DISSERTATION

APPLICATION OF NOVEL GENETIC METHODS FOR STUDIES OF UNSATURATED FATTY ACID SYNTHESIS IN *Pseudomonas aeruginosa*

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WE HEREBY RECOMMEND THAT THIS DISSERTATION PREPARED UNDER SUPERVISION BY KYOUNG-HEE CHOI ENTITLED "APPLICATION OF NOVEL GENETIC METHODS FOR STUDIES OF UNSATURATED FATTY ACID SYNTHESIS IN *Pseudomonas aeruginosa*" BE ACCEPED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

APPLICATION OF NOVEL GENETIC METHODS FOR STUDIES OF UNSATURATED FATTY ACID SYNTHESIS IN *Pseudomonas aeruginosa*

As with most bacteria, the development of genetic tools for *Pseudomonas aeruginosa* and related bacteria has not kept up with the pace of sequencing and high-throughput post-genomic technologies, e.g. microarrays. In the course of this dissertation, new genetic tools were developed that accelerate post-genomic studies. The utility of these tools was then tested for studies on the mechanism and regulation of unsaturated fatty acid (UFA) biosynthesis in *P. aeruginosa.*

The tools developed here include a broad-host-range transposon Tn*7*-based site-specific gene integration system, a rapid and efficient bacterial transformation method, and a rapid gene replacement method. Traditional methods used for chromosomal insertion of exogenous DNA rely mostly on either species-specific methods (e.g., phage integration systems) or on randomly integrating transposons. To overcome these and other limitations of traditional methods, a Tn*7*-based integration system was developed. This transposon inserts site- and orientation-specifically downstream of the essential *glmS* gene in *P. aeruginosa* and other bacteria. Since insertions occur at a naturally-evolved, neutral intergenic chromosomal site so that bacterial fitness is not affected. Mini-Tn*7* vectors were developed for single-copy genetic complementation, gene and protein fusion analyses, reporter gene tagging and gene expression studies. Because of its broad-host-range, the mini-Tn*7* system was shown to be applicable in many other

gram-negative organisms including *Burkholderia* spp. and *Yersinia pestis*. A rapid transformation method was developed in support of the mini-Tn*7* system. This method is generally applicable for delivery of genetic elements such as replicative and non-replicative plasmids, suicide delivery vectors, gene replacement vectors and chromosomal fragments containing selection markers. In the absence of a reliable transduction method, the latter procedure is especially useful for strain construction because it allows transfer of mutations tagged with an antibiotic selection marker between different strains. To facilitate high-throughput mutant construction, a combination of rapid transformation and Gateway recombinational cloning was implemented to greatly improve and accelerate a relatively tedious traditional gene replacement procedure.

UFAs play an essential role in cellular physiology by maintaining proper membrane fluidity in response to changes in various environmental conditions such as temperature, oxygen, toxic compounds, growth, osmotic pressure, and hydrostatic pressure. Generally, microbial UFAs are synthesized via two different pathways: 1) enzymes encoded by the *fabA*, *fabB* and other genes which belong to the type II fatty acid biosynthetic pathways. This pathway is found in many bacteria, including *Escherichia coli* and *Streptococcus pneumoniae*; and 2) by aerobic desaturation of saturated fatty acids (SFAs) by an inducible desaturase activity. This pathway is found in gram-positive bacteria (e.g., *Bacillus subtilis*), mycobacteria and *Saccharomyces cerevisiae*. These organisms typically lack FabA and FabB homologs. In this study, we showed that UFA synthesis in *P. aeruginosa* is unique in that this bacterium uses both of the pathways mentioned above. Even though two pathways exist for UFA production and the FabAB

pathway is not required for aerobic growth, the FabA and FabB proteins are indispensable for anaerobic growth because of the oxygen-dependency of the enzymes of the aerobic pathway. There are two distinct desaturase genes in *P. aeruginosa* that are *desA* and *desB* encoding acyl-lipid desaturase and acyl-CoA desaturase, respectively. During aerobic growth, DesA, but not DesB, is capable of complementing the UFA-auxotrophy of *fabAB* mutants by introducing the double bond into the fatty acyl chain of phospholipids. In contrast, DesB acts on the CoA-esterified form of exogenous long chain fatty acids. The *desB* gene forms an operon with *desC*, encoding a putative oxidoreductase which is thought to be involved in providing electrons from the electron transport chain for the DesB-catalyzed reaction. Microarray and RT-PCR analyses demonstrated that the *desBC* operon is under DesT repressor control. Under anaerobic conditions, UFAs are derived from *de novo* fatty acid synthesis, and FabA and FabB are essential players in this process. Previous studies showed that the *P. aeruginosa fabA* and *fabB* genes form an operon whose expression was up-regulated in biofilm-grown cells and repressed by exogenous fatty acids, especially oleic acid. Since the molecular mechanism underlying these observations remained a mystery, the newly developed genetic tools were employed to study regulation of *fabAB* operon expression. These studies revealed that transcription of the *fabAB* operon is complex. While it is most likely co-transcribed with the upstream *PA1612-PA1611* operon, it is also transcribed by a second promoter which is either located in the *PA1611-fabA* intergenic region or somewhere within the *PA1612-PA1611* operon. The *PA1611-fabA* intergenic region contains a 30 bp regulatory element which is highly conserved and present only in *Pseudomonas* spp. Deletion of this sequence greatly reduced *fabA* expression and

abolished repression by exogenous UFAs, strongly suggesting a role as an important regulatory DNA sequence. However, repeated genetic and biochemical attempts failed to identify *fabAB* regulatory protein(s). Gene deletion experiments demonstrated that none of the targeted regulatory genes, including all of the transcriptional regulators of the GntR family to which the *E. coli fabA* and *fabB* activator belongs, encodes an activator of *fabAB* expression. While the combination of random *mariner* transposon mutagenesis and gene fusion technology revealed several genes, including *rpoN*, *desA*, *anr* and several of unknown function, that exhibited an effect on *fabAB* expression, follow up studies revealed only an indirect and in most cases moderate role of these genes in regulation of *fabAB* expression. Biochemical attempts involved DNA affinity chromatography with 30 bp concatamer or *PA1611-fabA* intergenic region substrates, but all of these attempts failed to identify specific DNA binding proteins. In summary, the data reveal that transcriptional regulation of *fabAB* operon expression is complex, involving several promoters, a conserved DNA regulatory element and probably several unidentified regulatory factors. The inability to identify a specific DNA binding protein by powerful genetic and biochemical methods suggests that *fabAB* operon expression may not simply be governed by protein-DNA interactions, but rather by a more complex regulatory mechanism(s), e.g. involvement of a riboregulatory element.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

$A_{540\ nm}$	absorbance at 540 nm
$A_{600\ nm}$	absorbance at 600 nm
ACP	acyl carrier protein
AHL	acylated homoserine lactone(s)
Ap	Ampicillin
Asp	Aspartate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
att	locus for phage attachment
B.	*Burkholderia* or *Bacillus*
β-Gal	β-galactosidase
bla	β-lactamase-encoding gene
bp	base pair(s)
C10	acyl chain ten carbons long
C12	acyl chain twelve carbons long
C16	acyl chain sixteen carbons long
C18	acyl chain eighteen carbons long
C4	acyl chain four carbons long
C6	acyl chain six carbons long
C8	acyl chain eight carbons long
Cb	Carbenicillin
CFA	cyclopropane fatty acid
CoA	coenzyme A
CTX	Cytotoxin
Δ	gene deletion or double bond
DIG	Digoxigenin

DMDS	dimethyl disulfide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid(s)
dNTP	deoxyribonucleoside triphosphate(s)
DTT	Dithiothreitol
E.	*Escherichia*
EDTA	ethylenediamine tetraacetic acid
ϕ	Phage
FA	fatty acid(s)
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
FabI	enoyl-ACP reductase
FabZ	β-hydroxyacyl-ACP dehydratase
FACS	florescence activated cell sorter
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
GC-MS	gas chromatography-mass spectrometry
GenBank	the NIH genetic sequence database
GFP	green fluorescent protein
glmS	glucosamine-6-phosphate synthetase
Gm	gentamycin
GW	Gateway compatible cassette
h	hour(s)

His	histidine
HMG	High mobility group
HSL	homoserine lactone(s)
IHF	integration host factor
int	phage CTX integrase gene
IPTG	isopropyl-β-D-thiogalactopyranoside
IR-L	left terminal inverted repeat
IR-R	right terminal inverted repeat
IS	insertion sequence
K.	*Klebsiella*
kb	kilobase(s) or 1000 bp
kDa	kilodalton(s)
Km	kanamycin
l	liter(s)
lacI	*E. coli lac* repressor structural gene
$lacI^q$	Promoter-up mutation of *lacI* gene
lacY	lactose permease-encoding gene
lacZ	β-galactosidase-encoding gene
LasI	*P. aeruginosa* N-[3-oxododecanoyl]-L-HSL synthase
LB	Luria-Bertani medium
log	logarithmic growth phase
LPS	lipopolysaccharide(s)
lux	Luciferase
M	molar
M.	*Mycobacterium*
m/z	mass-to-charge-ratio
MCS	multiple cloning site(s)
mg	milligram(s)
MIC	minimal inhibitory concentrations
min	minute(s)
μl	microgram(s)

µl	microliter(s)
ml	milliliter(s)
µM	micromolar
mM	millimolar
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
ng	nanogram(s)
nm	nanometer(s)
nt	nucleotide(s)
^oC	degree(s) Celcius
ori	origin of replication
oriT	origin of transfer
p	para
P.	*Pseudomonas*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
P_{tac}	*E. coli trp-lac* hybrid promoter
PtdEtn	phosphatidylethanolamine
PtdGro	phosphatidylglycerol
^{r}	resistance/resistant
R6K	R6Kγ origin of replication
RBS	ribosome-binding site
Rep	replication protein
RhlI	*P. aeruginosa* C_4-HSL synthase
^{s}	sensitive/susceptible
S.	*Staphylococcus, Salmonella* or *Streptococcus*

sacB	*Bacillus subtilis* gene encoding levansucrase
SDS	sodium dodecyl sulfate
SFA	saturated fatty acid
T_0	phage λ terminator T_0
T_1	the *E. coli rrnB* terminator T_1
TLC	thin layer chromatography
Tm	melting temperature
Tn	Transposon
Tns	Transposase
Ts	temperature sensitive
u	unit(s)
UFA	unsaturated fatty acid
V.	*Vibrio*
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
xylE	catechol-2,3-dioxygenase-encoding gene

List of Genbank submissions

AY737005	Cloning vector pUC18-mini-Tn*7*T-Tp
AY884832	Cloning and suicide delivery vector pUC18R6KT
AY884834	Cloning and Tn*7* delivery vector pUC18R6K-mini-Tn*7*T-Km
AY962893	Transcriptional fusion vector pUC18-mini-Tn*7*T-Gm-*lux*
AY597269	*FRT* source vector pFRT2
AY597270	Gentamycin resistance *FRT* vector pFGM1
AY597271	Chloramphenicol resistance *FRT* vector pFCM1
AY597272	Kanamycin resistance *FRT* vector pFKM1
AY597273	Flp expression vector pFLP3
AY599226	Cloning vector pUC18-mini-Tn*7*
AY599227	Cloning vector pUC18-mini-Tn*7*T
AY599228	Cloning vector pUC18-R6K-mini-Tn*7*T

AY599229	Cloning vector pUC18Pv-mini-Tn*7*T
AY599230	Cloning vector pUC18T-mini-Tn*7*T
AY599231	Cloning vector pUC18-mini-Tn*7*T-Gm
AY599232	Cloning vector pUC18T-mini-Tn*7*T-Gm
AY599233	Cloning vector pUC18-mini-Tn*7*T-Gm-*lacZ*
AY599234	Cloning and expression vector pUC18-mini-Tn*7*T-LAC
AY619004	Cloning vector pUC18-mini-Tn*7*-Gm
AY619005	Cloning vector pUC18-mini-Tn*7*-LACM15
AY619006	Cloning vector pUC18R6K-mini-Tn*7*T-Km
AY619007	*Pseudomonas aeruginosa PA5548* (*PA5548*) gene, partial cds; transposon mini-Tn*7*, complete sequence; and glucosamine-6-phosphate synthetase (*glmS*) gene, partial cds
AY712950	Tetracycline resistance *FRT* vector pFTC1
AY712951	Trimethoprim resistance *FRT* vector pFTP1
AY712952	Cloning vector pUC18T-mini-Tn*7*T-Gm-REP
AY712953	Mini-Tn7 delivery vector pUC18R6KT-mini-Tn*7*T
AY737004	Cloning vector pUC18-mini-Tn*7*T-Gm-Gateway
AY737006	Cloning vector pUC18-mini-Tn*7*T-Tp-Gateway
AY884833	Tn*7* transposase expression vector pTNS2
AY928469	Cloning vector pEX18ApGW
DQ153108	Cloning vector pUC18R6K-mini-Tn*7*T-Gm

LIST OF PUBLICATIONS

Parts of this dissertation have been published in the following articles:

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Kun Zhu, Kyoung-Hee Choi, Herbert P. Schweizer, Charles O. Rock and Yong-Mei Zhang (2006). Two Aerobic Pathways for the Formation of Unsaturated Fatty Acids in *Pseudomonas aeruginosa. Mol Microbiol* **60**:260-273.

Kyoung-Hee Choi and Herbert P. Schweizer (2006). mini-Tn*7* insertion in bacteria with single *att*Tn*7* sites: example *Pseudomonas aeruginosa. Nature Protocols* **1**:153-161.

Kyoung-Hee Choi, David DeShazer and Herbert P. Schweizer (2006). mini-Tn7 insertion in bacteria with multiple *glmS*-linked *att*Tn*7* sites: example *Burkholderia mallei* ATCC 23344. *Nature Protocols*, **1**:162 - 169.

Kyoung-Hee Choi and H.P. Schweizer (2006). mini-Tn*7* insertion in bacteria with secondary, non-*glmS*-linked *att*Tn*7* sites: example *Proteus mirabilis* HI4320. *Nature Protocols*, **1**:170 - 178.

CHAPTER 1

Introduction

1 Gene integration systems: study of gene expression as nature intended it to be

1.1 Necessity for versatile gene integration systems

With the advent of high-throughput methods for bacterial genomic and proteomic analyses, more rapid and elegant molecular genetic methods are also required to take full advantage of the wealth of information revealed by genome-wide analyses. Examples of such methods include means of mutational and transcriptional analyses for verification of data obtained with e.g. microarray analysis, as well as construction of genetically modified organisms for diverse microbiological and biotechnological applications. Many pathogenic bacteria form biofilms or live in environments, e.g. in animals or on plants, that are not easily amenable to study using conventional methods. These include plasmid-based technologies, such as complementation analyses and other studies necessitating the introduction and stable maintenance of novel genetic elements, for instance, reporter gene fusions. The main problem is that plasmids are normally maintained by antibiotic selection and such selection is not feasible in many instances, e.g during biofilm and animal studies. A few years ago, we therefore started to investigate chromosomal integration systems for gene delivery systems as alternatives to plasmids in several respiratory pathogens, mostly *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia*. These opportunistic bacteria cause life-threatening infections in

compromised patients, and are responsible for especially high morbidity and mortality in cystic fibrosis patients (Denton et al., 1998, Govan et al., 1996, Valdezate et al., 2001). The goal was to develop a versatile, robust method for targeted chromosomal gene delivery method that is easy to use and transferable between different bacteria. Special emphasis was placed on developing vectors that allow rapid construction of single-copy, chromosomal fusions to reporter genes. The overall aim of the proposed studies was therefore to derive a set of strains and plasmids that will allow researchers working with diverse organisms to use the same basic genetic system for chromosomal delivery of reporter gene fusions and other functional elements. I anticipate that the tools developed in this study with the proposed model organisms will be applicable in many other bacteria, including some closely related potential bioterrorism agents such as *B. mallei* and *B. pseudomallei.* Many of the previously developed broad-host-range plasmid vectors developed in the Schweizer laboratory are applicable for the study of these organisms (Burtnick et al., 2001, DeShazer et al., 1997, Moore et al., 1999, Schweizer et al., 1996b).

1.2 Reporter gene technology

Gene fusion technology offers a means of analyzing expression of the many bacterial genes and also plays an important role in the development of biosensors. While many reporter genes are available (Schenborn et al., 1999), *lacZ* encoding β-galactosidase (βGal), *GFP* encoding green fluorescent protein (GFP) and *lux* encoding bacterial luciferase are now most commonly used. Plasmid-based reporter genes facilitate construction of gene fusions and are easily introduced into bacterial cells by transformation or conjugation. However, they suffer from some potentially serious drawbacks. First, increased plasmid copy number does not reflect the natural

situation and expression from plasmid-localized promoters may be influenced by supercoiling (Dorman, 1991). Second, most plasmids are maintained in cells by antibiotic selection and such selection may either not be feasible in some environments or not desirable for fear of spreading antibiotic resistance markers (de Lorenzo et al., 1992, de Lorenzo, 1994). Third, background expression can be a problem in instances where weak signals are being analyzed. To overcome these drawbacks, several methods were developed to reduce the copy number of the fusions or to transfer the fusions in single copy to the chromosome.

1.2.1 Methods to reduce the copy number of plasmid-borne reporters

To derive broad-host-range reporter gene plasmid constructs expressing low background levels of the reporters, several methods are available. Some examples: (i) employ starting vectors that are based on lower copy number replicons (Santos et al., 2001, Schweizer et al., 2001a); (ii) use an *in vivo* shuttling method to transfer cloned DNA between high-copy number *E. coli* plasmids and low-copy number broad-host range plasmids (Ouimet et al., 2000); and (iii) use host strains that lower plasmid copy number due to mutations in chromosomal genes (e.g., *Escherichia coli pcnB* strains; *pcnB* mutations reduce plasmid copy number of pBR322 and its derivatives because PcnB is required for the rapid degradation of RNAI, the antisense RNA that controls the copy number of ColE1-related plasmids (He et al., 1993)).

1.2.2 Methods and delivery vehicles for chromosomal integration of reporter genes

Methods to transfer gene fusions into bacterial chromosomes involve (i) integration via homologous recombination; (ii) transfer to specific sites using phage-

based methods and (iii) random integration using transposon delivery. The diverse delivery vehicles underlying these methods can be divided into two classes, (i) those that integrate randomly into the chromosome and (ii) those that integrate at specific sites within the chromosome. In the following, I will briefly discuss the different methods and highlight applicability, as well as their advantages and disadvantages.

1.2.2.1 Chromosomal insertions via homologous recombination

These methods can either be dependent on the host's recombination system or other engineered recombination systems, e.g. phage integrase systems. There are basically three methods to insert gene fusions via homologous recombination.

First, a reporter gene can be inserted into the coding sequence of the gene to be analyzed in the same transcriptional orientation as the promoter. The resulting construct is then used to replace the chromosomal copy of the gene via gene replacement. An advantage of this method is that it can be employed in any recombination-proficient host strain. Disadvantages of this method are numerous, e.g. it is not applicable for analysis of essential genes and it is limited to screenable reporters or selectable markers.

Second, reporters can be integrated into the chromosome via merodiploid formation. In this method, only the 5' portion of the gene to be studied is fused to the reporter gene on a non-replicative plasmid. This plasmid is then introduced into the respective host strain and integrants are obtained via homologous recombination between the 5' portions of the cloned and chromosomal gene sequences, respectively, and by selecting for the antibiotic resistance marker contained on the plasmid backbone. Advantages of this method include that it does not result in a mutant of the studied gene and that integrants can be selected and maintained via antibiotic

selection. An obvious disadvantage is that integrants are only stable in the presence of antibiotic selection.

Third, site-specific recombinases can be used to target heterologous DNA sequences at predetermined locations in a bacterial chromosome. This necessitates the natural or artificial presence of recombinase target sites in the chromosome. A relatively simple site-specific recombinase system to use in bacteria is the Flp recombinase-Flp recombinase target (*FRT*) site system from *Saccharomyces cerevisiae* (Schweizer, 2003). In *E. coli* and *Salmonella enterica* serovar Typhimurium, chromosomal *FRT* sites can be inserted into a known location and in an orientation-specific manner using short PCR homing primers and the λ RED recombination system (Datsenko et al., 2000). Single-copy chromosomal *lacZ* fusions to specific *Salmonella* promoters were isolated by first incorporating a chromosomal *FRT* site following the promoter of choice and then incorporating a plasmid-borne promoter-less *lacZ* gene at this site in a Flp recombinase-mediated reaction (Ellermeier et al., 2002). Advantages of this approach include that the fusions can be rapidly isolated in specific, predetermined locations on the chromosome of even recombination-deficient hosts and the integrated sequences are stably maintained in the absence of antibiotic selection. The procedure has obvious disadvantages, including that fusions cannot be isolated to essential genes because the promoter of the studied gene is uncoupled from the rest of the gene's coding sequence and although the Flp-*FRT* technology works in most bacteria studied to date (Schweizer, 2003), insertion of *FRT* sequences in bacteria other than *E. coli* and *Salmonella* sp. is not a trivial task, unless they are delivered by randomly inserting transposons with all the drawbacks of random delivery (see section 1.2.2.3).

1.2.2.2 Phage-based methods for chromosomal integration

In *E. coli*, plasmid-borne *lacZ* gene fusions can be recombined *in vivo* on to a specialized transducing λ phage, which is then used to lysogenize host strains in which the fusion is replicated in single copy along with the chromosome (Hand et al., 2000). For use in *P. aeruginosa*, the Schweizer laboratory previously developed a phage mini-CTX system, which does not involve phage CTX particles but rather its integrase, which catalyzes site-specific integration between *att* sites located on the integration plasmid and the chromosome, respectively (Hoang et al., 2000) (reviewed in the appended paper). These ϕCTX-based integration-proficient mini-CTX vectors allow insertion of gene cassettes at a defined 30 bp chromosomal *attB* sequence (Hayashi et al., 1993). The *attB* site is a phage attachment site that has naturally evolved to allow phage integration without causing a mutant phenotype and without compromising fitness. Following site-specific vector integration at the chromosomal *attB* site, unwanted sequences, including plasmid-associated promoters and antibiotic markers, can be removed *in vivo* by yeast Flp recombinase (Hoang et al., 1998). Specialized mini-CTX vectors have been used to construct *P. aeruginosa* host strains allowing regulated expression from the T7 and *lac* promoters, as well as for studying gene expression using *GFP*-, *lacZ*- and *lux*-based reporter genes (Becher et al., 2000, Schweizer et al., 2001b). Multiple applications have shown that single-copy reporter genes integrated via mini-CTX are versatile tools for studies of gene expression in biofilms (Sauer et al., 2002, Wyckoff et al., 2001). The main drawback is that the mini-CTX system can only be used in *P. aeruginosa*. For example, although *P. putida* strain KT2440 contains a putative *attB* site which over 30 bp is 86% identical to *attB* from *P. aeruginosa*, and is located within the same genomic content as that found in *P. aeruginosa* (Schweizer et al., 2001b), the mini-CTX vectors cannot use it

as an integration site (unpublished observations made in the Schweizer laboratory). A similar, but less-sophisticated system was previously described for use in *Mycobacterium tuberculosis* BCG (Stover et al., 1991). Advantages of phage-based systems include that they are easy to use and that relatively large (at least 50 kb) heterologous, foreign DNA fragments are integrated at naturally evolved chromosomal locations, thus not compromising the fitness of the resulting bacterium. The obvious disadvantages of phage-based systems are that they are species-specific.

1.2.2.3 Transposon-based methods for chromosomal integration

A commonly used method for single-copy chromosomal insertion of reporter genes involves the use of mini-Tn*5* transposons as delivery vehicles (de Lorenzo et al., 1994). This is a proven technology and is applicable to a wide-range of bacteria (Hansen et al., 2000a, b, Ramos et al., 2000, Valls et al., 2000, Whiteley et al., 2000). Mini-transposons have also been used to develop different reporter gene tools for analyzing gene expression in biofilms (Christensen et al., 1999). A phage D3112-based method for construction of random single copy reporter gene insertions in the *P. aeruginosa* chromosome has also been described but it is limited to this bacterium (Karkhoff-Schweizer et al., 1994, Schweizer et al., 1996a). The advantage of the mini-Tn*5*-based technology is that it is applicable to a wide-range of bacteria. However, this methodology also has several drawbacks. These include: (i) insertions occur randomly in the genome and position effects cannot easily be controlled. Consequently, considerable efforts need to be invested to determine the transposon insertions sites and the fitness of the bacteria (Ramos et al., 2000); (ii) analysis of gene expression or fitness in different mutant backgrounds is difficult since the transposable elements will be integrated in different chromosomal locations, and (iii)

the integrated transposable elements contain antibiotic markers that prevent use of vectors with the same selectable markers or may render the microbes unsuitable for use in environments where antibiotics are unusable or undesirable.

1.3 Tn*7*-based site-specific integration

Common bacterial composite transposons contain a variable number of antibiotic-resistance genes flanked by insertion sequences which are either oriented as direct or indirect repeats (Fig. 1.1, panel A). Unlike other transposons, Tn*7* contains distinct left (Tn*7*L) and right (Tn*7*R) ends which result in an orientation-specific transposon insertion. Tn*7*L and Tn*7*R flank DNA sequences which consist of an integron with an antibiotic-resistance cassette, and the *tns* operon encoding the TnsA, TnsB, TnsC, TnsD and TnsE proteins which form the Tn*7* transposase complex (Fig. 1.1, panel B).

Whereas most transposable elements move at low frequency and insert into many different target sites, displaying little target site-selectivity, Tn*7* is distinguished by its ability to insert at a high frequency into bacterial chromosomes with a defined orientation at a specific insertion site, *att*Tn*7* (Craig, 1996, Peters et al., 2001b). Tn*7* attachment sites are found in the chromosomes of many bacteria (Craig, 1989, Koch et al., 2001) and are located downstream of the 3' ends of the respective *glmS* genes, encoding glucosamine-6-phosphate synthetase. For *E. coli* it has been well established that the sequences required for Tn*7* insertion extend into the 3' *glmS* coding sequence, which is highly conserved in different bacterial species, indicating that site-specific Tn*7* insertion reflects utilization of its transposase pathways and substantial conservation of at least critical portions of *glmS* (Peters et al., 2001a). Since the transposon itself is inserted downstream of *glmS* in the intergenic region

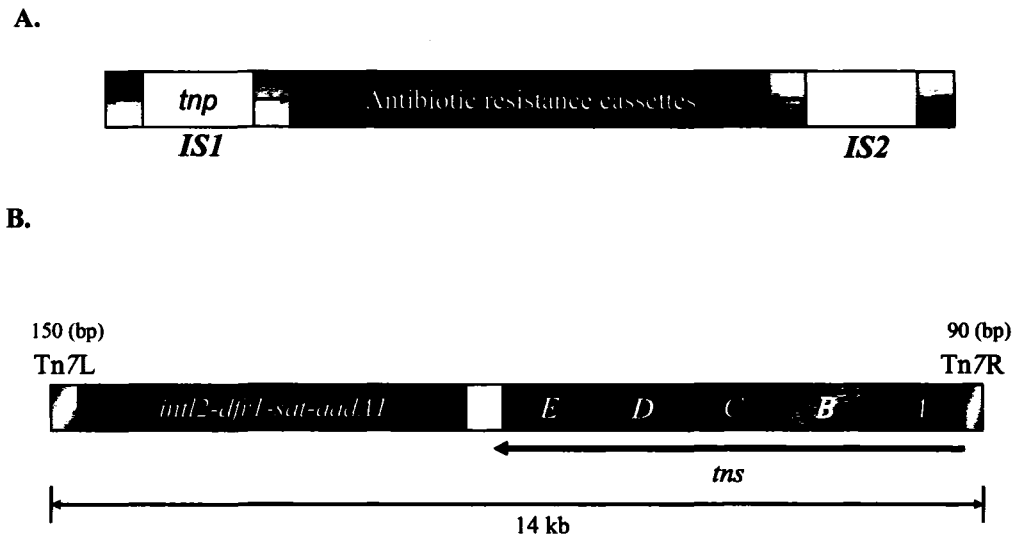


Fig. 1.1. General structure of a common bacterial composite transposon and transposon Tn*7*. **A)** A composite transposon consists of insertion sequences (IS) that flank DNA sequences encoding a transposase (Tnp) and antibiotic resistance genes. The IS sequence which contains two inverted repeats can either be in the same or inverted orientation. **B)** Tn*7* contains distinct left (Tn*7*L) and right (Tn*7*R) ends which flank DNA sequences of integron containing integrase gene *intI2* and antibiotic-resistance gene cassettes, *dfrI* (trimethoprim), *sat* (streptothricin) and *aadA1* (spectinomycin), and the Tn*7* transposase complex-encoding operon, *tnsABCDE*.

with the downstream gene, its insertion does not affect expression of either gene (Peters et al., 2001a). Transposition of Tn*7* minimally requires the Tn*7* left (Tn*7*L) and Tn*7* right (Tn7R) ends, and the transposase complex, which is comprised of the products of five genes, *tnsABCDE* (Peters et al., 2001a). The TnsD pathway (consisting of TnsABC+D) catalyzes site-specific insertion at *att*Tn*7*, whereas the TnsE pathway (consisting of TnsABC+E) catalyzes random transposition (Peters et al., 2001a). Several Tn*7*-based mini-transposons were designed (Bao et al., 1991, Hojberg et al., 1999, Koch et al., 2001, McKown et al., 1988, Shen et al., 1992) and used for construction of bacterial reporter strains. Some of them contain less than 200 bp of Tn*7*L and Tn*7*R, and transpose at relatively high (10^{-3} to 10^{-4}) frequencies in the presence of a helper plasmid transiently expressing the transposase (McKown et al., 1988). However, existing plasmids contain few cloning sites, they only allow marked insertions, their complete sequences are unknown, they are unstable in certain media due to their size and presence of redundant sequences, and are thus unsuited for routine and high-throughput use.

1.4 Other uses of chromosomal gene integration systems

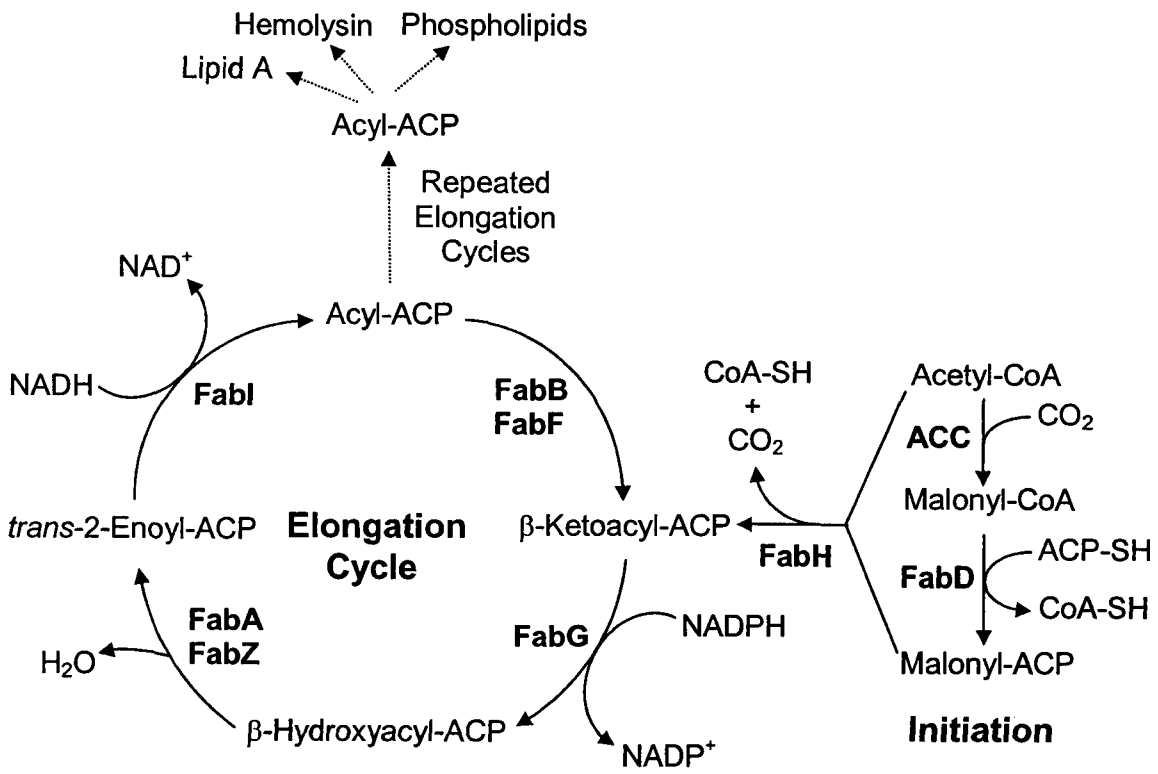
Although the preceding paragraphs were written to explain the specific application of various gene integration systems for the construction and application of reporter gene fusions, most of the described technologies are also applicable for (i) single-copy complementation analyses, i.e. as nature intended it to be, and (ii) engineering of other genetic traits into the respective bacterial hosts, including regulatory elements (Karkhoff-Schweizer et al., 1994, Schweizer, 1994, 2001, Schweizer et al., 2001b).

2 Unsaturated fatty acid biosynthesis and physiological roles in Gram-negative and Gram-positive bacteria, mycobacteria, and yeast

2.1 Saturated and unsaturated fatty acid synthesis in Gram-negative bacteria

Fatty acid biosynthetic pathways are divided into type I and II systems. Type I fatty acid synthase systems are found in mammals, birds and yeasts. They consist of a large multifunctional protein composed of distinct domains that catalyze the individual fatty acid synthesis reaction steps. In most bacteria and plant plastids, fatty acid synthesis is catalyzed by a type II or dissociated fatty acid synthase system, where distinct enzymes catalyze the individual fatty acid synthesis reactions. Although some of the enzymes involved in type II fatty acid synthesis possess overlapping functions, they have different substrate specificities (Lai et al., 2003).

The type II fatty acid biosynthesis pathway of *E. coli* is summarized in Fig. 1.2. In the initiation step acetyl-CoA is carboxylated by the acetyl-CoA carboxylase (Acc) enzyme complex which is composed of AccA, AccB, AccC, and AccD, resulting in a formation of malonyl-CoA. The malonyl group is then transferred to the sulfhydryl group of holo-ACP by malonyl-CoA:ACP transacylase (FabD). It should be noted that apo-ACP, the *acpP* gene product, must be converted into its active form, holo-ACP by ACP synthase (AcpS) before it serves as an acyl group carrier. In the initial reaction of the elongation cycle, malonyl-ACP undergoes a condensation with acetyl-CoA, a reaction which is catalyzed by β-ketoacyl ACP synthase III (FabH). *E. coli* FabH is highly selective for acetyl-CoA, whereas in many gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*, FabH preferentially utilizes branched-chain fatty acids over acetyl-CoA for synthesis of iso- and anteiso-branched fatty acids (Choi et al., 2000a, Heath et al., 1996a, Qiu



et al., 2005). Moreover, FabH in *Mycobacteria tuberculosis* prefers long chain acyl-CoAs to produce long-chain mycolic acids (Choi et al., 2000b). The residual reactions of the elongation cycle are catalyzed by an NADH-dependent β-ketoacyl-ACP reductase (FabG) resulting in formation of β-hydroxyacyl-ACP, two β-hydroxyacyl-ACP dehydratases (FabA or FabZ) and a NADH-dependent enoyl-ACP reductase (FabI). Two enzymes, β-ketoacyl ACP synthase III (FabB or FabF), initiate subsequent elongation cycles by condensation of the acyl-ACPs resulting from each previous cycle with malonyl-ACP (White et al., 2005). Saturated acyl-ACPs of desired acyl chain lengths, mostly C12, C14 and C16, serve as substrates for synthesis of other essential macromolecules, including phospholipids and lipid A, or are used for modification of proteins, e.g. hemolysin.

Unsaturated fatty acids (UFAs) play a critical role in maintaining the fluidity of bacterial membranes. Three different fatty acids are commonly found in *E. coli* membrane phospholipids: the saturated fatty acid (SFA) palmitic (hexadecanoic) acid and the two unsaturated fatty acids (UFAs) palmitoleic (*cis*-9-hexadecenoic) acid and *cis*-vaccenic (octadecenoic) acid (Mansilla et al., 2004). In cells grown at 37 °C, palmitic acid is only found in position 1 of phospholipids and palmitoleic acid is found in position 2. However, upon a temperature downshift, *E. coli* adapts by replacing the palmitic acid at position 1 with *cis*-vaccenic (Cronan et al., 1973). In other words, the synthesis of *cis*-vaccenic acid increases, whereas the synthesis rate of palmitoleic acid and other unsaturated fatty acids is similarly adjusted in response to the lower growth temperature (Cronan, 1975).

In *E. coli*, three enzymes, FabA, FabB and FabF, are required for the synthesis of UFAs (Mansilla et al., 2004). FabA, encoding β-hydroxydecanoyl dehydratase dehydrates the 10 carbon fatty acid synthesis intermediate β-hydroxydecanoyl-ACP to

trans-3-decenoyl-ACP, followed by isomerizing *trans*-3-decenoyl-ACP to *cis*-2-decenoyl-ACP (Block, 1971) (Fig. 1.3.). In addition to FabA, another dehydratase isoform, FabZ, can also catalyze the dehydration reaction, but not the isomerization of the double bond of decenoyl-ACP (Heath et al., 1996b). It was shown that UFA auxotrophs could be isolated that contained mutations in a gene encoding a β-hydroxydecanoyl thioester dehydrase, demonstrating the requirement of the FabA isomerase activity for UFA synthesis (Silbert et al., 1967). The crystal structure of FabA dehydrase indicated that the enzyme is composed of a symmetric dimer containing a α+β hot dog fold structure, each of which has an active site histidine or aspartic acid residue located at the interface of the two subunits (Leesong et al., 1996). During the dehydration reaction, His70 acts as a catalytic base to abstract a proton from the C2 of the acyl-ACP and Asp84 contributes to remove the hydroxyl group from the C3 of the same molecule, resulting in enoyl-ACP intermediates. The *trans*-3-decenoyl-ACP is then isomerized to *cis*-2-decenoyl-ACP in a reaction during which Asp84 abstracts a proton from C4 of the substrate and the proton is subsequently transferred to His70 (Kimber et al., 2004, Leesong et al., 1996). The histidine residue in the active site is placed in a tunnel that accommodates a 10-carbon acyl chain length, explaining its preference for β-hydroxydecanoyl-ACP as a substrate, although FabA reacts with all β-hydroxyl-ACP intermediates during fatty acid synthesis (Heath et al., 1996b). In contrast to FabA, FabZ forms a hexamer (a trimer of dimers) with a distinct active site residue, glutamic acid (Kimber et al., 2004).

Two condensing enzymes, FabB and FabF, are required for the elongation of *cis* unsaturated acyl-ACP to form the UFAs found in membrane phospholipids (de Mendoza et al., 1983). FabB and FabF are 3-ketoacyl-ACP synthases which are responsible for the elongation of fatty acid chains by adding C2 units from malonyl-

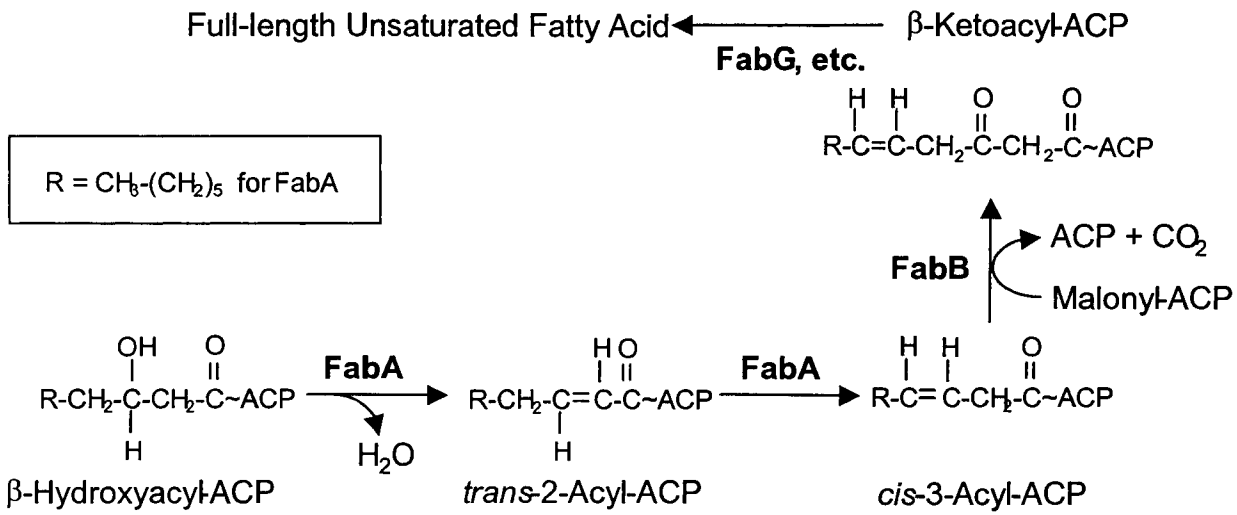


Fig. 1.3. The anaerobic pathway for synthesis of UFAs. This pathway is found in the α and γ Proteobacteria. In the reactions shown, FabA catalyzes the isomerization of the *trans*-2 double bond to a *cis*-3 double bond at the 10 carbon stage of FA synthesis. The *cis*-3 product is then condensed with malonyl-ACP to form a β-ketoacyl-ACP, essentially bypassing the enoyl-ACP reductase (FabI) step Fig. 1.2) and thus maintaining the double bond. In subsequent rounds of elongation, the acyl chain is then elongated to the full-length UFA.

ACP, resulting in the formation of C12, C14, C16 and C18 fatty acids, which are then incorporated into membrane lipids (Mansilla et al., 2004). Since both *fabA* and *fabB* mutants are UFA auxotrophs, both enzymes are essential for UFA synthesis (Birge et al., 1972). Since a *fabF* mutant cannot synthesize *cis*-vaccenic acid from palmitoleic acid, FabF has a role different from FabB as an elongation enzyme during UFA synthesis (Garwin et al., 1980a). This suggested that FabB is exclusively involved in the elongation of C10 unsaturated chains to C12 intemediates (Cronan et al., 1996), while FabF plays a critical role in converting C16 to C18 molecules (Garwin et al., 1980b). As a consequence, *fabF* mutants contain significantly decreased levels of *cis*-vaccenic acid which is normally synthesized to maintain membrane fluidity at low temperatures, and are thus impaired in thermal phospholipids modulation (Garwin et al., 1980a). Even though FabB can also catalyze the elongation of palmitoleic acid to *cis*-vaccenic acid and its overexpression in *fabF* mutants harboring a FabB-encoding plasmid significantly increased *cis*-vaccenic acid levels, its activity is not regulated by growth temperature (de Mendoza et al., 1983). The γ-proteobacteria *Haemophilus influenzae* and *Pasteurella multicida* possess a single FabB / FabF homologue which is 74 % identical to *E. coli* FabB, suggesting that this is a protein with FabB activity (Wang et al., 2003). Other bacteria also seem to lack FabF homologues, including other *Pasteurellaceae* such as *Actinobacillus actinomycetemcomitans*, *Haemophilus ducreyi*, *Mannheimia haemoltica* (Wang et al., 2003). Thermal modulation at low growth temperature occurs mainly in free-living bacteria, but members of the *Pasteurellaceae* use mostly warm-blooded animals as hosts and, thus, probably do not require the thermal control achieved by changing fatty acid composition (Wang et al., 2003). This is the most likely explaination as to why these bacteria possess FabB, but no FabF homologs. Despite the fact that FabF is considered as an essential thermal

modulator of membrane fluidity in *E. coli,* and *fabF* mutants are incapable of regulating their UFA to SFA ratios, they can still grow at low temperature (Cronan et al., 1987). In contrast, a mutant of the deep-sea bacterium *Photobacterium profundus* SS9 containing a disrupted *fabF* gene was growth-impaired at elevated hydrostatic pressure and the addition of *cis*-vaccenic acid restored its growth rate to that observed with wild-type. This demonstrated that in this bacterium FabF is required for viability at high hydrostatic pressure and that it functions as a modulator of high pressure adaptation through membrane phospholipid changes, i.e., by increasing the production of *cis*-vaccenic acids (Allen et al., 2000, Macdonald et al., 1985).

Besides the thermal regulation of elongation of palmitoleic acid to *cis*-vaccenic acid by FabF, a second temperature-dependent system was found to adjust fatty acid composition of lipid A, the hydrophobic anchor of the outer membrane lipopolysaccharide. When *E. coli* is grown at 37 °C, palmitoleic acid is absent from its lipid A. Upon a temperature downshift to 12 °C, its levels are increased to about 11% of total fatty acids found in lipid A by replacing lauric acid (C_{12}) with palmitoleic acid ($C_{16:1}$) (Van Alphen et al., 1979). Changes in the fatty acid composition of the lipid A also occur in *Proteus mirabilis*, where the palmitic acid content of the LPS decreases and the palmitoleic acid increases in cells grown at 15 °C (Rottem et al., 1978). The increases in palmitoleic acid resulted from significant accumulation of *lpxP* mRNA, which encodes the cold shock-induced enzyme, palmitoleoyltransferase (Carty et al., 1999). This enzyme catalyzes the transfer of a palmitoleoyl acyl group from palmitoleoyl-ACP to the lipid A intermediate Kdo_2-lipid IV_A. At normal growth temperature, two transferase enzymes, LpxL (HtrB) and LpxM (MsbB) play an important role in the incorporation of laurate (C_{12}) and myristate (C_{14}) into the lipid A precursor during late stage synthesis (Clementz et al.,

1996, Clementz et al., 1997). A mutation in the *lpxL* homolog *lpxP* resulted in greater antibiotic sensitivity during a temperature downshift due to altered membrane integrity. Thus, the optimization of the membrane fluidity during cold shock might be achieved through the acylation of an unsaturated palmitoleoyl chain by LpxP rather than incorporation of a saturated lauroyl acyl group into lipid A (Vorachek-Warren et al., 2002). Genes encoding cold-induced lipid A acyltransferases exist in many other bacteria that can live outside of animal hosts, such as *E. coli*, *Klebsiella pneumoniae* and all serovars of *Salmonella enterica*, whereas animal pathogens lack the thermal acyltransferase control system (Vorachek-Warren et al., 2002). *Yersinia pestis* also has a system responsible for cold-induced structural changes in lipid A. At 37 °C, the body temperature of mammalian hosts, *Y. pestis* primarily synthesizes tetra-acylated lipid A, whereas at 21 °C, the body temperature of the flea vector, the bacterium predominantly synthesizes the hexa-acylated form of lipid A through the modification with C_{12} and $C_{16:1}$ fatty acid chain by *lpxM* (*msbB*) and *lpxP* homologs, respectively (Rebeil et al., 2006). Although a *lpxM lpxP* double mutant of *Y. pestis* is unable to produce hexa-acylated lipid A at 21 °C, it is more susceptible to cecropin, but not to polymyxin B. It was also capable of flea infection, suggesting that survival in the flea gut does not necessarily rely on the low-temperature-dependent control by hexa-acylated lipid A synthesis (Rebeil et al., 2006). Interestingly, this hexa-acylated form of lipid A was found to stimulate human monocytes to produce more tumor necrosis factor-α (TNF-α) at 21 °C. In contrast, once *Y. pestis* is transmitted to humans where it expresses the predominantly producing tetra-acylated form, activation of human macrophages is suppressed which favors the infective process (Rebeil et al., 2004). Lastly, *Aeromonas* species also alter their membrane phospholipid composition in response to growth temperature and salinity by decreasing the ratio of UFA to SFA or

increasing the synthesis of branched fatty acid at high NaCl concentration and high temperature (Chihib et al., 2005).

2.2. Control of fatty acid synthesis

2.2.1 Metabolic control

Control of fatty acid biosynthesis is coordinated with phospholipid and macromolecular synthesis and partly also with growth rate in a response to altered conditions such as amino acid starvation and energy source depletion. The stringent response is regulated by changing the amounts of the alarmoneguanosine-3',5'-bispyrophosphate (ppGpp) (Cashel et al., 1996). This molecule is synthesized by the *relA* gene product, ppGpp synthetase I, which is stimulated by codon-specific binding of uncharged tRNA to the acceptor sites of ribosomes during the elongation step in protein synthesis (Justesen et al., 1986). SpoT also plays a role in catalyzing ppGpp synthesis by regulating its levels independent of RelA (Xiao et al., 1991). This guanosine tetraphosphate also is sensed by a *sn*-glycerol-3-phosphate acyltransferase (PlsB) and the activity of PlsB is inhibited in the presence of elevated concentrations of the ppGpp, causing an increase in the levels of long chain acyl-ACPs followed by repressing early steps such as AccABCD, FabH, and FabI in the fatty acid synthesis pathway (Cashel et al., 1996, Davis et al., 2001, Heath et al., 1994, Heath et al., 1996a, c).

2.2.2 Regulation of *fabA* and *fabB* gene expression in *E. coli*

Two distinct transcriptional regulators regulate *fabA* and *fabB* gene expression in *E. coli*. FadR (Fatty acid degradation Regulator) negatively regulates expression of

the *fad* genes which are involved in fatty acid transport and β-oxidation (Cronan et al., 1998), but it also acts as a positive transcriptional regulator of *fabA* and *fabB* gene expression (Campbell et al., 2001, Henry et al., 1992). FadR was initially discovered as result of a mutation which caused constitutive *fad* gene expression, indicating that it functions as a repressor of fatty acid β-oxidation gene expression (Cronan et al., 1998). FadR activity is regulated by long-chain acyl-CoAs that bind to the carboxy terminus of the protein, such that the amino terminal domain of FadR protein can no longer bind to its DNA target sites, resulting in transcription of the *fad* genes (van Aalten et al., 2001, Xu et al., 2001). It was also shown that the *fadR* mutant synthesized less UFAs than the wild type strain and *fabA*(Ts) *fadR* double mutants were incapable of growing without exogenous UFA supplementation, suggesting that FadR also functions as an activator of UFA synthesis (Nunn et al., 1983). This notion was supported by the finding that FadR binds to the -40 region of the *fabA* promoter to sequences similar to those found upstream of *fadL* and *fadBA*, leading to activation of *fabA* transcription; *fabA* activation is repressed by long chain acyl-CoAs derived from metabolism of exogenous fatty acids (Henry et al., 1992). Several experiments demonstrated that *fabB* is also positively controlled by FadR: i) microarray analysis of gene expression in wild-type vs. *fadR* mutant; ii) hypersensitivity of a *fadR* mutant to cerulenin which targets FabB; iii) *fadR fabB* double mutants exhibit synthetic lethal phenotypes (Campbell et al., 2001). Zhang and collegues suggested the existence of a second regulator based on the demonstration of additional regions of similarity in the *fabA* and *fabB* upstream regions besides FadR-binding sites by using a computational phylogenetic footprinting method to predict putative transcriptional factor binding sites in bacterial regulators (Zhang et al., 2002). The presence of these transcription factor binding sites was confirmed using a DNA affinity column, which identified

YijC as a regulator of *fabA* and *fabB* expression (McCue et al., 2001). The *yijC* gene was renamed *fabR* for Fatty acid biosynthesis Regulator (McCue et al., 2001). A strain harboring a *fabR* mutation produced significantly higher UFA levels, especially *cis*-vaccenic acid, and also had increased *fabA* and *fabB* mRNA levels, demonstrating that FabR functions as a repressor of *fabA* and *fabB* expression (Zhang et al., 2002).

