974. Extracellular toxins of *Pseudomonas aeruginosa*. J. Infect. Dis. **130**(Suppl.):S94-S99.

76. **Mader, J. T., A. Vibhagool, J. Mader, and J. H. Calhoun.** 1994. *Pseudomonas aeruginosa* Bone and Joint Infections, p. 293-325. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

77. **Martin, S. J., J. M. Meyer, S. K. Chuck, R. Jung, C. R. Messick, and S. L. Pendland.** 1998. Levofloxacin and Sparfloxacin: New Quinolone Antibiotics. Ann. of Pharmacother. **32:**320-336.

78. **McMurry, L. M., M. Oethinger, and S. B. Levy.** 1998. Triclosan targets lipid synthesis. Nature **394:**531-532.

79. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1-7.

80. **Miyakawa, S., K. Suzuki, T. Noto, Y. Harada, and H. Okazaki.** 1982. Thiolactomycin, a new antibiotic. IV. Biological properties and chemotherapeutic activity in mice. J. Antibiot. **35:**411-419.

81. **Moore, R. A., D. DeShazer, S. Reckseidler, A. Weissman, and D. E. Woods.** 1999. Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. Antimicrob. Agents Chemother. **43:**465-470.

82. **Moré, M. I., D. Finger, J. L. Stryker, C. Fuqua, A. Eberhard, and S. C. Winans.** 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science **272:**1655-1658.

83. **Mukamolova, G. V., A. S. Kaprelyants, D. I. Young, and M. Young.** 1998. A bacterial cytokine. Proc. Natl. Acad. Sci. USA **95:**8916-8921.

84. **Nakae, T.** 1997. Multiantibiotic resistance caused by active drug extrusion in *Pseudomonas aeruginosa* and other Gram-negative bacteria. Microbiol. SEM **13:**273-284.

85. **Nicas, T., and B. H. Iglewski.** 1985. The contribution of exoproducts to virulence of *Pseudomonas aeruginosa*. Can. J. Microbiol. **31:**387-392.

86. **Nikaido, H.** 1998. Antibiotic resistance by Gram-negative multidrug efflux pumps. Clin. Infect. Dis. **27**(Suppl. 1)**:**S32-S41.

87. **Nikaido, H.** 1996. Multidrug efflux pumps of Gram-negative bacteria. J. Bacteriol. **178:**5853-5859.

88. **Oishi, H., T. Noto, H. Sasaki, K. Suzuki, T. Hayashi, H. Okazaki, K. Ando, and M. Sawada.** 1982. Thiolactomycin, a new antibiotic. I. Taxonomy of the producing organism, fermentation and biological properties. J. Antibiot. **35:**391-395.

89. **Parsek, M. R., A. L. Schaefer, and E. P. Greenberg.** 1997. Analysis of random and site-directed mutations in *rhlI*, a *Pseudomonas aeruginosa* gene encoding an acylhomoserine lactone synthase. Mol. Microbiol. **26:**301-310.

90. **Parsek, M. R., D. L. Val, B. L. Hanzelka, J. E. Cronan, and E. P. Greeneberg.** 1999. Acyl homoserine-lactone quorum-sensing signal generation. Proc. Natl. Acad. Sci. USA. **96:**4360-4365.

91. **Pavlovskis, O. R., and A. H. Shackelford.** 1974. *Pseudomonas aeruginosa* exotoxin in mice: localization and effects on protein synthesis. Infect. Immun. **9:**540-546.

92. **Pavlovskis, O. R., and B. Wretlind.** 1989. Assessment of protease (elastase) as a *Pseudomonas aeruginosa* virulence factor in experimental mouse burn infection. Infect. Immun. **24:**181-187.

93. **Pearson, J., K. Gray, L. Passador, K. Tucker, A. Eberhard, B. Iglewski, and E. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA **91:**197-201.

94. **Pearson, J., L. Passador, B. Iglewski, and E. Greenberg.** 1995. A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:**1490-4.

95. **Pennington, J. E.** 1994. *Pseudomonas aeuginosa* Pneumonia and Other respiratory Tract Infections, p. 159-181. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

96. **Pesci, E. C., and B. H. Iglewski.** 1997. The chain of command in *Pseudomonas* quorum sensing. TIM **5:**132-134.

97. **Pesci, E. C., and B. H. Iglewski.** 1999. Quorum sensing in *Pseudomonas aeruginosa*, p. 147-155. *In* G. M. Dunny and S. C. Winans (ed.), Cell-Cell Signaling in Bacteria. Am. Soc. Microbiol., Washington, DC.

98. **Pesci, E. C., J. B. Milbank, J. P. Pearson, S. McKnight, A. S. Kende, E. P. Greenberg, and B. H. Iglewski.** 1999. Quinolone signaling in cell-to-cell communication system of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **96:**11229-11234.

99. **Pesci, E. C., J. P. Pearson, P. C. Seed, and B. H. Iglewski.** 1997. Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. **179:**3127-3132.

100. **Pier, G. B.** 1998. *Pseudomonas aeruginosa*: a key problem in cystic fibrosis. ASM News **64:**339-347.

101. **Pier, G. B., M. Grout, T. S. Zaidi, G. Meluleni, S. S. Mueschenborn, G. Banting, R. Ratcliff, M. J. Evans, and W. H. Colledge.** 1998. *Salmonella typhi* uses CFTR to enter intestinal epithelial cells. Nature **392:**79-82.

102. **Prandota, J.** 1987. Drug diposition in cystic fibrosis: progress in understanding pathophysiology and pharmacokinetics. Pediatr. Infect. Dis. J. **6:**1111-1126.

103. **Raetz, C. H. R.** 1996. Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. Brooks Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*. Am. Soc. Microbiol., Washington, DC.

104. **Rahme, L. G., E. J. Stevens, S. F. Wolfort, J. Shao, R. G. Tompkins, and F. M. Ausubel.** 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science **268:**1899-1902.

105. **Rock, C. O., and J. E. Cronan.** 1981. Acyl carrier protein from *Escherichia coli*. Methods Enzymol. **71:**341-351.

106. **Roos, K. L., and W. M. Scheld.** 1994. *Pseudomonas aeruginosa* Infections of the Central Nervous System, p. 257-291. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

107. **Rubin, J., L. Yu, and S. E. Stool.** 1988. Malignant external otitis externa. J. Pediatr. **113:**965-970.

108. **Rubin, J., and v. L. Yu.** 1988. Malignant external otitis: insights into pathogenesis, clinical manifestations, diagnosis and therapy. Am. J. Med. **85**:391-398.

109. **Sabath, L. D.** 1984. Biochemical and physiologic basis for susceptibility and resistance of *Pseudomonas aeruginosa* to antimicrobial agents. Rev. Infect. Dis. **6**(Suppl. 3):S643-S656.

110. **Saiman, L.** 1998. Further Insights Into Early Acquisition of *Pseudomonas aeruginosa*. Pediat. Pulmon. **26**:79-80.

111. **Schaberg, D. R., D. H. Culver, and R. P. Gaynes.** 1991. Major trends in the microbial etiology of nosocomial infection. Am. J. Med. **91** (Suppl. 3B): 72S-75S.

112. **Schimpff, S. C., W. H. Greene, V. M. Young, and P. H. Wiernik.** 1973. *Pseudomonas* septicemia: incidence, epidemiology, prevention and therapy in patients with advanced cancer. Eur. J. Cancer **9**:449-455.

113. **Schimpff, S. C., M. Moody, and V. M. Young.** 1970. Relationship of colonization with *Pseudomonas aeruginosa* to development of Pseudomonas bacteremia in cancer patients. Antimicrob. Agents Chemother. **10**:240-244.

114. **Schimpff, S. C., P. H. Wiernik, and J. B. Block.** 1972. Rectal abscesses in cancer patients. Lancet **2**:844-847.

115. **Shapiro, J. A.** 1998. Thinking about bacterial population as multicellular organism. Annu. Rev. Microbiol. **52**:81-104.

116. **Shimizu, K., T. Kumada, W.-C. Hsieh, H.-Y. Chung, Y. Chong, R. S. Hare, G. H. Miller, F. J. Sabatelli, and J. Howard.** 1985. Comparison of aminoglycoside resistance

paterns in Japan, Formosa, and Korea, Chile and the United States. Antimicrob. Agents Chemother. **28:**282-288.

117. **Shortridge, V. D., A. Lazdunski, and M. L. Vasil.** 1992. Osmoprotectants and phosphate regulate expression of phospholipase C in *Pseudomonas aeruginosa*. Mol. Microbiol. **6:**863-871.

118. **Smith, R. P.** 1994. Skin and Soft Tissue Infections Due to *Pseudomonas aeruginosa*, p. 327-369. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

119. **Soliman, A. E.** 1978. A rare case of malignant otitis externa in a non-diabetic patient. J. Laryngol. Otol. **92:**811-812.

120. **Speert, D. P.** 1994. *Pseudomonas aeruginosa* Infections in Patients with Cystic Fibrosis, p. 183-235. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

121. **Speert, D. P., S. W. Farmer, M. E. Campbell, J. M. Musser, R. K. Selander, and S. Kuo.** 1990. Conversion of *Pseudomonas aeruginosa* to the phenotype characteristic of strains from patients with cystic fibrosis. J. Clin. Microbiol. **28:**188-194.

122. **Srikumar, R., T. Kon, N. Gotoh, and K. Poole.** 1998. Expression of *Pseudomonas aeruginosa* multidrug efflux pumps MexA-MexB-OprM and MexC-MexD-OprJ in a multidrug-sensitive *Escherichia coli* strain. Antimicrob. Agents Chemother. **42:**65-71.

123. **Srikumar, R., X.-Z. Li, and K. Poole.** 1997. Inner membrane efflux components are responsible for the β-lactam specificity of multidrug efflux pumps in *Pseudomonas aeruginosa*. J. Bacteriol. **179:**7875-7881.

124. **Storey, D. G., E. E. Ujack, and H. R. Rabin.** 1992. Population transcript accumulation of *Pseudomonas aeruginosa* exotoxin A and elastase in sputa from patients with cystic fibrosis. Infect. Immun. **60:**4687-4694.

125. **Storey, D. G., E. E. Ujack, H. R. Rabin, and I. Mitchell.** 1998. *Pseudomonas aeruginosa lasR* transcription correlates with the transcription of *lasA*, *lasB* and *toxA* in chronic lung infections associated with cystic fibrosis. Infect. Immun. **66:**2521-2528.

126. **Terry, J. M., S. E. Pina, and S. J. Mattingly.** 1991. Environmental conditions which influence mucoid conversion in *Pseudomonas aeruginosa* PAO1. Infect. Immun. **59:**471-477.

127. **Thomassen, M. J., C. A. Demko, B. Boxerbaum, R. C. Stern, and P. J. Kuchenbrod.** 1979. Multiple isolates of *Pseudomonas aeruginosa* with differing antimicrobial susceptibility paterns from patients with cyctic fibrosis. J. Infect. Dis. **140:**873-880.

128. **Tomasz, A.** 1979. From penicillin-binding proteins to the lysis and death of bacteria. Rev. Infect. Dis. **1:**434-467.

129. **Tsay, J. T., C. O. Rock, and S. Jackowski.** 1992. Overproduction of beta-ketoacyl-acyl carrier protein synthase I imparts thiolactomycin resistance to *Escherichia coli* K-12. J. Bacteriol. **174:**508-513.

130. **Turnowsky, F., K. Fuchs, C. Jeschek, and G. Hoegenauer.** 1989. *envM* genes of *Salmonella typhimurium* and *Escherichia coli*. J. Bacteriol. **171:**6555-6565.

131. **Van Delden, C., and B. H. Iglewski.** 1998. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerg. Infect. Dis. **4:**551-560.

132. **Vance, D. E., I. Goldberg, O. Mitsuhashi, K. Bloch, and S. Omura.** 1972. Inhibition of fatty acid synthetases by the antibiotic cerulenin. Biochem. Biophys. Res. Commun. **48:**649-656.

133. **Vasil, M. L.** 1997. Pers. Commun.

134. **Vogelman, B., S. Gudmundsson, J. Turnidge, J. Leggett, and W. A. Craig.** 1998. In vivo postantibiotic effect in a thigh infection in neutropenic mice. J. Infect. Dis. **157:**287-298.

135. **Wilson, R., and R. B. Dowling.** 1998. *Pseudomonas aeruginosa* and other realed species. Thorax **53:**213-219.

136. **Woods, D. E., S. J. Cryz, R. L. Friedman, and B. H. Iglewski.** 1982. Contribution of toxin A and elastase to virulence of *Pseudomonas aeruginosa* in chronic lung infection of rats. Infect. Immun. **36:**1223-1228.

137. **Woods, D. E., W. S. Hwang, M. S. Shahrabadi, and J. U. Que.** 1988. Alteration of pulmonary structure by *Pseudomonas aeruginosa* exoenzyme S. J. Med. Microbiol. **26:**133-141.

138. **Woods, D. E., M. S. Schaffer, H. R. Rabin, G. D. Campbell, and P. A. Sokol.** 1986. Phenotypic comparison of *Pseudomonas aeruginosa* strains isolated from a variety of clinical sites. J. Clin. Microbiol. **24:**260-264.

139. **Woods, D. E., D. C. Straus, J. Johanson, W.G., and J. A. Bass.** 1981. Role of salivary protease activity in adherence of gram-negative bacilli to mammalian buccal epithelial cells *in vivo*. J. Clin. Invest. **68:**1435-1440.

140. **Woods, D. E., and M. L. Vasil.** 1994. Pathogenesis of *Pseudomonas aeruginosa* infections, p. 21-50. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

141. **Wright, A. J.** 1999. The Penicillins. Mayo Clin. Proc. **74:**290-307.

142. **Zloty, P., and M. W. Belin.** 1994. Occular Infections Caused by *Pseudomonas aeruginosa*, p. 371-399. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa*: infections and treatment. Marcel Dekker, NY.

CHAPTER 2

A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants

(Presented in Tung T. Hoang, RoxAnn R. Karkhoff-Schweizer, Alecksandr J. Kutchma and Herbert P. Schweizer. 1998. *Gene* **212**: 77-86)

The technologies described in this paper facilitate manipulations of the bacterial chromosome and are not only useful in *P. aeruginosa*, but also in many other bacteria. I have developed and utilized this technology for the purpose of making unmarked mutations of genes involved in the autoinducer and fatty acid biosynthetic pathways (refer to chapters 3 and 6). I acknowledge the contribution of Dr. RoxAnn Karkhoff-Schweizer for her help with testing of the gene-replacement vectors and Alecks Kutchma for sequencing the *pabC* gene.

2.1 ABSTRACT

An improved method for gene replacement in *Pseudomonas aeruginosa* was developed. The method employs several new gene replacement vectors that incorporate (*i*) the counterselectable *sacB* marker, (*ii*) a *lacZα* allele for blue-white screening, (*iii*) the pUC18/19 vectors multiple cloning site with 10 unique restriction sites, (*iv*) an *oriT* for conjugation-mediated plasmid transfer and (*v*) carbenicillin, gentamicin (Gm) and tetracycline selectable markers. A cassette was constructed that contains a Gm^r selectable marker next to the green fluorescent protein structural gene, with both markers being flanked by Flp recombinase target (*FRT*) sites. The *FRT* cassette was used to insertionally inactivate the cloned *P. aeruginosa pabC* gene encoding aminodeoxychorismate lyase. After conjugal

transfer into *P. aeruginosa*, plasmid integrants were selected and deletion of unwanted DNA sequences was promoted by sucrose counterselection. The *FRT* cassette was excised with high frequencies (close to 100%) from the chromosome after conjugal transfer of a Flp recombinase-expressing plasmid; this *sacB*-containing plasmid was subsequently cured by sucrose counterselection, resulting in an unmarked *P. aeruginosa ΔpabC* strain.

2.2 INTRODUCTION

Analyses of cloned genes and their products from bacteria in the environments of their native chromosomes necessitate methods that enable the genetic exchange of mutated, plasmid-borne DNA with the respective genomes. Our laboratory was the first to develop a gene replacement system for *Pseudomonas aeruginosa* that allowed for positive selection of all steps involved in the gene replacement process (18). This system included the *Bacillus subtilis sacB* gene as counter-selectable marker (18), allowing for positive selection of the segregation of true mutants from the more frequently occuring merodiploids on medium containing 5% sucrose. This selection process greatly facilitated the genetic exchange not only of selectable markers but also screenable markers, e.g., *lacZ*- or *xylE*-based gene fusions (22, 23), and Ts-conferring alleles (unpublished data). The latest improvement included a gene replacement vector, pEX100T, which was genetically engineered to facilitate the cloning and genetic manipulation of virtually any DNA fragment (22). This was achieved by incorporating into a single vector (*i*) the counterselectable *sacB* marker; (*ii*) a *lacZα* allele for blue-white screening on XGal-containing media; (*iii*) an *oriT* for conjugation-mediated plasmid transfer and (*iv*) unique cloning sites for *Sma*I and the rare-cutting meganuclease I-*Sce*I. By site-specific mutagenesis and subcloning, most restriction sites within the vector were eliminated giving greater access to restriction sites within the cloned fragment, thus facilitating the construction of insertions, deletions and frameshifts.

Although in its present form this vector is very versatile and has been used by many laboratories to introduce marked and unmarked mutant alleles into the *P. aeruginosa* chromosome, it suffers from some drawbacks: (*i*) even though the presence of very few restriction enzyme cleavage sites facilitates mutagenesis of cloned fragments, lack of a MCS hampers cloning of previously mutagenized fragments; (*ii*) the current replacement vector

utilizes Ap^r as the selectable marker and β-lactams are not applicable in many other bacteria. Being the sole selectable plasmid marker, it also precludes the use of Ap^r/Cb^r conferring cassettes in insertional mutagenesis experiments (23). One goal of the present studies was to further extend the usefulness of this vector system by addressing these shortcomings.

Besides construction of versatile suicide delivery vectors, efforts aimed at optimizations of existing gene replacement systems must also include development of methods that allow generation of chromosomal insertions of heterologous DNA segments eventually devoid of any selection marker, as is currently required for metabolic engineering of novel phenotypes for environmental applications and development of live vaccine strains (6). Useful methods previously employed in other bacteria to excise unwanted marker sequences from the chomosome involve various site-specific recombination systems, in particular the Cre-*lox* system of the phage/plasmid P1 (1, 24), the ParA-*res* system of RP4 (12), the TnpR-*res* system of the γδ transposon (2) and the *Saccharomyces cerevisiae* Flp-*FRT* system (3). These systems are useful tools for genetic engineering due to the high specificity of the respective recombinases, which enables directed operations *in vivo* on DNA molecules as large as bacterial or eukaryotic chromosomes. Of all of these systems, the Flp-*FRT* recombination system (16) is perhaps the most versatile, since it is the least restrictive in terms of host-range, functioning in bacteria (3), yeasts (16), plants (13) and mammalian cells (8). The Flp enzyme is a site-specific recombinase which promotes recombination at a specific 13-bp site within a 65-nt sequence, termed *FRT* (see figure 2.2B)(5).

In this study, we adapted the *S. cerevisiae* Flp-*FRT* system for use in bacteria other than *E. coli*. This included construction of a new Gm^r-*FRT* cassette and a broad-host-range (bhr) Flp recombinase-expressing vector.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains, plasmids, media and culture conditions. The bacterial strains and plasmids used in this study are listed in table 2.1. LB medium was used as the rich medium for both *E. coli* and *P. aeruginosa*. The minimal medium used for *P. aeruginosa* was VBMM. VBMM is VB medium containing 0.3% citrate and is selective for *P. aeruginosa*

since *E. coli* cannot utilize citrate (11). For growth of *pabC* mutants, VBMM was supplemented with 10 μg PABA per ml. Antibiotics were used in selection media at the following concentrations: for *E. coli*, Ap (100 μg/ml); Gm (10 μg/ml); Tc (10 μg/ml); and for *P. aeruginosa*, Cb (500 μg/ml); Gm (100-200 μg/ml); Tc (50 μg/ml). Lactose phenotypes were screened on LB plates containing 40 μg Xgal per ml. Hemolysin production of *P. aeruginosa* cells was screened on sheep blood agar plates (BBL Microbiology Systems, Cockeysville, MD). Sucrose resistant colonies were obtained by streaking *P. aeruginosa* merodiploids or cells containing plasmids with *sacB* on VBMM or LB or PIA (Difco, Detroit, MI) plates supplemented with 5% sucrose.

2.3.2 DNA manipulations. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase and T4 DNA Pol were purchased from Gibco-BRL (Gaithersburg, MD) and used as recommended by the supplier, except T4 DNA Pol which was used for blunt ending DNA fragments in the respective restriction enzyme incubation buffers in the presence of 100 μM dNTPs (17). *Taq* and *Taq*$^-$ Pol were purchased from Qiagen (Santa Clarita, CA) and Stratagene (La Jolla, CA), respectively, and were used as recommended by the suppliers. Small scale isolations of plasmid DNA from *E. coli* and *P. aeruginosa* were achieved utilizing the QIAprep spin miniprep kit (Qiagen, Santa Clarita, CA), and DNA transformations were done as previously described (11). DNA restriction fragments were eluted from agarose gels by utilizing the Geneclean procedure (BIO 101, San Diego, CA). For genomic Southern analysis, chromosomal *P. aeruginosa* DNA was isolated utilizing the IsoQuick nucleic acid extraction kit (ORCA Research, Bothell, WA), digested with various restriction endonucleases, electrophoresed on a 1% agarose gel in 0.5x TBE buffer and transferred to Photogene nylon membranes (Gibco BRL, Gaithersburg, MD) as previously described (17). Plasmid DNA was biotinylated by random hexamer priming following the NEBlot™ Phototype™ kit protocol (New England Biolabs, Beverly, MA). Following transfer and ultraviolet fixation (17), the membranes were probed with the biotinylated DNA fragment according to the Phototype™ detection kit protocol (New England Biolabs, Beverly, MA). Automated nucleotide sequencing was performed as previously described (11).

2.3.3 Plasmid constructions. Details on the construction of selected plasmids not evident from the information given in table 2.1 are as follows.

To obtain the gene replacement vectors pEX18Ap and pEX19Ap, 1,791-bp *Pvu*I fragments from pUC18 or pUC19 (26), respectively, were gel-purified and ligated to the large *Pvu*I fragment of pEX100T (22), from which the overlapping *Sph*I and *Nsi*I had been eliminated by digestion with *Nsi*I, followed by blunt-ending and ligation. The vectors pEX18Gm and pEX18Tc were obtained from pEX18Ap in several steps. First, two primers (APU2, 5'-GTTGAATACTCATACTCTTC and APD, 5'-catcgaTTGTCAGACCAAG-TTTACTCAT) were synthesized. These primers are complimentary to nt 5630-5650 (APU2) and 4780-4760 (capital letters of APD; lower case letters mark nt that were inserted to generate a *Cla*I site [underlined]) of pEX18Ap. Primers APD and APU2 were used to prime synthesis from pEX18Ap DNA in a 50 µl reaction mixture containing 1x *Taq*$^+$ buffer (Stratagene, LA Jolla, CA), 200 µM of each dNTP, 30 pmol of each primer, ~10 pmol plasmid DNA and 2.5 u *Taq*$^+$ Pol (Stratagene, La Jolla, CA). The reaction mixture was subjected to the following thermal cycles: 1 cycle at 96°C for 1.5 min; 30 cycles (95°C, 1 min; 58°C, 25 s; 72°C, 2.5 min) and a final extension at 72°C for 5 min. A similar reaction was set up with pEX19Ap template DNA. All reactions yielded single, linear 5-kb fragments which were treated (20 min; 37°C) with 2.5 u T4 DNA Pol in the presence of 100 µM dNTPs and purified by agarose gel electrophoresis. For construction of pEX18Gm/pEX19Gm and pEX18Tc/pEX19Tc, the 5-kb PCR fragments from the pEX18Ap/pEX19Ap reactions were ligated to a 830-bp blunt-ended *Sac*I fragment from pUCGM (20) or to a 1,348-bp blunt-ended *Cla*I-*Sty*I fragment from pALTER-1 (Promega, Madison, WI), respectively. Only those isolates containing the *aacC*1 and *tet* genes in the same transcriptional orientation as the originally present *bla* gene were retained.

Table 2.1. Bacterial strains and plasmids

		Releveant genotype or description	Source or reference
Strains			
E. coli	DH5αF'	[F$^+$ ϕ80 *lacZ*ΔM15] Δ(*lacZYA-argF*)U169 *recA*1 *endA*1 *hsdR*17 [$r_K^- m_K^+$] *supE*44 *thi*-1 *gyrA relA*1	Gibco-BRL, Gaithersburg, MD
	SM10	*thi*-1 *thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Km^r)	(7)
P. aeruginosa	PAO1	prototroph	B.H. Holloway
	PAO205	PAO1 with Δ(*pabC-orf*)::Gm^r-GFP *FRT*	This study
	PAO206	PAO1 with unmarked Δ(*pabC-orf*)	This study
Plasmids			
	pΔH-1	Ap^r; 3.4 kb *Bam*HI fragment with Δ*plcH*	M.L. Vasil
	pALB2	Ap^r, Tc^r; source of *sacB*' gene	(22)
	pALTER-1	Tc^r; source of *tet* gene	Promega, Madison, WI
	pCP16	Ap^r; source of *FRT* sequences	(3)
	pEX100T	Ap^r; *oriT*$^+$ *sacB*$^+$ gene replacement vector	(22)
	pGEM-T*oriT*	Ap^r; source of *oriT*	(23)
	pGFPmut1	Ap^r; source of *GFP* gene	(4)

Table 2.1. Bacterial strains and plasmids (cont.)

	Releveant genotype or description	Source or reference
pMMC6	Ap^r; source of inducible Flp recombinase	(5)
pUCGM	Ap^r, Gm^r; source of Gm^r cassette	(20)
pUC18/19	Ap^r; cloning vectors	(26)
pUC1918	Ap^r; cloning vector with duplicated pUC18/19 MCS	(21)
pUCP20T	Ap^r; mobilizable broad-host-range cloning vector	(23)
pEX18Ap[a]	Ap^r; *oriT*$^+$ *sacB*$^+$, gene replacement vector with MCS from pUC18	This study
pEX18Gm[a]	Gm^r; *oriT*$^+$ *sacB*$^+$, gene replacement vector with MCS from pUC18	This study
pEX18Tc[a]	Tc^r; *oriT*$^+$ *sacB*$^+$, gene replacement vector with MCS from pUC18	This study
pFGS1	Ap^r, Gm^r; replacement of a 1.9-kb *Hpa*I fragment from the *tet* genes of pCP16 with a 0.83-kb *Sma*I fragment from pUCGM	This study
pFLP1	Ap^r; ligation of a blunt-ended 2.5-kb pMMC6 *Cla*I+*Xba*I fragment into the blunt-ended *Afl*III site of pGEM-T*oriT*	This study
pFLP2	Ap^r; 2.6-kb *Bam*HI-*Sph*I fragment from pALB2 ligated between the same sites of pPS908	This study
pPS681	Ap^r; 6.7-kb fragment containing the *P. aeruginosa acpP* region	Laboratory collection

Table 2.1. Bacterial strains and plasmids (cont.)

	Releveant genotype or description	Source or reference
pPS698	Ap^r; 3-kb *Bam*HI-*Hin*dIII *pabC'-orf'* fragment from pPS681 ligated between the same sites of pUC18	This study
pPS747	Ap^r; pUCP20T with 0.7-kb *Pst*I-*Xba*I fragment from pGFPmut1	This study
pPS838	Ap^r, Gm^r; 1.5-kb blunt-ended pFGS1 *Hin*dIII-*Sma*I fragment ligated into the blunt-ended pUC1918 *Eco*RI	This study
pPS854	Ap^r; religated 2.9-kb reverse PCR fragment from pPS838	This study
pPS856	Ap^r, Gm^r; 0.83-kb blunt-ended *Sac*I fragment from pUCGM ligated into the *Eco*RV site of pPS854	This study
pPS858	Ap^r, Gm^r; blunt-ended pPS747 *Pst*I-*Xba*I fragment ligated into the blunt-ended *Eco*RI site of pPS856	This study
pPS908	Ap^r; 2.5-kb blunt-ended pMMC6 *Cla*I-*Xba*I fragment ligated into the *Sma*I site of pUCP20T	This study
pPS953	Ap^r, Gm^r; replacement of a 1.3-kb pPS698 *Stu*I fragment with a 1.8-kb blunt-ended pPS858 *Sac*I fragment	This study
pPS955	Ap^r, Gm^r; ligation of a 3.6-kb *Bam*HI-*Hin*dIII fragment from pPS953 between the same sites of pEX18Ap	This study

[a] The corresponding pEX19Ap, pEX19Gm and pEX19Tc vectors contain the pUC19 MCS.

The Gm^r-*GFP FRT* cassette contained on pPS858 was assembled in several steps. First, two primers (FSGA, 5'-tagaattcCATTGAGTAAGTTTTTAAGCACATC and FSGB, 5'-gatatcCATTGCT-GTTGACAAAGGGAATCA) were synthesized and phosphorylated by T4 polynucleotide kinase. These oligos primed just inside the *FRT* sequences contained on pPS838 and either introduced an *Eco*RI site (underlined lower case letters in FSGA) or an *Eco*RV site (underlined lower case letters in FGSB). The phosphorylated FSGA and FSGB oligos were used to prime synthesis from pPS838 DNA in a reverse PCR reaction as described above. However, the reaction mixtures were subjected to the following thermal cycles: 1 cycle at 96°C for 40 s; 30 cycles (95°C, 40 s; 54°C, 12 s; 72°C, 2 min and 20 s) and a final extension at 72°C for 5 min. The single 2.9-kb reaction product was gel-purified and then religated to form pPS854. The final vector, pPS858, was derived from pPS854 by insertion of the Gm^r-encoding cassette from pUCGM and the *GFP* cassette from pPS747 in successive steps, as detailed in table 2.1.

2.3.4 Gene replacement and in vivo marker excision in *P. aeruginosa*. For gene replacement, the previously *sacB*-based described strategy (22) was employed.

For isolation of Δ*plcH* mutants, a 3.4-kb *Bam*HI fragment from pΔH-1 containing an unmarked 2-kb *Sma*I-deletion of the *plcH* coding sequence (15) was cloned into the *Bam*HI site of all pEX vectors. The recombinant plasmids were conjugated from *E. coli* SM10 into PAO1 and Cb^r, Gm^r or Tc^r plasmid-integrants were selected on VBMM medium containing the appropriate antibiotic. Merodiploids were resolved by plating on VBMM medium containing 5% sucrose. The hemolysin phenotypes of sucrose^r mutants were screened on blood agar plates and putative Δ*plcH* mutants showing no zones of hemolysis after 36 h were counted.

For isolation of a Δ(*pabC-orf*) mutant, pPS955 was conjugally transferred from *E. coli* SM10 into PAO1 (18), followed by selection of Gm^{r}colonies on VBMM + Gm medium. The merodiploid colonies were plated on VBMM + PABA + Gm medium containing 5% sucrose. The chromosomal restriction patterns of sucrose^r, putative Δ(*pabC-orf*)::Gm^r-*GFP FRT* mutants was verified by genomic Southern analysis utilizing as the probes either the biotin-labeled 3-kb chromosomal insert of pPS698 (*pabC-orf* probe) or the biotin-labeled

0.83-kb insert of pUCGM (*aacC1* or Gm probe). Strain PAO205 containing the correct insertion was retained for the excision experiment.

Deletion of the chromosomally-integrated Gm^r-GFP markers by Flp recombinase-catalyzed excision was achieved by conjugally transferring the Flp-expressing pFLP2 from *E. coli* SM10 into *P. aeruginosa* PAO205 at 37°C. The mating mixtures were suspended in 1 ml PBS (14). Aliquots (100 µl) of the undiluted, 10^{-1}, 10^{-2} and 10^{-3} diluted (in PBS) cell suspension were plated on VBMM + PABA + Cb medium and incubated at 37°C until single colonies were discernable. 20 single colonies were screened for Gm^s on VBMM + PABA +/- Gm plates. Plasmid pFLP2 was cured by generously streaking cells from four patches containing Gm^s colonies on VBMM + PABA medium supplemented with 5% sucrose, followed by incubation at 37°C. Loss of pFLP2 was tested by patching 20 sucrose^r colonies on VBMM + PABA +/- Cb medium, and then verified by subjecting eight LB-grown sucrose^r, Cb^s and Gm^s cells to plasmid mini-prep isolation and agarose gel electrophoresis of 10 µl aliquots of "DNA" (using a replicative Cb^r-conferring plasmid as a positive control).

Excision events were initially assessed by performing colony PCR on four Gm^r isolates utilizing two *aacC1*-specific primers (Gm-Up, 5'-TGGAGCAGCAACGATGTTAC and Gm-Down, 5'-TGTTAGGTGGCGGTACTTGG). To do this, cells from an entire patch were transferred to a microfuge tube containing 50 µl H_2O and the cell suspension was boiled for 5 min. Lysates (5 µl) were then transferred to tubes containing (in 45 µl) 1x PCR buffer (Qiagen, Santa Clarita, CA), 200 µM of each dNTP and 30 pmoles of each Gm-Up and Gm-Down primer. The reaction mixtures were denatured in a thermal cycler at 96°C for 5 min before addition of 2.5 u *Taq* Pol (Qiagen, Chatsworth, CA) and subjecting the mixtures to 34 cycles of amplification (96°C, 35 s; 54°C, 20 s; 72°C, 1 min) followed by a final extension at 72°C for 5 min. Five µl of the 50 µl reaction mixtures were analyzed on an agarose gel.

The mutants were then further verified or by isolating chromosomal DNAs from the same four colonies used in PCR analysis and by performing genomic Southern analyses utilizing the above described biotin-labeled *pabC-orf* and *aacC1* probes.

2.4 RESULTS AND DISCUSSION

2.4.1 Construction of new gene replacement vectors. To extend the usefulness of the *sacB*-based gene replacement system, several new vectors were constructed (figure 2.1). Besides incorporating all the features of pEX100T, the vectors also contain a MCS in *lacZα* in two orientations with respect to *lacZpo*, each containing 10 unique restriction sites. To expand the use of the vectors to other bacteria where the Ap^r/Cb^r marker may not be applicable, derivatives were constructed that either contain Gm^r or Tc^r selectable markers.

To test the usefulness of these vectors for introduction of unmarked mutant alleles into the *P. aeruginosa* chromosome we decided to mutate *plcH*, encoding heat labile hemolysin, a phenotypic trait that can easily be scored on blood agar plates (15). The same unmarked Δ*plcH* mutant allele was cloned into all pEX vectors, the recombinant plasmids were conjugated into PAO1 and Cb^r, Gm^r or Tc^r plasmid-integrants were selected. $Sucrose^r$ selection yielded unmarked Δ*plcH* mutants at frequencies ranging from 5 to 25%, as indicated by lack of zones of hemolysis on blood agar plates. Selected putative mutants were analyzed by colony PCR utilizing nested primers and all of these lacked the *plcH* coding sequence (data not shown).

2.4.2 Design of an FRT cassette vector and construction of a Gm^r selectable FRT cassette. The recently described *FRT* cassettes (3) were not useful in *P. aeruginosa* for various reasons. First, the Km^r cassette was not useful because of the high intrinsic resistance of *P. aeruginosa* to Km. Second, the Tn*10*-derived Tc^r cassette, although potentially useful, does confer Tc^r only when present in single copy or in a few copies per cell. Thus, insertions of this cassette into multicopy plasmids cannot be monitored by Tc^r. In addition, both of these cassettes contain redundant DNA sequences and they cannot be easily isolated due to lack of duplicated flanking restriction sites.

Our goals therefore were to develop an *FRT* cassette vector that (*i*) would allow easy excision of the cassette from its vector and that (*ii*) would contain a basic *FRT* cassette possessing unique restriction sites for insertion of desired marker genes, thus allowing

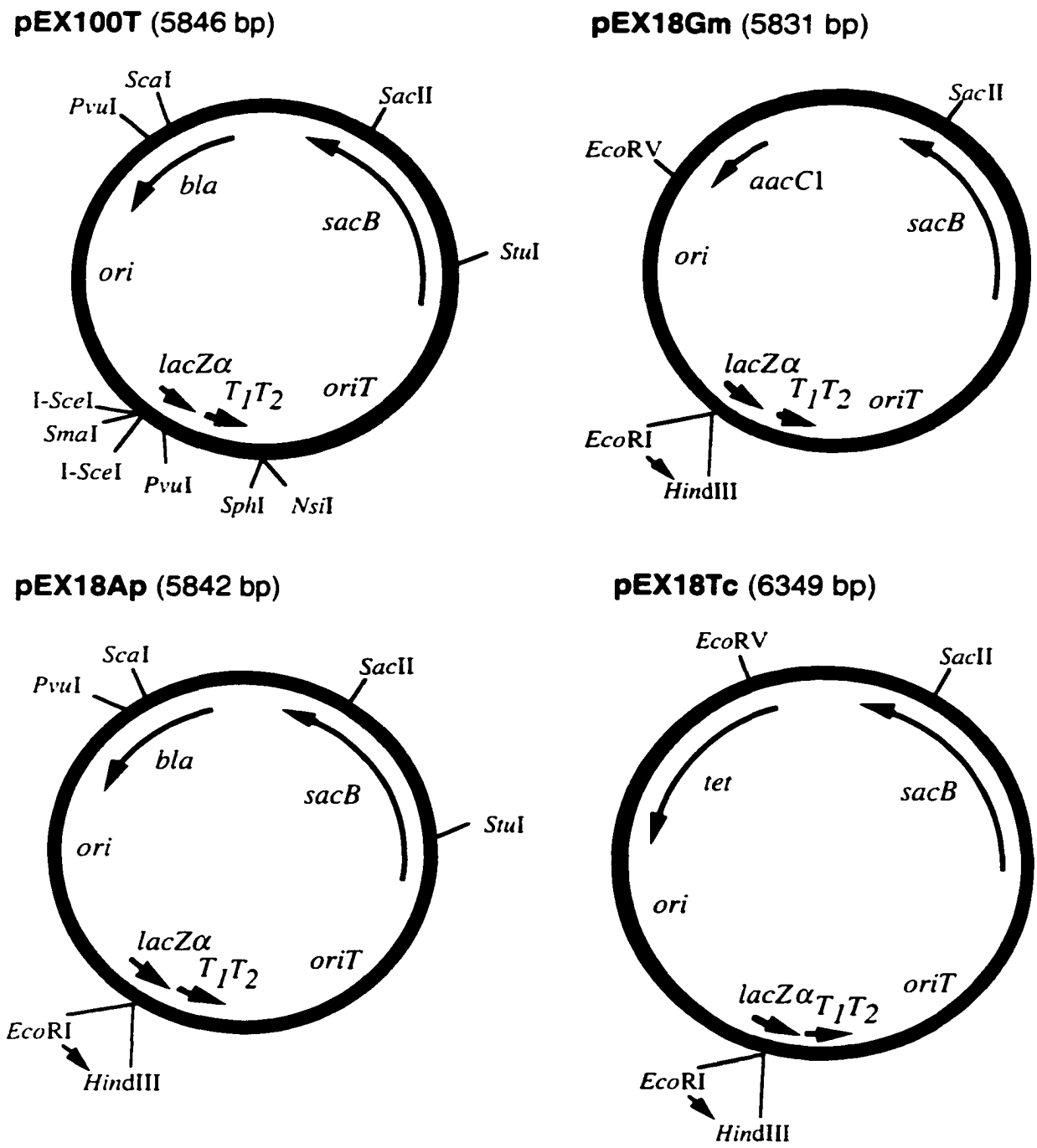


Figure 2.1. Maps of gene replacement vectors. Plasmids are drawn to scale. The locations of genes and their transcriptional orientations are shown, including the double transcriptional terminators T_1 and T_2, and 5S rRNA gene from *E. coli*. The restriction sites of the MCS in all plasmids, in counterclockwise order, are: *Eco*RI-*Sac*I-*Kpn*I-*Sma*I-*Bam*HI-*Xba*I-*Sal*I-*Pst*I-*Sph*I-*Hin*dIII. In the corresponding pEX19Ap, pEX19Gm, and pEX19Tc vectors, the order of the sites of the MCS is reversed. The nt sequences of pEX100T, pEX18Ap, pEX18Gm and pEX18Tc were deposited in GenBank and were assigned the accession numbers U17500, AF004910, AF047518, and AF047519, respectively.

construction of customized *FRT* cassettes. To do this, pPS854 (figure 2.2A) was constructed as detailed in Materials and Methods (section 2.3.3), and in table 2.1. This plasmid contains the *FRT* sites flanked, in inverted order, by unique recognition sites for eight enzymes that can be utilized to liberate the *FRT* cassette from the vector backbone.

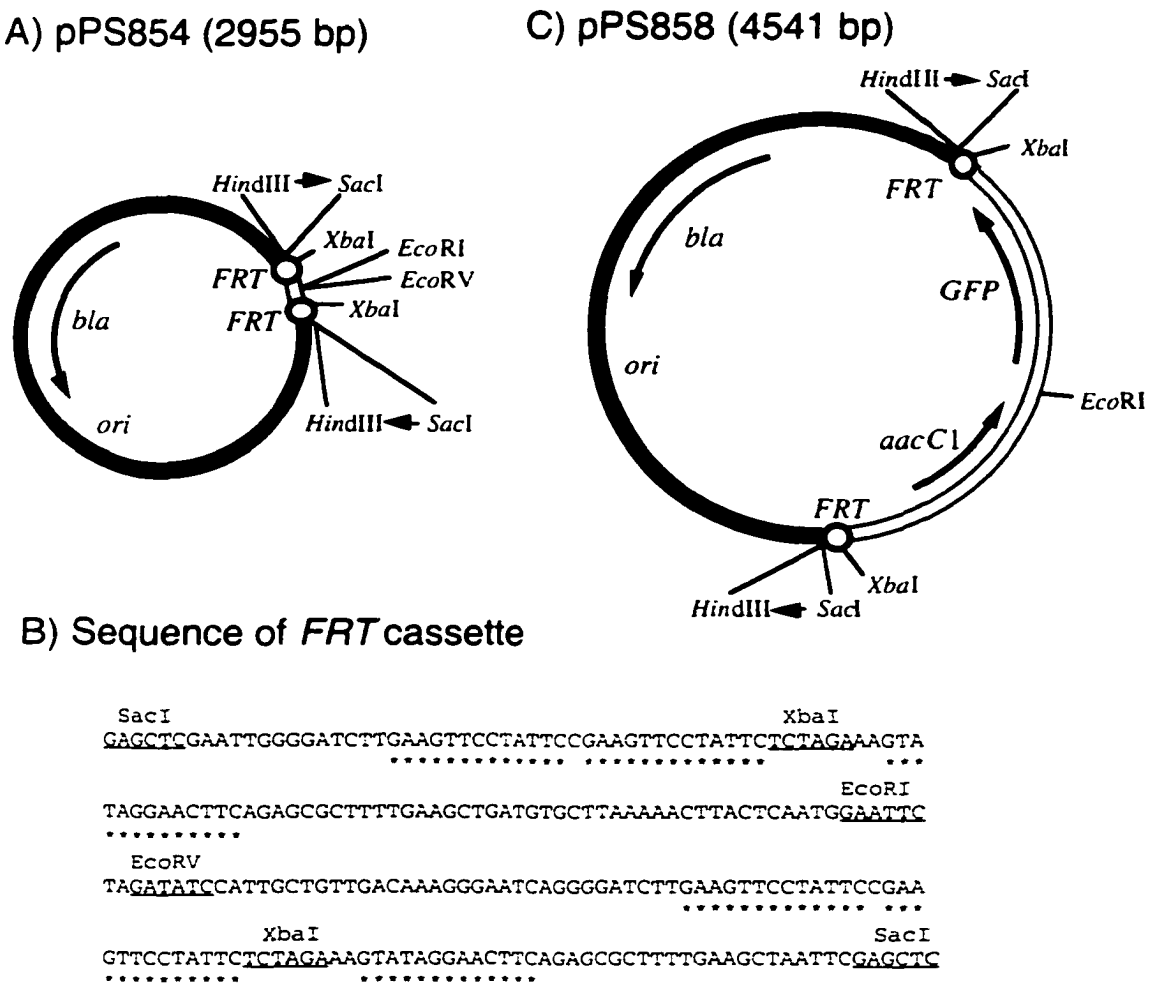


Figure 2.2. Physical maps of *FRT* cassette vectors and sequence of *FRT* cassette. Plasmids are drawn to scale. *(A)* The basic *FRT* cassette vector pPS854 was derived as described in Materials and Methods, section 2.3.3. The locations of selected restriction enzyme cleavage sites and genes are shown. *(B)* Nt sequence of the region located between the *Sac*I sites of pPS854. Asterisks underline the *FRT* sequences. *(C)* pPS858 was derived from pPS854 by insertion of the Gm^r determinant from pUCGM (20) and the *GFP* from pPS747 (table 2.1) into the *Eco*RI and *Eco*RV sites, respectively. *Hin*dIII --> *Sac*I, *Hin*dIII-*Sph*I-*Pst*I-*Sal*I-*Xba*I-*Bam*HI-*Sma*I-*Kpn*I-*Sac*I.

The region separating the *FRT* sites (68 bp) contains unique *Eco*RI and *Eco*RV restriction sites, separated by 2 bp (figure 2.2B). These two sites facilitate the construction of customized *FRT* cassettes by insertion of selectable and/or screenable markers, or addition of other desirable features, e.g., polystop codons or transcription termination/initiation sequences. The *FRT* cassette can easily be excised from its vector since the two *FRT* sites are flanked, in inverted order, by unique *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Sal*I, *Pst*I, *Sph*I and *Hin*dIII sites.

This *FRT* cassette vector was then used to construct pPS858 (section 2.3.3; figure 2.2C). Plasmid pPS858 carries a *FRT* cassette containing the selectable Gm^r marker and the screenable GFP; the GFP gene is transcribed from the *aacC1* promoter. It should be noted that although the GFP marker has proven useful to monitor loss of the entire Gm^r-GFP cassette in *E. coli*, its use in *P. aeruginosa* may be limited to cells grown under conditions where the production of endogenous fluorescent pigments is restricted, e.g., in the presence of high concentrations of iron. It proved not useful for the experiments described in this study due to the intense fluorescence of *P. aeruginosa* cells grown on VBMM medium. Alternatively, the *FRT* cassettes could be equipped with the widely applicable *xylE* screenable marker (21).

2.4.3 Construction of a bhr Flp recombinase-producing plasmid. None of the hitherto available Flp recombinase-producing vectors were either mobilizable or able to replicate in bacteria other than *E. coli*, thus necessating construction of such vectors.

Since some recombinases are sufficiently active to promote excision when transiently expressed in the bacterium of interest from a non-replicative, mobilizable plasmid (12), we first constructed pFLP1 (table 2.1). This plasmid contains the recombinase structural gene, *FLP*, under transcriptional control of the strong but regulatable rightward λ promoter, λp_R. In addition, *FLP* is fused to the initiation codon of the *cro* structural gene, encoding the efficiently translated λ Cro antirepressor. Since the insert also contains cI_{857}, the gene encoding the thermo-labile λ repressor, all the elements required for efficient and regulated transcription, and translation of Flp are present (5). When transformed into *E. coli* mobilizer

strain SM10, pFLP1 was efficiently transferred between *E. coli* strains. However, when utilizing this plasmid in excision experiments from the *P. aeruginosa* chromosome we observed excision of the *FRT* cassette only at very low frequencies (0.5 to 1%) (19). Although the exact reasons for this observation remain unknown, it was presumably because in the absence of any selective pressure we could not control the number of recipient cells that temporarily received the Flp-producing, non-replicative pFLP1. Since changes of various parameters known to effect conjugation efficiencies, e.g., time of conjugation, growth rate of bacteria, media composition, etc., did not lead to marked and consistent improvements of excision frequencies, we decided to construct pFLP2 (figure 2.3). This replicative bhr plasmid contains the Flp-expressing cassette from pMMC6, is mobilizable and curable from its host by sucrose^r selection since it carries the counterselectable *sacB* marker. Its use and effectiveness is described in the next paragraph.

2.4.4 Isolation of an unmarked *P. aeruginosa* Δ*pabC* mutant. To examine the usefulness of the tools described in this study, we decided to isolate an unmarked Δ*pabC* mutant. The putative *P. aeruginosa pabC* gene is one of the genes delimiting a fatty acid biosynthetic gene cluster that is currently being studied in our laboratory (chapter 5). In *E. coli*, the *pabC* gene product, aminodeoxychorismate lyase, is required for PABA synthesis and *pabC* mutants are PABA auxotrophs (9). As is the case in *E. coli*, the *P. aeruginosa pabC* gene lies next to a gene (*orf*) that encodes a putative protein of unknown function (figure 2.4A) and whose inactivation in *E. coli* did not result in a detectable phenotype (9).

A defined pPS955-borne Δ(*pabC-orf*) was constructed as described in table 2.1, and the deletion was returned to the *P. aeruginosa* chromosome as illustrated in figure 2.4A. After conjugal transfer of the non-replicative pPS955 from *E. coli* SM10 into PAO1, merodiploids were obtained by selecting Gm^r. From these, colonies having undergone Δ1 were selected as sucrose^r, Gm^r and Cb^r. The unmarked Δ(*pabC-orf*) mutant PAO206 was then derived from the Gm^r-*GFP* integrant PAO205 by Flp-catalyzed excision of the Gm^r-GFP markers. During its expression in the recipient, Flp recombinase acted at the *FRT* sites to catalyze excision of the Gm^r-*GFP* element (marked with Δ2) at very high frequencies (all 20

colonies tested were Gm^s), leaving behind a short *FRT*-containing sequence. It should be noted that although Flp production from pFLP2 is under control of the temperature-labile λ

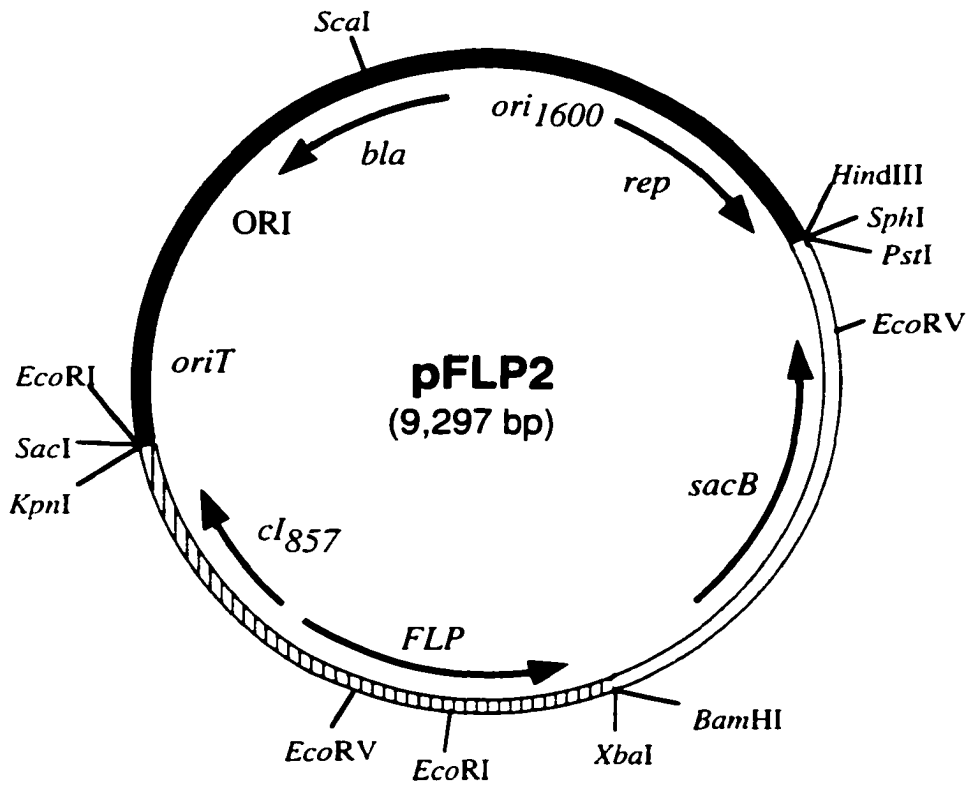


Figure 2.3. Physical map of the bhr Flp vector pFLP2. Only selected restriction enzyme cleavage sites are shown. Expression of *FLP* is driven by the strong, rightward λ promoter (located in the *FLP-cI*$_{857}$ intergenic region) and is regulated by the Ts, *cI*$_{857}$-encoded λ repressor. Efficient Flp translation is achieved by its fusion to the λ Cro ATG initiation codon (5). The nt sequence of pFLP2 was deposited in GenBank and assigned the accession number AF048702.

repressor (5), complete excision was observed even at 30°C, corroborating similar observations previously made by Cherepanov and Wackernagel (3). The pFLP2 vector was efficiently cured from the Gm^r cells by sucrose selection; all 20 of the Gm^r colonies tested had lost the plasmid as indicated by their Cb^s phenotypes and absence of DNA in plasmid mini-preps. Since basal levels of Flp expression seemed sufficient for complete excision, all steps

involved in the excision process, i.e., conjugal transfer of pFLP2 and selection of Cb^r exconjugants, were performed at 37°C.