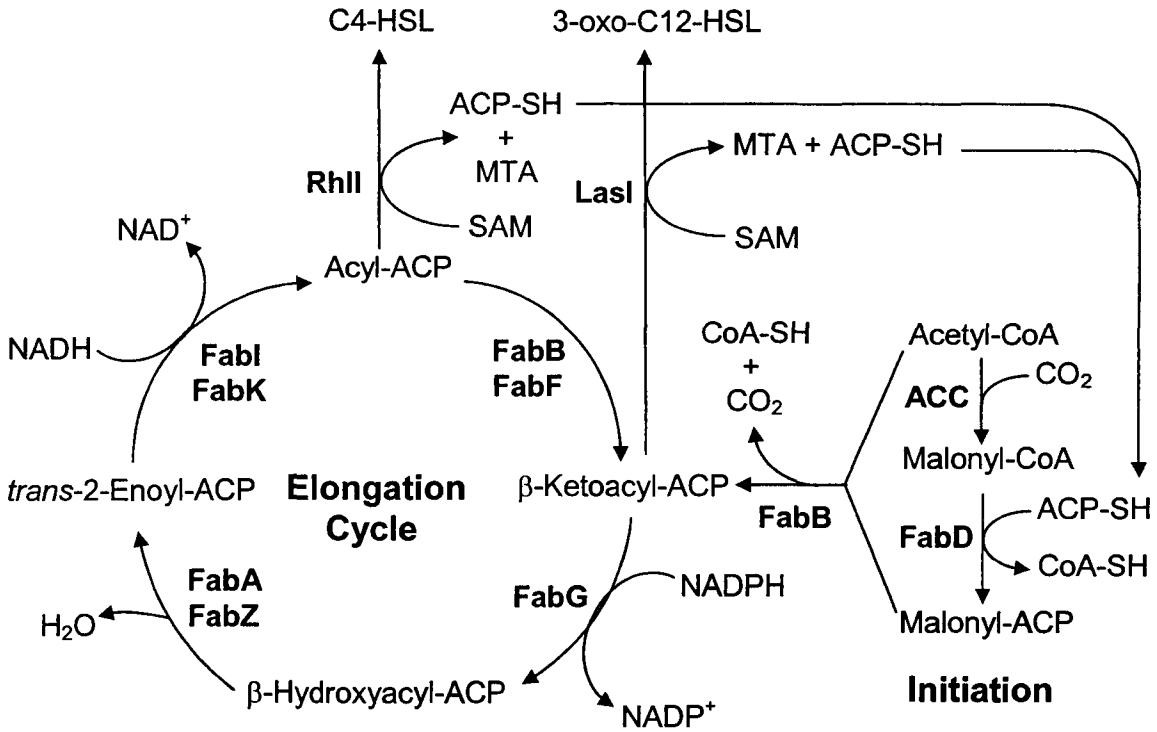
2.3 Other modulators of membrane fluidity: cyclopropane fatty acids

In addition to changes in UFA to SFA ratios, membrane fluidity can also be adjusted by synthesis of cyclopropane fatty acids (CFA) (Perly et al., 1985). CFAs are commonly found in Gram-positive (e.g. *Clostridium*, *Lactobacillus*, *Lactococcus*, and *Streptococcus*) and Gram-negative (e.g. *Campylobacter*, *Chromohalobacter*, *Citrobacter*, *Desulfobacter*, *Escherichia*, *Herbaspirillum*, *Helicobacter*, *Pseudomonas*, *Salmonella*, *Vibrio*, and *Yersinia*) bacteria (Bakholdina et al., 2004, Budin-Verneuil et al., 2005, Kaclikova et al., 2005, Kim et al., 2005, Konneke et al., 2003, Lechevalier, 1977, Martinez-Rodriguez et al., 2005, Munoz-Rojas et al., 2006, Suutari et al., 1994, Vargas et al., 2005). CFA synthesis is influenced by many environmental factors including temperature, salinity, growth phase and pH. Bacterial membrane phospholipids contain the *cis*-form of CFAs, predominantly *cis*-9,10-methylene hexadecanoic acid, *cis*-11,12-methylene octadecanoic acid (lactobacillic acid) and *cis*-9,10-methylene octadecanoic acid (dihydrosterculic) which are derived from palmitoleic (*cis*-9-hexadecenoic), *cis*-vaccenic (*cis*-11-octadecenoic), and oleic (*cis*-9-octadecenoic) acid, respectively (Grogan et al., 1997). Upregulation of CFA synthesis under certain conditions is accompanied by a decrease in cellular UFA content (Cronan et al., 1974). The CFA synthase encoded by the *cfa* gene in *E. coli* forms the cyclopropane ring via a carbocation reaction between a *S*-

adenoylmethionine and a linear monoene unit (Grogan et al., 1997). *cfa* transcription and CFA formation is induced by the starvation of cells for amino acids or the transition to the stationary phase (Grogan et al., 1997). Although growth phase is the predominant factor influencing CFA formation, the rate of cyclization decreased during temperature downshift in *E. coli*, *Pseudomonas* spp. and *Herbaspirillum* sp. strain K1, suggested that low growth temperature decreases CFA formation (Suutari et al., 1994). In *Lactobacillus fermentum*, synthesis of the CFA dihydrosterulic acid is increased at temperatures above 26 °C by converting vaccenic acid to dihydrosterulic acid via oleic acid, a reaction that is reversed at temperatures lower than 20 °C (Suutari et al., 1992). Although most sulphate-reducing psychrophiles such as *Desulfobacter* species synthesized high amounts of *cis*-unsaturated fatty acids in response to low growth temperature, *Desulfobacter hygrogenophilus* downregulates the production of the CFA *cis*-9,10-methylenehexadecanoic acid when grown at 12 °C (Konneke et al., 2003).

2.4. Unsaturated fatty acid synthesis in *P. aeruginosa* and other *Pseudomonas* spp.

E. coli and *P. aeruginosa* possess very similar fatty acid biosynthesis pathways with some notable distinctions. The fatty acid biosynthesis pathway in *P. aeruginosa* is outlined in Fig 1.4. For the initiation of fatty acid synthesis, acetyl-CoA is carboxylated to generate malonyl-CoA by an ATP-dependent acetyl-CoA carboxylase (ACC). The malonyl group is then transferred to ACP by malonyl-CoA:ACP acyltransferase (FabD). Unlike *E. coli* where the initial consensation reaction is catalyzed by FabH, malonyl-ACP is decarboxylated to acetyl-ACP by β-ketoacyl ACP synthase I (FabB), which then condenses these two molecules to



generate acetoacetyl-ACP which is then reduced by a NADH-dependent *β*-ketoacyl-ACP reductase (FabG). The resulting β-hydroxylbutyryl-ACP is subsequently dehydrated to crotonyl-ACP by one of two β-hydroxyacyl-ACP hydratases (FabA or FabZ). Finally, crotonyl-ACP is reduced to butyryl-ACP by one of two NADH-dependent enoyl-ACP reductases (FabI or FabK). As in *E. coli*, subsequent elongation cycles are initiated by condensation of β-ketoacyl-ACP and malonyl-ACP. *P. aeruginosa* has a well characterized quorum-sensing system which uses two acyl homoserine lactones, butyryl-homoserine lactone (C4-HSL) and 3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL) molecules as signaling molecules. These two molecules are synthesized by distinct homoserine lactone synthases using *S*-adenosyl methionine (SAM) as homoserine lactone donor and fatty acid synthesis acyl-ACP intermediates as acyl donors. C4-HSL is synthesized by RhlI from butyryl-ACP and SAM, and 3-oxo-C12-HSL by LasI from 3-oxo-C12-acyl-ACP and SAM.

Although the overall organization of the *fab* genes in *P. aeruginosa* is very similar to that of *E. coli*, there exist some differences. Unlike *E. coli* where the *fabA* and *fabB* genes are found in two separate chromosomal locations, these genes form an operon in *P. aeruginosa*. In most Gram-negative bacteria, the *fabH* gene is found in the *fabD-fabG-acpP-fabF* cluster, but in *P. aeruginosa* this gene is located elsewhere on the chromosome, if it does exist at all (Hoang et al., 1997, 1999). The different arrangement of these genes may imply the existence of distinct fatty acid biosynthesis regulatory mechanisms.

As in *E. coli*, the anaerobic pathway of UFA synthesis in *P. aeruginosa* is linked to the pathway of saturated fatty acid synthesis and experimental evidence suggests that it proceeds via the same enzymes and biosynthethic steps that are found in *E. coli*. However, there are several key differences: 1) In *E. coli*, the *fabA* and *fabB*

genes are known to be positively regulated by FadR, which functions as both a repressor for *fad* gene expression and activator of *fabA* and *fabB* gene expression (Campbell et al., 2001, DiRusso et al., 1993, Nunn et al., 1983). In addition, FadR is associated with growth phase-dependent regulation of the *fad*, *fab* and *uspA* genes for survival during stationary phase, such that gene expression of *fad* and *uspA* is increased during stationary phase, but *fab* gene expression is decreased immediately after exponential phase (Farewell et al., 1996). No significant homologue of *E. coli* FadR can be found in *P. aeruginosa*; 2) As a repressor of UFA biosynthesis, the FabR transcription factor controls UFA production through the regulation of *fabA* and *fabB* gene expression in *E. coli* (Zhang et al., 2002). Although *P. aeruginosa* contains two *fabR* homologues, *PA1539* and *PA4890*, their functions have not yet been characterized.

In addition to the anaerobic biosynthetic pathway of UFA biosynthesis, it was suggested that the psychrotrophic *Pseudomonas* sp. strain E-3 also possesses an aerobic fatty acid desaturase activity for formation of UFA (Wada et al., 1989). A palmitoyl-CoA desaturase from the membrane fraction introduced a double bond into stearoyl-CoA to form oleate and was inhibited by the addition of respiratory inhibitors such as NaN_3 and KCN, demonstrating that the desaturase system in *Pseudomonas* E-3 is dependent on molecular oxygen and NADPH or NADH and resembles acyl-CoA desaturase systems in other organisms (Hollowey, 1983, Wada et al., 1989). This finding suggested that the situation in some *Pseudomonas* spp. may be similar to that found in *Vibrio* ABE-1 where aerobic and anaerobic pathways coexist. Whereas some UFAs such as $C_{12:1}$ and $C_{14:1}$ are produced by an anaerobic system in *Vibrio* ABE-1, $C_{16:1}$ is formed by converting $C_{14:0}$ to $C_{16:1}$ via $C_{16:0}$ by aerobic desaturation (Morita et al., 1992).

2.5 Physiological roles and control of membrane fluidity in *Pseudomonas* spp. and other bacteria

Although most solvents are highly toxic to bacteria, several *Pseudomonas* species are able to grow in the presence of high concentrations of organic solvents such as alkanes, phenol, toluene and *p*-xylene (Chen et al., 1995, Cruden et al., 1992, Heipieper et al., 1992, Kim et al., 1998). Some of these *Pseudomonas* strains have the ability to use these toxic compounds as a carbon source, which provides a means of reducing their toxicity (Ramos et al., 1995). However, this mechanism plays a minor role in protecting cell membranes because most bacteria tolerant to organic compounds lack metabolic systems for these solvents (Ramos et al., 2002). For example, *E. coli* K12 strains tolerant to hexane do not biotransform the solvent and *P. putida* DOT-T1E strain degrades toluene, but does not metabolize it (Mosqueda et al., 1999, Ramos et al., 2002). In *Pseudomonas oleovorans* GP12, tolerance to octane is not associated with its metabolism because alkane is metabolized to alcohol, increasing its toxicity (Chen et al., 1995). Thus, it was suggested that other pathways may be invoved in protecting cells from toxic compounds. As a response to organic solvents, membrane phospholipid composition is altered to decrease membrane fluidity which in turn decreases membrane penetration for these solvents (Ramos et al., 2002).

For a short-term response, a *cis/trans* UFA isomerase in *Pseudomonas putida* P8 strain acts opposite to *fabA* isomerase activity to rapidly adapt to environments containing toxic solvents such as phenol, toluene and ethanol by changing the membrane fluidity (Heipieper et al., 1992). The structural gene for *cis/trans* isomerase (*cti)* was identified by Tn*5* transposon mutagenesis (Holtwick et al., 1997). A strain harboring a *cti* mutation did not produce *trans*-unsaturated fatty acids, but a

complemented strain was able to synthesize *trans*-unsaturated acids, resulting in normal growth at 28 °C. *trans* configured UFAs are the principal component of the membrane phospholipids of *P. putida* P8 (Heipieper et al., 1992). However, recently it was shown that *trans*-UFAs were significantly increased only during an abrupt environmental change, such as a higher growth temperature, and in the absence of other adaptive pathways, e.g. *de novo* synthesis of fatty acids (Hartig et al., 2005). Furthermore, the isomerization competed with CFA synthesis, which is elevated during the transition from exponential growth to stationary phase (Hartig et al., 2005). In addition, *cis/trans* isomerization was also shown in *Vibrio* sp. strain ABE-1 with increasing ambient temperature (Okuyama et al., 1991). Similarly, a psychrophilic bacterium, *Pseudomonas syringae* Lz4W strain also isomerizes *cis-* to *trans*-UFAs in response to increasing the growth temperature to 28 °C, causing a decrease in membrane fluidity (Kiran et al., 2004).

For a long-term adaptation on exposure to organic solvents, changes in phospholipid head groups occur to adjust for elevated membrane fluidity. The principal phospholipids in *P. putida* S-12 are phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphatidylglycerol or cardiolipin (CL). In the presence of toluene, the level of PE decreased and that of CL and PG increased (Weber, 1994). Similarly, cardiolipin significantly increased in *P. putida* DOT-T1 in environments containing toluene (Ramos et al., 1997). Cardiolipin has a higher melting temperature than PE, which causes lower membrane fluidity (Weber et al., 1996). Unlike other *Pseudomonas* species, *P. aeruginosa* utilizes multidrug efflux systems to tolerate organic solvents. Among several efflux systems, MexAB-OprM mainly contributes to the intrinsic solvent tolerance in an energy dependent manner (Li et al., 1998). *E. coli* compensates for changes in membrane fluidity resulting from

phenol exposure by increasing the saturated fatty acid content of its membrane phospholipid molecules (Keweloh et al., 1991).

In *Pseudomonas putida* and *Pseudomonas stutzeri*, addition of naphthalene and its degradation increased the ratio of SFA to UFA, and also changed the proportions of cyclopropane and branched-chain fatty acids (Mrozik et al., 2005). *Pseudomonas aeruginosa* is known to be highly resistant to antibiotics and disinfectants such as the quanternary ammonium compounds (QACs) (Russell, 1982). QACs are widely used for disinfecting foods and include didecyldimethylammonium bromide (DDAB) and benzyldimethyltetradecylammonium chloride (BC14). The intrinsic resistance to these disinfectants seems to be due to the cell wall and membrane and can be accomplished by several changes in fatty acids, outer membrane proteins and lipids (Tabata et al., 2003). In the presence of BC14, cells undergo adaptation through increases in hydroxylated fatty acids and lauric acid levels, with concomitant decreases in palmitoleic acid levels (Guerin-Mechin et al., 1999).

The membrane phospholipids in psychrotrophic marine *Pseudomonas* spp. predominantly contain hexadecenoic acid ($C_{16:0}$) and, to lesser amounts, octadecenoic ($C_{18:1}$) and few changes in fatty acid content are seen with decreasing growth temperatures (Bhakoo et al., 1980). This was also observed in most psychrophilic *Desulfobacter* species, where a minor increase in the production of *cis*-UFAs was noted during a downward temperature shift (Konneke et al., 2003). Furthermore, in psychrotolerant groundwater α- and β-proteobacteria including *Sphingomonas* sp., *Caulobacter* sp. and *Herbaspirillum* sp., unsaturation of SFAs occurred in response to decreasing temperature. The α-proteobacteria *Sphingomonas* sp. also has the ability of temperature-induced modification of lipid fatty acids such as methylation of $C_{18:1}$

for synthesizing a branched $C_{19:1}$ fatty acid. The β-proteobacterium *Herbaspirillum* sp. decreases CFA synthesis at low temperature, although growth phase had a stronger influence in *E. coli* and *Pseudomonas* spp. (Mannisto et al., 2001).

2.6 Unsaturated fatty acid synthesis in Gram-positive bacteria

2.6.1 *Bacillus subtilis*

Unlike most gram-negative bacteria, gram-positive organisms do not produce UFAs by *de novo* synthesis pathway and thus lack *fabA* and *fabB* homologs that are essential for UFA synthesis in Gram-negative bacteria (Heath et al., 2001). In contrast, UFAs are synthesized from existing saturated fatty acids by inducible desaturase activities. Fulco found that a fatty acid-desaturating pathway is induced upon a downshift of growth temperature in *B. megaterium* (Fulco, 1969). It was also shown that the levels of desaturase synthesis after culture transfer from 35 °C to 20 °C far exceeded the levels found in cultures grown constantly at 20 °C, a so called "hyperinduction" process, suggesting that a modulator protein is synthesized only at lower growth temperature (Fujii et al., 1977). As with *B. megaterium*, UFA synthesis in other bacilli such as *B. subtilis*, *B. pumilus*, *B. licheniformis* and *B. alvei* is induced upon a temperature downshift, while cells growing at 30 or 37 °C produce SFAs (Chang et al., 1973, Fulco, 1967, Grau et al., 1993). However, in *B. brevis*, similar palmitate desaturating activies were found in cells growing either at 20 or 30 °C (Fulco, 1967). A *des* gene encoding a desaturase was identified by complementation of *E. coli* strains harboring mutations in either *fabA* or *fabB* required for UFA synthesis (Aguilar et al., 1998). The *des* gene encodes a polypeptide of 352 amino acid residues which contains the conserved tripartite motif of histidine clusters

necessary for catalysis and the two membrane-spanning domains found in the well-known membrane desaturases of cyanobacteria and plants, even though the *Bacillus* enzyme shares only little sequence homology with them (Aguilar et al., 1998). It was demonstrated that the *B. subtilis* desaturase is a Δ5-Des acyl-lipid desaturase which introduces a *cis*-double bond at the Δ5 position into acyl groups of membrane phospholipids rather than into acyl-thioester intermediates, resulting in desaturation of palmitate of membrane lipids and failure of elongation of $C16:1^{\Delta5}$ to $C18:1^{\Delta7}$ (Aguilar et al., 1998, Altabe et al., 2003).

The desaturation system is inactivated by the addition of rifampicin or chloramphenicol before a sudden change to low growth temperature, demonstrating that it necessitates *de novo* synthesis of RNA and proteins (Grau et al., 1993). Transcriptional analysis showed that expression of the *des* gene is regulated by a temperature change. The *des* gene is not transcribed at 37 °C, whereas the transcription of the *des* gene is transiently upregulated upon a temperature-downshift (Aguilar et al., 1999a). The stability of transcripts from genes encoding desaturases such as Δ12 and ω from *Synechococcus* sp. strain PCC7000s and Δ6, Δ9 and ω from *Synechococcus* sp. strain PCC6803 is increased at low temperatures (Sakamoto et al., 1997, Los et al., 1997). Even though the instability of cold-shock protein transcript at 37 °C and its stability upon a cold-shock is a major factor of the transcription of cold-shock-inducible genes in bacteria (Gualerzi et al., 2003), the stabilities of *des* mRNAs from cells grown at 37 °C or a temperature downshift were not different (Aguilar et al., 1999a). Additionally, the *des* gene transcript in which the wild-type *des* promoter was switched to the *spac* promoter, a non-cold-shock promoter, was not abolished after continual bacterial growth at low temperature. Taken together, these results demonstrated that the hyperinduction process of the wild-type *des* gene at low growth

temperature is closely related to turn-off of transcription, but not to the instability of *des* mRNA (Aguilar et al., 1999b). The transcription of the *des* gene requires a transcriptional regulator which controls the desaturation system in a temperature-dependent manner because the *des* gene induction at low temperature is not associated with *de novo* protein synthesis (Mansilla et al., 2004). It was demonstrated that the transcription of *des* gene is regulated by two genes, *yocF* and *yocG*, which are located downstream of *des* and encode a two-component regulatory system (Aguilar et al., 2001). The products of *yocF* and *yocG* exhibit high structural similarity to histidine kinases and response regulators, respectively. It was found that *des* gene expression is dependent on *yocF/yocG* two component signal transduction system by showing that mutation of either *yocF* or *yocG* inhibited transcription from the *des* promoter and UFA production at low temperatures and these defects were complemented by transformation of the mutant with a plasmid containing expressing either gene. Consequently, *yocF* encoding a histidine kinase and *yocG* encoding a response regulator, were named as *desK* and *desR*, respectively (Aguilar et al., 2001). DesK is a membrane-associated histidine kinase undergoing autophosphorylation at His-188 by intracellular ATP, following by transferring the phosphate group to DesR, a cytoplasmic response regulator which then binds to the *des* promoter region (Albanesi et al., 2004, Cybulski et al., 2002). The C-terminal DNA binding domain of DesR contains a helix-turn-helix DNA-binding motif and belongs to the NarL family of transcriptional regulators (Fabret et al., 1999). Also, DesK determines the level of phophorylation of DesR by regulating the balance of both kinase and phosphatase activities, depending on whether membrane phopholipids are ordered or disordered. In other words, when membrane lipids are physically in an ordered state, kinase activity of DesK predominates, causing phosphorylation of DesR and inducing *desB*

transcription followed by increase in the production of Δ5-desaturase to maintain an appropriate degree of membrane fluidity. Conversely, a disordered membrane state results in dephosphorylation of the response regulator DesR by switching the sensor protein DesK to phosphatase-biased activity (Albanesi et al., 2004). Interestingly, even though the C-terminal domain of DesK (DesKC) contains both kinase and phosphatase activites, in the absence of transmembrane segments DesKC exhibits only kinase activity causing constitutive *des* gene expression, demonstrating that the transmembrane segments of DesK strongly affect the sensing of membrane fluidity by modulating the kinase and phosphatase activities (Albanesi et al., 2004).

In addition to desaurating membrane lipids, *B. subtilis* cells also adapt to a sudden temperature downshift by increasing ante-iso-branched fatty acid levels which possess a lower melting temperature than iso-branched fatty acids to adjust the liquid phase state of membrane (Kaneda, 1991, Suutari et al., 1994).

2.6.2 Branched chain fatty acid synthesis in Gram-positive bacteria

Many gram-positive bacteria, including *Bacillus*, *Staphylococcus*, *Arthrobacter* and *Listeria* synthesize branched-chain fatty acids (Annous et al., 1997, Kaneda, 1991), whereas *E. coli* and many other Gram-negative organisms produce only even-number saturated and unsaturated fatty acids (Choi et al., 2000a). The food-borne pathogen *Listeria monocytogenes* possesses a thermal adaptation system for growth at low temperature, and thus is able to survive in low temperature environments. Similar to *Bacillus* spp., anteiso-branched fatty acids, especially $C_{15:0}$, were increased in *L. monocytogenes* cells grown at temperatures lower than 10 °C to ensure proper membrane fluidity (Annous et al., 1997). Higher plants and animals exhibit a high positional preference in the fatty acids distribution in that saturated

fatty acids are placed at the 1-position of the phospholipid and unsaturated fatty acids at the 2-position (Kaneda, 1977). Since most species of *Bacillus* have saturated fatty acids, some of these must be located in the 2-position. It was shown that, for the most part, *Bacillus* spp. either contain anteiso- or iso-branched fatty acids are located at the 2-position (Kaneda, 1972a, b). Originally, branched-chain fatty acids are synthesized utilizing α-keto acids as a primer source. These α keto acids are derived from valine and leucine as the precursors of iso-branched chain fatty acids, and from isoleucine for ante-iso-branched chain fatty acids (Kaneda, 1991). The branched short-chain fatty acids isovaleric, isobutyric and 2-methylbutyric acids are synthesized from α-keto acids by two enzymes, α-keto-isovalerate dehyrogenase and an acyl-CoA hydrolase (Namba et al., 1969). FabH is known to catalyze the formation of acetoacetyl-ACP by the condensation of acetyl-coenzyme A (acetyl-CoA) with malonyl-acyl carrier protein (malonyl-ACP) during fatty acid biosynthesis in *E. coli* (Tsay et al., 1992). However, even though the two condensing enzymes, eFabH (*E. coli* FabH) and bFabH (*B. subtilis* FabH) share approximately 50% homology, they use different substrates for condensation with malonyl-ACP (Choi et al., 2000a). eFabH utilizes acetyl-CoA selectively and butyryl-CoA with low activity (Heath et al., 1996a), whereas bFabH (*B. subtilis* FabH) prefers branched-chain CoA thioesters for synthesis of branched-chain fatty acids (Choi et al., 2000a). The increased synthesis of anteiso-branched fatty acids at low growth temperatures is known to be due to the primer selection specificity of FabH, which is responsible for condensing malonyl-ACP with isovaleryl-CoA, isobutyryl-CoA and 2-methylbutyryl-CoA during anteiso-branched fatty acid synthesis (Choi et al., 2000a). Two *B. subtilis* FabH homologs, bFabH1 and bFabH2, were identified by searching the genome for related sequences

using *E. coli* FabH sequence (Choi et al., 2000a). Mutations in bFabH1 or bFabH2 resulted in strains with auxotrophy for branched fatty acids (Willecke et al., 1971).

Similarly, FabH in *Streptomyces glaucescens* also is associated with the synthesis of branched-chain fatty acids by using either butyryl-CoA or isobutyryl-CoA as a primer (Han et al., 1998). *Streptomyces* spp. produce most of their fatty acids from branched-chain primers including isobutyl isovaleryl, and anteisovaleryl groups with a methyl branch at ω-position (Revill et al., 2001). When *S. glaucescens* cells were grown in the presence of thiolactomycin, an inhibitor of fatty acid synthesis, branched-chain fatty acid synthesis was inhibited and the production of straight-chain fatty acids was upregulated, showing that the initiation of fatty acid synthesis is independent of FabH (Han et al., 1998). Consistent with other gram-positive bacteria, FabH in *Staphylococcus aureus* also utilizes both straight- and branched-chain (C4-C6) acyl-CoA primers. FabH substrate specificity in this bacterium was ranked in the order of isobutyryl-, hexanoyl-, butyryl-, isovaleryl- and acetyl-CoA (Qiu et al., 2005).

2.6.3 Regulation of fatty acid synthesis in Gram-positive bacteria

FapR (Fatty acid and phospholipid Regulator) was identified as a global bacterial transcriptional regulator for membrane lipid biosynthesis found in gram-positive bacteria, such as *Bacillus* spp., *Listeria* spp., *S. aureus*, *Clostridium difficile* and related genera (Schujman et al., 2003, Schujman et al., 2005). Unlike DesR and FabT controlling UFA levels in membrane phospholipids, FapR regulates almost all sets of genes associated with type II fatty acid biosynthesis (FAS II). Among genes involved in FAS II (the *fap* regulon) in *B. subtilis*, the *fabHAF* operon encoding the FabHA and FabF condensing enzymes, is highly expressed by inhibition of fatty acid synthesis, suggesting the existence of regulator sensing a decreased activity of FAS II

and controlling the synthesis of the FabHA and FabF condensing enzymes (Schujman et al., 2001). Both, FapR binding to an inverted repeat DNA fragment containing the promoter of *fabHAF* and upregulation of *fap* regulon in a *fapR* null mutant indicated that FapR functions as a global negative regulator of membrane phospholipid biosynthesis (Schujman et al., 2003).

The cyanobacterium *Synechocystis* sp. PCC 6803 was also reported to possess two histidine kinases and a response regulator controlling the transcription of *des* gene by low growth temperatures (Suzuki et al., 2000). Hik33 and Hik19, histidine kinases, were identified by inserting spectinomycin-resistance cassette into 43 putative genes (Hik1-Hik43) on chromosome of *pdesB*::*lux* cells. Mutation of Hik33 caused the deregulation of cold-shock inducible genes including *desD* encoding a Δ6 desaturase (Suzuki et al., 2001). Unlike the *B. subtilis* DesK/DesR regulatory machinery, this system also regulates many other cold inducible genes as a global cold sensor (Inaba et al., 2003). In addition to the two histidine kinases, Rer1, a response regulator, was identified by screening a *Synechocystis* library with random mutations that resulted in the inhibition of the induction of the *desB* gene at low temperature (Suzuki et al., 2000). To ameliorate the effects of temperature changes on the physical state of membrane phospholipids at low temperatures, Hik33, composed of a HAMP (histidine-kinase-adenylyl-cyclase-methyl-binding protein phosphatase) domain, a PAS (PER-ARNT-SIM) domain responsible for protein-protein interaction between HAMP and histidine kinase domain, and a histidine kinase domain, forms a dimer that autophosphorylates the histidine residue in the histidine kinase domain (Sakamoto et al., 2002). This is followed by transferring the phosphate group to an aspartate residue in the C-terminal receiver domain of Rer1. The N-terminal region of Rerl, which contains an HMG (high-mobility group) box and the transcriptional activator

domain of aryl hydrocarbon receptor nuclear translocator (ARNT) binds to the promoter region of cold-inducible genes (Sakamoto et al., 2002, Suzuki et al., 2000). Interestingly, Los suggested that, in response to low temperature shift, genomic supercoiling of DNA region containing the *desB* regulatory elements for the ω3 desaturase which is induced by cold-shock appears to act as temperature recognition mechanism in cyanobacterium *Synechocystis* (Los, 2004). This effect in DNA supercoiling was prevented by the addition of novobiocin, an inhibitor of DNA gyrase, following by changing in the expression levels of several desaturase genes, indicating that temperature-induced changes in genomic supercoiling might be essentially associated with a global regulation pathway for thermal control in mesophilic cyanobacterium (Los, 2004). Regulation of UFA synthesis by two-component systems in *B. subtilis* and *Synechocystis* spp. suggests that such systems may play key roles in the regulation of expression of aerobic fatty acid desaturation enzymes in prokaryotes (Mansilla et al., 2004).

2.6.4 Other unsaturated fatty acid synthesis pathways in anaerobic Gram-positive bacteria

Although *fabA* and *fabB* are responsible for UFA synthesis in most bacteria, many Gram-positive anaerobes that produce UFAs, including Streptococci and Clostridia, do not contain homologs of either *fabA* or *fabB* (Marrakchi et al., 2002a). Therefore, these organisms must possess different UFA synthesis mechanisms. Unlike *E. coli*, *S. pneumoniae* possesses a single dehydratase, FabZ, that introduces the *trans* double bond into C10 intermediates, but does not isomerize the *trans*-2 to *cis*-3 intermediate (Marrakchi et al., 2002a). After the formation of the *trans*-2 intermediate, SFAs and UFAs are synthesized by distinct mechanisms. For SFA

synthesis, the double bond is reduced by the FabK enoyl-ACP reductase, followed by elongation of the saturated enoyl-ACP by FabF (β-ketoacyl-ACP synthase II). For UFA synthesis, FabM isomerizes *trans*-2-decenoyl-ACP to the *cis*-3-decenoyl-ACP which is then elongated by FabF to full-length UFAs (Marrakchi et al., 2002a). The competition between FabK and FabM at branch point determines SFA or UFA formation. In *S. mutans* strain UA159, FabM was identified as a *trans*-2/*cis*-3-decenoyl-ACP isomerase by genetic and biochemical experiments (Fozo et al., 2004). However, the identification of FabM does not explain how UFAs are synthesized in other Gram-positive anaerobes since FabM homologs are only found in *Streptococcus* species (Marrakchi et al., 2002a). *S. pneumoniae* does not posess FadR, FabR or FapR homologs for the regulation of UFA synthesis. Recently, Fatty acid biosynthesis Transcriptional regulator (*fabT*), the second gene in the *fab* gene cluster, was identified as a regulator of UFA production and membrane fatty acid content (Lu et al., 2006). A *fabT* mutation caused elevated production of SFAs and displayed high sensitivity to low pH and detergents. FabT belongs to the MarR superfamily and acts as a repressor of *fab* gene cluster expression by binding to two palindromic sequences located in the *fabT* and *fabK* promoter regions. Even though a putative FabT-related sequence was found upstream of the *fabM* ribosomal binding site, microarray and RT-PCR analyse showed that *fabM* expression stays at similar levels in a *fabT* mutant, demonstrating that FabT does not function as a regulator of *fabM* expression (Lu et al., 2006). Other genera of gram-positive bacteria, such as *Clostridium*, *Enterococcus* and *Lactococcus*, contain comparable *fab* gene clusters with a *fabT* homolog and palindrome sequences in the *fabT* and *fabM* promoter region, suggesting that FabT is also responsible for *fab* gene cluster regulation in these bacteria (Lu et al., 2006). *Enterococcus faecium* contains two *fab* gene clusters including one similar to the *S.*

pneumoniae cluster except *fabM* and a second with three genes, *fabI*, *fabZ* and *fabF* (Paulsen et al., 2003). Although *Entercococcus faecalis* possesses a fatty acid content similar to *E. coli*, it does not have *fabA* and *fabB* for UFA synthesis. In contrast to *S. pneumoniae*, there are no *fabM* but rather two FabZ homologs, *Ef*FabN and *Ef*FabZ (Lu et al., 2005, Wang et al., 2004). *Ef*FabN acts as a bifunctional enzyme with dehydratase and isomerase activities and is capable of replacing *E. coli* FabA in spite of having higher homology to *E. coli* FabZ, whereas the latter, *Ef*FabZ, catalyzes only the dehydratase reaction (Wang et al., 2004).

S. mutans, a major dental pathogen, adapts to low pH environments because it rapidly metabolizes various sugars to acidic end products, lowering the pH of the environment (Belli et al., 1991). This necessitates specialized adaptive mechanisms such as increases in F_1F_0 ATPase activity, a low-pH-inducible DNA repair system and membrane fatty acid composition alterations (Quivey et al., 2000a, Quivey et al., 2000b). When *S. mutans* cells were grown at pH 5, the production of monounsaturated fatty acids was increased more than in cells grown at pH 7, showing that the alteration of membrane fatty acid content may enable oral bacteria to thrive in low pH conditions (Quivey et al., 2000a). However, in *E. coli*, the acid-adaptive response results in membranes with higher concentrations of cyclopropane fatty acids by inducing the cyclopropane fatty acid synthase gene (*cfa*) (Brown et al., 1997, Chang et al., 1999)

2.7 Fatty acid synthesis in *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is believed to be resistant to most antibiotics due to the low permeability of a cell wall bilayer composed of mycolic acids, arabinogalactan and peptidoglycan (Brennan et al., 1995). Unlike many other

prokaryotes and eukaryotes, Mycobacteria possess both type I and type II fatty acid synthesis systems. Fatty acids produced by the type I multifunctional synthase are elongated by the type II system, modified and condensed with α-branch fatty acids, resulting in the formation of mature mycolic acids, long-chain α-alkyl-β-hydroxy fatty acids containing 70 to 90 carbons in the acyl chain lengths (Brennan et al., 1995). Mycolic acids are found either covalently attached to the cell wall or as components of trehalose dimycolate (TDM), a glycolipid which exhibits immunemodulatory activities (Bloch, 1950).

Mycobacterial type II fatty acid synthase (FAS) is unable to synthesize mycolic acids from acetyl-CoA and, instead, elongates medium-chain-length C12 to C16 fatty acids to very-long-chain meromycolic acids (Schweizer et al., 2004). Bacterial type I fatty acid synthetases purified from mycobacterial strains are composed of homohexamers for fatty acid synthesis domains in a single polypeptide chain (Bloch et al., 1977, Fernandes et al., 1996, Schweizer et al., 2004, Wood et al., 1978). Compared to other type I fatty acid synthases, bacterial fatty acid synthesis multienzymes utilize different reductants such as NADPH and NADH, for their ketoacyl and enoyl reduction steps, respectively (Bloch et al., 1977, Schweizer et al., 2004, Seyama et al., 1987). In the mycobacterial FAS I system, the acetyl-group is elongated by two carbon units by combining acetyl-CoA and malonyl-CoA to generate butyryl thioesters bound to an ACP-like protein, followed by elongation cycles of condensation, β-ketoacyl reduction, dehydration and enoyl reduction to yield C_{16} and C_{18} thioesters. These fatty acid chains are then utilized in the CoA derivative form for the synthesis of membrane phospholipids. However, further elongation of these fatty acyl thioesters through fatty acid type I synthesis system leads to the C_{20} (eicosanoyl-CoA) and C_{26} thioesters (hexacosanoyl-CoA), which then

are converted to their CoA derivatives to synthesize mycolic acids (Takayama et al., 2005). It was suggested that the C_{20} fatty acid is the starting primer which fatty acid type II dissociated enzymes elongate to very long meromycolic acids, followed by modifications with cyclopropane rings, methyl-, methoxy- or keto-group (Takayama et al., 2005). Unlike the C_{20} fatty acid, the C_{26} fatty acid is responsible for the α-alkyl chain and methyl carboxyl part of all mycolic acids of *M. tuberculosis* (Takayama et al., 2005). The eicosanoyl-CoA derived from the fatty acid type I synthesis system undergoes the condensation with malonyl-ACP by β-ketoacyl-ACP synthase III (mtFabH). Even though mtFabH is 37.3 % identical to ecFabH and has the same Cys-His-Asn catalytic triad, it has a distinct substrate specificity such that mtFabH utilizes preferentially longer acyl-CoA chain over acetyl-CoA (Choi et al., 2000b). It was also found that even though mtFabH was susceptible to thiolactomycin, a well-known type II fatty acid synthase inhibitor, mtFabH overexpression in *Mycobacerium bovis* BCG did not confer thiolactomycin resistance, suggesting that mtFabH is not the main thiolactomycin target (Choi et al., 2000b). Taken together, in contrast to other bacterial known FabHs, mtFabH activity is an important link between the type I and type II fatty acid synthesis systems for production of long chain mycolic acids (Choi et al., 2000b, Scarsdale et al., 2001). To be utilized as a substrate for the condensation with acyl-CoA, malonyl-ACP is produced through the transacylation of malonyl-CoA by mtFabD encoding malonyl-CoA:ACP transacylase to form β-ketoacyl-ACP, following by the reduction of keto-form intermediates to β-hydroxyacyl-ACP by MabA with the oxidation of NAD(P)H (Schaeffer et al., 2001b). The ACP involved in FAS II system is encoded by the *acpM* (*Rv2244*) which is clustered with *fabD*, and *kasA* and *kasB* encoding β-ketoacyl synthases (Cole et al., 1998, Schaeffer et al., 2001a, Yuan et al., 1998a). The crystal structure of AcpM revealed that an amino

terminal region consists of four helices positioned in a bundle by interhelical hydrophobic interactions, which is similar to other bacterial ACPs and in addition, the carboxy terminal region extended from coil domain suggested the interaction with very long chain meromycolates as intermediates in mycolic acid biosynthesis (Wong et al., 2002). AcpM was easily overexpressed in and isolated from *E. coli* as a mixture of apo-AcpM, holo-AcpM and palmitoylated-AcpM (Kremer et al., 2001). It was demonstrated that holo-AcpM is a preferred substrate for mtFabD to catalyze the transacylation of malonate from malony-CoA to ACP for the formation of malonyl-ACP (Kremer et al., 2001).

As the second step of FAS II elongation cycle, the reduction of ketoacyl-ACP is carried out by MabA (β-ketoacyl reductase), also called FabG1, and requires NADPH as an electron donor. In agreement with the mtFabH preference for long-chain fatty acid substrates, MabA also prefers long-chain fatty acids such as C_8-C_{20}, unlike other bacterial known ketoacyl reductases (Choi et al., 2000b, Marrakchi et al., 2002b). Its crystal structure revealed that MabA exists as a tetramer and shares the conserved fold of short-chain dehydrogenases/reductases, but shows some significant rearrangements of the active-site loops having a unique tryptophan residue in the absence of a cofactor (Cohen-Gonsaud et al., 2002). Interestingly, since MabA is suggested to form an operon with *inhA,* encoding NADH-dependent 2-*trans*-enoyl-ACP reductase, and both genes are structurally and functionally related, isoniazid, a specific inhibitor of InhA, also targets MabA by a similar mechanism, in that Mn^{III}-oxidized isoniazid covalently binds to the oxidized form of the MabA cofactor, $NADP^+$, resulting in an isonicotinoyl-NADP adduct that tends to bind to the MabA active site (Banerjee et al., 1994, Ducasse-Cabanot et al., 2004, Quemard et al., 1995).

The β-hydroxyacyl-ACP is reduced to enoyl-ACP by an unidentified dehydratase. Like FabZ in *E. coli* and *S. pneumoniae*, β-hydroxyacyl-ACP dehydrase is responsible for the formation of 2-*trans*-enoyl-ACP from β-hydroxyacyl-ACP (Takayama et al., 2005). For synthesis of UFA, a *trans*-enoyl-ACP is converted to *cis*-enoyl-ACP, bypassing the reduction step by InhA reductase. No *fabA*-like homolog is found in the *M. tuberculosis* genome (Cole et al., 1998). However, more recently candidates for hydrase and isomerase were found by genome searching utilizing *S. pneumoniae* FabZ or FabM and the predicted acyl dehydratase of *Corynebacterium glutamicum* ATCC 13032 as queries (Takayama et al., 2005). The gene product encoded by *Rv0098,* which belongs to one of 219 core mycobacterial essential genes, has 36% identity and 54% similarity with *S. pneumoniae* FabZ (Marmiesse et al., 2004, Takayama et al., 2005). Moreover, it also contains the active site residues His^{72} and Glu^{97} conserved in the *S. pneumoniae* FabZ, and *E. coli* FabA and FabZ enzymes (Takayama et al., 2005). Interestingly, the *Rv0098* gene product is indispensable for the mycobacterial survival in mouse lung macrophages (Sassetti et al., 2003). Furthermore, genome blast searches with other *Mycobacterium* species showed that *Rv0098* homologs are found in *M. leprae*, *M. bovis* and *M. smegmatis* (Takayama et al., 2005). The second candidate for *M. tuberculosis* FabZ is *Rv0130*, the sequence of which is 44% identical with the putative acyl dehydratase (NCgl0284) of *C. glutamicum* (Takayama et al., 2005).

Like FabI , FabK and FabM, the sole enoyl-ACP reductase, InhA, reduces the previous intermediate to acyl-ACP, following by condensation with malonyl-ACP by KasA and/or KasB. The subsequent cycles are repeated to generate full-length meromycolates. *M. tuberculosis* InhA is responsible for the NADH-dependent 2-*trans*-enoyl-ACP reduction preferentially with long-chain substrates (Dessen et al.,

1995, Quemard et al., 1995). The InhA enzyme is known to be inhibited by covalent binding of activated isoniazid to NAD^+ ring, forming isoniazid-NAD complex (Rozwarski et al., 1998).

Two β-ketoacyl synthases encoded by *kasA* and *kasB* initiate the subsequent rounds of extending long chain by condensation of acyl-ACP with malonyl-ACP (Schaeffer et al., 2001b). However, the two enzymes lack total functional redundancy. KasA is suggested to play a role in initial chain elongation reactions whereas KasB may be involved in extension of the elongation products to full-lengths (Gao et al., 2003, Kremer et al., 2000b, Slayden et al., 2002). Mutation of either gene leads to significant growth defects, suggesting that both enzymes are required for normal growth of *Mycobacterium* spp. (Bhatt et al., 2005, Gao et al., 2003).

As mentioned above, meromyolates are modified by deaturation reactions and other postsynthetic modifications, resulting in a great diversity of mycolic acids (Cole et al., 1998). Introduction of the double bond to meromycolates is carried out by either an unknown FabA-like β-hydroxyacyl-ACP dehydrase or by aerobic desaturases such as DesA1, DesA2 and DesA3 which are oxygen- and NADPH-dependent (Cole et al., 1998). Both β-hydroxyacyl-ACP dehydrase and 2-*trans*-enoyl-ACP isomerase are suggested to be responsible for introducing double bonds into precursors of meromycolic acids, resulting in the synthesis of *cis*-unsaturated fatty acids (Takayama et al., 2005). Besides exhibiting β-hydroxyacyl-ACP dehydrase activity during the elongation of SFAs, the separate isomerase activity of same enzyme is also involved in UFA synthesis.

S. pneumoniae FabM, known to catalyze the isomerization of *trans*-enoyl-ACP, was used for a BLAST search of the *M. tuberculosis* genome to identify genes encoding potential 2-*trans*-enoyl-ACP isomerases (Marrakchi et al., 2002a, Takayama

et al., 2005). A group of 21 enzymes, annotated as probable enoyl-CoA hydratases showed strong sequence homology, and it was predicted that these enzymes belong to the hydratase/isomerase superfamily (Cole et al., 1998, Takayama et al., 2005). However, further studies will be necessary to establish their functions.

Besides dehydrase and isomerase activities, a desaturase system is also involved in the synthesis of UFAs. A stearoyl-CoA desaturase activity expressed by *M. phlei* was reduced when cells were grown in an iron-deficient medium and supplementation of iron restored normal activity levels, suggesting that iron is necessarily involved in synthesis and assembly of the desaturase system (Kashiwabara et al., 1975). Similarly, a very long-chain fatty acid desaturation system was isolated from *M. smegmatis* and it catalyzed the desaturation of lignoceryl-CoA to a Δ^{15}-monounsaturated fatty acid when molecular oxygen and NADPH were present (Kikuchi et al., 1986). Taken together, these results demonstrated that synthesis of UFAs is carried out by an oxidative desaturase system. Interestingly, a Des protein, with significant homology to plant stearoyl-ACP desaturase, was found to be a major antigenic protein in human patients infected with *M. tuberculosis* and *M. bovis* (Jackson et al., 1997). Genome sequencing showed that whereas three similar desaturase genes have little similarity to closely related vertebrate or yeast genes which use CoA esters, they are analogous to plant desaturases, stearoyl-ACP Δ^9 desaturases which utilize ACP thioesters as substrates, suggesting that acyl-ACP thioesters are substrates for the formation of unsaturated meromycolates (Cole et al., 1998). Two proteins encoded by *desA1* and *desA2* that are putative acyl-ACP desaturase which were also identified by Pfam analysis in other mycobacterial species such as *M. tuberculosis* strain CDC1551, *M. avium* subspecies *paratuberculosis*, *M. leprae*, *M. bovis* and *M. smegmatis* (Dyer et al., 2005). Recently, the DesA1 and

DesA2 proteins from *M. tuberculosis* H37Rv were overexpressed in *E. coli* BL21(DE3), and it was found that DesA1 was an insoluble protein, but that DesA2 was expressed as a soluble protein (Dyer et al., 2005). The X-ray structure of purified DesA2 showed that it is a homodimeric protein, which shares structural similarity with the castor plant stearoyl-ACP Δ9 desaturase (Dyer et al., 2005). PhoA translational fusions to DesA1 resulted in a $PhoA^+$ phenotype in *M. smegmatis*, suggesting that DesA1 is an exported protein (Lim et al., 1995). However, DesA1 and DesA2 lack typical N-terminal signal sequences, indicating that these proteins are exported by a *sec*-independent pathway (Downing et al., 1999). The third desaturase protein, DesA3, shares motifs containing three regions of primary conserved sequences, $HX_{(3\text{ or }4)}H$, $HX_{(2\text{ or }3)}HH$ and $HX_{(2\text{ or }3)}HH$ found in membrane-bound Δ9 acyl-lipid and acyl-CoA desaturases (Los et al., 1998, Phetsuksiri et al., 2003, Shanklin et al., 1994). However, DesA3 possesses higher sequence homology with the Δ6 acyl-lipid desaturase of cyanobacteria than with Δ9 acyl-CoA and acyl-lipid desaturases of animals and yeasts (Los et al., 1998, Phetsuksiri et al., 2003). Hydropathy analyses revealed that DesA3 has three hydrophobic membrane-spanning regions and three His motifs in hydrophilic domains, concluding that DesA3 is a membrane-bound Δ9-desaturase (Phetsuksiri et al., 2003). The thiourea isoxyl, an anti-tuberculosis drug, was found to inhibit the synthesis of oleic and mycolic acids by blocking DesA3 activity in a dose-dependent manner. The overexpression of DesA3, but not DesA1 or DesA2, in *M. bovis* BCG resulted in an increase in DesA3 desaturase activity and in the minimum inhibitory concentration for isoxyl, indicating that DesA3 is the target of isoxyl (Phetsuksiri et al., 2003).

The mycolic acid backbone is suggested to be derived from a Claisen-type condensation of two aliphatic molecules, long fatty acid thioesters, via reduction of

the keto group (Asselineau et al., 2002, Lee et al., 1997, Schweizer et al., 2004). Two long-chain fatty acids, the meromycolate chains of up to 56 carbons and a shorter chain of 22-26 carbons, are linked via a Claisen condensation enzyme, and the resulting molecule is then reduced to form mature mycolic acids (Gande et al., 2004, Kremer et al., 2000a). Similarly, mycolic acids from *Corynebacterium glutamicum* are produced via the condensation of two C16 fatty acids, resulting in a C32 mycolic acid (Gande et al., 2004). Formation of an UFA chain is essential for subsequent modifications such as cyclopropanation, epoxidation, hydroxylation, methoxylation and ketonization to generate the mature mycolates (Dubnau et al., 2000, Dyer et al., 2005, Yuan et al., 1995). Modifications of the cyclopropane rings and methyl branches are accomplished through a large family of an *S*-adenosyl methionine (SAM)-dependent methyl transferases which alter double bonds in the meromycolate fatty acid chain to *cis* or *trans*, or other chains (Glickman et al., 2001). Whereas the alpha-mycolates contain two *cis*-cyclopropane rings at distal and proximal positions, with farther or nearest β-hydroxyl group of mycolic acid, respectively, the oxygenated mycolic acids such as methoxy- and keto-mycolates possess either *cis*-cyclopropane or *trans*-cyclopropane rings at only their proximal position and a methoxy group or keto group adjacent to a methyl branch, respectively. Whereas keto-mycolates are most broadly distributed among oxygenated mycolates, methoxy-mycolates are more restrictive and mostly found in slow-growing species like *M. tuberculosis* (Barry et al., 1998). For an example, fast-growing *M. smegmatis* contains oxygenated mycolic acids with an epoxy function (Dubnau et al., 1997).

A strain with a mutation in *mmA2* gene had a cyclopropyl ring at proximal position in alpha-mycolates, but still contained a double bond at the distal position, showing that *mmA2* gene plays a critical role in converting the *cis* olefin into a *cis*

cyclopropyl group at the distal position (Glickman, 2003). Before the discovery of *mmA2*, *cmaA1* encoding a protein with 34% identity to the cyclopropane fatty acid synthase from *E. coli*, was suggested to be responsible for introducing the cyclopropane ring at the distal position of the alpha-mycolates (Yuan et al., 1995). However, it was shown that a *cmaA1* null mutation did not affect the modification of alpha-mycolates, demonstrating that *cmaA1* is not essential for the cyclopropanation at the distal position of alpha-mycolates (Glickman, 2003).

While PcaA (proximal cyclopropanation of alpha-mycolates) is required to modify the proximal cyclopropyl group of the alpha-mycolates, *cmaA2* (cyclopropane mycolic acid synthase 2) is responsible for encoding *trans*-cyclopropane synthase of the oxygenated mycolic acids (Glickman et al., 2001). In the case of the *trans* methoxy- or keto-mycolates, the *cis* olefin is initially converted into a *trans* olefin with formation of an allylic methyl group by *mmaA1*-encoded enzyme, subsequently following by producing a *trans* cyclopropane by a SAM-dependent *trans*-cyclopropane synthase encoded by *cmaA2* (Glickman, 2003, Yuan et al., 1998b). Full-length oxygenated mycolic acids are then formed by introducing the methyl branch together with an adjacent hydroxy group at the distal position by MmaA4. The hydroxyl group is then *O*-methylated in the hydroxy group by MmaA3 to produce methoxy-mycolates (Glickman, 2003, Yuan et al., 1998b). *M. bovis* BCG lacks *O*-methyltransferase MMAS-3 responsible for producing methoxy-mycolates and, as a consequence, this strain only produces alpha- and keto-mycolates (Yuan et al., 1998b).

2.8 Fatty acid synthesis in *Saccharomyces cerevisiae*

In *Saccharomyces cerevisiae*, SFAs are synthesized by a type I fatty acid synthase, producing primarily $C_{16:0}$ and $C_{18:0}$. These fatty acid CoA esters are changed into monounsaturated fatty acids by a Δ-9 fatty acid desaturase which introduces a double bond between carbons 9 and 10 of saturated acyl-CoA precursors (Martin et al., 2002). The *OLE1* gene encodes an endoplasmic reticulum membrane-bound acyl-CoA desaturase, Ole1p, which is comprised of a N-terminal domain containing a di-iron-oxo moiety at its catalytic core and a C-terminal domain with cytochrome b_5-like membrane-bound redox systems (Mitchell et al., 1995). *OLE1* gene expression is highly regulated at the transcriptional and post-transcriptional levels by extracellular fatty acids and oxygen availability. First, transcriptional regulation of *OLE1* is achieved by two distinct promoter elements which are a 111-bp fatty acid response element (FAR) region located 580 bp upstream of the start codon differentially regulated by the addition of saturated or unsaturated fatty acids (Choi et al., 1996), and a 50-bp O_2-regulated (O2R) element located approximately 360 bp upstream of the start codon (Nakagawa et al., 2001). Second, at the post-transcriptional level, the half-life of *OLE1* mRNA is reduced in the presence of UFAs (Gonzalez et al., 1996). *OLE1* mRNA levels were decreased substantially in the presence of UFAs whereas its transcription was induced by exogenous SFAs (Choi et al., 1996, Nakagawa et al., 2001). It was demonstrated that *OLE1* gene expression was derepressed under conditions of oxygen starvation in order to respond to reduced availability of oxygen as a substrate for the desaturase (Nakagawa et al., 2001). In addition, its expression is controlled by two homologous endoplasmic reticulum membrane-bound proteins, Spt23p and Mga2p. Disruption of either gene does not affect cell viability, whereas *spt23p mga2p* double mutants are UFA auxotrophs, suggesting that both independently activate *OLE1* gene expression for UFA synthesis

(Zhang et al., 1999). It was also shown that *OLE1* transcription is activated by the N-terminal domains of these two proteins released from the membrane through ubiquitin/proteosome-dependent processing and proteosome-dependent processing of Spt23 is repressed by UFAs (Hoppe et al., 2000). However, unlike Spt23p, Mga2p processing is highly induced under hypoxic condition through the O2R element (Jiang et al., 2001).

3. Goals of research and hypotheses

Fatty acids are essential for cellular function by providing precursors for synthesis of cellular components such as bacterial membrane lipids, lipopolysaccharide, quorum sensing molecules and virulence factors. To function properly, the fluidity of bacterial membranes must be maintained by controlling the SFA to UFA ratio in response to environmental cues. While the pathway for SFA biosynthesis had been established prior to this dissertation work, the mechanisms for UFA synthesis and its regulation remained largely unexplored. Although the complete genome sequence of *P. aeruginosa* had just been published at the onset of my studies, the arsenal of genetic tools available for this bacterium had not kept up with other post-genomic tools to fully take advantage of the genome sequence information. *The main hypothesis of this research was that the mechanisms and regulation of UFA biosynthesis in P. aeruginosa is complex and may be regulated by several proteins in response to various environmental conditions. A secondary hypothesis was that a combination of improved genetic methods and tools would facilitate genetic analyses of P. aeruginosa and provide the basis for elucidation of the UFA biosynthetic pathway(s) and their regulation.* An understanding of the fatty acid biosynthetic pathways and their regulation in response to environmental cues could then possibly lay the foundation for identification of new anti-*Pseudomonas* drugs.

The following specific aims were pursued to test these hypotheses:

1) To develop a highly-efficient bacterial site-specific gene integration system for construction of single-copy bacterial reporter gene constructs for studies of gene regulation (Chapter 2).
2) To develop a rapid DNA transformation method for high-throughput genetic manipulation of *P. aeruginosa* with non-replicative or replicative plasmids and chromosomal fragments (Chapter 3).
3) To characterize potential *P. aeruginosa* desaturases required for UFA synthesis suggested by genome sequence information (Chapter 4).
4) To characterize the regulation of expression of the *fabAB* operon encoding the enzymes for the anaerobic UFA synthesis pathway at the molecular level (Chapter 5).

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CHAPTER 2

A Tn*7*-based broad-range bacterial cloning and expression system

(Presented by Kyoung-Hee Choi, Jared B. Gaynor, Kimberley G. White, Carolina Lopez, Catharine M. Bosio, RoxAnn R. Karkhoff-Schweizer and Herbert P. Schweizer, Nature Methods 2005, 2:443-448)

The work presented in this article describes a novel Tn*7*-based site-specific gene integration system for use in various gram-negative bacteria. This new genetic tool will facilitate studies on bacterial gene expression and behavior in biofilm, plant and animal models.

2.1 ABSTRACT

For many bacteria, cloning and expression systems are either scarce or non-existing. A series of mini-Tn*7* vectors was constructed and their potential as broad-range cloning and expression systems evaluated. In bacteria with a single chromosome, including *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Yersinia pestis*, and in the presence of a helper plasmid encoding the site-specific transposition pathway, Tn*7* insertions occured site- and orientation specific at a single *att*Tn*7* site downstream of the *glmS* gene. *Burkholderia thailandensis* contains two chromosomes, each containing a *glmS* gene and an *att*Tn*7* site. The Tn*7* system allows engineering of diverse genetic traits into bacteria, as demonstrated by complementing a biofilm-growth defect of *P. aeruginosa*, establishment of expression systems in *P. aeruginosa* and *P. putida*, and GFP-tagging of *Y. pestis*. It will thus

find wide-spread biomedical and environmental applications, especially in environments where plasmids and antibiotic selection are not feasible, namely plant and animal models or biofilms.

2.2 INTRODUCTION

Over 150 bacterial genomes have been sequenced, but tools for genetic and expression analyses have not kept up with the "sequencing pace". Such tools are either scarce or non-existing for many of these bacteria, or exhibit a narrow host range. The result is that much of the information contained in bacterial genomes cannot be exploited to their fullest potential. Tn*7*-based vectors show the most promise bringing us a step closer to development of a broad-range bacterial cloning and expression system, both in terms of host-range capabilities (Tn*7* was shown to transpose in at least 20 bacterial species thus far ((Craig, 1989, 1996), Pubmed and our results) and ease of use. The molecular biology of Tn*7* is well understood (Craig, 1996, Peters et al., 2001). Whereas most transposable elements move at low frequencies and display little target site-selectivity, Tn*7* is distinguished by its ability to insert at a high frequency into bacterial chromosomes site- and orientation-specifically at Tn*7* attachment, or *att*Tn*7*, sites. These sites are located downstream of highly conserved *glmS* genes, encoding essential glucosamine-6-phosphate synthetase (Craig, 1996, Peters et al., 2001), but recognition by the transposase also requires critical portions of the *glmS* 3' region (Peters et al., 2001). Tn*7* transposition minimally requires the Tn*7* left (Tn*7*L) and Tn*7* right (Tn*7*R) ends, and the transposase complex, which is comprised of the products of five genes, *tnsABCDE*. TnsABC+D catalyzes high-frequency transposition to *att*Tn*7*, whereas TnsABC+E directs transposition at much lower frequencies to chromosomal non-*att*Tn*7* sites and, preferentially, conjugative

plasmids (Peters et al., 2001). Elements containing <200 bp of Tn*7*L and Tn*7*R still transpose at relatively high (10^{-3} to 10^{-4}) frequencies in the presence of a helper plasmid expressing the transposase (McKnown et al., 1988). Transposon delivery and transient expression of transposase is achieved with the help of suicide plasmids, which only persist transiently in the target bacterium because it cannot support plasmid replication. Here we describe a series of small, fully sequenced mini-Tn*7* vectors which contain selection markers that, after transposition, can be excised from the bacterial chromosome with yeast Flp recombinase (Hoang et al., 1998). We demonstrate efficient mini-Tn*7* transposition into diverse target bacteria for purposes of gene complementation and expression in biofilms and an animal model, as well as establishment of regulated gene expression systems.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains and media. We grew *P. aeruginosa* PAO1(Holloway, 1955), its Δ(*mexAB-oprM*) Δ(*mexCD-oprJ*) derivative PAO238 (Chuanchuen et al., 2001), *P. putida* KT2440 (American Type Culture Collection strain 47054) and *Burkholderia thailandensis* E264 (Brett et al., 1998) strains in LB broth (Becton Dickinson & Co.). *Yersinia pestis* strains Kim6+, its *pgm* derivative A1122 (biovar Medievalis) and AZ96-2456 (biovar Orientalis), all from the CDC-Fort Collins collection, were grown in heart infusion (HI) broth (Difco). We added antibiotics to media as follows (in μg ml^{-1}): for *E. coli*, ampicillin (Ap), 100; Gm, 15; Km, 35; for *P. aeruginosa*, carbenicillin (Cb), 200; Gm30; for *P. putida*, Cb, 1000; Gm, 30; for *B. thailandensis*, Gm, 450; and for *Y. pestis*, Km, 35. β-galactosidase phenotypes were assessed on agar plates supplemented with 40 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-

β–D-galactopyranoside (XGal), with or without 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). We grew biofilms in flow cells in FAB medium in the presence of 2 μg/ml azithromycin (AZM) and analyzed them as previously described(De Kievit et al., 2001).

2.3.2 Plasmid and transposon construction. Standard molecular biology procedures were used (Sambrook et al., 2001). Individual plasmids and transposons were constructed as following. The mini-Tn*7* base vector pUC18-mini-Tn*7* was constructed as follows: a blunt-ended 1,726-bp *Sac*I-*Xba*I fragment from pTJ1R (Hojberg et al., 1999, a gift from J. Wall, Univ. of Missouri, Columbia) was cloned between the *Pvu*II sites of pUC18 to form pUC18-mini-Tn*7*-Km. The nucleotide sequences of the pUC18-Tn*7* junctions were determined by using primers KM-up and KM-down. A blunt-ended 1,245-bp *Sal*I-*Pst*I fragment was then deleted from this plasmid and replaced with a synthetic polylinker to form pUC18-mini-Tn*7*. The polylinker was derived by annealing PL-1 and PL-2. To generate pUC18-mini-Tn*7*T, the phage λ T_o and the *E. coli rrnB* T_1 terminators were excised as a 352-bp *Xho*I-*Sac*I fragment from pPS1427 (laboratory collection), blunt-ended with T4 DNA polymerase and ligated into the blunt-ended *Nsi*I site of pUC18-mini-Tn*7*. A mobilizable pUC18-mini-Tn*7*T derivative was obtained in several steps: first a unique *Pvu*II site was engineered downstream of Tn*7*L using primers Pvu-up, Pvu-Down and inverse PCR with pUC18-mini-Tn7T as the template. Restriction of the 3.25 kb kb PCR fragment with *Pvu*II and self-ligation yielded pUC18Pv-mini-Tn*7*T. Next, a blunt-ended 268-bp *Sac*II-*Hin*dIII fragment containing the *oriT* from pGEM-T-*oriT* (laboratory collection) was inserted into the *Pvu*II site of pUC18Pv-mini-Tn7T to derive pUC18T-mini-Tn*7*T. pUC18R6K-mini-Tn*7*T was constructed as follows: the

ori$_{R6K}$ was amplified from pUX-BF13(Bao et al., 1991) using primers R6K-F-AgeI and R6K-R-SacII. The 394 bp PCR fragment was cloned into pCR2.1 (Invitrogen) to form pPS1444. A blunt-ended 381 bp *Age*I-*Sac*II fragment from pPS1444 was then ligated between the blunt-ended *Alw*NI-*Afl*III sites of pUC18-mini-Tn*7*T; this replaced the *ori*$_{pMB9}$ with *ori*$_{R6K}$. The mobilizable pUC18R6KT-mini-Tn*7*T was derived from pUC18R6K-mini-Tn*7*T by first creating a *Pvu*II site in the plasmid backbone, followed by insertion of *oriT*, utilizing the primers and strategies described above for creation of pUC18T-mini-Tn*7*T.

Several mini-Tn*7* derivatives with selectable markers were created as follows: the *aacC*1 gene was excised from pPS856 (Hoang et al., 1998) on a blunt-ended 1,053-bp *Sac*I fragment and cloned into the *Eco*RV sites of pUC18-mini-Tn*7*T or pUC18T-mini-Tn*7*T to form pUC18-mini-Tn*7*T-Gm and pUC18T-mini-Tn*7*T-Gm. To generate pUC18R6K-mini-Tn*7*-Km, a blunt-ended 1,206-bp *Sac*I Km^r *FRT* cassette from pFKM1 was inserted into the *Eco*RV site of pUC18R6K-mini-Tn*7*T. The Gateway®-compatible pUC18-mini-Tn*7*T-Gm-GW was constructed by inserting a 1,755 bp *Hin*dIII-*Kpn*I Gateway® conversion fragment (Invitrogen) between the same sites of pUC18-mini-Tn7T-Gm.

To obtain the pUC18-mini-Tn*7*T-Gm-*lacZ* fusion vector, a promoter-less 3,315 bp *lacZ* fragment from pUC18-*lacZ* (laboratory collection) was ligated into the *Stu*I site of mini-Tn*7*T-Gm. The mini-Tn*7*-*lacZ* construct contains a RNase III processing site between the MCS and *lacZ*, which allows construction of transcriptional fusions with independent translation of *lacZ* (Linn et al., 1990). The pUC18-mini-Tn*7*-Gm-*lux* fusion vector was derived in two steps. First, a 6,791 bp *Hin*dIII(blunt)-*Kpn*I fragment from pUC-*lux* (Schweizer et al., 2000) was cloned between the *Sma*I and *Kpn*I sites of pUC18-mini-Tn*7*-Gm to form pUC18-mini-Tn*7*-

Gm-*lux*. Second, pUC18-mini-Tn*7*T-Gm-*lux* was obtained by cloning a 7,006 bp *Bam*HI-*Kpn*I fragment from pUC18-mini-Tn*7*-Gm-*lux* between the same sites of pUC18-mini-Tn*7*T-Gm. For construction of the pUC18-mini-Tn*7*T-LAC expression vector, the *lacI*^{q}-P$_{tac}$ region was PCR-amplified from pVLT35 (de Lorenzo et al., 1993) using primers lacIqPtac-DN and lacIqPtac-UP. The 1,497 bp PCR fragment was cloned into pCR2.1 to create pPS1473. Next, a 1,487 bp *Nsi*I-*Sac*I fragment from pPS1473 was cloned between the *Nsi*I and *Sac*I sites of pUC18-mini-Tn*7*T-Gm to generate pUC18-mini-Tn*7*T-LAC. pPS1477 containing the *xylE* reporter gene under *lacI*^{q}-P$_{tac}$ control was obtained by cloning a 1,365 bp *Kpn*I-*Xho*I fragment from pX1918 (Schweizer, 1993a) between the same sites of pUC18-mini-Tn*7*T-LAC. pPS1450 contains the PAO1 *nfxB*$^+$-*mexC*$^+$*D*$^+$-*oprJ*$^+$ genes on a 9,923 bp *Bam*HI fragment from pKMJ002 (Chuanchuen et al., 2001) cloned into the *Bam*HI site of pUC18-mini-Tn*7*T-Gm. pUC18-mini-Tn*7*-Gm-LacZΔM15 was created by subcloning a 4,400 bp *Hin*dIII-*Spe*I fragment from mini-CTX-LacZΔM15 (Schweizer et al., 2001) between the same sites of pUC18T-mini-Tn*7*-Gm. pUC18T-mini-Tn*7*T-Gm-REP containing the *ori*$_{R6K}$ replicon on the transposon was obtained by ligating a 454 bp *Bam*HI-*Eco*RV fragment from pPS1444 between the *Bam*HI-*Stu*I sites of pUC18-mini-Tn*7*T-Gm. To derive pPS1504 containing GFP transcribed from the promoter of the *neo* (Km^r) gene, a 789 bp *Hin*dIII-*Sac*I fragment containing GFP*mut3* (Cormack et al., 1996) was cloned between the same sites of pPS1418. Next, pPS1513 carrying a mini-Tn*7*T-Km-GFP was constructed by ligating a 1,845 bp *Eco*RV-*Hin*dIII fragment between the same sites of pUC18R6K-mini-Tn*7*T.

We assembled the mini-Tn*7* elements on pUC18 or its mobilizable derivatives which contain either the original ColE1-derived (pUC18T) or the R6K replicon (pUC18R6KT). The Tn*7* helper plasmid pTNS1 was derived from pUX-BF13 (Bao

et al., 1991) by deletion of a 909 bp *Cla*I fragment from within the *tnsE* 5' coding sequences. For pTNS2, we cloned a 6,698 bp *Eco*RI-*Cla*I(blunt) fragment from pTNS1 between the *Eco*RI-*Sma*I sites of pUC18R6KT, in which the *tnsA,B,C* and *D* genes are constitutively transcribed from the *E. coli lac* operon promoter.

The *FRT* cassette vector pFRT2 was derived from pPS854 (Hoang et al., 1998) by filling in the *Eco*RI site with T4 DNA polymerase. To derive pFCM1, the *cat* gene was PCR amplified from pUC18CMR (Schweizer, 1988) on a 906 bp PCR fragment using CAT-Up and CAT-Down and cloned into pCR2.1 (Invitrogen) to yield pPS1484. The *Eco*RI site located in *cat* was mutated using the Stratagene QuikChange® site-directed mutagenesis kit and mutagenic primer ECO-CAT. Finally, a 892-bp *Sma*I fragment from the resulting pPS1381 was cloned into the *Eco*RV site of pFRT2 to yield pFCM1. To construct pFKM1, the *Hin*dIII, *Nru*I, *Sma*I and *Xho*I sites were eliminated from the *neo* gene of pUC4K (Taylor et al., 1988) by site-directed mutagenesis and using HIN-KM, NRU-KM, SM-KM and XHO-KM. The *neo* gene was then PCR-amplified from the resulting pPS1428 on a 992-bp fragment using Kan-Up and Kan-Down. This 992 bp fragment was cloned into pCR2.1 to yield pPS1418. Finally, a 980 bp *Sma*I fragment from pPS1418 was ligated into the *Eco*RV site of pPS1387 to form pFKM1. The Gm^r conferring pFGM1 was constructed by ligating a 830 bp blunt-ended *Sac*I fragment from pUCGM (Schweizer, 1993b) into the *Eco*RV site of pFRT2. pFTC1, containing a Tc^r marker was constructed by subcloning a blunt-ended 1,775-bp *Hin*dIII-*Pst*I fragment from pPS875 (laboratory collection) into the *Eco*RV site of pFRT2. Similarly, the Tp^r cassette vector pFTP1 was obtained by cloning a blunt-ended 611-bp *Eco*RI fragment from p34E-Tp (Schweizer et al., 1996) into the *Eco*RV site of pFRT2. To create the Flp expression vector pFLP3, a 1,633 bp *Xba*I fragment from pPS880 containing the *tet* gene was

cloned between into the *Xba*I site of pFLP2 (Hoang et al., 1998). In *E. coli*, plasmids were selected on media supplemented with 25 μg ml^{-1} chloramphenicol (pFCM1), 35 μg ml^{-1} kanamycin (pFKM1); 15 μg ml^{-1} gentamycin (pFGM1); 10 μg ml^{-1} tetracycline (pFTC1) and 100 μg ml^{-1} trimethoprim (pFTP1).

2.3.3 Tn*7* delivery and stability of recombinant traits. Mini-Tn*7* vectors were either delivered by four-parental matings or by co-electroporation of bacteria with mini-Tn*7* delivery vector and helper plasmid. For delivery of mini-Tn*7* vectors by four-parental matings, SM10(λ*pir*) (Miller et al., 1988) /pTNS1, HB101/pRK2013 (Km^r) (Figurski et al., 1979), *E. coli* harboring the respective mini-Tn*7* delivery plasmid and the respective recipient strains were grown overnight at 37°C in LB, supplemented with antibiotics for plasmid-containing strains. 0.1 ml of each culture was added to a microcentrifuge tube with 0.6 ml LB, the cells were pelleted, washed twice with 1 ml LB and then resuspended in 30 μl LB medium. The conjugation mixture was placed on a filter membrane on nonselective LB agar and incubated at 37°C for ~5 h. The filter was then placed in 0.2 ml of 0.9% (w/vol) NaCl, cells were dislodged by vortexing and 0.1 ml aliquots were plated on selective agar plates. For Tn*7* delivery into *P. aeruginosa*, competent cells were prepared and electroporated utilizing a method whose details will be published elsewhere (Choi et al., 2006). Briefly, 6 ml of an overnight culture grown in LB medium was harvested by centrifugation at room temperature for 1 min. After washing each cell pellet twice with 1 ml of room temperature 300 mM sucrose, they were resuspended in a combined total of 100 μl 300 mM sucrose. For electroporation, 50 ng of suicide delivery and helper plasmid DNA was added to 100 μl of electrocompetent cells and the mixture was transferred to a 2 mm gap width electroporation cuvette. After applying a pulse (settings: 25 μF;

200 Ohm; 2.5 kV), 1 ml of LB medium was added, cells were transferred to a tube and shaken for 2 h at 37°C. The entire mixture was then plated on two LB plates with 30 μg ml^{-1} Gm. Published procedures were used for electroporation of *P. putida* (Smith et al., 1989) and *Y. pestis* (Conchas et al., 1990).

A published procedure was used for Flp recombinase-mediated marker excision (Hoang et al., 1998) with minor modification. Instead of biparental mating with pFLP2-harboring conjugable *E. coli* SM10, pFLP2 plasmid was electroporated into the Tn*7* integrants containing antibiotic resistance marker flanked by *FRT* sites. The stability of recombinant traits was monitored as detailed in the legend to Fig. 2.8.

2.3.4 Determination of Tn*7* insertion sites. We used colony PCR (Hoang et al., 1998) to verify chromosomal Tn*7* insertions in bacteria for which the genome sequences were known. Common primers used for all bacteria were Tn*7*L and Tn*7*R. Their locations of priming sites are shown in Fig. 2.5 and primer sequences are provided in Table 2.1. Bacteria-specific primers were *glmS*-down and *glmS*-up for *P. aeruginosa*; put-*glmS*UP and put-*glmS*DN for *P. putida*; and GlmSDn2 and PstSUp2 for *Y. pestis*. To determine the mini-Tn*7* insertion site in *B. thailandensis* E264 (Brett et al., 1998), for which no genome sequence was available at the beginning of this study, the following strategy was used. First, mini-Tn*7*T-Gm-REP containing the ori_{R6K} on the transposon was transposed to the chromosome of strain E264R, a spontaneous rifampicin-resistant E264 derivative, after conjugal transfer from *E. coli* via four-parental mating. Second, genomic DNA was isolated from Gm^r colonies and digested with either *Pst*I or *Xho*I, which do not cut within the transposed sequences. Third, after self-ligation of chromosomal fragments, *E. coli* CC118(λ*pir*) was transformed to Gm^r to recover ori_{R6K}-containing plasmids. Fourth, flanking

Table 2.1. Primers used in this study

Primer Name	Sequences (5' ---- 3')
Km-up	CAGACAGTTTTATTGTTCATGATG
Km-down	ACCAACTGGTCCACCTACAAG
PL-1	TCGACGATATCATGCATGAGCTCACTAGTGGATCCCCCGGGCTGCAGGAATTCCTCGAGAAGCTTGGGCC CGGTACCTCGCGAAGGCCTTGCA
PL-2	AGGCCTTCGCGAGGTACCGGGCCCAAGCTTCTCGAGGAATTCCTGCAGCCCGGGGGATCCACTAGTGAGC TCATGCATGATATCG
Pvu-up	CACAGAATCAGCTGATAACGCAGGA (*Pvu*II site is underlined)
Pvu-down	GCCTTTGAGTCAGCTGATACCGCT (*Pvu*II site is underlined)
R6K-F-AgeI	ATTAACCGGTTGTCAGCCGTTAAGTGTTCCT (the *Age*I site is underlined)
R6K-R-SacII	ATTACCGCGGCAGTTCAACCTGTTGATAGTAC (the *Sac*II site is underlined)
lacIqPtac-DN	CTTACATGCATTGCGTTGCGC (the *Nsi*I site is underlined)
lacIqPtac-UP	GGTACCGAGCTCGAATTCTGT (the *Sac*I site is underlined)
Cat-Up	AGCCCCGGGCCAACTTTTGG (*Sma*I site is underlined)
Cat-Down	CGACGCCCGGGTCGAATTTG (*Sma*I site is underlined)
ECO-CAT	GAATGCTCATCCGGAGTTCCGTATGGCA (single base change is underlined)
HIN-KM	AATGCATAAGCTCTTGCCATTCTCACCGG (single base change is underlined)

Table 2.1. Primers used in this study (cont.)

Primer Name	Sequences (5' ---- 3')
NRU-KM	GGTATAAATGGGCACGCGATAATGTCGGG (single base change is underlined)
SM-KM	CTGCGATCCCCGGCAAAACAGCATTC (single base change is underlined)
XHO-KM	AAACGTCTTGCTCCAGGCCGCGATTAAATTC (single base change is underlined)
Kan-Up	GGGGGCCCGGGAAAGCCAC (*Sma*I site is underlined)
Kan-Down	TCCCGGGAAGTCAGCGTAATG (*Sma*I site is underlined)
Tn*7*L	ATTAGCTTACGACGCTACACCC
Tn*7*R	CACAGCATAACTGGACTGATTTC
glmS-down	GCACATCGGCGACGTGCTCTC
glmS-up	CTGTGCGACTGCTGGAGCTGA
put-*glmS*UP	AGTCAGAGTTACGGAATTGTAGG
put-*glmS*DN	TTACGTGGCCGTGCTAAAGGG
GlmSDn2	ACGCCACCGGAAGAACCGATACCT
PstSUp2	GCTATACGTGTTTGCTGATCAAGATGC
*glmS*1-DN	GTTCGTCGTCCACTGGGATCA
*glmS*2-DN	AGATCGGATGGAATTCGTGGAG

sequences were determined by nucleotide sequencing using these plasmids as templates, and Tn*7*L and Tn*7*R as sequencing primers. After determination of the flanking sequences for the two *glmS* genes present in this bacterium, the two PCR primers *glmS*1-DN and *glmS*2-DN were designed to determine Tn*7* insertions either downstream of *glmS1* or *glmS2* in combination with Tn*7*L.

2.3.5 Animal experiments and FACS analysis.

We injected mice IP with 3 x 10^5 A1122-GFP or phosphate-buffered saline (PBS; control). After 30 min, we harvested peritoneal exudate cells and washed them extensively in FACS buffer (PBS, 2% fetal bovine serum, 0.05% sodium azide). Cells were fixed in 1% paraformaldehyde, then washed and resuspended in FACS buffer. We analyzed viable cells, as determined by forward and side scatter, on a Cyan MLE flow cytometer using Summit software (Dako Instrumentation, Fort Collins, CO).

2.3.6 Catechol-2,3-dioxygenase expression. For measurement of C23O activity, we grew cells in LB medium to mid-logarithmic phase (A_{600nm} ~ 0.5), then measured and calculated C23O activity as previously described (Karkhoff-Schweizer et al., 1994).

2.3.7 GenBank accession numbers. GenBank accession numbers for all plasmids are provided in Table 2.2.

2.4 RESULTS

Table 2.2. Plasmids vectors derived in this study and GenBank accession numbers

Plasmid	Accession No.	Pertinent Features
Flp Expression vector		
pFLP3	AY597273	Ap^r, Tc^r; Source of Flp recombinase
***FRT* cassette vectors**		
pFRT2	AY597269	Ap^r; *FRT* cassette base vector
pFCM1	AY597271	Ap^r, Cm^r; Source of Cm^r *FRT* cassette
pFGM1	AY597270	Ap^r, Gm^r; source of Gm^r *FRT* cassette
pFKM1	AY597272	Ap^r, Km^r; source of Km^r *FRT* cassette
pFTC1	AY712950	Ap^r, Tc^r; source of Tc^r *FRT*cassette
pFTP1	AY712951	Ap^r, Tp^r; source of Tp^r *FRT* cassette
mini-Tn*7* vectors		
pUC18-mini-Tn*7*	AY599226	Ap^r; ColE1 replicon; mini-Tn*7* base vector
pUC18-mini-Tn*7*T	AY599227	Ap^r; ColE1 replicon
pUC18Pv-mini-Tn*7*T	AY599229	Ap^r; pUC18-mini-Tn7T with unique *Pvu*II site in pUC18 DNA
pUC18T-mini-Tn*7*T	AY599230	Ap^r; pUC18Pv-mini-Tn7T with *oriT* in *Pvu*II site
pUC18R6K-mini-Tn*7*T	AY599228	Ap^r; R6K replicon
pUC18R6KT-mini-Tn*7*T	AY712953	Ap^r; R6K replicon and *oriT*

Table 2.2. Plasmids vectors derived in this study and GenBank accession numbers (cont.).

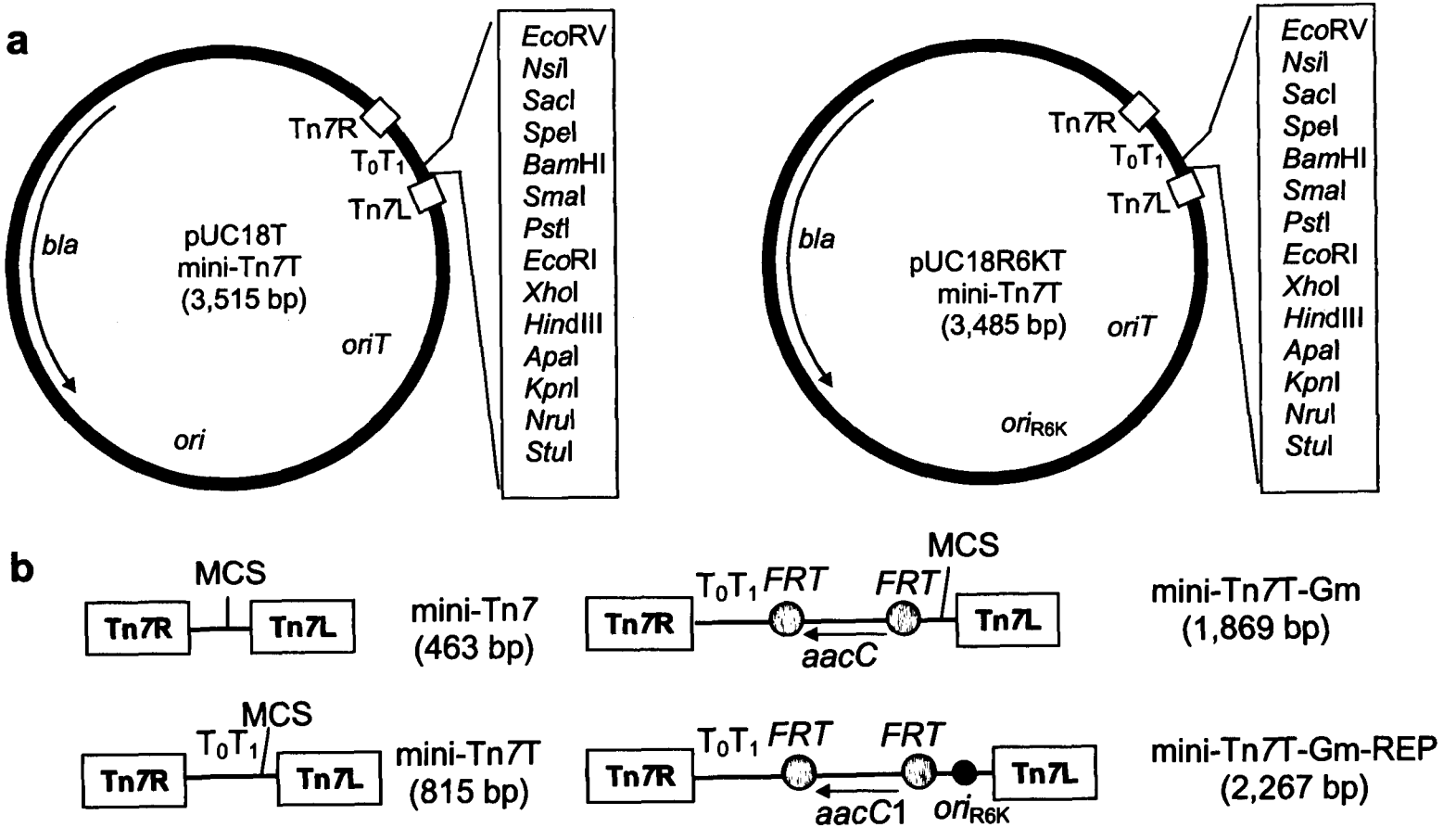
Plasmid	Accession No.	Pertinent Features
pUC18-mini-Tn*7*-Gm	AY619004	Ap^r; Gm^r on mini-Tn*7*
pUC18-mini-Tn*7*T-Gm	AY599231	Ap^r; Gm^r on mini-Tn*7*T
pUC18T-mini-Tn*7*T-Gm	AY599232	Ap^r; Gm^r on mini-Tn*7*T; *oriT* on pUC18
pUC18-mini-Tn*7*T-Gm-GW	AY737004	Ap^r; Gm^r, Cm^r on mini-Tn*7*T; Gateway® destination vector
pUC18R6K-mini-Tn*7*T-Km	AY619006	Ap^r; Km^r on mini-Tn*7*T
pUC18-mini-Tn*7*T-Gm-*lacZ*	AY599233	Ap^r; Gm^r on mini-Tn*7*T; *lacZ* transcriptional fusion vector
pUC18-mini-Tn*7*T-Gm-*lux*	AY962893	Ap^r; Gm^r on mini-Tn*7*T; *luxCDABE* transcriptional fusion vector
pUC18-mini-Tn*7*-Gm-LacZΔM15	AY619005	Ap^r; Gm^r on mini-Tn*7*T; construction of host strains for P_{lac}-containing plasmids and blue/white screening
pUC18-mini-Tn*7*T-LAC	AY599234	Ap^r; Gm^r on mini-Tn*7*T; construction of strains with chromosomal P_{tac} expression cassette
pUC18T-mini-Tn*7*T-Gm-REP	AY712952	Ap^r; Gm^r and ori_{R6K} on mini-Tn*7*T; *oriT* on pUC18
Helper plasmid		
pTNS1	NA[a]	Ap^r; R6K replicon; encodes the TnsABC+D specific transposition pathway
pTNS2	AY884833	Ap^r; R6K replicon; encodes the TnsABC+D specific transposition pathway

[a]NA, not applicable; complete sequence unknown

2.4.1 Delivery vectors, mini-Tn*7* elements and helper plasmids. The fullest potential of previously devised Tn*7* systems could not be fully exploited because their individual components were not engineered for general applicability (Bao et al., 1991, Hojberg et al., 1999, Klausen et al., 2003, Koch et al., 2001). We therefore developed optimized delivery vectors, mini-Tn*7* elements and helper plasmids.

We constructed two suicide mini-Tn*7* delivery vectors in this study (Fig. 2.1a). The pUC18T-based vectors contain the ColE1 replicon and do not replicate outside of the *Enterobacteriaceae*. They thus serve as efficient suicide delivery vectors in many bacteria. On the other hand, pUC18TR6K-based vectors contain the R6Kγ origin of replication and, therefore, depend on the presence of the *pir* gene for replication. Thus, they allow suicide delivery in all bacteria, including the *Enterobacteriaceae* and closely related species(Miller et al., 1988).

We derived mini-Tn*7*T (Fig. 2.1b) from mini-Tn*7* (Fig. 2.2) by insertion of two strong transcriptional terminators to prevent read-through from the *glmS* promoter after chromosomal insertion and a multiple cloning site (MCS) for cloning of antibiotic selection markers, reporter genes and other DNA fragments destined for chromosomal insertion. Based on mini-Tn*7* and mini-Tn*7*T, an entire family of mini-Tn*7* elements was derived (Fig. 2.3 and Table 2.2), ranging from those containing simple selection markers (mini-Tn*7*T-Gm, Fig. 2.1b), to those allowing construction of *lacZ* and *lux* transcriptional fusions, strains for regulated expression from the *tac* promoter and self-cloning of *att*Tn*7* flanking sequences (mini-Tn*7*T-Gm-REP, Fig. 2.1b). The selection markers contained on the mini-Tn*7* elements are flanked by *FRT* sites and can therefore be excised from the chromosome with Flp recombinase after mini-Tn*7* insertion. Although we only tested gentamycin (Gm) and kanamycin (Km) resistance markers in this study, we generated a series of improved *FRT* cassettes with



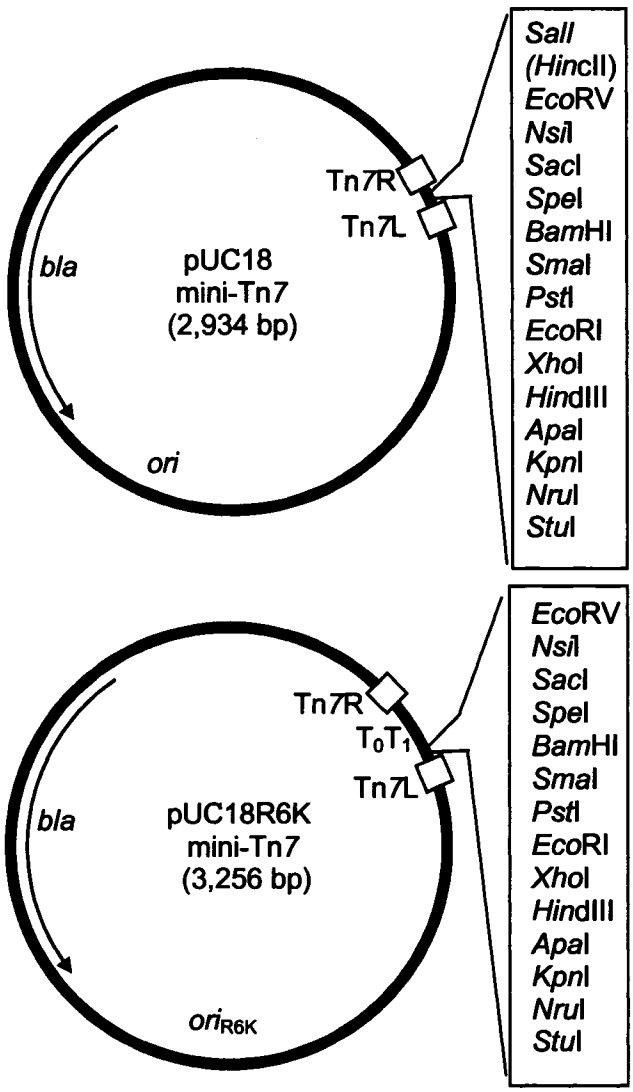
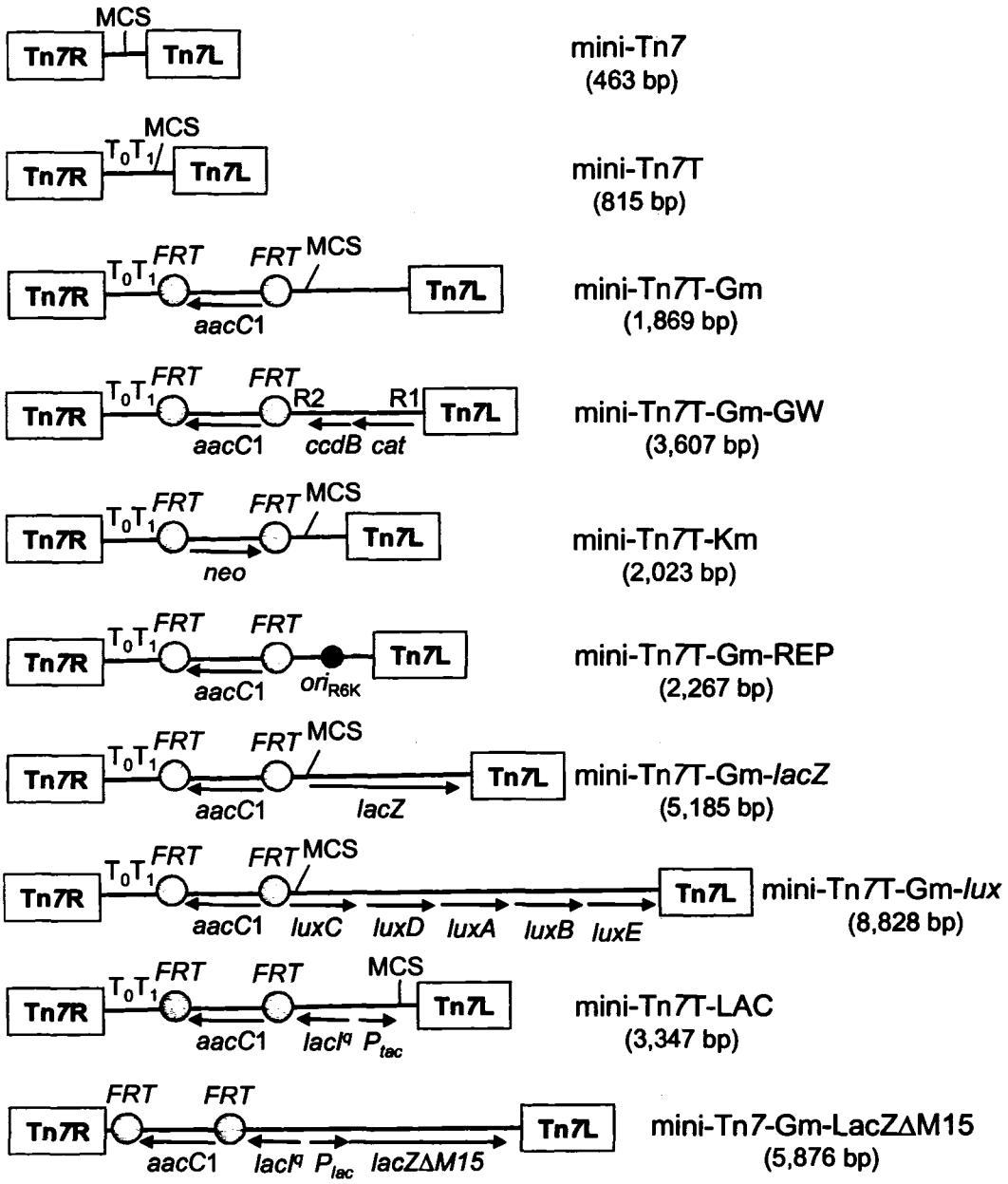


Fig. 2.2. Maps of mini-Tn*7* suicide delivery base vectors. The nucleotide sequences of both Tn*7* ends of a mini-Tn*7* element contained on the previously constructed pTJ1R (Hojberg et al. *Appl. Environm. Microbiol.* **65**, 4085-4093 (1999)) were determined and the sequences located between the Tn*7* ends were then replaced with a synthetic polylinker to generate the base vector pUC18-mini-Tn*7*. pUC18R6K was generated by replacing the ColE1 origin of replication (*ori*) with the R6K replication origin (ori_{R6K}). Other abbreviations: *bla*, β-lactamase encoding gene; Tn*7*L and Tn*7*R, left and right end of Tn*7*, respectively.



chloramphenicol (Cm), tetracycline (Tc) and trimethoprim (Tp) markers for use in other bacteria (Table 2.2).

The most commonly used helper plasmid pUX-BF13 encodes the TnsABC+D specific, as well as the TnsABC+E non-specific transposition pathways. To eliminate potential complications caused by the superfluous non-specific pathway, we constructed pTNS1, a mobilizable helper plasmid encoding only the specific TnsABC+D transposition pathway. Although we mostly used pTNS1 for the studies described here, we are now also using a pTNS1 derivative, pTNS2, because its entire sequence is known and transposition efficiencies are slightly higher when compared to pTNS1.

2.4.2 Mini-Tn7 transposition in *P. aeruginosa*. A previous report indicated the use of Tn*7* derivatives for GFP-tagging in this bacterium (Klausen et al., 2003), but the vectors were very specialized, they did not allow for marker excision and the insertion site was not reported. In this study we used *P. aeruginosa* as a tester strain for our newly developed vectors, but methods for Tn*7* delivery, transposition into *att*Tn*7* and verification of transposition events are similar for other bacteria.

Electroporation delivery routinely yields >500 transformants per 50 ng each of mini-Tn*7* delivery vector and helper plasmid input DNAs. Southern analysis of 10 transformants confirmed that all Tn*7* insertions occurred in the same chromosomal DNA fragment (not shown) and we suspected the Tn*7* insertion site to lie in the 54 bp *glmS-PA5548* intergenic region (Fig. 2.4), as had been previously suggested (Koch et al., 2001). PCR analysis utilizing various primer pairs designed to the *glmS* region and Tn*7* ends verified this location and yielded the PCR fragments indicated in Fig. 2.4. Sequence analysis of PCR fragments obtained from two separate transformants

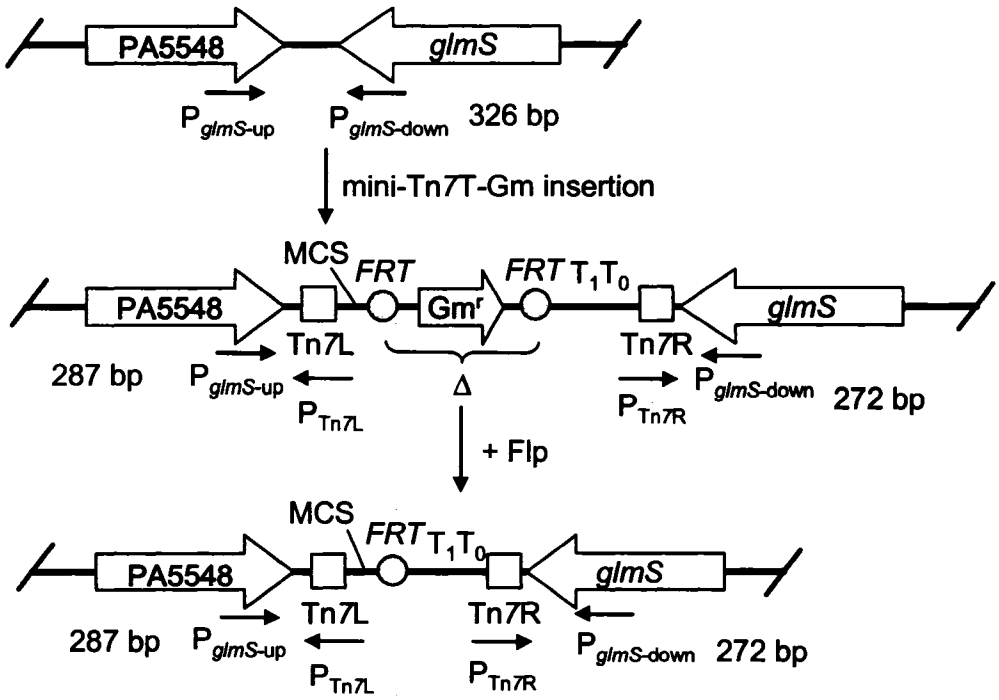


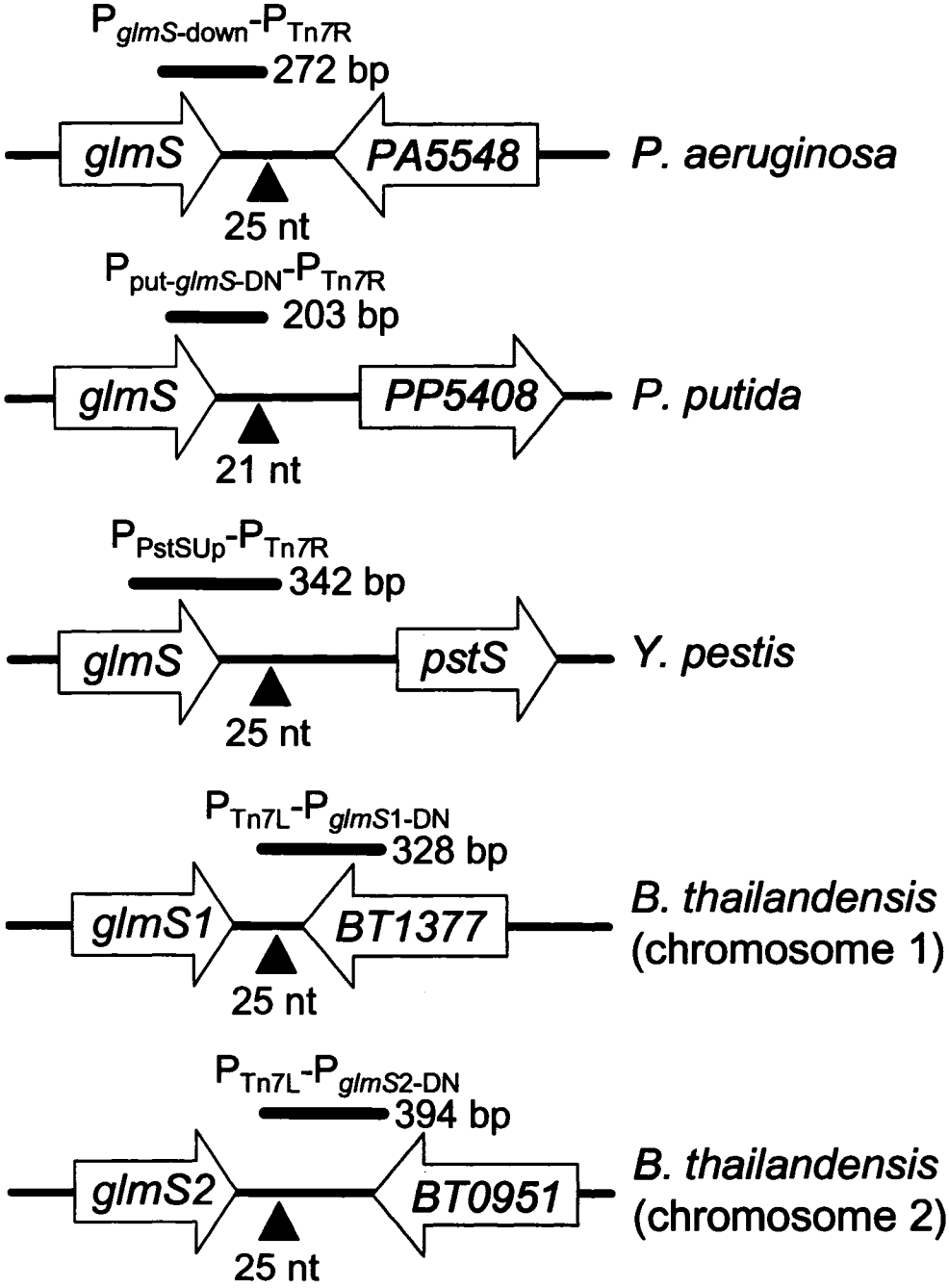
Fig. 2.4. Integration of mini-Tn*7* in *P. aeruginosa*. Mini-Tn*7*T-Gm was transposed into the PAO1 chromosome after co-electroporation of pUC18-mini-Tn*7*T-Gm and pTNS1, and Gm^r transformants resulting from transposition of mini-Tn*7*T-Gm into the *PA5548-glmS* intergenic region were selected. In most instances, the Gm^r marker was removed by Flp-mediated excision resulting in strains with unmarked mini-Tn*7* insertions.

Verification of transposition events by PCR using the primer pairs shown by convergent arrows yields PCR fragments whose sizes are indicated in bp. Integration of mini-Tn*7* in other bacteria follows the same general scheme and integration sites are schematically illustrated in Fig. 2.5.

after Flp excision of Gm^r revealed the insertions between nucleotides 24 and 25, and 25 and 26 downstream of the *glmS* stop codon (for details see GenBank accession number AY619007). These sites seem to lie in one of the inverted repeats of the putative *glmS* transcriptional terminator and most likely render it dysfunctional. We confirmed this by transposition of either mini-Tn*7*-Gm-*lacZ* (containing no transcriptional terminators) or mini-Tn*7*T-Gm-*lacZ* (containing the T_0 and T_1 terminators) into strain PAO1. While significant (267 Miller units) *lacZ* transcription could be observed in the strain containing mini-Tn*7*-*lacZ*, only background levels of transcription (10 Miller units) were measured in the strain containing mini-Tn*7*T-*lacZ*.

2.4.3 Use of mini-Tn*7* in other bacteria. The versatility of the various mini-Tn*7* constructs was tested by transposition into bacteria posing diverse challenges.

Since a previous report indicated that in *P. putida* strains Tn*7* derivatives did not transpose to a unique site (Staley et al., 1997), we chose this strain to address the issue of site-specific versus random transposition. Transposition of mini-Tn*7*-Gm into the *P. putida* chromosome was achieved by co-electroporation of competent KT2440 cells with pUC18-mini-Tn*7*-Gm and either pUX-BF13 or pTNS1 helper DNA. Genomic Southern and PCR analyses revealed that with pUX-BF13, of 10 Gm^r transformants tested 3 (or 30%) had clean insertions at the putative *att*Tn*7* site downstream of *glmS* in the 171 bp *glmS-PP5408* intergenic region (Fig. 2.5); 5 of 10 transformants (or 50%) had insertions at *att*Tn*7*, but some chromosome rearrangements around *att*Tn*7* were evident. Only 2 (or 20%) of the insertions tested were located outside of the *glmS* region. In contrast, when we used pTNS1 all insertions occurred at *att*Tn*7* and 60% of them were clean insertions 21+/-1 bp downstream of *glmS*, i.e., there was no evidence of chromosome rearrangements in



the *att*Tn*7* region, which were observed in the other 40%. However, the 60% clean *att*Tn*7* insertion success rate achieved is more than satisfactory given the fact that no site-specific chromosomal gene integration currently exists for this bacterium.

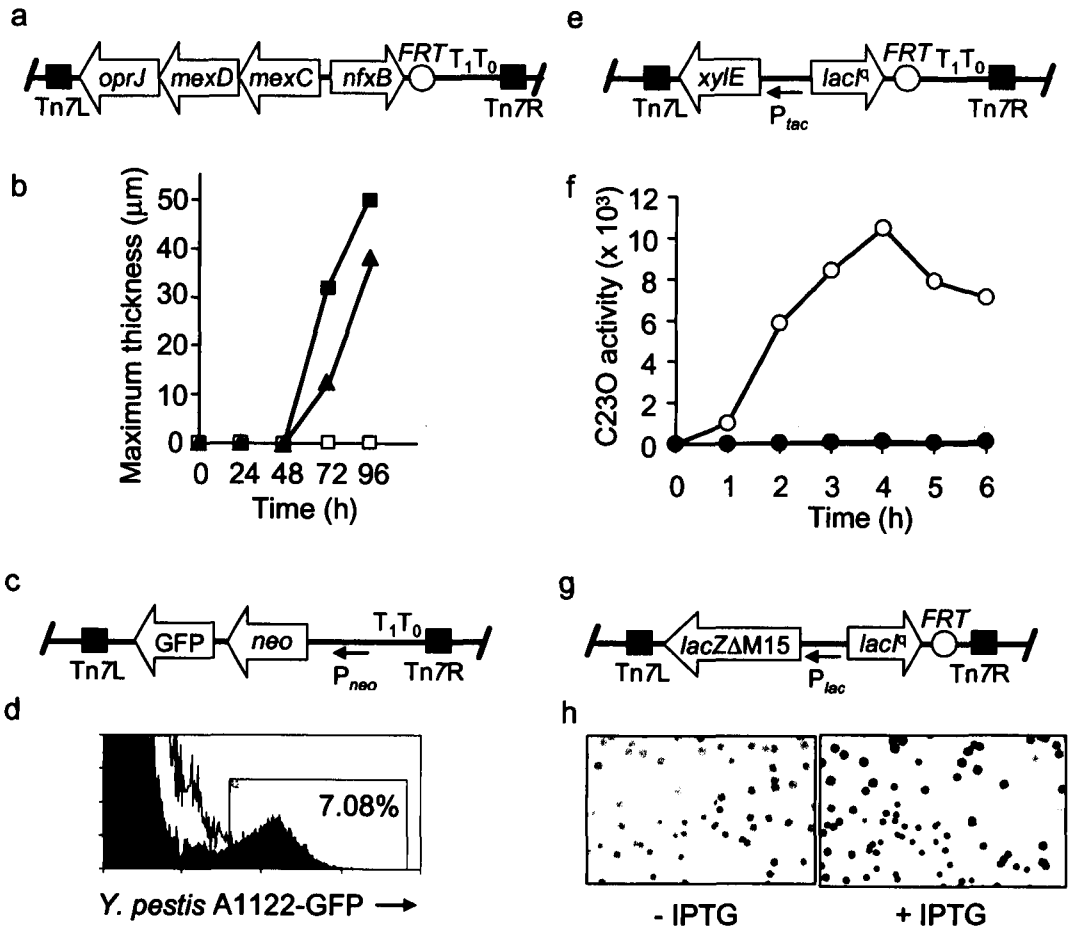
Y. pestis is on the select agent list because of bioterrorism and biowarfare concerns (Prevention, 2000). Although this bacterium can be genetically manipulated with relative ease, no site-specific gene integration system is currently available, which would be desirable for pathogenesis studies. We co-electroporated cells of two different biovars, KIM6 (Medievalis) and AZ96-2456 (Orientalis), with pUC18R6K-mini-Tn*7*T-Km and pTNS1. Genomic Southern and PCR analyses of 10 Km^r isolates of each strain indicated that Tn*7* transposed to a unique site in the chromosome of either biovar. Sequence analysis revealed that the insertion site was identical in each strain and located 25 nucleotides downstream of *glmS* in the 482 bp *glmS-pstS* intergenic region (Fig. 2.5).

Lastly, to prove the general usefulness of the new vectors for bacteria for which the genome sequences are yet unknown, incomplete or unpublished, we transposed a mini-Tn*7*T-Gm-REP derivative containing the ori_{R6K} into the genome of *B. thailandensis* strain E264. *B. thailandensis* is closely related to *B. pseudomallei* and *B. mallei*, the causative agents of melioidosis (Dance, 1996) and glanders (Howe, 1950), respectively, and thus can serve as a surrogate for these select agent bacteria. After self-ligation of DNA fragments containing the mini-Tn*7* element, thus forming plasmids containing ori_{R6K} and a Gm^r marker, we determined the *att*Tn*7* sites by sequencing the Tn*7*-chromosomal DNA junction sequences using P_{Tn7L} and P_{Tn7R} as sequencing primers. All insertions thus verified demonstrated that the *B. thailandensis* genome contains two closely-related *glmS* genes, *glmS*1 on chromosome 1 and *glmS*2 on chromosome 2. Tn*7* insertion occurred 25 bp

downstream of either *glmS*1 or *glmS*2 (Fig. 2.5), but none of the four transformants tested had insertions at both loci. We then verified the insertion sites by using Tn*7*L and a $glmS_{Bt}$-specific primer, i.e., either *glmS*1-DN or *glmS*2-DN. Despite two separate insertion sites, site-specific insertions can therefore be rapidly screened utilizing specific PCR primers which yield PCR fragments of defined sizes, depending on which *att* site is used.