Successful execution of the steps labeled Δ1 and Δ2 in figure 2.4A was monitored by colony PCR analysis utilizing *aacC1*-specific primers, as detailed in section 2.3.4.

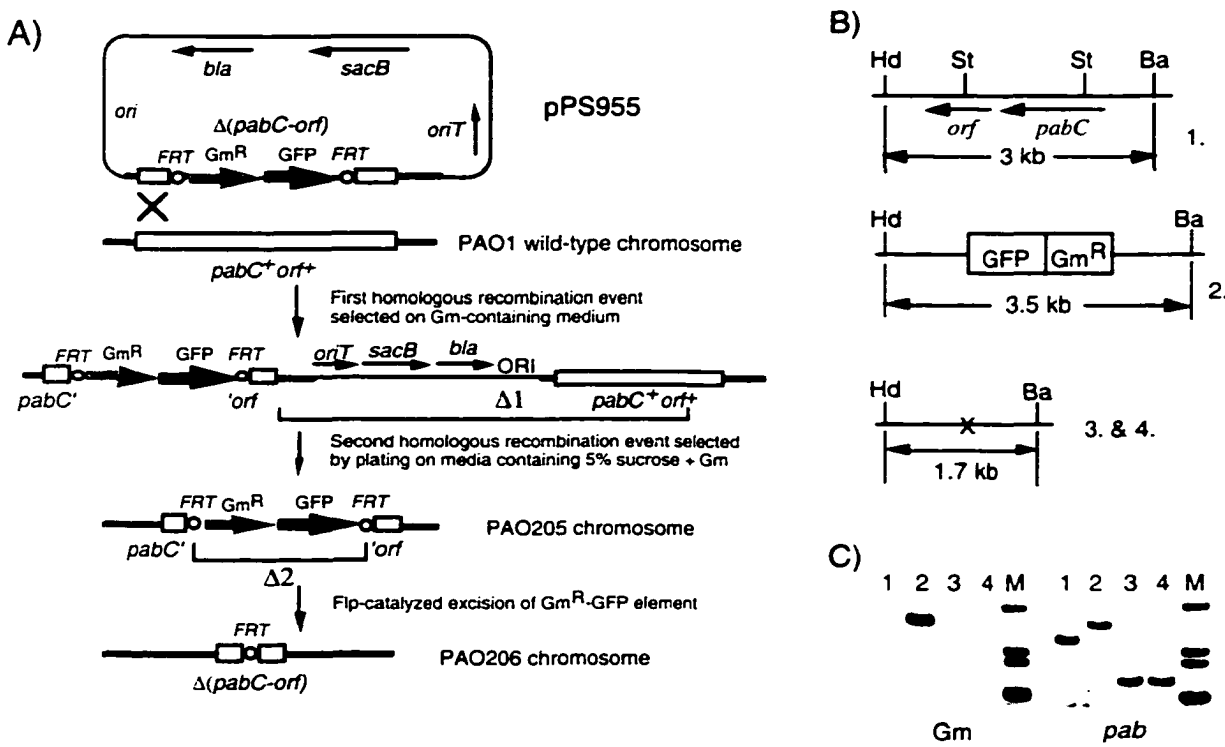


Figure 2.4. Strategy for isolation of an unmarked Δ(*pabC-orf*) mutation. *(A)* For gene replacement, the previously described *sacB*-based strategy (22) was employed, as detailed in Materials and Methods, section 2.3.4. Colonies having undergone Δ1 were screened as sucrose^r, Gm^r and Cb^s. The unmarked Δ(*pabC-orf*) mutant was then derived from the Gm^r-*GFP* integrant by Flp-catalyzed excision (Δ2) of the Gm^r-GFP markers. The *orf* indicates a *P. aeruginosa* gene of unknown function. *(B)* Genomic organization of the PAO1 *pabC*$^+$-*orf*$^+$ region (1.) and of Δ(*pabC-orf*) mutants PAO205 (2.) and PAO206 (isolates 3. and 4.). The relative positions of *Bam*HI (Ba) and *Hin*dIII (Hd) sites, as well as the lengths of *Bam*HI-*Hin*dIII fragments expected after digestion of the respective chromosomal DNAs are shown. The *Stu*I (St) sites shown in (1.) delimit the Δ(*pabC-orf*) mutation and the X in (3. and 4.) marks the position of the *FRT* sequence in the unmarked Δ(*pabC-orf*) strain. *(C)* Genomic Southern analysis. Nylon membranes containing electrophoretically separated genomic DNA fragments from the isolates depicted in panel *B* were either probed with a biotinylated DNA *pabC-orf* fragment (panel labeled *pab*) or with a *aacC1* fragment (panel labeled Gm), as described in Materials and Methods. DNA in lanes 1, 2, 3 and 4 correspond to *Bam*HI-*Hin*dIII fragments from the strains shown in B). Lane M shows (top to bottom) biotinylated λ *Hin*dIII (4.3, 2.4, and 2.04 kb) markers and a biotinylated 1.35-kb ϕX *Hae*III marker.

All four PAO205 colonies analyzed showed a single fragment of ~550 bp, which was absent from the parental strain PAO1 and two isolates of the putative PAO206 excision mutants (data not shown). To further verify the genetic make up of the the putative unmarked Δ(*pabC-orf*) mutants, genomic Southern analysis was performed (figure 2.4B and C). From the results presented in figure 2.4C it is evident that both deletion events produced the desired restriction patterns. Probing with a *pabC-orf* specific probe (panel labeled *pab*) revealed deletion of a 1.3-kb region from the PAO1 wt chromosome in both the Δ(*pabC-orf*)::Gm^r-*GFP* insertion mutant PAO205 (lane 2) and the unmarked Δ(*pabC-orf*) mutant PAO206 (lanes 3 and 4). The size of the 3-kb *Bam*HI-*Hin*dIII fragment observed in wt PAO1 (lane 1) was increased to a 3.5-kb fragment (3 kb minus 1.3-kb genomic DNA plus 1.8-kb Gm^r-*GFP* fragment) (lane 2) in the insertion mutant PAO205, and reduced to 1.7 kb (lanes 3 and 4) in the unmarked deletion mutant PAO206. Probing with a *aacC*1-specific probe (panel labeled Gm) revealed the presence of the 1.8-kb Gm^r-*GFP FRT* cassette only in the Δ(*pabC-orf*)::Gm^r-*GFP* insertion mutant PAO205 on a 3.5-kb *Bam*HI-*Hin*dIII fragment (lane 2). As expected, the *aacC*1 sequences were absent from wt PAO1 genomic DNA (lane 1) and they were deleted from the excision mutant PAO206 (lanes 3 and 4).

2.4.5 Isolation of other mutations in *P. aeruginosa* and *E. coli*. Clearly, the efficiency and adaptibility of the procedure make it ideal for routine isolation of unmarked chromosomal mutants in various bacteria. To date, utilizing the gene replacement vectors and strategies described above, we have utilized it for isolation of unmarked *P. aeruginosa* Δ(*pabC-orf*) (this study), Δ*asd* (10) and Δ(*mexAB-oprM*) (19) mutants. The same procedure was also utilized for construction of an unmarked *E. coli* Δ*asd* mutant strain (our unpublished data), except that a plasmid with a Ts *ori* was utilized as the suicide delivery vector.

2.5 CONCLUSIONS

(i) This paper describes an efficient procedure for isolation of unmarked mutant alleles in the *P. aeruginosa* chromosome, utilizing a combination of versatile gene replacement

vectors and a bhr version of the *S. cerevisiae* Flp-*FRT* recombination system. With a few modifications, the method is also applicable in other bacteria, e.g., *E. coli*.

(ii) Supporting this system are several new mobilizable gene replacement vectors. They contain the MCS and the *lacZα* from the popular pUC18/19 cloning vectors and facilitate the cloning and manipulation of DNA fragments, followed by their transfer into the *P. aeruginosa* chromosome. Since they are available with Ap (Cb), Gm and Tc selectable markers, they will be applicable in a wide range of bacteria.

(iii) A versatile *FRT* cassette vector was constructed. This high-copy number, pUC-based vector contains unique *Eco*RI and *Eco*RV restriction sites flanked by two *FRT* sites. Based on this vector, a *FRT* cassette was constructed that provides a Gm^r selectable and a GFP screenable marker. In concert with a newly constructed Flp recombinase-expressing bhr vector, several unmarked *P. aeruginosa* mutants, as well as an unmarked *E. coli* mutant, were constructed.

(iv) The technique described in this paper provides a method for gene replacement/*in vivo* excision that complements existing, similar techniques (1, 12) but has the added advantage that the Flp-*FRT* system is functional in all bacterial systems studied to date.

(v) In bacteria, such as the pseudomonads, that exhibit high intrinsic resistance to many antibiotics one could envision employing a single *FRT* cassette with a workable selectable marker for isolation of consecutive chromosomal mutations in the same strain. The only possible drawback to such an approach may be that recombination between *FRT* sites placed in the same chromosome could lead to a deletion or inversion of a large chromosomal segment, depending on the orientation of the *FRT* sites and the distance between them (25). Although such large rearrangements are unlikely, especially when selecting mutants on minimal media, it is advisable when integrating a cassette into a second gene in the same host to place the second cassette in the same orientation as the first. With the advent of genome sequencing such experiments will be possible and optimized *FRT* cassettes will be valuable tools for micro- and macro-manipulations of entire bacterial chromosomes (25).

2.6 ACKNOWLEDGMENTS

This work was supported by a grant from the Research Council of the College of Veterinary Medicine and Biomedical Sciences at Colorado State University and, in part, by start up funds from the Department of Microbiology. We wish to thank Dr. W. Wackernagel, Univ. of Oldenburg, and Dr. M.L. Vasil, Univ. of Colorado Health Sciences Center, for the provision of bacterial plasmids.

2.7 REFERENCES

1. **Ayres, E., K., V. J. Thomson, G. Merino, D. Balderes, and D. Figurski.** 1993. Precise deletions in large bacterial genomes by vector-mediated excision (VEX): the *trfA* gene of promiscous plasmi RK2 is essential for replication in several Gram-negative hosts. J. Mol. Biol. **230:**174-185.

2. **Camilli, A., D. T. Beattie, and J. Mekalanos.** 1994. Use of genetic recombination as a reporter of gene expression. Proc. Natl. Acad. Sci. USA **91:**2634-2638.

3. **Cherepanov, P. P., and W. Wackernagel.** 1995. Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158:**9-14.

4. **Cormack, B., R. Valdivia, and S. Falkow.** 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene **173:**33-38.

5. **Cox, M. M.** 1983. The FLP protein of the yeast 2-μm plasmid: expression of a eukaryotic genetic recombination system in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **80:**4223-4227.

6. **De Lorenzo, V.** 1994. Designing microbial systems for gene expression in the field. Trends Biotechnol. **12**:365-371.

7. **De Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn*5* and Tn*10*-derived transposons. Methods Enzymol. **235**:386-405.

8. **Dymecki, S. M.** 1996. A modular set of Flp, FRT and *lacZ* fusion vectors for manipulating genes by site-specific recombination. Gene **171**:197-201.

9. **Green, J. M., W. K. Merkel, and B. P. Nichols.** 1992. Characterization and sequence of *Escherichia coli pabC*, the gene encoding aminodeoxychorismate lyase, a pyridoxal phosphate-containing enzyme. J. Bacteriol. **174**:5317-5323.

10. **Hoang, T., S. Williams, and H. P. Schweizer.** 1997. Molecular genetic analysis of the region containing the essential *Pseudomonas aeruginosa asd* gene encoding aspartate-β-semialdehyde dehydrogenase. Microbiol. **143**:899-907.

11. **Hoang, T. T., and H. P. Schweizer.** 1997. Fatty acid biosynthesis in *Pseudomonas aeruginosa*: cloning and characterization of the *fabAB* operon encoding β-hydroxydecanoyl-acyl carrier protein dehydratase (FabA) and β-ketoacyl-acyl carrier protein synthase I (FabB). J. Bacteriol. **179**:5326-5332.

12. **Kristensen, C. S., L. Eberl, J. M. Sanchez-Romero, M. Givskov, S. Molin, and V. De Lorenzo.** 1995. Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid RP4. J. Bacteriol. **177**:52-58-0.

13. **Lyznik, L. A., K. V. Rao, and T. K. Hodges.** 1996. Flp-mediated recombination of FRT sites in the maize genome. Nucleic Acids Res. **24**:3784-3789.

14. **Miller, J. H.** 1992. A Short Course in Bacterial Genetics. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

15. **Pritchard, A. E., and M. L. Vasil.** 1986. Nucleotide sequence and expression of a phosphate-regulated gene encoding a secreted hemolysin of *Pseudomonas aeruginosa*. J. Bacteriol. **167:**291-298.

16. **Sadowski, P.** 1995. The Flp recombinase of the 2-microns plasmid of *Saccharomyces cerevisiae*. Prog. in Nucleic Acids Res. & Mol. Biol. **51:**53-91.

17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

18. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6:**1195-1204.

19. **Schweizer, H. P.** 1998. Intrinsic resistance to inhibitors of fatty acid biosynthesis in *Pseudomonas aeruginosa* is due to efflux: application of a novel technique for generation of unmarked chromosomal mutations for the study of efflux systems. Antimicrob. Agents Chemother. **42:**394-398.

20. **Schweizer, H. P.** 1993. Small broad-host-range gentamycin resistance cassettes for site-specific insertion and deletion mutagenesis. BioTechniques **15:**831-833.

21. **Schweizer, H. P.** 1993. Two plasmids, X1918 and Z1918, for easy recovery of the *xylE* and *lacZ* reporter genes. Gene **134:**89-91.

22. **Schweizer, H. P., and T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:**15-22.

23. **Schweizer, H. P., T. R. Klassen, and T. Hoang.** 1996. Improved methods for gene analysis and expression in *Pseudomonas*, p. 229-237. *In* T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (ed.), Molecular biology of Pseudomonads. Am. Soc. Microbiol., Washington, DC.

24. **Sternberg, N., D. Hamilton, and R. Hoess.** 1981. Bacteriophage P1 site-specific recombination. II. Recombination between *loxP* and the bacterial chromosome. J. Mol. Biol. **150:**487-507.

25. **Wild, J., Z. Hradecna, G. Posfai, and W. Szybalski.** 1996. A broad-host-range *in vivo* pop-out and amplification system for generating large quantities of 50- 100-kb genomic fragments for direct DNA sequencing. Gene **179:**181-188.

26. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-119.

CHAPTER 3

Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains.

(Presented in Tung T. Hoang, Alecks J. Kutchma, Anna Becher, and Herbert P. Schweizer. 2000. *Plasmid* **43**:59-72)

The work presented in this paper describes for the first time a method that I developed to integrate genes into *P. aeruginosa* chromosome for purposes of studying gene regulation, using stably integrated single copy chromosomal gene fusions. Besides from the various possible applications of this technology, this paper demonstrates the utilization of the technology for studying autoinducer regulated genes and protein expression in *P. aeruginosa*. I thank A.J. Kutchma for help with the construction of chromosomal *rhlA::lacZ* fusions and Anna Becher for help with construction of mini-CTX-*lacZ*.

3.1 ABSTRACT

An improved method for integration of exogenous DNA fragments at a defined site within the genome of *Pseudomonas aeruginosa* was developed. The method relies on two integration-proficient vectors, mini-CTX1 and mini-CTX2. These two vectors contain (i) a tetracycline (*tet*) selectable marker, (ii) an *oriT*-for conjugation-mediated plasmid transfer, (iii) the pMB1-derived origin of replication, (iv) a modified ϕCTX integrase (*int*) gene, (v) a versatile multiple cloning site (MCS) flanked by T4 transcriptional termination sequences (Ω elements) and (vi) the ϕCTX attachment site. The MCS and Ω elements are flanked by yeast Flp recombinase target sites which allow *in vivo* excision of unwanted plasmid backbone sequences, including *tet* and *int*, from the genome of integrants by Flp recombinase. In the

mini-CTX2 vector *int* transcription is driven from the strong *trc* promoter which is regulated by the Lac repressor that is encoded by *lacI*^{q} also contained on the plasmid. Upon conjugal transfer, mini-CTX1 and mini-CTX2 integrated at frequencies of 10^{-8} and 10^{-7}, respectively. The usefulness of the integration vectors for gene fusion analyses was demonstrated by chromosomal insertion of autoinducer (AI)-regulated *lasB-lacZ* and *rhlA-lacZ* fusions into wild-type and AI synthase mutants. In wild-type, the fusions responded in a cell density-dependent manner and expression of both fusions was either greatly reduced or abolished in AI synthase mutants. Lastly, an expression cassette containing the T7 polymerase gene under Lac repressor control was constructed, integrated into the *P. aeruginosa* chromosome and used to express the hexahistidine-tagged *P. aeruginosa* AI synthase RhlI.

3.2 INTRODUCTION

With the advent of genome sequencing, new and improved technologies are needed for analysis of the many unknown gene sequences that are rapidly emerging from these projects. Gene fusion analyses provide a useful technology for initial transcriptional analyses of genes of unknown function or of genes whose products are difficult to assay (26, 29). Many plasmid-based systems are available for constructing such gene fusions (15, 28) but plasmid systems suffer from inherent problems. Although higher-copy number vectors may be useful for identification of weak promoters, their use inherently does not reflect the natural situation and titration effects involving single-copy, chromosomally-encoded transcriptional regulators may lead to improperly regulated gene expression. Furthermore, genomic DNA and plasmid DNA differ in their extent of supercoiling, a state of DNA which has been shown to play a major role in regulation of gene expression (10). To circumvent such problems, in *E. coli* λ-based systems have therefore been developed for single-copy chromosomal integration of gene fusions (15, 28). Although chromosomal gene integration can be achieved in bacteria other than *E. coli* with the help of suicide vectors, such attempts suffer from some potentially serious drawbacks. First, integration of non-replicative plasmids leads to merodiploid formation and they are unstable unless selective pressure is maintained. Second, although stable integrants can be obtained by gene replacement (24) such strategies are not applicable when analyzing essential genes. Third, integration via homologous

recombination does not allow for routine integration of DNA sequences at a defined, neutral site. The *E. coli* λ system has also proven to be useful for genetic engineering of strains, e.g. the construction of host strains for regulated expression from the T7 promoter (31). Initial attempts at developing a phage-based system for efficient and stable integration of exogenous sequences into the *P. aeruginosa* genome were based on ϕCTX (33). Although this system allowed efficient integration of the *attP*-containing plasmid into the genome via a suicide plasmid containing *attP*, it was not very userfriendly because it involved co-transformation with an integrase-expressing plasmid, it did not allow for easy cloning of exogenous DNA due to lack of a versatile MCS and it did not allow for removal of unwanted plasmid-backbone sequences. To develop a more versatile integration-proficient, ϕCTX-based system for *P. aeruginosa*, we adapted an approach that was previously described for mycobacterial integration vectors (30). This approach includes (i) inclusion of the *int* gene on the same vector containing *attP* and (ii) inclusion of a MCS for facilitated cloning of DNA fragments. We further improved our system by the engineering of *FRT* sites that allow subsequent *in vivo* removal of unwanted plasmid sequences from the genome (11).

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strains, plasmids, PCR primers, media and culture conditions. The bacterial plasmids and PCR primers used in this study are listed in table 3.1. DH5αF' (Gibco BRL, Gaithersburg, MD) and JM109 (35) were used as cloning hosts and MC4100Δ*asd* (laboratory collection) as host for *asd*-based vectors. Strains SM10 (9), SM10*lacI*^{q} [SM10 made *lacI*$^{q+}$ using a previously described method (23)] or MOB1 (MC4100Δ*asd* with the transduced mobilizer region from SM10) were used as mobilizer strains. *P. aeruginosa* isolates were derived from wild-type PAO1. The unmarked Δ*rhlI* mutant PAO210 was derived by deletion of an internal 245-bp *Nco*I fragment from the PAO1 chromosome utilizing the pPS856-derived *FRT* cassette and the Flp-recombinase procedure previously described (11). The same procedure was used to derive Δ*lasI* mutant PAO214 by transfer of a 187-bp *Eco*RI-*Pst*I deletion to the PAO1 chromosome and to finally construct the Δ*lasI* Δ*rhlI* mutant PAO216 by recombining the 245-bp Δ*rhlI* mutation into the chromosome of Δ*lasI* strain

Table 3.1. Plasmids and Primers

Plasmid or primer	Relevant properties	Reference or origin
Plasmids		
pALTER-1	Tc^r; site-directed mutagenesis vector	Promega
pEB1	Ap^r; bhr vector containing T7 polymerase structural gene under LacI control	(3)
pFLP2	Ap^r; source of Flp recombinase	(11)
p1000	Ap^r; source of ϕCTX *attP* site	(33)
pIBH	Ap^r; source of ϕCTX integrase	(33)
pSE380	Ap^r; source of $lacI^q$ and *trc* promoter	(2)
pUC19	Ap^r; cloning vector	(35)
pUCP20T	Ap^r; bhr cloning vector	(25)
pUCP20T*asd*	Asd^+; pUCP20T with *P. aeruginosa asd* gene as sole selectable marker	Laboratory collection
pUO58-24	Gm^r; source of *P. aeruginosa rhlAB* and *rhlRI* genes	U. Ochsner
pPS1001	Ap^r, Tc^r; 1,292-bp blunt-ended *Hin*dIII-*Bam*HI fragment containing *int* from pIBH cloned into *Sma*I site of pALTER-1; *Eco*RI, *Pst*I and *Sma*I sites removed from *int* with CTX-Eco, CTX-Pst and CTX-Sma mutagenic primers	This study

Table 3.1. Plasmids and primers (cont.)

PCR Primers[4]	
CTX-Eco	GAACCTGGCGGAgTTCATGATCTGG
CTX-EcoV	CTCTGGCTCGAcATCAAACGCAACC
CTX-Kpn	ATCCTCGGTACtGAAACCGCGCGCA
CTX-Pst	GCTGCGCGCACTcCAGCAGCAGGC
CTX-Sma	TCCCGGACTCACCgCGGGTGAAAAGC
CTX-Xho	CCATTCACtCGTGAGGAAATCGACC
DE3-D	(*Bam*HI)-TTTGGATCCTTACGCGAACGCGAAGTCCGA
DE3-U	(*Bam*HI)-CCCGGATCCACAATGGTGCAAAAAAGAGAGT
Int-Start	(*Nco*I)-GAACCATGGCAGACGGAGTCGAGGTACGCG
$P_{ser\text{-}up}$	CGAGTGGTTTAAGGCAACGGTCTTGA
$P_{ser\text{-}down}$	AGTTCGGCCTGGTGGAACAACTCG
RhlI-D2	(*Bam*HI)-AGggAtCCTCACACCGCCATCGACAGCGTAC
RhlI-U2	(*Nde*I)-GGcatATGATCGAATTCCTCTCTGAATCGC
Tet-Down	(*Nco*I)-CTTCCATGGTCAGGTCGAGGTGGCCCGGCTCC
Tet-EcoV	TTGCGGGATATtGTCCATTCCGAC

[4] Primers are printed 5' to 3'; lowercase letters either indicate non-matching oligonucleotides to form the indicated motif as underlined or oligonucleotides that introduce other non-motif changes.

PAO214. All deletion constructs were verified by genomic Southern analyses and by PCR amplification of the chromosomal segments containing the deletion, followed by automated nucleotide sequencing. The Δ*asd* cloning host HPS11 was derived from HPS1 by P1 transduction (27) of the Δ(*malA-asd*) mutation from strain SH309 (21) into HPS1 (23), which had been temporarily made *recA*$^+$ using a previously described method (5). The *P.*

aeruginosa expression strain PAO-T7 was created by integration of mini-CTX-T7 at the *attB* site of Δ*asd* strain PAO186 (12), followed by Flp-mediated excision of the plasmid backbone. *E. coli* and *P. aeruginosa* strains were routinely grown in LB medium (Gibco BRL) or peptone-trypticase soy broth (PTSB; 5% peptone, 0.25% tryptic soy broth) (Becton Dickinson, Sparks, MD) at 37°C with shaking to ensure adequate aeration. *P. aeruginosa* exconjugants were selected on PIA (Becton Dickinson). Antibiotics were used in selection media at the following concentrations: for *E. coli*, ampicillin (Ap, 100 μg/ml); gentamicin (Gm, 10 μg/ml); tetracycline (Tc, 5 μg/ml); and for *P. aeruginosa*, carbenicillin (Cb, 500 μg/ml); Gm (100-200 μg/ml); Tc (50-200 μg/ml). Lactose phenotypes were screened on LB or PIA plates containing 40 μg/ml XGal.

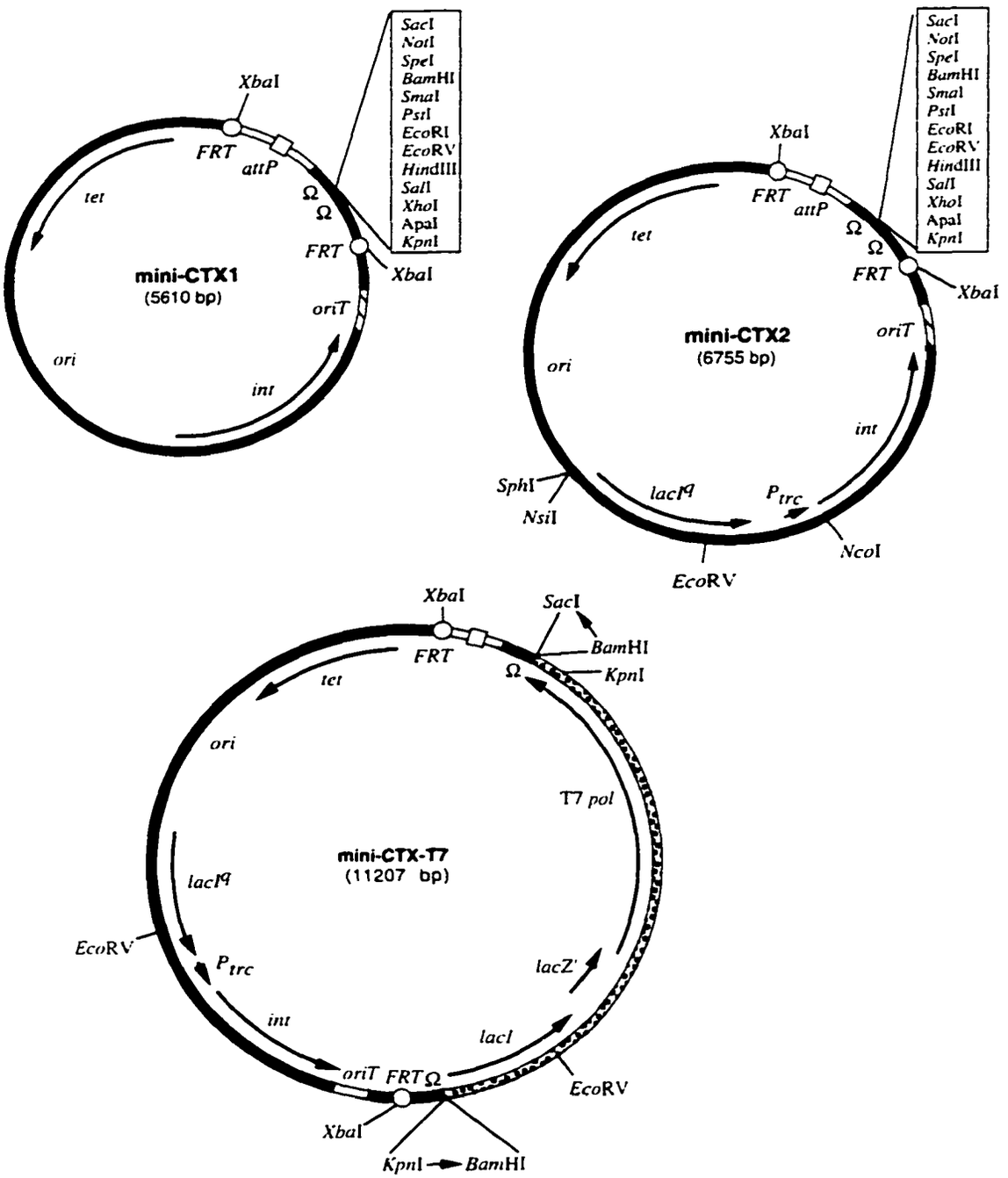
3.3.2 DNA manipulations and genetic techniques. Routine DNA procedures including PCR, restriction enzyme digestions, purification of DNA fragments, ligations, and electroporation and transformation were done as previously described (11). Plasmid DNAs were purified using the QIAprep spin miniprep kit (Qiagen, Santa Clarita, CA). The *sacB* sequences were cured from *P. aeruginosa* by plating on LB or PIA medium containing 5% sucrose and DNA sequences were excised *in vivo* from the chromosome by Flp recombinase encoded on pFLP2 (11). Plasmids were transferred from *E. coli* mobilizer strains to *P. aeruginosa* in biparental matings (24). For determination of integration efficiencies, the donor strain *E. coli* SM10 harboring mini-CTX1 or mini-CTX2 was grown overnight in LB medium containing 5 μg/ml Tc and the recipient PAO1 was grown in LB medium. Donor and recipient cultures were diluted 50-fold into fresh LB medium without antibiotics and grown to exponential phase. Equal volumes (0.7 ml) of donor and recipient were mixed, the cells pelleted in a microfuge and resuspended in a minimal volume (~25 μl) of LB. The mixtures were pipetted on a small (1 cm diameter) cellulose acetate filter (Sartorius, Edgewood, NY) placed on a LB plate with or without 1 mM IPTG. After overnight incubation at 37°C, the filter was transferred to 1 ml of LB medium and the cells were suspended off the support by vigorous vortexing. Exconjugants were selected by plating 100 μl aliquots of the undiluted cell suspension on PIA medium containing 200 μg/ml Tc. Total *P. aeruginosa* counts in the cell suspension were determined by plating serial dilutions (in

LB) on PIA medium. Insertion at the *attB* locus was verified by colony PCR or genomic Southern analysis. Colony PCR was performed as previously described (11). For genomic Southern analysis, chromosomal *P. aeruginosa* DNA was isolated utilizing a genomic isolation kit (Orca Research, Bothell, WA), digested with various restriction endonucleases, electrophoresed on a 1% agarose gel in 0.5x TBE buffer and transferred to Photogene nylon membranes (Gibco BRL) as previously described (20). Plasmid DNA was biotinylated by random hexamer priming following the NEBlot™ Phototype™ kit protocol (New England Biolabs, Beverly, MA). Following transfer and ultraviolet fixation (20), the membranes were probed with the biotinylated DNA fragment according to the Phototype™ detection kit protocol (New England Biolabs). For verification of mini-CTX1 integration at the *attB* locus, the $tRNA^{Ser}$ region of two isolates was amplified from 100 ng of genomic DNA. The PCR fragments were gel-purified using Geneclean and automated nucleotide sequencing was performed as previously described (13).

3.3.3 Plasmid constructions. Details on the construction of selected plasmids not evident from the information given in table 3.1 are as follows.

Several plasmids served as sources for the functional elements of the integration vectors mini-CTX1 and mini-CTX2 (figure 3.1)(details can be found in table 3.1). The plasmid backbone including the *tet* gene and *ori* were derived from pUCP26 (35) (the N-terminal sequences of the *tet* gene located between the *Cla*I and *Nru*I sites were derived from pPS1010 to eliminate the *Eco*RV site) and the *oriT* (origin of transfer) was obtained from pGEM-T-*oriT* (25). The *FRT* sequences, MCS and Ω transcriptional terminators were derived by combining sequences from pPS854 (11), pWSK29 (32) and pACΩ (24), respectively. Finally, p1000 and pIBH served as a sources for the ϕCTX *attP* site and *int*, respectively (33). The *Eco*RI, *Eco*RV, *Kpn*I, *Pst*I, *Sma*I and *Xho*I sites found in the *int* gene were eliminated by site-specific mutagenesis using the Altered Sites™ mutagenesis kit (Promega, Madison, WI) and CTX *int* mutagenic primers introducing single base changes (table 3.1) while not altering the integrase amino acid primary sequence. The segment containing the $lacI^q$, P_{trc} and the *ori*

Figure 3.1. Maps of the integration vectors mini-CTX1, mini-CTX2 and mini-CTX-T7. Plasmids are not drawn to scale. *Bam*HI --> *Sac*I depicts the location and orientation of a *Bam*HI-*Kpn*I-*Sac*I-*Eco*RI-*Pst*I-*Sma*I-*Bam*HI-*Spe*I-*Xba*I-*Not*I-*Sac*I MCS downstream of T7 *pol*. Similarly, *Kpn*I --> *Bam*HI shows a *Kpn*I-*Apa*I-*Xho*I-*Sal*I-*Cla*I-*Hin*dIII-*Spe*I-*Pst*I-*Sal*I-*Xba*I-*Bam*HI MCS upstream of *lacI*. The locations of genes and their transcriptional orientations are shown, including the T4 transcription terminators (Ω); *lacZ'*, which encodes the 161 NH_2-terminal amino acids of *lacZ* which exhibit β-Gal α peptide activity; and T7 *pol*, the structural gene for T7 polymerase. The nucleotide sequences of mini-CTX1 and mini-CTX2 have been deposited in GenBank, and were assigned the accession numbers AF140576 and AF140577, respectively.



of mini-CTX2 was excised from pSE380 (2) on a 2,652-bp *Nco*I-*Rca*I fragment. The mini-CTX-T7 vector was derived in two steps. First, a 4,419-bp fragment containing *lacI*, P_{lacUV5} and the T7 *pol* was PCR amplified from pEB1 template DNA and subcloned into the *Bam*HI site of pUC19 after digestion with *Bam*HI. Second, the *lacI*-P_{lacUV5}-T7 *pol* gene cassette was subcloned into mini-CTX2 as an *Eco*RI-*Hin*dIII fragment to form mini-CTX-T7. The *asd*-based expression vector pPS1032 was constructed by ligation of a 4,214-bp *Ase*I-*Hin*dIII fragment derived from pUCP20T*asd* to a 449-bp *Ase*I *Hin*dIII fragment from pET-15b (Novagen, Madison, WI). This generated an expression vector that contained *asd* as sole selectable marker, an *ori* for maintenance in *E. coli*, an ori_{1600} and Rep for replication in *P. aeruginosa*, an *oriT* for interspecies conjugal transfer, and the T7 promoter and His6-encoding domain with unique cloning sites. The source of the *lasB-lacZ* transcriptional fusion used in this study was pLPBL (B.H. Iglewski and L. Passador, University of Rochester). A *rhlA-lacZ* transcriptional fusion was derived from pLPBL in several steps. First, a *Bgl*II site was introduced upstream of the *lasB* regulatory region on pLPBL by tailing an *Eco*RI site with a non-phosphorylated *Bgl*II linker (5'- CAGATCTGAATT) (22); this procedure resulted in pPS959 which contains a unique *Bgl*II site flanked by two *Eco*RI sites. Second, the region containing the *rhlA* regulatory region (18) was excised from pUO58-24 on a 623-bp *Bgl*II-*Bam*HI fragment and cloned into pLITMUS29 (New England Biolabs) to form pPS963. Finally, a *rhlA-lacZ* transcriptional fusion was derived by replacing a 434-bp *Bgl*II-*Bam*HI fragment on pPS959 containing the *lasB* regulatory region with a 624-bp *Bgl*II-*Hin*dIII fragment, yielding pPS964. For transfer into the *P. aeruginosa* genome, the *lasB-lacZ* and *rhlA-lacZ* fusions were then subcloned between between the *Eco*RI and *Eco*RV sites of mini-CTX1 on ~3.8-kb *Eco*RI-*Dra*I fragments (the *Dra*I site is located 59-bp downstream of *lacZ* early in *lacY*), yielding pPS1041 and pPS1052, respectively.

3.3.4 β-Galactosidase assays. For β-Gal activity assays, samples were prepared similarly to a previously described method (1). Briefly, cells were grown overnight at 37°C in PTSB medium with shaking. Overnight cultures were subcultured into fresh PTSB medium at a ratio of 1:200 and were grown under the same conditions. At the indicated time points, aliquots were removed and centrifuged at 13,000 rpm in a microfuge for 5 min. Cell pellets

were resuspended in an appropriate volume of 0.1 M phosphate buffer (pH 7.0) to obtain a final optical density at 540 nm of 0.2 to 0.5 and then stored at -20°C until use. Samples were assayed in triplicate after SDS/chloroform permeabilization of the cells and β–Gal activities were assayed and units calculated as previously described (17). The *t* distribution was used to establish 95% confidence limits.

3.3.5 Stability of *lasB-lacZ* integrants. A single colony of strain PAO230 was picked and used to inoculate a LB culture which was grown for 24 h at 37°C. The culture was diluted 10^6-fold into fresh LB medium and grown at 37°C for 24 h. This procedure was repeated until the cells had grown for an estimated 100 generations. The cells were then plated on LB agar for single colonies and genomic DNAs were isolated from 10 different colonies. The presence of the *lasB-lacZ* fusion was verified by using the biotinylated ~270-bp PCR fragment obtained by amplification with $P_{ser\text{-}up}$ and $P_{ser\text{-}down}$ as the probe in genomic Southern analyses. The isolates were also plated on LB-XGal agar to phenotypically verify the presence of *lasB-lacZ*.

3.3.6 Protein techniques. Expression of recombinant proteins from T7 promoter-based expression vectors in *P. aeruginosa* by IPTG induction was achieved and whole cell extracts were prepared as previously described (14). Proteins were analyzed by electrophoresis on 0.1% SDS-10% PAGE using a previously described method (16), and visualized by quick staining with Coomassie Brillant Blue R-250 (4).

3.4 RESULTS

3.4.1 Construction and properties of integration vectors. Our intent was to develop a gene integration system for *P. aeruginosa* that would allow for routine integration of genetic elements in single-copy at a defined location within the chromosome without disruption of any functional sequence. Moreover, the integrated genetic element should be devoid of any antibiotic selectable marker and transcription originating from within the integrated sequences should not influence neighboring chromosomal genes. To this end, the mini-CTX

vectors were constructed (figure 3.1). These small vectors contain a φCTX attachment site and the cognate *int* gene. These two elements direct the non-replicative plasmid to the corresponding *attB* site at a defined location in the *P. aeruginosa* chromosome either after *oriT*-mediated conjugation, or by electroporation or transformation. A versatile MCS not only facilitates cloning of DNA segments but also allows chromosomal integration of these segments in a defined and predictable orientation. Flanking of the MCS by strong T4 transcriptional terminators (19) not only minimizes transcription of neighboring chromosomal DNA from promoters located within integrated DNA segments but our evidence also indicates that they probably also prevent transcription of integrated genes from chromosomal promoters (see below). Finally, the presence of *FRT* sites on both sides of the cloned DNA segment allows for efficient *in vivo* Flp recombinase-mediated excision of unwanted DNA sequences. Since it is known that increased transcription of transposase or integrase genes can lead to increased transposition or integration efficiencies, we also constructed mini-CTX2. Besides possessing all the pertinent features of mini-CTX1, in this vector the *int* gene is transcribed from the strong P_{trc}, its transcription is regulated by the Lac repressor and its translation initiation is facilitated by a strong consensus RBS.

3.4.2 Chromosomal integration of mini-CTX vectors. To test their integration efficiencies, the mini-CTX vectors were conjugally transferred into *P. aeruginosa* strain PAO1. Since they cannot replicate in *P. aeruginosa*, selection of Tc^r exconjugants yielded derivatives containing the plasmid integrated into the chromosome, presumably via integrase-mediated recombination at the *attB* site (figure 3.2, step 1). The integration frequencies obtained with mini-CTX1 were about 1.2×10^{-8} (table 3.2). As expected, increased transcription of the *int* gene from P_{trc} and/or improved translation of the integrase from an optimized RBS increased the integration efficieny about 10-fold to around 1×10^{-7}. The addition of IPTG to the mating mixture showed no effect on integration efficieny, presumably since the non-replicative plasmid does not persist long enough in *P. aeruginosa* to allow accumulation of repressing amounts of Lac repressor. Finally, excision of the unwanted plasmid DNA sequences located between the *FRT* sites was achieved by expressing Flp recombinase from a conjugative plasmid, pFLP2 (figure 3.2, step 2). This step results in

an unmarked integrant, ensures that plasmid-associated promoters - that could potentially interfere with transcription of integrated genes - are deleted, and potentially contributes to the stability of integrated DNA segments by concomitant deletion of the *int* gene. However, integrants were stably maintained in the absence of Tc selection without excision of the plasmid backbone.

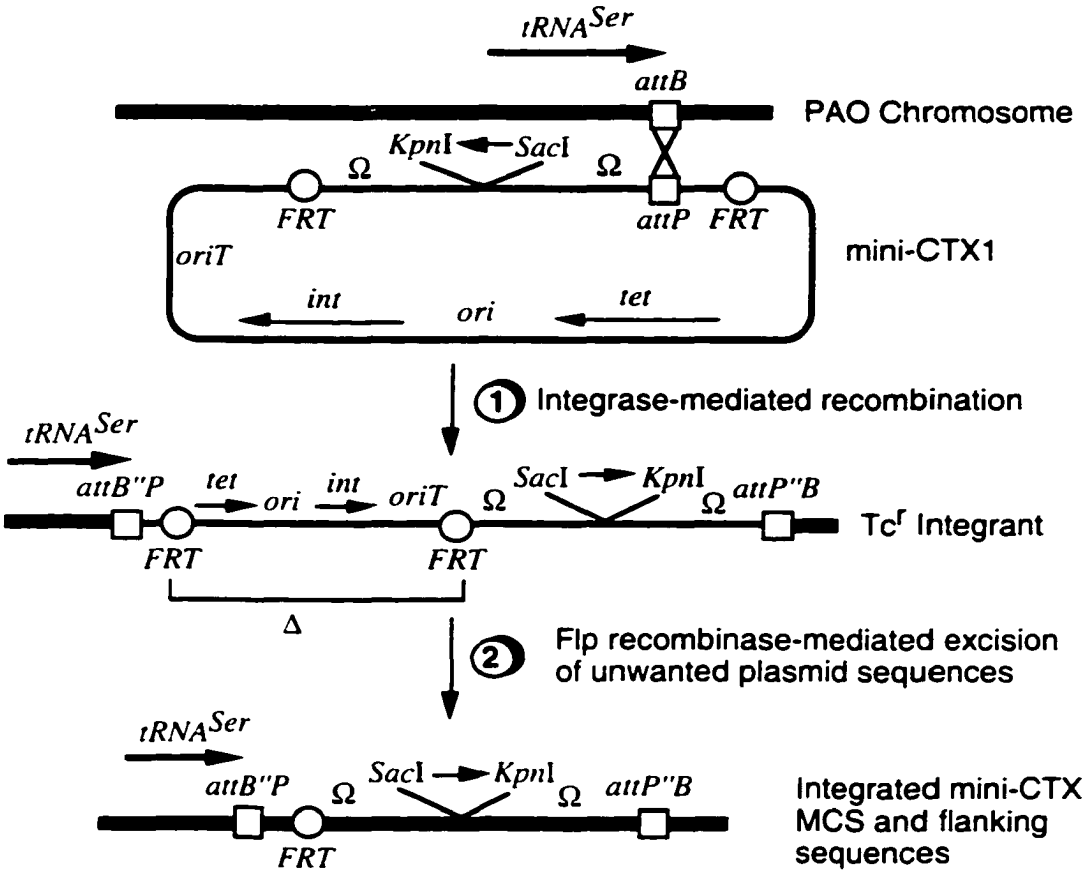


Figure 3.2. Mini-CTX-mediated integration at the *attB* site of *P. aeruginosa*. The two steps leading to isolation of unmarked integrants are illustrated. Step 1 depicts the integrase-mediated integration of the non-replicative mini-CTX plasmid at the *attB* site after transfer into a *P. aeruginosa* recipient strain. This event is selected for by plating on Tc-containing selective medium. Note that the $tRNA^{Ser}$ sequence is not interrupted by the integration event (see figure 3.3). Step 2 shows the Flp-mediated excision of plasmid sequences leading to removal of genes and associated promoter sequences that might interfere with expression of genes cloned into the MCS, especially gene fusions. *Sac*I --> *Kpn*I depicts the location and orientation of the mini-CTX1 MCS (for details see figure 3.1).

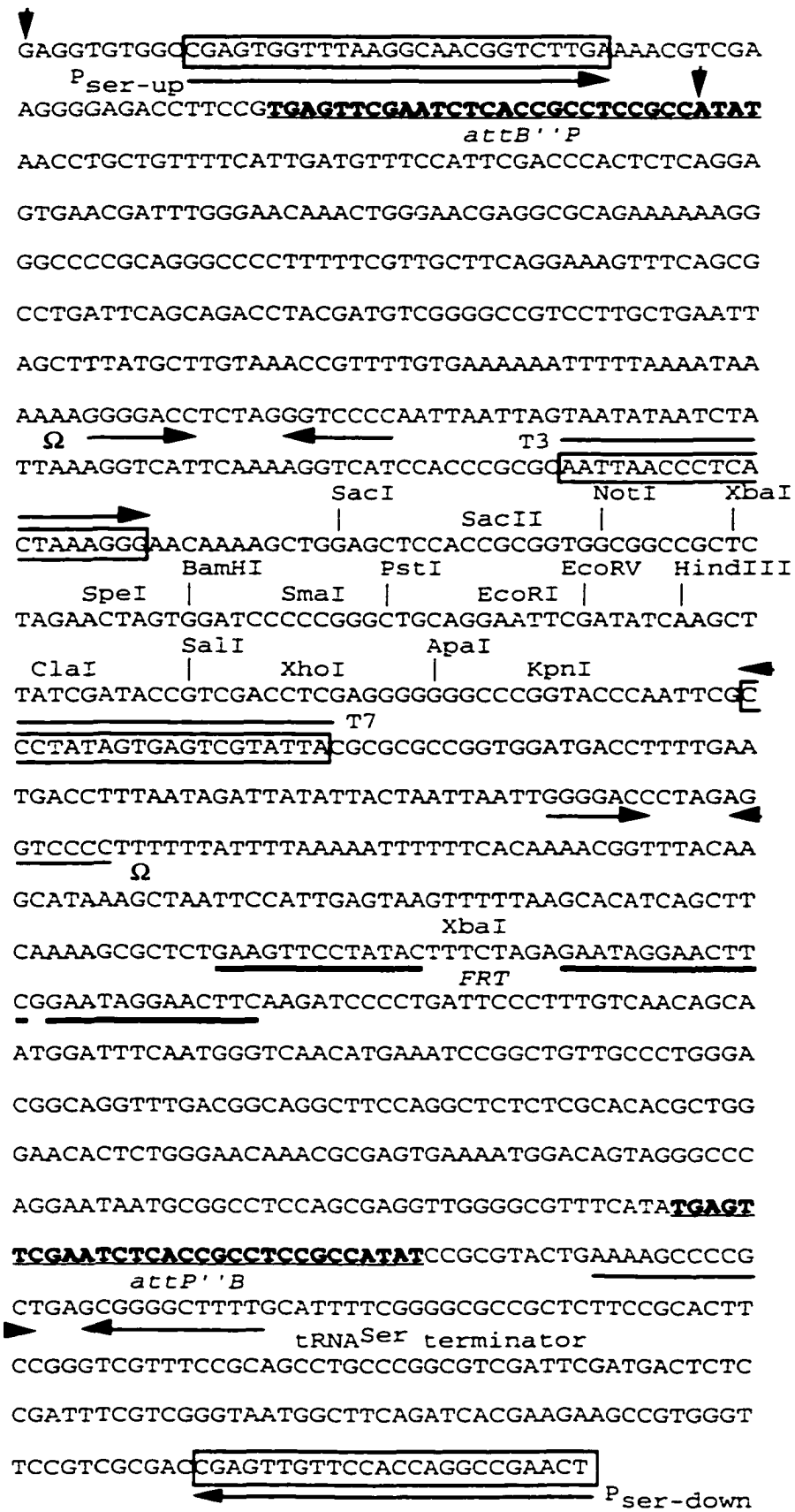
Table 3.2. Frequencies of mini-CTX chromosomal integration

	Frequencies of Tc^r integrants	
	- IPTG	+ IPTG
mini-CTX1	1.2×10^{-8}	ND[a]
mini-CTX2	1.46×10^{-7}	0.97×10^{-7}

[a] ND, not determined

3.4.3 Characterization of chromosomally-integrated mini-CTX sequences. In order to verify integration of mini-CTX1 sequences at the *P. aeruginosa attB* region, this region was PCR-amplified from chromosomal DNA isolated from wild-type PAO1 and several of the mini-CTX integrants after Flp-mediated excision by employing primers $P_{ser\text{-}up}$ and $P_{ser\text{-}down}$ (figure 3.3). Whereas these primers amplified a ~270-bp fragment from PAO1, they amplified a ~1,160-bp fragment from the integrants. This 1,164-bp PCR fragment was sequenced in its entirety (figure 3.3). As expected, the mini-CTX-derived sequences were integrated at the *attB* region, which is overlapping the end of the $tRNA^{Ser}$ gene. The mini-CTX MCS was flanked by the transcriptional terminators from the Ω element, and the T3 and T7 primer binding sites which were derived from pWSK29. The mini-CTX-derived sequences extend past the single *FRT* site end with the *attP/B* site. The sequences beyond *attP/B* contain the chromosomal $tRNA^{Ser}$ transcriptional terminator. The same analysis was performed with mini-CTX2 integrants and the same results were obtained (data not shown). These results confirmed the events depicted in figure 3.2 - i.e. that the mini-CTX sequences integrated at the *attB* region and that Flp recombinase removed the unwanted plasmid sequences.

Figure 3.3. Sequence of the *attB* region after mini-CTX1 integration and Flp-mediated excision. The integration vector mini-CTX1 was integrated and the plasmid-backbone was excised using Flp recombinase as depicted in figure 3.2. The *attB* region was PCR amplified from two integrants using the primers $P_{ser\text{-}up}$ and $P_{ser\text{-}down}$ (boxed) and the nucleotide sequences of the fragments were determined using the same primers except for 10 nucleotides upstream of $P_{ser\text{-}up}$ which were taken from GenBank accession number D13407 to illustrate the beginning of $tRNA^{Ser}$ whose sequence is delimited by vertical arrows. Primer binding sites are boxed and the directions of synthesis using these primers are indicated with arrows. Inverted arrows mark the locations of the T4 transcription termination signals (Ω) and the $tRNA^{Ser}$ terminator. Hybrid *attB''P* sequences are bold-faced and the *FRT* sequence is underlined. The restriction sites of the mini-CTX1 MCS are also marked.



3.4.4 Chromosomal integration and characterization of gene fusions. To test the feasibility of the mini-CTX system for construction of single-copy, chromosomally located gene fusions, we first integrated a *rhlA-lacZ* transcriptional fusion into wild-type PAO1. To test the effects of orientation in the chromosome on transcription, the *rhlA-lacZ* fusion was cloned as a *Eco*RI-*Dra*I fragment between the *Eco*RI-*Eco*RV or *Eco*RI-*Sma*I sites of mini-CTX1, yielding pPS1052 and pPS1053, respectively. Upon integration of these two plasmids into the *P. aeruginosa* chromosome and excision of the plasmid sequences, the fusion constructs were therefore either oriented in the same transcriptional orientation as $tRNA^{Ser}$ or in the opposite orientation, respectively (for orientation of the MCS see figure 3.3). When β–Gal activities were measured in the two fusion constructs and plotted as a function of cell-density, the two graphs were superimposed indicating that *lacZ* transcription is neither significantly influenced by the $tRNA^{Ser}$ promoter nor by any other chromosomally-located promoter (data not shown). The correct physiological response pattern of a *lasB-lacZ* fusion was assessed by integrating it into wild-type or into strains containing mutations in either PAI synthase I (Δ*lasI*), PAI synthase II (Δ*rhlI*) or both (Δ*lasI* Δ*rhlI*), and β–Gal expression was measured as a function of cell density (figure 4.4). In the *lasI*$^+$ *rhlI*$^+$ wild-type background, *lasB-lacZ* was expressed in the typical cell-density dependent manner. The *lasB-lacZ* fusion showed very low activity in the Δ*lasI* mutant and even less in the Δ*rhlI* mutant. This finding is consistent with previous studies that showed that *rhlI* insertion mutants showed very little elastolytic activity in plate assays as well as in elastase assays (18). As expected, no *lasB-lacZ* activity was present in the Δ*lasI* Δ*rhlI* mutant background. Although the *lasB-lacZ* fusion was oriented in the same orientation as $tRNA^{Ser}$, it showed barely detectable activity in the Δ*lasI* and Δ*lasI* ΔrhlI mutant backgrounds, indicating that the T4 transcriptional terminators effectively terminate transcription originating from the $tRNA^{Ser}$ gene promoter. When integrated into the same strains, a *rhlA-lacZ* fusion responded in a similar manner, although - as expected - its expression was not impaired in a Δ*lasI* mutant (data not shown).