2.4.4 Applications of mini-Tn*7* elements. Two major utilities for site-specific insertion elements are the ability to perform experiments in environments where antibiotic selection is not feasible and to expand the range of well-characterized gene expression systems to bacteria other than *E. coli* (Schweizer et al., 2004).

To prove stable complementation in the absence of selection, we performed two experiments. First, we integrated a mini-Tn*7* containing the cloned *nfxB*$^+$-*mexC*$^+$*D*$^+$-*oprJ*$^+$ genes into the chromosome of the Δ(*mexCD-oprJ*) *P. aeruginosa* strain PAO238 (Fig. 2.6, panel a) and showed that the cloned genes complemented its biofilm-deficient growth phenotype in the presence of subinhibitory azithromycin concentrations (Fig. 2.6, panel b; details to be presented elsewhere). Second, to demonstrate the utility of mini-Tn*7* for generation of GFP-tagged bacteria for *in vivo* tracking, we transposed the mini-Tn*7*T-Km-GFP into the *Y. pestis* A1122 chromosome (Fig. 2.6, panel c) and monitored the fate of A1122-GFP cells after intraperitoneal (IP) injection. It is evident that only 30 min after IP injection, 7% of peritoneal exudate cells (PECs) are positive for A1122-GFP (Fig. 2.6, panel d). This result shows that GFP-tagged *Y. pestis* bacteria rapidly associate with mouse PECs and that the signal from single-copy, chromosomally-located GFP is sufficiently strong for FACS detection.



To obtain proof-of-principle for the utility of mini-Tn*7* vectors for establishment of expression systems, we constructed several *P. aeruginosa* expression strains. To establish a regulated expression system which is entirely chromosomally-located, we cloned a promoter-less *xylE* reporter gene into the MCS of mini-Tn*7*T-Gm-LAC. After integration at PAO1 *att*Tn*7* and following Flp-mediated excision of Gm^r, we obtained a *P. aeruginosa* strain which contained a chromosomal P_{tac}-*xylE* expression cassette under control of the neighboring $lacI^q$ (Fig. 2.6, panel e). We then measured C23O expression in IPTG-induced and uninduced cells of this strain. Whereas uninduced cells expressed little C23O activity over the entire testing period, this activity was readily inducible and measurable in cells induced for as short as 1 h (Fig. 2.6, panel f). As previously demonstrated in *E. coli* and *P. aeruginosa* (Karkhoff-Schweizer et al., 1994, Schweizer, 1994), transposable elements and site-specific gene integration systems are also useful for construction of host strains enabling establishment of a regulated α-complementation and expression systems. When we transposed mini-Tn*7*-Gm-LacZΔM15 into the *P. aeruginosa* PAO1 chromosome (Fig. 2.6, panel g), the resulting strain allowed IPTG-dependent blue/white screening (Fig. 2.6, panel h) and IPTG-inducible *xylE* expression from P_{lac} (data not shown) after transformation with pUCP19 (vector) or its recombinant derivative pCDO (Karkhoff-Schweizer et al., 1994). We obtained similar data after transposition of mini-Tn*7*-Gm-LAC into *P. putida* KT2440 (data not shown).

2.5 DISCUSSION

Most existing bacterial chromosome integration systems are narrow-host range because they are either based on phage *attB* (Hoang et al., 2000, Kieser et al., 2000, Stover et al., 1991, Trieu-Cuot et al., 1991) or species-specific (Charpentier et

al., 2004) chromosomal integration sites. Though transposon-based systems can be broad-host-range, the resulting insertions tend to be random, e.g., Tn*1545* which integrates in various Gram-positive bacteria at diverse chromosomal *att* sites (Trieu-Cuot et al., 1991). Although random transposon delivery, e.g. via mini-Tn*5* (de Lorenzo et al., 1990), is generally widely applicable, it has several drawbacks. First, insertions occur randomly in the genome, most often within coding sequences. Consequently, considerable efforts need to be invested to determine the transposon insertion sites and the fitness of the mutant bacteria. Second, since most bacteria lack efficient chromosomal gene transfer procedures, integrated transposons cannot easily be transferred between different mutant backgrounds for meaningful comparative analyses. Site-specific integration vectors, like mini-Tn*7*, overcome many of these hurdles. First, because they integrate at an intergenic, naturally evolved site, compromise of bacterial fitness as a consequence of gene integration is generally of little or no concern. Second, the same constructs can be rapidly integrated in a site- and orientation specific manner into many different mutant backgrounds, thus facilitating comparative gene expression analyses. Possible drawbacks of site-specific integration systems include lack of integration sites in some bacteria or absence of host factors needed for integration.

The family of mini-Tn*7* vectors described here provides the ultimate in versatility in terms of suicide vector delivery (ori_{pMB9} and ori_{R6K}), delivery options (electroporation or conjugation), vector capabilities (small size with carefully engineered MCS and terminators to avoid read-through from *glmS*), choice of selection markers (engineered to be devoid of restriction sites and to be excisable *in vivo*) and availability of vector sequences from GenBank. Although we have not constructed all possible combinations of suicide delivery vectors and mini-Tn*7*

elements with diverse selection markers, we provide all the tools and strategies necessary to derive the appropriate vectors for the bacterium of choice.

When we used pTNS1 or pTNS2, insertions in bacteria with single chromosomes always occurred site-specifically at *att*Tn*7* sites associated with single *glmS* genes. *B. thailandensis* provided an example of a bacterium with two chromosomes, revealing two *glmS* genes and two *att*Tn*7* sites, further highlighting the close association of *glmS* and *att*Tn*7*. The presence of two *att*Tn*7* sites may offer the possibility of sequentially integrating two separate mini-Tn*7* constructs. This should be possible because although secondary Tn*7* transposition is subject to target immunity, Tn*7* immunity is not a global immunity, but is rather dependent on distance between separate insertion events (Stellwagen et al., 1997). However, insertion of two Tn*7*s into the same genome may lead to recombination events, which could be deleterious to the cell. Furthermore, immunity and structural similarities may complicate usage of Tn*7* elements in bacteria naturally harboring transposons of the superfamily that Tn*7* is a member of, but this situation has not yet been encountered.

Mini-Tn*7* elements integrate at an intergenic site and therefore probably have little effect on bacterial fitness. However, since *att*Tn*7* sites are located in the 3' end of the *glmS* mRNA, insertions could have an adverse effect on GlmS expression which possible could affect peptidoglycan synthesis. Fortunately, this does not seem to be the case because *P. aeruginosa* and *Y. pestis* wild-type and integrant strains recovered with equal efficiency from stationary phase, indicating that rates of peptidoglycan synthesis were not affected by the insertions (Fig. 2.7). Once integrated into the chromosome, expression or reporter gene constructs and complementing fragments are stable, and do not require continued antibiotic selection. When we grew *P. aeruginosa* and *Y. pestis* integrants in the absence of selection for 100 generations,

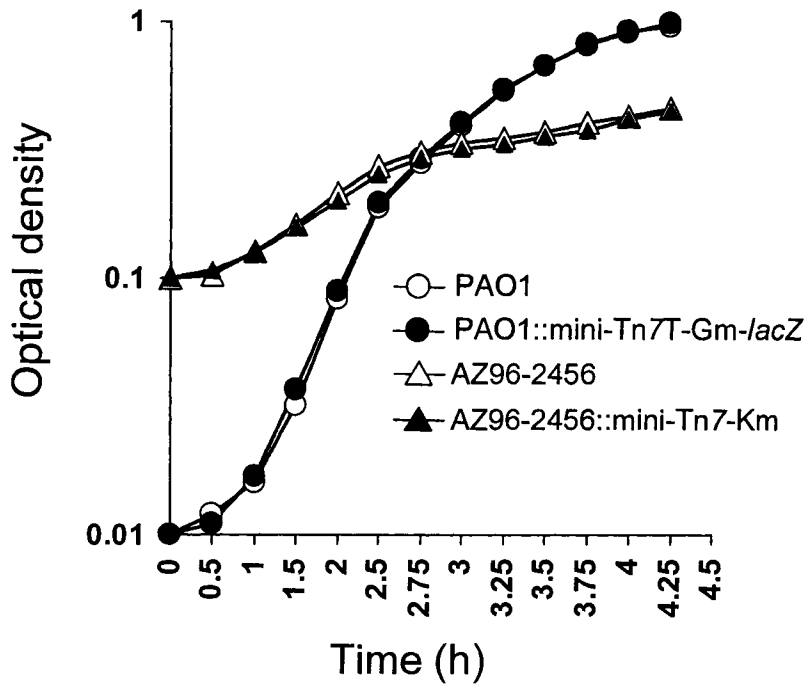


Fig. 2.7. Mini-Tn*7* insertion does not affect GlmS expression. *P. aeruginosa* PAO1 with or without a mini-Tn*7*T-Gm-*lacZ* insertion and *Y. pestis* AZ96-2456 with and without a mini-Tn*7*-Km insertion were pre-grown to saturation overnight in either LB at 37°C (*P. aeruginosa*) or HI at 30°C (*Y. pestis*) medium. The cultures were diluted into the same media to the indicated optical densities and growth was monitored by reading the optical densities at 540 nm (*P. aeruginosa*) or 600 nm (*Y. pestis*). The results show that the respective growth curves for were indistinguishable, indicating that mini-Tn*7* insertion does not significantly affect *glmS* mRNA stability and/or GlmS expression. GlmS expression is required for peptidoglycan synthesis and its impaired expression would therefore most likely be detectable by a difficulty of the respective insertions strains to recover from stationary phase and transition to exponential phase, a period of active peptidoglycan synthesis.

insertions were 100% stable (Fig. 2.8). This facilitates applications in environments where antibiotic selection is not feasible (e.g., animal and plant models, biofilms, etc.). Optional removal of the antibiotic resistance markers facilitates downstream applications, such as transformation with complementing plasmids or expression constructs, or allows construction of unmarked strains for applications where the presence of antibiotic resistance markers is disallowed or undesirable (e.g., vaccine strains and strains destined for environmental release).

The new vectors allow engineering of bacteria for various environmental and biomedical applications. First, we demonstrated the use of mini-Tn*7* for single-copy, i.e., as nature intended it to be, complementation in a biofilm, an environment in which plasmids are difficult to select. Second, we used the method for stable GFP-tagging of *Y. pestis* for studying dissemination of this bacterium from the initial site of infection. Third, as we demonstrated with *P. aeruginosa* and *P. putida,* the mini-Tn*7* vectors allow expansion of well-characterized and familiar host and regulated expression systems to bacteria other than *E. coli*, even those for which the necessary plasmid vectors are not available because the entire expression system can be engineered into the chromosome.

In this study, we focused on Gram-negative bacteria. However, since *glmS* genes are present in all sequenced bacterial chromosomes (Table 2.3), we predict that mini-Tn*7* elements will be applicable in many other species. Despite the universal presence of *glmS* genes in bacteria, however, Tn*7* transposition has not yet been documented in Gram-positive bacteria. This could be an indication of a possible limitation of the Tn*7* system or simply a lack of application and/or proper engineering of the system for use in these bacteria. In a few genomes, *glmS* either overlaps with or is separated by an intergenic region from the downstream gene that is shorter than the

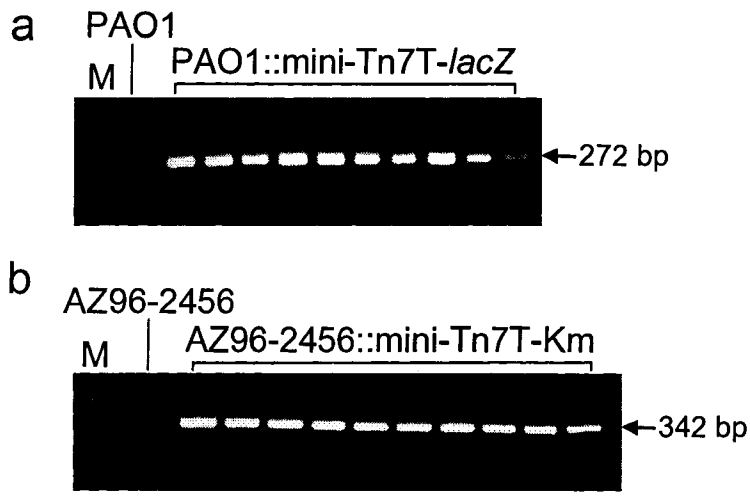


Fig. 2.8. Stability of mini-Tn*7* insertions in the absence of selection. *P. aeruginosa* PAO1 without and with a mini-Tn*7*T-Gm-*lacZ* insertion, and *Y. pestis* AZ96-2456 without and with a mini-Tn*7*-Km insertion were pre-grown overnight in LB-gentamycin medium at 37°C or in HI-kanamycin medium 30°C, respectively, to ensure starting with saturated cultures which retained 100% of the selection marker. The cultures were then diluted 1,000-fold and regrown to saturation in media without antibiotics. The procedure was repeated 5 times until the cells had gone through an estimated 100 generations. Here we assume that a saturated culture contains about 10^9 cells/ml. A 1,000-fold dilution and regrowth to saturation equals 20 generation (since $10^6 = 2^{20}$). The cultures were then plated for single colonies on non-selective plates and 100 colonies of each strain were tested for their ability to grow on selective plates. For PAO1::mini-Tn*7*T-Gm-*lacZ*, presence of *lacZ* was checked by plating ~1,000 cells on LB-gentamycin plates containing 40 μg/ml X-Gal. 100% of *P. aeruginosa* and *Y. pestis* recombinants maintained the expected phenotypic traits, i.e., Gm^r and $LacZ^+$ for PAO1::mini-Tn*7*T-Gm-*lacZ* and Km^r for AZ96-2456::mini-Tn*7*-Km. The physical presence of the respective mini-Tn*7* elements was then tested in 10 randomly selected integrants of each strain by colony PCR utilizing the primer pairs shown in Fig. 2.5 of the main text. The results show that even after prolonged absence of antibiotic selection the mini-Tn*7* elements are present in all derivatives tested. Lane M contained molecular weight markers. The positions and sizes of the expected PCR fragments are marked.

Table 2.3. *glmS* genes in annotated bacterial genomes.

Bacteria	*glmS*	Intergenic region (bp)[a]	Downstream gene (name)
Acinetobacter sp. ADP1	3,498,186..3,500,024	3,500,025..35,000,267 (243)	3,500,268..3,500,654 (*ACIAD3577*)
Agrobacterium tumefaciens str. C58 chromosome circular	1,770,656..1,772,482[b]	overlapping	1,770,156..1,770,659[b](*Atu1785*)
Anaplasma marginale str. St. Maries	916,492..918,318	918,319..918,684 (366)	918,685..919,824[b] (*dnaN*)
Aquifex aeolicus VF5	194,591..196,369	196,369 (1)	196,370..196,714[b] (*aq_302*)
Azoarcus sp. EbN1	1,314,666..1,316,492[b]	1,314,449..1,314,665 (217)	1,312,928..1,314,448 (*dhaL*)
Bacillus anthracis str. Ames	157,874..159,676	159,677..160,039 (363)	160,040..160,849 (*BA0160*)
Bacillus anthracis str. 'Ames Ancestor'	157,874..159,676	159,677..160,039 (363)	160,040..160,849 (*GBAA0160*)
Bacillus cereus ATCC 10987	157,876..159,678	159,679..160,104 (426)	160,105..162,642 (*BCE0159*)
Bacillus cereus ZK	157,864..159,666	159,667..160,002 (336)	160,003..160,839 (*BCZK0153*)
Bacillus clausii KSM-K16	271,118..272,920	272,921..273,164 (244)	273,165..273,989 (*ABC0246*)
Bacillus halodurans C-125	289,008..290,810	290,811..291,038 (228)	291,039..291,509 (*BH0269*)
Bacillus licheniformis ATCC 14580 (NC_006270)[c]	183,613..185,415	185,416..185,605 (190)	185,606..185,929 (*BL02705*)
Bacillus licheniformis ATCC 14580 (NC_006322)[c]	183,421..185,223	185,224..185,413 (190)	185,414..185,737 (*BLi00205*)
Bacillus subtilis subsp. *subtilis* str. 168	200,263..202,065	202,066..202,111 (46)	202,112..202,252 (*ybbU*)
Bacillus thuringiensis serovar konkukian str. 97-27	163,596..165,398	165,399..165,627 (229)	165,628..167,076[b] (*bla*)
Bacteroides fragilis NCTC 9343	3,098,428..3,098,589[b] (fragment?)	3,098,029..3,098,427 (399)	3,096,136..3,098,019[b] (*BF2659*)
Bartonella henselae str. Houston-1	1,093,818..1,095,641[b]	1,093,327..1,093,817 (491)	1,092,612..1,093,326 (*BH09910*)
Bartonella quintana str. Toulouse	904,333..906,156[b]	903,951..904,332 (382)	901,842..903,950 (*recG*)
Bdellovibrio bacteriovorus HD100	3,335,698..3,337,584[b]	overlapping	3,335,269..3,335,715 (*Bd3423*)
Bifidobacterium longum NCC2705	1,029,857..1,031,749[b]	1,029,781..1,029,856 (76)	1,029,079..1,029,780[b] (*BL1174*)
Bordetella bronchiseptica RB50	5,109,569..5,111,401[b]	5,109,370..5,109,568 (199)	5,108,389..5,109,369 (*BB4801*)

Table 2.3. *glmS* genes in annotated bacterial genomes (cont.)

Bacteria	*glmS*	Intergenic region (bp)[a]	Downstream gene (name)
Bordetella parapertussis 12822	4,546,564..4,548,396[b]	4,546,429..4,546,563 (135)	4,545,385..4,546,428 (*BPP4213*)
Bradyrhizobium japonicum USDA 110	5,102,052..5,103,878b[b]	5,101,973..5,102,051 (79)	5,101,202..5,101,972[b] (*bll4606*)
Brucella abortus biovar 1 str. 9-941 chromosome II	651,255..653,078	653,079..653,232 (154)	653,233..655,353[b] (*recG*)
Brucella suis 1330 chromosome II	563,998..565,821[b]	563,844..563,997 (154)	561,723..563,843 (*recG*)
Buchnera aphidicola str. APS (*Acyrthosiphon pisum*)	25,945..27,774[b]	25,805..25,944 (140)	24,950..25,804 (*rpoH*)
Buchnera aphidicola str. Bp (*Baizongia pistaciae*)	27,630..29,462	27,533..27,629 (97)	26,669..27,532 (*rpoH*)
Buchnera aphidicola str. Sg *(Schizaphis graminum*)	26,225..28,051[b]	26,072..26,224 (153)	25,214..26,071 (*rpoH*)
Burkholderia pseudomallei K96243 chromosome 1	331,614..333,446[a] (*glmS*1)	331,540..331,613 (74)	331,093..331,539 (*BPSL0311*)
	1,531,255..1,533,156 (*glmS*2)	1,533,157..1,533,365 (209)	1,533,366..1,534,697[b] (*BPSL1313*)
Burkholderia pseudomallei K96243 chromosome 2	2,714,171..2,715,988[b] (*glmS*3)	2,713,950..2,714,170 (221)	2,713,323..2,713,949 (*BPSS2008*)
Campylobacter jejuni RM1221	1,456,083..1,457,879[b]	1,456,081..1,456,082 (2)	1,452,955..1,456,080[b] (*CJE1557*)
Campylobacter jejuni subsp. *jejuni* NCTC 11168	1,300,819..1,302,615[b]	1,300,817..1,300,818 (2)	1,297,691..1,300,816[b] (*Cj1365c*)
Chlamydia muridarum Nigg	237,665..239,485	239,486..239,588 (103)	239,589..240,785 (*TC0204*)
Chlamydia trachomatis D/UW-3/CX	958,567..960,387	960,388..960,489 (102)	960,490..961,686 (*tyrP_1*)
Chlamydophila abortus S26/3	874,394..876,223[b]	874,325..874,393 (69)	873,137..874,324[b] (*CAB755*)
Chlamydophila caviae GPIC	902,654..904,483[b]	902,586..902,653 (68)	901,398..902,585[b] (*CCA00787*)
Chlamydophila pneumoniae CWL029	1,109,895..1,111,724	1,111,725..1,111,811 (87)	1,111,812..1,113,002 (*tyrP_1*)
Chlamydophila pneumoniae J138	1,109,244..1,111,073	1,111,074..1,111,160 (87)	1,111,161..1,112,351 (*tyrP_1*)
Chlorobium tepidum TLS	124,386..126,230	126,231..126,477 (247)	126,478..127,419 (*CT0131*)
Chromobacterium violaceum ATCC 12472	704,054..705,883	705,884..705,928 (45)	705,929..706,330[b] (*CV0678*)
Clostridium acetobutylicum ATCC 824	180,165..181,991	181,992..182,365 (374)	182,366..182,713 (*CAC0159*)

Table 2.3. *glmS* genes in annotated bacterial genomes (cont.)

Bacteria	*glmS*	Intergenic region (bp)[a]	Downstream gene (name)
Clostridium perfringens str. 13	2,677,051..2,678,883[b]	2,677,030..2,677,050 (21)	2,676,861..2,677,031[b] (*CPE2326*)
Corynebacterium glutamicum ATCC 13032	2,373,816..2,375,693[b]	2,373,759..2,373,815 (57)	2,373,513..2,373,758 (*cg2491*)
Coxiella burnetii RSA 493	1,718,365..1,720,200	1,720,201..1,720,202 (2)	1,720,203..1,720,787 (*CBU1788*)
Dehalococcoides ethenogenes 195	484,507..486,288	486,289..486,295 (7)	486,296..486,748[b] (*DET0532*)
Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough	3,311,611..3,313,434	3,313,435..3,313,722 (288)	3,313,723..3,315,165 (*DVU3157*)
Enterococcus faecalis V583	2,053,427..2,055,235[b]	2,053,159..2,053,426 (268)	2,051,878..2,053,158[b] (*EF2150*)
Erwinia carotovora subsp. *atroseptica* SCRI1043	5,047,222..5,049,054[b]	5,046,951..5,047,221 (271)	5,046,558..5,046,950[b] (*ECA4507*)
Escherichia coli CFT073	4,412,872..4,414,701[b]	4,412,559..4,412,871 (313)	4,411,518..4,412,558[b] (*pstS*)
Escherichia coli K12	3,909,862..3,911,691[b]	3,909,549..3,909,863 (315)	3,908,508..3,909,548[b] (*pstS*)
Escherichia coli O157:H7	4,705,284..4,707,113[b]	4,704,585..4,705,283 (699)	4,703,982..4,704,584[b] (*ECs4670*)
Escherichia coli O157:H7 EDL933	4774,265..4,776,094[b]	4,773,967..4,774,264 (298)	4,773,877..4,773,966[b] (*Z5226*)
Francisella tularensis subsp. *tularensis* Schu 4	390,379..392,217	392,218..392,300 (83)	392,301..392,825 (*FTT0389*)
Geobacter sulfurreducens PCA	276,632..278,461[b]	276,534..276,631 (98)	276,219..276,533 (*GSU0269*)
Haemophilus ducreyi 35000HP	1,591,723..1,593,555[b]	1,591,369..1,591,722 (354)	1,591,072..1,591,368[b] (*HD1893*)
Haemophilus influenzae Rd KW20	451,754..453,586[b]	451,701..451,753 (53)	451,167..451,700 (*dsbB*)
Halobacterium sp. NRC-1	5,646..7,451[b]	5,644..5,645 (2)	3,322..5,643 (*VNG0005H*)
Helicobacter hepaticus ATCC 51449	832,635..834,488	overlapping	834,475..834,894[b] (*HH0855*)
Helicobacter pylori 26695	1,609,975..1,611,768	overlapping	1,611,722..1,612,417 (*HP1533*)
Helicobacter pylori J99	1,560,142..1,561,935	1,561,936..1,561,958 (23)	1,561,959..1,562,585 (*jhp1421*)
Idiomarina loihiensis L2TR	2,816,695..2,818,527[b]	2816299..2816694 (396)	2,815,876..2,816,298[b] (*IL2615*)
Lactococcus lactis subsp. *lactis* Il1403	1,033,815..1,035,632	1035633..1035762 (130)	1,035,763..1,036,443 (*radC*)
Legionella pneumophila str. Lens	3,144,315..3,146,129[b]	overlapping	3,143,653..3,144,318[b](*lpl2747*)

Table 2.3. *glmS* genes in annotated bacterial genomes (cont.)

Bacteria	*glmS*	Intergenic region (bp)[a]	Downstream gene (name)
Legionella pneumophila str. Paris	3,304,270..3,306,084[b]	3,303,609..3,304,269 (661)	3,303,608..3,304,273[b] (*lpp2892*)
Legionella pneumophila subsp. *pneumophila* str. Philadelphia 1	3,209,200..3,211,014[b]	overlapping	3,208,538..3,209,203[b] (*tpm*)
Leifsonia xyli subsp. *xyli* str. CTCB07	2,057,988..2,059,838[b]	2,057,983..2,057,987 (5)	2,057,626..2,057,982[b] (*acpS*)
Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130 chromosome I	508,027..509,859[b]	508,022..508,026 (5)	506,975..508,021[b] (*LIC10444*)
Leptospira interrogans serovar Lai str. 56601 chromosome I	3,764,133..3,765,965	3,765,966..3,765,970 (5)	3,765,971..3,767,017 (*rfbA*)
Listeria monocytogenes str. 4b F2365	771,348..773,153	773,154..773,265 (112)	773,266..774,006 (*LMOf2365_0763*)
Mannheimia succiniciproducens MBEL55E	161,342..161,596 (*glmS*1)	161,597..161,630 (34)	161,631..163,175 (*glmS*2)
	161,631..163,175 (*glmS*2)	163,176..163,188 (13)	163,189..163,371 (*MS0190*)
Methylococcus capsulatus str. Bath	12,615..14,447	14,448..14,533 (86)	14,534..16,618 (*metG*)
Mycobacterium avium subsp. *paratuberculosis* str. k10	4,721,192..4,723,066	4,723,067..4,723,181 (115)	4,723,182..4,724,081 (*MAP4254*)
Mycobacterium bovis AF2122/97	3,802,717..3,804,591[b]	3,802,496..3,802,716 (221)	3,801,641..3,802,495[b] (*Mb3465c*)
Mycobacterium leprae TN	462,979..464,856	464,857..464,950 (94)	464,951..465,073[b] (*ML0372*)
Mycobacterium tuberculosis CDC1551	3,840,965..3,842,839[b]	3,840,828..3,840,964 (137)	3,839,889..3,840,827[b] (*MT3541*)
Mycobacterium tuberculosis H37Rv	3,855,015..3,856,889[b]	3,854,794..3,855,014 (221)	3,853,939..3,854,793[b] (*Rv3435c*)
Neisseria meningitidis Z2491	260,980..262,818[b]	260,865..260,979 (115)	258,807..260,864[b] (*metG*)
Nitrosomonas europaea ATCC 19718	244,066..245,913	245,914..246,048 (135)	246,049..246,774 (*NE0210*)
Nocardia farcinica IFM 10152	976,813..978,693	978,694..978,696 (3)	978,697..979,116[b] (*nfa8770*)
Parachlamydia sp. UWE25	1,561,129..1,562,952[b]	1,561,103..1,561,128 (26)	1,559,888..1,561,102[b] (*tyrP_4*)
Pasteurella multocida subsp. *multocida* str. Pm70	1,947,165..1,948,997[b]	1,946,448..1,947,164 (717)	1,945,617..1,946,447 (*plpB*)

Table 2.3. *glmS* genes in annotated bacterial genomes (cont.)

Bacteria	*glmS*	Intergenic region (bp)[a]	Downstream gene (name)
Photorhabdus luminescens subsp. *laumondii* TTO1	30,923..32,819[b]	30,829..30,922 (94)	30,574..30,828[b] (*rpmE*)
Prochlorococcus marinus str. MIT 9313	2,063,076..2,064,980[b]	2,063,069..2,063,075 (7)	2,061,614..2,063,068 (*manC*)
Prochlorococcus marinus subsp. *marinus* str. CCMP1375	1,620,235..1,622,139[b]	1,620,212..1,620,234 (23)	1,618,775..1,620,211 (*manC*)
Prochlorococcus marinus subsp. *pastoris* str. CCMP1986	1,535,851..1,537,746[b]	1,535,831..1,535,850 (30)	1,535,291..1,535,830 (*rimM*)
Pseudomonas aeruginosa PAO1	6,243,110..6,244,945[b]	6,243,056..6,243,109 (54)	6,241,850..6,243,055[b] (*PA5548*)
Pseudomonas putida KT2440	6,170,456..6,172,291[b]	6,170,285..6,170,455 (171)	6,169,103..6,170,284[b] (*PP5408*)
Pseudomonas syringae pv. tomato str. DC3000	6,376,404..6,378,239[b]	6,376,039..6,376,403 (365)	6,374,515..6,376,038[b] (*PSPTO5594*)
Ralstonia solanacearum GMI1000	200,835..202,673	202,674..203,510 (837)	203,511..203,966[b] (*RSc0179*)
Rhodopirellula baltica SH 1	6,810,910..6,812,694	6,812,695..6,812,797 (103)	6,812,798..6,813,016 (*RB12653*)
Rhodopseudomonas palustris CGA009	3,027,638..3,029,464	3,029,465..3,029,722 (258)	3,029,723..3,030,526 (*RPA2661*)
Salmonella enterica subsp. *enterica* serovar Choleraesuis str. SC-B67	4,004,093..4,005,922[b]	4,003,943..4,004,092 (150)	4,002,278..4,003,942 (*yabN*)
Salmonella enterica subsp. *enterica* serovar Paratyphi A str. ATCC 9150	3,844,054..3,845,883[b]	3,843,905..3,844,053 (149)	3,842,594..3,843,904 (*SPA3699*)
Salmonella enterica subsp. *enterica* serovar Typhi str. CT18	3,778,356..3,780,185	3,780,186..3,780,595 (410)	3,780,596..3,781,171 (*stgA*)
Salmonella enterica subsp. *enterica* serovar Typhi Ty2	3,763,847..3,765,676	3,765,677..3,766,086 (410)	3,766,087..3,766,662 (*stgA*)
Salmonella typhimurium LT2	4,069,035..4,070,864[b]	4,068,885..4,069,034 (150)	4,067,127..4,068,884 (*STM3860*)
Shewanella oneidensis MR-1	4,951,368..4,953,197[b]	4,951,288..4,951,367 (80)	4,951,084..4,951,287 (*SO4740*)
Shigella flexneri 2a str. 2457T	3,852,356..3,854,185	3,854,186..3,854,305 (120)	3,854,306..3,855,634 (*S3960*)
Shigella flexneri 2a str. 301	3,919,109..3,920,938[b]	3,919,007..3,919,108 (102)	3,917,660..3,919,006[b] (*SF3808*)
Silicibacter pomeroyi DSS-3 megaplasmid	149,971..151,794[b]	149,947..149,970 (24)	149,467..149,946 (*SPOA0139*)
Sinorhizobium meliloti 1021	1,794,802..1,796,628[b]	overlapping	1,794,308..1,794,805[b] (*SMc00230*)

Table 2.3. *glmS* genes in annotated bacterial genomes (cont.)

Bacteria	*glmS*	Intergenic region (bp)[a]	Downstream gene (name)
Staphylococcus aureus subsp. *aureus* COL	2,206,327..2,208,132[b]	2,206,096..2,206,326 (231)	2,205,313..2,206,095[b] (*SACOL2144*)
Staphylococcus aureus subsp. *Aureus* MRSA252	2,309,650..2,311,455[b]	2,309,419..2,309,649 (231)	2,308,636..2,309,418[b] (*SAR2241*)
Staphylococcus aureus subsp. *aureus* Mu50	2,279,803..2,281,608[b]	2,279,572..2,279,802 (231)	2,278,789..2,279,571[b] (*SAV2153*)
Staphylococcus aureus subsp. *aureus* MW2	2,230,020..2,231,825[b]	2,229,789..2,230,019 (231)	2,229,006..2,229,788[b] (*MW2079*)
Staphylococcus aureus subsp. *aureus* N315	2,210,083..2,211,888[b]	2,209,852..2,210,082 (231)	2,209,069..2,209,851[b] (*SA1958*)
Staphylococcus epidermidis RP62A	1,797,037..1,798,842[b]	1,796,555…1,797,036 (482)	1,795,685..1,796,554[b] (*SERP1759*)
Streptococcus agalactiae 2603V/R	950,302..952,116	952,117..952,292 (176)	952,293..953,426 (*SAG0945*)
Streptococcus mutans UA159	1,127,608..1,129,422[b]	1,127,061..1,127,607 (547)	1,125,351..1,127,060[b] (*mtlA1*)
Streptococcus pneumoniae R6	240,298..242,106	242,107..242,224 (118)	242,225..243,274 (*spr0246*)
Streptococcus pyogenes M1 GAS	1,053,614..1,055,428[b]	1,053,419..1,053,613 (195)	1,053,083..1,053,418[b](*SPy1277*)
Streptococcus pyogenes MGAS315	965,519..967,282[b]	965,324..965,518 (195)	964,988..965,323[b] (*SpyM3_0909*)
Streptococcus pyogenes MGAS8232	1,029,398..1,031,212[b]	1,029,203..1,029,397 (195)	1,028,867..1,029,202[b] (*spyM18_1226*)
Streptococcus thermophilus CNRZ1066	801,958..803,766	803,767..803,883 (117)	803,884..804,213 (*phnA*)
Streptococcus thermophilus LMG 18311	795,534..797,342	797,343..797,459 (117)	797,460..797,789 (*phnA*)
Streptomyces coelicolor A3(2)	3,043,786..3,045,603 (*glmS*2)	3,045,604..3,045,618 (15)	3,045,619..3,046,146[b] (*SCO2790*)
	5,153,334..5,155,181 (*glmS*1)	5,155,182..5,155,212 (31)	5,155,213..5,155,785[b] (*SCO4741*)
Sulfolobus solfataricus P2	325,069..326,844[b] (*glmS*1)	overlapping	323,985..325,076[b] (*SSO0381*)
	1,091,187..1,092,941[b] (*glmS*2)	1,091,147..1,091,186 (40)	1,090,286..1,091,146[b] (*SSO1266*)
Synechocystis sp. PCC 6803	161,768..163,663[b]	161,643..161,767 (125)	161,088..161,642[b] (*bcp*)
Thermoanaerobacter tengcongensis MB4	2,099,588..2,101,426[b]	2,099,484..2,099,587 (104)	2,099,076..2,099,483[b] (*TTE2189*)

Table 2.3. *glmS* genes in annotated bacterial genomes (cont.)

Bacteria	*glmS*	Intergenic region (bp)[a]	Downstream gene (name)
Thermosynechococcus elongatus BP-1	1,281,346..1,283,259[b]	1,281,246..1,281,345 (100)	1,280,919..1,281,245 (*tlr1236*)
Treponema pallidum subsp. *Pallidum* str. Nichols	937,274..939,181[b]	937,200..937,273 (74)	936,477..937,199 (*TP0860*)
Tropheryma whipplei str. Twist	709,829..711,679[b]	707,471..709,828 (2,358)	706,067..707,470 (*ispE*)
Tropheryma whipplei TW08/27	184,178..186,028	186,029..187,877 (1,849)	187,878..189,107[b] (*ispE*)
Wigglesworthia glossinidia endosymbiont of *Glossina brevipalpis*	10,399..12,237	12,238..12,242 (5)	12,243..13,604[b] (*thdF*)
Wolbachia endosymbiont of *Drosophila melanogaster*	520,123..521,943	521,944..522,252 (309)	522,251..522,694 (*WD0536*)
Wolinella succinogenes DSM 1740	23,584..25,371	25,372..25,376 (5)	25,378..27,207 (*WS0023*)
Xanthomonas axonopodis pv. Citri str. 306	848,221..849,219	849,220..849,221 (2)	849,222..850,376 (*nagA*)
Xanthomonas campestris pv. *campestris* str. ATCC 33913	685,495..687,324	687,325..687,636 (312)	687,637..689,061 (*XCC0570*)
Xanthomonas oryzae pv. oryzae KACC10331	760,789..762,756	762,757..763,137 (381)	763,138..763,611 (*XOO0747*)
Xylella fastidiosa Temecula1	139,554..141,383	141,384..141,656 (273)	141,657..142,808 (*PD0111*)
Yersinia pestis biovar Medievalis str. 91001	4,580,283..4,582,112[b]	4,579,801..4,580,282 (482)	4,578,760..4,579,800[b] (*pstS2*)
Yersinia pestis CO92	4,639,195..4,641,024[b]	4,638,713..4,639,194 (482)	4,637,672..4,638,712[b] (*pstS*)
Yersinia pestis KIM	4,585,973..4,587,802[b]	4,585,491..4,585,972 (482)	4,584,450..4,585,490[b] (*pstS*)
Yersinia pseudotuberculosis IP 32953	4,730,141..4,731,970[b]	4,729,659..4,730,140 (482)	4,728,618..4,729,658[b] (*pstS*)
Zymomonas mobilis subsp. *mobilis* ZM4	50,970..52,793[b]	50,787..50,969 (183)	49,995..50,786[b] (*ZMO0055*)

glmS, downstram gene and intergenic sequences and their genome coordinates were obtained from GenBank (www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html). Only eubacteria are listed, but Archaeal genomes also contain *glmS* genes. [a]Distance in bp between *glmS* and the downstream gene. [b]Complementary sequence; [c]Sequences obtained from the same strain, but by two different consortia (accession numbers are in parentheses).

average distance of *att*Tn*7* from *glmS* (25 bp). In these cases, Tn*7* may actually insert within a coding sequence, but since *att*Tn*7* sequences do not possess signature motifs and can only be experimentally determined, it is unknown whether or not this will actually occur or have any consequences in these bacteria.

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CHAPTER 3

An improved method for rapid transformation and generation of unmarked *Pseudomonas aeruginosa* deletion mutants

(Presented by Kyoung-Hee Choi, and Herbert P. Schweizer, *BMC Microbiol.* 2005 23;5:30., and Kyoung-Hee Choi, Ayush Kumar and Herbert P. Schweizer. *Journal of Microbiol. Methods* 2006 64:391-397)

The work presented in these articles established a rapid and efficient method for transformation of *P. aeruginosa* with various types of DNA elements such as non-replicative or replicative plasmids and chromosomal DNA fragments. This method was exploited for development of a rapid allele replacement method.

3.1 ABSTRACT

A rapid microcentrifuge-based method is described for preparation of *Pseudomonas aeruginosa* electrocompetent cells with up to 10,000-fold increased transformation efficiencies over existing procedures. This increased efficiency now enables the use of transformation for all applications requiring DNA transfer. These include transfer of chromosomal mutations marked with antibiotic resistance genes between *P. aeruginosa* strains, which solves the riddle of not having an efficient and reliable transduction procedure for this bacterium. Not surprisingly, the method also allows for very efficient transformation with replicative plasmids, with transformation efficiencies ranging from 10^7 to $> 10^{11}$ transformants per microgram of DNA. With

efficiencies of up to > 10^3 transformants per microgram of DNA the method replaces in most instances conjugation for the transfer of non-replicative plasmids used in gene replacement, site-specific gene integration and transposon mutagenesis experiments. Lastly, this method allows faster creation of many deletion mutants.

Traditional gene replacement procedures are still time-consuming. They usually necessitate cloning of the gene to be mutated, insertional inactivation of the gene with an antibiotic resistance cassette and exchange of the plasmid-borne mutant allele with the bacterial chromosome. PCR and recombinational technologies can be exploited to substantially accelerate virtually all steps involved in the gene replacement process. We describe a method for rapid generation of unmarked *P. aeruginosa* deletion mutants. Three partially overlapping DNA fragments are amplified and then spliced together *in vitro* by overlap extension PCR. The resulting DNA fragment is cloned *in vitro* into the Gateway vector pDONR221 and then recombined into the Gateway-compatible gene replacement vector pEX18ÄpGW. The plasmid-borne deletions are next transferred to the *P. aeruginosa* chromosome by homologous recombination. Unmarked deletion mutants are finally obtained by Flp-mediated excision of the antibiotic resistance marker. The method was applied to deletion of 25 *P. aeruginosa* genes encoding transcriptional regulators of the GntR family. While maintaining the key features of traditional gene replacement procedures, for example, suicide delivery vectors, antibiotic resistance selection and sucrose counterselection, the method described here is considerably faster due to streamlining of some of the key steps involved in the process, especially plasmid-borne mutant allele construction and its transfer into the target host. With appropriate modifications, the method should be applicable to other bacteria.

3.2 INTRODUCTION

Since the initial description of phage transduction in *Pseudomonas aeruginosa* in 1959 (Holloway et al., 1959), repeated attempts were made to develop transduction into a viable, reliable, repeatable and efficient gene transfer method (Budzik et al., 2004, Darzins et al., 1989, Krishnapillai, 1972, Morgan, 1979). However, despite isolation of various transducing phage, the latest as recently as 2004 (Budzik et al., 2004), such attempts never materialized for mostly unknown reasons. As a result, routine transfers of mutations within a given strain lineage (e.g., PAO1 (Holloway, 1955)) or between different *P. aeruginosa* isolates (e.g., between PAO1 and PAK (Ishimoto et al., 1989) or PA14 (Rahme et al., 1995)) were complicated and mostly relegated to relatively time-consuming and cumbersome allele replacement procedures, which are driven by recombination of plasmid-borne alleles into the various chromosomes of interest. The less than desirable outcome of the lack of efficient gene transfer procedures was that over the years much comparative work was conducted with different *P. aeruginosa* strain lineages or even with non-isogenetic strains, for example in different PAO1 strain backgrounds, which are now known not to be isogenetic (Stover et al., 2000).

A comprehensive PAO1 transposon library was recently established (Jacobs et al., 2003) but the transfer of transposon insertions into different strains is still a relatively tedious, multi-step procedure (Gallagher et al., 2002), which diminishes the value of a great resource. Here we describe a simple, rapid and inexpensive solution to the problem of efficient chromosomal DNA fragment transfer between *P. aeruginosa* strains. Additionally, its efficiency makes this now the method of choice for transformation not only with replicative plasmids but also with non-replicative plasmids used for gene replacement, site-specific gene integration and transposon

delivery experiments.

The elucidation of the *P. aeruginosa* strain PAO1 genome sequence in 2000 (Stover et al., 2000) opened the doors for genome- and proteome-scale research with this important opportunistic pathogen. These include the availability of commercial (Affymetrix, Santa Clara, CA) *P. aeruginosa* GeneChips® for transcriptome analyses (Goodman et al., 2004), as well as the *P. aeruginosa* PAO1 gene collection for high-throughput expression and purification of recombinant proteins (LaBaer et al., 2004). A comprehensive transposon mutant library was generated containing over 30,000 sequence-defined mutants, corresponding to an average of five insertions per gene (Jacobs et al., 2003).

However, despite the development of many genetic tools for *P. aeruginosa* over the past decade (Schweizer et al., 2004, Suh et al., 2004), isolation of defined deletion mutants is still a relatively tedious process, which relies on construction of deletion alleles, most often tagged with an antibiotic resistance gene, on a suicide plasmid, followed by recombination of the plasmid-borne deletions into the chromosome, usually after conjugal transfer of the suicide plasmid (reviewed in (Schweizer et al., 2004)). Merodiploids arising from chromosomal integration of the suicide plasmid are resolved by utilization of a counterselectable marker, most often *Bacillus subtilis sacB* (sucrose counterselection) (Schweizer, 1992) or, less frequently, *rpsL* (streptomycin counterselection) (Stibitz, 1994). In cases where the antibiotic selection markers are flanked by site specific recombination sites, e.g., the Flp recombinase target (*FRT*) (Hoang et al., 1998) or Cre recombinase (*loxP*) (Quenee et al., 2005) sites, they can subsequently be deleted from the chromosome, resulting in unmarked deletion mutants.

Unfortunately, the previously described method for one-step inactivation of chromosomal genes in *Escherichia coli* using PCR products (Datsenko et al., 2000) does not work in *P. aeruginosa*, although we have used a modification (Gust et al., 2003) of this approach where the target gene is first cloned into a plasmid, followed by λ RED-mediated recombination of a PCR-generated mutated copy of the gene (Chuanchuen et al., 2005). Here, we describe a more streamlined method for generation of chromosomal deletion mutants. In this method, a "mutant" fragment containing the 5' and 3' flanking regions of the target gene plus the antibiotic resistance gene is assembled by a modified (Herring et al., 2004) PCR overlap extension protocol (Horton et al., 1990) and then cloned into a suicide vector using the Gateway recombinational cloning system, similarly to a previously described method (Wolfgang et al., 2003). The plasmid-borne deletion allele is then transferred to *P. aeruginosa* utilizing a rapid transformation procedure and recombined into the chromosome. The transformants are often merodiploids which are then resolved by sucrose counterselection against the undesired wild-type sequences. The antibiotic resistance marker is subsequently deleted from the chromosome by Flp recombinase.

3.3 MATERIALS AND METHODS

3.3.1 Growth media, plasmids, strains and growth conditions. Luria–Bertani (LB; 10 g/l tryptone, 5 g/l yeast extract per liter, 10 g/l NaCl; Becton, Dickinson and Co.) medium was used in all experiments. For plasmid maintenance in *E. coli*, the medium was supplemented with 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, 35 μg/ml kanamycin or 15 μg/ml gentamycin. For selection of *P. aeruginosa* transformants, carbenicillin (Cb), gentamycin (Gm) and tetracycline (Tc) were added to LB agar plates at final concentrations of 200, 30 and 50 μg/ml, respectively, as

appropriate. The following plasmids were used in this study (Table 3.1): pUCP20, pUCP24, pUCP26 (West et al., 1994) and pFLP2 (Hoang et al., 1998), broad-host-range plasmids that replicate in *P. aeruginosa*; pBT20 (Kulasekara et al., 2005), a *mariner* transposon delivery plasmid which does not replicate in *P. aeruginosa* because it contains the R6Kγ origin of replication and, therefore, depends on the presence of the *pir* gene for replication; pPS1527 and pPS1596, two pEX18Ap (Hoang et al., 1998) based plasmids which also do not replicate in *P. aeruginosa*. All plasmids were propagated in and isolated from *Escherichia coli*. The 7656-bp mini-Tn*7* delivery vector pUC18-mini-Tn*7*T-Gm-*lacZ* (GenBank accession number AY599233) and its helper plasmid pTNS1 (Choi et al., 2005a) were used for Tn*7* delivery experiments. pTNS1 was derived from pUX-BF13 (Bao et al., 1991) by deletion of the *tnsE* gene and therefore expresses only the TnsABC + D site-specific transposition pathway. The *P. aeruginosa* strains used in this study were PAO1 (Holloway, 1955), PAK (Ishimoto et al., 1989) and PA14 (Rahme et al., 1995) and cultures of these strains were routinely grown at 37°C.

3.3.2 Preparation of competent cells and transformation by electroporation. *P. aeruginosa* cells were made competent utilizing a microcentrifuge-based procedure. Six milliliters of an overnight culture grown in LB medium were equally distributed into 4 microcentrifuge tubes and the cells were harvested by centrifugation at room temperature for 1–2 min at 16,000 ×g. The cell pellet in each tube was washed twice with 1 ml of room temperature 300 mM sucrose and the 4 cell pellets were then resuspended in a combined total of 100 μl 300 mM sucrose, which contained on average 10^9–10^{10} viable bacteria.

For electroporation, 500 ng of chromosomal DNA purified via the QIAamp kit was

Table 3.1. Bacterial strains used in this study

Strains or plasmids	Relevant properties	Reference or source
E. coli		
DB3.1	F⁻ *gyrA462 endA1 glnV44* Δ(*sr1-recA*) *mcrB mrr hsdS20*(r_B^-, m_B^-) *ara14 galK2 lacY1 proA2 rpsL20*(Sm^r) *xyl5* Δ*leu mtl1*	Invitrogen
P. aeruginosa		
PAO1	Prototroph	Holloway, 1955
PAK	Prototroph	Ishimoto et al., 1989
PA14	Prototroph	Rahme et al., 1995
PAO460	PAO1 with mini-Tn*7*T-p*fabA*Δ30'-*lacZ*	This study
PAO435	PAO1 with mini-Tn*7*T-p*fabA'-lacZ*	This study
PAO518	PAO435 with Δ*PA0120*::Gm	This study
PAO519	PAO435 with Δ*PA0121*::Gm	This study
PAO520	PAO435 with Δ*PA0268*::Gm	This study
PAO521	PAO435 with Δ*PA0275*::Gm	This study
PAO522	PAO435 with Δ*PA0797*::Gm	This study
PAO523	PAO435 with Δ*PA1142*::Gm	This study
PAO524	PAO435 with Δ*PA1269*::Gm	This study
PAO511	PAO435 with Δ*PA1520*::Gm	This study
PAO526	PAO435 with Δ*PA1526*::Gm	This study
PAO527	PAO435 with Δ*PA2032*::Gm	This study
PAO528	PAO435 with Δ*PA2100*::Gm	This study
PAO530	PAO435 with Δ*PA2692*::Gm	This study
PAO531	PAO435 with Δ*PA2802*::Gm	This study
PAO532	PAO435 with Δ*PA2825*::Gm	This study
PAO533	PAO435 with Δ*PA2897*::Gm	This study
PAO534	PAO435 with Δ*PA3249*::Gm	This study
PAO535	PAO435 with Δ*PA3381*::Gm	This study
PAO536	PAO435 with Δ*PA3757*::Gm	This study

Table 3.1. Bacterial strains used in this study (cont.).

Strains or plasmids	Relevant properties	Reference or source
PAO537	PAO435 with Δ*PA4165*::Gm	This study
PAO538	PAO435 with Δ*PA4185*::Gm	This study
PAO539	PAO435 with Δ*PA4769*::Gm	This study
PAO540	PAO435 with Δ*PA4906*::Gm	This study
PAO541	PAO435 with Δ*PA5283*::Gm	This study
PAO542	PAO435 with Δ*PA5431*::Gm	This study
PAO543	PAO435 with Δ*PA5525*::Gm	This study
Plasmids or DNA elements		
pUCP20	Ap^r; broad-host range vector	West et al., 1994
pUCP24	Gm^r; broad-host range vector	West et al., 1994
pUCP26	Tc^r; broad-host range vector	West et al., 1994
pFLP2	Ap^r; source for Flp recombinase	Hoang et al., 1998
pPS1596	Ap^r, Gm^r; pEX18ApGW-Δ*PA3676*::Gm	A. Kumar
pPS1453	Ap^r, Gm^r; pUC18-mini-Tn*7*T-Gm-*lacZ*	Choi et al., 2005
pTNS1	Ap^r; ori_{R6K} replicon, Tn*7* transposase-expressing helper plasmid.	Choi et al., 2005
pBT20	Ap^r, Gm^r; ori_{R6K} replicon, *mariner* transposon	S. Lory
pPS1460	Ap^r, Gm^r; pUC18-mini-Tn*7*T-Gm-p*fabA'-lacZ*	This study
pPS1492	Ap^r, Gm^r; pUC18-mini-Tn*7*T-Gm-p*fabAΔ30'-lacZ*	This study
pDONR221	Km^r; Gateway donor vector	Invitrogen
pEX18ApGW	Ap^r; Gateway destination vector	This study

mixed with 100 µl of electrocompetent cells and the mixture was transferred to a 2 mm gap width electroporation cuvette. After applying a pulse (settings: 25 µF; 200 Ω; 2.5 kV on a Bio-Rad GenePulserXcell™; Bio-Rad), 1 ml of room temperature LB medium was added at once, cells were transferred to a small (13 × 100 mm) glass tube and shaken for 0, 1 and 2 h at 37°C. The cells were then harvested in a microcentrifuge tube. A total of 900 µl of the supernatant was discarded and the cell pellet was resuspended in the residual medium. The entire mixture was then plated on an LB + Gm30 plate. The plates were incubated at 37°C until colonies appeared (usually within 24 h). Controls included cells pulsed without added DNA.

For transformation experiments with plasmids the same electroporation conditions were used and either dilutions (for replicative plasmids) or the entire mixtures (for non-replicative plasmids) after concentration as described above were plated on selective plates. When pEX-based vectors were used, transformants were patched on LB + Gm30 and LB + Cb200 plates and only those colonies that grew well on the LB + Gm30 plates were further considered. Gm^r and Cb^s colonies were counted as double cross-over events. In contrast, Gm^r and Cb^r colonies indicated merodiploids, which resulted from single cross-over events. Typical DNA input concentrations were 10–50 ng for replicative and 300–500 ng for non-replicative plasmids.

3.3.3 DNA manipulations. Routine procedures were employed for manipulation of DNA (Sambrook et al., 2001). Plasmid DNAs were isolated using the QIAprep Mini-spin kit (Qiagen). *P. aeruginosa* chromosomal DNA fragments (20–30 kb) were isolated using the QIAamp DNA Mini Kit and the DNA was suspended in 200 µl of buffer AE (10 mM Tris–HCl, 0.5 M EDTA, pH 9). Plasmid DNA fragments were

purified from agarose gels utilizing the QIAquick gel extraction kit. For colony PCR, a single, large colony (or the equivalent from a cell patch) was picked from a plate, transferred to 30 µl H_2O in a microcentrifuge tube and boiled for 5 min. Cell debris was removed by centrifugation in a microcentrifuge (2 min; 16,000 ×g) and the supernatant was transferred to a fresh tube which was placed on ice. Five microliters of the supernatant were used as source of template DNA in a 50 µl PCR reaction containing *Taq* buffer, 1.5 mM $MgSO_4$, 5% DMSO, 0.2 µM each of two primers (e.g., P1 (5'-AAGGTCGCCTCGAATTCTCCGAGGTTT) and P2 (5'-ACCAGCAAGCCG ATGGCCAGCACCAT) for verification of the Δ(*mexAB*::Gm^r) mutation, 200 µM dNTPs and 5 units *Taq* polymerase (Fermentas). Cycle conditions were 96°C for 5 min, followed by 30 cycles of 96°C for 45 s, 60°C for 45 s, 62.5°C for 45 s, 65°C for 45 s, 67.5°C for 45 s, 70°C for 45 s and 72°C for 1.5 min, and a final extension at 72°C for 10 min. After PCR, 5 µl aliquots were analyzed by agarose gel electrophoresis in undigested form or after digestion with *Bgl*II.