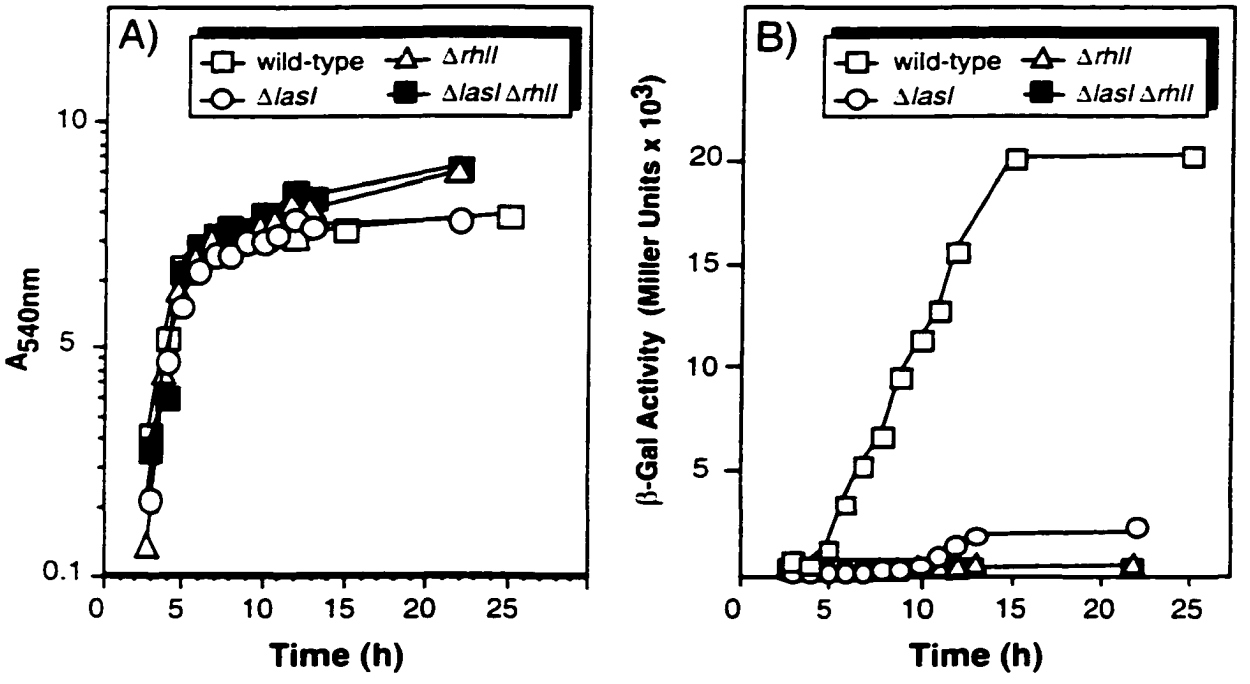


Figure 3.4. Activity of a *lasB-lacZ* transcriptional fusion integrated in various mutant strains. The indicated strains harboring the *lasB-lacZ* fusion were grown in PTSB medium. At the times shown, samples were withdrawn and after the A_{540nm} was read (panel **A**) β-Gal activities (panel **B**) were measured. Samples were assayed in triplicate and the *t* distribution was used to establish 95% confidence limits.

3.4.5 Stability of chromosomally-integrated sequences. For assessment of the stability of chromosomally integrated sequences in the absence of selective pressure, strain PAO230 (*lasB-lacZ*) was grown in LB medium for 100 generations at 37°C. When plated on LB + XGal indicator medium, all colonies tested after 100 generations were blue indicating the presence of the *lasB-lacZ* fusions. To verify the presence of the *lasB-lacZ* sequences, genomic DNA was isolated from ten Lac$^+$ isolates and hybridized with a probe spanning the *P. aeruginosa attB* site. The results shown in figure 3.5 show that all ten isolates tested still had the *lasB-lacZ* fusion integrated at the *attB* site, as indicated by the ~7.5-kb *Sph*I fragment seen in the digests of the mutant DNAs when compared to the ~3.4 kb *Sph*I fragment seen in the DNA derived from wild-type PAO1. This result demonstrated that the DNA segments integrated at the chromosomal *attB* site via the mini-CTX vector were stably maintained in the absence of selective presssure.

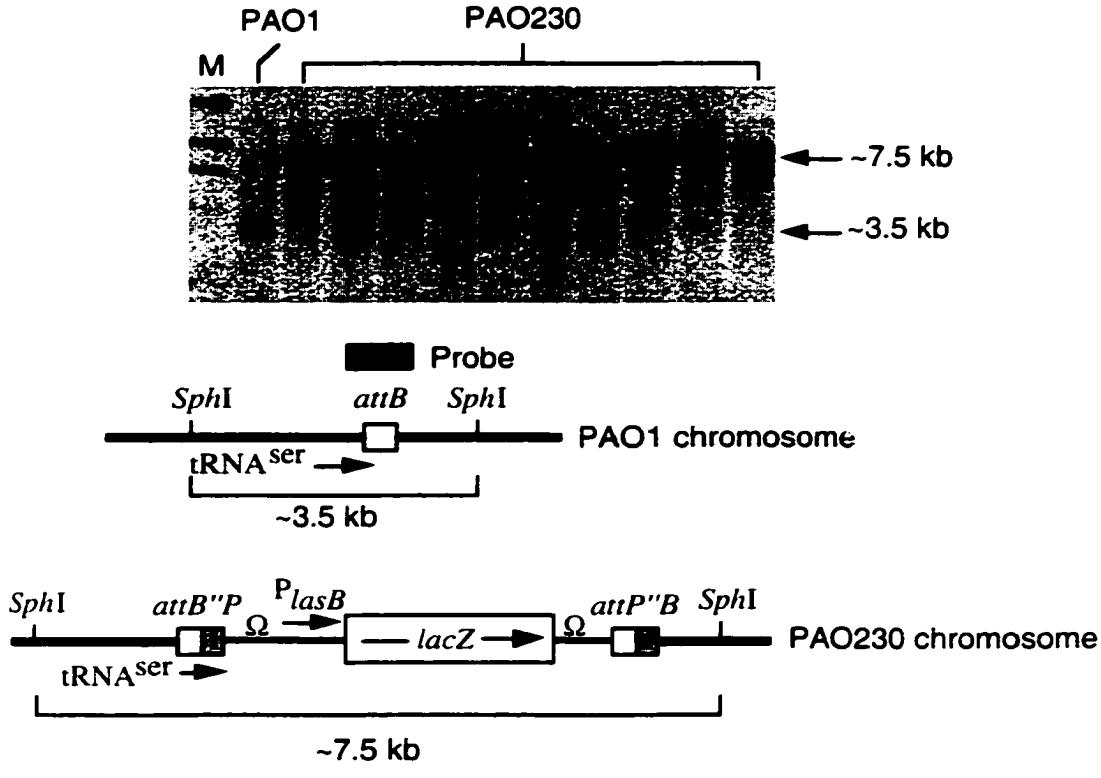


Figure 3.5. Stability of a *lasB-lacZ* fusion in the absence of selection. Nylon membranes containing electrophoretically separated genomic *Sph*I DNA fragments were probed with a biotinylated PCR fragment spanning the *attB* region. PAO1 denotes the lane containing DNA from wild-type and PAO230 denotes the lanes containing DNA from PAO1 *lasB-lacZ* fusion strains grown for 100 generations in the absence of selection. Lane M shows (top to bottom) biotinylated λ *Hin*dIII (23.1, 9.4, 6.6, 4.4 and 2.3 kb - the latter two are barely visible) markers.

3.4.6 Construction of a T7 polymerase-expressing *P. aeruginosa* host strain and expression of a fusion protein. To provide another example for the usefulness of the mini-CTX system, we constructed an expression cassette and integrated it into the *P. aeruginosa* genome. This cassette contains (i) a T7 *pol* gene which is transcribed from P_{lacUV5} and (ii) the *lacI* gene encoding Lac repressor for regulated expression of T7 *pol*. The cassette was cloned into mini-CTX2 and integrated into the genome of Δ*asd* strain PAO186, followed by Flp-mediated excision of the plasmid-backbone. The resulting strain PAO-T7 was transformed with pPS1049, a T7 promoter-based expression vector containing the *P.*

aeruginosa rhlI gene fused to a His6-encoding domain. As evidenced from figure 3.6, His6-RhlI expression was readily induced by addition of IPTG to the growth medium and no expression was detected in uninduced cultures. During the characterization of the cloned *lacI*-P_{lacUV5}-T7 *pol* segment, we discovered that the *lacI* gene was not described from the $lacI^q$ promoter but instead most likely from a promoter that was assembled during construction of pEB1 (3) or its precursors. Nonetheless, *lacI* transcription in the integrated cassette must be effcient as evidenced by tight regulation of *rhlI* gene expression in the absence of IPTG (figure 3.6).

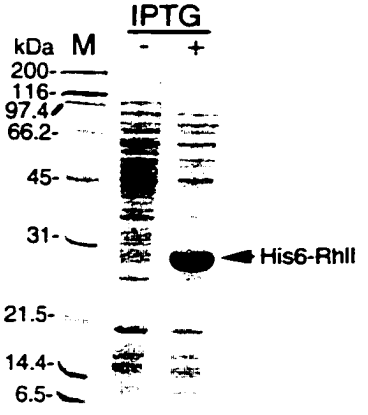


Figure 3.6. Expression of a His6-RhlI fusion protein in *P. aeruginosa*. Cells of PAO-T7 harboring the expression vector pPS1049 were grown in LB medium. When cells reached mid-log phase ($A_{540\ nm}$ ~ 0.8), the cultures were divided and either left uninduced (-IPTG) or were induced by addition of 1 mM IPTG (+IPTG). Growth was continued for another 3 h before whole cell extracts were prepared and separated by SDS-PAGE electrophoresis. The position of the His6-RhlI fusion protein is indicated. Lane M shows (top to bottom) molecular weight markers from Bio Rad Laboratories (myosin, β-Gal, phosphorylase b, bovine serum albumin, ovalbumin, cabonic anhydrase, trypsin inhibitor, lysozyme and aprotinin) and their sizes are given in kDa.

3.5 CONCLUSIONS

We have developed several integration-proficient vectors designed for efficient and stable integration of exogenous sequences at a defined location within the *P. aeruginosa* genome. The vectors include versatile MCS for cloning of DNA fragments. Because the ϕCTX *xis*

gene is not present in the integration vectors, integrated sequences are stably maintained in the absence of selection.

The usefulness of the integration vectors for gene fusion studies was demonstrated by constructing PAI-regulated *lasB-lacZ* and *rhlA-lacZ* fusions. When integrated in various defined PAI synthase mutants, these gene fusions showed the expected expression patterns. Unlike the parental multicopy vector-based fusions, the integrated, single-copy fusions showed no detectable background activities, irrespective of their orientations in the genome. A chromosomally-integrated *lasB-lacZ* fusion was shown to be stably maintained for 100 generations in the absence of selective pressure.

Though integration of mini-CTX plasmids containing chromosomal DNA fragments could also occur via RecA-mediated homologous recombination, we have not yet observed integration of many mini-CTX-based plasmids via merodiploid formation. This may be because Int-mediated integration of *attP*-containing plasmids is more efficient than RecA-mediated recombination of such plasmids at the homologous chromosomal loci, or because the *lasB-lacZ* and *rhlA-lacZ* segments tested in this study contained relatively short (500-600 bp) homologous chromosomal sequences. Nonetheless, we always check putative integrants via colony PCR utilizing the $P_{ser\text{-}up}$ and $P_{ser\text{-}down}$ primer pair, and combinations of these primers with primers designed to the integrated DNA sequences.

The general usefulness of these integration vectors for strain construction was demonstrated by construction of a cassette containing *lacI* and the T7 *pol* gene that allows for regulated expression from T7 promoter-containing bhr vectors in *P. aeruginosa* strains. Although we have previously described the construction and use of a similar expression cassette in *P. aeruginosa* for regulated expression from P_{tac}-containing plasmids, it was based on a transposable element that could only be randomly integrated into the genome (14). Similarly, the only other T7 promoter-based expression system described for *P. aeruginosa* was less userfriendly because it necessitated a two-plasmid system and antibiotic selection (3). We anticipate that the mini-CTX plasmids will facilitate engineering of various *P. aeruginosa* host strains for other expression systems that are currently only available for *E. coli*.

In conjunction with an *asd*-based expression vector and a Δ*asd* host strain, His6-RhlI could be expressed in a system that necessitated no antibiotic selection. This system may be

useful for large-scale expression of recombinant proteins or for expression of proteins in situations where antibiotic selection is not feasible. The newly described integration system will be especially useful for introducing β–Gal, GFP or other reporters during studies of biological systems in which plasmid constructs cannot be easily maintained by antibiotic selection, e.g. animal models or bacterial biofilms (6).

To facilitate construction of chromosomally-located, single-copy GFP and β–Gal transcriptional fusions, we have constructed the mini-CTX-*GFP* (GenBank accession number AF140578) and mini-CTX-*lacZ* (GenBank accession number AF140579) vectors. Both of these vectors are currently used by us and others for construction of single-copy, chromosomally-located *GFP* and *lacZ* fusions, respectively, and details will be presented elsewhere.

Preliminary results from our laboratory indicated that the *attP* based vectors may integrate into other *Pseudomonas* sp.. With appropriate modifications, they may thus be useful for the genetic engineering of strains destined for enviromental release. Since our integration vectors are equipped with *FRT* sites, they will allow engineering of strains eventually devoid of any selection marker, as is currently required for metabolic engineering of novel phenotypes for environmental applications and development of live vaccine strains (7,8).

3.6 ACKNOWLEDGMENTS

This work was supported by grant GM56685 from the National Institutes of Health and a grant from the Research Council of the College of Veterinary Medicine at Colorado State University to H.P.S. T.T.H. was supported by Student Traineeship HOANG99H0 from the American Cystic Fibrosis Foundation. We thank B.H. Iglewski and L. Passador for providing the *lasB-lacZ* fusion plasmid pLPBL, U. Ochsner for pUO58-24, and F. Lutz for p1000 and pIBH.

3.7 REFERENCES

1. **Albus, A. M., E. C. Pesci, L. J. Runyen-Janecky, S. E. H. West, and B. H. Iglewski.** 1997. Vfr controls quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. **179:**3928-3935.

2. **Brosius, J.** 1989. Superpolylinkers in cloning and expression vectors. DNA **8:**759-777.

3. **Brunschwig, E., and A. Darzins.** 1992. A two-component T7 system for the overexpression of genes in *Pseudomonas aeruginosa*. Gene **111:**35-41.

4. **Chen, H., H. Cheng, and M. Bjerkness.** 1993. One-step Coomassie Brillant Blue R-250 staining of proteins in polycarylamide gel. Anal. Biochem. **212:**295-296.

5. **Chippaux, M., J. Feutrier, M. Lepelletier, D. Touati-Schwartz, and M. C. Pascal.** 1982. Selection of *recA*$^+$ recombinant cosmids: an easy method for making *recA* strains temporarily Rec$^+$, permitting P1-mediated transduction in a *recA*$^-$ background and transduction of a *recA* mutation. Biochim. Biophys. Res. Commun. **106:**1269-1271.

6. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295-298.

7. **De Lorenzo, V.** 1994. Designing microbial systems for gene expression in the field. Trends Biotechnol. **12:**365-371.

8. **De Lorenzo, V., and K. Timmis.** 1992. Specialized host-vector systems for the engineering of *Pseudomonas* strains destined for environmental release, p. 151-174. *In* S. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas*: Molecular biology and biotechnology. Am. Soc. Microbiol. Washington, DC.

9. **De Lorenzo, V. and Timmis, K.N.** 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn*5* and Tn*10*-derived transposons. Methods Enzymol. **235**: 386-405.

10. **Dorman, C. J.** 1991. DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. Infect. Immun. **59:**745-749.

11. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77-86.

12. **Hoang,T., S. Williams, H.P. Schweizer and Lam, J.S.** 1997. Molecular genetic analysis of the region containing the essential *Pseudomonas aeruginosa asd* gene encoding aspartate-β-semialdehyde dehydrogenase. Microbiol. **143**: 899-907.

13. **Hoang, T. T., and H. P. Schweizer.** 1997. Fatty acid biosynthesis in *Pseudomonas aeruginosa*: cloning and characterization of the *fabAB* operon encoding β-hydroxydecanoyl-acyl carrier protein dehydratase (FabA) and β-ketoacyl-acyl carrier protein synthase I (FabB). J. Bacteriol. **179:**5326-5332.

14. **Karkhoff-Schweizer, R. R., and H. P. Schweizer.** 1994. Utilization of mini-D*lac* transposable element to create an α-complementation and regulated expression system for molecular cloning in *Pseudomonas aeruginosa*. Gene **140:**7-15.

15. **Linn, T., and R. St. Pierre.** 1990. Improved vectors system for constructing transcriptional fusions that ensures independent translation of *lacZ*. J. Bacteriol. **172:**1077-1084.

16. **Makowski, G. S., and M. L. Ramsby.** 1993. pH modification to enhance the molecular sieving properties of sodium dodecyl sulfate-10% polyacrylamide gel. Anal. Biochem. **212:**283-285.

17. **Miller, J. H.** 1972. Experiments in Molecular Genetics, Cold Spring Habor Laboratory Press, Cold Spring Habor, NY.

18. **Pearson, J. P., E. C. Pesci, and B. H. Iglewski.** 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systerms in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. **179:**5756-5767.

19. **Prentki, P. and Krisch, H.** (1984). In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29**: 303-313.

20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

21. **Schweizer, H., Sweet, G. and Larson, T.J.** 1986. Physical and genetic structure of the *glpD-malT* interval of the *Escherichia coli* K-12 chromosome. Mol. Gen. Genet. **202**: 488-492.

22. **Schweizer, H.P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6**: 1195-1204.

23. **Schweizer, H.P.** 1994. A method for construction of bacterial hosts for *lac*-based cloning and expression vectors: α complementation and regulated expression. BioTechniques **17**: 452-456.

24. **Schweizer, H. P., and T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:**15-22.

25. **Schweizer, H. P., T. R. Klassen, and T. Hoang.** 1996. Improved methods for gene analysis and expression in *Pseudomonas*, p. 229-237. *In* T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (ed.), Molecular biology of Pseudomonads. Am. Soc. Microbiol., Washington, DC.

26. **Silhavy, T. J., and J. R. Beckwith.** 1985. Uses of lac fusions for the study of biological problems. Microbiol. Rev. **49:**398-418.

27. **Silhavy, T.J., Berman, M.L. and Enquist, L.W.** 1984. Experiments with Gene Fusions, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

28. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy lac-based cloning vectors for protein and operon fusions. Gene **53:**85-96.

29. **Slauch, J. M., and T. J. Silhavy.** 1991. Genetic fusions as experimental tools. Methods Enzymol. **204:**213-248.

30. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, J. Jacobs, W.R., and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature **351:**456-460.

31. **Tabor, S.** 1995. Expression using the T7 RNA polymerase/promoter system. p. 16.2-16.9. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Short protocols in Molecular Biology, 3 ed., John Wiley & Sons, NY.

32. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195-199.

33. **Wang, Z., G. Xiong, and F. Lutz.** 1995. Site-specific integration of the PHICTX genome into the *Pseudomonas aeruginosa* chromosome:characterization of the functional integrase gene located close to and upstream of *attP*. Mol. Gen. Genet. **246:**72-79.

35. **West, S. E. H., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and the sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **128:**81-86.

35. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-119.

CHAPTER 4

Construction and use of low-copy number T7 expression vectors for purification of problem proteins: purification of *Mycobacterium tuberculosis* RmlD and *Pseudomonas aeruginosa* LasI and RhlI proteins, and functional analysis of purified RhlI

(Presented in Tung T. Hoang, Yufang Ma, Richard J. Stern, Michael R. McNeil and Herbert P. Schweizer. 1999. *Gene* **237**: 361-371)

This work established technologies for expression and purification of very insoluble proteins. The two *P. aeruginosa* autoinducer synthases were purified utilizing newly engineered expression strains and vectors. In addition, it demonstrated that the preferred substrates for the short chain autoinducer synthase (RhlI) are derived from the fatty acid biosynthetic pathway. I thank Dr. Yufang Ma for amplifying and cloning of RmlD, Dr. Richard Stern for help with the engineering of an expression strain and Dr. Michael McNeil and for assistance with GC-MS.

4.1 ABSTRACT

Purification of proteins from *Escherichia coli* under native conditions is often hampered by inclusion body formation after overexpression from T7 promoter-based expression vectors. This is probably due to the relatively high-copy number of the ColE1-based expression vectors. To circumvent these problems, the low-copy number pViet and pNam expression vectors were constructed. These vectors contain the pSC101 origin of replication and allow the expression of oligohistidine and intein chitin-binding domain fusion proteins, respectively. Since pViet and pNam do not replicate in *E. coli* B strains, an *E. coli* K-12 host

strain [SA1503(DE3)] was constructed. This strain is defective in the Lon and OmpT proteases and allows IPTG-inducible expression of recombinant proteins from the T7 promoter. The new vectors were successfully tested by purification of three very insoluble proteins (RmlD, LasI and RhlI) under non-denaturing conditions, and all three proteins retained enzymatic activity. The purified hexahistidine (His6)-tagged *Pseudomonas aeruginosa* RhlI protein was subjected to more detailed analyses which indicated that (i) only butyryl-acyl carrier protein (ACP) and *S*-adenosylmethionine (SAM) were required for synthesis of *N*-butyryl-L-homoserine lactone; (ii) when present at physiological concentrations, butyryl-coenzyme A and NADPH were not substrates for RhlI; (iii) RhlI was able to synthesize *N*-hexanoyl-L-homoserine lactone from hexanoyl-ACP and SAM; (iv) RhlI was able to direct synthesis of *N*-butyryl-L-homoserine lactone from crotonyl-ACP in a reaction coupled to purified *P. aeruginosa* FabI (enoyl-ACP reductase).

4.2 INTRODUCTION

With the advent of genomic sequencing (for a comprehensive listing of finished and unfinished microbial genomes see the TIGR web site at www.tigr.org), functional analyses of many annotated and unknown proteins in the database are essential to confirm or identify the role of these proteins. Often, an important step toward achieving these goals is the construction and use of expression vectors for obtaining purified and functional proteins. Having obtained the genomic sequence, this can easily be done by designing primers, followed by PCR amplification and cloning of the gene of interest into various expression vectors that allow subsequent purification via affinity chromatography, e.g. Ni^{2+} agarose chromatography of oligohistidine-tagged proteins (32) or chitin agarose chromatography of proteins containing fused CBDs (6). The promoter of choice in the expression vectors is often the T7 RNA polymerase-dependent T7 promoter (32). With the complete sequence of *M. tuberculosis* (7) available and the *P. aeruginosa* genome sequence nearing completion, we have been interested in purifying various proteins by overexpression in *E. coli* to study their function in the respective pathways.

In an effort to define the pathways for AHL biosynthesis in *P. aeruginosa* and L-rhamnose biosynthesis in *M. tuberculosis*, our laboratories began purifying proteins involved in these pathways by overexpression in *E. coli*, utilizing various commercially available T7 promoter-based expression systems, including the pET vectors from Novagen (Milwaukee, WI), allowing expression and purification of His6-tagged fusion proteins, and the pTYB vectors from New England Biolabs (Beverly, MA), which allow expression and purification of untagged proteins via intein-CBD-tagged fusion proteins. Whereas many of these T7 promoter-based (32) expression systems have been successfully used by us for expression and purification of several *M. tuberculosis* and *P. aeruginosa* recombinant proteins in *E. coli*, overexpression of some proteins in these systems presented significant problems with inclusion-bodies, presumably resulting by expression from these relatively high-copy number vectors. When present in inclusion bodies, most of the expressed proteins were lost by co-pelleting with other cellular debris during centrifugation of the cell lysates. Although some of the proteins could be solubilized, recovered and purified from the inclusion bodies (27), they had little to no activity. To solve the insolubility problem, we attempted various recommended conditions to reduce the level of expression, including expression for shorter time and expression at room temperature, reducing the levels of the inducer IPTG, and adding up to 10% glycerol or 0.5% Triton X-100 to the cells before lysis. Despite many repeated attempts, we failed to purify active *M. tuberculosis* dTDP-L-rhamnosyl synthase RmlD and the two *P. aeruginosa* AHL synthases, LasI and RhlI, under non-denaturing conditions, using either pET-15b and pET-16b from Novagen or pTYB1 from New England Biolabs. We attributed at least part of this problem to the high copy number of the expression vectors. Typically, the T7-promoter based expression vectors contain the ColE1-derived replicon and therefore each cell will harbor from 20 to >100 plasmid copies. Upon induction with IPTG of the commonly used *E. coli* B host strain BL21(DE3), sufficiently large amounts of T7 RNA Pol are expressed to transcribe the inserts of most, if not all, of these plasmids.

During our attempts at RhlI purification, we confirmed previous observations by Jiang et al. (15) who had shown that RhlI becomes insoluble when overexpressed in a pET system. These authors were only able to purify RhlI in inclusion bodies and obtained protein with

little activity, as evidenced by the necessity for adding large amounts (>8 mg) of RhlI protein to their assays. Other laboratories have reported the purification of two AHL synthases, LuxI from *V. fischeri* (29) and TraI from *A. tumefaciens* (22, 29), but only as fusions to maltose binding protein and in the presence of strong denaturants, i.e. urea or guanidine hydrochloride, respectively. Parsek et al. (24) recently reported the purification of active recombinant RhlI from its native host, *P. aeruginosa*, but the yield was rather low. Although functional proteins can be obtained using these purification methods, they are not applicable to all proteins and the results may be variable for some proteins.

In this study, we successfully purified three otherwise highly insoluble proteins under native conditions, by expression from the pSC101-based low-copy number plasmid pViet in our newly engineered *E. coli* K-12 host strain SA1503(DE3).

We utilized the purified His6-tagged RhlI to address some controversial issues regarding RhlI substrates and co-factor dependency that may have arisen from experiments utilizing protein derived from inclusion bodies. Previous studies by other laboratories with AHL synthases from various bacteria have indicated that the respective enzymes only require an acyl donor, most likely acyl groups carried by ACP, and SAM as a source of the HL moiety (22, 29, 33). In a recent report, Jiang et al. (15) claimed that the *P. aeruginosa* BHL synthase, RhlI, was an exception in that it could utilize butyryl-CoA as the acyl donor and that NADPH was required for this reaction. These results were subsequently disputed by using RhlI purified from *P. aeruginosa* (24). To further address the substrate requirements of RhlI, we tested His6-tagged RhlI purified from *E. coli* with physiologically relevant concentrations of various substrates and co-factors, and showed that only SAM and butyryl-ACP are required for BHL synthesis. In addition, RhlI catalyzed the synthesis of HHL from hexanoyl-ACP and SAM. When coupled to purified enoyl-ACP reductase (FabI), RhlI was able to direct synthesis of BHL from crotonyl-ACP, indicating that the crotonyl-ACP derived from malonyl-ACP and acetyl-CoA via the fatty acid biosynthetic pathway is the most likely substrate for RhlI.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strains, plasmids, media and culture conditions. The bacterial strains and plasmids used in this study are listed in table 4.1. Strains DH5α and JM109 were used as the routine cloning hosts. LB medium (Difco, Detroit, MI) was the rich medium used in all experiments. Ampicillin (Ap; Sigma, St. Louis, MO) was added to liquid and solid media at 100 μg/ml. Routine expression for each protein was done by inoculating 0.5 to 1 l of LB + Ap broth with 5 to 10 ml of an overnight culture grown at 37°C. The new cultures were grown at 37°C with shaking at 220 rpm to an A_{600nm} of 0.5-0.8 (~3 h). The cultures were then transferred to RT (~25°C) and shaken for another 10 to 15 min before addition of IPTG to final concentrations of 0.25 to 0.7 mM. Expression was allowed to continue at RT for 6 to 8 h.

4.3.2 DNA manipulations and vector constructions. Plasmid DNA was prepared using Qiaprep Spin Miniprep columns or Qiafilter Plasmid Midi columns (Qiagen, Santa Clarita, CA). Restriction enzymes, dNTPs, T4 DNA Pol, T4 DNA ligase and primers were purchased from Gibco-BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA) and used according to the manufacturer's recommendations. *Taq* Pol was purchased from Qiagen. Previously described procedures were used for preparation of *P. aeruginosa* and *M. tuberculosis* chromosomal DNAs (2, 11), and for performing DNA transformations (10, 18). DNA fragments were purified from agarose using the Geneclean III procedure (BIO 101, San Diego, CA).

The expression vectors pViet and pNam were constructed in three steps (figure 4.1). First, the 2.1 kb *Nru*I-*Ssp*I fragment of pET-15b, containing the T7 promoter, the His6-tag region with cloning sites, the T7 transcription terminator and the *lacI* gene, was ligated to the 3.2 kb *Ssp*I fragment of pWSK29 (36), containing the pSC101 *ori* (21, 35) and the *bla* gene to yield pET-101 (figure 4.1, step 1). Second, the 3.3 kb *Nde*I-*Bgl*II fragment, blunt-ended with T4 DNA Pol (containing the pSC101 origin of replication and the *lacI* gene) and the 1.7 kb *Bgl*II fragment, also blunt-ended with T4 DNA Pol (containing the T7 promoter, His6-tag region, T7 transcription terminator and the *bla* gene) of pET-101 were individually isolated.

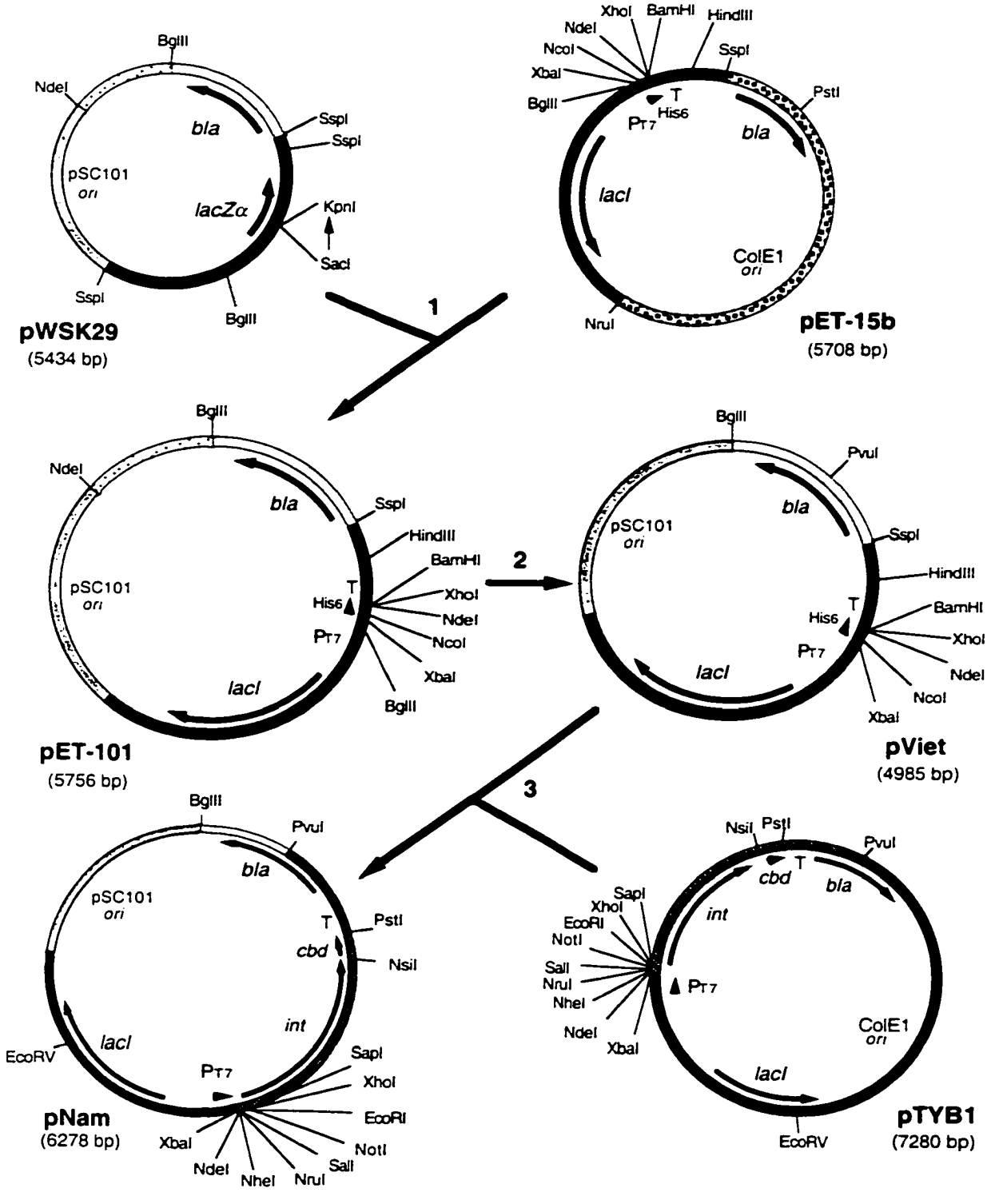
Table 4.1. Bacterial strains and plasmids

Strains or Plasmids	Relevant properties	Reference or source
E. coli		
AD202	*araD139* Δ(*argF-lac*) *ompT*::Km^r *flhD5301* *fruA25 relA1 rpsL150 rbsR22 deoC1*	(1)
BL21(DE3)	{λ(DE3)} F$^-$ *ompT hsdS$_B$*, ($r_B^-m_B^-$) *dcm gal*	(32)
DH5α	{Φ80Δ*lacZ*ΔM15} Δ(*lacZYA-argF*)U169 *recA1* *endA1 hsdR17* [$r_K^-m_K^+$] *supE44 thi-1 gyrA relA1*	Gibco-BRL
JM109	[F', *traD36*, *proAB*, *lacI^q*] *endA1 recA1 gyrA96 thi* *hsdR17* (r_K^-, m_K^+) *relA1 supE44* Δ(*lac-proAB*)	(38)
SA1500	*lon-100*(del?) λ$^-$ *his-87 relA1 rspL181* *spoT1 thi-1*	(9)
SA1503	*lon-100*(del?) λ$^-$ *his-87 ompT*::Km^r *relA1* *rspL181 spoT1 thi-1*	This study
SA1503(DE3)	{λ(DE3)} *lon-100*(del?) *his-87 ompT*::Km^r *relA1* *rspL181 spoT1 thi-1*	This study
Sφ874	Δ(*sbcB-rfb*)	(23)
Sφ874(DE3)	{λ(DE3)} Δ(*sbcB-rfb*)	This study
C. violaceum		
CV026	mini-Tn*5* mutant of ATCC strain 31532	(20)
P. aeruginosa		
PAO1	prototrophic	(14)

Table 4.1. Bacterial strains and plasmids (cont.)

Strains or Plasmids	Relevant properties	Reference or sources
M. tuberculosis		
H37Rv	virulent	ATCC
Plasmids		
pET-15b	Ap^r; NH_2-terminal His6 tag vector	Novagen
pET-16b	Ap^r; NH_2-terminal His6 tag vector	Novagen
pTYB1	Ap^r; intein-CDB-based expression vector for purification of untagged proteins	New England-Biolabs
pWSK29	Ap^r; pSC101-based low-copy number cloning vector	(36)
pET-16b-*rmlD*	Ap^r; PCR-amplified *Nde*I-*Xho*I *rmlD* fragment cloned into *Nde*I + *Xho*I cut pET-16b	This study
pViet	Ap^r; low-copy number His6 tag expression vector	This study
pNam	Ap^r; low-copy number intein-CDB expression vector	This study
pViet-*rmlD*	Ap^r; *Nde*I-*Xho*I *rmlD* fragment of pET-16b-*rmlD* subcloned into pViet cut with the same enzymes	This study
pPS1074	Ap^r; PCR-amplified *Nde*I-*Sap*I *rhlI* fragment cloned into pNam cut with the same enzymes	This study
pPS1077	Ap^r; PCR-amplified *Nde*I-*Bam*HI *lasI* fragment cloned into pViet cut with the same enzymes	This study
pPS1079	Ap^r; PCR-amplified *Nde*I-*Bam*HI *rhlI* fragment cloned into pViet cut with the same enzymes	This study

Figure 4.1. Construction of pViet and pNam. Details on the constructions of these plasmids are given in chapter 4.3.2. Step 1: replacement of a 2.1-kb *Nru*I-*Ssp*I fragment of pET-15b (containing the ColE1-derived *ori*) with a 3.2-kb *Ssp*I fragment from pSC101 (containing the pSC101-derived *ori*). Step 2: deletion of the *Bgl*II-*Nde*I fragment from pET-101 (to ensure a unique *Nde*I site in the MCS). In the process of ligating the blunt-ended *Nde*I and *Bgl*II ends, one *Bgl*II site was regenerated, and orientation of the *bla* gene and the His6 tag with respect to *lacI* was confirmed by restriction digests. Step 3: subcloning of the intein-CBD region from pTYB1 on a 2.3-kb *Pvu*I-*Xba*I fragment. Only important restriction sites are indicated for each plasmid. Plasmid segments are patterned for easier distinction of the derivation of individual fragments, but are not drawn to scale. The *Sac*I->*Kpn*I MCS on pWSK29 is equivalent to the one found on pBluescript-SK (Stratagene, La Jolla, CA). Abbreviations: *bla*, β-lactamase encoding gene; *cbd*, gene encoding the CBD from *Bacillus circulans*; His6, His6 tag-encoding region from pET-15b; *lacZ*α, gene encoding α-peptide of β-galactosidase; *lacI*, gene encoding *lac* repressor; *ori*, origin of replication; *int*, intein-encoding gene from *Saccharomyces cerevisiae*; P_{T7}, phage T7 gene 10 promoter; T, T7 transcription terminator. The nucleotide sequences of pNam and pViet were deposited in GenBank and assigned the accession numbers AF147463 and AF147464, respectively.



These fragments were then ligated together to give pViet, eliminating the small *Nde*I-*Bgl*II fragment of pET-101 (figure 4.1, step 2). The 2.3 kb *Pvu*I-*Xba*I fragment of pTYB1, containing the MCS and intein-CDB and T7 transcription terminator, was subcloned into the 4 kb *Pvu*I-*Xba*I fragment of pViet to yield pNam.

The *M. tuberculosis rmlD* gene and the *P. aeruginosa lasI* and *rhlI* genes were amplified from genomic DNA templates by PCR. For amplification of *rmlD*, two primers (rs83, 5'-GGAATTCCATATGGCGGGCAGGTCAGAA and rs112, 5'-CCGCTCGAGT CAGTC ACGCGTTGAGGGTAA) introducing *Nde*I and *Xho*I sites (underlined), respectively, were synthesized. PCR reactions contained in a 50 µl reaction mixture 1x *Taq* buffer (Qiagen), 200 µM of each dNTP, 30 pmol of each primer, 0.5 µg of genomic *M. tuberculosis* H37Rv DNA and 2.5 u *Taq* polymerase. The reaction mixture was subjected to the following thermal cycles: 1 cycle at 94°C for 5 min; 35 cycles (94°C, 1 min; 58°C, 25 s; 72°C, 1 min) and a final extension at 72°C for 10 min. The PCR product was gel-purified, digested with *Nde*I + *Xho*I and ligated into pET-16b to yield pET-16b-*rmlD*. For amplification of the *P. aeruginosa lasI* and *rhlI* genes, mutagenic primers were designed that would introduce *Nde*I sites at the respective ATG initiation codons and *Bam*HI sites downstream of the respective coding regions. The primer sets were lasIU, 5'-GTGCATATGATCGTACAAATTGGTC GGCG (the *Nde*I site is underlined) and lasID, 5'-ACA GGATCCCCGTCATGAAACCGC CAG (the *Bam*HI site is underlined) for *lasI*, and rhlI-NdeI, 5'-CACGGGGACTTGCATATG ATCGAATT (the *Nde*I site is underlined) and rhlID, 5'-AGGGATCCTCACACCGCCATC GACAGCGGT (the *Bam*HI site is underlined) for *rhlI*. PCR conditions were: 1 cycle at 96°C for 2 min; 35 cycles (95°C, 1 min; 58°C, 25 s; 72°C, 45 s) and a final extension at 72°C for 10 min. Individual PCR fragments were gel-purified, digested with *Nde*I + *Bam*HI and ligated into pViet to yield pPS1077 and pPS1079, respectively. Plasmid pPS1074 was constructed by PCR amplifying the *rhlI* gene from PAO1 chromosomal DNA, using two primers, the rhlI-NdeI primer described above and rhlI-Sap2 5'-ACGGCGCTCTTCCGCACACCGCCATCG ACAGCGGTAC (introducing the underlined *Sap*I site). The PCR fragment was then purified, digested with *Nde*I + *Sap*I and cloned into pNam cut with the same enzymes.

4.3.3 Strain constructions. *E. coli* SA1503(DE3) was engineered for the expression of genes from pViet and pNam. A P1 lysate (30) was prepared of *E.coli* K-12 strain AD202 and used to transduce the *ompT*::Km^r mutation into strain SA1500, which already contains the *lon*-100 mutation. The resulting strain SA1503 was lysogenized with λDE3 using the λDE3 lysogenization kit from Novagen (Milwaukee, WI) to yield SA1503(DE3). Strain Sϕ874 was also lysogenized with λDE3 using the same kit.

4.3.4 Purification of RmlD, LasI and RhlI. Protein purification was done as previously described (27). Briefly, cells from a 1 l induced culture of Sϕ874(DE3)/pViet-*rmlD* (RmlD), SA1503(DE3)/pPS1077 (LasI) or SA1503(DE3)/pPS1079 (RhlI) were harvested and resuspended in 10 ml of 20 mM Tris-HCl, 0.5 M NaCl, pH 7.9 (MCAC-0 buffer), with 1 mM PMSF (Sigma, St. Louis, MO). Cell suspensions were lysed by 2 to 3 passes through a pre-chilled French Press minicell at 19,000 psi. Cell lysates were ultracentrifuged at 260,000 x g (1 h, 4°C) and the supernatants were applied to columns with 1.5 ml bed volumes of Ni^{2+}-NTA agarose resin from Qiagen, which had been equilibrated with 5 bed volumes of MCAC-0 buffer. The columns were washed with a minimum of 15 bed volumes of MCAC-0 buffer, and then with a minimum of 15 bed volumes of MCAC-0 buffer containing 40 mM imidazole (Sigma). Proteins were eluted with three bed volumes of MCAC-0 containing 200 mM imidazole, and 1 ml aliquots were collected. Glycerol was added to between 15 and 30% (v/v), depending on the enzyme, before small aliquots were frozen at -70°C for future use. Although washing with 40 mM imidazole was the standard for most of our proteins, the actual concentration may have to be adjusted 50 mM for other proteins. All columns steps were performed at 4°C.

For purification of an untagged RhlI protein using the intein-CBD system, a 500 ml SA1503(DE3)/pPS1074 culture was grown in LB + Ap medium, induced and a clarified lysate was prepared as described above. Untagged RhlI was eluted from a chitin agarose column using the cleavage buffer (20 mM Tris-HCl at pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 40 mM DTT) recommended by the manufacturer (New England Biolabs), but contained 0.4% (v/v) Triton X-100 and 5% glycerol.

4.3.5 Protein methods. For protein analysis, whole cell extracts of uninduced and IPTG-induced cultures were prepared as previously described (16). Protein concentrations were determined using the Bradford dye-binding assay (Bio Rad Laboratories, Hercules, CA), using bovine serum albumin as the standard. Proteins were analyzed by electrophoresis on 0.1% SDS-10% PAGE as previously described (19) and visualized by quick staining with Coomassie Brilliant Blue R-250 (5).

4.3.6 AHL in vitro synthesis reactions. *P. aeruginosa* ACP was purified as previopusly described (17; chapter 5). Acyl-ACPs were synthesized by incubation of ACP with butyric-anhydride, crotonic-anhydride or hexanoic-anhydride (Sigma) to obtain butyryl-ACP, crotonyl-ACP or hexanoyl-ACP, respectively (12, 37). The acyl-ACPs were quantitated using the Ellman reagent (3, 12). The purification and characterization of the *P. aeruginosa* FabI was described elsewhere (12).

RhlI reactions (500 µl) contained buffer (22) [10 mM Tris-HCl, pH 7.4, 330 mM NaCl, 15% glycerol, 0.7 mM DTT, 2 mM EDTA, 25 mM $MgSO_4$, 0.1 mM $FeSO_4$] and, unless indicated otherwise, contained 10 µM of acyl-CoA or acyl-ACP, 100 µM SAM, 200 µM NADPH and 1 µg of RhlI. The RhlI-FabI coupled reactions also contained 200 µM NADH and 1 µg of FabI. Reactions were carried out for 1 h at 37°C, stopped by addition of 250 µl of ethyl acetate and extracted three times with 250 µl of ethyl acetate. The ethyl acetate was then evaporated by rotatory vacuum evaporation. The residues were dissolved in 10 µl of acetonitrile and 3 µl aliquots were spotted onto reverse-phase C_{18} TLC plates (Whatman, Clifton, NJ). 50 pmoles of BHL or 10 pmoles of HHL standards were applied to each TLC plate as positive controls. When necessary, TLC plates were developed in 60% (v/v) methanol in water to separate BHL and HHL as previously described (15). For detection of AHLs, 5 ml of an overnight culture of strain CV026 grown at RT in LB medium was diluted into 50 ml of 0.3% LB agar kept at 45°C and the agar mixture was used to overlay the dried TLC. After solidification of the overlay, the TLC plate was incubated at RT in a wet box until spots were clearly discernable. The presence of AHLs in the extracted reactions was scored by appearance of a violet spot (20).

HPLC and GC-MS were used to confirm the identity of BHL. Large scale (10 ml) reactions were performed in Moré buffer, 10 μM butyryl-ACP, 100 μM SAM, 200 μM NADH and 1 μg RhlI. The samples were extracted three times with 0.5 volumes of ethyl acetate and dried as described above. C_{18} reverse-phase HLPC was utilized with a 10-60% (v/v) methanol-water gradient to fractionate BHL from other components of the reaction. HPLC fractions were spotted on TLC and the presence of BHL in the fractions was detected using the CV026 overlay method as described above. BHL-containing fractions were pooled and subjected to HPLC developed isocratically with 48% methanol. Fractions were again analyzed using the CV026 bioassay, and samples containing the most BHL were then subjected to positive-ion GC-MS using a Hewlett-Packard HP5970B mass selective detector as described previously (25).

4.4 RESULTS AND DISCUSSION

4.4.1 Construction of low-copy number expression vectors and host strains. To construct low-copy number expression vectors, we equipped the pET-15b and pTYB1 vectors with the pSC101 replicon. This replicon was chosen (i) because it is well characterized (21, 35), (ii) because plasmids containing this replicon can be manipulated using standard procedures, even though the copy number is only ~5 (36), and (iii) because Sankar et al. (28) had suggested that low-copy number T7 expression vectors helped minimize toxic effects of proteins expressed in *E. coli*. The resulting pViet and pNam expression vectors (figure 4.1) still contain all the features of the parental plasmids including unique MCS that allow in-frame fusions of target proteins to His6 or intein-CBD domains, and the T7 transcription terminator for increased plasmid stability. The decreased copy number of the new vectors leads to decreased β-lactamase production, thus minimizing destruction of the Ap in the growth medium which can pose selection problems when using high-copy number T7 expression vectors (32). Although pSC101-based plasmids, including the parental pWSK29 vector, normally replicate in *E. coli* B strains such as BL21(DE3)(28), we noted that our pSC101-based vectors no longer replicated in this commonly used expression strain, presumably due to deletion of a region that is essential for replication in this strain. This

necessitated engineering of a new *E. coli* K-12 host strain, SA1503(DE3) (table 4.1). This was achieved in two steps. First, an *ompT* mutation was transduced into the *lon* strain SA1500 to derive the *ompT lon* strain SA1503. Next, the strain SA1503 was lysogenized with λDE3 to derive the T7 expression strain SA1503 (DE3). By lysogenization with λDE3, expression strains can be tailored to individual needs, e.g. we constructed Δ(*sbcB-rfb*) strain Sφ874(DE3) (table 4.1) as a host for purification of the mycobacterial RmlD to minimize potential contamination with the *E. coli* homologue.

4.4.2 Expression and purification of His6-tagged RmlD, LasI and RhlI, and untagged RhlI. To evaluate the new expression vectors, they were used to express and purify the *lasI*, *rhlI* and *rmlD* gene products. These genes encode proteins that are extremely insoluble when overexpressed in *E. coli* and lose activity when purified from inclusion bodies (15) (our observations) using standard procedures (27).

When the resulting His6 expression vectors pPS1077 and pPS1079 were transformed into SA1503(DE3) and pViet-*rmlD* into Sφ874(DE3), the LasI, RhlI and RmlD His6 fusion proteins were expressed to significant levels following IPTG induction (figure 4.2, panel A). When necessary, the modest level of leakyness observed under uninduced conditions with some constructs, e.g. RhlI (figure 4.2A), could be controlled by transforming SA1503(DE3) with pLysS or pLysE, plasmids expressing different levels of the T7 RNA polymerase inhibitor lysozyme (32). All three His6-tagged proteins were successfully purified from cell extracts by Ni^{2+} agarose affinity chromatography under native conditions (figure 4.2B), although modest inclusion body formation was sometimes still observed, mainly with RhlI. When necessary, inclusion body formation in at least the *lacY*$^+$ strains could be further minimized by reducing IPTG levels in the presence of glucose to inhibit expression of the chromosomal *lacY* gene by catabolite repression. Upon elution from the column, the proteins precipitated within 3 h at 4°C, unless glycerol was added to between 15 and 30% (final concentration). After addition of glycerol immediately following elution, small aliquots of each protein could be stored at -70°C for extended periods of time without evidence of precipitation. The yield for these proteins ranged from 0.6 to 1.2 mg per 1 l of culture after 6-8 h of induction at RT. We obtained 1 mg of RhlI from 1 l of *E. coli* culture,

which is a great improvement over the 0.36 mg of RhlI obtained from 1 l of *P. aeruginosa* culture (24).

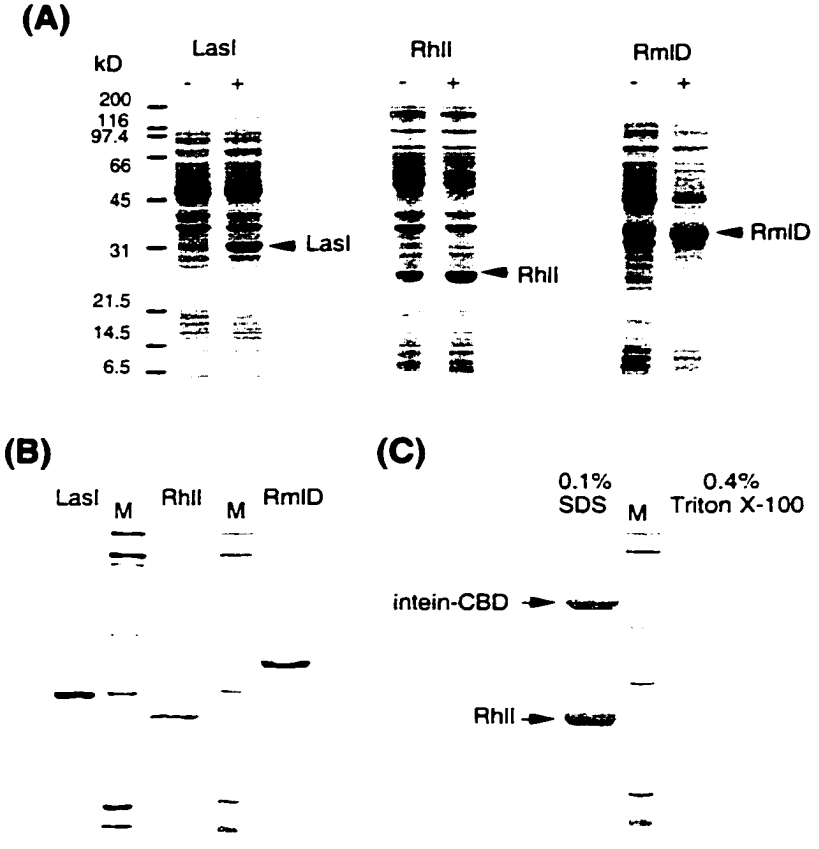


Figure 4.2. Expression and purification of LasI, RhlI and RmlD. LasI and RhlI were expressed in SA1503(DE3) and RmlD was expressed in Sϕ874(DE3). Protein patterns were analyzed by electrophoresis on 0.1% SDS-10% PAGE and proteins were stained with Coomassie Blue. **(A)** Total cellular proteins in cell extracts from uninduced (-) versus IPTG induced (+) cultures containing a plasmid expressing the respective protein. The relative positions of broad-range protein markers [from Bio-Rad (Hercules, CA)] are indicated on the left in kDa and the proteins were (top to bottom) myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme, aprotinin; the same markers were used in lanes marked M in panels **(B)** and **(C)**. **(B)** Purified His6-tagged LasI, RhlI, and RmlD. Approximately 1 μg of each protein was loaded. **(C)** SDS and 0.4% Triton X-100 eluted fractions of RhlI from chitin agarose columns. SDS elutes all proteins, including the intein-CBD fusion protein and untagged RhlI. Triton X-100 elutes untagged RhlI only.