3.3.4 Construction of the Gateway-compatible gene replacement vector pEX18ApGW. The previously described gene replacement vector pEX18Ap (Hoang et al., 1998) was modified by cloning of a 1,755 bp *Hin*dIII-*Kpn*I fragment from pUC18-mini-Tn*7*T-Gm-GW (GenBank accession number AY737004), followed by transformation into the *gyrA* strain DB3.1 (Invitrogen).

3.3.5 Creation of a deletion allele. For PCR-amplification of the gentamycin resistance gene cassette, a 50 µl PCR reaction contained 5 ng pPS856 (Hoang et al., 1998) template DNA, 1x HiFi Platinum *Taq* buffer, 2 mM $MgSO_4$, 200 µM dNTPs,

0.2 μM of Gm-F and Gm-R (Table 3.2) and 5 units of HiFi Platinum *Taq* polymerase (Invitrogen). Cycle conditions were 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 1 min 30 s, and a final extension at 68°C for 7 min. The resulting 1,053 bp PCR product was purified by agarose gel electrophoresis and its concentration determined spectrophotometrically ($A_{260\ nm}$) in an Eppendorf Biophotometer (Hamburg, Germany) and using the 50-2000 μl Eppendorf UVettes. For amplification of 5' and 3' gene fragments, two 50 μl PCR reactions were prepared. The first reaction contained 20 ng chromosomal template DNA, 1x HiFi Platinum *Taq* buffer, 2 mM $MgSO_4$, 5% DMSO, 200 μM dNTPs, 0.8 μM of PA1520-UpF-GWL and PA1520-UpR-Gm (Table 3.2) and 5 units of Platinum *Taq* polymerase. The second reaction contained the same components as the first, but 0.8 μM of PA1520-DnF-Gm and PA1520-DnR-GWR (Table 3.2). Sequences of primers sets used for deletion of other genes are shown in Table 3.3. Cycle conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 30 s, and a final extension at 68°C for 10 min. The resulting PCR products were purified by agarose gel electrophoresis and their concentrations determined spectrophotometrically ($A_{260\ nm}$). Lastly, to generate the fragment of *Gene'-FRT*-Gm-*FRT-'Gene*, a 50 μl PCR reaction contained 50 ng each of the *PA1520* 5' and 3' purified template DNAs, and 50 ng of *FRT*-Gm-*FRT* template DNA prepared during 1st round PCR. The reaction mix also contained 1x HiFi Platinum *Taq* buffer, 2 mM $MgSO_4$, 5% DMSO and 200 μM dNTPs, and 5 units of HiFi Platinum *Taq* polymerase. After an initial denaturation at 94°C for 2 min, 3 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min were run without added primers. The third cycle was paused at 30 s of the 68°C extension, primers GW-*attB1* and GW-*attB2* were added to 0.2 μM each, and the cycle was then finished by another 30 s extension at 68°C. The PCR was

Table 3.2. PCR oligonucleotides

Name	Sequence (5' → 3')[1]
Gene-specific primers	
G-UpF-GWL[2]	TACAAAAAAGCAGGCTccgatcagttgcaacacatc
G-UpR-Gm[2]	TCAGAGCGCTTTTGAAGCTAATTCGttgttcctgcaccaccatct
G-DnF-Gm[2]	AGGAACTTCAAGATCCCCAATTCGccggcgagttccacctgaa
G-DnR-GWR[2]	TACAAGAAAGCTGGGTggttgagcttgctgtcgata
Common primers	
Gm-F	CGAATTAGCTTCAAAAGCGCTCTGA
Gm-R	CGAATTGGGGATCTTGAAGTTCCT
GW-*attB*1	GGGGACAAGTTTGTACAAAAAAGCAGGCT
GW-*attB*2	GGGGACCACTTTGTACAAGAAAGCTGGGT

[1]Sequences in capital letters are common for all genes amplified and overlap with the Gm or *attB* primer sequences. Lower-case letters indicate gene-specific sequences, here *PA1520*. PCR oligonucleotide sequences for all 27 genes amplified and cloned in this study are given in Table 3.3.

[2]G, gene specific sequence, here *PA1520*.

Table 3.3. Sequences of PCR primers used to amplify genes encoding transcriptional regulators of the GntR family

Name	Sequence (5' → 3')[1]
PA0120-UpF-GWL	TACAAAAAAGCAGGCTtcttcgtccaagctggtcaa
PA0120-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGatgaacggctccagcgcata
PA0120-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGatcagcgaactgcggctgtt
PA0120-DnR-GWR	TACAAGAAAGCTGGGTttgaggatgtgctggcgcat
PA0121-UpF-GWL	TACAAAAAAGCAGGCTattcgaagcacgtacccagc
PA0121-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGcacttcgctttccaccagca
PA0121-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGaactgaccgagaatcccttg
PA0121-DnR-GWR	TACAAGAAAGCTGGGTccttcatgccgaattcctcg
PA0268-UpF-GWL	TACAAAAAAGCAGGCTaactgcaactgatcctcgac
PA0268-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGgtagaccagcttggcgttgt
PA0268-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGgatcatcgaggacgactacg
PA0268-DnR-GWR	TACAAGAAAGCTGGGTtcgatgtcgtcccgttcgat
PA0275-UpF-GWL	TACAAAAAAGCAGGCTtgaacgagcgtaccgactac
PA0275-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGtacctggagtagctgggtgt
PA0275-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGctgctgatgagccacaagct
PA0275-DnR-GWR	TACAAGAAAGCTGGGTaggtcgagaatctcgatgcc
PA0797-UpF-GWL	TACAAAAAAGCAGGCTattcgaagcacgtacccagc
PA0797-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGcacttcgctttccaccagca
PA0797-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGagaatcccttgctgcagggt
PA0797-DnR-GWR	TACAAGAAAGCTGGGTgccttcatgacgaatgcctc
PA1142-UpF-GWL	TACAAAAAAGCAGGCTtgacggtgatctaccagtcc
PA1142-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGaccatcgagtggaagtggct
PA1142-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGgtgctgtacgtcgagcacta
PA1142-DnR-GWR	TACAAGAAAGCTGGGTacctcgacgttgacctgtag

Table 3.3. Sequences of PCR primers used to amplify genes encoding transcriptional regulators of the GntR family (cont.).

Name	Sequence (5' → 3')[1]
PA1269-UpF-GWL	TACAAAAAAGCAGGCTatgctcaacgcccctaacct
PA1269-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGttcggaaatacgcgtcacca
PA1269-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGagatgttcgaagtgcgggtg
PA1269-DnR-GWR	TACAAGAAAGCTGGGTcgacgatatgtgcctcgatc
PA1285-UpF-GWL	TACAAAAAAGCAGGCTcgacgagaggtacgggtata
PA1285-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGtcggcgagttcggaaatgct
PA1285-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGgcaatctgcgagtgctggaa
PA1285-DnR-GWR	TACAAGAAAGCTGGGTtcagtccagtcgctccagtt
PA1520-UpF-GWL	TACAAAAAAGCAGGCTccgatcagttgcaacacatc
PA1520-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGttgttcctgcaccaccatct
PA1520-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGccggcgagttccacctgaa
PA1520-DnR-GWR	TACAAGAAAGCTGGGTggttgagcttgctgtcgata
PA1526-UpF-GWL	TACAAAAAAGCAGGCTcaaggaacgggaacgtatcc
PA1526-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGgcgtagaggctttccacatg
PA1526-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGcgttcagctctacatcctgc
PA1526-DnR-GWR	TACAAGAAAGCTGGGTactgccgaggaactggttca
PA2032-UpF-GWL	TACAAAAAAGCAGGCTaacaggcatacaggctgctg
PA2032-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGgcctttcagcgattgcatgg
PA2032-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGtggaacagtggccgatcaag
PA2032-DnR-GWR	TACAAGAAAGCTGGGTcgcaggcagttccgatactt
PA2100-UpF-GWL	TACAAAAAAGCAGGCTctgaagtacaaacggctggc
PA2100-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGgcacagaatctgctggtgct
PA2100-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGtggatgaagaggggctactg
PA2100-DnR-GWR	TACAAGAAAGCTGGGTgtactaaaggcctctgcggt

Table 3.3. Sequences of PCR primers used to amplify genes encoding transcriptional regulators of the GntR family (cont.).

Name	Sequence (5' → 3')[1]
PA2299-UpF-GWL	TACAAAAAAGCAGGCTtgtacagccagctcaaggag
PA2299-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGtagcccatctgcgtcatgga
PA2299-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGttcgctggaagtcacctacc
PA2299-DnR-GWR	TACAAGAAAGCTGGGTgaggtattcgaagtccagcg
PA2692-UpF-GWL	TACAAAAAAGCAGGCTtcgaatacggcatccactgc
PA2692-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGgagcacgctgatttcgttgg
PA2692-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGtcgacggcgacaaatcgatc
PA2692-DnR-GWR	TACAAGAAAGCTGGGTcatccagcgcagcacttctt
PA2802-UpF-GWL	TACAAAAAAGCAGGCTcaatctcgtcagtacctgcg
PA2802-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGggcgctgacgtattccatga
PA2802-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGtgctgatcgaactgatcgcc
PA2802-DnR-GWR	TACAAGAAAGCTGGGTccagaactcctggtcgaact
PA2825-UpF-GWL	TACAAAAAAGCAGGCTgatggcgtcgaagaatcgct
PA2825-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGagcaccagcatgaccaggta
PA2825-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGtcaagcgtctcgaacagctc
PA2825-DnR-GWR	TACAAGAAAGCTGGGTctggtgcaggtcgaacatct
PA2897-UpF-GWL	TACAAAAAAGCAGGCTgctgctttaccaacgcatcg
PA2897-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGatgtggatcgcatccacgca
PA2897-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGcgaactgatgttcgaccagg
PA2897-DnR-GWR	TACAAGAAAGCTGGGTgaactgctcggtgttgctga
PA3249-UpF-GWL	TACAAAAAAGCAGGCTtcagcgagcagatcgtccat
PA3249-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGctgaccatcgcatggaagtg
PA3249-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGtgtccagcgtctaccagatc
PA3249-DnR-GWR	TACAAGAAAGCTGGGTaactccaggtcgcagtcgat

Table 3.3. Sequences of PCR primers used to amplify genes encoding transcriptional regulators of the GntR family (cont.).

Name	Sequence (5' → 3')[1]
PA3381-UpF-GWL	TACAAAAAAGCAGGCTgcacttgtctagacaaagcg
PA3381-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGttcgagcaccagttcgtcga
PA3381-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGaactgaccaccctgcgttac
PA3381-DnR-GWR	TACAAGAAAGCTGGGTgacctggtactggaagcgat
PA3757-UpF-GWL	TACAAAAAAGCAGGCTatgaagacagcccacgacct
PA3757-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGagacgcggcgtaatgaaggt
PA3757-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGctctccagcttcagcgagat
PA3757-DnR-GWR	TACAAGAAAGCTGGGTatccgggtcatcagcagcat
PA4165-UpF-GWL	TACAAAAAAGCAGGCTtcactgctcgagtcggtcaa
PA4165-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGacgctccaggttgacgtagt
PA4165-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGgcatggatcatcgaggacga
PA4165-DnR-GWR	TACAAGAAAGCTGGGTtcgccgtagagattgcgcat
PA4185-UpF-GWL	TACAAAAAAGCAGGCTgatcctgagcatggaactgg
PA4185-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGcgtggtggcgatgtagatca
PA4185-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGccaggaagatttccgcaagg
PA4185-DnR-GWR	TACAAGAAAGCTGGGTatggcatcgacgatctggtc
PA4769-UpF-GWL	TACAAAAAAGCAGGCTctgtcggatgacatcgttgc
PA4769-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGtagtaggcacaggacccttc
PA4769-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGacaccatcaagggcctgttc
PA4769-DnR-GWR	TACAAGAAAGCTGGGTcaggacctcctgcacatagt
PA4906-UpF-GWL	TACAAAAAAGCAGGCTagctgcacaagctgatcgct
PA4906-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGgccgttgtcgaacaatcggt
PA4906-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGtgaacatgcgtttccaccgc
PA4906-DnR-GWR	TACAAGAAAGCTGGGTatcgaagtagtcggcgtagc

Table 3.3. Sequences of PCR primers used to amplify genes encoding transcriptional regulators of the GntR family (cont.).

Name	Sequence (5' → 3')[1]
PA5283-UpF-GWL	TACAAAAAAGCAGGCTgattccgtcgatacgccagt
PA5283-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGcatggatatcgatcagccgc
PA5283-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGttccacggggtgaagatgct
PA5283-DnR-GWR	TACAAGAAAGCTGGGTtgcaccagcttgcggtaact
PA5431-UpF-GWL	TACAAAAAAGCAGGCTgcctatgaacagttgcacgc
PA5431-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGtgggtgacgatcacctgttc
PA5431-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGtatgtcacgccgtcacacca
PA5431-DnR-GWR	TACAAGAAAGCTGGGTctggagtcgtccatccagta
PA5525-UpF-GWL	TACAAAAAAGCAGGCTagctgaccatggccgagaat
PA5525-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGaggatctgcaggaaacgctc
PA5525-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGaggcactggaaggactcgaa
PA5525-DnR-GWR	TACAAGAAAGCTGGGTtccaggaggttcagc

[1]Sequences in capital letters are common for all genes amplified and overlap with the Gm or *attB* primer sequences (see Table 3.2. in main text). Lower-case letters indicate gene-specific sequences used for amplification of genes indicated by their PA annotation number.

completed by 25 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 5 min, and a final extension at 68°C for 10 min. The resulting major PCR product was purified by agarose gel electrophoresis and its concentrations determined spectrophotometrically ($A_{260\ nm}$). The identity of the PCR fragment was confirmed by *Xba*I digestion (each *FRT* site of the *FRT*-Gm-*FRT* fragment contains a *Xba*I site).

3.3.6 BP and LR clonase reactions. The BP and LR clonase reactions for recombinational transfer of the PCR product into pDONR221 and pEX18ApGW, respectively, were performed as described in Invitrogen's Gateway cloning manual, but using only half of the recommended amounts of BP and LR clonase mixes and either *E. coli* DH5α (Liss, 1987) or HPS1 (Schweizer, 1994) as host strains. The presence of the correct fragments in transformants obtained with DNA from either clonase reaction was verified by digestion with *Xba*I because each *FRT* site flanking the Gm^r gene contains a *Xba*I site. However, before plasmid isolation from transformants obtained with DNA from the LR clonase reaction, 25-50 transformants were i) patched on LB+Km and LB+Ap plates, and ii) simultaneously purified for single colonies on LB+Ap plates. This was necessary to distinguish between those colonies containing only the desired pEX18ApGW-Δ*Gene*::Gm from those containing this plasmid and the frequently contaminating pDONR-Δ*Gene*::Gm (pEX18Ap-derived plasmids confer Ap^r and pDONR plasmids confer Km^r). Plasmids were then isolated from Ap^r Km^s transformants and analyzed by *Xba*I digestion. In those extremely rare instances where all patched transformants contained a mixed plasmid population (<1% of to date performed LR clonase reactions), retransformation with Ap^r selection was necessary to obtain colonies containing only pEX18ApGW-Δ*Gene*::Gm.

3.3.7 Transfer of plasmid-borne deletions to the *P. aeruginosa* chromosome.

The rapid electroporation method (Section 3.3.2.) was used to transfer the pEX18ApGW-borne deletion mutations to *P. aeruginosa*. For electroporation, 300-500 ng of plasmid DNA was mixed with 100 µl of electrocompetent cells prepared as above and transferred to a 2 mm gap width electroporation cuvette. After applying a pulse (settings: 25 µF; 200 Ohm; 2.5 kV on a Bio-Rad GenePulserXcell™), 1 ml of LB medium was added at once, and the cells were transferred to a 17 x 100 mm glass or polystyrene tube and shaken for 2 h at 37°C. However, to minimize emergence of spontaneous *sacB* mutations in pEX-based plasmids due to sucrose exposure, we keep post-pulse recovery times with these plasmids to a minimum (0 to 1 h). The cells were then harvested in a microcentrifuge tube. 800 µl of the supernatant was discarded and the cell pellet resuspended in the residual medium. The entire mixture was then plated on two LB plates containing 30 µg per ml Gm (LB+Gm30). The plates were incubated at 37°C until colonies appeared (usually within 24 h). Under these conditions, the transformation efficiencies were generally 30-100 transformants per µg of DNA. A few colonies were patched on LB+Gm30 plates and LB+Cb200 plates to differentiate single- from double cross-over events. To ascertain resolution of merodiploids, Gm^r colonies were struck for single colonies on LB+Gm30 plates containing 5% sucrose. Gm^r colonies from the LB-Gm-sucrose plates were patched onto LB+Gm30+5% sucrose, as well as LB plates with 200 µg per ml carbenicillin (LB+Cb200). Colonies growing on the LB-Gm-sucrose, but not on the LB-carbenicillin plates were considered putative deletion mutants. The presence of the correct mutations was verified by colony PCR. To do this, a single, large colony (or the equivalent from a cell patch) was picked from a LB-Gm-sucrose plate, transferred to 30 µl H_2O in a microcentrifuge tube and boiled for 5 min. Cell debris was removed

by centrifugation in a microcentrifuge (2 min; 16,000x*g*), and the supernatant was transferred to a fresh tube which was placed on ice. 5 µl of the supernatant was used as source of template DNA in a 50 µl PCR reaction containing *Taq* buffer, 1.5 mM MgSO4, 5% DMSO, 0.6 µM each of PA1520-UpF-GWL and PA1520-DnR-GWR, 200 µM dNTPs and 5 units *Taq* polymerase (Fermentas, Hanover, MD). Cycle conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 55°C for 30 s, and 72°C for 2 min s, and a final extension at 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis.

3.3.8 Flp-mediated marker excision. Electrocompetent cells of the newly constructed mutant strain were prepared as described in the preceding paragraph and transformed with 20 ng of pFLP2 (Hoang et al., 1998) DNA as described above. After phenotypic expression at 37°C for 1 h, the cell suspension was diluted 1:1,000 and 1:10,000 with either LB or 0.9% NaCl, and 50 µl aliquots were plated on LB+Cb200 plates and incubated at 37°C until colonies appeared. Transformants were purified for single colonies on LB+Cb200 plates. Ten single colonies were tested for antibiotic-susceptibility on LB±Gm30 plates and on a LB+Cb200 plate. Two Gm^s Cb^r isolates were struck for single colonies onto a LB+5% sucrose plate and incubated at 37°C until sucrose-resistant colonies appeared. Ten sucrose-resistant colonies were retested on a LB+5% sucrose (master) plate and a LB+Cb200 plate. Finally, two sucrose-resistant and Cb^s colonies were struck on LB plates without antibiotics, and their Cb^s and Gm^s phenotypes confirmed by patching on LB±Cb200 and LB±Gm30 plates. Deletion of the Gm^r marker was assessed by colony PCR utilizing the conditions and primers described in the preceding paragraph.

3.3.9 β-galactosidase activity assays. Cells were grown at 37°C with shaking to exponential phase (optical density at 540 nm ~0.4-0.8) in LB medium with 0.05% Brij 58 +/- 0.05% oleic acid. β-galactosidase activity was measured in chloroform/sodium dodecyl sulfate-permeabilized cells and its activity calculated as previously described (Miller, 1992).

3.4 RESULTS

3.4.1. Transfer of chromosomal antibiotic resistance markers. Using the newly developed method, we were able to routinely transfer mutations marked with a Gm^r marker, either a ~1.1 kb cassette (Hoang et al., 1998) or a ~1.8 kb *mariner*-based transposon (Wong et al., 2000), between PAO1 strains. Depending on the incubation times after electroporation, transformation efficiencies ranged from < 10 to ~50 colonies per 500 ng of input DNA (Table 3.4) and higher DNA concentrations did not result in more transformants (not shown). Fig. 3.1 illustrates successful transfer of a Δ(*mexAB*)::Gm^r mutation into PAO1. We also tested the method for transferring the same mutation from the PAO1 background to PAK and PA14. Although transformation of PAK or PA14 with PAO1-derived DNA resulted in a 10–100-fold lower transformation efficiency, presumably due to restriction barriers, the Δ(*mexAB*)::Gm^r mutation was nonetheless successfully transferred (Fig. 3.1). Again, input DNA concentrations > 500 ng did not improve transformation efficiencies. Since colonies were routinely obtained and all tested transformants contained the correct mutations, the observed efficiencies are sufficient for routine marker transfer between different *P. aeruginosa* strains.

Although we mostly concentrated on transfer of small (1–2 kb) Gm^r selection markers between PAO1 strains, we also managed to transfer a ~6.2 kb tetracycline

Table 3.4. Effects of various parameters on PAO1 transformation efficiencies

DNA	Replicative	Size	Amount (ng)	Selection	Transformation efficiency (transformants/microgram of DNA)		
					Recovery time (h)		
					0	1	2
Chromosomal fragments	No[a]	20–30 kb	500	Gm^r	8	28	48
pUCP20	Yes[b]	3898 bp	20	Cb^r	1.7×10^9	4.0×10^{10}	2.8×10^{11}
pUCP24	Yes[b]	4036 bp	20	Gm^r	5.6×10^8	1.7×10^9	5.6×10^{10}
pUCP26	Yes[b]	4977 bp	20	Tc^r	5.6×10^8	4.6×10^9	3.5×10^{11}
pPS1527	No[c]	8174 bp	300	Gm^r	3	27	30
pPS1596	No[c]	7850 bp	300	Gm^r	13	33	110

In all cases, 100 µl aliquots of electrocompetent cells were transformed with the indicated amounts of DNA. One milliliter of LB medium was added and the cells were either immediately ($T = 0$) plated on selective medium or after 1 or 2 h shaking at 37 °C. Selective medium consisted of LB with 200 µg/ml Cb, or 30 µg/ml Gm or 50 µg/ml Tc. Transformants were counted after overnight incubation at 37 °C.
[a] After incubation for the indicated amount of time, cells were concentrated 1 : 10 and the entire mixture was plated on a single LB + Gm30 plate. Antibiotic resistant colonies were the result of homologous recombination between chromosomal DNA fragments flanking the Gm^r marker in a

Δ(*mexAB*)::Gm^r allele. The numbers shown are from a single experiment but similar numbers were obtained with other chromosomally inserted Gm^r markers.

[b] After incubation for the indicated amount of time, cells were diluted up to 10^9-fold in LB medium and 100 µl aliquots were plated on selective plates to yield single colonies. The numbers shown are averages from two separate experiments.

[c] Since these pEX18Ap-based plasmids do not replicate in *P. aeruginosa*, antibiotic-resistant colonies after transformation were the result of either plasmid-integration (merodiploid formation) into the chromosome via a single cross-over event or exchange of plasmid sequences for chromosomal sequences via a double cross-over event. Both possible scenarios are mediated by homologous recombination between the ~500 bp flanking sequences present on the plasmids and the corresponding chromosomal sequences. After incubation for the indicated amount of time, cells were concentrated 1 : 10 and the entire mixture was plated on a single LB + Gm30 plate. Transformants were patched on LB + Gm30 and LB + Cb200 plates to distinguish double from single cross-over events. Data shown are the total numbers of Gm^r colonies after patching from single experiments but transformation into different PAO1 mutant derivatives yielded similar numbers.

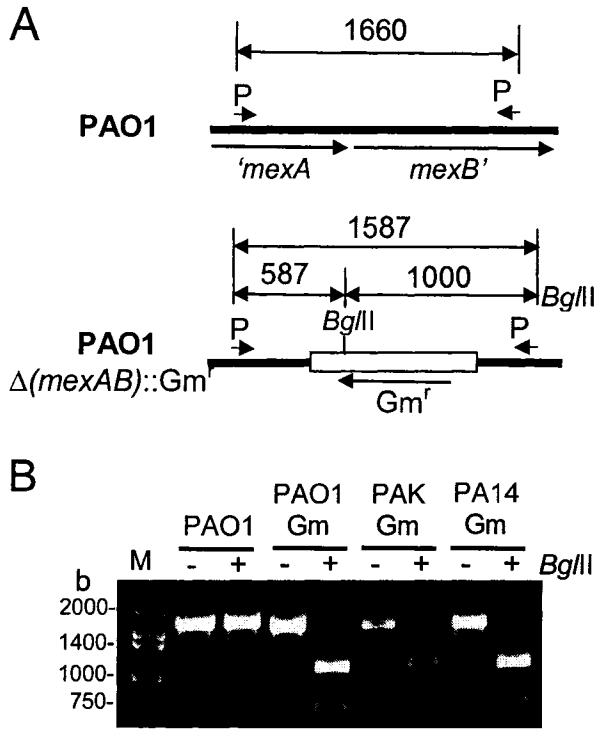


Fig.3.1. Transfer of a Δ(*mexAB*)::Gm^r mutation between different *P. aeruginosa* strains. **A.** Genetic make up of the *P. aeruginosa* PAO1 wild-type (top) and mutant (bottom) *mexAB* region with the approximate location and transcription direction of the Gm^r selection marker. A total of 534 bp of the *mexAB* genes was deleted and replaced with a 1053 bp Gm^r-encoding fragment. The locations of PCR primer sites, a unique *Bgl*II site, and expected sizes of DNA fragments before and after *Bgl*II digestion are also indicated. Although only shown for PAO1, this information is the same for PAK and PA14. **B.** Chromosomal DNAs prepared by boiling of cells were used as templates in PCR reactions primed with P1 and P2. As expected, the PCR fragment obtained from PAO1 (as well as PAK and PA14, not shown) DNA templates was not cleaved by *Bgl*II, whereas the PCR fragments obtained with DNA from Gm^r transformants were cleaved with *Bgl*II due to the presence of a *Bgl*II site within the Gm^r-encoding *aacC1* gene. Lane M contained Hi-Lo molecular size markers from Minnesota Molecular.

resistance-encoding transposon insertion from a strain of the comprehensive PAO1 transposon library (Jacobs et al., 2003) into our PAO1 laboratory strain. However, efficiencies were pretty poor (2 to 8 transformants per microgram of input DNA) and for unknown reasons tetracycline resistance markers were inherently more difficult to work with, partially because a fairly high antibiotic concentration (100 μg per ml) was required for marker selection to avoid a background of untransformed colonies.

3.4.2. Transformation with replicative plasmids. *E. coli–P. aeruginosa* shuttle plasmids are mostly used for complementation or expression experiments. For plasmid transformation, the same conditions described above were used, except that dilutions suitable to obtain single colonies were plated. When cells were plated on selective media immediately after electroporation, transformation efficiencies ranged from 5.6×10^8 to 1.7×10^9 per microgram input DNA when using a plasmid with the same backbone, but containing different selection markers (Table 3.4). Incubation for 1 h or 2 h further improved the transformation efficiencies by two to three logs. A similar efficiency (3.7×10^8/μg) was obtained when 50 ng of the larger (9297 bp) pFLP2 were electroporated into PAO1 and cells were incubated for 1 h before plating. With PAO1, the observed transformation efficiencies are 100–10,000-fold higher when compared to various published *P. aeruginosa* electroporation protocols (Dennis et al., 1995, Diver et al., 1990, Enderle et al., 1998, Farinha et al., 1990, Irani et al., 1997, Smith et al., 1989). Although we only tested plasmids within a relatively small size range (3.8–9.3 kb), input DNA size had no obvious effect on transformation efficiencies. Similar observations were previously made utilizing a traditional electroporation method where transformation efficiencies obtained with 4.6 and 21.6 kb plasmids were indistinguishable (Diver et al., 1990). To test the efficiency of the

method with other *P. aeruginosa* strains, PA14 and PAK competent cells were prepared and electroporated with 20 ng of pUCP20 DNA. In a typical experiment, the transformation efficiencies were 2.2×10^6 and 6.7×10^8 transformants per microgram of DNA for PA14 and PAK, respectively.

3.4.3 Transformation with non-replicative plasmids. Non-replicative plasmids are widely used in gene replacement experiments and for transposon mutagenesis. To assess the utility of the procedure for gene replacement experiments, pPS1527 and pPS1596, which are based on the gene replacement vector pEX18Ap (Hoang et al., 1998), were used. These plasmids contain *PA1520* and *PA3676* gene deletions, respectively, and ~500 bp of chromosomal DNA flanking the Gm^r cassette inserted in each gene. With a 2 h post-electroporation incubation each plasmid yielded 30–110 transformants per microgram of input DNA (Table 3.4), resulting from homologous recombination between plasmid-borne sequences and the chromosome, either mediated by single (44% for pPS1527; 30% for pPS1596) or double (56% for pPS1527; 70% for pPS1596) cross-over events.

Finally, we determined the efficiency of the procedure for transposon delivery. Site-specific integration of a mini-Tn*7* derivative at its *att*Tn*7* site (Peters et al., 2001) was very efficient and with 50 ng of helper plasmid and transposon delivery vector input DNAs we routinely obtained > 500 transformants (Fig. 3.2). In a typical experiment, transformation of PAO1 cells with 500 ng of the 6587-bp *mariner*-transposon delivery plasmid pBT20 (Kulasekara et al., 2005) yielded 1.5×10^3 transformants per microgram of input DNA following a 2 h post-electroporation incubation. These transformants were the result of *mariner* transposition into random positions in the chromosome. Typically, plating a couple of 1 ml transformation

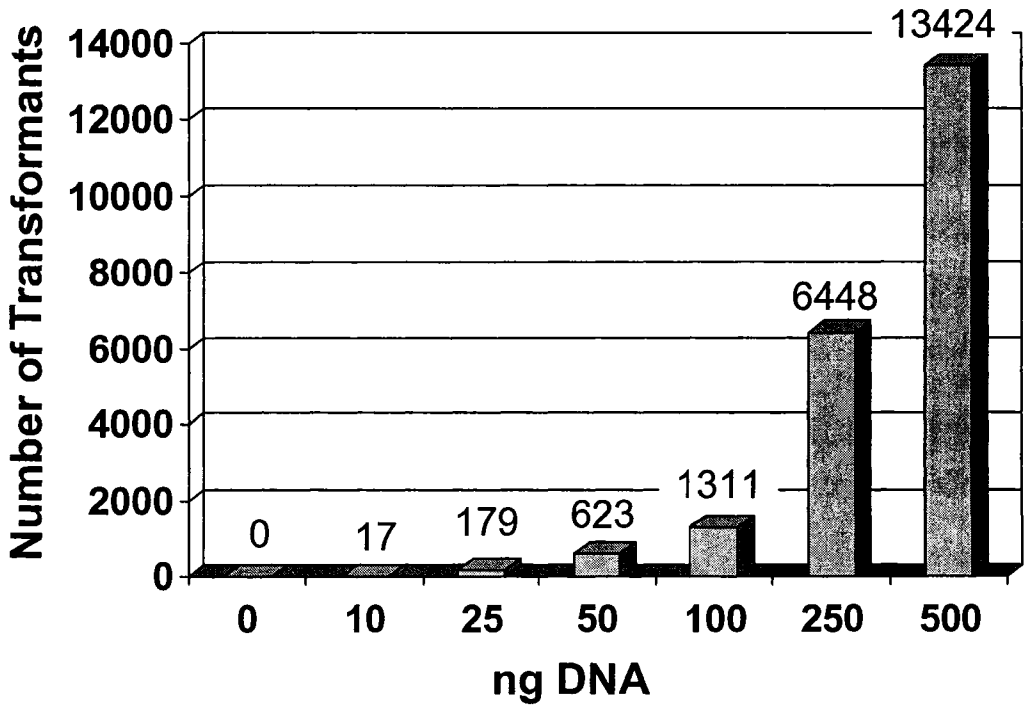


Fig. 3.2. Efficiency of Tn*7* delivery into PAO1 by co-electroporation of non-replicative helper plasmid and mini-Tn*7* delivery vector DNA. One hundred microliter aliquots of electrocompetent PAO1 cells prepared by the rapid method were transformed with equal amounts of pTNS1 (~10.8 kb) and mini-Tn*7* delivery vector pUC18-mini-Tn*7*T-Gm-*lacZ* DNA (7656 bp). After electroporation, 1 ml of LB medium was added to the samples and the cell suspensions were incubated at 37 °C for 2 h. Aliquots were then plated on LB + Gm30 plates at dilutions that would yield single colonies. Gm^r transformants were counted after overnight incubation at 37 °C. The numbers above the bars represent actual colony counts from a single experiment, but these results were reproducible in different experiments.

mixes resulting from 100 µl competent cells and 500 ng of pBT20 each yielded upwards of 25,000 transposon insertions, which was sufficient for most routine transposon mutagenesis experiments. The mini-Tn*7* transposon system yielded significantly fewer transformants per unit input DNA when compared to the *mariner* system because the former necessitates simultaneous transformation of recipient cells with two plasmids, the transposon delivery plasmid and transposase-expressing helper plasmid, whereas the latter contains the transposon and transposase on a single plasmid.

3.4.4 Construction of a Gateway-compatible suicide vector. We previously described the pEX family of relatively small, fully sequenced suicide plasmids which are now widely used for gene replacement experiments in *P. aeruginosa* and related bacteria (Hoang et al., 1998). Wolfgang et al. (Wolfgang et al., 2003) recently described a Gateway-compatible pEX18Gm derivative. Here, we modified pEX18Ap for use as a Gateway destination vector for cloning of mutations marked with a gentamycin resistance (Gm^r) encoding gene. We prefer using the Gm^r marker because it is more easily transferable between *P. aeruginosa* chromosomes by transformation with chromosomal DNA fragments when compared to other selection markers (Choi et al., 2005b). The resulting vector pEX18ApGW (Fig. 3.3) contains the functional sequences for recombination-based cloning (*attR1* and *attR2*) and the *ccdB* counterselection marker, while maintaining the *sacB* counterselection marker for downstream resolution of merodiploids.

3.4.5 Generation of a mutant fragment by PCR overlap extension. The amplification of each mutant fragment was achieved in a two step PCR that required 8

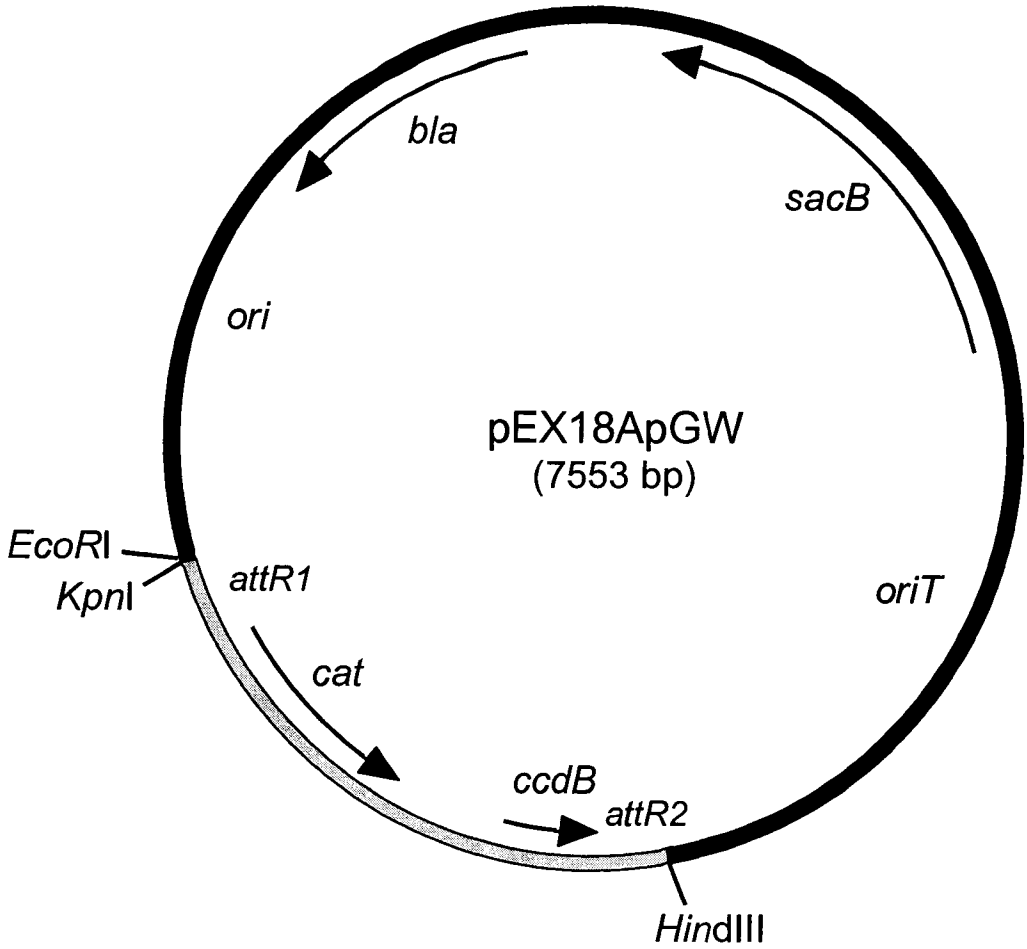


Fig. 3.3. Map of the Gateway-compatible pEX18ApGW. This vector was derived by cloning a Gateway conversion fragment (grey) into the multiple cloning site of pEX18Ap (black). Only selected restriction enzyme cleavage sites are shown. Abbreviations: *attR1* and *attR2*, bacteriophage λ recombination sites; *bla*, β-lactamase-encoding gene; *cat*, chloramphenicol acetyl transferase-encoding gene; *ccdB*, gene encoding gyrase-modifying enzyme (CcdB poisons host DNA gyrase by forming a covalent complex with the DNA gyrase A subunit and thus serves as a counter-selectable marker in $gyrA^+$ cloning hosts [Bernard et al., 1994]); *ori*, ColE1-derived replication origin; *oriT*, origin of conjugal transfer; *sacB*, *Bacillus subtilis* levansucrase-encoding gene. The sequence of this plasmid was deposited in GenBank and assigned accession number AY928469.

PCR primers, 4 of which were unique for each fragment to be mutated and 4 of which were common primers (Table 3.2). The gene-specific portions of the unique primers were designed to yield T_m's of 55-57°C and PCR fragments ranging from 200 to 600 bp. These parameters enabled utilization of the same PCR conditions for different target genes, while at the same time provided for amplification of sufficient chromosomal DNA to allow for efficient homologous recombination of plasmid-borne sequence with the chromosome.

The basic strategy for generation of the mutant fragment by PCR overlap extension is illustrated in Fig. 3.4. In PCR1, the four gene-specific primers are used to amplify the 5' and 3' regions of the target gene. Simultaneously, two of the common primers, Gm-F and Gm-R, are used to amplify the Gm^r cassette flanked by *FRT* sites from a pPS856 template. Since the Gm^r fragment is used for all constructs, it can be prepared in larger amounts ahead of time and stored until needed. Fig. 3.5A illustrates typical results of PCR1 utilizing the example of *P. aeruginosa* gene *PA1520*, encoding a putative transcriptional regulator of the GntR family. When we adhered to the prescribed PCR conditions, the amplified fragments were very clean. It was especially prudent to follow the conditions for PCR amplification of the Gm^r *FRT* fragment since the *FRT* sites possess significant secondary structures.

The PCR fragments were gel-purified and equal amounts (50 ng) of each were combined for PCR2. PCR2 was allowed to proceed for 3 cycles in the absence of exogenous primers, which for *PA1520* yields the intermittent 1,709 bp '*attB1-PA1520*'-*FRT*-Gm-*FRT*-'*PA1520-attB2*' fragment illustrated in Fig 3.5A. The common Gateway primers GW-*attB1* and GW-*attB2* were then added and the PCR continued for another 25 cycles to obtain the final, recombination-proficient DNA fragment, which constituted the predominant PCR product at this stage (Fig. 3.5B).

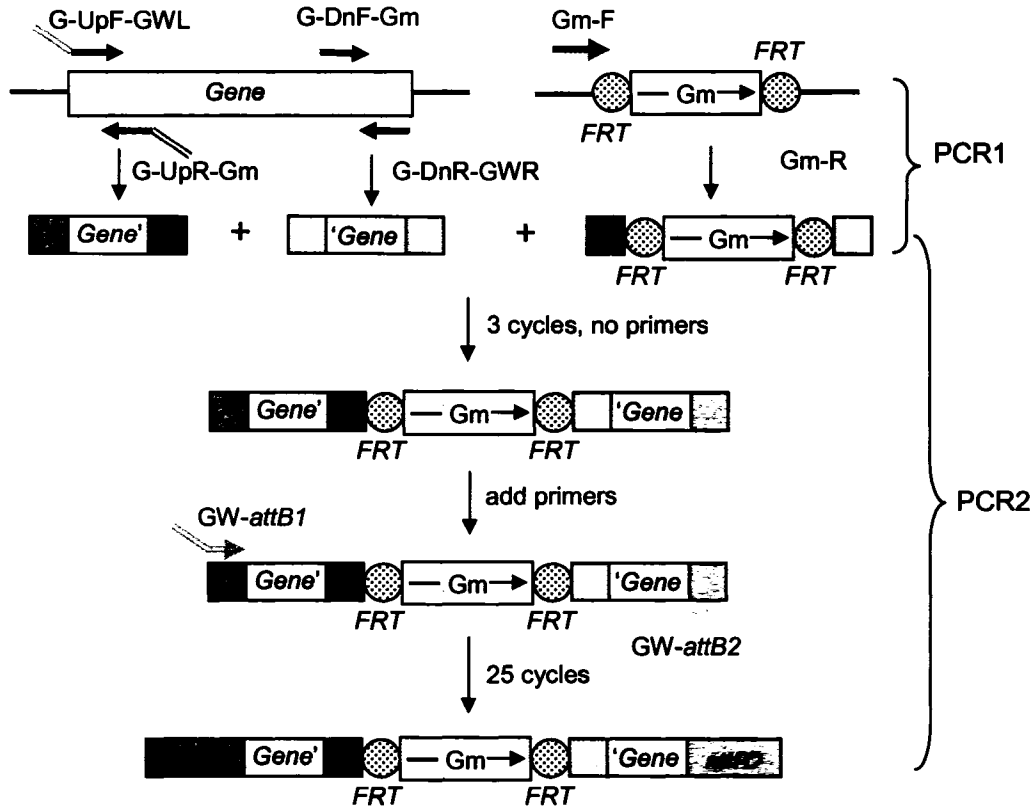


Fig. 3.4. Schematic illustration of mutant fragment generation by overlap extension PCR. During first round PCR (PCR1), the 5' and 3' ends of the target genes, as well as the gentamycin (Gm) resistance cassette are amplified using four gene-specific primers (G-UpF-GWL, G-UpR-Gm, G-DnF-Gm and G-DnR-GWR) and the common Gm-specific primers (Gm-F and Gm-R). This generates three fragments with partial overlaps either to each other (indicated by the blue boxes signifying Gm overlap) or the *attB1* and *attB2* λ recombination sites (indicated by the green and pink boxes). These purified fragments are then assembled *in vitro* by overlap extension during second round PCR (PCR2) using common primers GW-*attB*1 and GW-*attB*2, resulting in a recombination-proficient mutant PCR fragment.

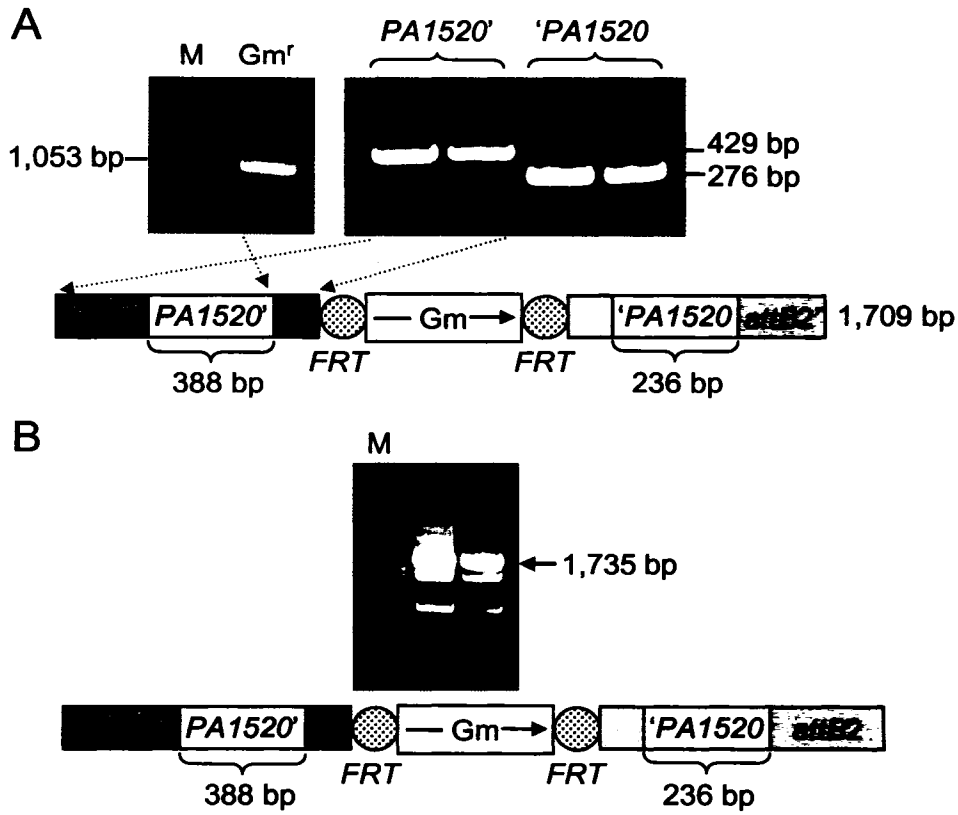
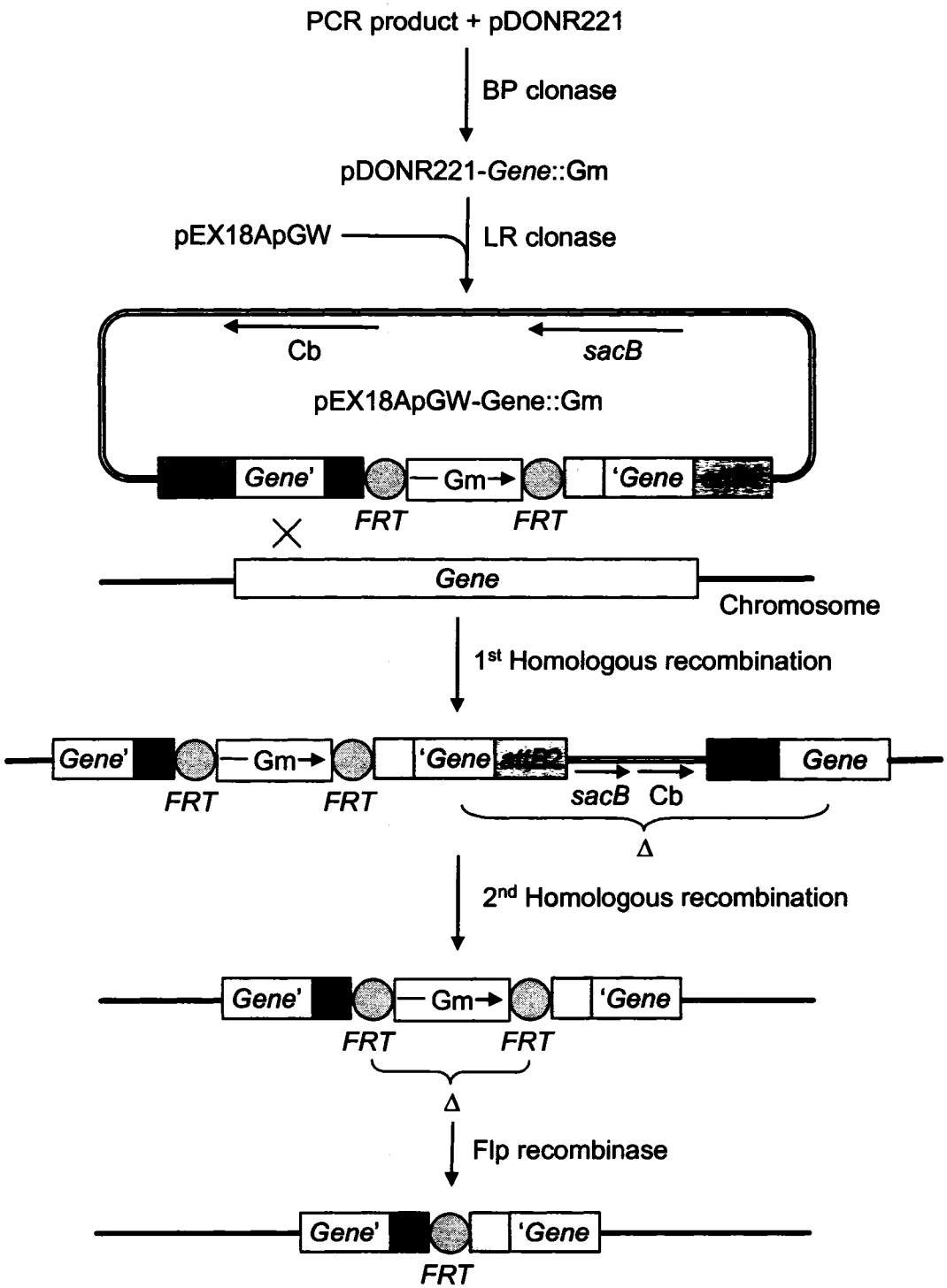


Fig. 3.5. PCR amplification of the Gm *FRT* cassette, *PA1520* gene fragments and the overlap extension product. **A.** First round PCR fragments. The left panel illustrates amplification of the 1,053-bp Gm^r fragment from pPS856 which contains 24 bp (right) and 25 bp (left) overlaps with the *PA1520'* and *'PA1520* fragments (blue boxes). The right panel illustrates amplification of the *PA1520'* and *'PA1520* fragments. The 5' fragment contains 388 bp of chromosomal DNA, 25 bp overlapping the left side of the Gm^r fragment and 16 bp overlapping the GW-*attB1* primer. Similarly, the 3' fragment contains 236 bp of chromosomal DNA, 24 bp overlapping the right side of the Gm^r fragment and 16 bp overlapping the GW-*attB2* primer. The sequences of the gene-specific and common primers are listed in Table 3.2. **B.** Second round PCR. The purified fragments shown in panel **A** were used in the second round PCR illustrated in Fig. 3.4 to derive the indicated *attB1-PA1520'-FRT*-Gm-*FRT-'PA1520-attB2* fragment. The entire 50 µl second round PCR reaction was subjected to agarose gel electrophoresis. The desired DNA fragment constituting the major product marked by the arrow was excised from the gel, purified and then used for the BP clonase reaction. Lanes labeled M in both panels contained Hi-Lo molecular size markers from Minnesota Molecular (Minneapolis, MN).

For *PA1520*, the resulting 1,735 bp *attB1-PA1520'-FRT*-Gm-*FRT*-'*PA1520-attB2* fragment was gel-purified and used for the BP clonase reaction for construction of an entry clone.

3.4.6 BP and LR clonase reactions. The BP and LR clonase reactions (Fig. 3.6) were performed according to Invitrogen's (Carlsbad, CA) Gateway cloning manual. For the BP clonase reaction pDONR221 was used, but only half of the recommended BP clonase enzyme mix. Gm^r and kanamycin resistant (Km^r) DH5α or HPS1 transformants were selected and the presence of the correct plasmids was confirmed by *Xba*I digestion (the *FRT* sites flanking the Gm^r gene each contain a single *Xba*I site). The insert of a correct pDONR-Δ*Gene*::Gm entry clone was then recombined into the destination vector pEX18ApGW using the LR clonase protocol, again using only half of the recommended amount of LR clonase mix. Recombinants were transformed into DH5α and Ap^r colonies were selected. We noticed that, at least under the conditions we used, many transformants exhibited a Ap^r Km^r phenotype, indicating that they either contained both pDONR-Δ*Gene*::Gm and pEX18ApGW-Δ*Gene*::Gm or, more likely, a cointegrate of both plasmids. The presence of the correct pEX18ApGW-Δ*Gene*::Gm in transformants that were only Ap^r was confirmed by *Xba*I digestion.

3.4.7 Gene replacement. To expedite gene replacement, an improved electroporation method (Choi et al., 2005b) was used for transfer of the suicide plasmid pEX18ApGW-Δ*Gene*::Gm into *P. aeruginosa* instead of the traditionally employed conjugation method. Although after transfer into *P. aeruginosa* double cross-over events can occur frequently (>50%), they can also be rare (<1-5%). In the



latter instance, merodiploids formed via integration of the suicide plasmid by a single cross-over event (Fig. 3.6). The merodiploid state was then resolved via sucrose selection in the presence of gentamycin, resulting in deletion of the wild type gene. For generation of unmarked deletion mutants, the Gm^r marker was subsequently removed by deletion of a 967 bp fragment using Flp recombinase. The presence of the desired deletion in either the marked or unmarked *PA1520* mutant was verified by colony PCR utilizing the gene-specific up and down primers (Fig. 3.7).

3.4.8 Deletion of 25 genes encoding transcriptional regulators of the GntR family. We are interested in characterizing the activator involved in regulation of expression of the *fabAB* operon, which encodes two essential enzymes of *de novo* fatty acid biosynthesis (Hoang et al., 1997). Expression of the *fabAB* operon is repressed by exogenously added fatty acids, most effectively by oleic acid, and preliminary results from our laboratory indicate that this may be due to relief of activation by a transcriptional regulator which binds to a 30 nucleotide site located in the *fabAB* promoter region (Choi and Schweizer, unpublished results). This 30 bp region is only found and conserved in *Pseudomonas* spp. Its deletion dramatically reduces transcription of the *fabAB* operon and the residual transcriptional activity is no longer fatty acid responsive (Fig. 3.8). In *E. coli*, transcriptional activation of the unlinked *fabA* and *fabB* genes is achieved by FadR, a regulator belonging to a group of transcriptional regulators with the GntR family signature (van Aalten et al., 2000, van Aalten et al., 2001). The PAO1 chromosome encodes more than 30 regulatory proteins with this signature. Of these, 27 have not been assigned function. Using the methodology described above for one of these genes, *PA1520*, we deleted 25 of the 27 genes (we could not delete the presumably essential *PA1285* and *PA2299*) in less than

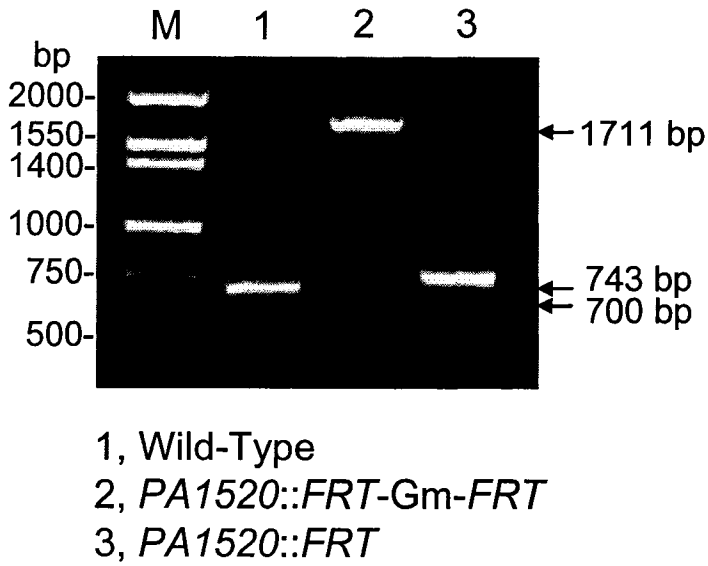


Fig 3.7. PCR analysis of marked and unmarked *P. aeruginosa PA1520* deletion strains. Colony PCR was performed on either wild-type PAO1 and its *PA1520* mutant derivatives, either containing a marked (Δ*PA1520*::*FRT*-Gm-*FRT*) or unmarked (Δ*PA1520*::*FRT*) *PA1520* deletion. The sizes of the expected PCR fragments are indicated. Note that the short deletion removes 41 bp of the *PA1520* coding sequence, corresponding to codons 131 to 145, but replaces these sequences with a 85 bp *FRT* scar. Lane M contained Hi-Lo molecular size markers of the indicated sizes from Minnesota Molecular.

4 weeks in strain PAO1 with a chromosomally integrated *fabA'-lacZ* transcriptional fusion and analyzed β-galactosidase expression in the resulting mutant strains (Fig. 3.8). In exponential phase cells, the overall pattern of *fabA* expression was indistinguishable from the parental strain in all 25 mutants, indicating that none of these genes encodes the *fabAB* activator. We would have expected an expression pattern similar to that observed in the PAO1::*fabA*Δ30'-*lacZ* control strain which contains a deletion of the 30 nucleotide putative activator-binding site.

3.5 DISCUSSION

The main advantages of the method for preparation of electrocompetent cells described here are its simplicity and speed, without compromising efficiency. The entire procedure can be performed with overnight cultures, in a microcentrifuge, at room temperature, with a simple 300 mM aqueous sucrose solution and takes less than 10 min to complete. This is in contrast to traditional procedures which require exponentially growing cells, time-consuming centrifugation steps, vessels and sometimes complex buffers that need to be refrigerated, and take hours to complete. Despite its speed and simplicity, transformation efficiencies are either comparable to or exceed those of traditional methods. Although a microfuge-based method was previously described which used freshly plated *P. aeruginosa* cells, the transformation efficiency was at least 1,000-fold lower than what we observe, probably because of considerable cell lysis due to the water used for cell suspension, and the procedure was not elaborated beyond transformation with a single, small replicative plasmid (Enderle et al., 1998).

The substantially increased transformation efficiencies achievable with the newly described method for preparation of electrocompetent cells provide a solution

to the 45-year-old problem of transduction in *P. aeruginosa*, at least for the transfer of antibiotic resistance markers. We did not test transfer of metabolic markers between chromosomes. From start (isolation of fragmented chromosomal DNA) to finish (plating on selective media) the entire procedure takes only about 3 h to complete. Colonies are ready for testing within a day, as compared to several days to over a week for transduction or gene replacement strategies, respectively. The ability to routinely transfer mutations between different *P. aeruginosa* mutants and strains will undoubtedly expedite proper genetic analyses of this medically important pathogen and provide a means for maximizing the use of the valuable genetic resources, including comprehensive mutant collections, developed for this bacterium. The method is especially powerful in conjunction with previously described methods for marker excision (Flp/*FRT* (Hoang et al., 1998) or Cre/*loxP* (Quenee et al., 2005)), which allows recycling of the same selection marker in the same strain for construction of mutants containing multiple lesions in the same chromosome. This simple procedure clearly proves that some times easy does it and may thus provide useful hints for remediation of similar problems faced with other bacteria. For example, we have found that the method works quite well for transposition of mini-Tn*7* elements into the *Proteus mirabilis* chromosome.

The simple method for preparation of highly electrocompetent *P. aeruginosa* cells now serves all of our DNA transfer needs, not only for DNA fragment transfer between chromosomes, but also for transformation with replicative and non-replicative plasmids. The rapid procedure accelerates routine complementation experiments with replicative plasmids, especially in situations requiring simultaneous transformation into diverse genetic backgrounds. The observed transformation efficiencies with replicative plasmids approach, and with some plasmids even exceed,

those of commercially available *E. coli* competent cells. They are even more remarkable considering that all of the plasmids used in these experiments were isolated from *E. coli* and thus face a considerable restriction barrier upon transformation into *P. aeruginosa*. This is not due to a significant higher input of viable cells, since our method employs 10^9–10^{10} colony forming units (cfu) of cells per 100 µl of electroporation mix, which is comparable to the 10^7–10^{10} cfu used in traditional electroporation methods (Diver et al., 1990, Farinha et al., 1990, Smith et al., 1989). For non-replicative plasmids, it replaces the traditional, relatively cumbersome and time-consuming conjugal transfer methods. The observed transformation efficiencies are more than sufficient for routine gene replacement, site-specific gene integration and transposon mutagenesis experiments.

Utilizing the methods described here we have now transferred more than 25 mutations between PAO1 mutant chromosomes, complemented and expressed numerous genes, isolated over 30 mutants by gene replacement, prepared several *mariner* transposon mutant libraries, and delivered mini-Tn*7* (Choi et al., 2005a) and mini-CTX (Hoang et al., 2000) derivatives to their respective integration sites. In our experience, the method described here for generation of unmarked deletion mutants is the fastest available to date. From start to finish, an individual unmarked *P. aeruginosa* mutant can be generated and characterized in ~14 days, which is up to one week faster than using our previously described method (Hoang et al., 1998). However, the main advantage of the method does not lie in individual mutant generation but in the speed by which collective deletion mutant pools can be generated. For example, we isolated unmarked deletions in 25 of the 27 genes encoding transcriptional regulators of the GntR family in less than 4 weeks. Two

genes could not be deleted, presumably because they encode essential proteins. Besides speed, what are some of the other unique features of this method?

First, the deletion of gene sequences is not restricted by the natural availability or synthetic generation of restriction sites that are subsequently used to delete intervening sequences. With proper primer location, the PCR-based method allows clean deletion of more-or-less the entire coding sequence without fear of generating truncated genes which potentially produce products with undesired side effects.

Second, Flp excision of the antibiotic resistance gene cassette leaves behind a short 85 bp *FRT* scar which does not exhibit any known polar effects on downstream genes. The method is therefore uniquely suited to study genes organized in polycistronic units. Flp excision is a very straightforward procedure with efficiencies approaching 100% (Hoang et al., 1998).

Third, the method is economical, especially in a more high-throughput format. PCR overlap extension requires only four gene specific primers of reasonable length which keeps oligonucleotide costs to a minimum. In comparison to traditional methods, it also requires fewer hands-on steps and gene-specific reagents (e.g., restriction enzymes).

3.6 CONCLUSIONS

Although not yet as fast as the PCR fragment-directed gene replacement method described for *E. coli*, the PCR- and recombinational cloning based method described opens the door for high-throughput generation of *P. aeruginosa* deletion mutants on a genome-wide scale. With appropriate modifications, i.e., choice of appropriate selection markers and Gateway-compatible gene replacement vectors, the

method should be applicable to the many other bacteria in which the pEX vector system can be applied (Schweizer et al., 2004).

3.7 ACKNOWLEDGMENTS

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CHAPTER 4

Two aerobic pathways for the formation of unsaturated fatty acids in *Pseudomonas aeruginosa*

(Presented by Kun Zhu, Kyoung-Hee Choi, Herbert P. Schweizer, Charles O. Rock and Yong-Mei Zhang. *Mol. Microbiol.* 2006 60:260-273)

The work presented in this article demonstrated that unsaturated fatty acids in *P. aeruginosa* are synthesized by two pathways: 1) anaerobic biosynthesis via the FabAB pathway, and 2) aerobic synthesis via the DesA and DesBC pathways. My major contributions to this work consisted of intensive mutant constructions, genetic verification and initial characterization of *fabAB* expression. I acknowledge Dr. Yong-Mei Zhang for the permission to use information contained in this article for my dissertation.

4.1 ABSTRACT

The double bond in anaerobic unsaturated fatty acid (UFA) biosynthesis is introduced by the FabA dehydratase/isomerase of the bacterial type II fatty acid biosynthetic pathway. A Δ*fabA* mutant of *Pseudomonas aeruginosa* grew aerobically, but required a UFA supplement for anaerobic growth. Wild-type cells produced $18{:}1^{\Delta 11}$ as the principal UFA, whereas the Δ*fabA* strain produced only $16{:}1^{\Delta 9}$. The double bond in the $16{:}1^{\Delta 9}$ was introduced after phospholipid formation and was localized in the *sn*-2 position. Two predicted membrane proteins, DesA and DesB, possessed the conserved histidine clusters characteristic of fatty acid desaturases. The

Δ*fabA*Δ*desA* double mutant required exogenous fatty acids for growth but the Δ*fabAdesB* double mutant did not. Exogenous stearate was converted to $18:1^{\Delta 9}$ and supported the growth of Δ*fabA*Δ*desA* double mutant. A Δ*fabA*Δ*desAdesB* triple mutant was unable to desaturate exogenous stearate and was an UFA auxotroph. We detected a 2.5-fold increase in *desA* expression in Δ*fabA* mutants, whereas *desB* expression was derepressed by the deletion of the gene encoding a transcriptional repressor DesT. These data add two aerobic desaturases to the enzymes used for fatty acid metabolism in proteobacteria: DesA, a 2-position phospholipid Δ9-desaturase that supplements the anaerobic FabA pathway, and DesB, an inducible acyl-CoA Δ9-desaturase whose expression is repressed by DesT.

4.2 INTRODUCTION

Biological membranes are required to maintain the appropriate fluidity to support normal membrane structure and function under various growth conditions. Bacterial membranes generally lack sterols and contain mainly phospholipids such as phosphatidylethanolamine (PtdEtn) and phosphatidylglycerol (PtdGro). To achieve the correct physical state of the membrane lipids, bacteria stringently control the production of a variety of fatty acids with different melting temperatures (Mansilla et al., 2004). When adapting to an environment that requires more rigidity in membrane lipids, cells produce more saturated fatty acids (SFA). When increased fluidity is needed, more unsaturated fatty acids (UFA) or branched-chain fatty acids (BFA) are produced.

Two unique biochemical reactions are required to produce UFAs in the model type II fatty acid biosynthesis system of *Escherichia coli* (Cronan et al., 1969, Rock et al., 2002). When the growing acyl chain reaches the β-hydroxydecanoyl-ACP stage,

FabA carries out the dehydration and isomerization reactions to produce *cis*-3-decenoyl-ACP to divert the nascent acyl chain into the UFA synthetic pathway. The *cis*-3-enoyl intermediate is then elongated by FabB, the primary determining factor in cellular UFA content in *E. coli* (Clark et al., 1983, Cronan et al., 1969). Some Gram-positive bacteria, such as Streptococci, utilize a combination of FabZ and FabM to introduce the *cis*-3 double bond into the growing acyl chain (Marrakchi et al., 2002) and FabF to carry out the elongation of UFA. The second mechanism for UFA synthesis in bacteria is aerobic desaturation of the existing fatty acids by desaturases, a group of iron-containing transmembrane enzymes found in bacilli (Aguilar et al., 1998), *Mycobacterium tuberculosis* (Phetsuksiri et al., 2003) and photosynthetic cyanobacteria (Los et al., 1998). In addition to UFAs, BFA can also lower the melting temperature or increase the fluidity of biological membranes. The BFA synthesis shares the same pathway as SFA except that the initiation condensing enzyme FabH prefers isobutyryl-CoA and isovaleryl-CoA as the primers for BFA synthesis (Choi et al., 2000, Han et al., 1998).

Fatty acid biosynthesis is coordinately regulated at the transcriptional level as part of the response to changes in the environment. In *E. coli*, transcriptional factors FadR and FabR work together to regulate UFA formation, with FadR being the activator (Henry et al., 1991) and FabR the repressor (Zhang et al., 2002) of the key genes *fabA* and *fabB*. FapR and FabT are global transcriptional repressors that simultaneously control the expression of many genes involved in fatty acid synthesis in Gram-positive bacteria (Lu et al., 2004, Schujman et al., 2003). A two-component signal transduction system, DesK–DesR, regulates the expression of the desaturase in *Bacillus subtilis* (Aguilar et al., 2001). This mechanism allows *B. subtilis* to adapt to an abrupt downshift of the growth temperature.

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen and its membrane contains straight-chain SFAs and UFAs (Kropinski et al., 1987). The two key components for UFA production using the dissociated fatty acid biosynthetic pathway are cotranscribed in a *fabAB* operon in *P. aeruginosa* (Hoang et al., 1997). Previous studies on psychrotrophic *Pseudomonas* (Wada et al., 1989) and *Vibrio* (Morita et al., 1992, Wada et al., 1989) strains implied that an aerobic mechanism for UFA formation was present in these strains. Here, we have identified and characterized two oxygen-dependent desaturases in *P. aeruginosa*, namely DesA (PA0286) and DesB (PA4888). Our results describe these two oxidative desaturases with different substrate specificities that supplement the anaerobic mechanism for UFA synthesis in a Gram-negative proteobacterium.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strains and growth condition. Bacterial strains and plasmids used in this study were listed in Table 4.1. *P. aeruginosa* strains were grown in rich medium LB or M9 medium with 0.4% glycerol as carbon source (Miller, 1972, Rock et al., 1985). Anaerobic growth on LB agar plates with 1% potassium nitrate (Hassett, 1996) was performed in an anaerobic bag (Becton Dickinson, Sparks, MD). When added, the oleate concentration was 0.1%, the stearate concentration was 0.01%, and the Brij-58 concentration was 0.5%. [1-^{14}C]Acetic acid (specific activity, 55 mCi mmol^{-1}) and [1-^{14}C]stearic acid (specific activity, 55 mCi mmol^{-1}) were purchased from American Radiolabelled Chemicals. *Naja mossambica* phospholipase A2 was purchased from Sigma.

4.3.2 Plasmid constructions and mutant generation. The Δ*desT* deletion mutant

Table 4.1. Strains and plasmids used in this study.

Strains and plasmids	Description	Source
P. aeruginosa		
PAO1	Wild-type	Holloway et al., 1955
PAO482	PAO1 with Δ*desT*::*FRT*	
PAO640	Gm^r; PAO1 with Δ*fabA*::*FRT*-Gm^r-*FRT*	This study
PAO641	Gm^r; PAO1 with Δ*desA*::*FRT*-Gm^r-*FRT*	This study
PAO651	PAO1 with Δ*desA*::*FRT*	This study
PAO652	PAO1 with Δ*fabA*::*FRT*	This study
PAO682	PAO1 with Δ*fabA*::*FRT*Δ*desA*::*FRT*	This study
13272	Tc^r; *desB*::IS*lacZ*/hah	Jacobs et al., 2003
PAO739	Tc^r; 13272 with Δ*fabA*::*FRT desB*::IS*lacZ*/hah	This study
PAO754	Tc^r; PAO739 with Δ*fabA*::*FRT*Δ*desA*::*FRT desB*::IS*lacZ*/hah	This study

Table 4.1. Strains and plasmids used in this study (cont.).

Strains and plasmids	Description	Source
Plasmids		
pDONR221	Km^r; Gateway donor vector	Invitrogen
pEX18Ap	Ap^r; Gene replacement vector with MCS from pUC18	Hoang et al., 1998
pEX18ApGW	Ap^r; Gateway destination vector	Choi et al., 2005
pFLP2	Ap^r; source for Flp recombinase	Hoang et al., 1998
pUCP20	Ap^r; broad-host range vector	West et al., 1994
p*desA*	Ap^r; *desA* from PAO1 inserted within *Xba*I-*Hin*dIII of pUCP20	This study
pPS1505	Ap^r, Gm^r; pEX18Ap with *Xho*I-*Sma*IΔ*desT*::Gm^r PCR fragment inserted between *Sal*I and *Sma*I sites	This study
pPS1604	Gm^r, Km^r; pDONR221 with *attB1-fabA'-FRT-*Gm^r*-FRT-'fabA-attB2*	This study
pPS1605	Gm^r, Km^r; pDONR221 with *attB1-desA'-FRT-*Gm^r*-FRT-'desA-attB2*	This study
pPS1606	Ap^r, Gm^r; pEX18ApGW with *attL1-fabA'-FRT-*Gm^r*-FRT-'fabA-attL2*	This study
pPS1607	Ap^r, Gm^r; pEX18ApGW with *attL1-desA'-FRT-*Gm^r*-FRT-'desA-attL2*	This study

PAO482 was created by gene replacement technology using pEX18Ap (Hoang et al., 1998). A Δ*desT*::Gm fragment (containing a deletion of 191 codons in *desT* marked with a gentamicin resistance (Gm^r) gene) was obtained by splicing overlap extension PCR and was cloned into pEX18Ap to yield pPS1505. PAO482 was created by mating *E. coli* SM10*lacI*^{q} harboring pPS1505 with PAO1 followed by Flp mediated marker excision. The Δ*fabA* and Δ*desA* deletion mutants marked with a Gm^r marker were created by a recently described method using Gateway technology for accelerated mutant construction (Choi et al., 2005). Briefly, recombination-proficient *attB1-fabA'-FRT-*Gm^r*-FRT-'fabA-attB2* and *attB1-desA'-FRT-*Gm^r*-FRT-'desA-attB2* fragments were generated in two steps by overlap extension PCR using four gene-specific primers and two common primers GW-*attB1* and GW-*attB2* (Table 4.2). BP (*att*B-*att*P) and LR (*att*L-*att*R) recombination reactions were performed to transfer the PCR product into pDONR221 (Invitrogen) and pEX18ApGW (Choi et al., 2005), followed by transfer of the plasmid-borne *fabA* and *desA* deletion alleles to the chromosome (Choi et al., 2005). This resulted in strains PAO640 (Δ*fabA*::Gm^r) and PAO641 (Δ*desA*::Gm^r) containing deletions of 151 codons (*fabA*) and 258 codons (*desA*) respectively. Unmarked deletion mutant strains PAO651 and PAO652 were generated by Flp-mediated marker excision utilizing pFLP2 (Hoang et al., 1998) as described (Choi et al., 2005). Strain PAO682 (Δ*fabA*Δ*desA*) was created by transferring chromosomal DNA fragments containing Δ*fabA*::Gm^r from PAO640 into the unmarked Δ*desA* mutant strain PAO651 as previously described following by Flp-mediated marker excision (Choi et al., 2006). Similarly, the same chromosomal DNA fragment from strain PAO640 was transferred into the *desB* transposon insertional mutant strain 13272 (Jacobs et al., 2003), to yield the Δ*fabAdesB* strain PAO739. Finally, utilizing the same procedures the Δ*desA*::Gm^r mutation from strain PAO641

Table 4.2. Primers for mutant construction.

Primer	Sequence (5'-3')[a]
fabA-specific primers	
fabA-UpF-GWL	TACAAAAAAGCAGGCTgcaacgcatactgcaacgct
fabA-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGagcaggtcttctcgggtgaa
fabA-DwnF-Gm	AGGAACTTCAAGATCCCCAATTCGctgttcacctccactgacag
fabA-DwnR-GWR	TACAAGAAAGCTGGGTgatcgatcagctcttcgagg
desA-specific primers	
desA-UpF-GWL	TACAAAAAAGCAGGCTcagcgagtaggtcttcagtt
desA-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGgaaggtgatctacgagaagc
desA-DwnF-Gm	AGGAACTTCAAGATCCCCAATTCGcatggtgcttacggtggata
desA-DwnR-GWR	TACAAGAAAGCTGGGTgtgctgacgcatatcaccat
desT-specific primers	
desT SOE-A	tgaaggattccgtctgcaagcc
desT SOE-B	TCAGAGCGCTTTTGAAGCTAATTCGgaggacatacggcttcctttgg
desT SOE-C	AGGAACTTCAAGATCCCCAATTCGgctgcgtttcatcatgatcggc
desT SOE-D	tagttgaactccgcctcgccat
Common primers	
Gm-F	CGAATTAGCTTCAAAAGCGCTCTGA
Gm-R	CGAATTGGGGATCTTGAAGTTCCT
GW-*attB*1	GGGGACAAGTTTGTACAAAAAAGCAGGCT
GW-*attB*2	GGGGACCACTTTGTACAAGAAAGCTGGGT

a. Sequences in capital letters are common for all genes amplified and overlap either with the Gm or *attB* primer sequences. Lower-case letters indicate gene-specific sequences for *fabA*, *desA*, or *desT*.

was transferred into the Δ*fabAdesB* strain PAO739 to yield the Δ*fabA*Δ*desAdesB* mutant PAO754.

The wild-type *desA* gene was amplified from strain PAO1 using primers *desA*-X (5'-atttctagataagccaggctcaggcg) and *desA*-H (5'-agtaagcttgcggaggaagagaag). After ligation into *Xba*I/*Hin*dIII site of shuttle vector pUCP20 (West et al., 1994), the resultant plasmid p*desA* was transformed into *E. coli* INVαF' (Invitrogen) and confirmed by double strand sequencing. The *desA* expression plasmid p*desA* was electroporated into *P. aeruginosa* at 1.8 kV with *E. coli* Pulser (Bio-Rad) and selected by carbenicillin (200 μg ml^{-1}).

4.3.3 Fatty acid composition analysis. Cultures (10 ml) of *P. aeruginosa* strains were grown to mid-log phase in LB medium and the lipids were extracted (Bligh et al., 1959). The fatty acid methyl esters were prepared and identified using Hewlett-Packard Model 5890 gas chromatograph as described previously (Zhang et al., 2002).

4.3.4 Acetate pulse-chase experiment. The desaturation of fatty acids as a function of time following *de novo* biosynthesis was measured using a [1-^{14}C]acetic acid pulse-chase experiment. Cells were grown in M9 medium to mid-exponential phase and pulsed for 15 min with 4 μCi ml^{-1} of [1-^{14}C]acetic acid to label newly synthesized fatty acids. The cells were then collected, washed twice with warm growth medium and resuspended in fresh medium without labelled acetate to initiate the chase period. At timed intervals, a 2 ml sample was withdrawn, immediately cooled to 4°C, and the lipids were extracted (Bligh et al., 1959). The acyl chains were converted to methyl esters and separated into unsaturated and saturated fraction by chromatography on 20% silver nitrate-impregnated Silica Gel H thin layer plates (Aguilar et al., 1998).

The plates were visualized using a PhosphoImager screen, and UFA and SFA were quantified using a Typhoon 9200 and ImageQuant 5.2 software (Amersham Biosciences).

4.3.5 Positional analysis. *Naja mossambica* phospholipase A2 was used for positional analyses of fatty acyl chains on phospholipids (Nagiec et al., 2004). Total lipids were separated on Silica Gel H thin layers (Analtech, Newark, DE) using chloroform:methanol:acetic acid (65:25:10, v/v). The PtdEtn spot was scraped off the plate and extracted with chloroform:methanol:water (5:5:1, v/v). The purified PtdEtn was resuspended in 1 ml of 1 M NaCl:0.1 M $CaCl_2$:H_2O (15:5:80, v/v; pH 8.5) and 200 μg of phospholipase A2. The mixture was stirred for 2 h at 25°C, and extracted with 3 ml of chloroform:methanol (2:1, v/v). The resulting organic phase was dried and the products separated on Silica Gel H layers using chloroform:methanol:water (70:30:3, v/v). The regions corresponding to free fatty acids and 1-acylglycerophosphoethanolamine were scraped from the plate and converted to fatty acid methyl esters for composition determination by gas chromatography.

4.3.6 Gas chromatography-mass spectroscopy (GC-MS). The double bond position of UFAs was determined by gas chromatography-mass spectrometry (Moss et al., 1989). Fatty acid methyl esters were treated with 1 ml of DMDS and 0.2 ml of 6% (w/v) iodine solution (in diethyl ether). The mixture was stirred for 24 h at room temperature, and then extracted with 2 ml of 10% (w/v) sodium thiosulphate solution and 5 ml of hexanes. The organic layer that contained the fatty acids and DMDS adducts was concentrated to 30 μl before GC-MS analysis using Hewlett-Packard 5890 series II gas chromatograph and Hewlett-Packard mass detector 5972.

4.3.7 Phospholipid mass spectrometry. Mass spectrometry of intact PtdEtn was performed by the Hartwell Center for Bioinformatics and Biotechnology at St Jude Children's Research Hospital. Approximately 50 μg of total lipid was dissolved into 0.25 ml of 45:45:10 (v/v) methylene chloride:methanol:water. One microlitre of formic acid was added to 100 μl aliquot before analysis. Mass spectrometry of PtdEtn was performed using a Finnigan™ TSQ® Quantum (Thermo Electron, San Jose, CA) triple quadrupole mass spectrometer equipped with the Nanospray Ion Source. Samples were introduced via static nanospray using EconTips™ (New Ojective, Woburn, MA). The instrument was operated in the positive ion mode using neutral loss scanning for the mass corresponding to the loss of the phosphoethanolamine headgroup from PtdEtn. Ion source parameters were as follows: spray voltage 1000 V, capillary temperature 270°C, capillary offset 35 V, and tube lens offset was set by infusion of the polytyrosine tuning and calibration solution (Thermo Electron, San Jose, CA) in electrospray mode. Acquisition parameters were: scan range 600–900 *m/z*, scan time 0.3 s, neutral loss mass 141.0 Da, collision energy 32 V, peak width Q1 and Q3 0.7 FWHM, and Q2 CID gas 0.5 mTorr. Mass spectrometry of PtdGro was performed with the instrument operating in the negative ion mode using single MS (Q1) scanning and/or parent ion scanning corresponding to the loss of phosphoryl glycerol minus one H_2O. Ion source parameters were the same as PtdEtn except that spray voltage was 800 V. Acquisition parameters for Q1 scanning were: scan range 700–800 *m/z*, scan time 0.1 s, peak width Q1 0.7 FWHM. Acquisition parameters for parent ion scanning were: scan range 700–800 *m/z*, scan time 0.1 s, product mass 153.0 *m/z*, collision energy 45 V, peak width Q1 and Q3 0.7 FWHM, and Q2 CID gas 0.5 mTorr. Instrument control and data acquisition were performed with the Finnigan Xcalibur™ software.

4.3.8 RNA isolation. Strains of *P. aeruginosa* were grown under different conditions as indicated to mid-log phase before the cells were harvested by centrifugation and total RNA was isolated using Ambion RNAqueous purification kit according to the manufacturer's instructions (Ambion). The purified RNA was mixed with 0.5 volume of LiCl precipitation solution (Ambion) and incubated at −20°C for 30 min. The precipitated RNA sample was treated with Turbo DNase (Ambion) at 37°C for 30 min with 1 U of DNase for 5 μg of RNA. Agarose gel electrophoresis and the Agilent Technologies 2100 Bioanalyzer Laboratory-on-a-chip system were used to assess the integrity of the purified RNA before it was used for real-time PCR analyses or further processed for microarray experiments.

4.3.9 Real-time PCR. The expression levels of *desA* and *desB* were measured by real-time PCR as described previously (Zhang et al., 2002). The oligonucleotide primers and probes were synthesized at the Hartwell Center for Bioinformatics and Biotechnology at St Jude Children's Research Hospital. Sequences of the primers and probes used to assess expression of *desA*, *desB* and housekeeping gene *rpoD* were listed in Table 4.3. The reverse transcription mixture (20 μl) contained 500 ng of total RNA, 12.5 ng μl^{-1} random hexamers (Invitrogen), 10 mM dNTPs (Sigma), 40 U of RNaseout (Invitrogen) and 10 U μl^{-1} of Superscript II reverse transcriptase (Invitrogen). Aliquots (2 μl) of the reverse transcription reaction were added in the real-time PCR reaction (30 μl) containing 1× of Taqman universal PCR master mixes (Applied Biosystems), 660 nM of each forward and reverse primer, and 150 nM of probe. Amplification and detection of specific products were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) with the following profile: one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, 40 cycles at 95°C

Table 4.3. Primers and probes for real-time PCR.

Gene	Forward primer	Reverse primer	Probe
desA	ggttgaccacagggatgaaca	ctcgacggtctcgcacttg	FAM-cgcgaatggaccgctatcc-BHQ1
desB	ggcaagatcctcgagaacatg	ggatcgttcatccagtcgtact	FAM-ctgggccacaacgtcatgc-BHQ1
rpoD	cacctgccggaggatatttc	gatccccatgtcgttgatcat	FAM-tgatgtcttccacctgttccgg-BHQ1

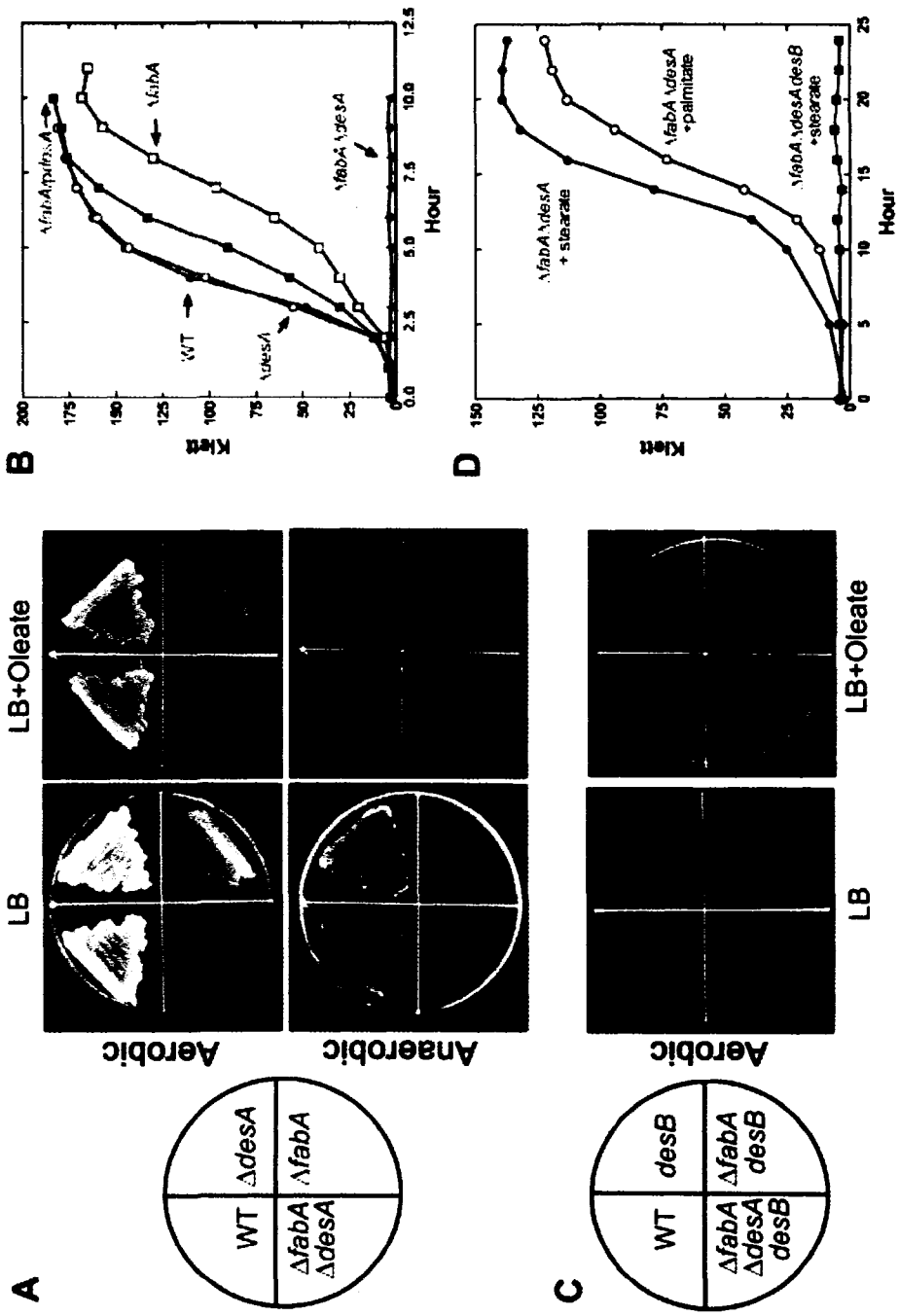
for 15 s followed by 60°C for 1 min. Experiments were performed with three replicas for each cDNA preparation, and no-reverse transcriptase and no-template controls were included for each reaction on the same 96-well plate. The critical threshold cycle (C_T) is defined as the cycle at which the fluorescence becomes detectable above background and is inversely proportional to the logarithm of the initial template molecules. All of the real-time values were compared using the C_T method, where the amount of *desA* (or *desB*) cDNA ($2^{-\Delta\Delta C_T}$) was calculated by normalizing C_T to the housekeeping gene *rpoD* (ΔC_T) before being compared with the amount of *desA* cDNA under a control condition ($\Delta\Delta C_T$), which was set as the calibrator at 1.0.

4.3.10 Affymetrix microarray analysis. Affymetrix *P. aeruginosa* genome arrays were used to assess the effect of *desT* deletion on the global gene expression. The mRNA in the isolated total RNA sample was converted to cDNA using procedures recommended by Affymetrix. RNA was then removed by alkaline hydrolysis and cDNA purified using MiniElute PCR purification kit (Qiagen). The cDNA was fragmented with DNase I (Amersham) at 0.6 U per μg cDNA. The 3' termini of fragmented cDNA were labelled with biotin using GeneChip DNA labelling reagent (Affymetrix) and the labelling efficiency was determined by a gel shift assay based on the retardation of the biotinylated cDNA upon addition of NeutrAvidin (Pierce). The fragmented labelled cDNA (1.5–4.0 μg) was hybridized for 16 h at 50°C to *P. aeruginosa* genome arrays (Affymetrix). Each array represents the annotated genome of *P. aeruginosa* strain PAO1 and includes 5549 protein-coding sequences, 18 tRNA genes, a representative of the ribosomal RNA cluster and 117 genes present in strains other than PAO1. After washing, staining and scanning of the arrays were performed according to the manufacturer's protocol (Affymetrix), the arrays were

analysed using the GeneChip Operating Software (GCOS) and global scaling was used to normalize the data from different arrays.

4.4 RESULTS

4.4.1 Growth and UFA formation in a *fabA* deletion strain. To verify the existence of an aerobic pathway in *P. aeruginosa*, a Δ*fabA* mutant was generated. The Δ*fabA* strain PAO652 was viable (Fig. 4.1A) although it grew significantly slower than the wild-type strain without exogenous UFA (Fig. 4.1B). The doubling time of strain PAO652 was 2 h compared with 50 min for the wild-type strain. This significant difference in growth rate and different experimental conditions may explain why a *fabA* mutant was previously described as auxotrophic for UFA (Hoang et al., 1997). In the present study, strain PAO652 was grown in Luria–Bertani (LB) + oleate medium before being switched to LB to obtain a seed culture which was used as inoculants for the experiments shown in Fig. 4.1A and B. In previous studies, however, the *fabA* mutant was pregrown on a rich broth + oleate plate before being patched onto RB plates with and without oleate to test for UFA auxotrophy. We noticed that the *fabA* mutant exhibited a lag phase (about 12 h in liquid medium) when switched from medium supplemented with oleate to that without. One possible explanation for this lag phase is that *desA* expression was repressed by oleate (see below and Fig. 4.7A). One could therefore interpret the slow growth phenotype of a *fabA* mutant directly transferred from +UFA to –UFA medium as UFA auxotrophy, especially when the incubation time was less than 24 h before reading of the results. Secondly, in the previous study oxygen limitation due to heavy inoculation by patching colonies from the seed plate may have rendered the desaturases (see below) less active thus unable to support robust growth.



Wild-type strain PAO1 contained 64% UFA in its membrane lipids (Table 4.4). The membrane lipids of strain PAO652 contained 20% UFA (Table 4.4), suggesting there was a second pathway for UFA synthesis when FabA was absent. Moreover, strain PAO652 produced C16:1, but not C18:1, the principle fatty acid in strain PAO1 (Table 4.4). The only other mechanism for UFA formation found in bacteria is the desaturation of phospholipid fatty acids which requires molecular oxygen (Aguilar et al., 1998). So we examined the anaerobic growth of the Δ*fabA* strain. Wild-type cells were viable under anaerobic condition and maintained the same fatty acid composition as those grown aerobically (Table 4.4). In contrast, strain PAO652 could not grow anaerobically without an oleate supplement (Fig. 4.1A), indicating that the second UFA pathway, unlike the FabA pathway, was oxygen-dependent.

4.4.2 Identification of DesA and DesB. The acyl-CoA desaturase (Ole1p) in *Saccharomyces cerevisiae* and the phospholipid desaturase (Δ5-Des) in *B. subtilis* have been well studied (Aguilar et al., 1998, Diaz et al., 2002, Stukey et al., 1990). Using these two sequences to query the *P. aeruginosa* genome database revealed two similar proteins encoded by the *PA0286* and *PA4888* genes. The two predicted proteins possessed several transmembrane domains and three appropriately spaced histidine-rich clusters characteristic of membrane-bound desaturases (Fig. 4.2) (Los et al., 1998, Shanklin et al., 1994). These histidine residues are located outside of transmembrane regions and are essential for catalysis (Diaz et al., 2002, Shanklin et al., 1994). Particularly, the histidine-rich regions of the *PA0286* gene product were closely related to those found in Ole1p (Fig. 4.2). The predicted protein sequence of the *PA4888* gene was 47% identical to the recently described acyl-CoA desaturase DesA in *M. tuberculosis* (Phetsuksiri et al., 2003) (Fig. 4.2). Moreover, the genes

Table 4.4. Fatty acid composition of *P. aeruginosa* wild-type and mutant strains.

Strain	C16:0[a]	C16:1[b]	C18:0	C18:1[b]	UFA:SFA ratio
PAO1 (wild-type)	33.1 ± 1.4	13.1 ± 0.7	2.8 ± 0.5	51.0 ± 2.9	1.8
PAO1 (anaerobic)	33.2 ± 1.2	12.2 ± 1.1	1.0 ± 0.1	53.6 ± 2.1	1.9
PAO652 (Δ*fabA*)	74.4 ± 1.8	19.1 ± 0.9	5.6 ± 0.5	< 1	0.24
PAO651 (Δ*desA*)	35.2 ± 0.8	16.4 ± 1.0	1.0 ± 0.1	47.1 ± 1.2	1.8
13272 (*desB*)	33.7 ± 0.4	17.5 ± 0.4	1.3 ± 0.2	47.5 ± 1.0	1.9
PAO739 (Δ*fabA*Δ*desB*)	73.5 ± 1.3	18.5 ± 0.7	7.5 ± 0.3	< 1	0.23
PAO682 (Δ*fabA*Δ*desA*)	–[c]	–	–	–	–
+ palmitate	73.8 ± 1.3	24.3 ± 1.4	1.3 ± 0.1	< 1	0.33
+ stearate	60.0 ± 5.1	5.5 ± 0.4	14.1 ± 2.1	20.4 ± 2.9	0.35
/p*desA*	55.5 ± 1.5	41.2 ± 1.0	3.3 ± 1.2	< 1	0.70

a. Data presented were in percentages of total fatty acids and were means ± standard error for three independent determinations.

b. The contributions of respective cyclopropane derivatives were included.

c. Strain PAO682 did not grow without a fatty acid supplement or a *desA* expression plasmid.

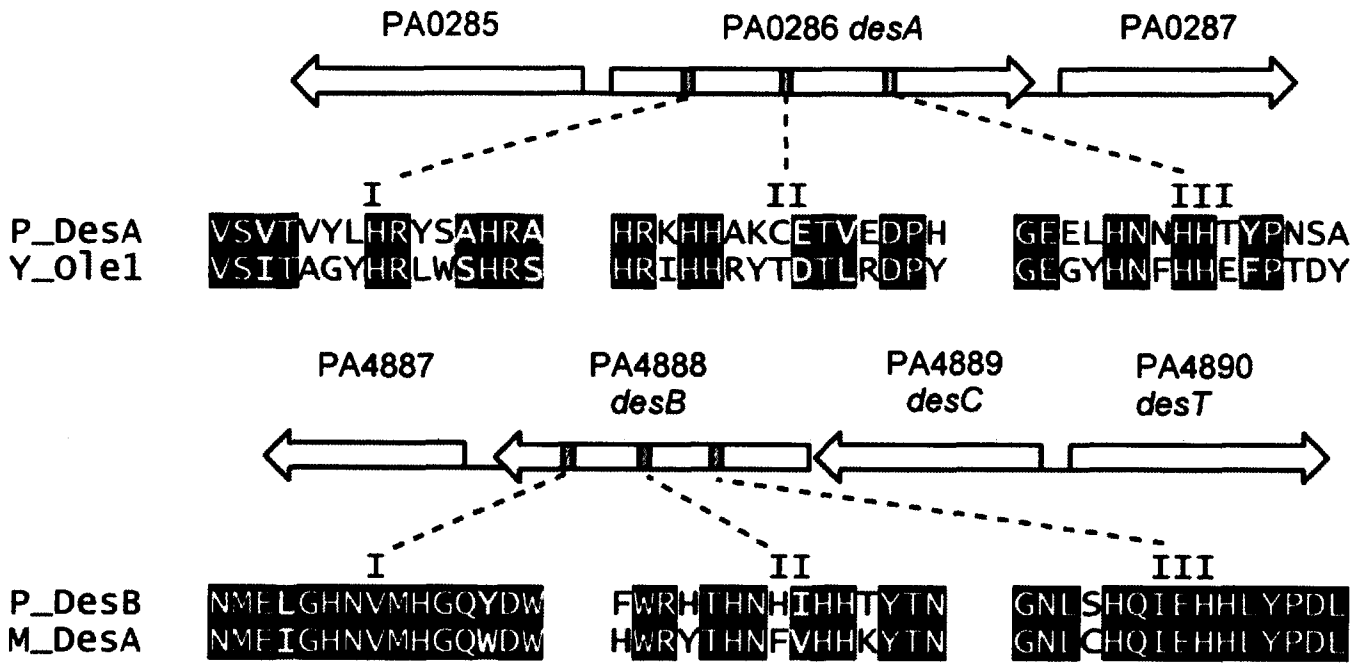


Fig. 4.2. Genetic organization and comparison of the fatty acyl desaturase histidine clusters of DesA and DesB. There are three histidine-rich regions (I, II and III) that are characteristic of fatty acid desaturases. *S. cerevisiae* acyl-CoA desaturase (Y_Ole1) and the putative DesA desaturase (P_DesA) are aligned, and *M. tuberculosis* desaturase (M_DesA) and the putative DesB desaturase (P_DesB) are aligned. Identical amino acids are highlighted in black and similar amino acids are highlighted in grey.

adjacent to *PA4888* in *P. aeruginosa* and *desA* in *M. tuberculosis* are predicted oxidoreductases, which are involved in the electron transport chain that supports the fatty acid desaturation reaction (Shanklin et al., 1994). These characteristics implied that the *PA0286* and *PA4888* genes encoded fatty acyl desaturases in *P. aeruginosa*, therefore they were designated *desA* and *desB* respectively. We designated the oxidoreductase in the *desB* operon as *desC*. To determine if similar desaturation mechanisms were present in other bacteria, we performed sequence homology search of both *P. aeruginosa* desaturases against microbial genomes. DesA-like desaturase sequences were relatively wide-spread and found in many beta- and gamma-proteobacteria species, but not in *E. coli* and *Salmonella*. In contrast, DesBC was only present in actinobacteria and few proteobacteria species. Importantly, DesB homologues were not present in other *Pseudomonas* species, such as *P. putida*, *P. syringae* and *P. fluorescens*.

The single knockout strains with either deleted *desA* (strain PAO651) or transposon insertional mutation of *desB* (strain 13272) did not exhibit a growth phenotype (Fig. 4.1), and did not have a significantly altered fatty acid composition compared with the wild-type strain (Table 4.4). Also, wild-type cells produced almost identical amounts of UFAs under aerobic and anaerobic conditions (Table 4.4). These data suggested that UFAs were mainly synthesized via FabA in the wild-type strain, and that the type II fatty acid biosynthetic pathway was the predominant source for UFA in *P. aeruginosa* regardless of oxygen availability.

The relationship between FabA and the putative desaturases was examined by constructing double knockout mutants. A *desA* deletion was introduced into strain PAO652 (Δ*fabA*) to generate the Δ*fabA*Δ*desA* double mutant, strain PAO682, and this strain failed to grow in the absence of an exogenous UFA supplement (Fig. 4.1A and

B). In contrast, the Δ*fabAdesB* double mutant strain PAO739 grew without an oleate supplement (Fig. 4.1C). These observations illustrated that DesA was able to generate sufficient UFAs in the absence of FabA to support aerobic growth, whereas DesB was not. To verify that the *desA* deletion caused the auxotrophic phenotype, a plasmid expressing *desA* was transformed into strain PAO682 (Δ*fabA*Δ*desA*). The expression of *desA* rescued strain PAO682 from the requirement for exogenous fatty acid and increased the proportion of 16:1 in the membrane phospholipids to 41% (Table 4.4), significantly higher than the amount of 16:1 produced in strain PAO652 (Δ*fabA*). These data tied the production of 16:1 in strain PAO652 to the activity of DesA. In addition, expression of *desA* in strain PAO652 (Δ*fabA*) elevated the UFA (mainly 16:1) level to 40% and resulted in a decrease of the doubling time from 2 h to 70 min (Fig. 4.1B), indicating that UFA synthesis, rather than the production of 18:1, was a limiting factor for growth.

4.4.3 UFA formation by DesA. One known substrate for bacterial desaturases is the fatty acyl chain esterified to lipid, as shown in *B. subtilis* and the cyanobacteria (Aguilar et al., 1998, Los et al., 1998). The desaturases from these strains introduce the double bond after SFAs are synthesized and esterified to glycerolphosphate, therefore formation of UFA takes place after the formation of SFA. An acetate pulse-chase experiment was conducted to monitor the change of UFA:SFA ratio in strain PAO652 (Δ*fabA*), where formation of 16:1 was dependent on DesA, and in strain PAO651 (Δ*desA*), where DesA activity was eliminated. The UFA:SFA ratio following the 15-min pulse was 2.1 in strain PAO651, and this ratio did not change significantly up to 2 h after the removal of [1-^{14}C]acetate (Fig. 4.3). This was also the case in the wild-type strain (data not shown). The consistent UFA:SFA ratio indicated

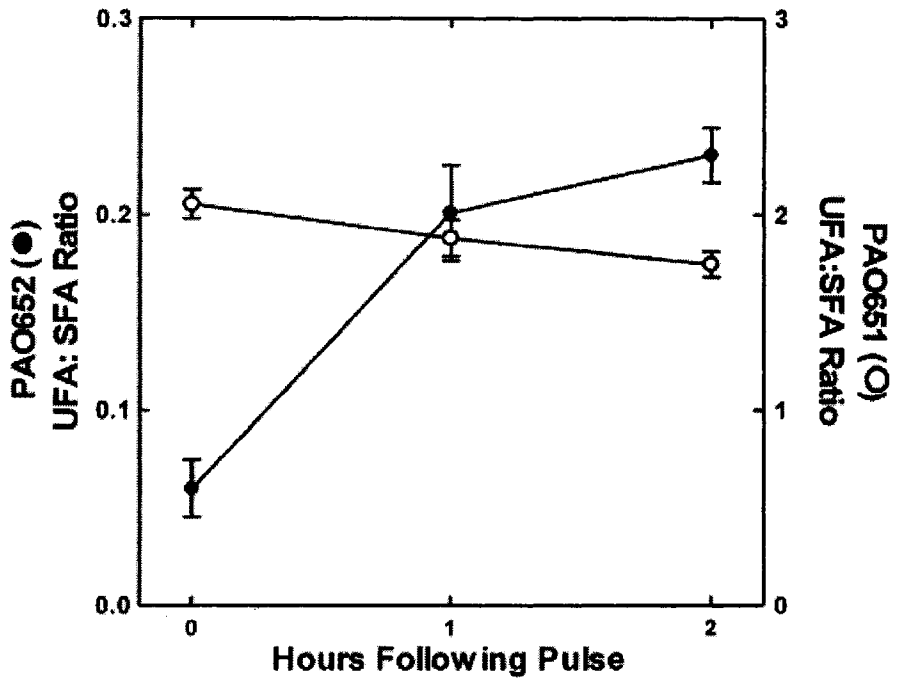


Fig. 4.3. Post-biosynthetic introduction of double bonds into fatty acids in the absence of *fabA*. Strains PAO651 (Δ*desA*) and PAO652 (Δ*fabA*) were grown in M9 medium supplemented with glycerol to mid-exponential phase and pulsed-labelled with 4 μCi ml^{-1} of [1-^{14}C]acetic acid for 15 min. The cells were washed to remove unincorporated [1-^{14}C]acetic acid and harvested at timed intervals for lipid extraction. The radiolabelled UFA and SFA methyl esters were separated by thin layer chromatography and quantified as described in MATERIALS AND METHODS. For strain PAO652, the ratio is indicated on left *Y*-axis (•). For strain PAO651, the ratio is indicated on right *Y*-axis (○). Data presented were means of three independent assays.

that the UFA and SFA were synthesized at the same time in strain PAO651, through the FabA pathway. In contrast, the UFA:SFA ratio in strain PAO652 was close to zero (0.06) after the 15 min acetate pulse (Fig. 4.3), indicating very small amount of UFA was synthesized at that point. The ratio was elevated to 0.20 at 1 h and to 0.23 at 2 h after the removal of [1-^{14}C]acetate (Fig. 4.3), which was similar to the UFA:SFA ratio found in compositional analysis of this strain (Table 4.4). Therefore, 16:1 formation by DesA occurred after the *de novo* biosynthesis of 16:0 by the type II pathway. These data argued against acyl-ACP as the DesA substrate, because there was no evidence for the formation of 18:1 in strain PAO652 (Δ*fabA*), in contrast to 18:1 being the most abundant fatty acid in the wild-type strain. If 16:0-ACP were converted to 16:1-ACP by DesA, then this intermediate would be elongated to 18:1-ACP by the type II pathway. Thus, *P. aeruginosa* DesA, like *B. subtilis*Δ5-Des, introduced double bonds into existing fatty acyl chains esterified to lipids.

4.4.4 DesA substrate specificity. A characteristic of acyl-phospholipid desaturases is that they recognize an acyl chain in only a single position on the glycerol backbone and introduce the double bond at a specific location in the hydrocarbon chain (Murata et al., 1995). PtdEtn is the major *P. aeruginosa* membrane phospholipid and was purified for fatty acid positional analysis. In strain PAO652 (Δ*fabA*), the *sn*-1 position of PtdEtn contained primarily 16:0 (83.6%) and the *sn*-2 position contained a mixture of 16:0 (55.6%) and 16:1 (37.3%). The observation that all of the 16:1 was located on the *sn*-2 position indicated that DesA only desaturated the *sn*-2 fatty acyl chain.

The double bond position in 16:1 produced by DesA in strain PAO652 (Δ*fabA*) was determined by converting the UFA methyl esters to their dimethyl disulphide (DMDS) adducts. These adducts were resolved by gas chromatography, and mass spectrometry was used to determine the fragmentation pattern of the 16:1-

DMDS derivative (Moss et al., 1989). The mass spectrum of the 16:1-DMDS adduct showed a weak peak at *m/z* = 362, corresponding to the mass of the intact molecular ion (Fig. 4.4A). The two major peaks at *m/z* = 145 and *m/z* = 217 corresponded to two ions arising from fragmentation between the two CH_3S groups located at Δ9 position (Fig. 4.4A). This fragmentation pattern clearly demonstrated that *P. aeruginosa* DesA was a Δ9 acyl-phospholipid desaturase.

The mass spectrum of PtdEtn from the wild-type strain showed that the most abundant PtdEtn species contained 18:1 and 16:0 acyl chains (*m/z* = 718; Fig. 4.5A). Three minor PtdEtn species were also found in wild-type cells: di16:0 (*m/z* = 692), 16:0/16:1 (*m/z* = 690) and 18:1/16:1 (*m/z* = 716) PtdEtn (Fig. 4.5A). Although wild-type cells expressing *desA* showed the same molecular species of PtdEtn, the ratio of 18:1/16:1-PtdEtn to 18:1/16:0-PtdEtn increased (Fig. 4.5B). The same was true for the ratio of 16:0/16:1-PtdEtn to di16:0-PtdEtn, consistent with phospholipids containing 16:0 acyl chain at *sn*-2 position being the substrate for DesA. The mass spectrum of PtdEtn from strain PAO652 (Δ*fabA*) revealed di16:0 and 16:0/16:1-PtdEtn as the two major molecular species (Fig. 4.5C). Because 16:1 was only present at *sn*-2 position in strain PAO652, the unsaturated PtdEtn molecular species was 1-palmitoyl-2-palmitoleoyl-PtdEtn. Similar to the effect of p*desA* in wild-type cells, expression of *desA* in strain PAO652 led to the conversion of dipalmitoyl-PtdEtn to 16:0/16:1-PtdEtn (Fig. 4.5D). Deletion of *desA* had only a modest effect on the abundance of the 16:0/16:1-PtdEtn species (Fig. 4.5E). The primary PtdGro molecular species in the Δ*fabA* strain PAO652 were di16:0 and 16:0/16:1, same as those of PtdEtn (Fig. 4.5F). These data, together with the positional analyses, indicated that DesA was a Δ9 fatty acid desaturase and introduced double bond exclusively into the *sn*-2 fatty acyl chain of phospholipids regardless of the head group.