We also assessed the use of pNam as an intein-CBD expression vector but concentrated on RhlI. After transformation of SA1503(DE3) with pPS1074, expression of

RhlI-intein-CBD was induced with IPTG and cell extracts were prepared. RhlI-intein-CBD was efficiently bound by the chitin agarose column. However, cleavage and elution of RhlI could not be achieved with the recommended cleavage buffer (20 mM Tris-HCl at pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 40 mM DTT) unless it was supplemented with 0.4% Triton X-100 and 5% glycerol. Some RhlI eluted from the column in the presence of Triton X-100 and glycerol (figure 4.2C); it was active and removal of the nonionic detergent was unnecessary since it did not affect the enzyme's activity (data not shown). The low yield of RhlI was most likely not due to inefficient DTT-mediated cleavage of the RhlI-intein-CBD since elution with 0.1% SDS yielded large amounts of RhlI (figure 4.2C). Because purified His6-tagged RhlI readily precipitated unless supplemented with glycerol (see above), the most likely explanation was that after overnight cleavage from the intein-CBD fusion, RhlI precipitated on the column, unless the cleavage buffer was supplemented with Triton X-100 and glycerol. Similar problems were also observed with LasI and RmlD, two other very insoluble proteins, but not with ACP, a very soluble protein who was successfully purified using pNam (12). Efforts are currently under way to minimize precipitation of cleaved protein on the column.

4.4.3 Functional analysis of His6-tagged RhlI. Although the purified LasI was active in *in vitro* *N*-(3-oxo)-dodecanoyl-L-homoserine lactone synthesis assays (13) and the activity of purified RmlD was shown by its ability to convert dTDP-glucose to dTDP-rhamnose when incubated with RmlB, RmlC and $NADP^+$ (31), we concentrated on characterization of RhlI in this study.

As shown in figure 4.3A, purified RhlI was capable of synthesizing BHL from low-concentrations (10 μM) of butyryl-ACP (spot 12). When butyryl-ACP was substituted with 10 μM butyryl-CoA, no BHL was synthesized (spot 5). The presence of excess (500 μM) butyryl-CoA resulted in barely detectable levels of BHL formation (spot 7). No BHL synthesis was observed with 10 μM butyryl-CoA (spot 1), 10 μM butyryl-CoA and no SAM (spot 2), no butyryl-CoA (spot 3), and 10 μM butyryl-CoA but no RhlI (spot 4). BHL synthesis from butyryl-ACP did not require NADPH (spot 11). However, BHL synthesis required RhlI (spot 8), SAM (spot 9) and an acyl-ACP donor (spot 10).

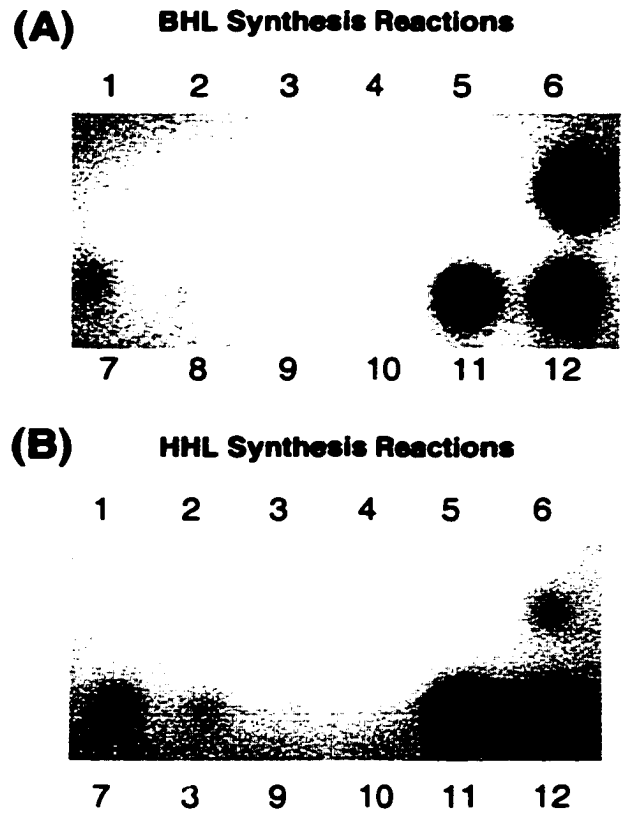


Figure 4.3. *In vitro* synthesis of butyryl homoserine lactone (BHL) and hexanoyl homoserine lactone (HHL) from defined substrates using purified RhlI. The extracted reactions were spotted onto C_{18} reverse phase TLC plates and AHLs were detected by the CV026 agar overlay method (see chapter 4.3.6 for details). **(A)** BHL synthesis reactions from butyryl-CoA or butyryl-ACP. Standard reactions contained in Moré buffer (see chapter 4.3.6) 100 μM SAM, 200 μM NADPH, varied concentrations of butyryl-CoA or 10 μM butyryl-ACP and 1 μg RhlI. Individual reactions contained: spot 1, 10 μM butyryl-CoA and no NADPH; spot 2, 10 μM butyryl-CoA and no SAM; spot 3, no butyryl-CoA; spot 4, 10 μM butyryl-CoA and no RhlI; spot 5, 10 μM butyryl-CoA; spot 6, 50 pmoles of BHL standard; spot 7, 500 μM butyryl-CoA; spot 8, 10 μM butyryl-ACP and no RhlI; spot 9, 10 μM butyryl-ACP and no SAM; spot 10, no butyryl-ACP; spot 11, 10 μM butyryl-ACP and no NADPH; spot 12, 10 μM butyryl-ACP. **(B)** HHL synthesis from hexanoyl-CoA or hexanoyl-ACP. The reactions contained in Moré buffer 100 μM SAM, 200 μM NADPH, varied concentrations of hexanoyl-CoA or 10 μM hexanoyl-ACP and 1 μg RhlI. In addition, individual reactions contained: spot 1, 10 μM hexanoyl-CoA and no NADPH; spot 2, 10 μM hexanoyl-CoA and no SAM; spot 3, no hexanoyl-CoA; spot 4, 10 μM hexanoyl-CoA and no RhlI; spot 5, 10 μM hexanoyl-CoA; spot 6, 500 μM hexanoyl-CoA; spot 7, 10 pmoles of HHL standard; spot 8, 10 μM hexanoyl-ACP and no RhlI; spot 9, 10 μM hexanoyl-ACP and no SAM; spot 10, no hexanoyl-ACP; spot 11, 10 μM hexanoyl-ACP and no NADPH; spot 12, 10 μM hexanoyl-ACP.

When provided with 10 μM hexanoyl-ACP as a substrate, purified RhlI could synthesize HHL (figure 4.3B, spot 12). Again, hexanoyl-ACP was the preferred substrate over hexanoyl-CoA since similar concentrations of hexanoyl-CoA did not lead to synthesis of detectable HHL (spot 5), and excess (500 μM) hexanoyl-CoA led to synthesis of barely detectable levels of HHL (spot 6). No HHL synthesis was observed with 10 μM hexanoyl-CoA (spot 1), 10 μM hexanoyl-CoA and no SAM (spot 2), no hexanoyl-CoA (spot 3), and 10 μM hexanoyl-CoA but no RhlI (spot 4). HHL formation required an acyl-ACP donor (spot 10), SAM (spot 9) and RhlI (spot 8), but not NADPH (spot 11).

The *in vivo* source for acyl-ACPs is most likely the fatty acid biosynthetic pathway. To test this notion, we set up a coupled *in vitro* system for synthesis of BHL from crotonyl-ACP and SAM by enoyl-ACP reductase (FabI) and RhlI (figure 4.4A). Crotonyl-ACP is derived by condensation of malonyl-ACP with acetyl-CoA by β-ketoacyl-ACP synthase III, followed by consecutive reduction and dehydration reactions (8). The enoyl-ACP reductase reaction is the last step in each cycle of fatty acid elongation. When coupled to FabI, RhlI could synthesize BHL from crotonyl-ACP (figure 4.4B, spots 8 and 9). This reaction required crotonyl-ACP (spot 1), SAM (spot 2), NADH (spot 3), RhlI (spot 4) and FabI (spot 6). In agreement with previous results which showed that NADH is a co-factor for purified FabI but not NADPH (12), NADH could serve as a reductant (spot 8) but NADPH could not (spot 7).

TLC and GC-MS were utilized to further confirm the identity of the *in vitro* synthesized AHL molecules. On C_{18} reverse-phase TLC plates, the *in vitro* synthesized BHL and HHL co-migrated with the BHL and HHL standards, respectively (figure 4.5). When compared to BHL, HHL produced more intense spots since HHL is the natural AHL required for violacein production in *C. violaceum* (20). To further demonstrate that the *in vitro* synthesized product was identical to BHL, BHL was HPLC-purified and then subjected to GC-MS (figure 4.6). The mass spectra obtained for synthetic BHL and BHL synthesized *in vitro* from butyryl-ACP by the action of RhlI were indistinguishable.

While our studies neared completion, Parsek et al. (24) published a detailed kinetic analysis of RhlI, using various acyl-CoA and acyl-ACP substrates for RhlI. These authors quantitatively showed that RhlI had higher affinity for butyryl-ACP than hexanoyl-ACP or

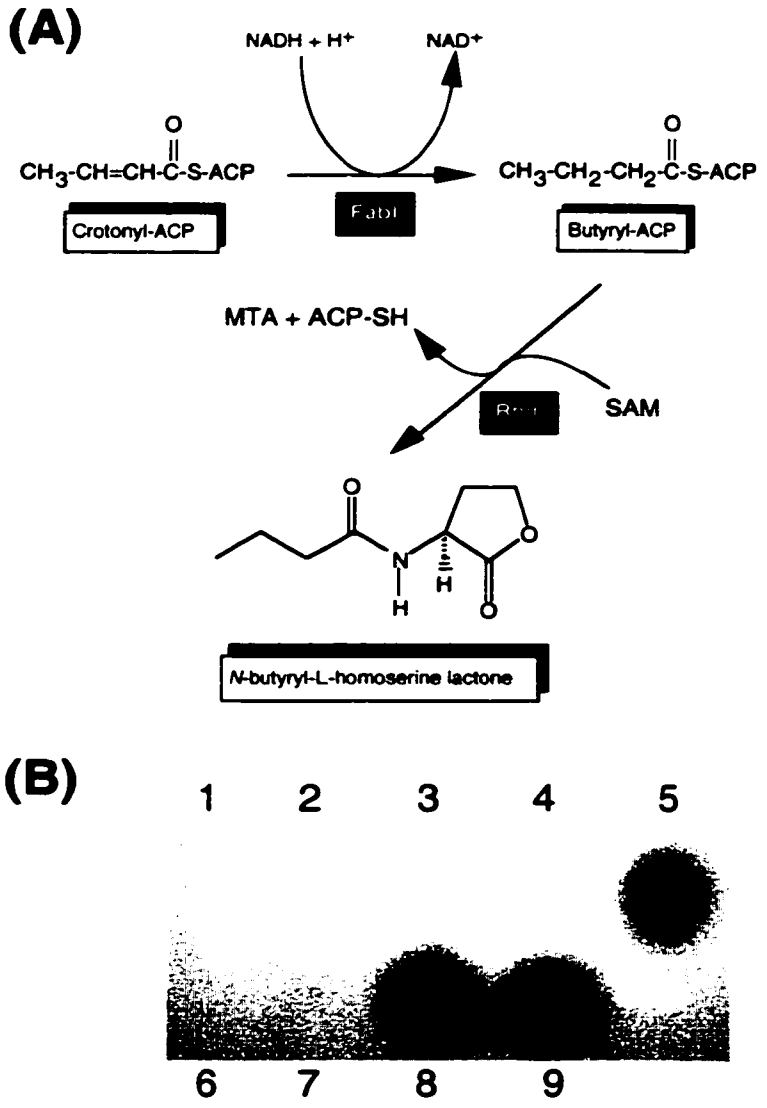


Figure 4.4. BHL synthesis in a FabI-RhlI coupled reaction. (A) Diagram depicting steps of the proposed physiological roles of enoyl-ACP reductase (FabI) and RhlI in BHL synthesis, and the requirements for NADH and SAM. (B) BHL synthesis from crotonyl-ACP via FabI and RhlI. The extracted reaction products were spotted onto reverse phase C_{18} TLC plates and BHL was detected with the CV026 agar overlay method. The standard reaction contained in Moré buffer 100 μM SAM, 200 μM NADPH and NADH, 1 μg of each RhlI and FabI, and 20 μM crotonyl-ACP. Individual reactions contained: spot 1, no crotonyl-ACP; spot 2, no SAM; spot 3, no NADH or NADPH; spot 4, no RhlI; spot 5, 50 pmoles of BHL standard; spot 6, no FabI; spot 7, NADPH only; spot 8, NADH only; spot 9, NADH and NADPH.

other acyl-ACPs. Although the indicator strain CV026 responded to both BHL and HHL in our assays, the amounts of BHL synthesized relative to HHL could not be quantitated with these assays since CV026 responded more intensely to HHL, which is the natural inducer for violacein production in *C. violaceum* (20), but is only a minor AHL produced by *P. aeruginosa* (24, 26).

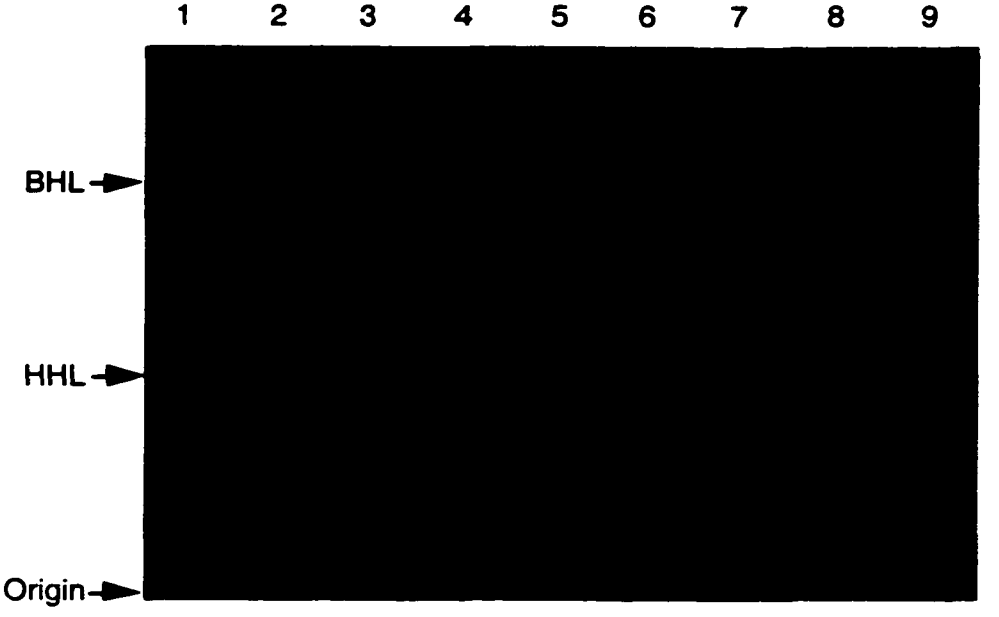


Figure 4.5. C_{18} reverse-phase TLC analysis of BHL and HHL synthesis reactions. The extracted reaction products were spotted onto reverse phase C_{18} TLC plates, the plates were developed with 60% (v/v) methanol in water and dried. BHL and HHL were detected with the CV026 agar overlay method. Synthesis reactions contained in Moré buffer 100 μM SAM, and 1 μg RhlI. In addition, the reactions contained: 10 μM butyryl-ACP (lane 1); 10 μM butyryl-ACP and 200 μM NADPH (lane 2); 20 μM crotonyl-ACP, 200 μM NADH and 1 μg FabI (lane 4); 20 μM crotonyl-ACP, 200 μM NADH, 200 μM NADPH and 1 μg FabI plus (lane 5); 10 μM hexanoyl-ACP (lane 8); 10 μM hexanoyl-ACP plus 200 μM NADPH (lane 9). Lane 3 contained approximately 50 pmoles of BHL standard. Lanes 6 and 7 contained approximately 30 pmoles of HHL standard.

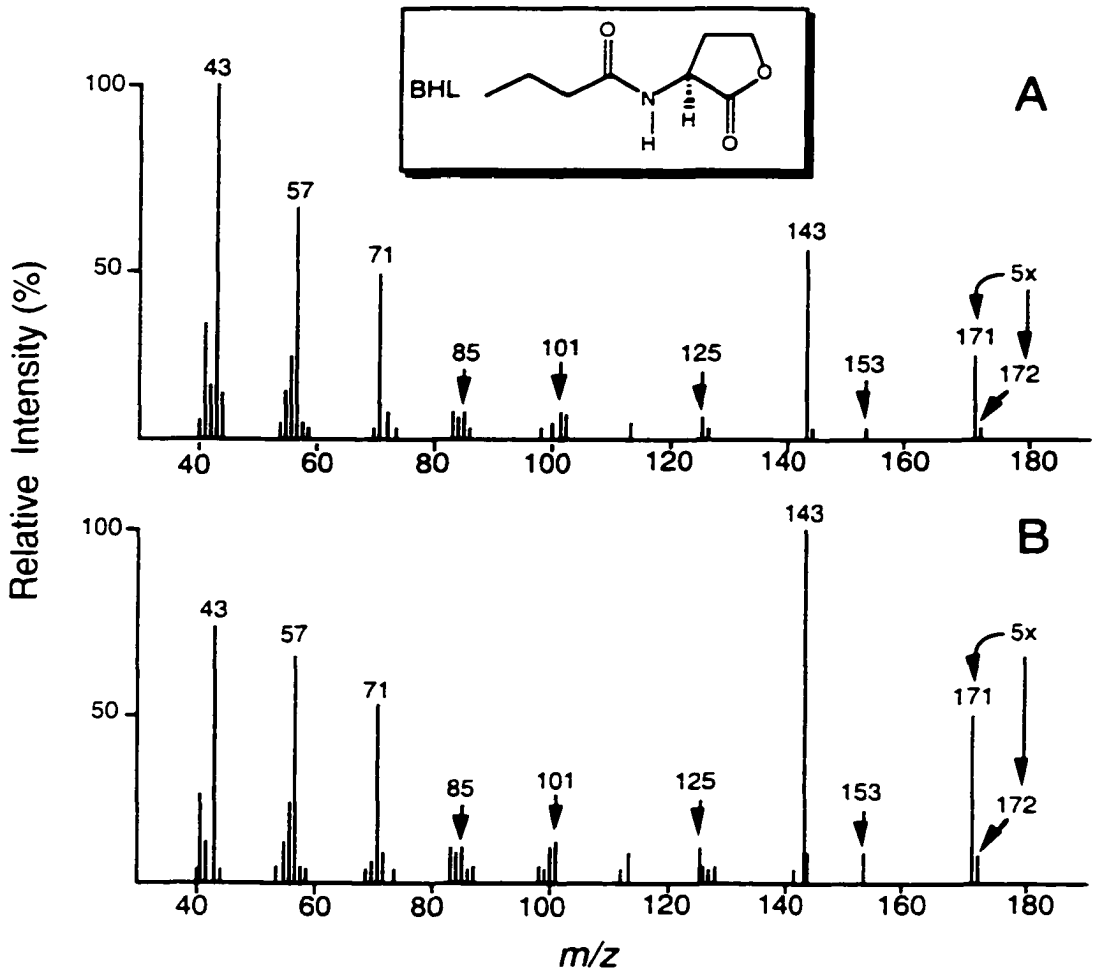


Figure 4.6. Identification of BHL by GC-MS. Mass spectra of the regions of interest from synthetic BHL (**A**) and BHL extracted from an *in vitro* reaction containing Moré buffer, 100 μM SAM, 20 μM butyryl-ACP and 1 μg RhlI (**B**) are shown. The molecular ion at 171 and the ^{13}C isotope peak of the molecular ion at 172 are enlarged 5-fold.

In summary, our results corroborate previous observations made by other investigators (4, 22, 29, 33), but contradict those of Jiang et al. (15) who reported that RhlI could efficiently synthesize BHL and HHL from SAM, and butyryl-CoA and hexanoyl-CoA,

respectively, and that NADPH was required for these reactions. Using recombinant purified RhlI, we not only showed that acyl-ACPs are the acyl donors for AHLs, but also demonstrated that when coupled to FabI, RhlI was capable of synthesizing BHL from very low concentrations of the natural acyl donor crotonyl-ACP. This is to date perhaps the most compelling evidence that the source for the acyl groups found in AHLs is the fatty acid biosynthetic pathway.

4.5 CONCLUSIONS

(i) We have developed versatile low-copy number T7 promoter-based expression vectors that allow expression and purification of His6- and intein-CBD tagged fusion proteins from *E. coli* K-12 hosts. These vectors will facilitate purification and characterization of many proteins, especially the ones that are insoluble upon overexpression. They will also be helpful to biochemically characterize the many unknown proteins that have been and continue to be identified in numerous bacteria, whose genomes either have already been sequenced or whose genome sequences are currently being determined.

(ii) Since for unknown reasons the newly constructed plasmids no longer replicated in *E. coli* B strain BL21(DE3), a new *E. coli* K-12 expression strain SA1503(DE3) was constructed. This strain preserves the most desirable traits of BL21(DE3), i.e. it does not produce the Lon and OmpT proteases, but has the added advantage that due to lack of a type barrier it is transformable with higher efficiencies with DNA isolated from commonly used K-12 cloning hosts, such as DH5α and JM109.

(iii) We have demonstrated the utility of these new expression vectors by expression and affinity purification of three extremely insoluble proteins from either *M. tuberculosis* or *P. aeruginosa*. These proteins could not be purified in their active form utilizing commercially available T7 expression vectors. We purified three His6-tagged proteins, LasI, RhlI and RmlD, by Ni^{2+} agarose chromatography utilizing non-denaturing conditions and an untagged RhlI protein via a RhlI-intein-CBD fusion protein by chitin agarose chromatography. All His6-tagged proteins retained

enzymatic activity after purification and they remained soluble when stored in the presence of 15 to 30% glyerol.

(iv) Purified RhlI protein was used to further define its role in AHL synthesis. The enzyme could utilize butyryl-ACP and hexanoyl-ACP, but not the respective CoA derivatives as substrates. Low levels of BHL and HHL synthesis were observed with high (500 μM), and therefore most likely non-physiological concentrations of butyryl-CoA and hexanoyl-CoA, respectively. For BHL and HHL synthesis, RhlI only required SAM and the respective acyl-ACPs but not NADPH. When coupled to purified enoyl-ACP reductase, RhlI was also capable of synthesizing BHL from crotonoyl-ACP in the presence of NADH and SAM, further indicating that the bacterial fatty acid biosynthetic pathway provides the acyl-ACPs that serve as the acyl donors for the HSL synthases. BHL synthesized by purified RhlI was indistinguishable from synthetic BHL as assessed by TLC and GC-MS analyses.

(v) The expression and purification procedure described in this paper will facilitate further studies with RhlI, including efforts geared at determination of its crystal structure. Futhermore, since the AHLs of *P. aeruginosa* play determinant roles in expression of numerous virulence factors (34), purified AHL synthases will facilitate studies aimed at identification of novel anti-virulence drugs.

(vi) In contrast to all other previous studies that employed components from heterologous sources, our study provides the first example where all of the protein components of an AHL synthesis system, i.e. ACP, RhlI, and enoyl-ACP reductase, were derived from the same bacterium.

4.6 ACKNOWLEDGMENTS

This work was suported by NIH grants GM56685 to H.P.S and AI33706 to M.R.M. T.T.H. was supported by Student Traineeship HOANG99H0 from the American Cystic Fibrosis Foundation. We thank Kelly Motichka and Delphi Chatterjee for their assistance with HPLC analysis of AHL samples. We are grateful to Dr. Barbara Iglewski for the gift of synthetic

BHL, to Dr. Anatol Eberhard for his sample of HHL, and to Dr. Mary Berlyn at the *E. coli* Genetic Stock Center for providing strains.

4.7 REFERENCES

1. **Akiyama, Y., and K. Ito.** 1990. SecY protein, a membrane-embedded secretion factor of *E. coli*, is cleaved by the OmpT protease *in vitro*. Biochem. Biophys. Res. Commun. **167**:711-715.

2. **Belisle, J. T., L. Pascopella, J. M. Inamine, P. J. Brennam, and W. R. Jacobs.** 1991. Isolation and expression of a gene cluster responsible for the biosynthesis fo the glycopeptidolipid antigens of *Mycobacterium avium*. J. Bacteriol. **173**:6991-6997.

3. **Bergler, H., P. Wallner, A. Ebeling, B. Leitinger, S. Fuchsbichler, H. Aschauer, G. Kollenz, G. Hoegenauer, and F. Turnowsky.** 1994. Protein *envM* is the NADH-dependent enoyl-ACP reductase *fabI* of *Escherichia coli*. J. Biol. Chem. **269**:5493-5496.

4. **Cao, J.-G., and E. A. Meighen.** 1993. Biosynthesis and stereochemistry of the autoinducer controlling luminescence in *Vibrio harveyi*. J. Bacteriol. **175**:3856-3862.

5. **Chen, H., H. Cheng, and M. Bjerkness.** 1993. One-step Coomassie Brillant Blue R-250 staining of proteins in polycarylamide gel. Anal. Biochem. **212**:295-296.

6. **Chong, S., F. B. Mersha, D. G. Comb, M. E. Scott, D. Landry, L. V. Vence, F. B. Perler, J. Benner, R. B. Kucera, C. A. Hirvonen, J. J. Pelletier, H. Paulus, and M.-Q. Xu.** 1997. Single-column purification of free recombinant proteins using a self-cleavable affinity tag dervived from a protein splicing element. Gene **192**:271-281.

7. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D.**

Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M.-A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Square, S. Square, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537-544.

8. **Cronan, J. E., and C. O. Rock.** 1996. Biosynthesis of membrane lipids, p. 612-636. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. Brooks Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*. Am. Soc. Microbiol., Washington, DC.

9. **Gottesman, S., and D. Zipser.** 1978. Deg phenotype of *Escherichia coli lon* mutants. J. Bacteriol. **133:**844-851.

10. **Hoang, T., S. Williams, and H. P. Schweizer.** 1997. Molecular genetic analysis of the region containing the essential *Pseudomonas aeruginosa asd* gene encoding aspartate-β-semialdehyde dehydrogenase. Microbiol. **143:**899-907.

11. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77-86.

12. **Hoang, T. T., and H. P. Schweizer.** 1999. Characterization of the *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase: a traget for triclosan and its role in acylated homoserine lactone synthesis. J. Bacteriol. **181:**5489-5497.

13. **Hoang, T. T., and H. P. Schweizer.** 1999. Synthesis of the *Pseudomonas aeruginosa* autoinducer *N*-[3-oxo-dodecanoyl]-L-homoserine lactone from metabolic precursors in a

reconstituted enzyme system. Abstract B/D309, Am. Soc. Microbiol., 99th Annual Meeting. Chicago, IL.

14. **Holloway, B. W., U. Roemling, and B. Tuemmler.** 1994. Genomic mapping of *Pseudomonas aeruginosa* PAO. Microbiol. **140:**2907-2929.

15. **Jiang, Y., M. Camara, S. R. Chhabra, K. R. Hardie, B. W. Bycroft, A. Lazdunski, G. P. C. Salmond, G. S. A. B. Stewart, and P. Williams.** 1998. In vitro biosynthesis of the *Pseudomonas aeruginosa* quorum-sensing signal molecule *N*-butanoyl-L-homoserine lactone. Mol. Microbiol. **28:**193-203.

16. **Karkhoff-Schweizer, R. R., and H. P. Schweizer.** 1994. Utilization of mini-D*lac* transposable element to create an α-complementation and regulated expression system for molecular cloning in *Pseudomonas aeruginosa*. Gene **140:**7-15.

17. **Kutchma, A. J., T. T. Hoang, and H. P. Schweizer.** 1999. Characterization of a *Pseudomonas aeruginosa* fatty acid biosynthetic gene cluster: purification of acyl carrier protein (ACP) and malonyl-coenzyme A:ACP transacylase (FabD). J. Bacteriol. **181:**5498-5504.

18. **Ma, Y., J. A. Mills, J. T. Belisle, V. Vissa, M. Howell, K. Bowlin, M. S. Scherman, and M. McNiel.** 1997. Determination of the pathway for rhamnose biosynthesis in mycobacteria: cloning, sequencing, and expression of the *Mycobacterium tuberculosis* gene encoding α-D-glucose-1-phosphate thymidylyltransferase. Microbiol. **143:**937-945.

19. **Makowski, G. S., and M. L. Ramsby.** 1993. pH modification to enhance the molecular sieving properties of sodium dodecyl sulfate-10% polyacrylamide gel. Anal. Biochem. **212:**283-285.

20. **McClean, K. H., M. K. Winson, L. Fish, A. taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. lamb, S. Swift, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production for the detection of *N*-acylhomoserine lactones. Microbiol. **143:**3703-3711.

21. **Miller, C. A., W. T. Tucker, P. A. Meacock, P. Gustafsson, and S. N. Cohen.** 1983. Nucleotide sequence of the partition locus of *Escherichia coli* plasmid pSC101. Gene **24:**309-315.

22. **Moré, M. I., D. Finger, J. L. Stryker, C. Fuqua, A. Eberhard, and S. C. Winans.** 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science **272:**1655-1658.

23. **Neuhard, J., and E. Thomassen.** 1976. Altered deoxyribonucleotides pools in P2 eductants of *Escherichia coli* K-12 due to deletion of the *dcd* gene. J. Bacteriol. **126:**999-1001.

24. **Parsek, M. R., D. L. Val, B. L. Hanzelka, J. E. Cronan, and E. P. Greenberg.** 1999. Acyl homoserine-lactone quorum-sensing signal generation. Proc. Natl. Acad. Sci. USA. **96:**4360-4365.

25. **Pearson, J., L. Passador, B. Iglewski, and E. Greenberg.** 1995. A second N-acylhomoserine lactone signal produced by Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. USA. **92:**1490-1494.

26. **Pearson, J. P., E. C. Pesci, and B. H. Iglewski.** 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. **179:**5756-5767.

27. **Petty, K. J.** 1994. Metal-chelate affinity chromatography, p. 10.11.8-10.11-22. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Strugh (ed.). Current Protocols in Molecular Biology. John Wiley and Sons, NY.

28. **Sankar, P., M. E. Hutton, R. A. VanBogelen, R. L. Clark, and F. C. Neidhardt.** 1993. Expression analysis of cloned chromosomal segments of *Escherichia coli*. J. Bacteriol. **175:**5145-5152.

29. **Schaefer, A. L., D. L. Val, B. L. Hanzelka, J. E. Cronan, and E. P. Greenberg.** 1996. Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. Proc. Natl. Acad. Sci. USA **93:**9505-9509.

30. **Schweizer, H., and W. Boos.** 1984. Characterization of the *ugp* region containing the genes for the *phoB* dependent *sn*-glycerol-3-phosphate transport system of *Escherichia coli*. Mol. Gen. Genet. **197:**161-168.

31. **Stern, R. J., T. Y. Lee, T. J. Lee, W. Yan, M. S. Scherman, V. D. Vissa, S. K. Kim, and M. R. McNeil.** 1999. Conversion of dTDP-4-keto-6-deoxyglucose to free dTDP-4-keto-rhamnose by the *rmlC* gene products of *Escherichia coli* and *Mycobacterium tuberculosis*. Microbiol. **145:**663-671.

32. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60-89.

33.**Val, D. L., and J. E. Cronan.** 1998. In vivo evidence that *S*-adenosylmethionine and fatty acid intermediates are the substrates for the LuxI family of autoinducer synthases. J. Bacteriol. **180:**2644-2651.

34. **Van Delden, C., and B. H. Iglewski.** 1998. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Em. Infect. Dis. **4:**551-560.

35. **Vocke, C., and D. Bastia.** 1983. Primary structure of the essential replicon of the plasmid pSC101. Proc. Natl. Acad. Sci. USA. **80:**6557-6561.

36. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195-199.

37. **Weeks, G., and S. J. Wakil.** 1968. Studies on the mechanisms of fatty acid synthesis. J. Biol. Chem. **243:**1180-1189.

38. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-119.

CHAPTER 5

Characterization of a *Pseudomonas aeruginosa* fatty acid biosynthetic gene cluster: purification of acyl carrier protein (ACP) and malonyl-CoA:ACP transacylase (FabD)

(Presented in Alecksandr J. Kutchma, Tung T. Hoang, and Herbert P. Schweizer. 1999. *Journal of Bacteriology* **181**: 5498-5504)

The work presented in this paper demonstrated that expression of a functional holo-ACP from a recombinant plasmid was possible. A. J. Kutchma and I contributed equally to this work. I cloned the fatty acid gene cluster and performed all FabD and ACP expression, characterization, and labeling presented in this paper. A.J. Kutchma assisted with sequencing of the fatty acid gene cluster and cloning the missing fragment that contained part of *fabF* and complete *papC* genes.

5.1 ABSTRACT

A DNA fragment containing the *Pseudomonas aeruginosa fabD* (encoding malonyl-coenzyme A (CoA):acyl carrier protein (ACP) transacylase), *fabG* (encoding β-ketoacyl-ACP reductase), *acpP* (encoding ACP), and *fabF* (encoding β-ketoacyl-ACP synthase II) genes was cloned and sequenced. This *fab* gene cluster is delimited by the *plsX* (encoding a poorly understood enzyme of phospholipid metabolism) and *pabC* (encoding 4-amino-4-deoxychorismate lyase) genes; the *fabF* and *pabC* genes seem to be translationally coupled. The *fabH* gene (encoding β-ketoacyl-ACP synthase III), which in most Gram-negative bacteria is located between *plsX* and *fabD*, is absent from this gene cluster. A chromosomal temperature-sensitive *fabD* mutant was obtained by site-directed mutagenesis that resulted in a

W258Q change. A chromosomal *fabF* insertion mutant was generated and the resulting mutant strain contained substantially reduced levels of *cis*-vaccenic acid. Multiple attempts aimed at disruption of the chromosomal *fabG* gene were unsuccessful. FabD was purified as an oligohistidine fusion protein (H_6-FabD) and ACP in its native form via an ACP-intein-chitin binding domain fusion protein, utilizing a novel expression and purification scheme that should be applicable to ACP from other bacteria. Matrix-assisted laser desorption/ionization spectrospcopy, native polyacrylamide electrophoresis, and amino-terminal sequencing revealed that (i) most of the purified ACP was properly modified with its 4'-phosphopantetheine functional group, (ii) it was not acylated, and (iii) the amino-terminal methionine was removed. In an *in vitro* system, purified ACP functioned as acyl acceptor and H_6-FabD exhibited malonyl-CoA:ACP transacylase activity.

5.2 INTRODUCTION

Fatty acid biosynthesis (Fab) in eukaryotes is catalyzed by a multienzyme complex encoded by a single gene and known as the type I system. In contrast, eubacteria contain a type II or dissociated Fab system, where the reactions are carried out by proteins encoded by multiple genes [for a review see (6)]. Bacterial fatty acid biosynthesis necessitates a three-carbon precursor, malonyl-CoA, which is derived from acetyl-CoA by the action of acetyl-CoA carboxylase. The malonyl-CoA is transferred to the acyl carrier protein (ACP) by malonyl-CoA:ACP acyltransferase (FabD). Fatty acid elongation involves four reactions: (i) a condensation reaction catalyzed by one of three β-ketoacyl-ACP synthase, FabB, FabF or FabH; (ii) a reduction involving a NADPH-dependent β-ketoacyl-ACP reducatase (FabG); (iii) a dehydration reaction catalyzed by either FabA or FabZ, β-hydroxyacyl-ACP dehyratases with broad, overlapping chain length specificities (15); and (iv) a second reduction reaction catalyzed by NADH-dependent enoyl-ACP reductase (FabI).

In *Escherichia coli* (28) and other bacteria (9, 25, 38), the genes *acpP*, *fabD*, *FabF*, *fabG* and *fabH*, encoding ACP, FabD, FabF, FabG and FabH, respectively, are contained in a *fab* gene cluster. In *Bacillus subtilis*, *fabH* is not part of the major *fab* gene cluster (25).

Besides the role of ACP in phospholipid (6) and rhamnolipid (3, 27) synthesis, ACPs play central roles in a broad range of other biosynthetic pathways that depend on acyl transfer reactions, including polyketide (35), non-ribosomal peptide (1), and depsipeptide biosynthesis (31), as well as in the transacylation of oligosaccharides (8, 11) and proteins (22). More recently, acyl-ACPs derived from the fatty acid biosynthetic pathway have been proposed to be the acyl donors for synthesis of acylated homoserine lactones (AHL) (26, 32, 40). The AHLs (or autoinducers) have received considerable attention in recent years since they are required for a regulatory phenomenon termed quorum sensing (10). Characterization of the two known AHL producing systems of *P. aeruginosa*, *las* and *rhl*, has shown that the respective autoinducers, *N*-butyryl homoserine lactone (C_4-HSL) and *N*-(3-oxo)-dodecanoyl homoserine lactone (3-oxo-C_{12}-HSL) are required for expression of a multitude of virulence factors and secondary metabolites (41).

We recently began characterization of the unique aspects of the *P. aeruginosa* Fab system (20), and in this report we describe the characterization of the *P. aeruginosa fabD-fabG-acpP-fabF* gene cluster, as well as the purification and characterization of FabD and ACP.

5.3 METHODS, RESULTS, AND DISCUSSION

5.3.1 Cloning and characterization of the *acpP*-containing region. When we initiated these experiments, the sequence of only a fragmented *acpP*-containing region was available from the *P. aeruginosa* genome database and it contained no intact *acpP* gene. This *acpP*-containing gene cluster was cloned into M13 which led to expression of toxic levels of apo-ACP. Previous attempts to clone intact *acpP* from *E. coli* (42), as well as from a number of other bacteria (25, 36), into multi-copy plasmids were hampered by the toxic effects of overexpressed apo-ACP (23). We therefore chose to reclone the chromosomal *acpP*-containing region into the low copy-number pWSK vectors [typically exhibiting only 5-8 copies/cell (44)]. A 147-bp sequence containing a partial *acpP* coding sequence PCR-amplified from *P. aeruginosa* chromosomal DNA using the two primers ACP1 and ACP2 (table 5.1). These partially degenerate primers were modeled after conserved amino acid

sequence regions found in the *E. coli*, *Haemophilus influenzae* and *Vibrio harveyi* ACP homologs (figure 5.2). PCR was performed on a PTC-100 PCR system thermocycler (MJ Research, Watertown, MA) using *Taq* polymerase from Qiagen (Santa Clarita, CA) and previously described conditions (19). The PCR fragment was cloned into pGEM-T (Promega, Madison, WI) to form pPS831. Nucleotide sequence analysis revealed the presence of a partial *acpP* gene on the PCR fragment. This fragment was used as a probe to generate a partial restriction map of the PAO1 chromosomal *acpP* region, which revealed a 3.4-kb *Eco*RV-*Bam*HI fragment which was cloned into the low-copy number cloning vector pWKS30 using previously described strategies (17, 20), yielding pPS840 (figure 5.1A).

The entire nucleotide sequence of both strands of a 3,433-bp chromosomal *Eco*RV-*Bam*HI fragment was determined (relevant segments of the sequence are shown in figure 5.1B). BLAST searches revealed the presence of the 3' end of *plsX*, complete sequences of *fabD*, *fabG*, *acpP*, and the 5' end of *fabF* (figure 5.1A). In order to obtain the 3' end of *fabF*, a 1.1-kb *Bam*HI-*Eco*RI fragment from pPS840 was used as a probe to identify a ~4-kb *Eco*RI-*Hin*dIII fragment which was cloned into pWSK29 to generate pPS671 (figure 5.1A).

Sequence analyses of pPS671 and several subclones derived from it established the entire sequence of both strands from the *Bam*HI site within *fabF* to the *Sal*I site immediately downstream of *fabF* (figure 5.1A). The *fab* gene cluster contains *fabF*, *acpP*, *fabG* and *fabD*, but not *fabH*. Many bacteria contain similarly organized *fab* gene clusters, i.e. *B. subtilis* (25), *V. harveyi* (36), *H. influenzae* (9) and *Streptomyces glaucascens* (38) but *P. aeruginosa* provides the first example of a Gram-negative bacterium where the *fabH* gene is absent from the *acpP*-containing cluster; the only other known example is *B. subtilis* (25).

The *fab* cluster is delimited by *pabC* and *plsX* homologs, encoding 4-amino-deoxychorismate lyase and a protein of unknown function in phospholipid synthesis, respectively. We recently confirmed that *pabC* encodes *P. aeruginosa* aminodeoxychorismate lyase (13), which is required for *p*-aminobenzoic acid (PABA) synthesis, since *pabC* mutants are PABA auxotrophs (18). Unlike *E. coli*, the ATG start codon of the *P. aeruginosa pabC* gene overlaps the TGA stop codon of *fabF*.

Table 5.1. Strains, plasmids and primers used in this study

Strain, plasmid, or primer	Relevant properties	Reference or origin
E. coli		
BL21(DE3)	*E. coli* B; F⁻ *ompT* r_B^- m_B^- (λDE3)	(37)
SA1503(DE3)	*lon-100 his-87 ompT*::Km^r *relA1 spoT1 thi-1* (λDE3)	(19a)
P. aeruginosa		
PAO1	Prototroph	B. H. Holloway
PAO198	PAO1 with *fabF*::Gm^r	This study
PAO204	PAO1 with *fabD*(Ts)	This study
Plasmids		
pEX100T	Ap^r *sacB*$^+$; gene replacement vector	(34)
pUCGM	Ap^r, Gm^r; source of Gm^r cassette	(33)
pWSK29/ pWKS30	Ap^r; low-copy number cloning and T7 expression vectors	(44)
pCYB1	Ap^r; intein-chitin binding domain expression vector	New England Biolabs
pET-15b	Ap^r; hexahistidine fusion and expression vector	Novagen
pGEM-T	Ap^r; TA-cloning vector	Promega
pNam	Ap^r; low-copy number intein-chitin binding domain expression vector	(19a)
pT7-7	Ap^r; T7 promoter expression vector	(39)
pPS671	Ap^r; *fabF*$^+$ *pabC*$^+$ (ligation of chromosomal 4-kb *Eco*RI-*Hin*dIII fragment between the same sites of pWSK29)	This study
pPS681	Ap^r; *fabD*$^+$ *fabG*$^+$ *acpP*$^+$ *fabF*$^+$ *pabC*$^+$ (ligation of 3.4-kb *Bam*HI-*Eco*RV fragment from pPS840 between the *Bam*HI and *Xba*I [blunt-ended] sites of pPS671)	This study

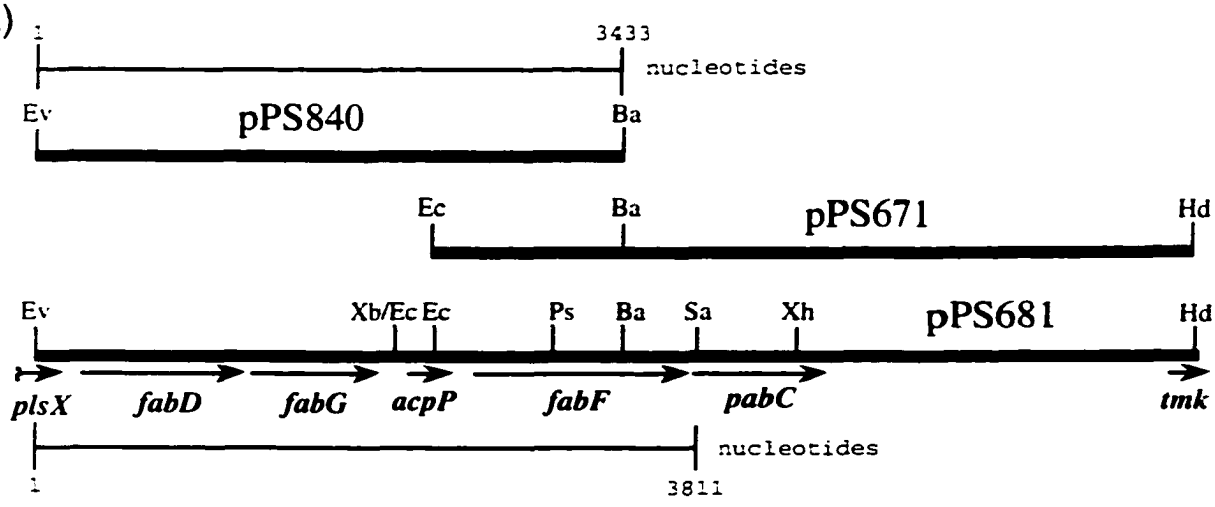
Table 5.1. Strains, plasmids and primers used in this study (cont.)

Strain, plasmid, or primer	Relevant properties	Reference or origin
pPS831	Ap^r; PCR-amplified 147-bp genomic segment from PAO1 cloned into pGEM-T	This study
pPS840	Ap^r; *fabD*$^+$ *fabG*$^+$ *acpP*$^+$ (3.7-kb chromosomal *Eco*RV-*Bam*HI fragment cloned between the same sites of pWSK29)	This study
pPS966	Ap^r; ligation of 1.8 kb *Nde*I-*Pst*I fragment from pPS981 between the same sites of pT7-7	This study
pPS981	Ap^r, *acpP*$^+$; ligation of *Nde*I+*Sap*I digested PCR fragment between same sites of pCYB1	This study
pPS1096	Ap^r *acpP*$^+$ (ligation of 735-bp *Hin*dIII-*Xba*I fragment from pPS966 between same sites of pNam)	This study
Primer[a]		
ACP1	ATCGAGGAGCG(A/C/T/G)GT(G/C)AAGAAGAT(A/C)AT	
ACP2	GTCGAACTCCTC(G/C)TC(A/C/T/G)AG(G/C)GCCAT	
FabD1	TGGATGCTCTCCACCtgGCGACCCGGGCTGT	
ACP-Nde	(*Nde*I)-TGAAAACAACATatgAGCACCATCGAAGAACGCGTT	
ACP-Sap	(*Sap*I)-ATCCGGCTCTTCCGCAttgCTGGGGAGC	
FabD-Nde	(*Nde*I)-GGGACCTATcatATGTCTGCATCCCTCGCATTCGTC	
FabD-Bam	(*Bam*HI)-CCCAGGatCCCCAGTTCCAGCGCAATCGCC	

[a] Primers are printed 5' to 3'; lowercase letters either indicate non-matching oligonucleotides to form the indicated motif as underlined or introduce other non-motif changes indicated in lowercase letters

Figure 5.1. The *P. aeruginosa acpP* region. **A)** Maps of plasmids containing a *fab* gene cluster and flanking genes. The chromosomal inserts of pPS840 and pPS671 were cloned individually and fusion of these two clones at the common *Bam*HI site yielded pPS681, containing a continuous sequence of this region. Abbreviations: Ba, *Bam*HI; Ec, *Eco*RI; Ev, *Eco*RV; Hd, *Hin*dIII; Ps, *Pst*I; Sa, *Sal*I; Xb, *Xba*I; Xh, *Xho*I. The following genes and their products were identified by nucleotide sequencing: *plsX*, encoding a poorly understood protein involved in phospholipid biosynthesis; *fabD*, malonyl-CoA:ACP transacylase; *fabG*, β-ketoacyl-ACP reductase; *acpP*, ACP; and *fabF*, β-ketoacyl-ACP synthase II; *pabC*, 4-amino-deoxychorismate lyase and *tmk*, thymidylate kinase. **B)** Partial nucleotide sequences of the *P. aeruginosa plsX*, *fabD*, *fabG*, *acpP*, *fabF* and *pabC* genes. Most of the sequences within the structural genes are omitted, as marked by the dashes. The complete sequence of the 3811-bp *Eco*RV-*Sal*I fragment (indicated in **A**) was deposited in GenBank and assigned accession number U91631. The deduced amino acid sequences are given in one-letter code below the nucleotide sequence and RBS denote putative ribosome-binding sites. Numbers above the sequence mark the first nucleotides of the initiation and last codons, respectively, and stop codons are marked with asterisks. The TG to CA nucleotide changes in *fabD* that were introduced by site-directed mutagenesis and resulted in a W258Q change and a FabD(Ts) phenotype are indicated above the nucleotide sequence.

A)



B)

```
EcoRV                                   175
GATATCGCTCCGGCACGCTA --- CTCGAGCATCTGCTGCTCTAATGTGTGACCGGGCGGTCCAGGCAGCCATC
 D  I  A  P  A  R    ---  L  E  H  L  L  L  *
 'plsX -->
CAACACTCAGTTTCCTGCGCCAGTCGCGGCTGGCGCGTTATCTCCGACGAGCAAAAGACCACAAGGGACCTATTC
                                                                  RBS
                            CA fabD(Ts) (W258Q)
 286                         ||                  1219
AATGTCTGCATCCCTC --- GTTCGCTGGGTGGAG --- GCCCTGGCCTGAGAGAGAGAAAGGAGAGAATCCC
  M  S  A  S  L  ---  V  R  W  V  E  ---  A  L  A  *          RBS
  fabD -->
1247                          1985
ATGAGTCTGCAAGGT --- ATGTACATGAGCTGAATGTGACGTTACCTTTCTCGGGTCTGTCATAAGCGCTGTC
 M  S  L  Q  G  ---  M  Y  M  S  *
 fabG -->
XbaI/EcoRI
TAGAATTCGTTGCGGAATTGCAGCCGGGTTATTAGATAGAATTATCGAAGTGGCCGTGGGAAGGTGAGGCGTTCA

GCTTGAAAAGCAGAAAACCCTTTCTATACACTTACCCGCAGCCCAGCTGCTCGGATTTTCCATAGGAGTGAAAAC
                                                                        RBS
     2186                          2417
AAGGTATGAGCACCATCGAA --- GCTCCCCAGCAATAAGTAGTCGTCGGATTTTCCGACGTGGAAAAGCCGCA
      M  S  T  I  E  ---  A  P  Q  Q  *
      acp -->
CAGCCTGTAAGGGCGTGCGGCTTTTCTTTAGCGCCTTACTACTCTGGTAGTCTGTCGGCGCAACCCCGTGAAATG

                 2544                                          3789 3794
AGAAAGAGGAAGTCGTAGTGTCGCGTAGACGCGTCGTCATTACTGGCATG --- TTCGCCGACTGATGCTGGAC
       RBS        M  S  R  R  R  V  V  I  T  G  M  ---  F  A  D  *  M  L  D
                  fabF -->                                          pabC -->
   SalI
TGGGTCGAC 3811
 W  V  D
```

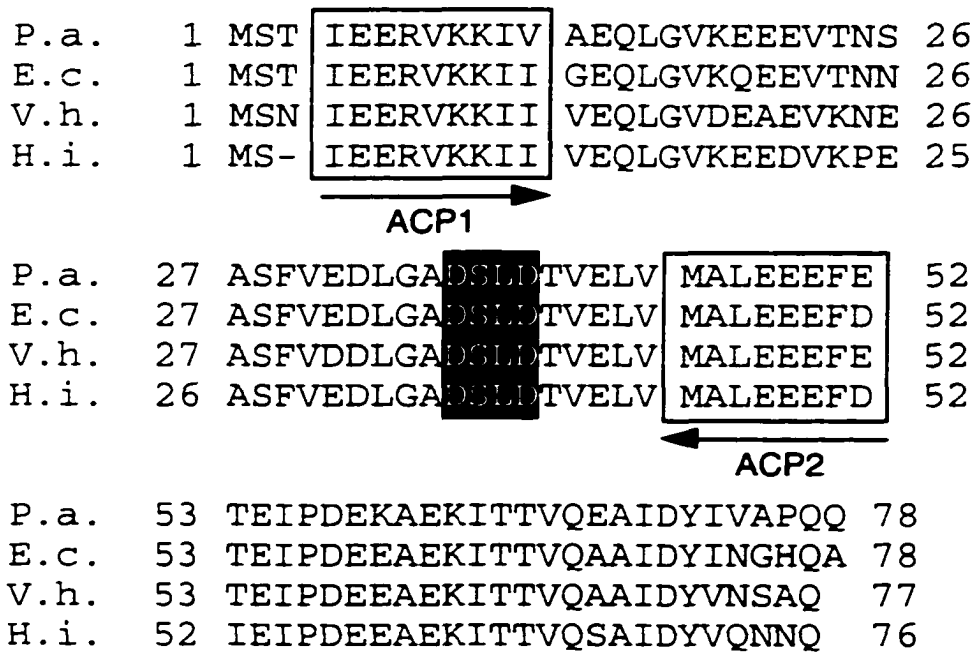


Figure 5.2. Alignments of Gram-negative ACPs. Alignments of *P. aeruginosa* (P.a.) ACP with its homologs from *E. coli* (E.c.), *V. harveyi* (V.h.) and *H. influenzae* (H.i.). Residues indicated in the boxed regions were used for design of the partially degenerate primers ACP1 and ACP2, which were used to PCR-amplify a partial *P. aeruginosa acpP* gene. The consensus sequences, DSLD, for attachment of the 4'-phosphopantetheine prostethic group are highlighted. The *acpP* nucleotide sequence deposited in the *P. aeruginosa* genome database was identical to our sequence with the exception of a single base change. It contained an A at position 2412 in place of a G found in our sequence (see Fig. 2B) and this changes the Pro CCC codon to a His CAC codon.

Although the two genes are translationally coupled, it is unclear whether both genes are co-transcribed from a single promoter upstream of *fabF*. The *fabF* mutant PAO198 (table 5.1), containing a non-polar Gm^r cassette in a transcriptional orientation opposite to *fabF* and *pabC*, was not a PABA auxotroph and we have not yet isolated mutants containing polar *fabF* mutations.

After our studies were completed, a contig appeared in the *Pseudomonas* Genome Project database which, albeit not annotated, confirmed our results and the gene order *plsX-fabD-fabG-acpP-fabF-pabC*, as well as the absence of *fabH* from this cluster. Searches of this database revealed several possible *fabH* homologs, which are located elsewhere in the chromosome and we are in the process of characterization of the most likely *fabH* homolog.

The deduced amino acid sequences of *fabD*, *fabG*, *acpP*, and *fabF* genes were analyzed, and, in general, the deduced amino acid sequences were most similar to the respective *E. coli* homologs. The identities were 66% for FabF, 65% for FabG and 56% for FabD. However, other bacterial Fab proteins also exhibited considerable similarities to the deduced *P. aeruginosa* protein sequences.

The high degree of primary amino acid sequence conservation was especially evident with ACP (figure 5.2). *P. aeruginosa* ACP was 90% identical to *E. coli* ACP, 83% identical to *H. influenzae* ACP and 80% identical to *V. harveyi* ACP. All ACP proteins consitsed of between 76 to 78 amino acids, and they contained the consensus sequence, DSLD, for attachment of the 4'-phosphopantetheine and a large proportion of acidic amino acids. The calculated isoelectric point of PAO1 ACP (pH 3.8) confirmed the acidic nature this protein.

5.3.2 Characteristics of *fab* genes and their products. Several lines of evidence indicated that the *fabF*, *fabD* and *fabG* genes cloned in this work encode β-ketoacyl-ACP synthase II (FabF), malonyl CoA-ACP transacylase (FabD) and β-ketoacyl-ACP reductase (FabG), respectively.

The evidence for FabF includes: (*i*) a high degree of similarity/identity to the well characterized homologs from *E. coli*; (*ii*) translation of both *E. coli* and *P. aeruginosa* FabF coding sequences is apparently initiated at a GTG because the first Met codon found in the *P. aeruginosa* FabF protein is not preceded by a suitable Shine-Dalgarno sequence and the first 10 NH_2-terminal amino acids of the *P. aeruginosa* FabF protein (MSRRRVVITG) are 80% identical to *the E. coli* FabF protein (MSKRRVVVTG), which has a GTG start (GenBank accession number AE000210); (*iii*) a *P. aeruginosa fabF* mutant was constructed by deletion of a 597-bp *Nco*I fragment from within the *fabF* gene, and replacing it with a 830-bp blunt-ended Gm^r-encoding cassette from plasmid pUCGM (33). The mutated *fabF* sequence was returned to the PAO1 chromosome via the gene replacement vector pEX100T (34). Gas chromatography-mass spectrometric (12) analysis of the fatty acid content in cell extracts of the *fabF* knockout mutant PAO198 (*fabF*::Gm^r) demonstrated eight-fold reduced levels of *cis*-vaccenic acid when compared to wild-type PAO1 (data not shown). This compares

favorably with a ten-fold reduction of *cis*-vaccenic acid levels seen in an *E. coli fabF* mutant (12).

The following evidence suggests that the *fabD* gene encodes malonyl CoA-ACP transacylase (FabD): (i) FabD is not only 56% identical (78% similar) to its counterpart from *E. coli* but contains the critical catalytic domain, the conserved pentapeptide GHSLG, which is the GXSXG signature motif of serine-dependent acylhydrolases (2); (ii) characterization of an *E. coli fabD*(Ts) mutant showed that the temperature-sensitive (Ts) phenotype resulted from a mutation that led to a W257Q substitution (43). We changed a conserved Trp^{258} to a Gln residue in the *P. aeruginosa* FabD protein by mutating two nucleotides (figure 5.1B) by site-specific mutagenesis using the Altered Sites Mutagenesis system (Promega). Primer FabD1 (table 5.1) introduced a double TG to CA mismatch that resulted in a W258Q change in the FabD amino acid sequence, which was confirmed by nucleotide sequence analysis. The return of the *fabD*(Ts) allele into the PAO1 chromosome was achieved utilizing a previously described strategy (20). FabD(Ts) mutants, including PAO204, were obtained at a frequency of 23%, demonstrating the essentiality of FabD in *P. aeruginosa*; and (iii) a H_6-FabD protein was overexpressed and purified (figure 5.3A), and its oberserved molecular weight of ~33-kDa matches closely that of the calculated molecular weight for FabD (32,442 Da) plus the H_6-containing NH_2-terminal extension (2,181 Da); and (iv) the purified FabD protein exhibits malonyl-CoA:ACP transacylase activity (see below).

Besides the high homology to other bacterial FabG proteins, and the presence of the amino acids of the NADPH binding signature $AX_2GXGX_2AX_6G$ near the NH_2-terminus, other evidence suggests its essential role in the cycles of fatty acid elongation in *P. aeruginosa*. First, despite repeated attempts, we were unable to isolate *fabG* knockout mutation indicating its essential nature. Second, the purified FabG protein is essential for fatty acid elongation in a reconstituted *in vitro* Fab synthesis system (21). A separate NADPH-dependent β-ketoacyl-ACP reductase, RhlG, has recently been described that is specifically involved in rhamnolipid synthesis (3), although no biochemical data supporting its presumed function were presented.

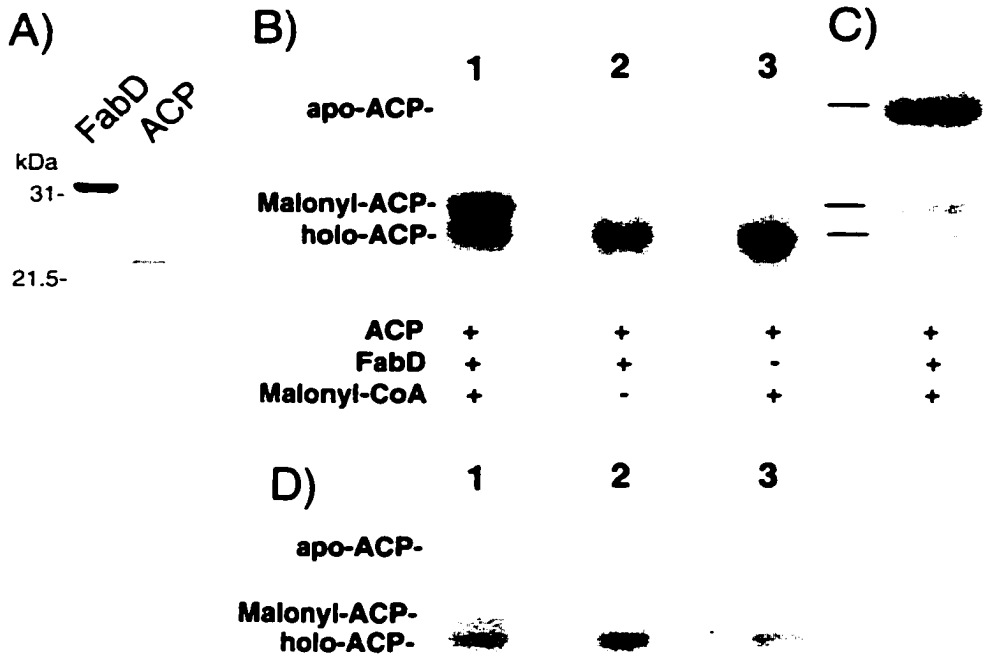


Figure 5.3. Purification of ACP, FabD, and assay of FabD activity. **A)** The FabD protein was expressed as an hexahistidine (H_6) FabD fusion protein and purified by Ni^{2+} agarose affinity chromatography. ACP was expressed as an ACP-intein-chitin binding domain fusion protein which was adsorbed to a chitin agarose affinity column, from which the ACP was eluted after self-cleavage of the fusion protein by DTT. Three μg of each protein were analyzed by electrophoresis on a 0.1% SDS-10% polyacrylamide gel, and protein bands were visualized by Coomassie Blue staining. The positions of two molecular weight markers, and their size in kilodaltons (kDa), are indicated on the left: carbonic anhydrase (31 kDa) and trypsin inhibitor (21.5 kDa). **B)** Acylation of ACP by H_6-FabD. The reaction mixtures (20 μl) contained ACP (2 μg), H_6-FabD (0.25 μg) and 0.1 mM malonyl-CoA in the indicated combinations. The products were analyzed by electrophoresis on 20% native polyacrylamide gels followed by staining with Commassie Blue. **C)** The acylation reaction and detection was performed as described in B) but the ACP was purified from cultures grown under conditions that did not allow for proper modification of apo-ACP with 4'-phosphopantetheine (see text for details). Panel **D)** illustrates the same experiment shown in panel B) except that the ACP was purified from a low-copy number expression construct.

5.3.3 Expression and purification of ACP. The ACP was overexpressed and purified utilizing the intein-CBD system. An ACP-intein-chitin binding domain (CDB) fusion protein was constructed following the basic protocol provided by New England Biolabs (Beverly, MA). Two PCR primers, ACP-Nde and ACP-Sap, were designed to introduce an *Nde*I site at

the ACP initiation codon and a *Sap*I site immediately downstream of the last codon of *acpP*. These primers were used to amplify a ~270-bp fragment utilizing pPS681 (table 5.1) DNA as the template and standard PCR conditions (18). The PCR fragment was cloned into pCYB1 DNA. This procedure yielded pPS981. When utilizing pPS981, various *E. coli* host strains, and different induction conditions, no detectable expression of ACP-intein-CBD was observed, unless the *lac* promoter was replaced with the more powerful T7 promoter from pT7-7 (39), a step that yielded pPS966. Optimization of the expression conditions using BL21(DE3)/pPS966 revealed that an overnight induction and growth at room temperature (RT) in LB medium (Gibco-BRL, Gaithersburg, MD) led to substantial overproduction of the ~66,000-*M*, ACP-intein-CBD fusion protein.

For purification of ACP-intein-CBD from a high-copy number expression vector, 4 l LB-ampicillin (100 μg/ml) cultures of BL21(DE3)/pPS966 were grown at 37°C temperature to log phase (A_{600nm} ~1.0) and gene expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were shaken at room temperature (RT) and harvested after a 9 h induction period. The cells were resuspended in 4 l of fresh LB medium without IPTG and further incubated for 1.5 h at 37°C before they were harvested. During this recovery period, most of the apo-ACP was converted to holo-ACP by ligation of the 4'-phosphopantetheine. When this period was omitted and the cells were harvested after a 13 h incubation at RT in the presence of IPTG, ~90-95% of the ACP preparation was apo-ACP which did not serve as a FabD substrate. The cell pellet was suspended in 50 ml of column buffer (20 mM Tris-HCl, pH 8.0; 500 mM NaCl; 0.1 mM EDTA; 0.1% Triton X100; 1 mM PMSF) and disrupted by French Press treatment (19,000 psi). All subsequent steps were performed at 4°C. Cell debris was removed by ultracentrifugation for 1 h at 260,000 x g. The cell extract was applied to a 10 ml bed volume of chitin beads (New England Biolabs) in a 30 ml column and washed with 15 volumes of column buffer. The column buffer was exchanged with cleavage buffer (20 mM Tris-HCl, pH 8.0; 50 mM NaCl; 0.1 mM EDTA; 30 mM DTT) and cleavage of the fusion protein on the column was achieved by overnight incubation. The cleaved ACP was eluted with 30 ml of cleavage buffer without DTT and fractions were collected. The ACP content of the fractions was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (24) and protein concentrations were

determined using the Bradford dye-binding assay from Bio Rad Laboratories with bovine serum albumin as the standard.

For purification of ACP from *E. coli* cells containing the low-copy number expression vector pPS1096, 250 ml LB-ampicillin cultures of SA1503(DE3)/pPS1096 were grown at 37°C to log phase (A_{600nm} ~1.0) and gene expression was induced by addition of 0.5 mM IPTG. The cells were shaken at RT for 12 h and the ACP protein was purified as described above. The low-copy number expression vector allowed for a simpler induction scheme and yielded an ACP preparation with a holo-ACP content of ~95%. This amount could not be boosted by growing the cells in the presence of pantothenic acid. In contrast to expression experiments using the high-copy number expression construct, the low-copy number expression vector did not lead to an observable cessation of cell growth.

SDS-PAGE analysis revealed that ACP had been purified to near homogeneity (figure 5.3A). On this gel, ACP migrated at the position of a 22.5-kDa protein, significantly larger than its calculated molecular weight of 8.7 kDa. The anomolous migration of PAO1 ACP on SDS-PAGE is consistent with observations for ACPs from other bacteria (25, 28, 35), and has been attributed to the protein's high charge to mass ratio (with ACP being highly acidic; calculated pI 3.8), as well as its low hydrophobic amino acid content, two factors that have a considerable influence on SDS binding (30). When the same ACP preparation was analyzed on a 0.1% SDS-13% PAGE containing 5 M urea, a single protein band of ~9 kDa was observed (data not shown), a value that closely matches its calculated mass of 8.7 kDa.

5.3.4 Characterization of ACP. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometric analysis (4) (performed at the Colorado State University Macromolecular Resource Facility) of the purified ACP fraction revealed three species with molecular weights of 8,583 Da (minor peak), 8,934 Da (major peak) and 17,844 Da (minor peak)(figure 5.4). These values correspond to the three ACP species commonly found in ACP preparations, apo-ACP (without 4'-phosphopantetheine), holo-ACP (with 4'-phosphopantetheine) and the ACP dimer. ACP dimerization was an artifact of the precipitation step used in MALDI sample preparation as no dimers were present in our ACP preparation, as judged by native PAGE (data not shown).

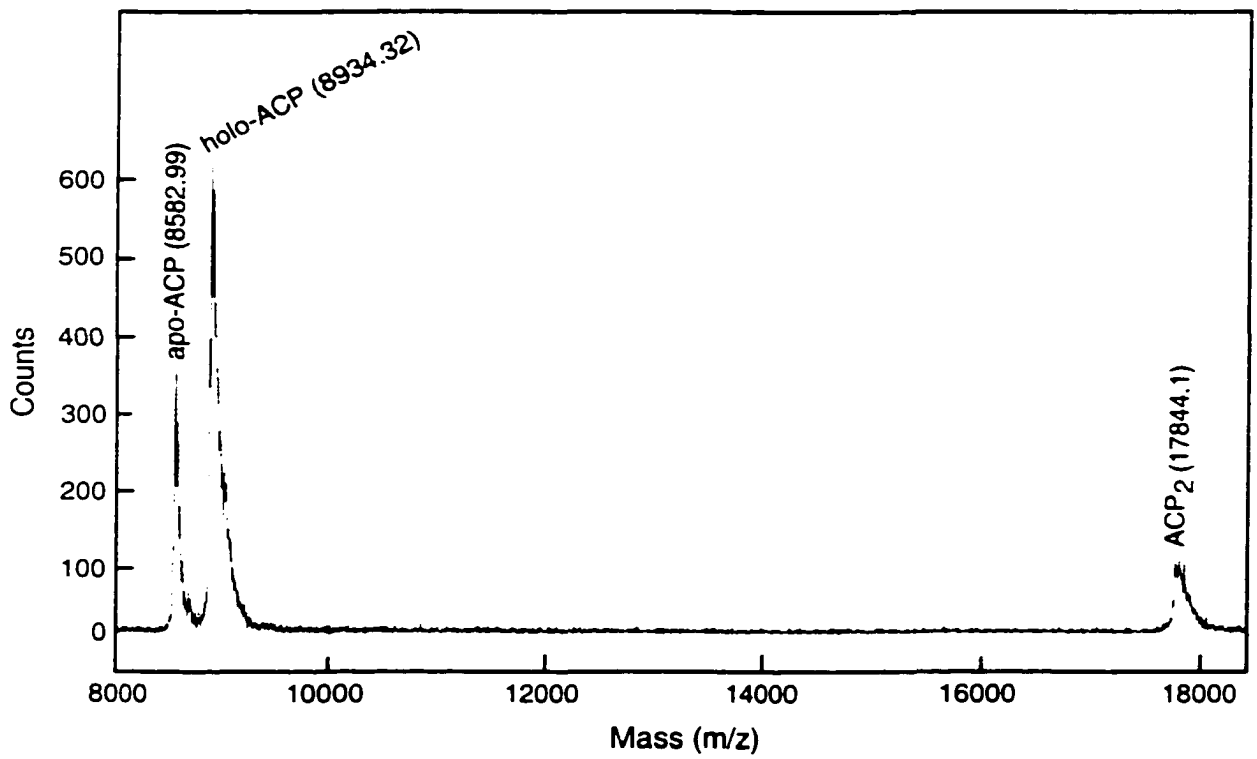


Figure 5.4. MALDI analysis of purified ACP. 25 pmoles of purified ACP were analyzed by matrix- assisted laser desorption/ionization (MALDI) spectroscopy using time-of-flight (TOF) detection. The MALDI/TOF trace indicate the presence of peaks corresponding to apo-ACP (8582.99), holo-ACP (8934.32) and ACP dimer (17844.1; ACP_2).

The data confirm that the majority of our recombinant ACP (the major peak at 8,934 Da) contains the 4'-phosphopantetheine group required for its activity, presumably attached to Ser^{36}, and that the NH_2-terminal methionine of ACP is post-translationally removed by an aminopeptidase (16), as has been observed with other ACPs (25, 28, 35). The latter was verified by NH_2-terminal amino acid sequence analysis of purified *P. aeruginosa* ACP (performed at the Peptide Sequencing Facility at the University of Victoria, Victoria, Canada), which revealed the sequence STIEE corresponding to amino acids 2-6 deduced from the nucleotide sequence (figure 5.1B).

Native PAGE is a powerful means for characterization of modified and unmodified ACP (29). It can be utilized to assess ACP dimer content and the ratio of apo-ACP to holo-ACP-SH. Since acylation alters the Stoke's radius of ACP, it can also be utilized to assess the

fraction of acyl-ACP in ACP preparations (29). Utilizing native PAGE, we showed that our ACP preparation (*i*) contained ~95% holo-ACP versus ~5% apo-ACP and (*ii*) contained no detectable acyl-ACPs (figure 5.3B). We do not know whether non-acylated ACP is due to masking of the 4'-phosphopantetheine prosthetic group in the ACP-intein-CBD fusion protein or due to efficient removal of acyl groups by the DTT used for cleavage of the fusion protein.

The identity of the holo-ACP was confirmed in a malonyl-CoA:ACP transferase assay by incubation of our ACP preparation with malonyl-CoA and purified FabD. For purification of FabD, a hexahistidine-FabD (H_6-FabD) expression vector was constructed. The *fabD* coding sequence was PCR-amplified from PAO1 genomic DNA with primers FabD-Nde, creating a *Nde*I site at the *fabD* ATG initiation codon, and FabD-Bam, which creates a *Bam*HI site immediately downstream of *fabD*. The PCR fragment was cloned into pET-15b (Novagen, Madison, WI) to form pPS979, which was then transformed into BL21(DE3). Expression of H_6-FabD, cell lysis and purification of the soluble fusion protein on a Ni^{2+}-agarose column (Qiagen) was performed as previously described (14), except that the cells were grown in LB-ampicillin medium. Purified FabD was used in acylation reactions (20 µl) that contained 2 µg ACP, 0.25 µg FabD, 0.1 mM malonyl-CoA in 20 mM Tris-HCl (pH 7.2), 100 mM NaCl, 10% glycerol, 1 mM DTT, 2 mM EDTA, 25 mM $MgSO_4$ and 0.1 mM $FeSO_4$. The mixtures were incubated at RT for 5 min, and products were analyzed by electrophoresis on 20% native PAGE, followed by staining with Commassie Blue R-250, as previously described (29). In this assay, at least 50% of the holo-ACP was converted into malonyl-ACP by FabD (figure 5.3B, lane 1); conversion was not complete since the reaction catalyzed by FabD is reversible. This reaction required FabD and malonyl-CoA since neither FabD alone (lane 2) nor malonyl-CoA alone (lane 1) led to formation of malonyl-ACP after a 5 min incubation period. Longer incubation times and higher malonyl-CoA concentrations resulted in some spontaneous acylation of ACP by malonyl-CoA alone. As shown in figure 5.3C, apo-ACP did not serve as a FabD substrate. Although of the ~10% holo-ACP present about half was converted to malonyl-ACP, the ACP preparation contained ~90% apo-ACP which did not serve as a FabD substrate. Similar results were obtained with ACP purified from a strain carrying a low-copy number ACP overproducer (figure 5.3D).

5.4 CONCLUSIONS

The ACP purification procedure described herein has several distinct advantages over other purification methods (7, 8): (i) it is rapid and yields are comparable to other methods (15, 25, 29). We routinely isolate in excess of 5 mg of ACP from 4 l of induced culture, containing either low- or high-copy number expression vectors, using a single 5 to 10 ml chitin agarose affinity column on which all the washing steps and the cleavage step are performed. We only know of one other method that yields more ACP when expressed from an overproducer but it is much more involved both in labor, as well as in instrumentation requirements (15); (ii) our purification procedure yields >95% holo-ACP, whereas other procedures relying on expression constructs yield mostly apo-ACP and little to almost no holo-ACP (7, 8). Although apo-ACP can efficiently be converted into holo-ACP by purified holo-ACP synthase (8), this step contaminates the ACP preparation and thus additional steps are required to purify the holo-ACP before further use; (iii) ACP purified by our procedure is not acylated and therefore does not require the deacylation steps prior to its use as holo-ACP in the synthesis of defined acyl-ACP substrates, when compared to ACP obtained with more traditional purification procedures (5); (iv) our purification method is inexpensive and can be performed in laboratories that neither have the expertise nor the equipment necessary for traditional protein purification schemes.

We have succesfully used the *P. aeruginosa* ACP prepared in this manner for the synthesis of defined acyl-ACP substrates (19); together with purified H_6-FabD and H_6-FabG, it functions in a reconstituted enzyme system leading to synthesis of biologically active 3-oxo-C_{12}-HSL from simple metabolic precursors (21).

Nucleotide sequence accession number. The complete sequence of the 3,811-bp *Eco*RV-*Sal*I fragment (figure 5.1A) was deposited in GeneBank and assigned accesssion no. U91631.

5.5 ACKNOWLEDGMEMTS

This work was suported by Public Health Service Grant GM56685 from the National Institutes of Health and by a grant from the CSU College of Veterinary Medicine and Biomedical Sciences. A.J.K. was supported by a Graduate Teaching Assistantship from the Department of Microbiology. A.J.K. and T.T.H. contributed equally to this work.

5.6 REFERENCES

1. **Baldwin, J. E., J. W. Bird, R. A. Field, N. M. O'Callaghan, C. J. Schofield, and A. C. Willis.** 1991. Isolation and partial characterization of ACV synthetase from *Cephalosporium acremonium* and *Streptomyces clavilugerus*. Evidence for the presence of phosphopantothenate in ACV synthase. J. Antibiot. **44:**241-248.

2. **Brenner, S.** 1988. The molecular evolution of genes and proteins: a tale of two serines. Nature **334:**528-530.

3. **Campos-Garcia, J., A. D. Caro, R. Najera, R. M. Miller-Maier, R. A. Al-Tahhan, and G. Soberon-Chavez.** 1998. The *Pseudomonas aeruginosa rhlG gene* encodes an NADPH-dependent β-ketoacyl reductase which is specifically involved in rhamnolipid synthesis. J. Bacteriol. **180:**4442-4451.

4. **Cottrell, J. S., and C. W. Sutton.** 1998. The identification of electrophoretically separated proteins by peptide mass fingerprinting, p. 1-16. *In* J. M. Walker (ed.), Protein Protocols on CD-ROM. Humana Press, Totowa, NJ.

5. **Cronan, J. E., and A. L. Klages.** 1981. Chemical synthesis of acyl thioesters of acyl carrier protein with native structure. Proc. Natl. Acad. Sci. USA. **78:**5440-5444.

6. **Cronan, J. E., and C. O. Rock.** 1996. Biosynthesis of membrane lipids, p. 612-636. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. Brooks Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*. Am. Soc. Microbiol., Washington, DC.

7. **Crosby, J., D. H. Sherman, M.J. Bibb, W. P. Revill, D. A. Hopwood, and T. J. Simpson.** 1995. Polyketide synthase acyl carrier proteins from *Streptomyces*: expression in *Escherichia coli*, purification and partial characterization. Biochim. Biophys. Acta. **1251:**32-42.

8. **Epple, G., K. M. G. M. Van der Drift, J. E. Thomas-Oates, and O. Geiger.** 1998. Characterization of a novel acyl carrier protein, RkpF, encoded by an operon involved in capsular polysaccharide biosynthesis in *Sinrhizobium meliloti*. J. Bacteriol. **180:**4950-4954.

9. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J.-F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudeck, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496-512.

10. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. **50:**727-751.

11. **Geiger, O., H. P. Spaink, and E. P. Kennedy.** 1991. Isolation of the *Rhizobium leguminosarum* NodF nodulation protein: NodF carries a 4'-phosphopantetheine prosthetic group. J. Bacteriol. **173:**2872-2878.

12. **Gelman, E. P., and J. E. Cronan.** 1972. Mutant of *Escherichia coli* deficient in the synthesis of *cis*-vaccenic acid. J. Bacteriol. **112:**381-387.

13. **Green, J. M., W. K. Merkel, and B. P. Nichols.** 1992. Characterization and sequence of *Escherichia coli pabC*, the gene encoding aminodeoxychorismate lyase, a pyridoxal phosphate-containing enzyme. J. Bacteriol. **174:**5317-5323.

14. **Heath, R. J., and C. O. Rock.** 1996. Roles of the FabA and FabZ β-hydroxyacyl-acyl carrier protein dehydratase in *Escherichia coli* fatty acid biosynthesis. J. Biol. Chem. **271:**27795-27801.

15. **Hill, R. B., K. R. MacKenzie, J. M. Flanagan, J. E. Cronan, and J. H. Prestegard.** 1995. Overexpression, purification and characterization of *Escherichia coli* acyl carrier protein and two mutant proteins. Protein Exp. Pur. **6:**394-400.

16. **Hirel, P. H., J. M. Schmitter, P. Dessen, K. Fayat, and T. S. Banque.** 1989. Extent of N-terminal methionine excision from *Escherichia coli* proteins is governed by the side-chain length of the penultimate amino acid. Proc. Natl. Acad. Sci. USA. **86:**8274-8251.

17. **Hoang, T., S. Williams, and H. P. Schweizer.** 1997. Molecular genetic analysis of the region containing the essential *Pseudomonas aeruginosa asd* gene encoding aspartate-β-semialdehyde dehydrogenase. Microbiol. **143:**899-907.

18. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77-86.

19. **Hoang, T. T., and H. P. Schweizer.** 1999. Characterization of the *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase: a target for triclosan and its role in acylated homoserine lactone synthesis. J. Bacteriol. **181:** 5489-5497.

19a. **Hoang, T.T., Y. Ma, R.J. Stern, M.R. McNeil, and H.P. Schweizer.** 1999. Construction and use of low-copy number T7 expression vectors: purification of *Mycobacterium tuberculosis* RmlD and *Pseudomonas aeruginosa* LasI and RhlI, and functional analysis of purified RhlI. Gene **237:** 361-371.

20. **Hoang, T. T., and H. P. Schweizer.** 1997. Fatty acid biosynthesis in *Pseudomonas aeruginosa*: Cloning and characterization of the *fabAB* operon encoding β-hydroxyacyl-acyl carrier protein dehydratase (*fabA*) and β-ketoacyl-acyl carrier protein synthase I (*fabB*). J. Bacteriol. **179:**5326-5332.

21. **Hoang, T. T., and H. P. Schweizer.** 1999. Synthesis of the *Pseudomonas aeruginosa* autoinducer *N*-[3-oxo-dodecanoyl]-L-homoserine lactone from metabolic precursors in a reconstituted enzyme system, abstr. B/D309, In Abstracts of the 99th General Meeting, of the American Society for Microbiology, Chicago, IL, p. 90.

22. **Issartel, J. P., V. Koronakis, and C. Hughes.** 1991. Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. Nature **351:**759-761.

23. **Keating, D. H., M. R. Carey, and J. E. Cronan.** 1995. The unmodified (apo) form of *Escherichia coli* acyl carrier protein is a potent inhibitor of cell growth. J. Biol. Chem. **270:**22229-22235.

24. **Makowski, G. S., and M. L. Ramsby.** 1993. pH modification to enhance the molecular sieving properties of sodium dodecyl sulfate-10% polyacrylamide gel. Anal. Biochem. **212:**283-285.

25. **Morbidoni, H. R., D. De Mendoza, and J. E. Cronan.** 1996. *Bacillus subtilis* acyl carrier protein is encoded in a cluster of lipid biosynthesis genes. J. Bacteriol. **178:**4794-4800.

26. **Moré, M. I., D. Finger, J. L. Stryker, C. Fuqua, A. Eberhard, and S. C. Winans.** 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science **272:**1655-1658.

27. **Ochsner, U. A., A. Fiechter, and J. Reiser.** 1994. Isolation, characterization , and expression in *Escherichia coli* of the *Pseudomonas aeruginosa rhlAB* genes encoding a rhmnosyltransferase involved in rhamnolipid biosurfactant synthesis. J. Biol. Chem. **269:**19787-19795.

28. **Rawlings, M., and J. E. Cronan.** 1992. The gene encoding *Escherichia coli* acyl carrier protein lies within a cluster of fatty acid biosynthetic genes. J. Biol. Chem. **267:**5751-5754.

29. **Rock, C. O., and J. E. Cronan.** 1981. Acyl carrier protein from *Escherichia coli.* Methods Enzymol. **71:**341-351.

30. **Rock, C. O., and J. E. Cronan.** 1979. Re-evaluation of the solution structure of acyl carrier protein. J. Biol. Chem. **254:**9778-9785.

31. **Rusnak, F., M. Sakaitani, D. Drueckhammer, J. Reichert, and C. T. Walsh.** 1991. Biosynthesis of the *Escherichia coli* siderophore enterobactin: sequence of the *entF* gene, expression and ourification of EntF, and analysis of covalent phosphopantetheine. Biochem. **30:**2916-2927.

32. **Schaefer, A. L., D. L. Val, B. L. Hanzelka, J. E. Cronan, and E. P. Greenberg.** 1996. Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. Proc. Natl. Acad. Sci. USA. **93:**9505-9509.

33. **Schweizer, H. P.** 1993. Small broad-host-range gentamycin resistance cassettes for site-specific insertion and deletion mutagenesis. BioTechniques **15:**831-833.

34. **Schweizer, H. P., and T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:**15-22.

35. **Shen, B., R. G. Summers, H. Gramajo, M. J. Bibb, and C. R. Hutchinson.** 1992. Purification and characterization of the acyl carrier protein of the *Streptomyces glaucescens* tetracenomycin C polyketide synthase. J. Bacteriol. **174:**3818-3821.

36. **Shen, Z., and D. M. Byers.** 1996. Isolation of *Vibrio harveyi* acyl carrier protein and the *fabG, acpP,* and *fabF* genes involved in fatty acid biosynthesis. J. Bacteriol. **178:**571-573.

37. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60-89.

38. **Summers, R. G., A. Ali, B. Shen, W. A. Wessel, and C. R. Hutchinson.** 1995. Malonyl-coenzyme A:acyl carrier protein acyltransferase of *Streptomyces glaucescens*: a possible link between fatty acid and polyketide biosynthesis. Biochemistry **34:**9389-9402.

39. **Tabor, S.** 1995. Expression using the T7 RNA polymerase/promoter system, p. 16.2-16.9. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Short protocols in Molecular Biology, 3 ed., John Wiley & Sons, NY.

40. **Val, D. L., and J. E. Cronan.** 1998. In vivo evidence that *S*-adenosylmethionine and fatty acid intermediates are the substrates for the LuxI family of autoinducer synthases. J. Bacteriol. **180:**2644-2651.

41. **Van Delden, C., and B. H. Iglewski.** 1998. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Em. Infect. Dis. **4:**551-560.

42. **Van Den Boom, T., and J. E. Cronan.** 1989. Genetics and regulation of bacterial lipid metabolism. Annu. Rev. Microbiol. **43:**317-343.

43. **Verwoert, I. G. S., E. F. Verhagen, K. H. van der Linden, H. Verbree, J. J. Nijkamp, and A. R. Stuitje.** 1994. Molecular characterization of an *Escherichia coli* mutant with a temperature-sensitive malonyl coenzyme A:acyl carrier protein transacylase. FEBS Letters **348:**311-316.

44. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195-199.

CHAPTER 6

Characterization of *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase (FabI): a target for the antimicrobial triclosan and its role in acylated homoserine lactone synthesis.

(Presented in Tung T. Hoang and Herbert P. Schweizer. 1999. *Journal of Bacteriology* **181**: 5489-5497)

The work presented in this article corroborated the notion that fatty acid biosynthetic enzymes are responsible for supplying the acyl moieties for the synthesis of butyryl-homoserine lactone autoinducer molecules. It also demonstrated that the *P. aeruginosa* FabI is a target for triclosan and, unlike any other bacterium looked at so far, that this bacterium contains at least one other enoyl-ACP reductase activity.

6.1 ABSTRACT

The *Pseudomonas aeruginosa fabI* structural gene, encoding enoyl-ACP reductase, was cloned and sequenced. Nucleotide sequence analysis revealed that *fabI* is probably the last gene in a transcriptional unit that includes a gene encoding an ATP-binding protein of an ABC transporter of unknown function. The FabI protein was similar in size and primary sequence to other bacterial enoyl-acyl carrier protein (ACP) reductases, and contained signature motifs for the FAD-dependent pyridine nucleotide reductase and glucose/ribitol dehydrogenase families, respectively. The chromosomal *fabI* gene was disrupted and the resulting mutant was viable but possessed only 62% of the total enoyl-ACP reductase activity

found in wild-type cell extracts. The *fabI*-encoded enoyl-ACP reductase activity was NADH-dependent and inhibited by triclosan; the residual activity in the *fabI* mutant was also NADH-dependent but not inhibited by triclosan. An oligohistidine-tagged FabI protein was purified and characterized. Purified FabI (i) could use NADH but not NADPH as co-factor; (ii) used both crotonyl-coenzyme A and crotonyl-ACP as substrates, although it was 6-fold more active with crotonyl-ACP; and (iii) was efficiently inhibited by low concentrations of triclosan. A FabI Gly^{95} to Val active site amino acid substitution was generated by site-directed mutagenesis and the mutant protein was purified. The mutant FabI protein retained normal enoyl-ACP reductase activity but was highly triclosan-resistant. When coupled to FabI, purified *P. aeruginosa N*-butyryl-L-homoserine lactone (C_4-HSL) synthase, RhlI, could synthesize C_4-HSL from crotonyl-ACP and *S*-adenosylmethionine. This reaction was NADH-dependent and inhibited by triclosan. The levels of C_4-HSL and *N*-[3-oxo-dodecanoyl]-L-homoserine lactones were reduced 50% in a *fabI* mutant, corroborating the role of FabI in acylated homoserine lactone synthesis *in vivo*.

6.2 INTRODUCTION

The important opportunistic pathogen *Pseudomonas aeruginosa* contains a type II or dissociated fatty acid synthetase system, in which the individual reactions are catalyzed by separate proteins (22, 24). Although the overall organization of the *P. aeruginosa fab* genes is similar to the one found in *E. coli* [for review see (9, 28)], some potentially significant differences exist. First, the *P. aeruginosa fabA and fabB* genes, ecoding β-hydroxyacyl-acyl carrier protein (ACP) dehydratase and β-ketoacyl-ACP synthase I, form an operon (22), whereas in *E. coli* these genes map to separate genetic loci. Second, unlike *E. coli* and several other Gram-negative bacteria, the *fabH* gene, encoding β-ketoacyl-synthase III, is absent from the *fabD-fabG-acpP-fabF* gene cluster, encoding malonyl-coenzyme A (CoA):ACP transacylase, β-ketoacyl-ACP reductase, ACP and β-ketoacyl-ACP synthase II (25). Searches of the *P. aeruginosa* genome database revealed several potential *fabH*-homologs located elsewhere on the genome.

Our current model for fatty acid biosynthesis in *P. aeruginosa* is shown in figure 6.1. Three β-ketoacyl-ACP synthases KAS I (FabB), KAS II (FabF) and KAS III (FabH) play pivotal roles in fatty acid synthesis. Initiation requires malonyl-CoA and malonyl-ACP. Malonyl-CoA is synthesized from acetyl-CoA via the acetyl carboxylase reaction (5). Malonyl-ACP is derived from malonyl-CoA and ACP by malonyl-CoA:ACP transacylase (FabD) (24, 25). Although a *P. aeruginosa fabH* homolog has only tentatively been identified, the first cycle of elongation is probably initiated by KAS III (FabH), which condenses malonyl-ACP with acetyl-CoA. Subsequent cycles are then initiated by condensation of malonyl-ACP with acyl-ACP, catalyzed by KAS I (FabB) for saturated fatty acids substrates and KAS II (FabF) for unsaturated fatty acid substrates. In the second step, the resulting β-ketoester is reduced to a β-hydroxyacyl-ACP by a NADPH-dependent β-ketoacyl-ACP reductase (FabG). It has recently been shown that *P. aeruginosa* contains a FabG homolog, RhlG, which presumably functions as a NADPH-dependent β-ketoacyl-ACP reductase specific for rhamnolipid synthesis (6). The third step in the cycle is either catalyzed by the *fabA*- or by *fabZ*-encoded β-hydroxyacyl-ACP dehydratase. The final step in each cycle involves conversion of *trans*-2-enoyl-ACP to acyl-ACP, a reaction catalyzed by NADH-dependent enoyl-ACP reductase (FabI). Physiologically, FabI is an important enzyme because (*i*) reduction of enoyl-ACP derivatives is thought to regulate the ratio of saturated versus unsaturated fatty acids, and coordinate fatty acid and phospholipid syntheses (16, 23); and (*ii*) it plays a determinant role in completing cycles of fatty acid elongation (15). FabI belongs to the short chain alcohol dehydrogenase family and in *E. coli* is the target of a group of antibacterial compounds, the diazoborines (44) and triclosan (19, 33). In *Mycobacterium tuberculosis* the FabI homolog InhA is one of the targets for the clinically used antimycobacterial isoniazid (2), and genetic evidence was obtained that *M. smegmatis* InhA is a triclosan target (32). More recently, it has been shown that *E. coli* mutants resistant to the antiseptic triclosan contain *fabI* mutations (33), and a subsequent study confirmed that triclosan and other related compounds indeed target FabI (19). Although *P. aeruginosa* strain PAO1 is resistant to triclosan due to constitutive expression of the *mexAB-oprM* encoded efflux pump, Δ(*mexAB-oprM*) mutants are susceptible to triclosan (41). The *E. coli* FabI protein has been co-crystallized with NADH and thienodiazoborine (18), or NAD^+ (26),

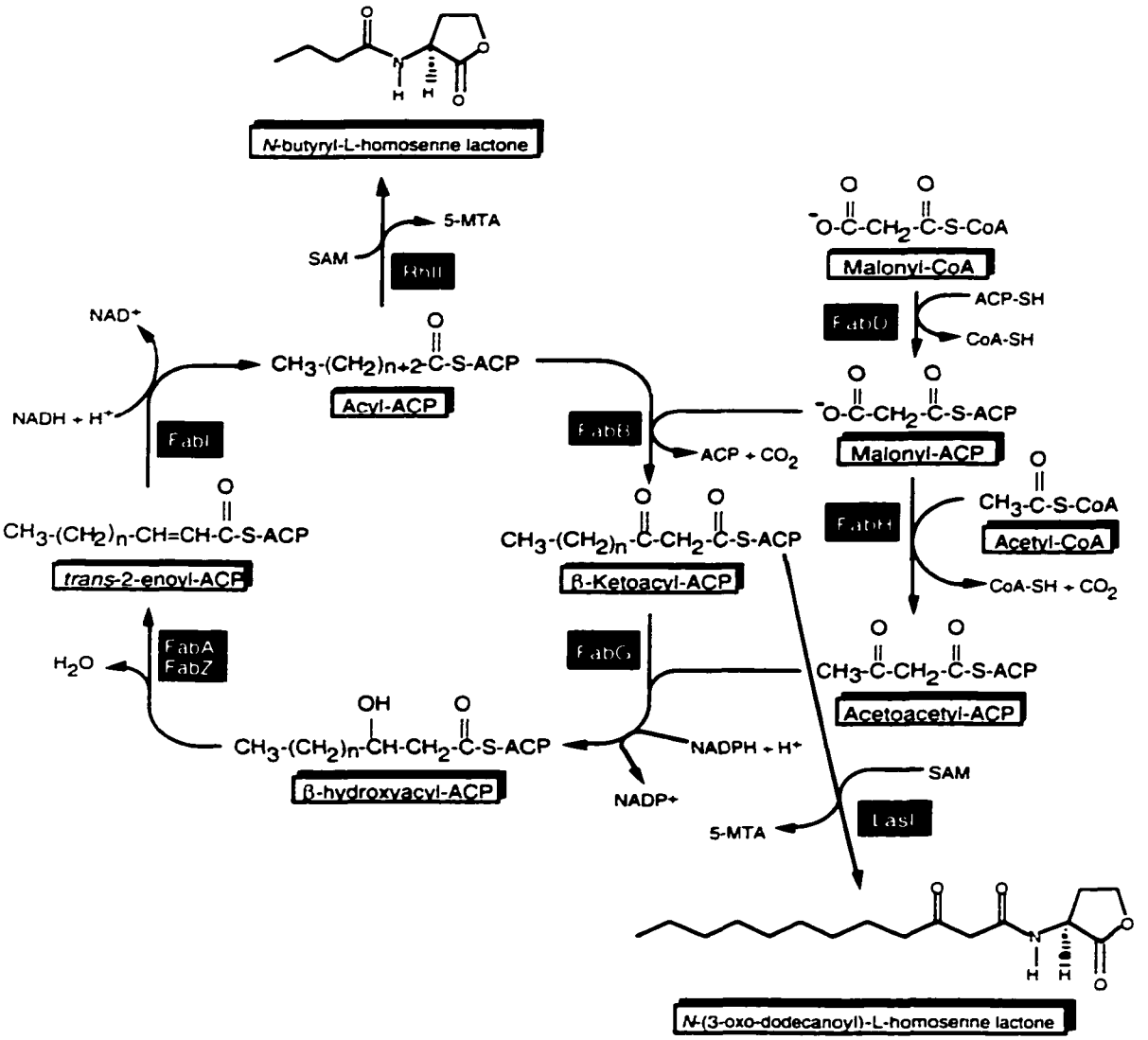


Figure 6.1. Current model for fatty acid biosynthesis in *P. aeruginosa* and the proposed role of butyryl-ACP as acyl donor in *N*-butyryl-L-homoserine lactone synthesis. The genes for all enzymes have been identified and characterized, except *fabH* for which only a tentative identification has been made among several paralogs. Abbreviations: ACP, acyl carrier protein; ACP-SH, ACP with 4'-phosphopantetheine co-factor; CoA-SH; coenzyme A; FabA (and FabZ), β-hydroxyacyl-ACP dehydratase; FabB, β-ketoacyl synthase I; FabD; malonyl-CoA:ACP transacylase; FabF, β-ketoacyl synthase II; FabG, β-ketoacyl-ACP reductase; FabH, β-ketoacyl synthase III; FabI; enoyl-ACP reductase; 5-MTA, 5-methylthioadenosine; SAM, *S*-adenosylmethionine; LasI, *N*-(3-oxo)-dodecanoyl homoserine lactone synthase; RhlI, *N*-butyryl homoserine lactone synthase.

and these studies allowed definition of the enzyme active site and amino acid residues important in substrate and drug interactions.

Besides providing fatty acid intermediates for a multitude of cellular constituents, i.e. phospholipids (9), lipid A (37), etc., the Fab pathway has been implicated in providing the acyl groups for the acylated homoserine lactones that are the signalling molecules in quorum-sensing (14, 35, 39, 45). In *P. aeruginosa*, quorum-sensing is a mechanism that regulates virulence factor gene expression (46), biofilm formation *in vitro* (10) and in natural settings (31), twitching motility (13), and other important cellular processes. According to the current model, quorum-sensing in *P. aeruginosa* involves two separate systems, LasR-LasI and RhlR-RhlI. These proteins are encoded by two separate, tandemly arranged transcriptional units. In these two systems, the LasI and RhlI proteins are homoserine lactone (HSL) synthases that direct the synthesis of *N*-(3-oxo) dodecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butyryl-L-homoserine lactone (C_4-HSL), respectively. According to the current model, the synthases react with acyl-ACPs and *S*-adenosylmethionine (SAM) to form the cognate homoserine lactones with concomitant release of 5-methylthioadenosine (5-MTA) (35, 39). For C_4-HSL synthesis, the *rhlI*-encoded *P. aeruginosa* HSL synthase RhlI catalyzes the formation of C_4-HSL in a reaction utilizing butyryl-ACP as the acyl donor and SAM as the nucleophile in a lactonization reaction (35, 39).

In this paper we describe the characterization of the *P. aeruginosa fabI* gene and its product, show evidence for the presence of at least one other enoyl-ACP reductase in this bacterium, provide biochemical evidence that FabI is a triclosan target and demonstrate its role in C_4-HSL synthesis.

6.3 MATERIALS AND METHODS

6.3.1 Bacterial strains, plasmids, primers and media. The relevant strains, plasmids and primers are shown in table 6.1. LB (Luria-Bertani) medium (34) was routinely used as the rich medium for all bacterial strains. For growth of the *fabI*(Ts) mutant JP1111 the NaCl concentration of LB medium was reduced to 0.05%. For some experiments, RB medium

Table 6.1. Bacterial strains, plasmids and primers

Strain, plasmid or primer	Description	Reference or source
E. coli		
DH5αF'	[F' ϕ80*lacZ*ΔM15] Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* [r_K^- m_K^+] *supE44 thi-1 gyrA relA1*	(27)
JP1111	Hfr *galE45 fabI392*(Ts) *relA1 spoT1*	(44)
BL21(DE3)	*E. coli* B; F' *ompT* r_B^- m_B^- (λDE3)	(43)
P. aeruginosa		
PAO1	PAO1	B. H. Holloway
PAO200	PAO1 with Δ(*mexAB-oprM*)	(41)
PAO234	PAO1 with *fabI*::Gm^r-*FRT*	This study
PAO235	PAO1 with *fabI*::*FRT*	This study
Plasmids		
pET-15b	Ap^r; hexahistidine fusion and expression vector	Novagen
pWSK29/30	Ap^r; low-copy number cloning and T7 expression vectors	(47)
pFLP2	Ap^r; source of Flp recombinase	(21)
pPS856	Ap^r, Gm^r; source of Gm^r-*FRT* cassette	(21)
pUCP21T	Ap^r; broad-host-range cloning vector	(42)
pEX18Tc	Tc^r; *sacB*-based gene replacement vector	(21)
pPS922	Ap^r; *fabI*$^+$ (ligation of a 880-bp *Bam*HI-*Hin*dIII PCR fragment between the same sites of pWSK30; *fabI*-transcription driven by P_{lac}^{a})	This study

Table 6.1. Bacterial strains, plasmids and primers (cont.)

Strain, plasmid or primer	Description	Reference or origin
pPS925	Ap^r; *fabI*::Gm^r-*FRT* (ligation of a 1,053-bp blunt-ended *Sac*I fragment from pPS856 into the *Sma*I site of pPS922)	This study
pPS933	Ap^r; *sacB*$^+$ *fabI*::Gm^r-*FRT* (ligation of a 1.95-kb *Bam*HI-*Hin*dIII fragment between the same sites of pEX18Tc)	This study
pPS935	Ap^r; H_6-FabI expression vector (PCR-amplified 0.85-kb *Nde*I-*Bam*HI fragment cloned between same sites of pET-15b)	This study
pPS967	Ap^r; *fabI*$^+$ (pUCP21T with *Bam*HI-*Hin*dIII fragment from pPS922; *fabI*-transcription driven by P_{lac})	This study
pPS1098	Ap^r; H_6-FabI expression vector (PCR-amplified 0.85-kb *Nde*I-*Bam*HI fragment cloned between same sites of pET-15b); expresses H_6-FabI mutant protein.	This study
Primers[b]		
FabIU	(*Hin*dIII)-GCAAGCttGCGGAAAACTGAGTAGCAGA	
FabID	(*Bam*HI)-ATGGatCCGCTTGAAGCGGCATTTTTC	
FabI-Nde	(*Nde*I)-GGcatATGGGATTTCTCACAGGAAAAC	
FabI G>V	CCACTCCGTCGtATTCGCTCCAG	

[a] P_{lac}, *E. coli lac* operon promoter
[b] Primers are printed 5' to 3'; lowercase letters either indicate non-matching oligonucleotides to form the indicated motif as underlined or introduce other non-motif changes indicated in lowercase letters

(20) was used instead of LB. The minimal medium employed for growth of *P. aeruginosa* was VBMM (40). Concentrations of antibiotics used in selection media were: *E. coli*, ampicillin (Ap), 100 μg/ml and gentamicin (Gm), 15 μg/ml; *P. aeruginosa*: carbenicillin (Cb), 500 μg/ml and Gm, 200 μg/ml.