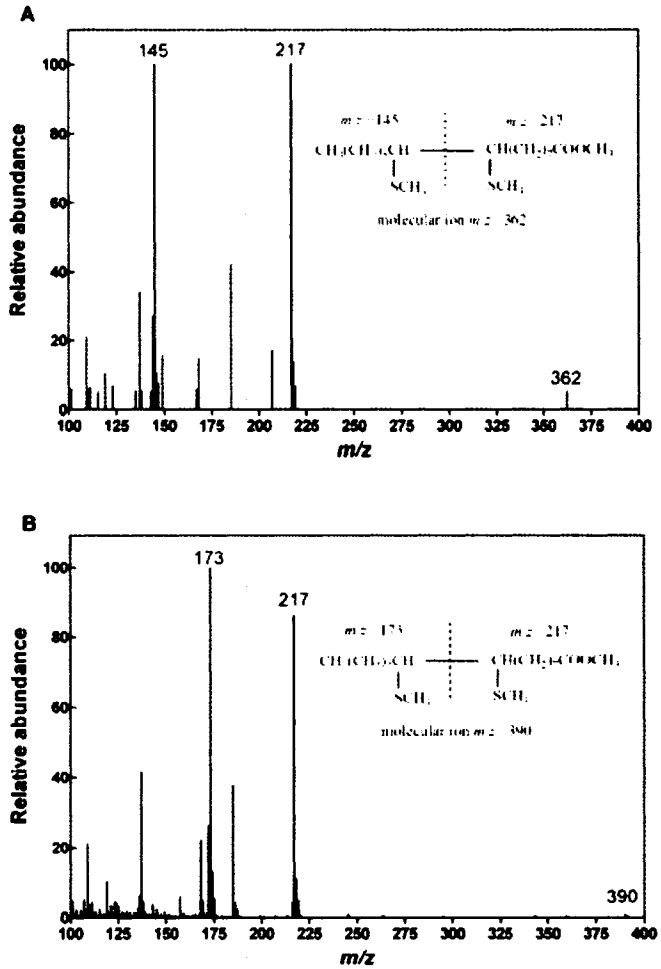


Fig. 4.4. Both DesA and DesB are Δ9 acyl desaturases.

A. Strain PAO652 (Δ*fabA*) was grown in LB broth at 37°C to mid-log phase, the lipids extracted, and the DMDS adduct of the 16:1 methyl ester was analysed by GC-MS as described in MATERIALS AND METHODS. The molecular ion was m/z = 362. The two major fragment ions m/z = 145 and m/z = 217 corresponded to the terminal methyl part and carboxyl end, respectively, and placed the double bond at the Δ9 position.

B. Strain PAO682 (Δ*fabA*Δ*desA*) was grown in LB broth supplemented with 0.01% stearate at 37°C to mid-log phase. The lipids were extracted, and the DMDS derivative of the 18:1 methyl ester was analysed by GC-MS. The molecular ion was m/z = 390. The two major fragment ions m/z = 173 and m/z = 217 corresponded to the terminal methyl part and carboxyl end of the 18:1 derivative, respectively, and placed the double bond at the Δ9 position.

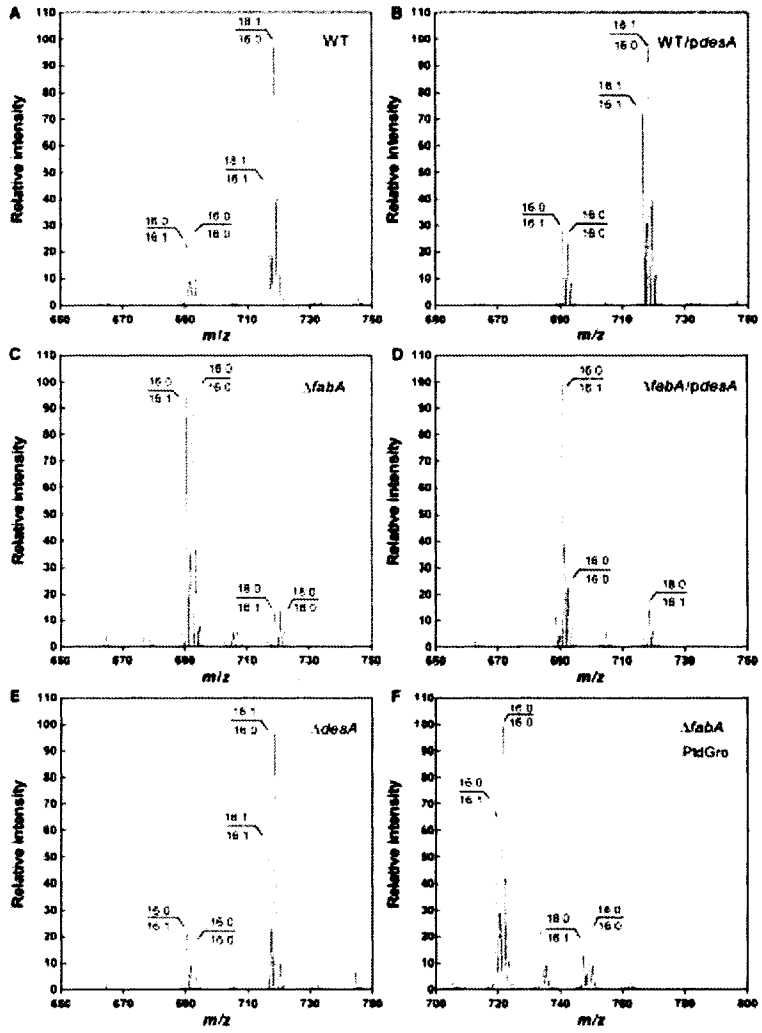


Fig. 4.5. Phospholipid molecular species analysis of wild-type and Δ*fabA* mutants. Mass spectra of PtdEtn and PtdGro were obtained as described in MATERIALS AND METHODS. The assignments of PtdEtn molecular species were based on the *m/z*-values: *m/z* = 692, di16:0; *m/z* = 690, 16:0/16:1; *m/z* = 720, 18:0/16:0; *m/z* = 718, 18:1/16:0; *m/z* = 716, 18:1/16:1. Mass spectra of PtdEtn from **(A)** wild-type strain PAO1, **(B)** strain PAO1/p*desA*, **(C)** strain PAO652 (Δ*fabA*), **(D)** strain PAO652/p*desA* and **(E)** strain PAO651 (Δ*desA*) were shown. **(F)** Mass spectrum of PtdGro from strain PAO652 (Δ*fabA*). The molecular species of PtdGro were di16:0, *m/z* = 722; 16:0/16:1, *m/z* = 720; 18:0/16:0, *m/z* = 750; 18:0/16:1, *m/z* = 748.

4.4.5 DesB-dependent UFA formation. Neither acyl-ACP nor phospholipid was a substrate for DesB because both although these molecules were present in strain PAO682 (Δ*fabA*Δ*desA*), this strain was unable to grow without an oleate supplement (Fig. 4.1A and B). The key observation was that SFA, either palmitate or stearate, was able to support the aerobic growth of strain PAO682 (Fig. 4.1D), indicating that DesB functioned to desaturate exogenous SFAs. Fatty acid compositional analysis showed that extracellular palmitate was converted to 16:1, but not elongated to 18:1 and stearate was converted to 18:1 (Table 4.4). The deletion of *desB* abolished the ability of SFA to support the growth of strain PAO754, a Δ*fabA*Δ*desA*Δ*desB* triple mutant, whereas this strain was able to grow in the presence of oleate (Fig. 4.1C and D). Exogenous ^{14}C-labelled stearate was converted to UFA by DesB in strain PAO682 (Δ*fabA*Δ*desA*) (Fig. 4.6, lane 4), while strain PAO682 growing in the presence of oleate did not desaturate exogenous stearate (Fig. 4.6, lane 5), indicating an inhibitory effect of oleate on DesB. ^{14}C-Acetate labelling of strain PAO682 growing with stearate showed that acetate was only incorporated into SFA (Fig. 4.6, lane 3), consistent with the substrate specificity of DesB for exogenous SFAs. ^{14}C-Acetate labelling of strain PAO739 (Δ*fabA*Δ*desB*) showed that both ^{14}C-labelled SFA and UFA were produced. A similar result was obtained when strain PAO739 was labelled with ^{14}C-palmitate, demonstrating that phospholipids produced from both *de novo* fatty acid synthesis and exogenous SFAs were substrates for DesA (data not shown). The mass spectrum of the 18:1-DMDS adduct from strain PAO682 (Δ*fabA*Δ*desA*) growing in stearate showed a weak peak at $m/z = 390$, corresponding to the mass of the intact molecular ion. The two major ions at $m/z = 173$ and $m/z = 217$ indicated fragmentation between the two CH_3S groups located at the Δ9 position (Fig. 4.4B), demonstrating that exogenous stearate was converted to $18{:}1^{\Delta 9}$ in strain PAO682

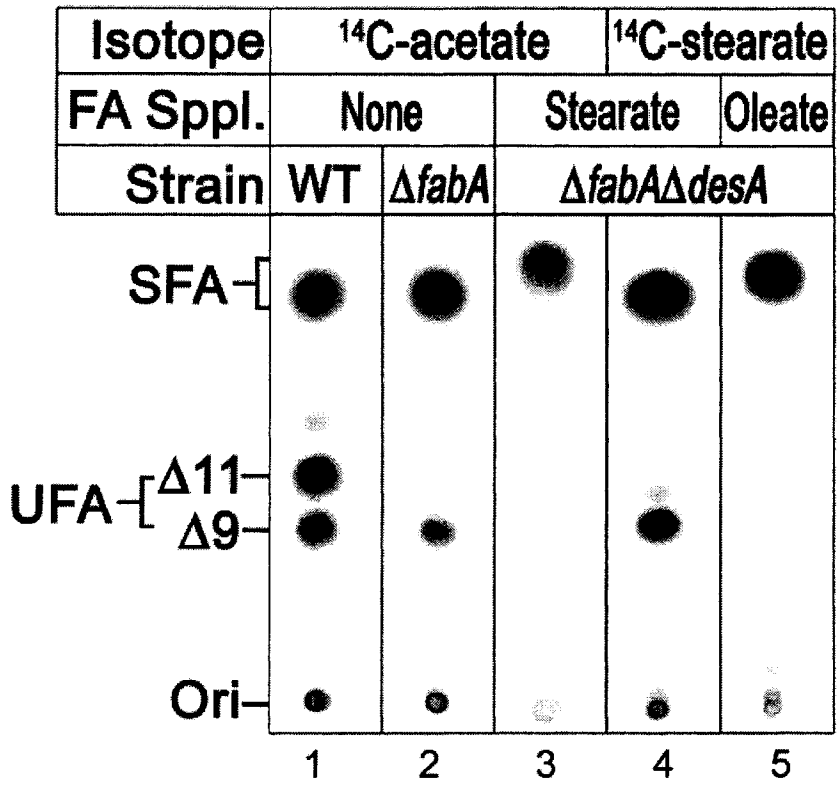


Fig. 4.6. Fatty acid biosynthesis and stearate desaturation in strain PAO682 (Δ*fabA*Δ*desA*). [1-^{14}C]Acetate or [1-^{14}C]stearate was added to the growth medium to monitor fatty acid biosynthesis and exogenous fatty acid desaturation in a panel of mutant strains. Following the 1-h labelling period, the cells were washed, lipids extracted, fatty acid methyl esters prepared and separated by argentation thin-layer chromatography as described in MATERIALS AND METHODS.

Lane 1. Wild-type strain PAO1 labelled with [1-^{14}C]acetate (2 μCi ml^{-1}).

Lane 2. Strain PAO652 (Δ*fabA*) labelled with [1-^{14}C]acetate (2 μCi ml^{-1}).

Lane 3. Strain PAO682 (Δ*fabA*Δ*desA*) grown in LB plus 0.01% stearate and labelled with [1-^{14}C]acetate (2 μCi ml^{-1}).

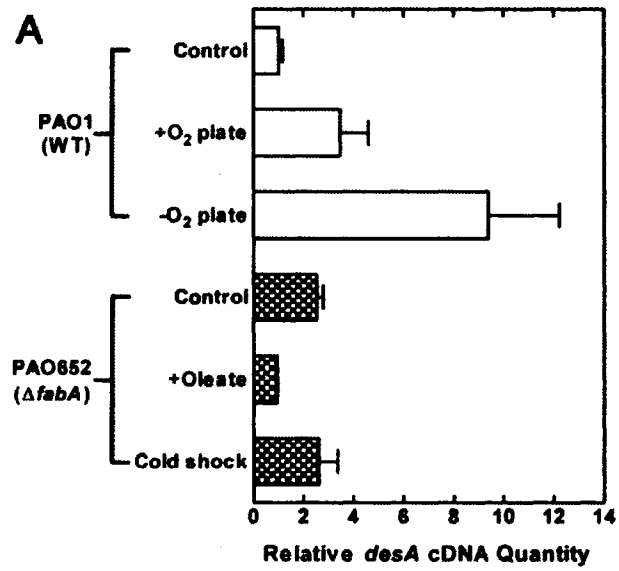
Lane 4. Strain PAO682 (Δ*fabA*Δ*desA*) grown in LB plus 0.01% stearate and labelled with [1-^{14}C]stearate (1 μCi ml^{-1}).

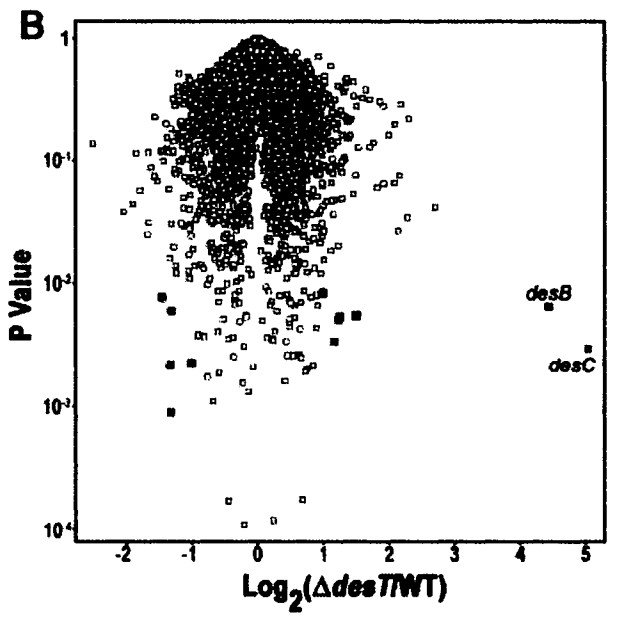
Lane 5. Strain PAO682 (Δ*fabA*Δ*desA*) grown in LB plus 0.1% oleate and labelled with [1-^{14}C]stearate (1 μCi ml^{-1}).

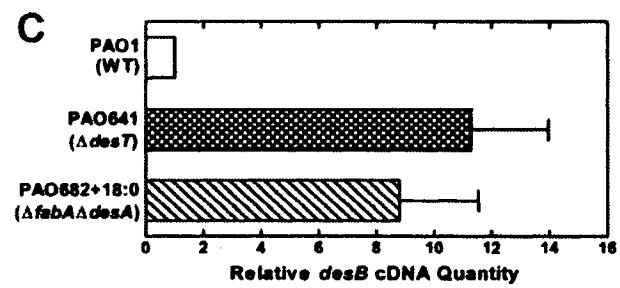
(Δ*fabA*Δ*desA*). Similarly, exogenous palmitate was converted to $16{:}1^{\Delta 9}$ in strain PAO682 (data not shown). The UFA product of the *de novo* biosynthetic pathway via FabA was $18{:}1^{\Delta 11}$, which was the sole octadecenoic acid detected with GC-MS in wild-type strain PAO1 (data not shown). These metabolic labelling and compositional experiments supported the conclusion that DesB selectively desaturates fatty acids imported from the growth medium.

4.4.6 Regulation of *desA* and *desB*. The dominant role of the FabA pathway in UFA synthesis implied that the DesA and DesB pathways were largely inactive when FabA was active, e.g. expression levels of *desA* and *desB* were repressed by UFA. Real-time polymerase chain reaction (PCR) was used to determine the relative expression levels of *desA* and *desB* under different growth conditions. The *desA* mRNA level in strain PAO1 grown in liquid medium was normalized to *rpoD* level and set at 1.0. Compared with growth with vigorous shaking in liquid broth, cells grown on an agar plate produced 3.4-fold more of *desA* mRNA (Fig. 4.7A). Anaerobic growth on agar plates further increased *desA* mRNA level to 9.5-fold higher than the control level (Fig. 4.7A). Thus, oxygen had a role in the regulation of *desA* expression and the increased expression on plates compared with shake flasks was attributable to reduced access to oxygen by colony formation. In strain PAO652 (Δ*fabA*), *desA* expression was 2.5-fold higher than the wild-type level (Fig. 4.7A). Exogenous oleate reduced *desA* mRNA in strain PAO652 to the same level as wild-type strain (Fig. 4.7A). Abrupt change in the growth temperature did not affect the *desA* expression in strain PAO652 (Fig. 4.7A).

Gene *PA4890* was located immediately upstream of *desCB* and was transcribed to the opposite direction. The deduced amino acid sequence encoded by *PA4890* indicated







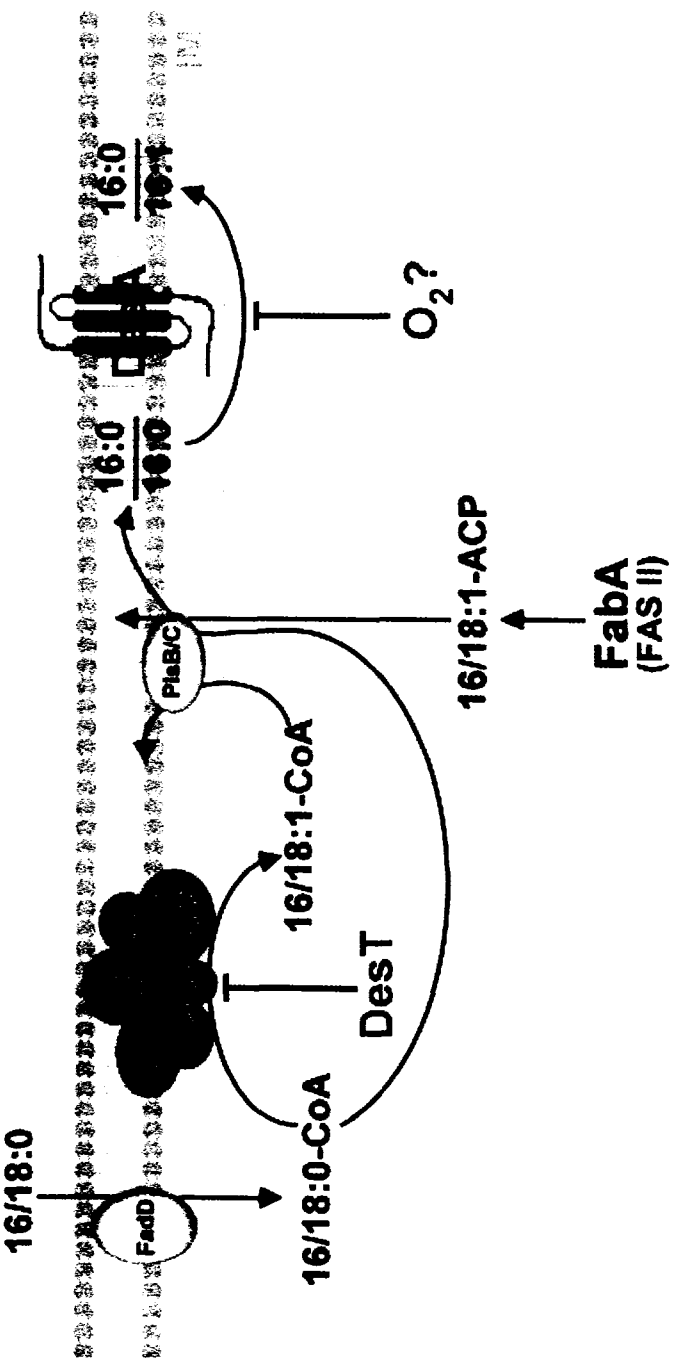
it belonged to the TetR family of transcriptional regulators. To investigate if *PA4890* was involved in the regulation of *desCB* expression, a *PA4890* deletion strain (PAO482) was generated, cDNA samples of strain PAO482 grown at 16°C and 37°C were prepared, and Affymetrix microarrays were used to determine the target genes of the *PA4890* encoded protein. Results from the microarray analysis showed that *PA4890* deletion had little global effect on gene expression (Fig. 4.7B). The two genes that were the most significantly deregulated in strain PAO482 were *desB* and *desC* (Fig. 4.7B). For example, microarray analysis using the 16°C sample showed expression levels of *desB* and *desC* genes were increased to 22- and 33-fold of the wild-type level respectively, suggesting that the *PA4890* encoded protein was a repressor of *desCB*. The expression level of *desB* in strain PAO482 determined by real-time PCR corroborated the microarray data (Fig. 4.7C). Thus, we designated *PA4890* as *desT*, a transcriptional repressor of *desCB* operon. A list of all genes that were deregulated by more than twofold in Δ*desT* strain PAO482 was summarized in Table 4.5.

4.5 DISSCUSSION

Our results lead to a model for the formation of UFA in *P. aeruginosa* outlined in Fig. 4.8. The anaerobic FAS II pathway utilizing the FabA isomerase to introduce the double bond into the growing acyl chain is the primary mechanism for UFA formation. Its principle products are $18{:}1^{\Delta 11}$- and $16{:}1^{\Delta 9}$-ACP thioesters that are incorporated into phospholipids by the glycerol-phosphate and acylglycerol-phosphate acyltransferases (PlsB and PlsC). The anaerobic UFA biosynthetic pathway is supplemented by two aerobic desaturases, and these alternate pathways provide a mechanism to modify existing membrane phospholipid fatty acids and to produce

Table 4.5. List of genes regulated in Δ*desT* strain PAO482.

Gene	Fold Change	Gene Description
PA0040	↓2.5	Conserved hypothetical protein
PA0707_*toxR*	↓2.5	Transcriptional regulator ToxR
PA2145	↑2	Hypothetical protein
PA2390	↓2.5	Probable ATP-binding/permease fusion ABC transporter
PA2555	↓2	Probable AMP-binding enzyme
PA3560_*fruA*	↑2.2	Phosphotransferase system, fructose-specific IIBC component
PA3933	↑2.3	Probable choline transporter
PA4861	↑2.8	Probable ATP-binding component of ABC transporter
PA4888_*desB*	↑21.8	Acyl-CoA desaturase
PA4889_*desC*	↑32.8	Oxidoreductase



UFA from exogenous SFA.

The DesA route introduces double bonds into the Δ9 position of acyl chains located at the 2-position of membrane phospholipids to produce the unsaturated PtdEtn (or PtdGro) molecular species. In the absence of FabA function, 16:0 is the exclusive fatty acid produced by FAS II, and there is an abundance of di16:0-phospholipid molecular species (Fig. 4.5C). The expression of *desA* is elevated in Δ*fabA* strains; however, the increase is rather modest (2.5-fold). Thus, DesA activity may be controlled by the membrane biophysical properties and inhibited by the high UFA content in wild-type membrane phospholipids. Expression of *desA* from a plasmid in the Δ*fabA* background elevates the UFA content reducing the di16:0-PtdEtn molecular species to the levels found in wild-type cells (Fig. 4.5D). The *desA* gene is not regulated significantly following an abrupt temperature shift. Therefore the position of the double bond and the lack of temperature regulation distinguish the regulation of *desA* expression in *P. aeruginosa* from that of the *des* phospholipid desaturase in *B. subtilis* (Aguilar et al., 2001). Rather than being regulated by a thermosenor like in *B. subtilis* (Aguilar et al., 2001), *P. aeruginosa desA* expression is most highly regulated by changes in oxygen availability (Fig. 4.7A), which is reminiscent of the induction of *OLE1* expression by Mga2p under hypoxic conditions (Jiang et al., 2001, Vasconcelles et al., 2001). Perhaps the cells induced *desA* expression in an attempt to scavenge traces of oxygen. However, the mechanism for *desA* regulation by oxygen is not known.

The second aerobic pathway of UFA synthesis in *P. aeruginosa* requires DesB which can desaturate exogenous stearate and palmitate, and the principle products are $18{:}1^{\Delta 9}$ and $16{:}1^{\Delta 9}$ respectively. Acyl-CoA synthetase (FadD) is the first and required step for the assimilation and metabolism of exogenous fatty acids in *E. coli* (Rock et

al., 1985), and *P. aeruginosa* contains several *fadD* homologues that are thought to be involved in fatty acid uptake. Some strains of *Pseudomonas* are able to oxidize long chain alkanes which provide another source of acyl-CoA for DesB (van Beilen et al., 1994). Acyl-CoAs are substrates for phospholipid synthesis and fatty acid β-oxidation and these two pathways may compete with DesB for acyl-CoA. The observation that exogenous 18:0 gave rise to both 18:1 and 16:1 fatty acids in the membrane phospholipids suggests that some of the 18:0-CoA undergoes one round of β-oxidation to form 16:0-CoA prior to the desaturation reaction by DesB, consistent with acyl-CoA being the substrate for DesB. In contrast, the observation that 16:0 was not converted to 18:1 indicates that DesB does not desaturate an acyl-ACP intermediate. The d*esB* gene is in an operon with *desC*, a gene encoding an oxidoreductase that is predicted to function in the electron transport coupled with the DesB desaturation reaction. The expression of the *desCB* operon is controlled by the DesT repressor as evidenced by the strong upregulation of both *desB* and *desC* genes in the Δ*desT* strain PAO482 (Fig. 4.7B and C). DesT appears to function as a sensor of exogenous fatty acid availability because real-time PCR showed that the *desCB* operon was also upregulated by exogenous SFA (Fig. 4.7C). Understanding the details of DesT-ligand-DNA interactions awaits purification of the DesT protein and identification of its DNA binding site(s).

4.6 ACKNOWLEDGMENTS

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CHAPTER 5

Studies of mechanisms governing *fabAB* transcription in *P. aeruginosa*

5.1 ABSTRACT

Pseudomonas aeruginosa contains three pathways for unsaturated fatty acid (UFA) biosynthesis, the aerobic DesA and DesBC pathways, and the anaerobic FabAB pathway. A previously developed mini-Tn*7*-based gene delivery system, which allows chromosomal integration of gene fusion constructs site-specifically without affecting the host's fitness, was utilized for the study of mechanisms of *fabAB* regulation. It was previously noted that this operon was highly expressed in biofilms and repressed by exogenous UFAs, in particular oleic acid (OA). Deletion of a 30 nt *fabA* upstream sequence, which is conserved in *P. aeruginosa*, *P. putida*, and *P. syringae,* led to a significant decrease in *fabA* transcription and loss of OA repression, suggesting positive regulation by an unknown positive regulatory mechanism. In the present studies, these observations were confirmed by *lacZ* fusion analyses using fusions containing progressively shorter *fabA* upstream fragments, starting with the entire *PA1611-fabA* intergenic region. Furthermore, RT-PCR analyses indicated that the *fabA* promoter may be located far upstream of the *fabAB* operon. *fabA* expression was less repressible in Δ*desT* mutant in the presence of OA, suggesting that *fabA* transcription is negatively regulated by DesT, a repressor of *desBC* expression. It was also shown that DesT binds to a site upstream of *fabA*. To identify a potential *fabAB* activator, genetic and biochemical approaches were employed. Deletion of candidate genes such as the *fabAB* upstream gene *PA1611*, which encodes a hybrid sensor-

response regulator protein, or the possible *fadR* homolog-encoding *PA1627* or all unidentified transcriptional regulators of the GntR family was performed to determine if any of these gene products function as a *fabAB* activator. However, none of these genes was involved in the regulation of *fabAB* transcription. Unconditional *fabA* mutants were generated by *mariner*-based random mutagenesis to screen for *fabA* activator(s). This approach was based on the premise that mutation of an activator converts a *fabA*(Ts) strain from a conditional mutant requiring OA supplementation only at 42 °C to an unconditional OA auxotroph requiring supplementation at all temperatures. β-galactosidase assays in unconditional mutants containing a *fabA'-lacZ* fusion demonstrated that besides several genes encoding unknown functions, *rpoN* and a gene encoding a fatty acid desaturase (designated as DesA in chapter 4) may be involved in *fabA* regulation, but probably via indirect mechanisms. Since *P. aeruginosa fabAB* operon regulation seems to be governed by a complex regulatory network, the identification of an activator was performed in an *E. coli* strain harboring a *fabA'-'lacZ* translational fusion by transforming a *P. aeruginosa* genomic library to avoid the interference with other factors indirectly involved in its regulation. This screen revealed that Anr slightly activated *fabA* expression in *E. coli*, but its effects were much less evident in *P. aeruginosa* and may result from global effects of Anr. Binding assays using the conserved 30 bp sequence did fail to isolate an activator, suggesting that *fabA* expression might not be regulated by protein-binding, but by a distinct mechanism.

5.2 INTRODUCTION

The common opportunistic human pathogen, *Pseudomonas aeruginosa,* causes serious infections in immunocompromised or cystic fibrosis patients. Fatty

acid synthesis is essential for cellular function by providing metabolic precursors for synthesis of many cellular components. The fatty acid biosynthetic enzymes are therefore potential targets for new antibacterials. It was previously established that the biosynthesis of fatty acids in *P. aeruginosa* closely resembles the pathway established in *E. coli*, and consists of two phases, initiation and elongation. In the initiation phase, malonyl-ACP is produced by condensation of CO_2 and acetyl-CoA, a step that is catalyzed by FabD which replaces the CoA with ACP. FabB then condenses the malonyl-ACP and acetyl-CoA, followed by decarboxylation. The resulting β-ketoacyl-ACP then enters the elongation cycle. After NADH-dependent reduction by FabG, the product β-hydroxyacyl-ACP is dehydrated by either FabA or FabZ to *trans*-enoyl-ACP, which is followed by a second reduction via the enoyl-ACP reductase FabI to produce an acyl-ACP. For further elongation, the acyl-ACP is condensed with malonyl-ACP in a reaction catalyzed by either FabB or FabF. The elongation cycle is repeated until full-length (C14-C16) chain fatty acids are synthesized (reviewed in (Hoang et al., 1999)). Besides exhibiting dehydratase activity, FabA also catalyzes the isomerization of *trans* fatty acids produced during *de novo* synthesis to fatty acids containing a *cis*-double bond. These UFAs are then condensed with malony-ACP by FabB, thus bypassing the FabI (enoyl-ACP reductase)-catalyzed step and maintaining the double bond. In subsequent rounds of elongation, full-length UFAs are formed (Hoang et al., 1997). This pathway is the main route for producing $16{:}1^{\Delta 9}$- and $18{:}1^{\Delta 11}$-ACP thioesters that are incorporated into phospholipids by the glycerol-phosphate and acyl-glycerol-phosphate acyltransferases (PlsB and PlsC) (Zhu et al., 2006). Since, UFAs are required for maintaining the fluidity of bacterial membranes, numerous studies on UFAs have

been performed in many prokaryotes, especially *Escherichia coli.* However, the pathways of UFA synthesis remained mostly uncharacterized in *P. aeruginosa.*

In a recent study, it was demonstrated that UFA biosynthesis in *P. aeruginosa* can occur via three different pathways depending on the availability of oxygen: 1) the aerobic pathways encoded by the fatty acid desaturase genes *desA* and *desBC* (Zhu et al., 2006), and 2) anaerobic pathway encoded by the *fabAB* operon (Hoang et al., 1997). It was shown that a Δ*fabA* mutant required UFA supplementation during anaerobic growth, but not during aerobic growth, demonstrating that although the aerobic pathway supports UFA formation, the *fabAB* operon is indispensable for UFA synthesis under anaerobic conditions (Zhu et al., 2006). In *E. coli*, two distinct transcriptional regulators regulate expression of the *fabA* and *fabB* genes involved in UFA synthesis. FadR, which negatively regulates expression of the *fad* genes involved in fatty acid transport and β-oxidation (Cronan et al., 1998), also acts as a positive transcriptional regulator of *fabA* and *fabB* gene expression (Campbell et al., 2001, Henry et al., 1992). It was shown that an *E. coli fadR* mutant synthesized less UFAs compared to wild-type strains and that *fabA*(Ts) *fadR* double mutants required UFA supplementation for growth, suggesting that FadR also functions as an activator of UFA synthesis (Nunn et al., 1983). In addition, *fabA* activation by FadR is repressed by exogenous long acyl-CoAs by binding to the FadR protein (Henry et al., 1992, van Aalten et al., 2000). Furthermore, *fabB* was also shown to be positively controlled by FadR because *fadR* mutants exhibit cerulenin hypersensitivity, and show conditional lethal phenotypes in *fadR fabB* double mutants (Campbell et al., 2001). In contrast, FabR negatively controls UFA synthesis in *E. coli* by binding to similar sequences in the *fabA* and *fabB* upstream regions (Zhang et al., 2002). Although a FabR homologue PA4890 showing 50 % similarity to the *E. coli* protein

was found in *P. aeruginosa*, it seems to play only a minor role, if any, in *fabA* regulation (Zhu et al., 2006).

Previous searches failed to identify a *P. aeruginosa* FadR homolog responsible for activation of *fabAB* transcription in *P. aeruginosa* and thus, the regulation of UFA synthesis in *P. aeruginosa* seems distinct from *E. coli.* Even though the activity of FabA and FabB from *P. aeruginosa* is very similar to that of the same enzymes from *E. coli*, there is one obvious distinction: whereas the *E. coli fabA* and *fabB* genes are located in two different regions of the chromosome, the *P. aeruginosa fabAB* genes form an operon (Hoang et al., 1997, 1999). The different organization of these genes may imply the existence of distinct regulatory pathways of UFA biosynthesis in *P. aeruginosa.*

Here, I demonstrate that *P. aeruginosa fabAB* operon expression seems to be controlled by a complex regulatory network composed of a combination of directly acting factors and indirectly acting transcriptional or translational regulators and other metabolic factors, rather than transcriptional regulators directly acting in the *fabAB* regulatory region.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial strains, plasmids, media and culture conditions. The bacterial strains and plasmids in this study are listed in Table 5.1. *Escherichia coli* and *P. aeruginosa* strains were maintained on Luria-Bertani medium (LB; 10 g per liter tryptone, 5 g per liter yeast extract, 10 g per liter NaCl; Becton, Dickinson & Co., Sparks, MD). Since *P. aeruginosa* cells are able to utilize citrate as a sole carbon source and energy source but not *E. coli*, citrate-based VBMM medium (Vogel-Bonner Minimal Medium; 3.0 g Na_3Citrate [citric acid Na_3 salt], 2.0 g Citric acid

Table 5.1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant properties	Reference or source
E. coli		
SM10*lacI*^{q}	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km *lacI*^{q}	T. Hoang
SM10 (λ*pir*)	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λ*pir*	Miller et al., 1988
DL291	F- *araD139* Δ(*argF-lac*)*U169 rpsL150 deoC1 relA1 rbsR ptsF25 flbB5301 glpR2 gyrA* Δ(*glpT-glpA*)593 *recA1*	Larson et al., 1982
DL291-Tn*7*[b]	Gm^{r}; DL291 derivative containing mini-Tn*7*T-*fabA'-'lacZY* gene fusion	This study
P. aeruginosa		
PAO1	Prototroph	Holloway et al., 1955
PAO434	PAO1 with mini-Tn*7*T-*lacZ*	This study
PAO435	PAO1 with mini-Tn*7*T-p*fabA'-lacZ*[a] resulted from integration of pPS1460	This study
PAO459	PAO1 with mini-Tn7T-p*fabA'-lacZ* resulted from integration of pPS1491	This study
PAO460	PAO1 with mini-Tn7T-p*fabAΔ30'-lacZ*	This study
PAO483	PAO434 with Δ*PA4890::FRT*[a]	This study
PAO484	PAO435 with Δ*PA4890::FRT*	This study
PAO491	PAO434 with Δ*PA1539::FRT*	This study

Table 5.1. Bacterial strains and plasmids used in this study (cont.).

Strain or plasmid	Relevant properties	Reference or source
PAO492	PAO435 with Δ*PA1539::FRT*	This study
PAO495	PAO460 with Δ*PA4890::FRT*	This study
PAO497	PAO460 with Δ*PA1539::FRT*	This study
PAO517	PAO435 with Δ*PA4890::FRT* Δ*PA1539::FRT*	This study
PAO194	PAO1 with temperature-sensitive *fabA*(Ts)	This study
PAO1010	PAO435 with Δ*anr*::*FRT*	This study
PAO1151	Gm^{ra}; PAO1 with mini-Tn*7*T-p*PA1612'-lacZ*	This study
Plasmid		
pPS752	Ap^r; $fabA^+$ $fabB^+$ (4.8 kb chromosomal *Bam*HI-*Eco*RI fragment cloned between the same sites of pUC18)	Hoang et al., 1997
pPS790	Ap^r; $fabA^+$ $fabB^+$ (2,039 bp PCR amplified *Bam*HI-*Kpn*I fragment from pPS752 cloned between the same sites of pUC19)	Hoang et al., 1997
pPS1450	Ap^r, Gm^r; mini-Tn*7* delivery vector	Choi et al., 2005
pPS1453	Ap^r, Gm^r; mini-Tn*7* delivery vector with a promoter-less *lacZ* gene	Choi et al., 2005
pPS1460	Ap^r, Gm^r; ligation of ~300 bp *Sal*I-*Sma*I fragment from pPS790 to *Xho*I-*Nru*I fragment of pPS1453	This study
pPS1482	Ap^r; pCR2.1 with PCR-amplified *PA1611-fabA* intergenic region from PAO1	This study

Table 5.1. Bacterial strains and plasmids used in this study (cont.).

Strain or plasmid	Relevant properties	Reference or source
pPS1488[b]	Ap^r; pCR-Blunt II-TOPO with PCR-amplified *PA1611-fabA* intergenic containing a 30 bp deletion	This study
pPS1491	Ap^r, Gm^r; pUC18-mini-Tn*7*T-p*fabA'-lacZ* delivery vector obtained by ligation of ~230 bp *Kpn*I-*Sma*I fragment of pPS1482 into the same sites of pPS1453	This study
pPS1492	Ap^r, Gm^r; pUC18-mini-Tn*7*T-p*fabAΔ30*::*lacZ* delivery vector obtained by ligation of ~230 bp *Kpn*I-*Sma*I fragment of pPS1488 into the same sites of pPS1453	This study
pDONR221	Km^r; Gateway donor vector	Invitrogen
pEX18Ap	Ap^r; gene replacement vector with MCS from pUC18	Hoang et al., 1998
pEX18ApGW	Ap^r; Gateway destination vector	Choi et al., 2005
pPS1669	Ap^r, Gm^r; pUC18-mini-Tn*7*T-Gm-*lacZ*-GW	Choi et al., 2005
pFLP2	Ap^r; source for Flp recombinase	Hoang et al., 1998
pPS856	Ap^r, Gm^r; Gm-*FRT* cassette	Hoang et al., 1998
pBT20	Ap^r, Gm^r; *mariner* transposon delivery vector	S. Lory
pPS1472	Ap^r, Km^r; pCR2.1 with amplified *PA1611* from PAO1	This study
pPS1476	Ap^r; pEX18Ap-*PA1611* (ligation of ~2.0 kb blunt-ended *Eco*RI fragment of pPS1472 to *Pst*I+*Eco*RI cleaved and blunt-ended pEX18Ap)	This study
pPS1479	Ap^r, Gm^r; pEX18Ap-Δ*PA1611*::Gm (ligation of blunt-ended *Kpn*I-*Nco*I fragment of pPS1476 to ~1.1 kb *Sma*I Gm^r::*FRT* fragment of pPS856)	This study

Table 5.1. Bacterial strains and plasmids used in this study (cont.).

Strain or plasmid	Relevant properties	Reference or source
pPS1503[b]	Ap^r, Gm^r; pEX18Ap-Δ*PA1627*::Gm (ligation of the *Stu*I-*Xho*I fragment of PCR-amplified Δ*PA1627*::Gm from PAO1 into the *Sma*I-*Sal*I sites of pEX18Ap)	This study
pPS1506[b]	Ap^r, Gm^r; pEX18Ap-Δ*PA1539*::Gm (ligation of the *Msc*I-*Xho*I fragment of PCR-amplified Δ*PA1539*::Gm into the *Sal*I-*Sma*I sites of pEX18Ap)	This study
pPS1505[b]	Ap^r, Gm^r; pEX18Ap-Δ*PA4890*::Gm (ligation of the *Xho*I-*Sma*I fragment of PCR amplified Δ*PA4890*::Gm into *Sal*I-*Sma*I sites of pEX18Ap	This study
pPS1634	Ap^r, Gm^r; pUC18R6K-mini-Tn*7*T-Gm-p*fabA::lacZY* (ligation of three fragments, *Bam*HI-*Xho*I fragment of pPS1633, ~250 bp *Bam*HI-*Eco*RV fragment of pPS790 and ~6.2 kb *Sma*I-*Sal*I fragment of pMC1403 into pPS1633)	This study
pPS1633	Ap^r, Gm^r; pUC18R6K-mini-Tn*7*T-Gm	Choi et al., 2005
pMC1403	Ap^r; *lacZY* fusion cloning vector for construction of translational fusions	Casadaban et al., 1980
pPS1733	Ap^r, Km^r ; *anr*$^+$ (ligation of 0.8 kb PCR fragment amplified with Anr-UP-KpnI and Anr-DN-BamHI to pCR2.1 vector)	This study
pPS1682	Sp^{ra}; *anr*$^+$ (ligation of 0.8 kb *Kpn*I-*Bam*HI fragment of pPS1733 into *Kpn*I-*Bam*HI cleaved pVLT35)	This study
pPS1684	Ap^r ; *anr*$^+$ (ligation of 0.8 kb *Kpn*I-*Bam*HI fragment of pPS1733 into *Kpn*I-*Bam*HI cleaved pUCP20)	This study
pPS1991[b]	Ap^r, Gm^r; pPS1669 with *att*L1-p*PA1612-att*L2 to promote *lacZ* expression	This study

[a] Abbreviations: Ap, ampicillin; *att*, λ attachment site (s); *FRT*, Flp recombinase target site; Gm, gentamycin; Km, kanamycin; MCS, multiple cloning site; p, promoters; ^{r}, resistance; Sp, spectinomycin

[b] see text for plasmid or strain construction details.

[free acid], 10.0 g K_2HPO_4, 3.5 g $NaNH_4PO_4$ x 4 H_2O, 1 mM $MgSO_4$ x 7 H_2O, and 0.1 mM $CaCl_2$) was used for counter-selection against *E. coli* after biparental matings between *E. coli* and *P. aeruginosa*.

For plasmid maintenance in *E. coli*, the media were supplemented with 100 μg per ml ampicillin, 25 μg per ml chloramphenicol, 35 μg per ml kanamycin or 15 μg per ml gentamycin. For marker selection in *P. aeruginosa*, 200 μg per ml carbenicillin, 30 μg per ml of gentamycin and 150 μg per ml of spectinomycin was used, as appropriate. If necessary, the monounsaturated fatty acid oleic acid (OA) was added together with 0.05 % Brij-58 to solubilize the fatty acid at final concentration of 0.05%.

5.3.2 DNA manipulations and vector constructions. Routine procedures were employed for manipulation of DNA (Sambrook et al., 2001). Plasmid DNA was isolated using the QIAprep Mini-spin kit (Qiagen, Valencia, CA) and *P. aeruginosa* chromosomal DNA was isolated using the QIAamp DNA Mini Kit. DNA fragments were purified from agarose gels utilizing the QIAquick gel extraction kit. The pUC18-mini-Tn*7*T-p*fabA'-lacZ* delivery vector was constructed by ligation of the ~300 bp *Sal*I+*Sma*I fragment of pPS790 to the *Xho*I+*Nru*I fragment of pPS1453.

The *lacZ* fusion plasmid containing a 30 bp deletion within the *fabA* upstream region was created in several steps. The *fabA* upstream region was PCR-amplified from PAO1 genomic DNA using primers IR187-UP and IR187-DN (sequences of primers used in this study are listed in Table 5.2.). These primers introduced *Sma*I and *Sal*I restriction enzyme cleavage sites at each end of the PCR fragment. The PCR fragment was purified and ligated into the TA cloning vector pCR2.1 (Invitrogen), resulting in pPS1482. This plasmid was used as a template in a two-step PCR

Table 5.2. Primers used in this study.

Primer Name	**Sequence (5' → 3')**
fabA upstream primers	
pfabA0	cgggaatgaacgattacctg
pfabA-attB1[a]	GGGGACAAGTTTGTACAAAAAAGCAGGCTcatgaccggatcgccttcgaa
pfabA1-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTctacgacaagggcggcaagc
pfabA2-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTggacgcgggaataaagtgaac
pfabA3-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTatctgttcgccggacactgtg
pfabA4-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTactttcaccgcaacgcaacag
pfabA4a-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTctgtgactttcaccgcaacg
pfabA5-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTctatgactaggctgccgctg
pfabA6-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTcgacgccgatacaataacccg
pfabA7-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTgcgcgacggccgctggacgaa
pfabA8-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTccgccacaaccctgcagttca
pfabA9-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTgggatttttgaggagctcgc
pfabA10-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTatgaccaaacaacacgccttc
pfabA11-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTatcagcgatgtcggcggcaag

Table 5.2. Primers used in this study (cont.).

Primer Name	**Sequence (5' → 3')**
pPA1612-UP-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTcgccacctgctctacttca
*fabA*Δ30	
PCRSOE-A	AGGTATCCGGTAAGCGGCAG
IR187SOE-B	TGTCCGGCGAACAGATGTTC
IR187SOE-C	GAACATCTGTTCGCCGGACATGACTAGGCTGCCGCTGCGA
PCRSOE-D	CGGTTCCTTTAGCAGCCCTT
PA1627 deletion	
1627SOE-A	gtgatcagttgcagcatcaccg
1627SOE-B	TCAGAGCGCTTTTGAAGCTAATTCGacccgggacagacccatgact
1627SOE-C	AGGAACTTCAAGATCCCCAATTCGggaagaaacatccaatcatcggat
1627SOE-D	accagcagtactaccaggaacc
PA4890 deletion	
4890SOE-A	tgaaggattccgtctgcaagcc
4890SOE-B	TCAGAGCGCTTTTGAAGCTAATTCGgaggacatacggcttcctttgg
4890SOE-C	AGGAACTTCAAGATCCCCAATTCGgctgcgtttcatcatgatcggc

Table 5.2. Primers used in this study (cont.).

Primer Name	**Sequence (5' → 3')**
4890SOE-D	tagttgaactccgcctcgccat
PA1539 deletion	
1539SOE-A	gcagggtagtagttgtgcgaca
1539SOE-B	TCAGAGCGCTTTTGAAGCTAATTCGtcgctcatgttccacctggttg
1539SOE-C	AGGAACTTCAAGATCCCCAATTCGcgagtgacagctcgatgtcctt
1539SOE-D	cgactaccgtttctgaatccgc
anr deletion	
anr-UpF-GWL	TACAAAAAAGCAGGCTttgacagggtgcgacaggta
anr-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGaatccttgcagtgtgcttgg
anr-DwnF-Gm	AGGAACTTCAAGATCCCCAATTCGggaagtgcacatcctcgact
anr-DwnR-GWR	TACAAGAAAGCTGGGTacgaagctgtccacggtcat
Nested PCR primers	
Rnd1-TnM	TATAATGTGTGGAATTGTGAGCGG
Rnd1-Pa1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
Rnd2-TnM	ACAGGAAACAGGACTCTAGAGG

Table 5.2. Primers used in this study (cont.)

Primer Name	**Sequences (5' → 3')**
Rnd2-Pa	GGCCACGCGTCGACTAGTAC
TnMSeq	CACCCAGCTTTCTTGTACAC
TnMRev	TGCACCGTGCAGTCGATGATAA
Other primers	
IR187-UP	GAGGGGAcCCGGgATGATCTACGACAA
IR187-DN	AGGTCTTCTCGGGTaccGGCGTGTTGT
Gm-UP	TGGAGCAGCAACGATGTTAC
Gm-DN	TGTTAGGTGGCGGTACTTGG
FabU	GGGATCCGGAATGATCTACGACAAGG
FabD	GGGTACCAAGTTTAGCCCGTTCATGC
PA1611-UP	AAATCCCTGAACTGCAGGGTTGTG
PA1611-DN-in	ACCTGCGCCAAGAATTCACCCATA
GmFRT-UP	CGAATTAGCTTCAAAAGCGCTCTGA
GmFRT-DN	CGAATTGGGGATCTTGAAGTTCCT
Anr-UP-KpnI	ATCGCGGCTGCGGTACCCTT
Anr-DN-BamHI	ATACAACGGATCCGCGCTGAG

Table 5.2. Primers used in this study (cont.)

Primer Name	Sequences (5' → 3')
EcglmS-DN	TGCAGCTGCTGGCTTACCATG
Tn*7*R	CACAGCATAACTGGACTGATTTC
DIG-fabA	DigN-ATGCGATCGATCATCAGCATGTTG

[a]Sequences in capital letters are common for all genes amplified in a particular experiment and overlap with the sequences of the other genes that they were spliced to, e.g. Gm or *attB* primer sequences. Lower-case letters indicate gene-specific sequences.

reaction to generate pPS1488 containing a 30 bp deletion in the *fabA* upstream region. In the first PCR reaction, two flanking DNA segments harboring overlapping sequences were amplified using two sets of primers (PCRSOE-A + IR187SOE-B) and (IR187SOE-C + PCRSOE-D) and the following cycle conditions: one cycle at 94°C for 2 min; 30 cycles of 94°C for 30s, 50°C for 2 min, 72°C for 1 min; a final extension at 72°C for 10 min. In the second PCR, each purified 1st round PCR fragment was used to perform splicing by overlap extension (SOE) PCR using primers PCRSOE-A and PCRSOE-D. The reaction mixture was subjected to the following thermal cycles: one cycle at 94°C for 2 min, 3 cycles of 94°C for 30 s, 50°C for 2 min, and 72°C for 1 min 40 s and a final extension at 72°C for 10 min. The resulting PCR product was cloned into pCR-Blunt II-TOPO vector (Invitrogen), resulting in a plasmid containing the *fabA* upstream sequences with a 30 bp deletion. Finally, pPS1491 and pPS1492 were constructed by ligating the *Kpn*I-*Sma*I fragments of pPS1482 and pPS1488, respectively, into pPS1453 cleaved with the same enzymes.

For promoter localization experiments, different portions of the *fabA* intergenic sequence were fused to a promoter-less *lacZ* contained on a mini-Tn*7* delivery vector utilizing Gateway universal cloning technology (Invitrogen) as described in chapter 3. The *fabA* upstream region was amplified using 11 different primer pairs consisting of combinations of pfabA-attB1 and pfabA1-attB2 to pfabA11-attB2, giving PCR segments containing progressively shorter lengths of the *fabA* upstream region at 20 ~21 bp intervals. PCR was performed using the following cycles: one cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 58°C for 30 s, 68°C for 20 s, and a final extension at 68°C for 10 min. To construct a *lacZ* fusion with the *PA1612* promoter, a fragment containing the *PA1612* promoter, the *PA1612-PA1611* operon, and the entire *PA1611-fabA* intergenic region was also amplified by PCR

using the primer pair pfabA-attB1 and pPA1612-UP-attB2 and the same cycling conditions except that the extension time was 3 min and 20 s. The individual PCR fragments thus obtained contain *att*B1 and *att*B2 sites which facilitate high-throughput cloning into the universal donor vector pDONR221 (Invitrogen) containing *att*P1 and *att*P2 sites by a site-specific recombination between *att*B and *att*P sites, resulting in plasmids harboring different inserts of *fabA* upstream sequences with flanking hybrid *att* sites, *att*L1 and *att*L2. This was followed by a second site-specific recombination between the *att*L and *att*R sites of the resulting plasmids and the destination vector pPS1669, respectively, resulting in mini-Tn*7* elements containing transcriptional *lacZ* fusions with different lengths of *fabA* upstream sequences.

5.3.3 Random *mariner*-based insertional mutagenesis. *mariner*-based random mutagenesis of PAO1 containing a temperature-sensitive *fabA* allele (PAO1 *fabA*(Ts)) (Hoang et al., 1997) was performed by electroporating pBT20 (Kulasekara et al., 2005) containing a *mariner* transposon with a Gm-resistance marker. It was reasoned that knock-out of potential *fabAB* activators would create unconditional mutants requiring OA supplementation at all temperatures. Identification of OA auxotrophs was performed by patching Gm^r transformants on LB containing 30 μg/ml gentamycin with and without OA. Chromosomal fragments from OA auxotrophs were prepared using the QIAamp DNA mini kit and transferred into the PAO1 *fabA*(Ts) parental strain containing a chromosomally integrated *fabA'-lacZ* fusion to assess whether the mutation generated by *mariner* transposon insertion directly affected *fabAB* expression. β-galactosidase assays were performed in cells grown in the presence of OA.

mariner-based random mutagenesis was also performed in PAO1-*fabA*(Ts) strain containing a chromosomally integrated *fabA'-lacZ* fusion. A knock-out of a potential activator would create an unconditional mutant requiring OA supplementation at all temperatures and result in lighter blue colonies. Lighter blue colonies were tested for OA auxotrophy by patching Gm^r transposon mutants on LB containing 30 μg/ml gentamycin with or without OA suplementation. In addition, β-galactosidase assays were performed in cells grown in the presence of OA.