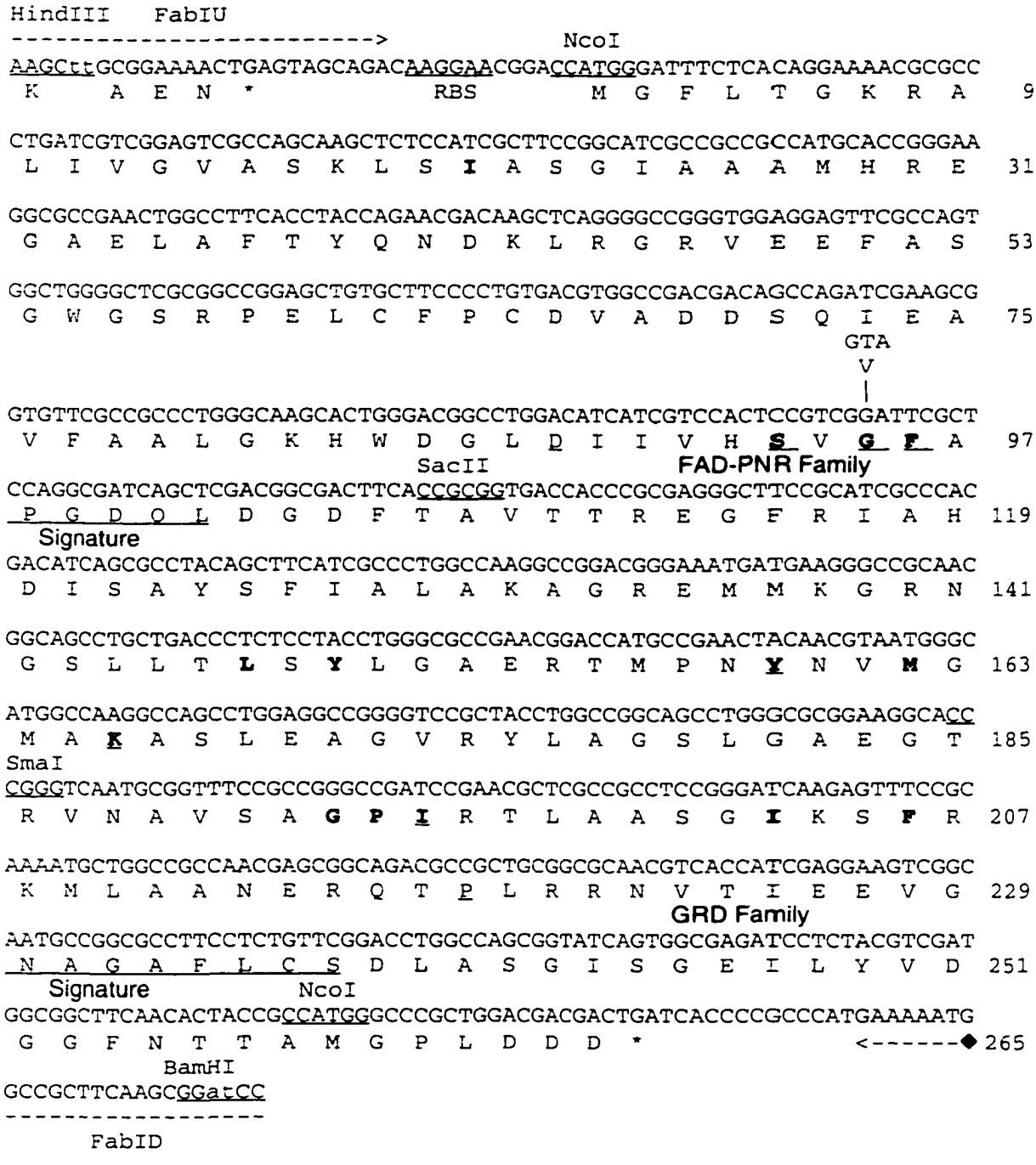
6.3.2 General DNA procedures. Routine DNA procedures were performed as previously described (21). The details underlying construction of pPS921 were as follows. For PCR amplification of a fragment from chromosomal DNA, two mutagenic oligonucleotides were synthesized. The FabIU and FabID primers were designed to introduce a *Hin*dIII site upstream of *fabI* and a *Bam*HI site downstream of *fabI*, respectively (table 6.1 and figure 6.2). These oligonucleotides were used to prime synthesis from PAO1 chromosomal DNA. PCR reactions were performed on a PTC-100 PCR system thermocycler (MJ Research, Watertown, MA), as previously described (21, 22). The ca. 870-bp PCR fragment was ligated to *Bam*HI+*Hin*dIII-digested pWSK30 (47) DNA to form pPS921. Nucleotide sequences were determined and analyzed as previously described (22). Motif searches were performed using the online E-Motif program provided by Stanford University, Palo Alto, CA.

A two-step method was used for site-directed mutagenesis for *fabI*. First, PCR reactions were set up as described above but using pPS935 DNA as the template, and DNA synthesis was primed using the T7 terminator primer (Novagen, Madison, WI) and a mutagenic primer, FabI G>V (table 6.1) that was designed (i) to introduce a single G to T change which leads to a Gly^{95} to Val amino acid change and (ii) to yield a PCR product that would properly prime to pPS935 DNA in a second round of PCR even when T-tailed by *Taq* DNA polymerase. The single 1,046-bp PCR product was digested with *Bam*HI+*Nde*I, and the resulting 840-bp *Bam*HI-*Nde*I fragment was gel-purified and ligated to *Bam*HI+*Nde*I-digested pET-15b DNA. The presence of the single G to T change was verified by nucleotide sequence analysis.

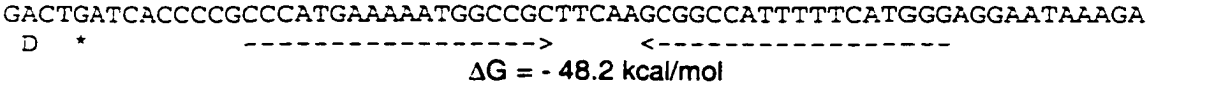
6.3.3 Disruption of the *fabI* gene. The *fabI* strain PAO235 was isolated in several steps. A plasmid-borne *fabI* mutation was constructed by insertion of the a blunt-ended 1053-bp Gm^r-*FRT* cassette from pPS856 (21) into the *Sma*I site located within the *fabI* coding sequence. The resulting mutation was returned to the *P. aeruginosa* chromosome after subcloning into the *sacB*-based suicide vector pEX18Tc (21) to derive the *fabI*::Gm^r-*FRT* mutant PAO234. From PAO234, the unmarked *fabI*::*FRT* mutant PAO235 was then derived by *in vivo* excision of the Gm^r-*FRT* cassette with Flp recombinase (21). The mutants were confirmed by performing colony PCR (21) utilizing the FabIU and FabID primers.

Figure 6.2. Nucleotide sequence of the *P. aeruginosa fabI*-containing region. **A)** The *fabI* region was PCR-amplified from PAO1 genomic DNA utilizing the mutagenic oligonucleotides labeled FabIU and FabID. The cloned *Bam*HI-*Hin*dIII fragment was sequenced on both strands. Listed below the nucleotide sequence is the deduced amino acid sequence of FabI and the extreme carboxy-terminal amino acids of an ORF with high similarity to the ATP-binding proteins of ABC transporters. The FAD-dependent pyridine nucleotide reductase (FAD-PNR) and glucose/ribitol dehydrogenase (GRD) family signatures found in the FabI coding sequence are underlined. Amino acids proposed to interact with NADH in the *E. coli* enzyme (1) via hydrogen bonds are printed in underlined bold-face letters and those residues that form hydrophobic contacts are printed in bold-face letters. The single nucleotide change introduced by site-directed mutagenesis that changed codon 95 and led to expression of a triclosan-resistant FabI protein is indicated above the sequence. A proposed consensus ribosome-binding site (RBS) is underlined. The priming sites for FabIU and FabID, and the directions of synthesis from the primers are marked by the broken arrows. Nucleotide changes incorporated into FabIU and and FabID to generate the indicated *Bam*HI and *Hin*dIII sites are printed in lower case letters. A diamond marks a nucleotide present in the *P. aeruginosa* genome database sequence that was deleted from the PCR-amplified fragment due to an error in FabID primer design. **B)** Nucleotide sequence of the *fabI* downstream region taken from the *P. aeruginosa* genome database. The last codon of the *fabI* coding sequence is shown and inverted arrows mark sequences capable of forming a stable RNA stem-loop structure with a calculated free energy (ΔG) of -48.2 kcal/mol.

A)



B)



6.3.4 Purification of an oligohistidine-tagged FabI fusion protein. A hexahistidine-FabI (H_6-FabI) expression vector was constructed by PCR-amplifying the *fabI* coding sequence from PAO1 genomic DNA with primers FabI-Nde, creating a *Nde*I site at the *fabI* ATG initiation codon and FabID, which creates a *Bam*HI site immediately downstream of *fabI*. The PCR fragment was digested with *Nde*I and *Bam*HI, and the resulting 840-bp *Bam*HI-*Nde*I fragment ligated between the same restriction sites of pET-15b to form pPS935, which was then transformed into BL21(DE3). Expression of H_6-FabI, cell lysis and purification of the soluble fusion protein on a Ni^{2+}-agarose column (Qiagen) was performed as previously described (17), except that the cells were grown in LB-Ap medium. The same procedure was used for purification of a mutant FabI protein.

Protein concentrations were determined using the Bradford dye-binding assay (Bio Rad Laboratories, Hercules, CA) and bovine serum albumin as the standard. Proteins were analyzed by electrophoresis on 0.1% sodium dodecyl sulfate-10% polyacrylamide gel (SDS-PAGE) as previously described (29). The proteins were visualized by quick staining with Coomassie Brillant Blue R-250 (7).

6.3.5 Enoyl-ACP reductase assays. *P. aeruginosa* cell extracts were prepared from exponentially growing cells (A_{540nm} ~ 0.8 to 1.0). Cells grown in LB medium at 37°C were harvested by centrifugation, and suspended and washed in 0.1 M sodium phosphate buffer (pH 6.5). Cell lysates were prepared by passing the cell suspensions three times through a French pressure cell (19,000 psi). Cell debris was removed by ultracentrifugation for 1 h at 260,000 x g and the supernatants were saved as cell extracts.

Enoyl-ACP reductase activity of purified H_6-FabI, as well as in cell extracts, was determined using crotonyl-ACP as the substrate. This was done by measuring spectrophotometrically the decrease in absorbance at 340 nm at room temperature due to consumption of NADH (or NADPH) during reduction of crotonyl-ACP (3). In 500 µl, a typical assay mixture contained 0.1 M sodium phosphate, pH 7.5, 0.1 mM NADH, and 1 µg of purified H_6-FabI. The reactions were initiated by addition of 20 µM crotonyl-ACP. In some instances, 0.1 mM NADPH was used as the reductant. For some experiments, enzyme and substrate concentrations were varied as indicated in the figure legends. A stock solution

of triclosan was prepared in 95% ethanol and appropriate amounts of ethanol were added to control experiments to assess possible inhibitory effects of the solvent on enzyme activities.

Crotonyl-ACP was prepared using a previously published procedure (48) with slight modifications. Briefly, 20 mg of purified *P. aeruginosa* ACP was precipitated as previously described (8), except that the hydroxylamine step was omitted. After precipitation of the protein with 10% trichloroacetic acid for 30 min on ice, the precipitate was pelleted in a microfuge, and washed twice with 0.2 M citrate buffer (pH 4.0) and water, respectively. The protein was resuspended in 0.1 M sodium phosphate buffer (pH 8.0) containing 2 µmoles of DTT and reacted with a 10-fold molar excess of crotonic anhydride (Aldrich, Milwaukee, WI) for 30 min at 4°C with constant stirring. The reaction mixture was loaded on a PD-10 column (Pharmacia Biotech, Uppsala, Sweden) that had been equilibrated with 0.1 M sodium phosphate buffer (pH 7.5), and the crotonyl-ACP was eluted with 3.5 ml of the same buffer. The crotonyl-ACP concentration was determined by measuring the difference in the number of free thiol groups of an untreated *versus* a hydroxylamine-treated sample by the Ellmann technique (12). The treatment of crotonyl-ACP (20 µl of the column eluate) was performed for 5 min at room temperature in a 1 M hydroxylamine solution in 0.1 M sodium phosphate buffer (pH 7.5). For thiol group determinations, hydroxylamine-treated or untreated crotonyl-ACP was reacted for 30 min at room temperature with 0.15 mM 5,5'-dithiobis (2-nitrobenzoic acid)(Aldrich) in a final volume of 0.8 ml 0.1 M phosphate buffer (pH 7.5), and the absorption was measured at 400 nm. According to this determination, 80% of the ACP was acylated.

6.3.6 Determination of acyl homoserine lactone concentrations in culture fluid. AHLs were detected in culture fluids of LB-grown *P. aeruginosa* strains as previously described (36), using *E. coli* XL1Blue/pECP61.5 for detection of C_4-HSL and *E. coli* MG4/pKDT17 for detection of 3-oxo-C_{12}-HSL. Standard curves were prepared using synthetic HSLs to ensure that bioassays were always performed in the linear range.

6.3.7 Butyryl homoserine lactone assays. The *in vitro* C_4-HSL synthesis reactions contained (in 500 µl) Moré buffer (10 mM Tris-HCl (pH 7.4), 330 mM NaCl, 15% glycerol,

0.7 mM DTT, 2 mM EDTA, 25 mM $MgSO_4$, 0.1 mM $FeSO_4$) (35), 0.1 mM SAM, 0.2 mM NADH, 1 μg purified H_6-RhlI (details to be published elsewhere), and various amounts of H_6-FabI. The reaction mixtures were incubated at 37°C for 1 h and then extracted 3 times with 250 μl ethyl acetate. The ethyl acetate extracts were vacuum-dried and the residue was suspended in 20 μl of HPLC-grade acetonitrile (Fisher Scientific, Fair Lawn, NJ). For detection of C_4-HSL, the *Chromobacterium violaceum* bioassay was used (30). Aliquots (5 μl) of the extracted reactions were spotted onto a Whatman (Clifton, NJ) C_{18} reverse phase thin layer chromatography (TLC) plate and allowed to air-dry. The solvent used for chromatography was 60% methanol/40% water (v/v). For detection, 2.5 ml of an overnight culture of strain CV026 grown at room temperature in LB medium was diluted into 25 ml of 0.3% LB agar kept at 45°C and the agar micture was used to overlay the dried TLC. After solidification of the overlay, the TLC plate was incubated overnight at room temperature in a wet box. The presence of C_4-HSL in the extracted reactions was scored by appearance of a violet spot on the TLC plates.

6.3.8 Nucleotide sequence accession number. The DNA sequence of the *fabI* region has been deposited in GenBank under accession number AF104262.

6.4 RESULTS

6.4.1 Cloning of the *fabI* gene of *P. aeruginosa*. At the onset of this project, an examination of the incomplete Pseudomonas Genome database release revealed two contigs which contained sequences that in BLAST searches showed extensive similarities to *E. coli fabI*. The amino and carboxy terminus, respectively, were located on two different contigs. The putative *fabI* coding sequence was successfully amplified from *P. aeruginosa* chromosomal DNA by employing two oligonucleotide primers (FabIU and FabID; table 6.1). These two primers were designed to introduce a unique *Hin*dIII site upstream of *fabI* and its putative ribosome-binding site, and to introduce a unique *Bam*HI site downstream of *fabI*. PCR amplification was very specific and yielded a 880 bp product that was digested with *Bam*HI + *Hin*dIII and ligated to similarly digested pWSK30 DNA to form pPS921. This

procedure placed *fabI* under transcriptional control of the *lac* promoter (P_{lac}) since the cloned *fabI* gene presumably lacks its own promoter. The low-copy number (5-8 copies per cell) cloning vector pWSK30 (47) was used to avoid potential toxicity problems that we had observed during the cloning of other *fab* genes (25). The *fabI*(Ts) *E. coli* strain JP1111 does not grow at 42°C and has a low-salt tolerance at the non-permissive temperature, e.g. it does not grow on regular LB medium containing 0.5% NaCl. This strain was transformed with pPS412 DNA and Ap^r transformants were selected at 30°C on LB-Ap medium. The transformants were then transferred to LB-Ap medium with and without 1 mM IPTG, and incubated at 42°C. Within 24 h, only the colonies plated on LB-Ap medium with IPTG grew under these conditions indicating that the cloned *fabI* (*i*) complemented the temperature-sensitive (Ts) and low-salt tolerance phenotype of the *E. coli fabI*(Ts) mutant and (*ii*) does not contain its own promoter since transcription from P_{lac} requires IPTG induction in the *lac* repressor producing strain JP1111. When JP1111 was transformed with pPS967, a multicopy plasmid expressing *fabI* from P_{lac} (table 6.1), complementation was observed in the absence of IPTG since the amounts of chromosomally encoded *lac* repressor were insufficient to fully repress *fabI* transcription.

6.4.2 Sequence analysis of the *fabI* gene. Since the *fabI* gene sequence in the incomplete genome database was contained on two separate contigs, we sequenced the cloned 874-bp *Hin*dIII-*Bam*HI fragment which contained a single open reading frame (ORF) and BLAST searches revealed that this protein shares significant identity to other FabI proteins. Alignments showed the greatest similarities with FabI proteins from *E. coli* and *Salmonella typhimurium* FabI (69%identity; 81% similarity), and *Haemophilus influenzae* (63% identity; 76% similarity). This similarity also extended to FabIs from Gram-positive bacteria, including *Bacillus subtilis* (49% identity; 66% similarity).

Analysis of the deduced FabI amino acid sequence using the E-Motif program revealed a glucose/ribitol dehydrogenase family signature motif (P..[KR].....[DE][FILVY].....[FWY].[AST]), as well as a signature motif for the FAD-dependent pyridine nucleotide reductase family (D..[FILMV]..[IV]G..P...L]. The latter sequence includes Gly^{95} which is equivalent to a glycine found in position 93 of *E. coli* FabI.

In E.coli, Gly^{93} to Ser and Gly^{93} to Ala changes, which lead to resistance to diazoborine (4) and triclosan (33), respectively, map close to the nucleotide binding site.

Examination of the latest *P. aeruginosa* genome database revealed a complete *fabI* gene sequence. Our sequence was identical to the database sequence with the exception of an additional G residue. This discrepancy arose from a mistake in the previous contig sequence after which our primer FabID was modeled. The *fabI* gene is followed by a sequence that could assume a stem-loop structure (ΔG = -48.2 kcal/mol) indicative of Rho-independent transcriptional terminators (38). Analysis of the *fabI* upstream sequence did not reveal any obvious promoter/regulatory sequences. Instead, the *fabI* gene is separated by 21 nt from another ORF whose gene product exhibits significant similarity to many bacterial ATP-binding proteins that are components of ABC transporters; this ORF is 58% identical and 74% similar to the hypothetical *E. coli* ABC transporter ATP-binding protein YejF (GenBank accession number U00008).

6.4.3 Disruption of the chromosomal *fabI* gene. A defined pPS933-borne *fabI*::Gm^r insertion was constructed as described in table 6.1, and the deletion was returned to the *P. aeruginosa* chromosome as illustrated in figure 6.3A. After conjugal transfer of the non-replicative pPS933 from *E. coli* SM10 into PAO1, merodiploids were obtained by selecting Gm^r. From these, colonies having undergone $\Delta 1$ were selected as $sucrose^r$, Gm^r and Cb^r. The unmarked *fabI*::*FRT* mutant PAO235 was then derived from PAO234 by Flp-catalyzed excision of the Gm^r marker. During its expression in the recipient, Flp recombinase acted at the *FRT* sites to catalyze excision of the Gm^r element (marked with $\Delta 2$), leaving behind a short *FRT*-containing sequence.

Successful execution of the steps labeled $\Delta 1$ and $\Delta 2$ in figure 6.3A was monitored by colony PCR analysis utilizing the FabIU and FabID primers. As expected, the primers amplified a 880-bp *fabI*-containing fragment from wild-type PAO1 DNA (figure 6.3C, lane 1). In PAO234 (lane 2) this fragment was increased to 1.93 kb by insertion of the 1,053-bp Gm^r-*FRT* cassette. After Flp-mediated excision of the Gm^r marker, the PCR fragment was reduced to ~970 bp (880 bp and one 86 bp *FRT*-containing sequence from pPS856)(lanes 3

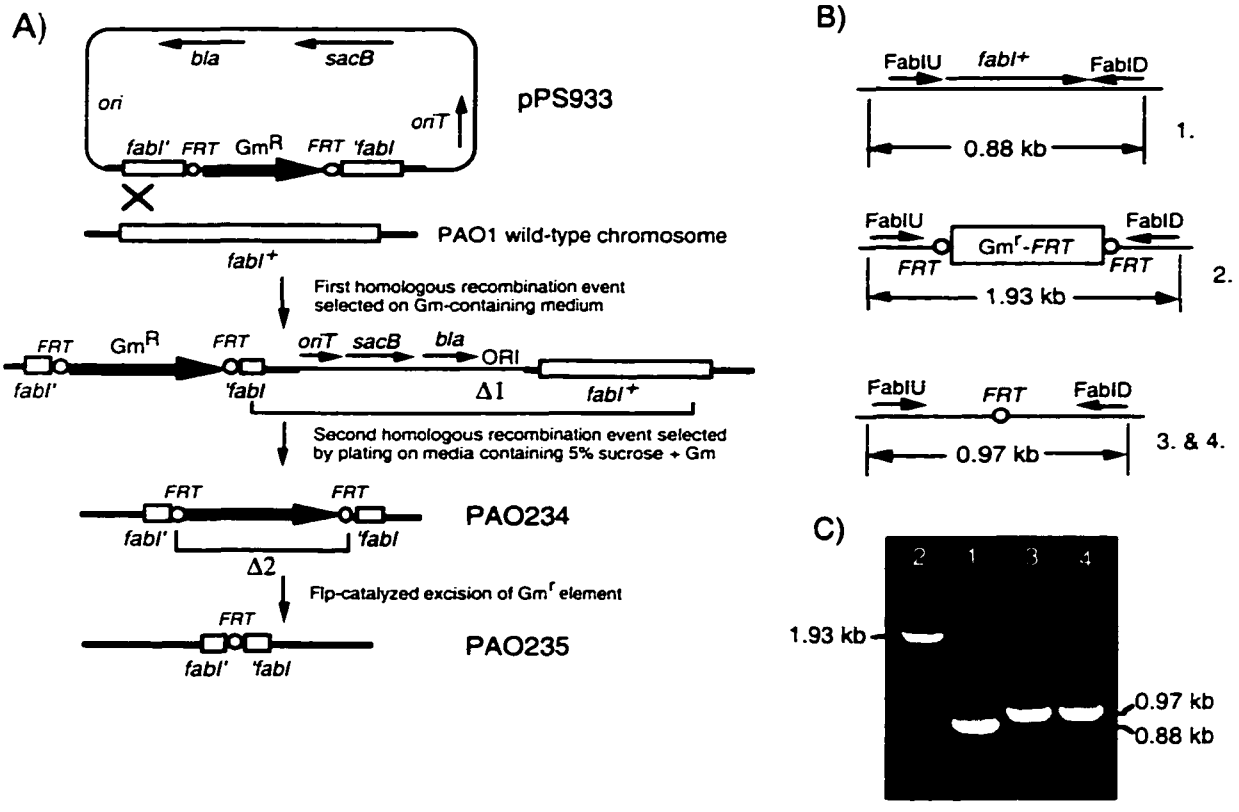


Figure 6.3. **A)** Strategy for isolation of an unmarked chromosomal *fabI* mutation via *sacB*-counterselected gene replacement (Δ1) and Flp-mediated excision of the gentamicin resistance marker (Δ2). **B)** Genetic organization of the *fabI* region in wild-type PAO1 (1.), the *fabI*::Gm^r-*FRT* insertion mutant (2.) and the unmarked *fabI* mutant after Flp-mediated excsision of the Gm^r marker (3. & 4.). **C)** PCR analysis of genomic DNA from the strains depicted in (B). The PCR reactions were primed utilizing the primers FabIU and FabID (figure 6.2A). Abbreviations: *bla*, β-lactamase-encoding gene; *FRT*, Flp recombinase target sites; Gm, Gm^r marker; *ori*, pMB1 origin of replication; *oriT*, origin of transfer; *sacB*, levansucrase-encoding gene.

and 4). These events were further verified by genomic Southern analyses utilizing Gm^r and *fabI* specific DNA segments as the probes (data not shown).

6.4.4 *fabI* mutants contain reduced levels of enoyl-ACP reductase activity. The growth rates of wild-type PAO1 and the *fabI* mutant PAO235 were superimposable when grown in

RB medium and other media (data not shown). Since PAO235 only contains a 86-bp non-polar insertion at a *Sma*I site that is located late in the *fabI* gene, we considered the possibility that this strain might still express a functional FabI protein. To rule out this possibility, we PCR-amplified the *fabI* region from PAO235 using the primers FabIU and FabID, and subcloned the resulting *Bam*HI-*Hin*dIII fragment into pWSK30. Under inducing conditions, this DNA segment no longer complemented the Ts and low-salt tolerance phenotypes of *E. coli fabI*(Ts) strain JP1111.

Determination of enzyme activities in cell-free extracts of wild-type and mutant PAO235 confirmed the existence of residual NADH-dependent activity in the mutant that amounted to 62% of the total activity observed in PAO1 extracts (figure 6.4). Whereas total

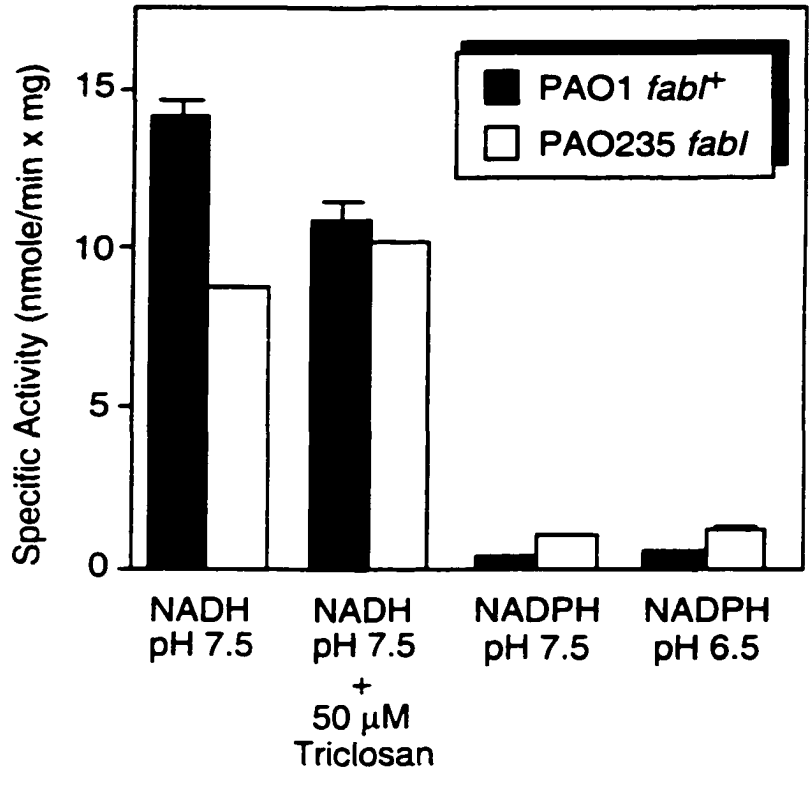


Figure 6.4. Enoyl-ACP reductase activities in cell extracts of wild type PAO1 and the *fabI* mutant PAO235. The 500 µl reactions contained in phosphate buffer with the indicated pH, 0.1 mM of NADH or NADPH, and 50 to 100 µg of PAO1 extract or 300 µg of PAO235 extract for determination of NADH- or NADPH-dependent enoyl-ACP reductase activities, respectively. The reactions were initiated by addition of 13 µM crotonyl-ACP, and the decrese in absorbance at 340 nm was recorded. The results shown represent the mean plus standard deviations of three experiments.

NADH-dependent enoyl-ACP reductase in PAO1 was inhibited by the addition of 50 μM triclosan, this inhibitor had no effect on the residual activity found in PAO235, even when its concentration was increased to 100 μM (data not shown). Cell extracts from both strains showed very little NADPH-dependent enoyl-ACP reductase activity when assayed at pH 7.5 or pH 6.5.

6.4.5 Purification and properties of purified FabI. An expression vector was constructed and FabI was purified as a H_6-FabI protein. When transformed into *E. coli* JP1111, the expression vector could complement the *fabI*(Ts) mutation of this strain, indicating expression of a functional H_6-FabI protein. The purified FabI protein (figure 6.5A) could utilize crotonyl-CoA and crotonyl-ACP as substrates (figure 6.5B) although it was 5.9-fold more active with crotonyl-ACP as the substrate.

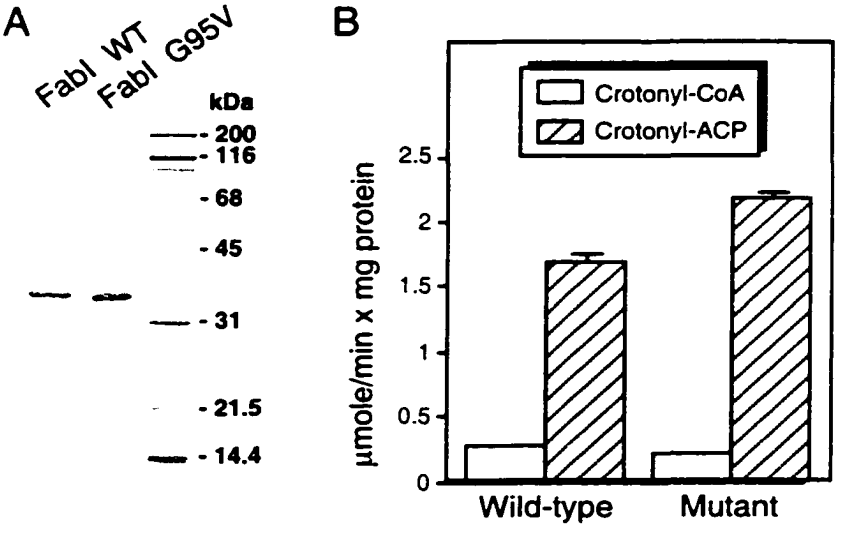


Figure 6.5. **(A)** Polyacrylamide gel electrohoresis of purified wild type FabI and FabI G95V mutant proteins and **(B)** substrate preference and specific activities of wild-type and mutant FabI proteins. **(A)** The proteins were expressed as H_6 fusion proteins and purified by Ni^{2+} affinity chromatography. One μg of each protein was analyzed by electrophoresis on a 0.1% SDS-10% polyacrylamide gel (pH 9.2) and proteins were detected by Coomassie Blue staining. The protein standards (Bio Rad Laboratories) in the rightmost lane were (top to bottom) myosin, β-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme; molecular weights are given in kilodaltons (kDa). **(B)** Enoyl-ACP reductase activities were measured using 20 μM crotonyl-CoA or 13 μM crotonyl-ACP as substrates and 0.1 mM NADH as co-factor. The reactions contained either 1 μg of wild-type FabI or 1 μg of mutant FabI protein. The results shown represent the mean plus standard deviations of three experiments.

The enzyme was unable to utilize NADPH as co-factor (not shown), which may be a consequence of its purification at alkaline pH, conditions that may inactivate its NADPH-dependent activity, as has been observed with the *E. coli* enzyme (3).

The enoyl-ACP reductase activity was inhibited by very low concentrations of triclosan (figure 6.6). Triclosan-resistant *E. coli* strains contain changes in amino acid residues that are thought to lign the enzyme active site (1, 33) and the most resistant strains contained Gly^{93} to Val substitutions (33). We therefore changed the corresponding Gly^{95} in the *P. aeruginosa* enzyme to a Val and purified the resulting H_6-tagged mutant protein. When compared to wild-type FabI, the mutant protein (figure 6.5A) was slightly less active when utilizing crotonyl-CoA as the substrate and slightly more active when utilizing crotonyl-ACP as the substrate (figure 6.5B). The mutant enzyme was significantly more resistant to triclosan (figure 6.6).

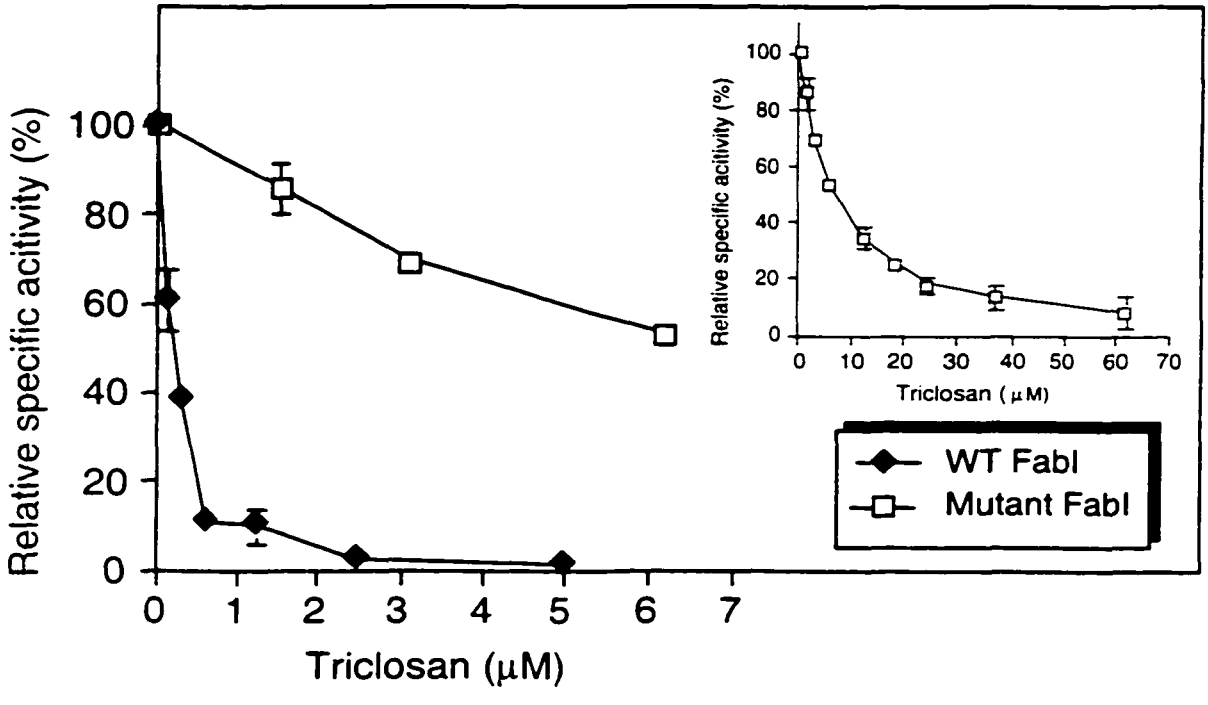


Figure 6.6. Inhibition of FabI activity by triclosan. The reactions (500 μl) in phosphate buffer (pH 7.5) contained either 1 μg of purified wild-type FabI or 1 μg of mutant FabI protein, 0.1 mM NADH, 13 μM crotonyl-ACP and increasing concentrations of triclosan. The insert shows the inhibition curve for the mutant FabI protein over a wider triclosan range; the first four values are identical to the ones plotted in the larger graph. Each value shown represents the mean plus standard deviations of three independent enzymatic reactions.

6.4.6 Role of FabI in HSL synthesis *in vitro* and *in vivo*. Reduction of crotonyl-ACP by FabI to form butyryl-ACP is the last step in the first cycle of fatty acid elongation following condensation of malonyl-ACP with acetyl-CoA (figure 6.1). Theoretically, butyryl-ACP generated by FabI could serve as a substrate for transacylation of RhlI, followed by a lactonization reaction utilizing SAM as the substrate to form C_4-HSL, and inhibiting FabI or mutational inactivation should therefore lead to reduced levels of HSL production.

To test this hypothesis, we first set up an *in vitro* C_4-HSL synthesis system with purified H_6-FabI and H_6-RhlI. In this system, RhlI synthesized C_4-HSL from crotonyl-ACP in the presence of FabI, NADH and SAM (figure 6.7, lane g). Under the same conditions, C_4-HSL synthesis was completely inhibited by 25 μM triclosan (lane b) and this inhibition was partially relieved by increasing concentrations of FabI (lane c to f). Inhibition of C_4-HSL synthesis by triclosan could not be relieved by increasing the RhlI concentration 7-fold (lane h).

To confirm the *in vivo* role of *fabI* in HSL synthesis, HSL levels were measured in culture fluids of wild-type PAO1 and *fabI* mutant PAO235. The *fabI* mutant produced only 50% of the 3-oxo-C_{12}-HSL and C_4-HSL levels of wild-type PAO1 (figure 6.8), paralleling the reduction of FabI enoyl-ACP reductase activity observed in this mutant (figure 6.4).

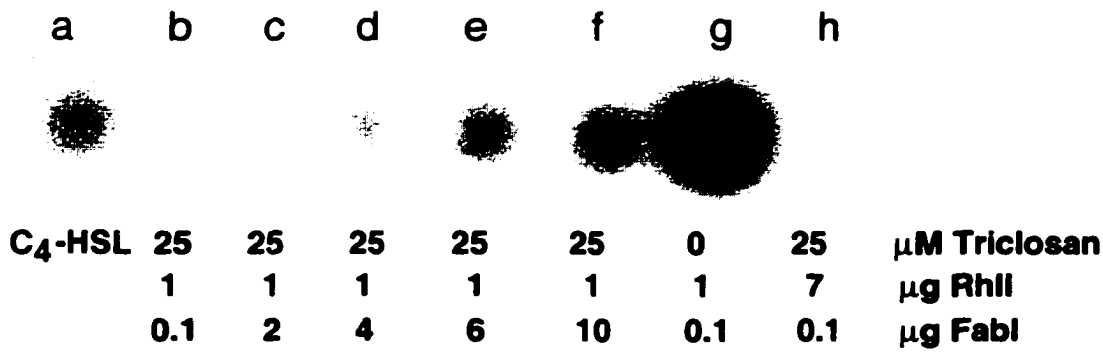


Figure 6.7. *In vitro* synthesis of butyryl homoserine lactone and inhibition by triclosan. *In vitro* synthesis reactions containing the indicated components plus 20 μM crotonyl-ACP, 0.1 mM SAM and 0.2 mM NADH were extracted with ethyl acetate, and portions of the concentrated residues were separated by TLC. C_4-HSL in the samples was detected by overlaying the TLC plate with the *C. violaceum* indicator strain CV026. Lane a contained 50 pmoles of synthetic C_4-HSL.

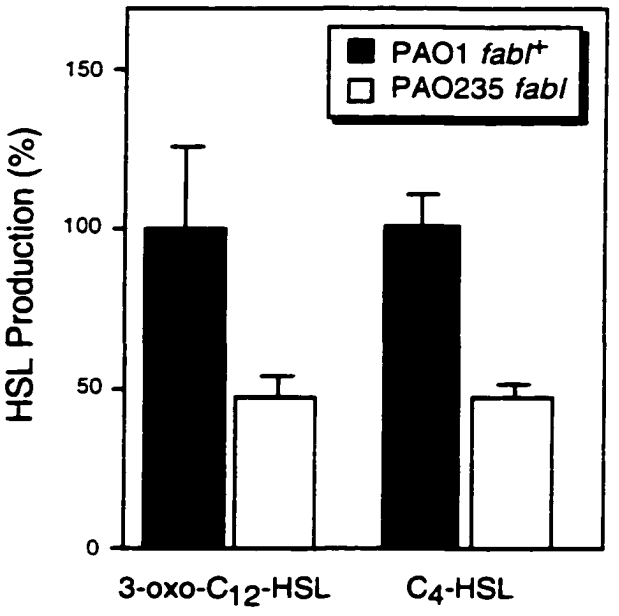


Figure 6.8. HSL levels in wild-type and a *fabI P. aeruginosa* mutant. HSLs were extracted from overnight culture supernatants and relative levels were determined using *E. coli*-based C_4-HSL and 3-oxo-C_{12}-HSL bioassays. Values were determined in triplicate and the values shown represent the means plus standard deviations.

6.5 DISCUSSSION

6.5.1 The *fabI* gene encodes an enoyl-ACP reductase. We have cloned and characterized a gene from *P. aeruginosa*, encoding an enoyl-ACP reductase, by PCR amplification from genomic DNA and complementation of a defined *E. coli fabI*(Ts) mutant. Sequence analysis revealed that this gene may be transcriptionally linked to an upstream ORF encoding an ATP-binding protein of an ABC transporter of unknown function, and the presence of strong downstream Rho-independent transcriptional terminator indicated that it is probably the last gene in a transcriptional unit.

Although our experiments showed that *P. aeruginosa* contains several enoyl-ACP reductases, several lines of evidence strongly support the notion that the cloned gene is *fabI*. First, the deduced FabI sequence is 69% identical to FabI from *E. coli* and *S. typhimurium* and contains the signature motif (D..[FILMV]..[IV]G..P...L]) for the FAD-dependent

pyridine nucleotide reductase family. Second, this motif contains the amino acid residues forming the enzyme active site that when mutationally altered confer resistance to specific inhibitors of FabI enoyl-ACP reductase activity. This included Gly^{95} which is equivalent to Gly^{93} found in *E. coli* FabI. *In E. coli*, Gly^{93} to Ser and Gly^{93} to Ala changes led to resistance to diazoborine (4) and triclosan (33), respectively. In *M. tuberculosis*, a Ser^{94} to Ala mutation, which leads to isoniazid resistance, lies the same region of the mycobacterial FabI homolog, InhA (11).

In *E. coli*, *fabI* is an essential gene and *fabI* knockout mutants are not viable. However, mutants containing single-amino acid substitutions can be isolated but they grow at reduced rates (33) or they contain Ts alleles (4) that allow growth only at the permissive temperature. We were able to disrupt the chromosomal *fabI* gene and showed that the disrupted gene no longer complemented an *E. coli fabI*(Ts) allele. This was expected since the insertion at a *Sma*I site disrupted amino acid residues that form an integral part of the enzyme active site (18, 26). Since we could isolate a *fabI* insertion mutant and since this mutant was not altered in its growth rate, we concluded that, unlike *E. coli*, *P. aeruginosa* must contain more than one enoyl-ACP reductase activity. This was corroborated by the presence of significant levels of NADH-dependent enoyl-ACP reductase activities in cell extracts of the *fabI* mutant PAO235 (figure 6.4). Since the activity found in the mutant amounted to 62% of the total activity found in wild-type, we concluded that FabI contributes a substantial part to the total enoyl-ACP reductase activity found in *P. aeruginosa*. This was corroborated by triclosan inhibition studies which indicated that ~23% of the total enoyl-ACP reductase activity in wild-type was inhibited by this drug, whereas the residual activity in PAO235 was triclosan-insensitive. Searches of the *P. aeruginosa* genome database revealed at least 18 possible paralogs exhibiting on average 25% identity and 40% similarity to FabI, but none of them contained the signature motifs found in FabI proteins.

When measured in cell extracts that were prepared at pH 6.5, *E. coli* FabI accepted both NADH and NADPH as co-factors but when cell extracts were prepared at 7.5, only the NADH-dependent enoyl-ACP reductase activity remained (3). However, the NADPH-dependent FabI activity amounted to only 14-22% of the NADH-dependent enzyme. Although we isolated our cell extracts at pH 6.5, the NADPH-dependent FabI activity in wild-

type or mutant cell extracts was very low, even when measured at pH 6.5, and required 3 to 6 times more enzyme for detection (figure 6.4). Under these conditions, the NADPH-dependent activities were 3.5% or 10% of the NADH-dependent acivities observed with wild-type and mutant extracts, respectively. We do not yet know what proportion, if any, of this activity is attributable to FabI since our purified FabI protein does not exhibit NADPH-dependent enoyl-ACP reductase activity (see below).

To assess the substrate specificity of FabI, we expressed and purified FabI as a H_6-fusion protein. As its *E. coli* counterpart, the purified FabI protein could use both crotonyl-CoA and crotonyl-ACP as substrates, although it was much more active with crotonyl-ACP (figure 6.5B). The purified enzyme could not use NADPH as co-factor (data not shown). We do not yet know whether this reflects an intrinsic property of the *P. aeruginosa* FabI enzyme or whether the NADPH-dependent activity was lost during the purification steps that involve buffers with alkaline pH. We were unable to differentiate between these two possibilities since H_6 fusion proteins cannot be purified at pH 6.5.

6.5.2 FabI is a triclosan target. It has recently been shown that in *E. coli* triclosan targets lipid synthesis and since triclosan-resistant mutants harbor mutations in *fabI*, it was concluded that the most likely target is enoyl-ACP reductase (33) and this was subsequently confirmed using purified FabI (19). To examine whether *P. aeruginosa* FabI is a triclosan target, we used the purified H_6-FabI in triclosan inhibition studies (figure 6.6). The purified protein was efficiently inhibited by very low concentrations of triclosan, showing 50% inhibition (IC_{50}) with 0.2 μM triclosan. McMurry et al. (33) and Heath et al. (18, 19) showed that their most triclosan-resistant mutants contained Gly^{93} to Val substitutions. To assess whether changing a glycine residue at the equivalent position 95 in *P. aeruginosa* FabI would lead to synthesis of a triclosan resistant FabI enzyme, we introduced a Gly^{95} to Val change by site-directed mutagenesis, and expressed and purified the mutant H_6-FabI protein. Although the mutant protein exhibited a slighly increased enoyl-ACP reductase activity (figure 6.5B), it showed an IC_{50} of 7 μM for triclosan and was not yet completely inhibited by the highest concentration (62 μM) tested in our experiments (figure 6.6, insert). While our studies neared completion, Heath et al. (18, 19) arrived at similar results comparing the purified *E. coli* FabI with Gly^{93} to

Ser and Gly^{93} to Val mutant enzymes; the triclosan IC_{50} was 2 μM for FabI and 8 μM for the Gly^{93} to Ser (19) or 10 μM for the Gly^{93} to Val mutant enzymes (18), respectively. Although both proteins were purified utilizing the same expression vector and same NH_2-terminal H_6 extension, *P. aeruginosa* FabI was ~10-fold more sensitive to triclosan when compared to the *E. coli* enzyme. Although we do not know the reason(s) for this observation, the differences may be partially due to the different enoyl-ACP reductase assays used for the inhibition studies. We used the natural substrate crotonyl-ACP whereas Heath et al. (18, 19) used the synthetic substrate *trans*-2-octadecenoyl-*N*-cysteamine, and the latter assay required substantially more FabI (12 μg versus 1 μg in our assays). The recent determination of the x-ray structure of the *E. coli* FabI-NAD^+-triclosan complex confirmed that triclosan interacts with both the NAD^+ and the protein via hydrogen and hydrophobic bonds, forming a stable ternary complex (18, 26). Mutations in the FabI active site interfere with the formation of a stable FabI-NAD^+-triclosan ternary complex and thus confer resistance to the drug.

We have previously shown that *P. aeruginosa* strain PAO1 is resistant to triclosan due to efflux by the tripartite MexAB-OprM multidrug efflux system but Δ(*mexAB-oprM*) mutants are susceptible to triclosan (41). The same efflux system seems also partially responsible for diazoborine resistance. Both wild-type PAO1 and *fabI* mutant PAO235 showed high levels of resistance to diazoborine (the minimal inhibitory concentration (MIC) in both strains was >160 μg/ml) and the MIC for diazoborine was significantly lower (80 μg/ml) in the Δ(*mexAB-opM*) mutant PAO200.

6.5.3 FabI participates in homoserine lactone synthesis. According to the current model, butyryl-ACP serves as the most likely acyl donor in C_4-HSL synthesis. To confirm that FabI can indeed provide butyryl-ACP for C_4-HSL synthesis by RhlI, we set up an *in vitro* synthesis system containing purified FabI, RhlI, the co-factor NADH and the substrates crotonyl-ACP and SAM. This reconstituted system allowed synthesis of biologically active C_4-HSL that co-migrated with synthetic C_4-HSL (figure 6.7). Synthesis of C_4-HSL was efficiently inhibited by triclosan. This inhibition was relieved by increasing concentrations of FabI but not RhlI, indicating that FabI is the triclosan target and not RhlI. Corroborating evidence that FabI plays a central role in HSL synthesis *in vivo* was provided by the observation that a *fabI*

mutant produced only 50% of the HSLs found in wild-trype cells. This is the first documented evidence that an enzyme of the fatty acid biosynthetic pathway plays a crucial role in HSL synthesis and the establishment of an *in vitro* HSL synthesis system will facilitate the search for novel antiomicrobials aimed at interfering with the pathways involved in HSL biosynthesis. The design and development of new FabI inhibitors will form an integral part of such strategies.

6.6 ACKNOWLEDGMENTS

This work was suported by Public Health Service Grant GM56685 from the National Institutes of Health. Additional funding to H.P.S. was provided by a grant from the Colorado State University Research Council of the College of Veterinary Medicine and Biomedical Sciences. We thank Drs. Barbara Iglewski and Anatol Eberhard for the generous gifts of synthetic HSLs, and Dr. Matt Parsek for providing the HSL bioassay strains and protocols. Triclosan (Irgasan™) was provided as a gift from Ciba corporation and diazoborine was provided by Dr. Friederike Turnowsky. We also thank Dr. Charles O. Rock for providing information on the mechanism of triclosan inhibition of FabI prior to publication.

6.7 REFERENCES

1. **Baldock, C., J. B. Rafferty, S. E. Sedelnikova, P. J. Baker, A. R. Stuitje, A. R. Slabas, T. R. Hawkes, and D. W. Rice.** 1996. A mechanism of drug action revealed by structural studies of enoyl reductase. Science **274:**2107-2110.

2. **Banerjee, A., E. Dubnau, A. Quemard, V. Balasubramanian, K. S. Um, T. Wilson, D. Collins, G. de Lisle, and W. R. Jacobs.** 1994. *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science **263:**227-230.

3. **Bergler, H., S. Fuchsbichler, G. Hoegenauer, and F. Turnowsky.** 1996. The enoyl-[acyl-carrier-protein] reductase (FabI) of *Escherichia coli*, which catalyzes a key regulatory

step in fatty acid biosynthesis, accepts NADH and NADPH as cofactors and is inhibited by palmitoyl-CoA. Eur. J. Biochem. **242:**689-694.

4. **Bergler, H., G. Hoegenauer, and F. Turnowski.** 1992. Sequences of the *envM* gene and of two mutated alleles in *Escherichia coli*. J. Gen. Microbiol. **138:**2093-2100.

5. **Best, E., and V. C. Knauf.** 1993. Organization and nucleotide sequence of the genes encoding the biotin carboxyl carrier protein and biotin carboxylase protein of *Pseudomonas aeruginosa* acetyl coenzyme A carboxylase. J. Bacteriol. **175:**6881-6889.

6. **Campos-Garcia, J., A. D. Caro, R. Najera, R. M. Miller-Maier, R. A. Al-Tahhan, and G. Soberon-Chavez.** 1998. The *Pseudomonas aeruginosa rhlG gene* encodes an NADPH-dependent β-ketoacyl reductase which is specifically involved in rhamnolipid synthesis. J. Bacteriol. **180:**4442-4451.

7. **Chen, H., H. Cheng, and M. Bjerkness.** 1993. One-step Coomassie Brillant Blue R-250 staining of proteins in polycarylamide gel. Anal. Biochem. **212:**295-296.

8. **Cronan, J. E., and A. L. Klages.** 1981. Chemical synthesis of acyl thioesters of acyl carrier protein with native structure. Proc. Natl. Acad. Sci. USA. **78:**5440-5444.

9. **Cronan, J. E., and C. O. Rock.** 1996. Biosynthesis of membrane lipids, p. 612-636. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. Brooks Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*. Am. Soc. Microbiol., Washington, DC.

10. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295-298.

11. **Dessen, A., A. Quemard, J. S. Blanchard, W. R. Jacobs, and J. C. Sacchettini.** 1995. Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*. Science **267:**1638-1641.

12. **Ellmann, G. L.** 1959. Tissue sulfhydryl groups. Anal. Biochem. **82:**70-77.

13. **Glessner, A., R. S. Smith, B. H. Iglewski, and J. B. Robinson.** 1999. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of twitching motility. J. Bacteriol. **181:**1623-1629.

14. **Greenberg, E. P.** 1997. Quorum sensing in Gram-negative bacteria. ASM News **63:**371-377.

15. **Heath, R. J., and C. O. Rock.** 1995. Enoyl-acyl carrier protein reductase (*fabI*) plays a determinant role in completing cycles of fatty acid elongation in *Escherichia Coli*. J. Biol. Chem. **270:**26538-26542.

16. **Heath, R. J., and C. O. Rock.** 1996. Regulation of fatty acid elongation and initiation by acyl-acyl carrier protein in *Escherichia coli*. J. Biol. Chem. **271:**1833-1836.

17. **Heath, R. J., and C. O. Rock.** 1996. Roles of the FabA and FabZ β-hydroxyacyl-acyl carrier protein dehydratase in *Escherichia coli* fatty acid biosynthesis. J. Biol. Chem. **271:**27795-27801.

18. **Heath, R. J., J. R. Rubin, D. R. Holland, E. Zhang, M. E. Snow, and C. O. Rock.** 1999. Mechanism of triclosan inhibition of bacterial fatty acid synthesis. J. Biol. Chem. **274:**11110-11114.

19. **Heath, R. J., Y.-T. Yu, M. A. Shapiro, E. Olson, and C. O. Rock.** 1998. Broad spectrum antimicrobial biocides target the FabI component of fatty acid biosynthesis. J. Biol. Chem. **273:**30316-30320.

20. **Henry, M. F., and J. E. Cronan.** 1992. A new mechanism of transcriptional regulation: release of an activator triggered by small molecule binding. Cell **70:**671-679.

21. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77-86.

22. **Hoang, T. T., and H. P. Schweizer.** 1997. Fatty acid biosynthesis in *Pseudomonas aeruginosa*: Cloning and characterization of the *fabAB* operon encoding β-hydroxyacyl-acyl carrier protein dehydratase (*fabA*) and β-ketoacyl-acyl carrier protein synthase I (*fabB*). J. Bacteriol. **179:**5326-5332.

23. **Kater, M. M., G. M. Konigstein, H. J. J. Nijkamp, and A. R. Stuitje.** 1994. The use of a hybrid genetic system to study the functional relationship between prokaryotic and plant multi-enzyme fatty acid synthase complexes. Plant Mol. Biol. **25:**771-790.

24. **Kutchma, A. J.** 1997. Characterization of a gene cluster containing the *Pseudomonas aeruginosa fabD*, *fabG*, *acpP* and *fabF* genes involved in fatty acid biosynthesis. M.S. Thesis, Colorado State University.

25. **Kutchma, A. J., T. T. Hoang, and H. P. Schweizer.** 1999. Characterization of a *Pseudomonas aeruginosa* fatty acid biosynthetic gene cluster: purification of acyl carrier protein (ACP) and malonyl-coenzyme A:ACP transacylase (FabD). J. Bacteriol. **181:** 5498-5504.

26. **Levy, C. W., A. Roujeinikova, S. Sedelnikova, P. J. Baker, A. R. Stuitje, A. R. Slabas, D. W. Rice, and J. B. Rafferty.** 1999. Molecular basis of triclosan activity. Nature **398:**383-384.

27. **Liss, L.** 1987. New M13 host: DH5αF' competent cells. Focus **9:**13.

28. **Magnuson, K., S. Jackowski, C. O. Rock, and J. E. Cronan.** 1993. Regulation of fatty acid biosynthesis in *Escherichia coli*. Microbiol. Rev. **57:**522-542.

29. **Makowski, G. S., and M. L. Ramsby.** 1993. pH modification to enhance the molecular sieving properties of sodium dodecyl sulfate-10% polyacrylamide gel. Anal. Biochem. **212:**283-285.

30. **McClean, K. H., M. K. Winson, L. Fish, A. taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. lamb, S. Swift, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1998. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production for the detection of *N*-acylhomoserine lactones. Microbiol. **143:**3703-3711.

31. **McLean, R. J. C., M. Whiteley, D. J. Stickler, and W. C. Fuqua.** 1997. Evidence of autoinducer activity in naturally occuring biofilms. FEMS Microbiol. Lett. **154:**259-263.

32. **McMurry, L. M., P. F. McDermott, and S. B. Levy.** 1999. Genetic evidence that InhA of *Mycobacterium smegmatis* is a target for triclosan. Antimicrob. Agents Chemother. **43:**711-713.

33. **McMurry, L. M., M. Oethinger, and S. B. Levy.** 1998. Triclosan targets lipid synthesis. Nature **394:**531-532.

34. **Miller, J. H.** 1992. A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

35. **Moré, M. I., D. Finger, J. L. Stryker, C. Fuqua, A. Eberhard, and S. C. Winans.** 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science **272:**1655-1658.

36. **Parsek, M. R., A. L. Schaefer, and E. P. Greenberg.** 1997. Analysis of random and site-directed mutations in *rhlI*, a *Pseudomonas aeruginosa* gene encoding an acylhomoserine lactone synthase. Mol. Microbiol. **26:**301-310.

37. **Raetz, C. H. R.** 1996. Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. Brooks Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*. Am. Soc. Microbiol., Washington, DC.

38. **Rosenberg, M. C., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13:**319-353.

39. **Schaefer, A. L., D. L. Val, B. L. Hanzelka, J. E. Cronan, and E. P. Greenberg.** 1996. Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. Proc. Natl. Acad. Sci. USA. **93:**9505-9509.

40. **Schweizer, H. P.** 1991. The *agmR* gene, an environmentally responsive gene, complements defective *glpR*, which encodes the putative activator for glycerol metabolism in *Pseudomonas aeruginosa*. J. Bacteriol. **173:**6798-6806.

41. **Schweizer, H. P.** 1998. Intrinsic resistance to inhibitors of fatty acid biosynthesis in *Pseudomonas aeruginosa* is due to efflux: application of a novel technique for generation of

unmarked chromosomal mutations for the study of efflux systems. Antimicrob. Agents Chemother. **42:**394-398.

42. **Schweizer, H. P., T. R. Klassen, and T. Hoang.** 1996. Improved methods for gene analysis and expression in *Pseudomonas*, p. 229-237. *In* T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (ed.), Molecular biology of Pseudomonads. Am. Soc. Microbiol., Washington, DC.

43. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60-89.

44. **Turnowsky, F., K. Fuchs, C. Jeschek, and G. Hoegenauer.** 1989. *envM* genes of *Salmonella typhimurium* and *Escherichia coli*. J. Bacteriol. **171:**6555-6565.

45. **Val, D. L., and J. E. Cronan.** 1998. In vivo evidence that *S*-adenosylmethionine and fatty acid intermediates are the substrates for the LuxI family of autoinducer synthases. J. Bacteriol. **180:**2644-2651.

46. **Van Delden, C., and B. H. Iglewski.** 1998. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Em. Infect. Dis. **4:**551-560.

47. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195-199.

48. **Weeks, G., and S. J. Wakil.** 1968. Studies on the mechanisms of fatty acid synthesis. J. Biol. Chem. **243:**1180-1189.

CHAPTER 7

Affinity purification of the *Escherichia coli* acyl-acyl carrier protein synthase: labeling of *Pseudomonas aeruginosa* acyl carrier protein and use in enzymatic synthesis of substrates for 3-oxo-homoserine lactone synthase

(To be submitted as a note by Sarah A. Sullivan, Tung T. Hoang, and Herbert P. Schweizer for publication in the journal *Analytical Biochemistry*)

I thank Sarah Sullivan for purifying several batches of acyl carrier protein (ACP) and labeling of ACP. The research presented in this chapter provides a method which will allow labeling of ACP with fatty acids of various chain lengths, using affinity-purified acyl-ACP synthase.

7.1 INTRODUCTION

Acyl carrier protein (ACP) preparations carrying defined acyl groups are important for studying various reactions *in vitro* (2, 8, 9). The *E. coli* acyl-ACP synthase (Aas) has combined 2-acyl-glycero-3-phosphoethanolamine acyltransferase and acyl-ACP synthase activities, but only the latter functions in the presence of ACP, free fatty acid, ATP, and Mg^{2+} (5). Therefore, the *E. coli* acyl-ACP synthase (Aas) offers one desirable way of producing various defined acyl-ACP metabolic intermediates (11, 14). The *Vibrio harveyi* Aas has also been purified and utilized to label ACP with various free fatty acids, but the gene for this enzyme has not yet been cloned (3, 16). Aas proteins from both organisms were previously purified using non-recombinant methods but the protocols, involving several steps, are quite laborious (3, 13). In this communication we demonstrate the overexpression of the *E. coli*

aas gene as an NH_2-terminal hexahistidine tagged fusion protein (H_6-Aas). The recombinant protein can be easily purified in one step via Ni^{2+}-NTA agarose (Qiagen, Santa Clarita, CA) column chromatography. Hexahistidine tagging offers the added advantage that H_6-Aas can easily be removed from the acyl-ACPs and other reaction components by passage through the same affinity column. Purified Aas has been used to successfully label ACP with hexanoic acid (C_6), octanoic acid (C_8), decanoic acid (C_{10}) and dodecanoic acid (C_{12}). A large scale decanoic acid labeling of the *P. aeruginosa* ACP was performed and the resulting decanoyl-ACP was demonstrated to be readily converted into *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL), utilizing coupled reactions of three purified *P. aeruginosa* enzymes, namely malonyl-CoA:ACP transacylase (FabD), β-ketoacyl-ACP synthase I (FabB), and 3-oxo-C_{12}-HSL synthase (LasI).

7.2 METHODS, RESULTS, AND DISCUSSION

To facilitate purification of ACP mainly in its active form (holo-ACP), we previously described two methods for its overexpression via an intein-chitin binding domain fusion protein (6). The first method involved regrowing ACP-expressing cells in fresh LB medium, and the second method involved expression from a low copy number plasmid. Both methods reduced the percentage of apo-ACP that is co-purified with the holo-ACP to ~5-10% (figure 7.2B). To achieve ACP preparations containing almost exclusively holo-ACP, routine *P. aeruginosa* ACP purification in our laboratory is now achieved by coexpression of *acpP* (encoding ACP) with cloned *E. coli acpS* (encoding holo-acyl carrier protein synthase; figure 7.1). To this end, the AcpS expressing pPS1118 was constructed by subcloning the *acpS* gene on a 470 bp *Ase*I-*Hin*dIII fragment from pDPJ (7) into pACYC184 (1) cut with the same restriction enzymes. Purification of ACP in the presence of AcpS was done as previously described (6), except that expression strains were grown in LB medium with ampicillin (Ap; 100 μg/ml) and chloramphenicol (Cm; 25 μg/ml) to maintain both the *acpP* and *acpS* containing plasmids. As evident from figure 7.2B, there was no more detectable apo-ACP in ACP preparations obtained from an strain coexpressing AcpS.

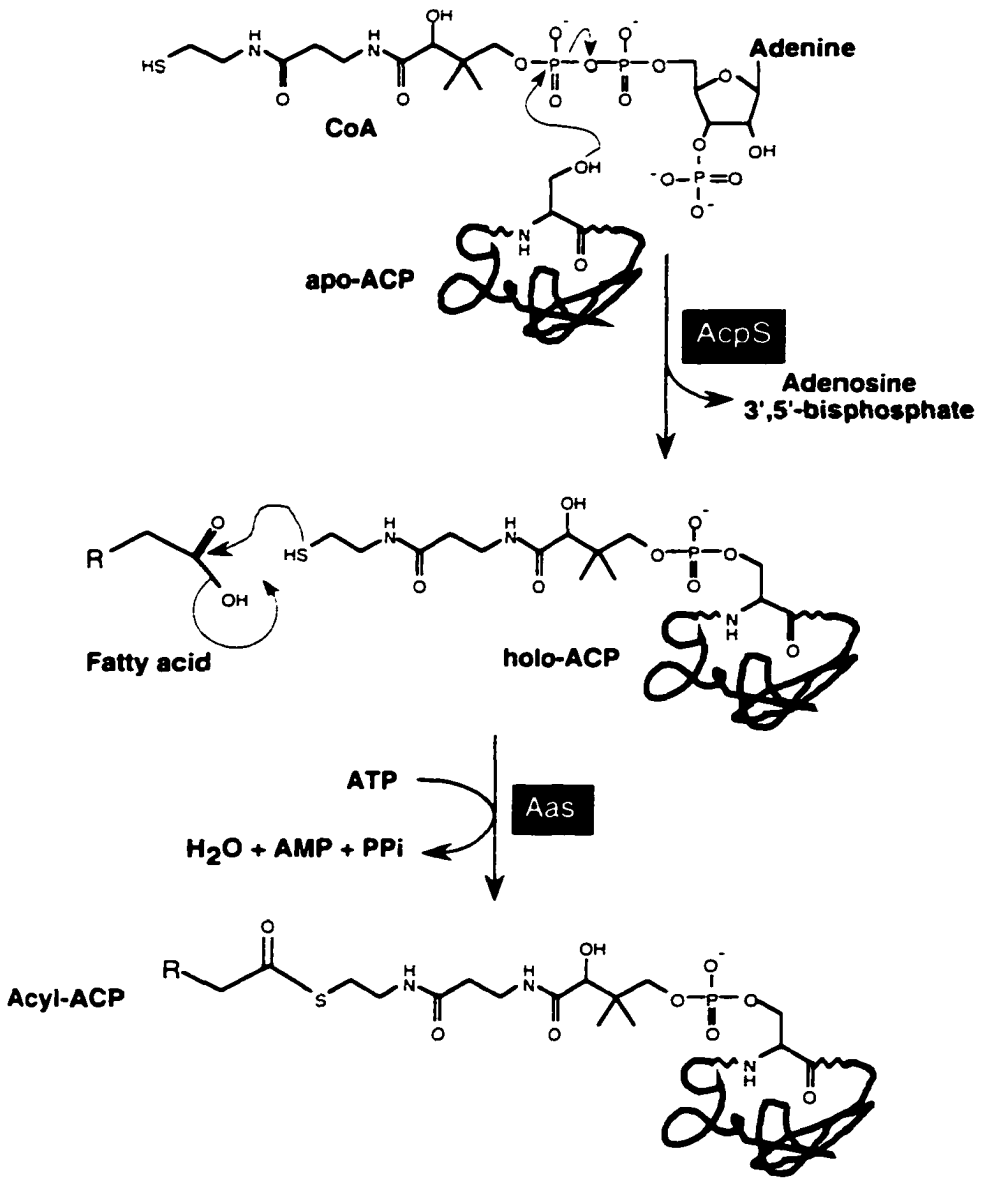


Figure 7.1. Post-translational modification and labeling of ACP. Since ACP does not contain a cysteine to serve as acyl carrier, ACP is post-translationally modified by acyl carrier protein synthase (AcpS) at the serine residue in the DSLD motif (see figure 5.2), utilizing the phosphopantetheine prosthetic group derived from Coenzyme A (CoA). This reaction can be achieved *in vivo* (figure 7.2B) or *in vitro* (7). Acylation of ACP via formation of a thioester bond is achieved in the presence of ATP by a cytoplasmic membrane protein, acyl-ACP synthase (Aas). Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; PP_i, pyrophosphate.

A H_6-Aas expression vector was constructed as follows. The *E. coli aas* gene was PCR amplified from genomic DNA, using two primers (aas-BamHI, 5'-CTTGGATCCCCTTCG ACCACAACGAAG [a unique *Bam*HI site is underlined] and aas-NdeI, 5'-GTTCATATGCT TTTTAGCTTTTTTCGAAA [a unique *Nde*I site is underlined] utilizing previously described conditions (4). The 2.2 kb PCR fragment was gel-purified, digested with *Nde*I + *Bam*HI and cloned between the same sites of pViet (4). After characterization by restriction enzyme mapping one clone, pPS1103, was retained and transformed into the *E. coli* expression strain SA1503(DE3)(4). Growth of this strain in LB medium followed by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) revealed the expression of the approximately 80 kDa H_6-Aas protein (figure 7.2A). Large scale purification of H_6-Aas was done by diluting an overnight culture grown at 37°C in LB with Ap (100 μg/ml) and Cm (25 μg/ml) 1:200 into 3 l of the same medium. The cells were then grown at 37°C to an A_{600nm} of ~0.7 and induced with 1 mM IPTG for 12 h at room temperature. Since Triton X-100 has been shown to facilitate solubilization of Aas (12), the harvested cells were resuspended in 50 ml of metal chelation affinity chromatography buffer with triton (MCACT) [2% Triton X-100, 20 mM Tris-HCl, 0.5 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.9]. A French press was used to lyse the cells with three passes at 19,000 psi. The lysate was clarified by ultracentrifugation at 100,000 x g for 30 min at 4°C, before addition of 100 units of DNaseI (Gibco-BRL, Gaithersburg, MD) and incubation on ice for 20 min to reduce viscosity. A portion (25 ml) of the clarified lysate was passed through Ni^{2+}-NTA agarose (Qiagen, Santa Clarita, CA)(1 ml bed volume) in a 2 ml Talon disposable column (Clontech Laboratories, Palo Alto, CA), which had been preequilibrated with 5 ml of MCACT. Five ml of MCACT containing 10 mM imidazole were passed through the column after application of the first 25 ml of clarified lysate to remove non-specifically bound proteins. After passing through the last 25 ml of clarified lysate, the column was washed with 10 ml of MCACT, followed by at least 100 ml of MCACT containing 50 mM imidazole. H_6-Aas was then eluted with 3 ml of MCACG buffer [20 mM Tris-HCl, 0.5 M NaCl, 0.5 mM PMSF, pH 7.9, 15% glycerol] containing 200 mM imidazole and small aliquots (200 μl) were frozen at -70°C. Figure 7.2A shows a SDS-PAGE analysis of a 3 μl aliquot of the purified Aas taken directly from the 3 ml

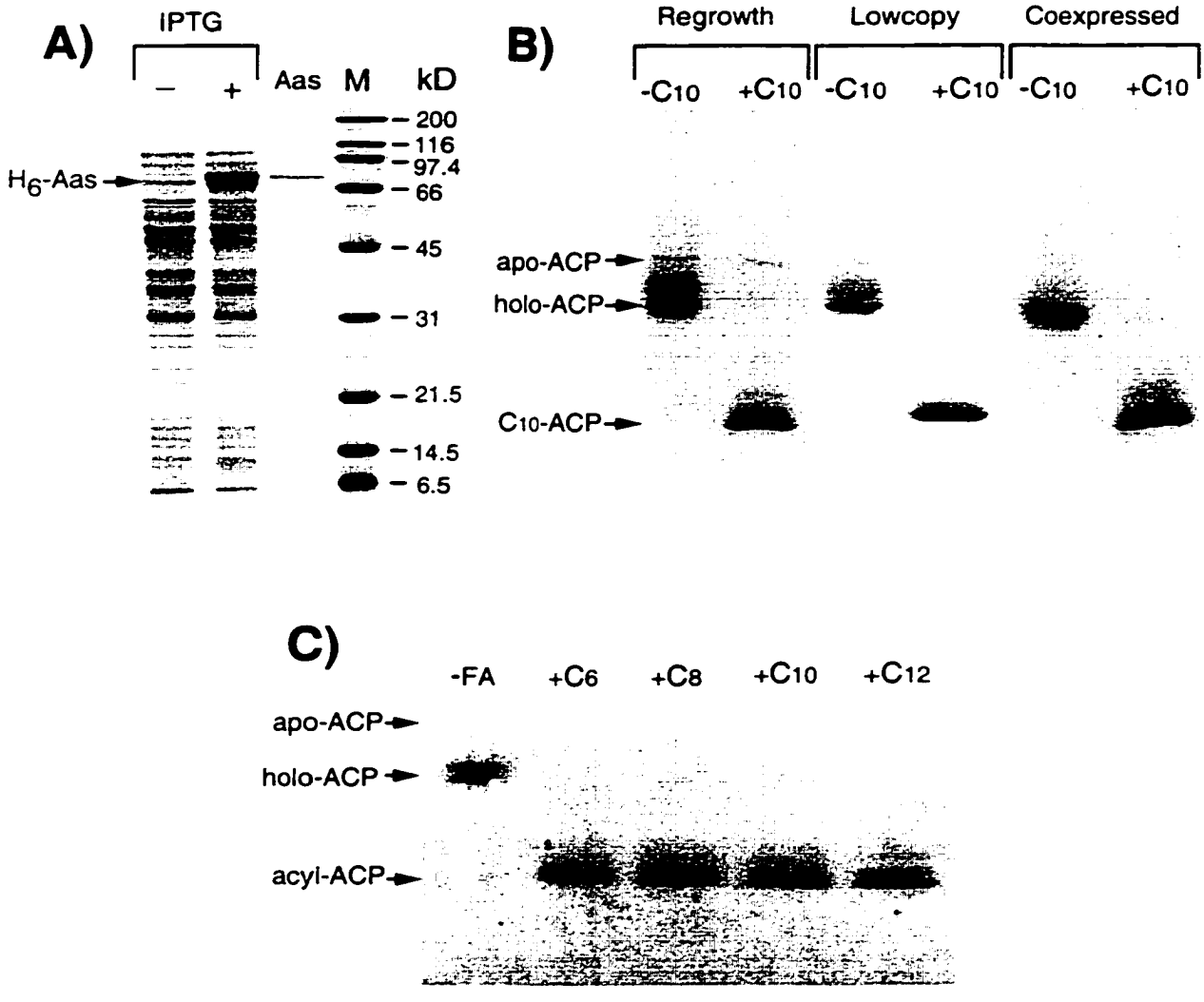


Figure 7.2. Purification and functional analyses of Aas. **(A)** Sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS-PAGE) analysis of cell extracts derived from uninduced (-IPTG) and induced (+ 1 mM IPTG) cells, as well as purified Aas. The 10% SDS-PAGE was stained with Coomassie blue. The sizes of broad-range protein markers (M) from Bio-Rad (Hercules, CA) are indicated in kDa (top to bottom: myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme, aprotinin). **(B)** Native PAGE analysis of unlabeled ($-C_{10}$) and decanoic acid labeled ($+C_{10}$) ACP. The various ACP preparations were incubated with purified Aas as described in the text, except the unlabeled reactions contained no C_{10}. The gel was stained with Coomassie blue. **(C)** Native PAGE analysis of unlabeled ACP (-FA; no fatty acids) and ACP labeled with fatty acids of various chain lengths: C_6, hexanoic acid; C_8, octanoic acid; C_{10}, decanoic acid; C_{12}, dodecanoic acid. The methods used for analyses of proteins for SDS-PAGE and native PAGE and Commassie blue staining were previously described (4, 12).

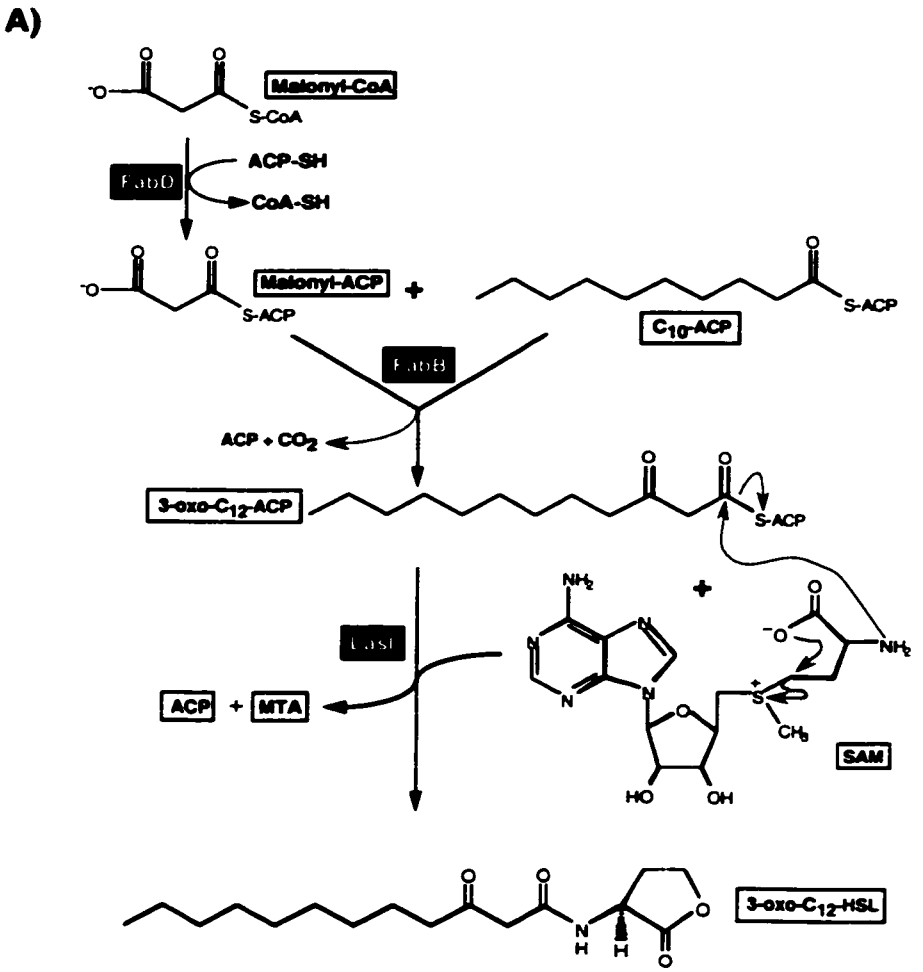
eluate from Ni^{2+}-NTA agarose column. Total yield was 0.35 mg of protein per liter induced culture.

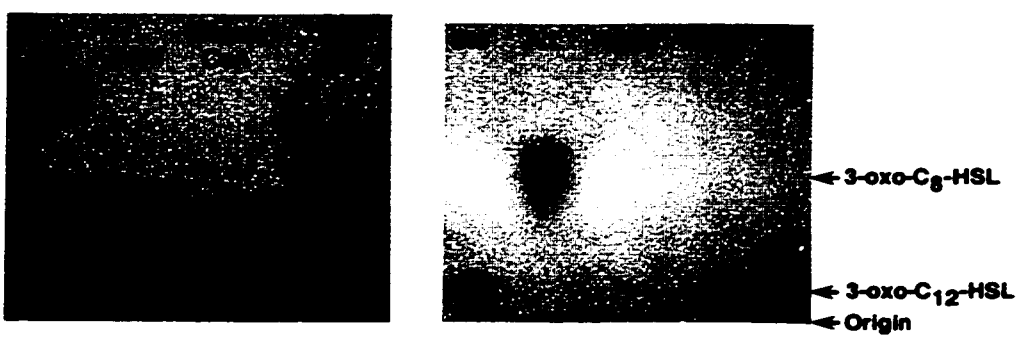
Figure 7.2B shows the results of a labeling experiment utilizing purified Aas. In 30 µl total volume, 10 µg ACP from various ACP preparations was incubated with 0.5 µg of Aas, and 0.5 mM decanoic acid in buffer (10 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM ATP, and 100 mM Tris-HCl, pH 8.0) for 18 h at 37°C. Purified H_6-Aas converted all of the holo-ACP present in the various ACP preparations to C_{10}-ACP but as expected did not act on apo-ACP.

To assess chain length specificities, we also labeled ACP with various other fatty acids using the same conditions as described above. From the results shown in figure 7.2C, it is evident that purified Aas efficiently labeled ACP with all the fatty acid substrates tested in this experiment - i.e. fatty acids with chain lengths of six to twelve carbons. Although high salt concentrations (0.4 M LiCl or NaCl) and Triton X-100 were shown to enhance the activity of *E. coli* Aas (12), these agents were not required in our hands, and they were also not required for ACP labeling using *V. harveyi* Aas (15).

To show that our acyl-labeled ACP was functional, we coupled three enzymes in an attempts to synthesize 3-oxo-C_{12}-HSL from decanoyl-ACP as diagrammed in figure 7.3A. Decanoyl-ACP was synthesized on a large scale by labeling ~10 mg of holo-ACP with Aas in the presence of 2 mM decanoic acid as described above. Typically, the amount of Aas obtained from a 3 l culture (~1 mg) was enough to label 20-30 mg of ACP. The three enzyme coupled reactions were performed in a 500 µl mixture containing 20 mM Tris-HCl (pH 7.5), 200 µM *S*-adenosylmethionine (SAM), 100 µM malonyl-CoA, ~50 µg of decanoyl ACP (C_{10}-ACP), and 1 µg of each FabD, FabB and LasI. The reaction mixtures were carried out at 37°C for 30 min and extracted three times with 250 µl of ethyl acetate. The ethyl acetate fractions were dried by rotary vacuum evaporation. The residues were resuspended in 30 µl of ethyl acetate and 10 µl aliquots were spotted on a C_{18} reverse phase thin layer chromatography (TLC) plate as previously described (3). 3-oxo-HSL containing fractions were detected by overlaying the dried TLC plate with the *Agrobacterium tumefaciens* detection strain NTL4(pZLR4) in the presence of 5-bromo-4-chloro-3-indolyl β-galactopyranoside (Xgal), as previously described (10, 15), and the results are shown in figure

Figure 7.3. **(A)** Synthesis of 3-oxo-C_{12}-HSL from C_{10}-ACP in FabD, FabB and LasI coupled reactions. According to this model, FabD (malonyl-CoA:ACP transacylase) transfers the activated malonyl group to ACP, which is then utilized in a condensation reaction with decanoyl-ACP (C_{10}-ACP), catalyzed by FabB (β-ketoacyl-ACP synthase I), to form 3-oxo-dodecanoyl-ACP (3-oxo-C_{12}-ACP). LasI (3-oxo-C_{12}-HSL synthase) then catalyzes the cyclization of the lactone ring from *S*-adenosylmethionine (SAM) and transfer of 3-oxo-C_{12} to produce 3-oxo-C_{12}-HSL and 5'-methylthioadenosine (MTA). **(B)** Detection of HSLs. After the extracted reactions mixtures were spotted on a C_{18} reverse phase TLC plate, it was overlaid with a *A. tumefaciens* HSL detection strain in the presence of 40 μg/ml Xgal to detect reactions that contained active HSLs. The reactions mixtures analyzed contained all reaction components (All) or were missing individual components. Synthetic 3-oxo-C_{12}-HSL was included as a standard (Std). **(C)** C_{18} reverse phase TLC analysis of HSL containing fractions. Selected reaction mixtures from the experiment **(B)** were spotted on a TLC plate, the plate was developed with 60% methanol in water and HSLs were detected with an *A. tumefaciens* detection strain. Although not visible in this picture, there was a faint spot on the original TLC plate in the 3-oxo-C_{12}-HSL position for the reaction containing no FabD. *A. tumefaciens* 3-oxo-C_8-HSL extracted from NT1/pTiC58Δ*accR* (10, 17) and synthetic 3-oxo-C_{12}-HSL standard (Std) were also applied as controls.





7.3B. The results show that the C_{10}-acyl ACP substrate is converted into biologically active 3-oxo-HSL via the combined actions of FabD, FabB and LasI. When malonyl-CoA, C_{10}-ACP, and FabB were individually excluded, no HSLs were detected, indicating that LasI cannot utilize C_{10}-ACP. Essential components for HSL synthesis (SAM and LasI) were also required. When FabD was excluded, some HSL was detected since some spontaneous transacylation of malonyl groups from malonyl-CoA to ACP occurs in the absence of FabD (unpublished data) (5). A minute amount of HSL was also detected in the absence of FabB but only if the reaction was carried out for a longer period (2 h at 37°C), due to spontaneous condensation of malonyl-ACP and C_{10}-ACP at very low rates (data not shown). To confirm the identity of 3-oxo-C_{12}-HSL, we separated HSL positive reaction mixtures by C_{18} reverse phase TLC in 60% methanol in water (v/v), and detected HSLs by overlaying the dried TLC plate with the NTL4/pZLR4 *A. tumefaciens* indicator strain in the presence of XGal. The results shown in figure 7.3C demonstrate that 3-oxo-C_{12}-HSL was indeed synthesized in the three enzyme coupled reactions.

7.3 CONCLUSIONS

We overexpressed and purified the *E. coli* acyl-ACP synthase (Aas) by affinity chromatography after histidine tagging the protein, and demonstrated its function for saturated fatty acids containing six to twelve carbons. The broad substrate specificity for *E. coli* Aas, including unsaturated (*cis* and *trans*) and saturated (C_8 to C_{18}) fatty acids, has previously been demonstrated (11, 14). We utilized the purified Aas to achieve large scale labeling of holo-ACP with decanoic acid. To demonstrate its activity, the labeled C_{10}-ACP was used in a three enzyme coupled reaction and shown to be converted into biologically active 3-oxo-C_{12}-HSL. This is the first example of *in vitro* enzymatic synthesis of a 3-oxo-acyl-ACP substrate required for studies of 3-oxo-HSL synthases. Although this was not necessary in the studies presented herein, the H_6-Aas can be easily removed from reaction mixtures by passage through a Ni^{2+}-NTA agarose column or microcon 30 microconcentrators (Amicon, Beverly, MA). Subsequent passage of the Ni^{2+} column eluate through a desalting

column (e.g. a PD-10 column from Pharmacia Biotech, Uppsala, Sweden) allows removal of other components (fatty acids, Mg^{2+}, DTT, AMP, and ATP) of the labeling reactions from the acyl-ACP preparations. The ease of purification and removal of Aas after labeling will greatly facilitate synthesis of various acyl-ACP substrates required for studies on the biosyntheses of fatty acids and acylated homoserine lactones, and other acyl-ACP requiring reactions.

7.4 ACKNOWLEDGMENTS

We thank Dr. Christopher Walsh for the gift of pDPJ, Dr. Stephen Farrand for providing *Agrobacterium* strains and detection protocols, and Dr. Barbara Iglewski for her generous gift of synthetic 3-oxo-C_{12}-HSL.

S. A. Sullivan and T .T. Hoang contributed equally to this work.

7.5 REFERENCES

1. **Chang, A.C.Y.C., and S.N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**: 1141-1156.

2. **Cronan, J. E., and A. L. Klages.** 1981. Chemical synthesis of acyl thioesters of acyl carrier protein with native structure. Proc. Natl. Acad. Sci. USA **78:**5440-5444.

3. **Fice, D., Z. Shen, and D. M. Byers.** 1993. Purification and characterization of fatty acyl-acyl carrier protein synthase from *Vibrio harveyi*. J. Bacteriol. **175:**1865-1870.

4. **Hoang, T. T., Y. Ma, R. J. Stern, M. R. McNeil, and H. P. Schweizer.** 1999. Construction and use of low-copy number T7 expression vectors for purification of problem

proteins: purification of *Mycobacterium tuberculosis* RmlD and *Pseudomonas aeruginosa* LasI and RhlI proteins, and functional analysis of RhlI. Gene **237:**361-371.

5. **Jackowski, S., P. D. Jackson, and C. O. Rock.** 1994. Sequencing and function of the *aas* gene in *Escherichia coli*. J. Biol. Chem. **269:**2921-2928.

6. **Kutchma, A. J., T. T. Hoang, and H. P. Schweizer.** 1999. Characterization of a *Pseudomonas aeruginosa* fatty acid biosynthetic gene cluster: purification of acyl carrier protein (ACP) and malonyl-coenzyme A:ACP transacylase (FabD). J. Bacteriol. **181:**5498-5504.

7. **Lambalot, R. H., and C. T. Walsh.** 1995. Cloning, overproduction, and characterization of the *Escherichia coli* holo-acyl carrier protein synthase. J. Biol. Chem. **270:**24658-24661.

8. **Moré, M. I., D. Finger, J. L. Stryker, C. Fuqua, A. Eberhard, and S. C. Winans.** 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science **272:**1655-1658.

9. **Parsek, M. R., D. L. Val, B. L. Hanzelka, J. E. Cronan, and E. P. Greeneberg.** 1999. Acyl homoserine-lactone quorum-sensing signal generation. Proc. Natl. Acad. Sci. USA **96:**4360-4365.

10. **Piper, K. R., S. B. von Bodman, and S. K. Farrand.** 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature **362:**448-450.

11. **Ray, T. K., and J. J. E. Cronan.** 1976. Activation of long chain fatty acids with acyl carrier protein: Demonstration of a new enzyme, acyl-acyl carrier protein synthase, in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **73:**4374-4378.

12. **Rock, C. O., and J. E. Cronan.** 1981. Acyl carrier protein from *Escherichia coli*. Methods Enzymol. **71:**341-351.

13. **Rock, C. O., and J. J. E. Cronan.** 1979. Solubilization, purification, and salt activation of acyl-acyl carrier protein synthase from *Escherichia coli*. J. Biol. Chem. **254:**7116-7122.

14. **Rock, C. O., and J. L. Garwin.** 1979. Preparative Enzymatic Synthesis and Hydrophobic Chromatography of Acyl-Acyl Carrierr Protein. J. Biol. Chem. **254:**7123-7128.

15. **Shaw, P. D., G. Ping, S. L. Daly, C. Cha, J. E. Cronan, K. L. Rinehart, and S. K. Farrand.** 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci. USA **94:**6036-6041.

16. **Shen, Z., D. Fice, and D. M. Byers.** 1992. Preparation of fatty-acylated derivatives of acyl carrier protein using *Vibrio harveyi* acyl-ACP synthase. Anal. Biochem. **204:**34-39.

17. **von Bodman, S. B., G. T. Hayman, and S. K. Farrand.** 1992. Opine catabolism and conjugal transfer of the nopaline Ti plasmid pTiC58 are coordinately regulated by a single repressor. Proc. Natl. Acad. Sci. USA **89:**643-647.

CHAPTER 8

In vitro synthesis of *N*-(3-oxo)-dodecanoyl-L-homoserine lactones from simple metabolic precursors using purified *Pseudomonas aeruginosa* Fab proteins and LasI

(part of a manuscript in preparation by Tung T. Hoang and Herbert P. Schweizer)

I acknowledge the assistance of Alecksandr J. Kutchma in PCR amplification and cloning of *fabG* into pET-15b.

8.1 ABSTRACT

Cell-to-cell communication in *P. aeruginosa* is mediated by mainly two diffusible autoinducers, *N*-butyryl-L-homoserine lactone (C_4-HSL) and *N*-[3-oxododecanoyl]-L-homoserine lactone (3-oxo-C_{12}-HSL). C_4-HSL and 3-oxo-C_{12}-HSL are produced by the *rhlI*- and *lasI*-encoded autoinducer synthases RhlI and LasI, respectively, using *S*-adenosylmethionine (SAM) and a source for the acyl side chains, either acylated acyl carrier proteins (acyl-ACPs) from the fatty acid biosynthetic (Fab) pathway or acyl-coenzyme A (acyl-CoA) molecules. However, it was hitherto unclear whether the Fab pathway was indeed a source of the acyl groups and whether it was sufficient to provide even the 3-oxo-acyl chains found in some HSLs. We used an *in vitro* system to assess the role of the Fab pathway in 3-oxo-C_{12}-HSL synthesis. Expression vectors were constructed and seven putative *P. aeruginosa* enzymes constituting the Fab cycle were purified as hexahistidine fusion proteins. In a reaction mixture containing ACP, acetyl-CoA, malonyl-CoA, SAM, NADH, NADPH, and the seven purified Fab proteins, LasI was able to synthesize biologically active 3-oxo-C_{12}-HSL. This reaction was inhibited by known Fab inhibitors. Omission of individual

components from the reactions allowed establishment of the minimal number of constituents required for a functional HSL biosynthetic pathway. Variations of enzymes and substrates of the Fab pathway provided evidence that this pathway can provide acyl chains for the syntheses of 3-oxo-C_8-HSL, 3-oxo-C_6-HSL, 3-oxo-C_{10}-HSL, and 3-oxo-C_{12}-HSL by LasI. These results provide (i) the first direct evidence that the Fab pathway is sufficient for synthesis of even the substituted acyl side chains found in the HSLs via acyl-ACP from cellular metabolites and (ii) that LasI is sufficient for synthesis of shorter-chain 3-oxo-acyl-HSLs.

8.2 INTRODUCTION

The expression of many extracellular *P. aeruginosa* virulence factors is regulated in a controlled, cell-density-dependent manner by a process called cell-to-cell communication (20, 22, 23). This process is mediated by the two *Pseudomonas* autoinducers (PAIs), *N*-[3-oxo-C_{12}]-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butyryl-L-homoserine lactone (C_4-HSL), and at least 3-oxo-C_{12}-HSL is required for biofilm maturation (3). Although PAI production and action has been studied in considerable detail at the genetic level, little is known about the biochemical pathways involved in PAI synthesis. It is now believed that the respective autoinducer synthases catalyze AI formation from acylated-acyl carrier proteins (acyl-ACPs) and *S*-adenosylmethionine (SAM) (18, 19, 28). However, even though several studies have postulated an involvement of the fatty acid biosynthetic (Fab) pathway by providing acyl-ACP as the acyl donor in heterologous *in vitro* reactions (18, 19, 28), no direct involvement of any of the Fab enzymes has yet been demonstrated. In addition, these studies were performed using protein components from heterologous sources (18).

According to the current model, the homoserine lactone moieties of the AIs are derived from SAM and their acyl moieties from the cellular acyl-ACP pool (figure 8.1A). This model predicts that the HSL synthases (LasI for 3-oxo-C_{12}-HSL and RhlI for C_4-HSL) are sufficient for catalysis of the acyl transfer and lactonization reactions, and a reaction mechanism has been proposed (18, 19) (see figure 1.5). We have previously obtained

evidence that the Fab pathway plays an important role in HSL synthesis since (i) FabI, when coupled to RhlI, was capable of synthesis of C_4-HSL from the Fab intermediate crotonyl-ACP, and that this reaction was inhibited by the FabI inhibitor triclosan (7, 8), and (ii) *fabI* mutants were greatly impaired in PAI synthesis (8).

We have also been interested in establishing the *P. aeruginosa* Fab pathway since it offers diverse sites for inhibition by various antimicrobial agents, including triclosan (14, 17) and thiolactomycin (4, 5, 11), and it is involved in diverse essential cellular pathways, including lipid A synthesis, phospholipid synthesis, lipoprotein synthesis, and the synthesis of protein-bound biotin and lipoic acid (2).

Since the Fab pathway is essential, conventional mutant analysis cannot be used to address its importance in HSL synthesis. To circumvent this problem, we purified the proteins implicated in synthesis of acyl-ACPs from simple metabolic precursors as hexahistidine (H_6) fusion proteins (figure 8.1) with the aim of employing them in an *in vitro* Fab synthesis system. These included FabA (longer chain-specific β-hydroxyacyl-ACP dehydratase), FabB (β-ketoacyl-ACP synthase I), FabD (malonyl-CoA: ACP transacylase), FabG, (NADPH-dependent β-ketoacyl-ACP reductase), FabH, (β-ketoacyl-ACP synthase III), FabI (NADH-dependent enoyl-ACP reductase), and FabZ (shorter chain-specific β-hydroxyacyl-ACP dehydratase). In addition, unmodified *P. aeruginosa* ACP was purified via an ACP-intein fusion protein (13) and LasI was purified as a H_6 fusion protein (7) (see also chapters 4 and 7).

Utilizing the purified enzymes, we demonstrated (i) that the Fab pathway, when coupled to LasI, is sufficient for 3-oxo-C_{12}-HSL synthesis from simple metabolic precursors, (ii) Fab components that are essential for HSL synthesis, (iii) provide evidence that Fab inhibitors may be effective inhibitors of HSL synthesis, and (iv) show that other 3-oxo-acyl-HSLs observed in *P. aeruginosa* culture supernatant can be synthesized via LasI with all acyl chains being derived from the Fab pathway.

8.3 MATERIALS AND METHODS

8.3.1 Strains and reagents

The following strains were used in this study: *E. coli* strains DH5α (15), BL21(DE3) (Novagen, Madison, WI); the wild-type *P. aeruginosa* strain PAO1 (10); and the mutant *P. aeruginosa* strains PAO191 (*fabA*) (9), PAO192 (*fabB*) and PAO204 (*fabD*(Ts)) (9, 12); *Agrobacterium tumefaciens* strains NTL4/pZLR4 (24) and NT1/pTiC58Δ*accR* (29); *Erwinia carotovora* strain EC14 (25). Unless otherwise indicated, bacterial strains were grown in LB medium (Difco, Detroit, MI) which was supplemented with 100 μg/ml ampicillin (Ap; Sigma, St. Louis, MO) where appropriate.

8.3.2 Construction of expression vectors and purification of Fab proteins. The genes for the individual enzymes were amplified from PAO1 genomic DNA essentially as previously described for FabD, FabI and LasI (7, 8). Primers (table 8.1) created novel restriction sites for *Nde*I at the NH_2-terminal methionine and *Bam*HI (or *Bgl*II for *fabG*) downstream of the stop codon. PCR was performed with *Taq* polymerase, the fragments were purified, digested with *Bam*HI+*Nde*I (or *Bgl*II+*Nde*I) and then ligated into *Bam*HI- and *Nde*I-digested pET15-b (Novagen, Madison, WI). The amplified *fabA* and *fabB* genes were first cloned into the TA-cloning vector pGEM-T (Promega, Madison, WI) before subcloning on an *Bam*HI+*Nde*I fragment into pET-15b. These procedures yielded the expression vectors listed in table 8.1. For expression, the plasmids were transformed into BL21(DE3). Screening of fusion protein-expressing transformants, expression of H_6-Fab proteins, cell lysis and purification of the soluble fusion proteins on Ni^{2+}-NTA agarose columns (Qiagen, Santa Clarita, CA) were performed as previously described (7), except for FabD. Since FabD eluted from Ni^{2+}-NTA agarose columns with 40 mM imidazole, washing of the column was done with at least 30 bed volumes of metal chelation affinity chromatography (MCAC) buffer with 20 mM imidazole instead of 40 mM imidazole.

Table 8.1. Plasmids, expression vectors and primers.

Plasmids/ Primers	Relevant properties	References or sources
pET-15b	Ap^r; T7-promoter base vector for expression of proteins with amino-terminal hexahistidine tag	Novagen
pGEM-T	Ap^r; TA cloning vector	Promega
pPS847	Ap^r; PCR amplified *fabA* gene (550-bp) cloned into pGEM-T	This study
pPS848	Ap^r; *fabA* gene from pPS847 was cloned into pET-15b on an *Nde*I-*Bam*HI fragment	This study
pPS836	Ap^r; PCR amplified *fabB* gene (1.2-kb) cloned into pGEM-T	This study
pPS837	Ap^r; *fabB* gene from pPS836 was cloned into pET-15b on an *Nde*I-*Bam*HI fragment	This study
pPS980	Ap^r; PCR amplified *fabG* gene (0.8-kb) cloned on an *Nde*I-*Bgl*II fragment into pET-15b cut with *Nde*I + *Bam*HI	(12)
pPS999	Ap^r; PCR amplified *fabH* gene (1.1-kb) was cloned into pET-15b on an *Nde*I-*Bam*HI fragment	This study
pPS937	Ap^r; PCR amplified *fabZ* gene (550-bp) was cloned into pET-15b on an *Nde*I-*Bam*HI fragment	This study
fabA-NdeI*	5'-TCATATGACCAAACAACACGCCTTCAC	This study
fabA-BamHI	5'-GGATCCCCCTTAGAAGCTGTCAGTGGAG	This study
fabB-NdeI	5'-TCCATATGCGTCGCGTCGTTATCACCGGTC	This study
fabB-BamHI	5'-ATGGATCCAATCAACCCTGCCAGCGCTTGAGGA	This study

Table 8.1. Plasmids, expression vectors and primers (continued).

fabG-NdeI	5'-GGAGAGAATCATATGAGTCTGCAAGGTAA GGTCGCA	This study
fabG-BglII	5'-GACAGATCTTATGACAGACCCGAGAAAGG TAAC	This study
fabZU	5'-CCTCATATGATGGACATCAACGAGATTCG	This study
fabZD	5'-GAGGATCCATCAAACTCATAGTTTGCGT	This study

* Novel restriction enzyme cleavage sites introduced by primers are underlined.

ACP was purified via as intein chitin-binding domain fusion protein as previously described (13) with the improvements detailed in chapter 7. LasI was purified as a fusion protein with an NH_2-terminal H_6-containing extension (7)(chapter 4).

Protein concentrations were determined using the Bradford dye-binding assay (Bio Rad Laboratories, Hercules, CA) and bovine serum albumin as the standard. Proteins were analyzed by electrophoresis on 0.1% sodium dodecyl sulfate-10% polyacrylamide gel (SDS-PAGE) as previously described (16). The proteins were visualized by quick staining with Coomassie Brilliant Blue R-250 (1).

8.3.3 Complementation with H_6-tagged Fab proteins. Wherever possible, the biological activity of H_6-fusion proteins was assessed by subcloning the fusion genes into an expression vector. To do this, the coding sequences for the H_6-tagged FabA, FabB and FabD proteins were subcloned into the broad-host range vector pUCP21 (26) on *Xba*I-*Bam*HI fragments (*Xba*I cleaves between the T7 promoter and the ribosome-binding site, and *Bam*HI cleaves immediately downstream of the respective *fab* coding sequences). Subcloning between the *Xba*I and *Bam*HI sites of pUCP21 placed the H_6-Fab coding sequences in the correct

transcriptional orientation with respect to the *lac* promoter contained on this cloning vector. As a result, the H_6-Fab coding sequences were constitutively transcribed in *P. aeruginosa* cells that are naturally devoid of *lac* repressor.

To test for expression of functional H_6-FabA and H_6-FabB proteins, the respective plasmid constructs were transformed into strains PAO191 (*fabA*::Gm^r) and PAO192 (*fabB*::Gm^r) (9). Since FabA and FabB are required for unsaturated Fab, these strains will not grow at 42°C unless supplemented with oleic acid or complemented with either a FabA- or FabB-expressing plasmid. Complementation was therefore scored as the ability of the transformed mutants to grow on RB rich medium at 42°C without oleate supplementation (9). To test for expression of a functional H_6-FabD protein, the respective plasmid construct was transformed into PAO204 (*fabD*(Ts)). Successful complementation was scored as the ability of the transformants to grow at 42°C.

8.3.4 Assembly of the Fab-HSL pathway and extraction of *N*-(3-oxo-acyl)-L-homoserine lactones. Complete reactions (total volume 500 µl) contained buffer (10 mM Tris-HCl (pH 7.4), 330 mM NaCl, 15% glycerol, 0.7 mM DTT, 2 mM EDTA, 25 mM $MgSO_4$, 0.1 mM $FeSO_4$) (18), with 2 µg of ACP, 1 µg each of FabA, FabB, FabD, FabH, FabI and FabZ, 0.5 µg of FabG, 5 µg of LasI, 0.25 mM SAM, 0.08 mM acetyl-CoA, 0.8 mM malonyl-CoA, and 0.6 mM each of NADH and NADPH. Reactions were incubated at 37°C for 1 h and extracted three times with 250 µl of ethyl acetate. Extracted HSLs were dried by rotary vacuum evaporation and resuspended in 20 µl of acetonitrile. For detection of fractions containing HSLs, 10 µl of each fraction was spotted on a C_{18} reverse-phase TLC plates (Whatman, Clifton, NJ) and the plates were dried at 37°C for 15 min before being overlaid with the detection strain. For TLC analysis of HSL fractions, the dried plates were developed in 60% methanol in water (v/v) (see chapter 6.3.7) and then dried for 20 min at 37°C prior to being overlaid with the detection strain.

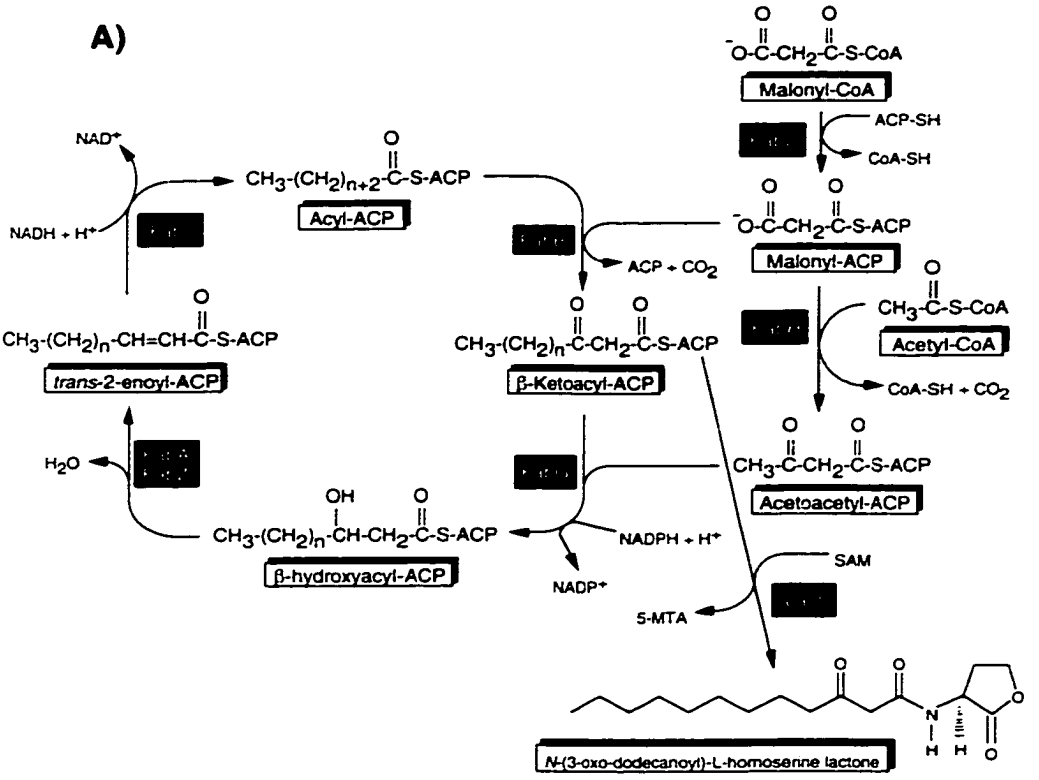
8.3.5 Detection and identification of HSLs. *Agrobacterium tumefaciens* detection strain (NTL4/pZLR4) was grown at 30°C for 48 hours in M9 medium with 1 mM $MgSO_4$, 0.1 mM

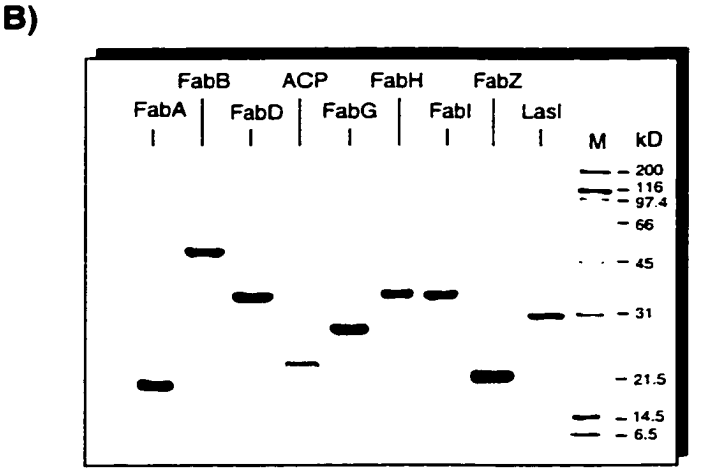
$CaCl_2$, 0.6 % glucose and 30 µg/ml gentamycin (27). Cells were harvested and resuspended in warm (~37°C) fresh M9 medium with 0.4 % agar, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.6 % glucose and 40 µg/ml Xgal (24). This suspension was used to overlay the TLC plates. The presence of HSLs was usually evident by the appearance of blue spots after incubation at room temperature for 36 to 48 h. Synthetic 3-oxo-C_{12}-HSL, *N*-(3-oxo-octanoyl)-HSL (3-oxo-C_8-HSL) and *N*-(3-oxo-hexanoyl)-HSL (3-oxo-C_6-HSL) were included as positive controls or standards. The latter two were extracted from 10 ml of stationary-phase clarified culture supernatants of *A. tumefaciens* strain NT1/pTiC58Δ*accR* (29) and *Erwinia carotovora* strain EC14 (25), respectively. To achieve this, cells were removed from cultures by centrifugation at 10,000 x g and the supernatants were filter-sterilized by passing them through a 0.2 µm filter (Millipore, Bedford, MA). The sterilized culture supernatants were extracted three times with 2.5 ml ethyl acetate. The pooled extracts were dried by rotary vacuum evaporation and resuspended in 0.5 ml of ethyl acetate. Usually, 10 µl aliquots of these HSL preparations were spotted onto each TLC plate. Extraction of total HSLs from *P. aeruginosa* strain PAO1 was performed using the same procedure.

8.4 RESULTS

8.4.1 Purification of proteins composing the Fab-AI pathway. According to our proposed model (8), at least seven Fab enzymes and ACP are required for providing the 3-oxo-C_{12}-acyl-ACP precursor required as an acyl donor for the LasI-catalyzed formation of 3-oxo-C_{12}-HSL (figure 8.1). In this study, we set out to complete our previously begun task of cloning, overexpressing and purifying all of these proteins. We previously described the cloning and characterization of the *fabAB* operon (9) and a gene cluster containing *fabD*, *fabG*, *acpP*, and *fabF*, as well as the overexpression and purification of FabD and ACP (13) (chapters 5 and 7). More recently, we presented a detailed characterization of purified FabI, encoding enoyl-ACP reductase, an important enzyme catalyzing the final reduction step of each cycle of fatty acid elongation (8)(chapter 6). We also reported the purification of the

Figure 8.1. Enzymes of the Fab-HSL biosynthetic pathway. **A)** The proposed reactions of fatty acid elongation in *P. aeruginosa*. The first cycle is initiated by FabH (β-ketoacyl-ACP synthase III), which condenses malonyl-ACP with acetyl-CoA; subsequent cycles are initiated by condensation of malonyl-ACP with acyl-ACP, catalyzed by FabB (β-ketoacyl ACP synthase I). The β-ketoacyl-ACP from the synthase reactions is reduced to β-hydroxyacyl-ACP by FabG (a NADPH-dependent β-ketoacyl-ACP reductase). The subsequent dehydration step is either catalyzed by FabA or FabZ, depending on the lengths of the β-hydroxyacyl-ACP substrates. The final step involves reduction of the dehydratase product to an acyl-ACP by action of FabI (a NADH-dependent enoyl-ACP reductase). The malonyl-ACP used for initiation of the cycles is derived from malonyl-CoA by malonyl-CoA:ACP acyltransferase (FabD). LasI (*N*-[3-oxododecanoyl]-L-homoserine lactone synthase) utilizes the 3-oxo-dodecanoyl-ACP from the Fab pathway for the synthesis of *N*-[3-oxo-dodecanoyl]-L-homoserine lactone. **B)** 0.1% sodium dodecylsulfate-13% polyacrylamide gel electrophoretic (SDS-PAGE) analysis of purified ACP, the various Fab proteins, and purified *N*-(3-oxododecanoyl)-L-homoserine lactone synthase (LasI). All proteins, except ACP, were purified with NH_2-terminal H_6-tag containing extensions. ACP was purified in its native form via an ACP-intein chitin-binding domain fusion protein (see chapters 5 and 7). The sizes of broad-range protein markers (M) from Bio-Rad (Hercules, CA) are indicated in kDa and were (top to bottom): myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme, aprotinin).





very insoluble LasI protein under native conditions using a novel expression vector (7)(chapter 4). In this study, we overexpressed and purified FabA, FabB and FabG based on our previous characterization of the *fabAB* operon (9) and the *fabG*-containing *fab* gene cluster (13). Amplification and cloning of the *fabH* gene was achieved by identification of the most likely homologous gene in the completed *P. aeruginosa* genome database (Pathogenesis Corporation) using other bacterial *fabH* genes as query sequences. To assess the biological activity of some H_6-fusion proteins, the genes encoding the H_6-tagged FabA, FabB and FabD proteins were subcloned into the broad-host range vector pUCP21 (26) where the expression of the respective genes was driven by the *lac* promoter. Complementation experiments were not possible for FabG, FabH and FabZ since no mutants are available. The plasmids complemented *P. aeruginosa fabA* and *fabB* mutations, as assessed by the ability of the transformants to grow in the absence of oleic acid, or by the ability of H_6-FabD to overcome the temperature sensitive phenotype of a *fabD*(Ts) mutant. We had previously shown that H_6-FabI was enzymatically active (8). Using non-denaturing conditions, all Fab proteins, ACP and LasI were purified to apparent homogeneity (figure 8.1B).

8.4.2 Assembly of the Fab-AI pathway. As shown in figure 8.2, when all theoretically required enzymes and substrates were included in the reaction mixture (spot labeled All), LasI was able to synthesize detectable 3-oxo-acyl-HSL. However, when either FabD, ACP, FabB, FabG, FabZ, FabI or LasI were omitted from the reaction mixture, no dectectable HSLs were synthesized, indicating that these enzymes are essential in the Fab-AI pathway and that they are functional as H_6-tagged proteins. Conversely, when FabA and FabH were omitted from the reaction mixture, significant amounts of HSLs were still synthesized, indicating that these enzymes were not absolutely required for the Fab reactions to occur. To assess substrate and co-factor requirements, reactions were set up missing either acetyl-CoA, or malonyl-CoA, or SAM, or NADH or NADPH. The results indicated that malonyl-CoA and SAM were absolutely required for HSL formation. Some HSL was still synthesized in the absence of acetyl-CoA and NADH, and omission of NADPH from the assay mixture had seemingly little effect. At the concentration tested (50 μM), the known Fab inhibitors cerulenin and triclosan

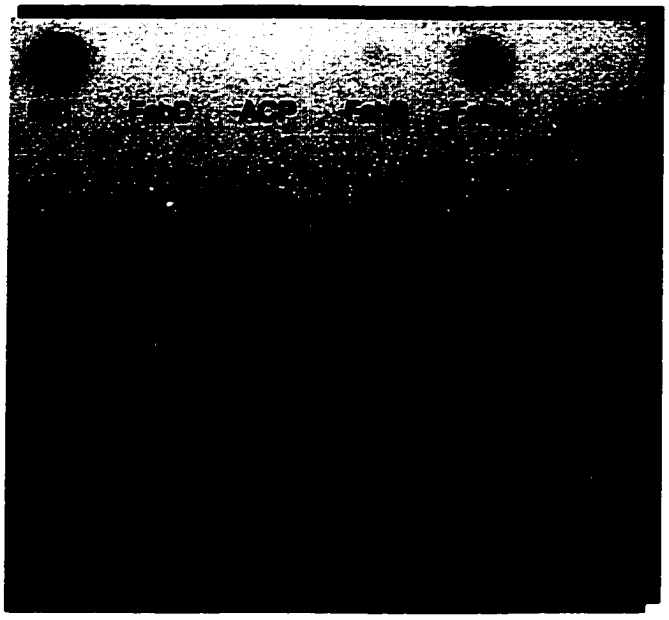
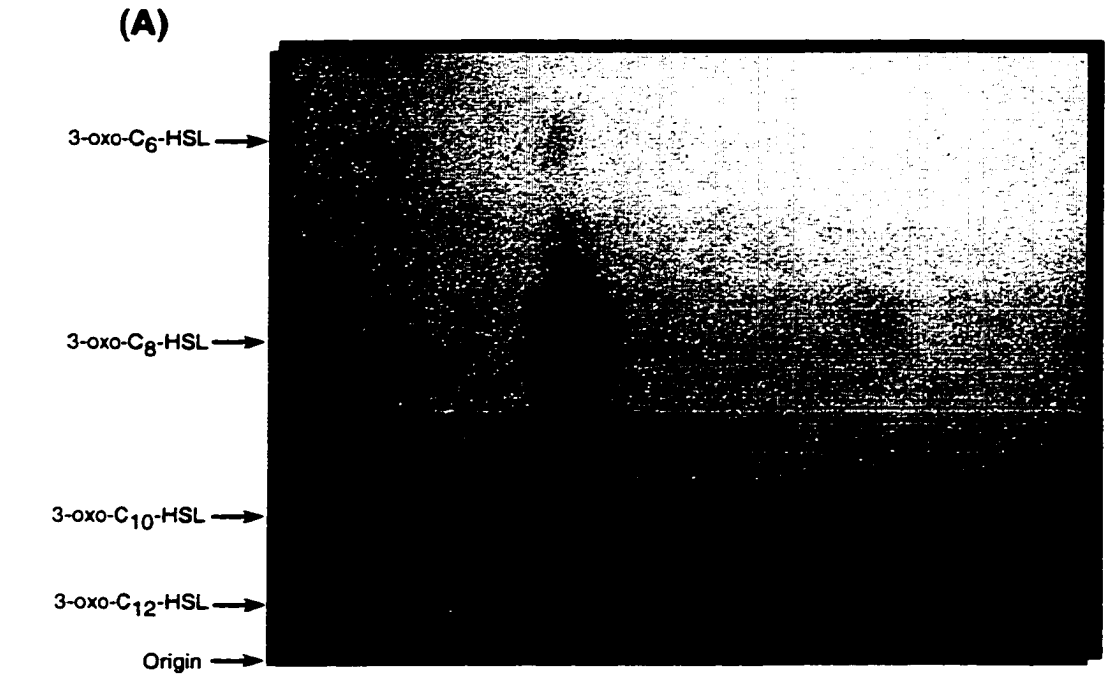


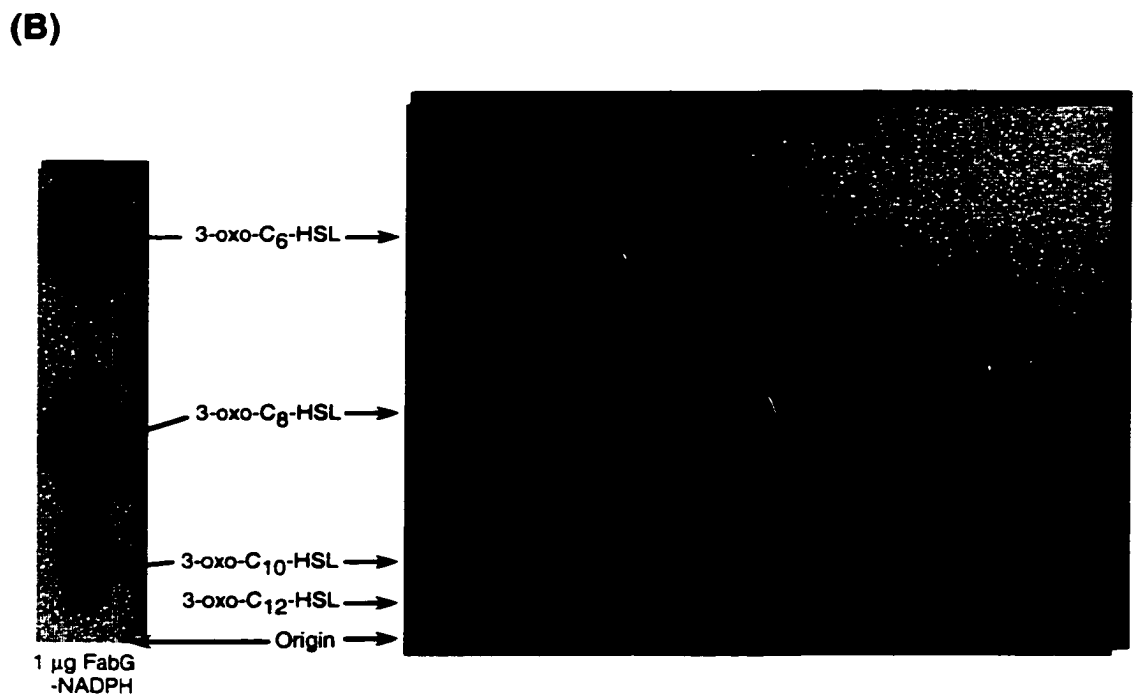
Figure 8.2. Synthesis of 3-oxo-acyl-HSLs in a reconstituted enzyme system from metabolic precursors. Ethyl acetate extracts of reactions were analyzed for the presence of HSLs by spotting samples on a C_{18}-reverse-phase thin layer chromatography (TLC) plate and overlaying it with the *Agrobacterium tumefaciens* HSL detection strain NTL4(pZLR4) in the presence of XGal. Spots indicate the presence of 3-oxo-acyl-HSLs and a 3-oxo-C_{12}-HSL standard (Std). The complete reaction (All) contained 2 μg of ACP, 1 μg of each FabA, FabB, FabD, FabH, FabI and FabZ, 0.5 μg of FabG, 5 μg of LasI, 0.25 mM SAM, 0.08 mM acetyl-CoA, 0.8 mM malonyl-CoA, and 0.6 mM of each NADH and NADPH. The other reactions either lacked the indicated enzymes, substrates or co-factors. Some reactions each contained 50 μM of the Fab inhibitors thiolactomycin (TLM), cerulenin (Cer), diazaborine (Dia) or triclosan (Tri). Enzyme abbreviations: ACP, acyl carrier protein; FabA, longer chain-specific β-hydroxyacyl-ACP dehydratase; FabB, β-ketoacyl-ACP synthase I; FabD, malonyl-CoA:ACP transacylase; FabG, NADPH-dependent β-ketoacyl-ACP reductase; FabH, β-ketoacyl-ACP synthase III; FabI, NADH-dependent enoyl-ACP reductase; FabZ, shorter chain-specific β-hydroxyacyl-ACP dehydratase; and LasI, *N*-[3-oxododecanoyl]-L-homoserine lactone synthase. Substrate and co-factor abbreviations: AcCoA, acetyl-CoA; NADH, reduced β-nicotinamide adenine dinucleotide; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; MaCoA, malonyl-CoA; SAM, *S*-adenosylmethionine.

efficiently inhibited HSL formation, and thiolactomycin and diazoborine showed some inhibitory effect.

8.4.3 Nature of homoserine lactone molecules synthesized *in vitro*. To putatively identify HSL molecule species contained in the reactions with biologically active HSLs (figure 8.2), the contents of these reaction mixtures were further characterized by TLC analysis in the presence of appropriate standards. As shown in figure 8.3A, all active reactions contained 3-oxo-C_{12}-HSL and trace amounts of 3-oxo-C_{10}-HSL, with the exception of the reaction containing no NADPH which produced mostly 3-oxo-C_8-HSL, 3-oxo-C_{10}-HSL, and trace amounts of 3-oxo-C_6-HSL. Since 3-oxo-C_8-HSL is the cognate *A. tumefaciens* autoinducer, its spot size is not indicative of a higher quantity of 3-oxo-C_8-HSL relative to the other 3-oxo-acyl-HSLs, but rather indicates a better response to its native HSL. Synthesis of shorter chain 3-oxo-acyl-HSLs was dependent on the co-factor present, as well as the concentration of FabG. When the Fab-AI pathway operated under optimal conditions (figure 8.3A, lane labeled All), the preferred substrate for LasI is 3-oxo-C_{12}-ACP since only minute amounts of shorter chain 3-oxo-acyl-HSLs were discernible. In the absence of NADPH and presence of NADH, i.e. conditions under which the FabG catalyzed reaction is greatly slowed down, LasI synthesized only small amounts of 3-oxo-C_{12}-HSL but large amounts of 3-oxo-C_8-HSL and 3-oxo-C_{10}-HSL (figure 8.3A, lane labeled -NADPH). Similar results were obtained when the amounts of FabG present in the reactions were manipulated in the absence of NADPH (figure 8.3B, lane labeled 0.25 μg FabG-NADPH). Increases in the amounts of FabG present in the reactions were accompanied by synthesis of higher levels of 3-oxo-C_6-HSL (figure 8.3B, lane labeled 1 μg FabG-NADPH), a finding consistent with the accumulation of increasing levels of 3-oxo-C_6-ACP due to a more efficient elongation of the first two fatty acid cycles at the FabG catalyzed step (figure 8.1). These results indicated that LasI alone is sufficient for synthesis of shorter chain 3-oxo-acyl-HSLs provided that the respective acyl donors are available and under conditions where LasI can compete with FabG for these substrates. When cells are grown under appropriate conditions, *P. aeruginosa* culture supernatants contain 3-oxo-C_8-HSL and 3-oxo-C_{10}-HSL (figure 8.3, lane labeled PAO1).

Figure 8.3. Identification of HSLs produced in *in vitro* synthesis reactions. The extracted and concentrated reaction products were spotted onto a C_{18}-reverse phase TLC plate, the plate was developed with 60% (v/v) methanol in water and overlaid with an *A. tumefaciens* detection strain in the presence of XGal. **(A)** All, complete reaction mixtures containing the components listed in the legend to figure 8.2; other reactions lacked the indicated enzymes, substrates or co-factors, or contained 50 μM of the Fab inhibitors thiolactomycin (TLM) or diazoborine (Diaz). The relative mobility of known 3-oxo-acyl-HSLs and the sample origin are marked on the left. **(B)** TLC analysis of HSLs extracted from culture supernatants of *P. aeruginosa* (PAO1), *Erwinia carotovora* (E.ca.) and *Agrobacterium tumefaciens* (A.t.), as well as from *in vitro* synthesis reactions lacking NADPH (-NADPH) and containing either 1 μg or 0.25 μg of FabG. The relative mobility of known 3-oxo-acyl-HSLs and the sample origin are indicated by arrows.





Although the *A. tumefaciens* detection strain used for this experiment can also detect straight chain HSLs which are unsubstituted at C3, the HSLs shown in figure 8.3A are most likely 3-oxo-acyl-HSLs because they migrate on TLC plates with a comet-like tail, as have been observed by other investigators (27). In addition, we have previously shown that LasI is unable to utilize 3-unsubstituted straight chain decanoyl-ACP as a substrate (chapter 7; figure 7.3B).

8.5 DISCUSSION

Using purified Fab enzymes and LasI, we established an *in vitro* system for the synthesis of 3-oxo-HSLs from simple metabolic precursors. This *in vitro* system defined the coupled *P. aeruginosa* Fab-3-oxo-acyl-HSL pathway and allowed determination of its critical enzymes and substrates.

The amounts of enzymes chosen for the initial establishment of the pathway were based on some preliminary experiments with the *in vitro* reconstituted *E. coli* Fab system (6). Since ACP is the most abundant protein in the cell (2), the amounts of ACP (2 μg) used in the reactions were twice than that of the other Fab proteins (1 μg), with the exception of FabG. The amounts of FabG (0.5 μg) were half than that for the other Fab proteins and only 10% of the amounts of LasI (5 μg) because FabG competes with LasI for 3-oxo-acyl-ACP intermediates. The amount of LasI utilized relative to FabG compensates for this competition, thus allowing more efficient production of 3-oxo-acyl-HSLs. A sufficient amount of SAM was used to ensure that it did not become the limiting factor for HSL synthesis by LasI. Excess malonyl-CoA and NADH/NADPH were included in the reaction relative to acetyl-CoA because five rounds of the Fab cyclic pathway are required for synthesis of 3-oxo-dodecanoyl-ACP, requiring five moles of malonyl-CoA and NADH/NADPH per mole of acetyl-CoA.

Omission of individual components allowed an assessment of their relative importance in the Fab-HSL pathways. Although some spontaneous transacylation of malonyl from CoA to ACP can occur in the absence of FabD (13)(chapter 7), no synthesis of HSL was detected

in the absence of FabD, because in order to complete the five cycles of the Fab pathway required for 3-oxo-C_{12}-acyl-ACP synthesis, a high concentration of malonyl-ACP is required, and spontaneous transacylation would not yield sufficient malonyl-ACP for synthesis of the 3-oxo-C_{12}-ACP intermediate. An absolute requirement for ACP demonstrated that ACP and not CoA serves as the acyl carrier in fatty acid and HSL syntheses. Since FabB is the major condensing enzyme, it was essential for HSL formation from malonyl-CoA. In contrast, FabH was not required presumably since FabB can decarboxylate malonyl-ACP to acetyl-ACP and then condenses these two molecules to initiate the cycle without FabH, as has been suggested for *E. coli* FabB (2). This would also explain the formation of HSLs in the reactions containing no acetyl-CoA (figure 8.2). Of the two dehydratases, only FabZ was essential for HSL formation but not FabA. This is probably due to the fact that FabZ is mostly required in the initial cycles since its *E. coli* counterpart has substrate specificity for C_4 to C_8 β-hydroxyacyl-ACP intermediates (6). In contrast, FabA acts preferably on C_{10} to C_{14} β-hydroxyacyl-ACP intermediates. As expected, SAM and malonyl-CoA were essential, corroborating the results of previous studies (18, 19). Exclusion of NADH led to the detectable HSL production but at much reduced levels. Since NADH is the reductant preferred by FabI (8), this result indicates that FabI can utilize NADPH but that this step becomes rate-limiting in the absence of NADH. Whereas in the absence of NADPH only minute amounts of 3-oxo-C_{12}-HSL were synthesized, the levels of 3-oxo-C_8-HSL and 3-oxo-C_{12}-HSL were greatly elevated (figure 8.3). Although FabG can utilize NADH, NADPH is the preferred cofactor for FabG and in its absence the FabG catalyzed step becomes rate limiting, leading to accumulation of shorter chain 3-oxo-acyl-ACPs. This enables LasI to compete for the shorter-chain 3-oxo-acyl-ACP substrates and use them for synthesis of the corresponding shorter chain 3-oxo-acyl-HSLs. An increase of FabG from 0.5 to 1 μg in the absence of NADPH allowed a more efficient elongation of fatty acids in during initial two cycles, leading to accumulation of 3-oxo-C_6-ACP which resulted in increased 3-oxo-C_6-HSL levels (figure 8.3B).

Since the natural HSL produced by *A. tumefaciens* is 3-oxo-C_8-HSL and this autoinducer preferentially binds to the TraR regulatory protein, levels of HSLs with different

acyl-chain lengths cannot easily be quantitatively compared using the results shown in figure 8.3. Based on minimal detection limits previously reported (27), TraR prefers 3-oxo-HSLs in the order 3-oxo-C_8-HSL >> 3-oxo-C_6-HSL > 3-oxo-C_{10}-HSL >> 3-oxo-C_{12}-HSL. Therefore, spots containing no NADPH and 0.5 μg of FabG appear bigger since they contain more 3-oxo-C_6-HSL and 3-oxo-C_8-HSL relative to the reaction containing all components (figure 8.2, spots labeled All and -NADPH). Although the HSLs shown in figure 8.3 are postulated to be 3-oxo-acyl-HSLs, they could also be 3-hydroxyacyl-HSLs. However, this unlikely since (i) the migratory patterns (tear drop shapes) resemble those of 3-oxo-HSLs and (ii) excluding FabA from the reaction slightly reduces HSL synthesis (figure 8.2 and 8.3A, spots labeled All and -FabA). The latter argument is founded on the fact that *E. coli* FabA prefers C_{10} to C_{14} β-hydroxyacyl-ACP substrates, and its exclusion should caused accumulation of 3-oxo-C_6-ACP, 3-oxo-C_8-ACP and 3-oxo-C_{10}-ACP for synthesis of the respective 3-oxo-acyl-HSL. Thus, if the observed spots were in fact 3-hydroxyacyl-HSLs, then we should have seen more HSLs detected in reactions labeled -FabA (figures 8.2 and 8.3A).

Although the predominant HSL normally found in *P. aeruginosa* culture supernatants is 3-oxo-C_{12}-HSL (21), others have also reported high levels of 3-oxo-C_6-HSL, 3-oxo-C_8-HSL, 3-oxo-C_{10}-HSL, and 3-oxo-C_{12}-HSL (27). These apparent discrepancies, coupled to our observations that LasI is sufficient for synthesis of these various other 3-oxo-HSLs leads to several conclusions. First, all acyl intermediates required for synthesis of 3-oxo-C_8-HSL, 3-oxo-C_6-HSL, 3-oxo-C_{10}-HSL, and 3-oxo-C_{12}-HSL are derived from the Fab pathway. Since we are using all purified components, this is to date the most compelling evidence that the Fab pathway indeed provides the acyl groups found in the AHLs. Second, LasI is able to synthesize all these HSLs and no additional autoinducer synthase(s) are required as previously thought. Lastly, the types of HSLs produced may depend on the metabolic state of the cell, i.e. the rate of flux through the Fab pathway and the availability of substrates and/or reductants. The flux of the Fab-AI pathway may also be controlled at the genetic level (e.g. by differential expression of the *fab* genes) or at the protein level (via substrate allosteric effects). It can be postulated that substrate flux through the Fab pathway also differs during the various stages of cell growth. This may explain why rapidly growing

cells, i.e. cells posing a high demand for acyl-ACP substrates for phospholipid synthesis, etc., express little, if any, HSLs. In contrast, when entering the stationary phase cell growth slows down and more acyl-ACP substrates become available for the increased levels of HSL synthesis. Extracts of total HSLs from stationary phase cultures (~36 h) of *P. aeruginosa* contained only 3-oxo-C_8-HSL and 3-oxo-C_{10}-HSL (figure 8.3B) and no 3-oxo-C_6-HSL and 3-oxo-C_{12}-HSL, as has previously been observed by other investigators (21, 27). Hence, it will be interesting to see if there are changes in the types and levels of different HSLs produced during various phases of cellular stationary growth. Thus, besides the well-known genetic regulation of autoinducer synthase expression, there may be hitherto unrecognized levels of regulation of autoinducer type expression. Since nothing is known about the regulation of the *P. aeruginosa* Fab pathway, an understanding of its regulatory mechanisms may yield some information about these yet unknown regulatory autoinducer synthesis mechanisms.

Finally, our *in vitro* Fab pathway coupled to the LasI autoinducer synthase provides the framework for establishment of a high-throughput screening method for drugs interfering with the Fab and/or HSL pathways. This method was validated by testing some known Fab inhibitors. Whereas cerulenin and triclosan efficiently inhibited HSL synthesis at the concentrations tested, others, i.e. thiolactomycin and diazoborine, had little effect. Although we did not further elaborate on studies with inhibitors, these initial studies nonetheless indicate the usefulness of a refined *in vitro* system for drug screening purposes.

8.6 REFERENCES

1. **Chen, H., H. Cheng, and M. Bjerkness.** 1993. One-step Coomassie brillant blue R-250 staining of proteins in polycarylamide gel. Anal. Biochem. **212:**295-296.

2. **Cronan, J. E., and C. O. Rock.** 1996. Biosynthesis of membrane lipids, p. 612-636. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. Brooks Low, B. Magasanik,

W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*. Am. Soc. Microbiol., Washington, DC.

3. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295-298.

4. **Hayashi, T., O. Yamamoto, H. Sasaki, A. Kawaguchi, and A. Okazaki.** 1983. Mechanism of action of the antibiotic thiolactomycin inhibition of fatty acid synthesis of *Escherichia coli*. Biochem. Biophys. Res. Comm. **115:**1108-113.

5. **Hayashi, T., O. Yamamoto, H. Sasaki, and H. Okazaki.** 1984. Inhibition of fatty acid synthesis by the antibiotic thiolactomycin. J. Antibiot. **37:**1456-1461.

6. **Heath, R. J., and C. O. Rock.** 1996. Roles of the FabA and FabZ β-hydroxyacyl-acyl carrier protein dehydratase in *Escherichia coli* fatty acid biosynthesis. J. Biol. Chem. **271:**27795-27801.

7. **Hoang, T. T., Y. Ma, R. J. Stern, M. R. McNeil, and H. P. Schweizer.** 1999. Construction and use of low-copy number T7 expression vectors for purification of problem proteins: purification of *Mycobacterium tuberculosis* RmlD and *Pseudomonas aeruginosa* LasI and RhlI proteins, and functional analysis of RhlI. Gene **237:**361-371.

8. **Hoang, T. T., and H. P. Schweizer.** 1999. Characterization of the *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase (FabI): a target for triclosan and its role in acylated homoserine lactone synthesis. J. Bacteriol. **181:**5489-5497.

9. **Hoang, T. T., and H. P. Schweizer.** 1997. Fatty acid biosynthesis in *Pseudomonas aeruginosa*: Cloning and characterization of the *fabAB* operon encoding β-hydroxyacyl-acyl

carrier protein dehydratase (*fabA*) and β-ketoacyl-acyl carrier protein synthase I (*fabB*). J. Bacteriol. **179:**5326-5332.

10. **Holloway, B. W., U. Roemling, and B. Tuemmler.** 1994. Genomic mapping of *Pseudomonas aeruginosa* PAO. Microbiol. **140:**2907-2929.

11. **Jackowski, S., C. M. Murphy, J. E. Cronan, and C. O. Rock.** 1989. Acetoacetyl-acyl carrier protein synthase. A target for the antibiotic thiolactomycin. J. Biol. Chem. **264:**7624-7629.

12. **Kutchma, A. J.** 1998. M.S. Thesis. Colorado State University, Fort Collins, CO.

13. **Kutchma, A. J., T. T. Hoang, and H. P. Schweizer.** 1999. Characterization of a *Pseudomonas aeruginosa* fatty acid biosynthetic gene cluster: purification of acyl carrier protein (ACP) and malonyl-coenzyme A:ACP transacylase (FabD). J. Bacteriol. **181:**5498-5504.

14. **Levy, C. W., A. Roujeinikova, S. Sedelnikova, P. J. Baker, A. R. Stuitje, A. R. Slabas, D. W. Rice, and J. B. Rafferty.** 1999. Molecular basis of triclosan activity. Nature **398:**383-384.

15. **Liss, L.** 1987. New M13 host: DH5αF' competent cells. Focus **9:**13.

16. **Makowski, G. S., and M. L. Ramsby.** 1993. pH modification to enhance the molecular sieving properties of sodium dodecyl sulfate-10% polyacrylamide gel. Anal. Biochem. **212:**283-285.

17. **McMurry, L. M., M. Oethinger, and S. B. Levy.** 1998. Triclosan targets lipid synthesis. Nature **394:**531-532.

18. **Moré, M. I., D. Finger, J. L. Stryker, C. Fuqua, A. Eberhard, and S. C. Winans.** 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science **272:**1655-1658.

19. **Parsek, M. R., D. L. Val, B. L. Hanzelka, J. E. Cronan, and E. P. Greeneberg.** 1999. Acyl homoserine-lactone quorum-sensing signal generation. Proc. Natl. Acad. Sci. USA **96:**4360-4365.

20. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260:**1127-1130.

21. **Pearson, J., K. Gray, L. Passador, K. Tucker, A. Eberhard, B. Iglewski, and E. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA **91:**197-201.

22. **Pesci, E. C., and B. H. Iglewski.** 1999. Quorum sensing in *Pseudomonas aeruginosa*, p. 147-155. *In* G. M. Dunny and S. C. Winans (ed.), Cell-Cell Signaling in Bacteria. Am. Soc. Microbiol., Washington, DC.

23. **Pesci, E. C., J. P. Pearson, P. C. Seed, and B. H. Iglewski.** 1997. Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. **179:**3127-3132.

24. **Piper, K. R., S. B. von Bodman, and S. K. Farrand.** 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature **362:**448-450.

25. **Schweizer, H. P.** 1994. Coordinate deprepression of alkaline phosphatase, and binding protein dependent transport systems for phosphate and *sn*-glycerol-3-phosphate by phosphate starvation in *Erwinia carotovora* subsp. *carotovora*. Can. J. Microbiol. **40:**310-313.

26. **Schweizer, H. P., T. R. Klassen, and T. Hoang.** 1996. Improved methods for gene analysis and expression in *Pseudomonas*, p. 229-237. *In* T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (ed.), Molecular biology of Pseudomonads, Am. Soc. Microbiol., Washington, DC.

27. **Shaw, P. D., G. Ping, S. L. Daly, C. Cha, J. E. Cronan, K. L. Rinehart, and S. K. Farrand.** 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal moleculesby thin-layer chromatography. Proc. Natl. Acad. Sci. USA **94:**6036-6041.

28. **Val, D. L., and J. E. Cronan.** 1998. In vivo evidence that *S*-adenosylmethionine and fatty acid intermediates are the substrates for the LuxI family of autoinducer synthases. J. Bacteriol. **180:**2644-2651.

29. **von Bodman, S. B., G. T. Hayman, and S. K. Farrand.** 1992. Opine catabolism and conjugal transfer of the nopaline Ti plasmid pTiC58 are coordinately regulated by a single repressor. Proc. Natl. Acad. Sci. USA **89:**643-647.

CHAPTER 9

9.1 CONCLUDING REMARKS

This study was initiated to define the pathways for fatty acid and acylated homoserine (HSL) biosynthesis in *Pseudomonas aeruginosa*. To facilitate these studies, improved genetic and biochemical approaches for analyzing fatty acid and HSL biosynthesis in this bacterium were developed. The genetic technologies described in chapter 2 facilitate construction of unmarked *fab* mutants and autoinducer synthase mutants, employing the yeast Flp-*FRT* system and several improved gene-replacement vectors. In chapter 3, I present a novel system for single-copy insertion of DNA at a defined location in the *P. aeruginosa* chromosome, facilitating studies of HSL-regulated genes using promoter-reporter gene fusions. Currently, nothing is known about the regulation of *fab* genes in *P. aeruginosa*. In *E. coli*, FadR plays a dual role in the regulation of fatty acid degradation (Fad) and biosynthesis (4-6). FadR acts a repressor of genes involved in β-oxidation and as an activator of *fabA*. In the presence of exogenous fatty acids, FadR complexes with fatty acids in the form of acyl-CoAs and the acyl-CoA-FadR complex leads to derepression of *fad* genes. However, this complex no longer activates *fabA*. Since an equivalent of eight ATP molecules is required for each cycle of Fab, this reciprocal action of FadR on Fad and Fab ensures that no energy is wasted in futile Fab cycles when exogenous fatty acids are available. Initial experiments in our laboratory using a merodiploid *fabA-xylE* transcriptional fusion demonstrated that transcription of *P. aeruginosa fabA* is downregulated in the presence of exogenous fatty acids (data not shown). The ease of manipulation of the *P. aeruginosa* chromosome by using the above mentioned novel technologies (chapters 2 and 3) for the introduction and deletion of DNA sequences will facilitate future genetic studies aimed at the regulation of *fab* and HSL regulated genes.

The procedures described in chapter 4 allowed the purification of very insoluble proteins (e.g. LasI, RhlI, and RmlD) by regulated expression from low-copy number plasmids. The reduction in copy number from 20-100 (ColE1 origin) copies per cell with commercially available T7 expression vectors to ≤5 copies (pSC101 origin) per cell with the new vector reduced the level of expression so that these very insoluble proteins remained largely soluble. Induction in late exponential growth (A_{600} of 0.7-0.8) and expression at room temperature for 6 h was sufficient; longer induction and incubation times compromised the outcome of the experiments and led to accumulation of these proteins inside the cells in inclusion bodies. Addition of inducer at higher cell densities ensured sufficient protein production.

Jiang et al. (9) previously argued that the FA degradative pathway (Fad) may be the major supplier of the acyl groups for *N*-butyryl-HSL synthesis by providing acyl-CoA substrates. The results presented in this dissertation indicated that this is not the case and my results corroborate those of Parsek et al. (10) who assayed RhlI synthase activity using radioactive *S*-adenosylmethionine (SAM). I have successfully developed a non-radioactive RhlI assay by coupling this enzyme to enoyl-ACP reductase (FabI), the enzyme catalyzing the last step in Fab. To date, these experiments perhaps provide the strongest evidence yet that the Fab and HSL biosynthetic pathways are tightly linked. I have also attempted to assay RhlI activity utilizing a nonradioactive substrate (Ellman's reagent, see chapter 4), by measuring the ACP-SH released during the synthase reaction (unpublished data) (figure 1.5). In contrast to previously proposed reaction mechanisms, the results obtained with this particular assay indicated that RhlI may possess thioesterase activity and cleave butyryl-ACP in the absence of SAM. Future experiments should be designed to confirm this result, since it could have a significant impact on our understanding of the physiology of *P. aeruginosa* free fatty acids, acyl-ACP and acyl-CoA pools during stationary phase growth. Cleaving of fatty acids off butyryl-ACP would yield free fatty acids which can be utilize in β-oxidation (via butyryl-CoA) during nutrient limiting conditions of stationary phase growth (3).

Purification of ACP in its functional form (holo-ACP) is essential for the synthesis of substrates required to study fatty acid and HSL biosynthesis. I developed a method for

purification of ACP containing close to 90% holo-ACP (chapter 5). This method was subsequently improved by coexpression of *acpP* from a low-copy number plasmid along with ACP synthase (AcpS, encoded by *acpS*) to achieve ACP preparations with close to 100% in the form of holo-ACP (chapter 7). This method yielded about 10 mg of holo-ACP from 4 l of culture. However, future experiments should be aimed at coexpression of *acpP* with *acpS* from high-copy number plasmids to further improve the yields of holo-ACP. This would allow an even more efficient production of acyl-ACPs, required as substrates for further studies of the Fab-HSL pathways involving synthesis of acyl-ACP substrates, by acyl-ACP synthase (Aas; chapter 7). Although Aas has previously been used for labeling of ACP with mostly saturated and unsaturated fatty acids (13, 14), labeling of ACP with substituted fatty acids (e.g. hydroxyl-substituted FA) using *E. coli* Aas may also be possible. However, this would require previous chemical synthesis of substituted fatty acids, e.g. 3-oxo- or 3-hydroxyl-FA, but this has hitherto remained a formidable challenge.

Evidence obtained in the course of the studies presented herein indicated that the last enzymatic step in Fab (catalyzed by FabI) may be accomplished by more than one enzyme in *P. aeruginosa* (chapter 6), although a second enoyl-ACP reductase has yet to be demonstrated in any bacterium. First, an unmarked *P. aeruginosa fabI* mutant was constructed using the FRT-Flp recombinase system and the presence of the desired mutation was confirmed by both PCR and Southern hybridization analysis. Second, this mutant grew equally well as the wild-type but in clarified cell lysates possessed only half of the total enoyl-ACP reductase measured in wild-type extracts. The remaining enoyl-ACP reductase activity in the *fabI* mutant was not inhibited by triclosan, indicating a different class of enoyl-ACP reductase. Third, even though there was a significant reduction of HSL levels in the mutant, it still produced 50% of the levels found in wild-type indicating again involvement of yet another enoyl-ACP reductase activity in *P. aeruginosa* HSL biosynthesis. Since this *fabI* mutation was obtained by insertional inactivation of the *fabI* gene in the middle of the gene and not by deletion, arguments have been raised that the resulting mutant could still produce a functional FabI fusion protein. However, when the mutant *fabI* gene was PCR amplified, cloned into an expression vector and transformed into an *E. coli fabI*(Ts) mutant, it was

unable to complement the FabI(Ts) phenotype. Ongoing experiments are aimed at PCR amplification and cloning of the inactivated *fabI* sequences for expression of this FabI fusion protein as a recombinant histidine-tagged protein, and testing whether this purified protein has enoyl-ACP reductase activity. If there is in fact another FabI homologue in *P. aeruginosa*, the other enoyl-ACP reductase activity (tentatively named FabJ) can be purified from the *P. aeruginosa fabI* mutant PAO235 isolated during my studies. Initial experiments indicated that ammonium sulfate precipitates of a clarified cell lysate of strain PAO235 contained an enoyl-ACP reductase activity in the 40-50% fractions. When coupled to RhlI, these dialyzed protein fractions led to the production of detectable butyryl-HSL from crotonyl-ACP, NADH and SAM. Thus, this assay can be used in future experiments aimed at purification of the FabJ-mediated enoyl-ACP reductase activity from the *fabI* mutant. Since the *P. aeruginosa* genome sequence is completed, further purification of this protein via ion-exchange chromatography and gel filtration, followed by amino-terminal sequencing, should allow rapid identification of FabJ and its coding sequence for further manipulation.

The ultimate goal of my studies was to assemble and define the complete Fab-HSL pathway *in vitro* leading to the synthesis of 3-oxo-C_{12}-HSL (chapter 8). With the exception of FabH, all enzymes of the Fab pathway were shown to be enzymatically active and to possess functions equivalent to those of previously described *E. coli* homologues. The HSLs produced in these reactions have not yet been quantitated and the efficiency of the *in vitro* reconstituted cycle has yet to be assessed. Ongoing experiments are aimed at quantitating the amounts of HSLs produced in these reactions using either one of two published methods (10, 17). Since there was no difference in the amounts of HSLs produced in the reactions that contained no FabH versus the reaction that contained all components (figure 8.2), I am not sure whether the purified FabH is functional. It is possible that *P. aeruginosa* FabB can also initiate the cycle, as has been observed with *E. coli* FabB. However, the initiation reaction may be much slower with FabB than that with FabH, and the amounts of HSLs produced in the reaction containing all components (figure 8.2) may only be a fraction of the pathway capability in the presence of a functional FabH homologue. In a three enzyme coupled reaction containing FabD, FabH and FabG, no FabG activity was detected by measuring

utilization of NADPH or NADH in the presence of ACP, and the substrates acetyl-CoA and malonyl-CoA (data not shown), a result that may be indicative of an inactive FabH. Thus, I am not yet certain whether I indeed purified the FabH homologue of *P. aeruginosa*. My current view is that the FabH purified in this study may not be the correct enzyme, especially since there is at least one other potential FabH in *P. aeruginosa* with high homology to the *E. coli* FabH. Efforts are underway to PCR amplify and clone the gene for this other FabH (tentatively named FabH2). The purification of a histidine-tagged FabH2 will allow elucidation of its function as condensing enzyme in the three enzyme coupled reaction described above, and its potential role in the Fab-HSL pathway by assessing levels of 3-oxo-C_{12}-HSL synthesis in the presence and absence of FabH2. Also, the function of FabH2 can be assessed in the Fab-HSL pathway leading to *N*-butyryl-HSL synthesis in the absence of FabB (discussed below).

The Fab-HSL pathway (figure 8.1) can produce all of the HSLs previously found in *P. aeruginosa* cell-free culture supernatants (10, 11, 16). During exponential growth of *E. coli*, there are significant pools of acetyl-ACP and malonyl-ACP, but acyl-ACPs with chain lengths of four carbons or longer are not major components (15). Thus, if this is the case for *P. aeruginosa*, the production of HSLs in *P. aeruginosa* during exponential phase may be insignificant, considering the availability of acyl-ACP substrates and the low levels of expression of autoinducer synthases. However, since HSLs are mostly made during stationary phase, the levels of acyl-ACPs with chain lengths of four carbons or longer could be significantly different in stationary phase. In addition, the expression of AI synthases is elevated during stationary phase to be able to compete with Fab enzymes for acyl-ACP substrates which could shift in the favor of HSL production. Previous experiments indicated that *P. aeruginosa* culture supernatants contained HSLs that comigrated on C_{18} reverse-phase TLC plates with 3-oxo-C_6-HSL, 3-oxo-C_8-HSL, 3-oxo-C_{10}-HSL, and 3-oxo-C_{12}-HSL standards (16). My results show that the Fab-HSL pathway can produce HSLs which comigrate with 3-oxo-C_6-HSL, 3-oxo-C_8-HSL, and 3-oxo-C_{12}-HSL standards on C_{18} reverse-phase TLC plates. Although I did not have 3-oxo-C_{10}-HSL standard available to me, I am pretty certain that 3-oxo-C_{10}-HSL was also produced from the pathway (figure 8.3). These experiments indicated

that all of the 3-oxo-acyl-HSLs observed in *P. aeruginosa* culture supernatants can be synthesized by 3-oxo-C_{12}-HSL synthase (LasI), depending on the availability of substrates, metabolic flux through the Fab pathway as discussed in chapter 8.5, and competition of HSL synthases during stationary phase with Fab enzymes for acyl-ACP substrates as discussed above. Future experiments should be directed towards purification and identification of the structures of the different HSLs observed in the experiments illustrated in figure 8.3 to confirm that they are indeed 3-oxo-acyl-HSLs and not 3-hydroxyacyl-HSLs. The possible production of 3-hydroxyacyl-HSLs from the Fab-AI pathway could be assessed by synthesizing the 3-hydroxyacyl-ACP substrates for LasI and assessing production of 3-hydroxyacyl-HSLs.

Although the Fab-HSL pathway has not yet been successfully assembled for the synthesis of *N*-butyryl-HSL (C_4-HSL), results described in chapter 4 indicated that the acyl group found in this HSL is indeed derived from the Fab pathway. This evidence was obtained by coupling the last step of the Fab pathway (FabI) with the *N*-butyryl-HSL synthase (RhlI). In addition, *N*-hexanoyl-HSL could be synthesized by RhlI from hexanoyl-ACP and SAM (chapter 4). Therefore, the results described in this dissertation indicate that LasI and RhlI are sufficient for synthesis of all HSLs previously observed in *P. aeruginosa* culture supernatants (8, 10, 11, 16). The assembly of the Fab-HSL pathway for the production of C_4-HSL was attempted using the same conditions as described in chapter 8.3.4 for 3-oxo-C_{12}-HSL synthesis, but using 0.3 mM of acetyl-CoA and malonyl-CoA, and 1 μg of FabG, and replacing LasI with RhlI. However, no C_4-HSL was detected in TLC overlays with *Chromobacterium violaceum* detection strain CV026 (chapter 4)(data not shown). This could be because the FabH used is not functional in initiation of the Fab cycle, and although FabB can initiate the cycle it may be too inefficient for the production of sufficient C_4-HSL detectable by CV026. Relative to the amount of 3-oxo-C_{12}-HSL required for detection by the *A. tumefaciens* strain NTL4(pZLR4) (12, 16), the amounts of C_4-HSL required for detection by CV026 are more than 200-fold higher (9). This is likely the reason why the low levels of 3-oxo-C_{12}-HSL synthesized in the 3-oxo-C_{12}-HSL pathway was detectable using the *A. tumefaciens* indicator strain, but similar amounts of C_4-HSL produced in the C_4-HSL pathway

was too low to be detected by CV026. Hence, the Fab-HSL pathway assembly for the synthesis of C_4-HSL awaits the identification of the true *P. aeruginosa* FabH homologue and perhaps a more sensitive detection strain. Regardless, this pathway leading to the synthesis of C_4-HSL can be used to screen for a functional FabH in the absence of FabB.

The Fab-HSL pathway established during my studies for this dissertation may be useful for future screening of novel antimicrobials. Despite considerable interest in finding new class(es) of antimicrobials inhibiting the Fab cycle, difficulties in synthesizing appropriate substrates and defining assays for Fab enzymes have limited the discovery of such inhibitors. The availability of a coupled *in vitro* Fab-HSL pathway leading to the production of detectable HSL molecules will undoubtedly facilitate screening procedures for inhibitors of this pathway. For example, the assay illustrated in figure 7.3 may potentially be useful for the screening of drugs that inhibit FabD, FabB, or LasI by screening for the absence of 3-oxo-C_{12}-HSL production. In addition, in the presence of an excess of FabB, FabD and all substrates, this coupled reaction can be used to assay for 3-oxo-HSL synthase activity, e.g. LasI. This is the first easy enzymatic assay for a 3-oxo-acyl-ACP synthase and nicely complements the RhlI assay which uses enzymatically synthesized butyryl-ACP and radiolabeled SAM (10). The availability of such an assay will greatly facilitate ongoing collaborative studies aimed at elucidation of the LasI crystal structure. Another potentially useful assay for drug discovery is the three enzyme coupled reaction involving FabD, FabH, and FabG, where NAD(P)H utilization by FabG can be assayed for the screening of drugs that inhibit this reaction, as well as the initial condensation reaction catalyzed by FabH. The availability of these assays will complement existing assays such as the ones previously established for FabI (1,2)(chapter 6), and FabA and FabZ (7). Finally, further development and refinement of assays for the individual enzymes of this pathway is only limited by the ability to synthesize the necessary substrates which are commercially not available. If stable saturated, unsaturated and substituted FAs can be chemically synthesized, then perhaps the recombinant acyl-ACP synthase (chapter 7) can be used for enzymatic synthesis of some of these substrates.

9.2 REFERENCES

1. **Bergler, H., S. Fuchsbichler, G. Hoegenauer, and F. Turnowsky.** 1996. The enoyl-[acyl-carrier-protein] reductase (FabI) of *Escherichia coli*, which catalyzes a key regulatory step in fatty acid biosynthesis, accepts NADH and NADPH as cofactors and is inhibited by palmitoyl-CoA. Eur. J. Biochem. **242:**689-694.

2. **Bergler, H., P. Wallner, A. Ebeling, B. Leitinger, S. Fuchsbichler, H. Aschauer, G. Kollenz, G. Hoegenauer, and F. Turnowsky.** 1994. Protein *envM* is the NADH-dependent enoyl-ACP reductase *fabI* of *Escherichia coli*. J. Biol. Chem. **269:**5493-5496.

3. **Clark, D. P., and J. E. Cronan Jr.** 1996. Two-carbon compoundss and fatty acids as carbon sources, p. 343-358. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. Brooks Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*. Am. Soc. Microbiol., Washington, DC.

4. **Cronan, J. E., and C. O. Rock.** 1996. Biosynthesis of membrane lipids, p. 612-636. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. Brooks Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*. Am. Soc. Microbiol., Washington, DC.

5. **DiRusso, C. C., and W. D. Nunn.** 1985. Cloning and characterization of a gene (*fadR*) invovlved in regulation of fatty acid metabolism in *Escherichia coli*. J. Bacteriol. **161:**583-588.

6. **Farewell, A., A. A. Diez, C. C. DiRusso, and T. Nystrom.** 1996. Role of the *Escherichia coli* FadR regulator in stasis survival and growth phase-dependent expression of the *uspA*, *fad*, and *fab* genes. J. Bacteriol. **178:**6443-6450.

7. **Heath, R. J., and C. O. Rock.** 1996. Roles of the FabA and FabZ β-hydroxyacyl-acyl carrier protein dehydratase in *Escherichia coli* fatty acid biosynthesis. J. Biol. Chem. **271:**27795-27801.

8. **Hoang, T. T., Y. Ma, R. J. Stern, M. R. McNeil, and H. P. Schweizer.** 1999. Construction and use of low-copy number T7 expression vectors for purification of problem proteins: purification of *Mycobacterium tuberculosis* RmlD and *Pseudomonas aeruginosa* LasI and RhlI proteins, and functional analysis of RhlI. Gene **237:**361-371.

9. **Jiang, Y., M. Camara, S. R. Chhabra, K. R. Hardie, B. W. Bycroft, A. Lazdunski, G. P. C. Salmond, G. S. A. B. Stewart, and P. Williams.** 1998. In vitro biosynthesis of the *Pseudomonas aeruginosa* quorum-sensing signal molecule *N*-butanoyl-L-homoserine lactone. Mol. Microbiol. **28:**193-203.

10. **Parsek, M. R., D. L. Val, B. L. Hanzelka, J. E. Cronan, and E. P. Greeneberg.** 1999. Acyl homoserine-lactone quorum-sensing signal generation. Proc. Natl. Acad. Sci. USA **96:**4360-4365.

11. **Pearson, J., L. Passador, B. Iglewski, and E. Greenberg.** 1995. A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA **92:**1490-4.

12. **Piper, K. R., S. B. von Bodman, and S. K. Farrand.** 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature **362:**448-450.

13. **Ray, T. K., and J. J. E. Cronan.** 1976. Activation of long chain fatty acids with acyl carrier protein: Demonstration of a new enzyme, acyl-acyl carrier protein synthase, in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **73:**4374-4378.

14. **Rock, C. O., and J. L. Garwin.** 1979. Preparative Enzymatic Synthesis and Hydrophobic Chromatography of Acyl-Acyl Carrierr Protein. J. Biol. Chem. **254:**7123-7128.

15. **Rock, C. O., and S. Jackowski.** 1982. Regulation of phospholipid synthesis in *Escherichia coli.* Composition of the acylacyl carrier protein pool in vivo. J. Biol. Chem. **257:**10759-10765.

16. **Shaw, P. D., G. Ping, S. L. Daly, C. Cha, J. E. Cronan, K. L. Rinehart, and S. K. Farrand.** 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal moleculesby thin-layer chromatography. Proc. Natl. Acad. Sci. USA **94:**6036-6041.

17. **Val, D. L., and J. E. Cronan.** 1998. In vivo evidence that *S*-adenosylmethionine and fatty acid intermediates are the substrates for the LuxI family of autoinducer synthases. J. Bacteriol. **180:**2644-2651.