The transposon insertion points in the mutants harboring an OA auxotrophy were first determined by PCR amplification using Gm-UP and Gm-DN primers to verify *mariner* transposon insertions in the PAO1 chromosome. PCR cycle conditions were as follows: one cycle of 96°C for 5 min, 30 cycles of 96°C for 45 s, 62°C for 30 s, and 72°C for 45 s and a final extension at 72°C for 10 min. Secondly, the absence of insertions in the *fabAB* operon was confirmed by PCR amplification using *fabAB* specific primers FabU and FabD. Its thermal cycles were one cycle of 96°C for 5 min, 30 cycles of 96°C for 45 s, 68°C for 30 s, and 72°C for 2 min 10 s and a final extension at 72°C for 10 min. The insertion sites of transposons located outside of the *fabAB* genes were determined by two-step random PCR amplifications using *mariner* specific primers (Rnd1-TnM and Rnd2-TnM), random PAO1 chromosomal primers (Rnd1-Pa1), and its nested primer Rnd2-Pa. In the first PCR reaction, 0.4 pmoles of each Rnd1-TnM and Rnd1-Pa1 primers were used and PCR cycle conditions were as follows: one cycle of 94°C for 2 min, 10 cycles of 94°C for 30 s, 48°C for 30 s reduced by 1°C per each round, and 72°C for 3 min, and 26 cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 3 min. In the subsequent PCR step, two microliters of 1st round PCR product were added to the PCR reaction mix with two nested primers Rnd2-TnM and Rnd2-Pa. The thermal cycles included one cycle of

94°C for 2 min, 31 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 3 min, and a final extension of 72°C for 5 min. The resulting PCR fragments were sequenced by using primer TnMSeq to determine chromosomal *mariner* transposon insertion sites (S. Lory, personal communication). An alternative method employed to map *mariner* insertion sites was as follows. Chromosomal DNAs from OA auxotrophs were digested with *Bam*HI which cuts the *mariner* transposon once, followed by self-ligation of chromosomal DNA fragments self-ligated and PCR amplification with primers Rnd2-TnM and TnMRev. The PCR conditions used were an initial denaturation at 94°C for 2 min, 30 cycles of 94°C for 30 s, 56°C for 30 s, 68°C for 6 min and a final extension at 68°C for 10 min. The resulting PCR fragments were sequenced as above.

5.3.4 Gene deletion. For construction of a gene replacement vector containing a deletion allele of the *PA1611* gene, this gene was amplified by using primers PA1611-UP and PA1611-DN-in. The PCR fragment was cloned into pCR2.1 to yield pPS1472 followed by the ligation of the ~2.0 kb blunt-ended *Eco*RI fragment of pPS1472 to *Pst*I+*Eco*RI cleaved and blunt-ended pEX18Ap vector. The *PA1611* coding sequence was deleted by replacement of a blunt-ended *Kpn*I-*Nco*I fragment of pPS1476 with a 1.1 kb *Sma*I Gm^r::*FRT* fragment of pPS856 (Hoang et al., 1998), resulting in pEX18Ap-Δ*PA1611*::Gm.

The plasmids pPS1503, pPS1505 and pPS1506 containing Δ*PA1627*::Gm, Δ*PA4890*::Gm and Δ*PA1539*::Gm, respectively, were constructed by SOEing PCR as described in the following procedure. Five ng of pPS856 plasmid was used for amplification of Gm-*FRT* cassette using primers GmFRT-UP and GmFRT-DN and the following cycle conditions: one cycle of 95°C for 2 min, 30 cycles of 94°C for 30

s, 50°C for 30 s, and 68°C for 1.5 min and a final extension at 68°C for 7 min. Flanking gene-specific DNA fragments were amplified using the following primer pairs: 1627SOE-A + 1627SOE-B and 1627SOE-C + 1627SOE-D; 4890SOE-A + 4890SOE-B and 4980SOE-C + 4890SOE-D; or 1539SOE-A + 1539SOE-B and 1539SOE-C + 1539SOE-D for *PA1627*, *PA4890*, or *PA1539*, respectively. Cycle conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 1 min, and 68°C for 10 min. Since each primer-B and primer-C contains sequences that overlap with the Gm-*FRT* fragment, the resulting three PCR fragments - i.e., the two flanking DNA fragments and the Gm-*FRT* segment - were annealed together and subjected to second round PCR amplification using each primer-A and primer-D. PCR was performed as follows: one cycle of 94°C for 2 min, 3 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 1 min (pause the PCR at last cycle 30 s before it finishes at 68°C to add primers, then continue), 25 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 5 min and a final extension at 68°C for 10 min. These steps resulted in the generation of a deletion allele of Δ*Gene*::Gm, which was subcloned into the gene replacement vector pEX18Ap (Hoang et al., 1998) by ligation of the respective *Stu*I-*Xho*I (*PA1627*), *Xho*I-*Sma*I (*PA4890*) or *Msc*I-*Xho*I (*PA1539*) digested PCR fragments containing a Gm-*FRT* segment flanked by part of chromosomal DNA sequences to *Sma*I+*Sal*I digested pEX18Ap vector. These gene replacement vectors with Δ*PA1611*::Gm, Δ*PA1627*::Gm, Δ*PA4890*::Gm and Δ*PA1539*::Gm were transformed into *E. coli* SM10*lacI*^{q} cells and then conjugally transferred to PAO1 and PAO1 harboring mini-Tn*7*T-*lacZ* or the mini-Tn*7*T-*fabA 'lacZ* chromosomal fusion by biparental mating for 5 h, followed by plating the recombinants on VBMM plates containing 30 μg/ml of gentamycin. Merodiploid recombinants were purified on the same medium and were resolved by streaking

individual Gm^r colonies on a LB plate containing 5% sucrose. Mutant constructions were verified by colony PCR, followed by Flp-mediated excision of Gm^r marker, which was performed by conjugal transfer of the Flp-producing pFLP2 into the recombinants (Hoang et al., 1998).

To identify potential *fabA* activator(s), the genes encoding unidentified transcriptional regulators of GntR family were deleted as described in chapter 3.

For creation of an *anr* deletion mutant, Gateway cloning technology was utilized. First, two flanking sequences were amplified by using the primer sets anr-UpF-GWL + anr-UpR-Gm and anr-DwnF-Gm + anr-DwnR-GWR. The cycle conditions were one cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 20 s, and a final extension at 68°C for 10 min. The resulting PCR fragments were used in second round PCR with the Gm-*FRT* fragment as described above, which yielded pPS1833 after subcloning of the SOEing fragment into pEX18ApGW.

For complementation experiments, *anr* was amplified by using primers Anr-UP-KpnI and Anr-DN-BamHI, the PCR fragment was digested with *Kpn*I+*Bam*HI followed by ligation into pVLT35 (Miller et al., 1988) or pUCP20 (West et al., 1994) digested with the same enzymes. This resulted in pPS1682 or pPS1684, respectively. The plasmids were individually transformed into the wild-type and Δ*anr* mutant strain containing a chromosomal p*fabA'-lacZ* transcriptional fusion by using the rapid electroporation method described in chapter 3. Transformants were selected on LB plates containing 150 μg/ml of spectinomycin or 200 μg/ml of carbenicillin for pVLT35 or pUCP20, respectively.

5.3.5 Construction of an *E. coli* host strain and a genomic library of *P.*

aeruginosa. For identification of an activator of *fabA* expression in a heterologous host, gene fusion technology was employed. First, the p*fabA*'-'*lacZY* translational gene fusion-containing Tn*7* delivery plasmid pPS1634 was constructed by ligation of three fragments: a *Bam*HI-*Xho*I fragment of pPS1633, an ~250 bp *Bam*HI-*Eco*RV fragment of pPS790 and an ~6.2 kb *Sma*I-*Sal*I fragment of pMC1403 (Casadaban et al., 1980), resulting in an in-frame fusion of a portion of the *fabA* coding sequence plus *fabA-PA1611* intergenic region to *lacZY.* Second, an *E. coli* strain containing a chromosomal insertion of the *fabA'-'lacZY* fusion construct was created by co-electroporating pPS1634 and pTNS1 into the Δ*lac E. coli* strain DL291 (Larson et al., 1982), resulting in the site-specific integration of *fabA'-lacZY* fusion at *att*Tn*7* downstream of *glmS* in the *E. coli* chromosome. The integration into *att*Tn*7* was verified by PCR amplification using primer pair EcglmS-DN and Tn*7*R and the following PCR cycle conditions: one cycle of 95°C for 5 min, 30 cycles of 95°C for 45 s, 59°C for 45 s and 72°C for 30 s, and a final extension at 72°C for 10 min. A PAO1 library was created by ligation of *Eco*RI+*Pst*I partially-digested chromosomal DNA fragments ranging from 0.75 kb to 5.0 kb into *Pst*I+*Eco*RI-digested pUC18. After transforming the library into *E. coli* containing the *fabA*'-'*lacZY* gene fusion, putative activator-encoding plasmids were selected as dark blue colonies on LB-ampicillin plates containing 40 μg/ml of X-Gal and 25 μM of IPTG. Plasmids isolated from blue colonies were retransformed into *endA*⁻ *E. coli* cells to facilitate recovery of high quality plasmid DNA. Plasmids from isolates showing dark blue color were mapped by digestion with restriction enzymes *Pst*I and *Eco*RI, *Bam*HI, *Hin*dIII or *Sph*I. For the identification of the putative *fabA*-activating gene encoded by these plasmids, vector-chromosomal DNA junction sequences were determined using the M13 forward and reverse sequencing primers.

5.3.6 β-galactosidase assays. Cells were grown at 37°C with shaking to exponential phase (optical density at 540 nm ~0.4-0.8) in LB medium with 0.05% Brij 58 +/- 0.05% oleic acid. β-galactosidase (βGal) activity was measured in chloroform/sodium dodecyl sulfate-permeabilized cells and its activity calculated as previously described (Miller, 1992).

5.3.7 RT-PCR analysis. Total RNA was extracted from PAO1 cells grown at 37°C and 16°C using the hot phenol method (Barton et al., 1996). Using 1 μg of RNA, cDNA was synthesized following the procedure described in the Invitrogen manual with minor modifications: total RNA was denatured at 65°C for 5 min in the presence of 0.2 μM primer DIG-fabA and 1 mM dNTP. After cooling for 2 min, 1x reverse transcriptase (RT) buffer, 5 mM $MgCl_2$, 10 μM DTT, 2 units of RNase inhibitor (RNase out), and 10 units of Superscriptase III enzyme were added and the mixture was incubated at 50°C for 50 min, then heat-inactivated at 85°C for 5 min. To remove DNA-RNA annealed complexes, RNase H was added and the tube was incubated at 37°C for 20 min. The cDNA was then used as a template for PCR amplification using different primer sets consisting of DIG-fabA, and primers annealing at various positions in the intergenic region, which included pfabA1-attB2 through pfabA9-attB2 or the primer pfabA0 within the *PA1611* coding region. The following cycle conditions were used: one cycle of 95°C for 30 s, 30 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 20 s and a final extension at 72°C for 10 min. PCR fragments were analyzed by agarose gel electrophoresis.

5.3.8 Biochemical studies

5.3.8.1 Attempts at purification of a FadR-like *fabA* activator using streptavidin beads. A biotinylated, double-stranded DNA oligonucleotide composed of the 30 bp putative regulatory region and 5 bp flanking sequences (5'-GGACACTGTGACTTTC ACCGCAACGCAACAGTCTATGACT-3', the 30 bp region is underlined) were prepared as follows: 1 μg of both oligonucleotides Bio-fadR-for (5'- Bio-GGACACTGTGACTTTCACCGCAACGCAACAGTCTATGACT) and fadR-Rev (5'-AGTCATAGACTGTTGCGTTGCGGTGAAAGTCACAGTGTCC) were denatured by incubation for 5 min at 95°C and then re-annealed either by slow cooling to room temperature or reducing the temperature to 25°C in 2.5°C increments in a thermal cycler. For preparation of crude cell extracts, three liters of *P. aeruginosa* PAO1 cells were grown at 37°C until they reached an optical density (540 nm) of 0.8. Cells were harvested by centrifugation, then passed through a French press three times in 27 ml of buffer (20 mM Tris-HCl [pH 7.9], 0.5 mM NaCl, 10% glycerol and 1 mM PMSF). Cell debris was removed by centrifugation at 12,000×g for 10 min followed by ultracentrifugation at 34,000×g for one hour. The supernatant was concentrated by addition of ammonium sulfate to 60 % saturation. The protein precipitate was collected by centrifugation and resuspended in 3 ml of 10 mM Tris-HCl (pH 7.5), 10% glycerol and then dialyzed at 4°C overnight against 3 liters of the same buffer. Three μg of the resulting protein extract was added to a DNA binding reaction composed of 1 μg of poly-dI-dC, 1 μg of dsDNA sequence, and 5× binding buffer (100 mM Hepes [pH 7.6], 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 50 mM DTT, 1% Tween 20 and 150 mM KCl). After incubation at room temperature for 15 min, 100 μl of μMACS streptavidin microbeads were added and the mixture was applied to a column which was equilibrated with 100 μl of protein equilibration buffer provided in the kit and rinsed twice with 100 μl of binding buffer in the magnetic separation unit.

After sample application, the column was washed six times with binding buffer before DNA-bound proteins were eluted by the addition of binding buffer containing 0.2-1.0 M NaCl. The protein contents of eluted samples were analyzed by 10% SDS-PAGE, followed by Coomassie blue staining.

5.3.8.2 Attempts at purification of FadR-like *fabA* activator using CNBr activated Sepharose beads. Similar to the method described above, a duplex of two oligonucleotides fadR-UP (5'-GAACCGGACACTGTGACTTTCACCGCAACGCA-ACAGTCTATGACTA) and fadR-DN (5'- TAGTCATAGACTGTTGCGTTGCGG-TGAAAGTCACAGTGTCCG) was created by denaturation of 0.5 nmoles of each primer at 95°C for 5 min, followed by re-annealing which was achieved by decreasing the temperature to 20°C in 2.5°C increments in a thermal cycler. For preparation of a cell-free lysate, PAO1 cells grown in 3 liters of LB to mid-log phase were harvested by centrifugation and suspended in buffer (20 mM Tris-HCl [pH 7.5], 10 mM NaCl, 1 mM EDTA and 1 mM DTT). Cells were lysed in a sonicator by administering ten short bursts of 10 s with 30 s pause intervals. The lysate was subjected to untracentrifugation (Beckman, SWTi28 rotor) at 45,000×g and 4°C for one hour. Ammonium sulfate was added to the supernatant to achieve 60% saturation. The precipitate was collected by centrifugation, dissolved in column buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100 mM NaCl, 0.1 mM DTT and 10 mM NaN_3) and then dialyzed overnight against 3 liters of the same buffer. The dialysed mixture was applied to a column packed with CNBr-activated Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ). Unbound proteins were removed by washing with three volumes of column buffer. DNA-bound protein(s) were eluted by the addition of TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, containing 0.2 - 0.8

M NaCl). The protein contents in eluted samples were anaylzed by electrophoresis on a 10% SDS-PAGE gel and the gel was stained with Coomassie blue or silver. All fractions showing distinct bands were collected and proteins were concentrated with microcon centrifugal filter units (Millipore). Protein bands were cut from the SDS-PAGE gel, subjected to in-gel trypsin digestion, and their identities confirmed using peptide mass fingerprinting.

5.3.8.3 Attempts at purification of DNA binding proteins using 30 bp concatamer affinity chromatography. A 30 bp concatamer was generated by PCR amplification using 30-concatamer-for (5'-(TAGACTGTTGCGTTGCGGTGAAAGTCACAG)$_3$) and its complementary 30-biotin-rev primer (5'-Bio-CTGTGACTTTCACCGCAAC GC). The cycle conditions were one cycle of 94°C for 2 min, 34 cycles of 94°C for 45 s, 55°C for 40 s with increasing 5 s every cycle, 68°C for 45 s, and a final extension at 68°C for 10 min. For DIG-labeled DNA, the concatamer was incubated with DIG-ddUTP in the presence of $CoCl_2$ and labeling buffer containing 200 mM potassium cacodylate, 25 mM Tris-HCl (pH 6.6), 0.25 mg/ml BSA at 37°C for 15 min, followed by stopping the reaction with EDTA as per the manufacturer's manual (Roche Applied Science, Cat. No. 3 353 591). Cell lysates from PAO1 cells grown overnight in 200 ml of LB medium were prepared by cell lysis in high salt buffer (20 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 1 mM PMSF, 1 mM EDTA, 0.1 % Triton X-100 and 10 μg/ml lysozyme) using freeze-thaw cycles as follows: 6 cycles of freezing in dry ice/ethanol for 5 min and thaw at 37°C in a shaker for 20 - 30 min. The cell lysates were subjected to ultracentrifugation at 34,000×g for 1 h (Beckman, SWTi28 rotor) followed by dialysis against 4.5 liters of buffer (10 mM Tris-HCl [pH 7.5], 10 % glycerol). DNA binding reactions contained 1 μg of poly-L-lysine, 1 μg of poly-dI-

dC, binding buffer (20 mM Hepes [pH 7.6], 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2 % Tween 20 and 30 mM KCl), 10-40 ng of concatamer DNA and 0.2-2 μg of protein extract, and the mixtures were incubated at room temperature for 30 min. The reaction mixtures were loaded onto a 6 % native polyacrylamide gel prepared and electrophoresed in 0.5× TBE buffer. DNA fragments were transferred to neutral or positively charged nylon membranes for biotin or DIG detection, respectively. The detection of biotin- or DIG-labeled DNA was performed following the protocols of the NEBlot Phototope detection kit (New England Biolabs, Beverly, MA) or the DIG gel shift kit (Roche Applied Science, Indianapolis, IN. Cat. No. 3 353 591), respectively.

5.4 RESULTS AND DISCUSSION

5.4.1 Characterization of *fabA* regulatory region. The 188 bp *PA1611-fabA* intergenic region contains several recognizable features. It contains a putative -10 region (TACAAT), but no corresponding -35 region, suggesting a promoter requiring an activator for RNA polymerase binding to initiate *fabAB* transcription (Fig.5.1). It also contains a 30 bp sequence which is conserved in all *Pseudomonas* species sequenced to date (Fig. 5.2). This conservation includes *PA1611* which encodes a probable sensor kinase/response regulator hybrid protein. This strong sequence conservation suggests that the 30 bp sequence may be an important *fabAB* regulatory region. A highly conserved Fatty acid biosynthesis repressor (FabR, or DesT in *P. aeruginosa*) binding site was also found upstream of the 30 bp sequence in *P. aeruginosa,* which may be involved in negative regulation of *fabA* expression. The DesT binding site is highly conserved in *fabA* upstream regions of several other bacteria (Fig. 5.3). I hypothesized that *fabA* regulation may be complex and mediated

Fig. 5.1. Sequence of the *PA1611-fabA* intergenic region. The last four *PA1611* codons and initial eight *fabA* codons are shown; arrows mark the transcription directions of *PA1611* and *fabA*, respectively. The conserved 30 bp sequence is boxed. A putative -10 region with good homology to the TATAAT σ_{70} consensus sequence is shown in underlined bold-faced letters. The DesT binding site is marked by inverted arrows.

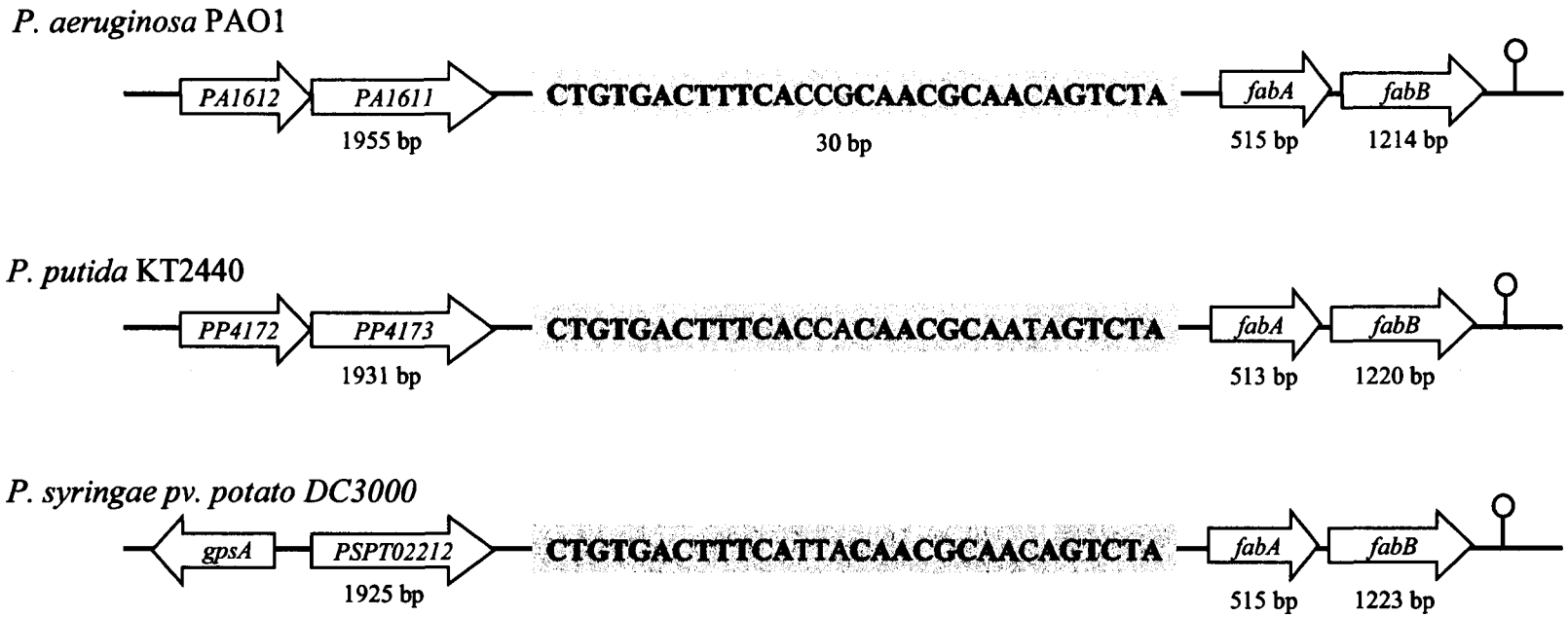


Fig. 5.2. The *fabA* upstream sequences are conserved in *P. aeruginosa*, *P. putida* and *P. syringae*. The *PA1611*, *PP4173* and *PSPT02212* encode a conserved sensor histidine kinase/response regulator and *gpsA* in *P. syringae* pv. *tomato* encodes a glycerol-3-phosphate dehydrogenase. The sizes of genes are indicated in bp. The highly conserved 30 bp sequence in the *fabA* upstream regions is shown in bold-faced letters in the grey box. A lollipop structure indicates the transcriptional terminators of the respective *fabAB* operons.

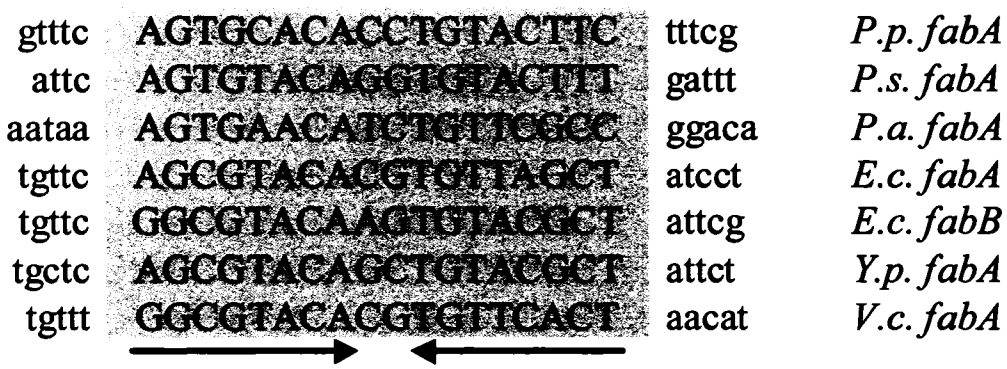


Fig. 5.3. FabR binding sites in the *fabA* or *fabB* upstream regions of several bacteria. The sequences in the gray box indicate conserved FabR binding sites. *P.p., P. putida*; *P.s., P. syringae*; *P.a., P. aeruginosa*; *E.c., E. coli*; *Y.p., Y. pestis*; *V.c., Vibrio cholerae.* Only the *P.a.* and *E.c.* sequences were experimentally shown to bind FabR (*E.c.*) and its *P.a.* homolog DesT.

by yet unknown regulatory protein(s) which act at conserved upstream regions. However, the organization of the *fabA* upstream regions of *E. coli* and *P. aeruginosa* is different. Whereas the *E. coli* FabR repressor binds at a site overlapping the -10 region and the FadR activator at a site overlapping the -40 region, *P. aeruginosa* contains a putative DesT repressor-binding site upstream of the 30 bp putative activator-binding site (Fig. 5.4). To assess whether the 30 bp sequence plays a role in *fabA* regulation, it was deleted and the corresponding region was fused to *lacZ*. After single copy insertion into the PAO1 chromosome, βGal activities were measured in the absence or presence of OA and compared to that expressed in the wild-type *fabA'-lacZ* fusion strain. As shown in Fig 5.5, OA repressed *fabAB* expression in the wild-type strain harboring a chromosomal *fabA'-lacZ* fusion, whereas *fabA*Δ30*'-lacZ* expression was significantly decreased compared to wild-type *fabA'-lacZ*. In addition, *fabAB* expression in the *fabA*Δ30 deletion construct was no longer repressible in the presence of OA. These results suggested that the 30 bp sequence is indeed involved in regulation of *fabA* expression and may be a binding site for an unknown activator or other effector molecule. This was confirmed by analyses using *lacZ* transcriptional fusions containing progressively shorter *fabA* upstream fragments (Fig. 5.6). The data shown in Fig. 5.7 demonstrate that deletion of the 30 bp sequence and beyond led to loss of *lacZ* activity and OA responsiveness. The 5 bp addition in primer 4a, which restores a complete 30 bp sequence, recovered *lacZ* expression indicating that this sequence is important for *fabA* transcription (Fig. 5.7B).

In order to further define the promoter region of *fabA*, RT- PCR was performed. RNA extracted from wild-type PAO1 cells grown at 37 ^{o}C was reverse-transcribed to cDNAs, which were then used as templates for PCR amplification utilizing primer R and the primers indicated in Fig. 5.8A. PCR products were

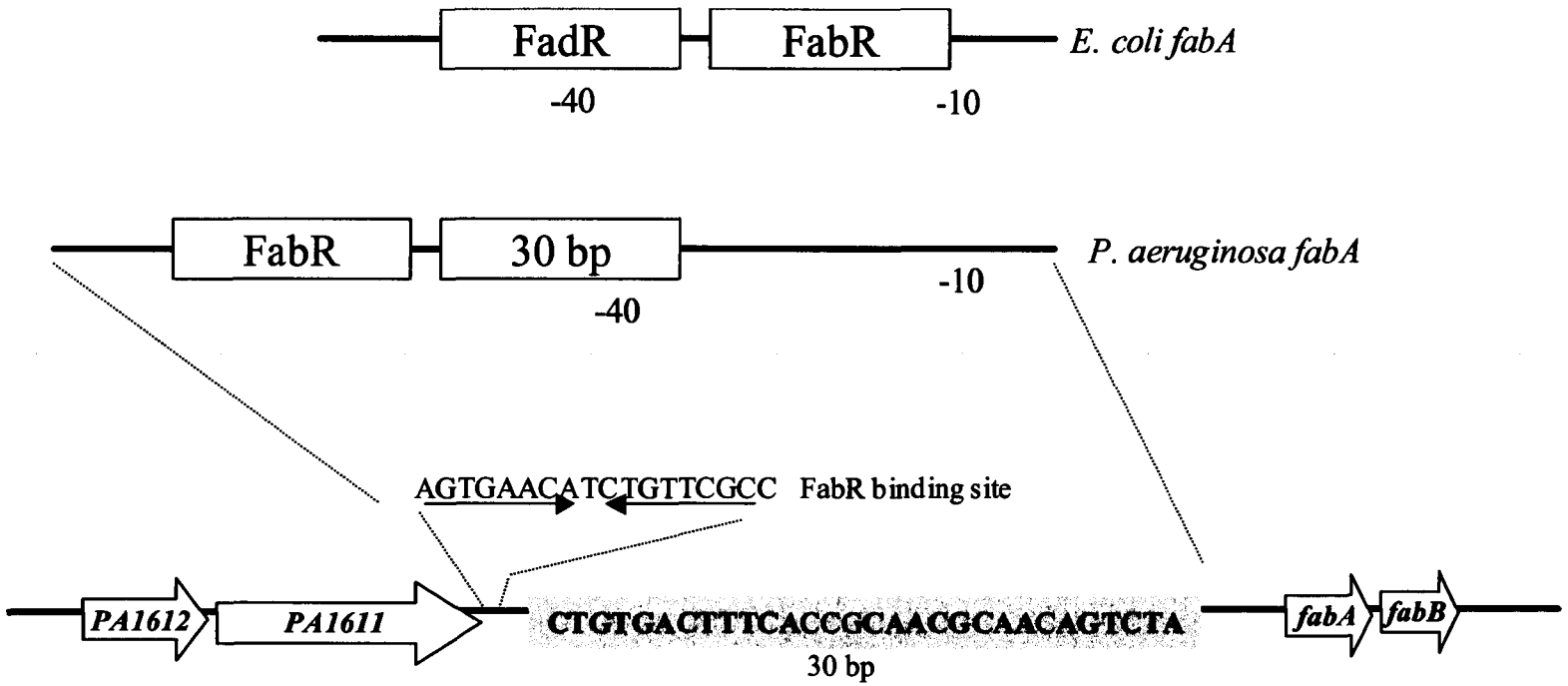


Fig. 5.4. Location of binding sites for *E. coli* FadR or *P. aeruginosa* FadR-like activator and FabR in the *E. coli fabA* and *P. aeruginosa* PAO1 *fabAB* upstream regions. Note that FabR in *P. aeruginosa* was recently renamed DesT.

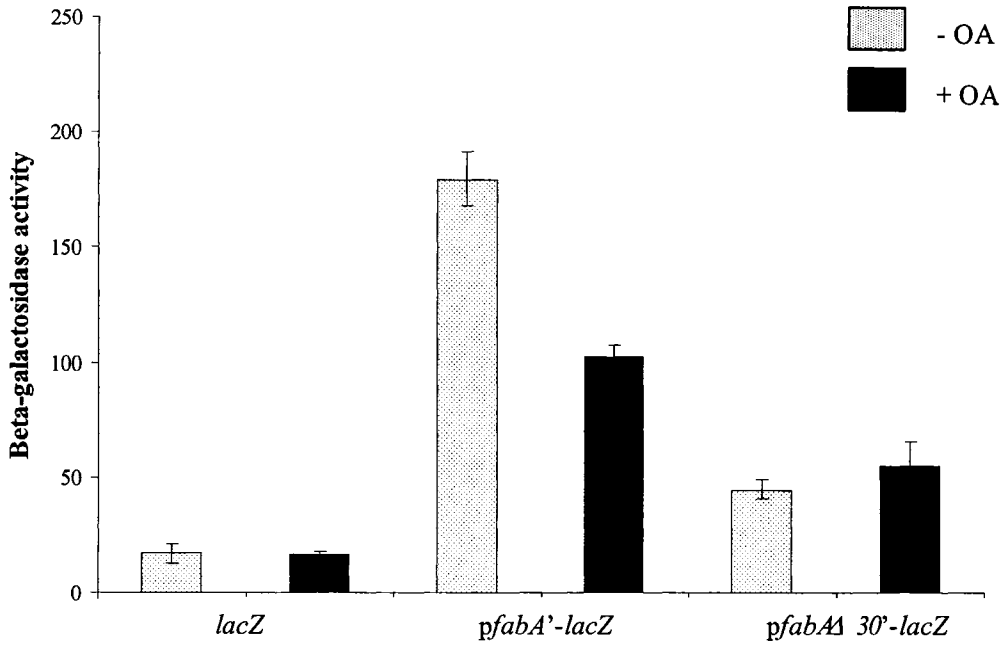


Fig. 5.5. *lacZ* expression in PAO1 containing chromosomally integrated *lacZ*, *fabA'-lacZ* or *fabA*Δ30*'-lacZ* transcriptional fusions. Stains were grown to mid-log phase in LB medium with or without 0.05% oleate (OA) supplementation and β-galactosidase activities were measured. β-galactosidase activities are expressed in Miller Units.

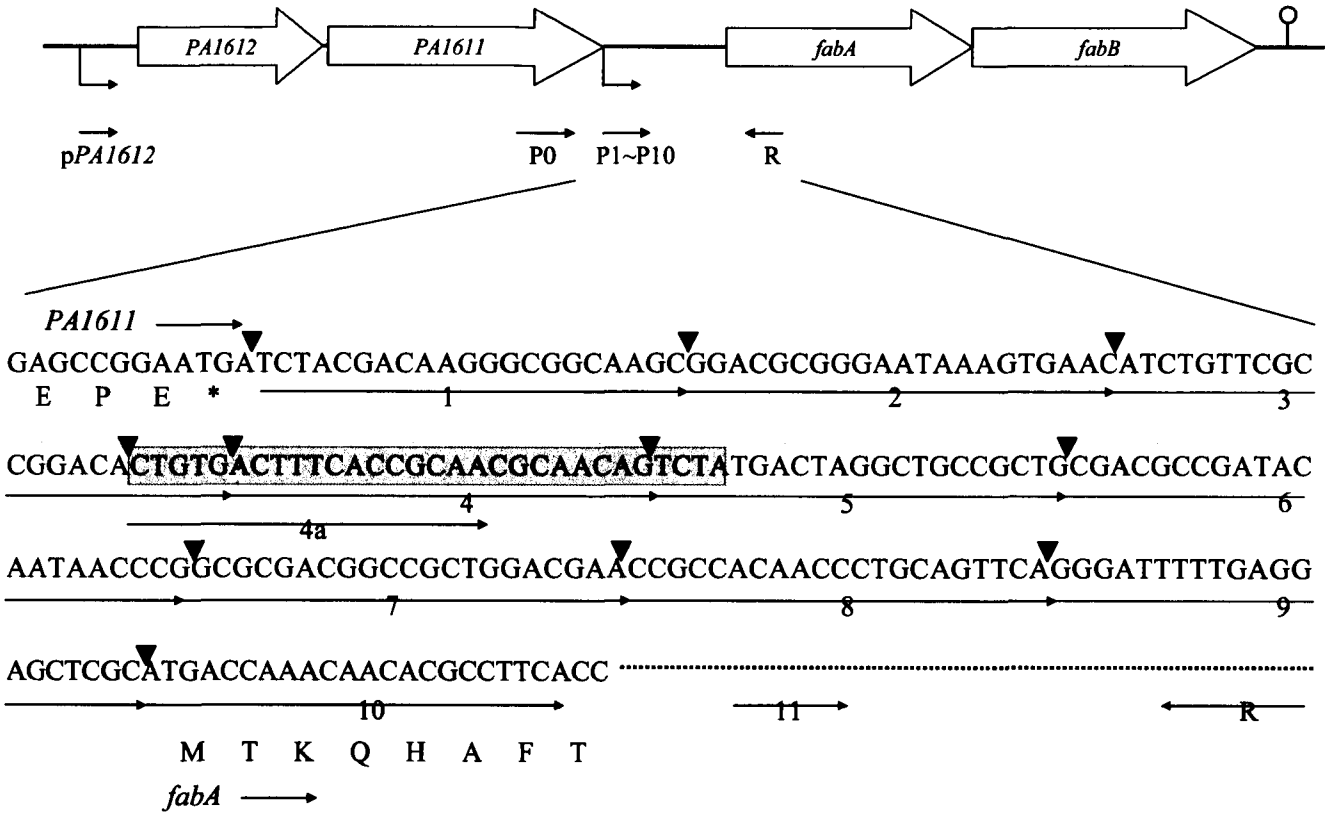


Fig. 5.6. Positions of primer-binding sites in the *PA1611-fabA* intergenic region. Primers are labeled P0, fabA0, P1-P11, fabA1-attB2 through fabA11-attB2, R, and fabA-attB1. The relative locations of primer-binding sites and priming directions are indicated by the arrows. The shaded sequence indicates the putative 30 bp regulatory element. Vertical arrow heads indicate the end points of sequences present in the *lacZ* fusion constructs analyzed in Fig. 5.7.

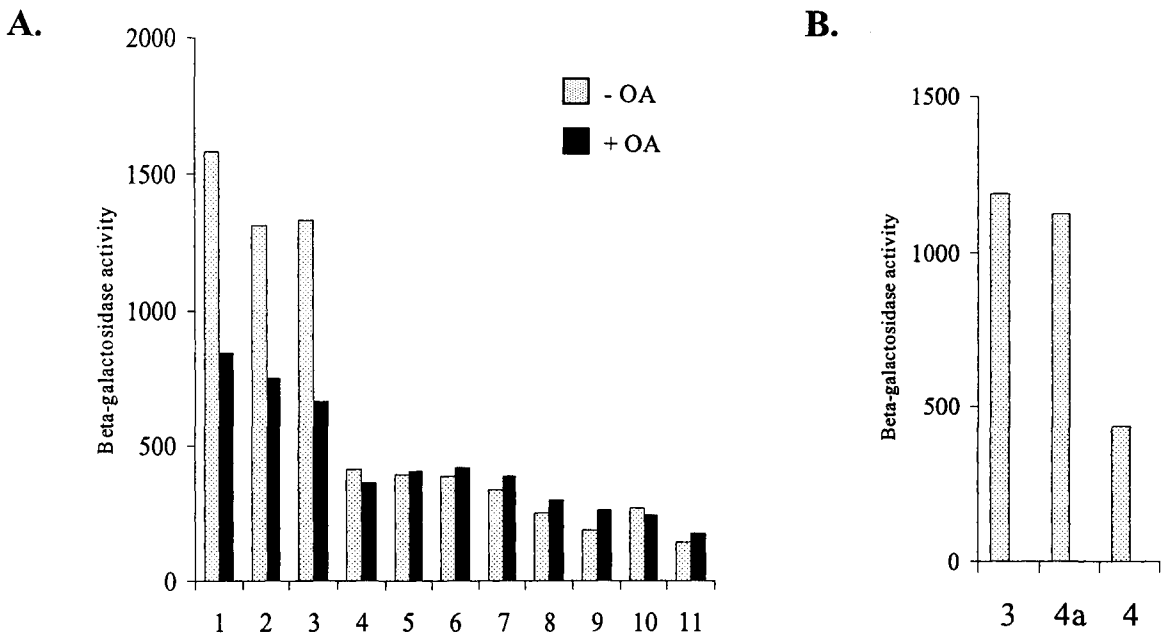


Fig. 5.7. The 30 bp sequence is important for *fabAB* expression. **A)** β-galactosidase activities were measured in PAO1 containing chromosomally integrated *fabA'-lacZ* fusions with progressively shorter *fabA* upstream regions (see Fig. 5.6 for sequence end points). **B)** The 5 bp addition in primer 4a, which restores a complete 30 bp sequence, restored *lacZ* expression to wild-type levels indicating that it is important for *fabA* transcription. Cells were grown with and without oleate (OA) supplementation, and β-galactosidase activities were measured as described in the legend to Fig. 5.5.

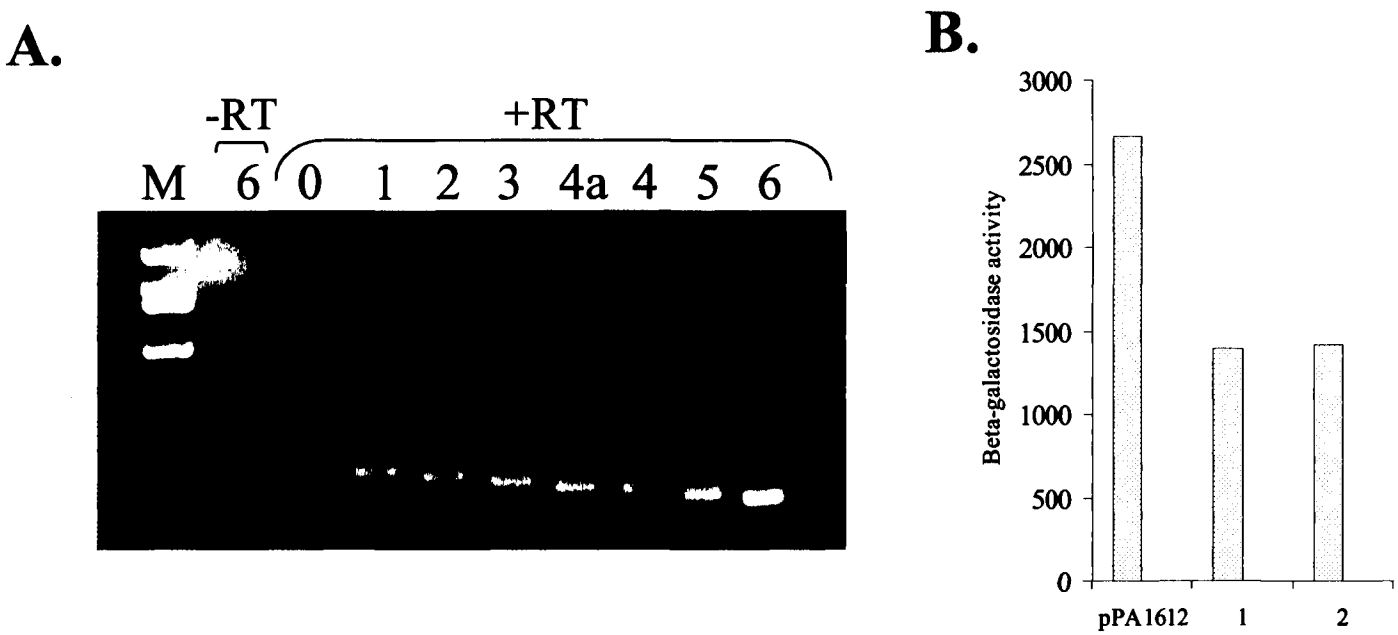


Fig. 5.8. Characterization of the *fabA* promoter region. **A)** RT-PCR analysis of *fabA* expression. RNA was extracted from PAO1 grown at 37 °C using the hot phenol extraction method. cDNA was synthesized using the Superscript III First-strand kit (Invitrogen) and primer R. The resulting cDNAs were used as templates for PCR amplification utilizing primer R and the indicated primers (see Fig. 5.6 for location of primer-binding sites). **B)** β-galactosidase activities in PAO1 containing *lacZ* fusions with various *fabA* upstream fragments. The upstream fragments were amplified with primer pair pPA1612 and pR, and primer pairs p1 and pR or p2 and pR (see Fig. 5.6 for primer-binding sites).

apparent in all reactions containing either primer 0 which primes within the *PA1611* coding region, or primers 1 through 6 which prime in the *fabA* intergenic region (Fig. 5.8A; see Fig 5.6 for location of *fabA* primer binding sites). To compare *fabA* transcription levels from promoters located in far upstream or *PA1611-fabA* intergenic sequences, βGal activities were measured in *lacZ* fusion strains harboring the putative *PA1612-PA1611* operon promoter, the entire *PA1612-PA1611* operon and the *PA1611-fabA* intergenic sequence (*PA1612'-lacZ*) or only the *PA1611-fabA* intergenic region (p1'-*lacZ* or p2'-*lacZ*) (Fig. 5.8B). It is evident that βGal levels expressed from *PA1612'-lacZ* were two times higher than those expressed from p1'-*lacZ* or p2'-*lacZ* (Fig. 5.8B). In summary, both RT-PCR and gene fusion analyses suggested that *fabA* may be co-transcribed with *PA1612-PA1611* or another promoter located outside of the *PA1611-fabA* intergenic region. This probably explains why identification of transcription start sites using primers with binding sites in the *fabA* 5' region failed (data not shown). Also, predictions of transcription terminators within *PA1613* through *fabB* using TransTerm software (http://cbcb.umd.edu/software/transterm/ttgenbank50/Pseudomonas aeruginosa.tt) indicated that there are no putative transcriptional terminators in the *PA1612* through *fabB* interval, but only downstream of *fabB*, indicating that the *fabA* operon may be co-transcribed *PA1612-PA1611*.

5.4.2 Identification of putative *fabAB* regulatory proteins

5.4.2.1 Negative regulators of *fabAB* expression. Besides FadR, an additional regulator of *fabA* and *fabB* gene expression in *E. coli* was originally suggested by a computational footprinting method. Actual binding of this "virtual" regulator was subsequently confirmed using DNA affinity chromatography (McCue et al., 2001, Zhang et al., 2002). Later, FabR (YijC) was found to function as a repressor of *fabB*

gene expression (Zhang et al., 2002). *P. aeruginosa* has two FabR homologs, *PA4890* and *PA1539*, which have about 50% sequence similarity to *E. coli* FabR. To assess whether these homologs repress *fabAB* expression in *P. aeruginosa*, β-galactosidase activities were measured in wild-type, Δ*PA4890* and Δ*PA1539* mutant strains containing a chromosomally-integrated *fabA'-lacZ* fusion. The addition of OA repressed *fabA* transcription in all three strains tested (Fig. 5.9). In the Δ*PA4890* strain, however, OA repressed *fabA* expression to a lesser extent than in wild-type and the Δ*PA1539* strain. However, deletion of *PA4890* did not affect *fabA* expression in the absence of OA, indicating that only PA4890 is involved in negative regulation of UFA synthesis in the presence of OA. In addition, gel shift assays utilizing purified protein showed that PA4890 binds to the putative FabR-binding site located upstream of *fabA* (Yong-Mei Zhang, unpublished data). Later, PA4890 was renamed DesT, a repressor *desBC* operon expression (chapter 4). My data suggest that DesT may also negatively regulate UFA synthesis via regulation of *fabAB* operon expression.

5.4.2.2 Positive regulation of *fabAB* expression. Previous studies preformed in the Schweizer laboratory and by others indicated that expression of the *fabAB* operon was up-regulated in biofilm-grown cells, probably because in biofilms bacteria grow under anaerobic conditions. Furthermore, *lacZ* expression in a wild-type strain containing a chromosomally integrated *fabA'-lacZ* fusion was repressed by 40%-50% in the presence of 0.05% of OA (Fig. 5.5). In other words, addition of exogenous UFA resulted in repression of *fabAB* transcription, indicating that its expression may be regulated by unknown activating factors. This situation is reminiscent to the one found in *E. coli* where in the presence of exogenous UFAs FadR binds acyl-CoAs and the acyl-CoA-FadR complex then binds to its cognate binding site, resulting in

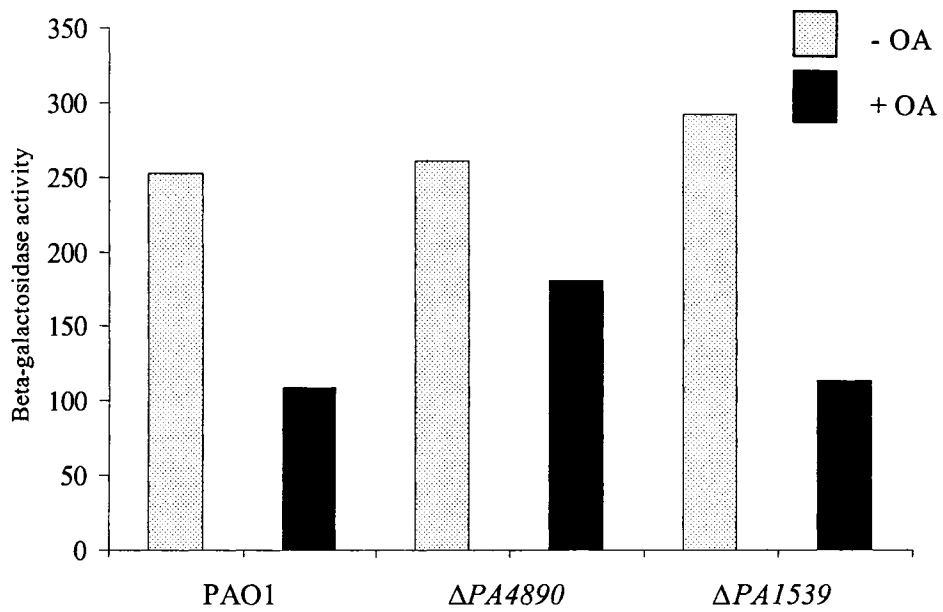


Fig. 5.9. Effects of FabR homologs on *fabA'-lacZ* expression. β-galactosidase activities were measured in wild-type PAO1 and Δ*PA4890* and Δ*PA1539* mutant strains containing a chromosomal *fabA'-lacZ* fusion. Cells were grown in LB medium. Where indicated, 0.05 % oleic acid (OA) was added to cells with 0.05 % Brij-58.

repression of *fabA* transcription. These results suggested the existence of a FadR-like *fabAB* regulatory gene in *P. aeruginosa.*

Three genetic approaches were used in an attempt to identify putative regulatory genes: 1) deletion of *PA1611*, *PA1627* and transcriptional regulators of the GntR family; 2) *mariner*-based random mutagenesis of a *fabA*(Ts) strain; and 3) use of *fabA'-'lacZ* gene fusion technology in an *E. coli* background.

1) Deletion of candidate genes. *PA1611* was considered as a candidate for a *fabAB* regulatory gene, because of its location upstream of *fabA* and because it encodes a two-component regulatory system (a probable sensor kinase/response regulator hybrid) and thus, may sense exogenous UFAs similar to what has been demonstrated in *B. subtilis* and other Gram-positive bacteria. However, deletion of *PA1611* had no effect on expression of a *fabA'-lacZ* fusion (data not shown).

The *PA1627* gene product is 26% homologous to *E. coli* FadR, which is known to positively regulate UFA synthesis, but deletion of *PA1627* had no effect on transcription of a *fabA'-lacZ* fusion (data not shown). Although UFA synthesis is increased when cells are grown under low temperature growth conditions in several bacteria, including *E. coli*, *PA1611* and *PA1627* deletion mutants grown at 16°C had similar levels of *fabA* transcription when compared to wild-type PAO1 (data not shown). Taken together, it can be concluded that neither *PA1611* nor *PA1627* are involved in regulation of *fabAB* transcription.

As described in detail in chapter 4, all unidentified transcriptional regulators of the GntR family were deleted in PAO1 containing a chromosomally inserted *fabA'-lacZ* fusion by employing a high-throughput Gateway cloning technology. Significant changes in *lacZ* expression levels in those knockout mutants would be indicative of positive regulation of *fabAB* transcription. However, none of the

mutations did affect *fabA* expression and, therefore, transcription factors of the GntR family do not seem to be involved in the regulation of *fabAB* expression in cells grown either at 37°C or 16°C.

2) *mariner*-based random insertional mutagenesis. *mariner*-based random mutagenesis of potential *fabAB* activators in strain PAO1 *fabA*(Ts), which is a conditional mutant requiring OA supplementation only at 42°C, will create unconditional mutants requiring OA supplementation at all temperatures. OA auxotrophs were identified by observing cell growth on LB without OA. Transposon insertions were transferred back to the *fabA*(Ts) strain to verify linkage between transposon insertion and the unconditional OA auxotrophy phenotype. Transposon insertion sites in mutants classified as OA auxotrophs were determined by nested PCR and DNA sequencing. *mariner* insertions causing OA auxotrophy due to inactivation of *fabAB* were excluded from further consideration. Several transposon insertions causing the desired phenotype were found and insertion sites were localized to the genes encoding the σ^{54} factor *rpoN*; *PA0286* (*desA*), encoding aerobic fatty acid desaturase; *PA1629*, encoding a probable enoyl-CoA hydratase/isomerase; *PA4760,* encoding a putative heat shock protein; *PA4233*, encoding a probable major facilitator superfamily (MFS) transporter; and several other genes – e.g. *PA0358*, *PA3649*, and *PA4476* - encoding proteins of unknown function (Table. 5.3). RpoN is a global regulator which regulates expression of nitrogen assimilation gene and fermentation gene expression (reviewed in (Reitzer, 2003)). However, there is no evidence that *rpoN* regulates UFA biosynthesis in *P. aeruginosa*. If it does, it may be due to global effects. As detailed in chapter 4, *PA0286* was shown to encode DesA, an aerobic fatty acid desaturase (Zhu et al., 2006). This gene might be closely linked to the regulation of the *fabAB* operon by affecting cellular UFA levels. Enoyl-CoA

Table 5.3. *mariner* insertions in genes causing unconditional UFA auxotrophy in *fabA*(Ts) PAO1.

Mutant	Gene with *mariner* (TnM) insertion	Function
# 6	*rpoN*::Tn*M*	σ^{54} transcription factor
# 16	*PA4760*::Tn*M*	Heat shock protein DnaJ
# 25	*PA1629*::Tn*M*	Probable enoyl-CoA hydratase/isomerase
# 30	*PA0358*::Tn*M*	Hypothetical protein
# 39	*PA3649*::Tn*M*	Hypothetical protein (63% similar to *E. coli yaeL*)
# 44	*PA4476*::Tn*M*	Hypothetical protein
# 75	*PA4233*::Tn*M*	Probable major facilitator superfamily (MFS) transporter
# 77	*PA0286* (*desA*)::Tn*M*	Fatty acid desaturase DesA

hydratase/isomerases are involved in fatty acid metabolism. These hydratase activities catalyze the hydratation of 2-*trans*-enoyl-CoA into 3-hydroxyacyl-CoA and the isomerase activities shift the 3-double bond of the intermediates of UFA oxidation to the 2-*trans* position.

To assess whether any given mutation generated by *mariner* transposon insertion directly affected *fabAB* expression, chromosomal DNA fragments of Gm^r auxotrophic integrants were transferred into the parental strain containing a chromosomally integrated *fabA'-lacZ* fusion and βGal activities were measured in cells grown in the presence of OA. These experiments showed that all transformants had similar levels of *fabA* expression when compared to the wild-type, indicating that the products of the mutated genes affected *fabAB* expression indirectly (data not shown). These data also indicated that a considerable number of genes and metabolic pathways seem to affect *fabAB* expression.

To directly screen for mutants affecting *fabA* transcription, the PAO1 *fabA*(Ts) strain containing a chromosomally integrated *fabA'-lacZ* fusion was used for *mariner*-based random mutagenesis. *mariner* insertion in an activator encoding gene would not only create an unconditional mutant requiring OA supplementation at all temperatures, but also result in lighter blue colonies caused by decreased *fabA'-lacZ* expression. As shown in Fig. 5.10., panel A, a significant decrease in βGal activities was observed in 14 mutants among the 62 OA auxotrophs that were identified in this screen. Nested PCR amplification and sequencing revealed that mutants #17, #18 and #43 contained *mariner* insertions in the *PA2222-PA2223* locus encoding hypothetical protein and hypothetical outer membrane protein (Fig. 5.10B). However, since the *PA2222-PA2223* operon could not be deleted, a further characterization of this operon was not pursued. However, for future studies transposon-generated *PA2222-PA2223*

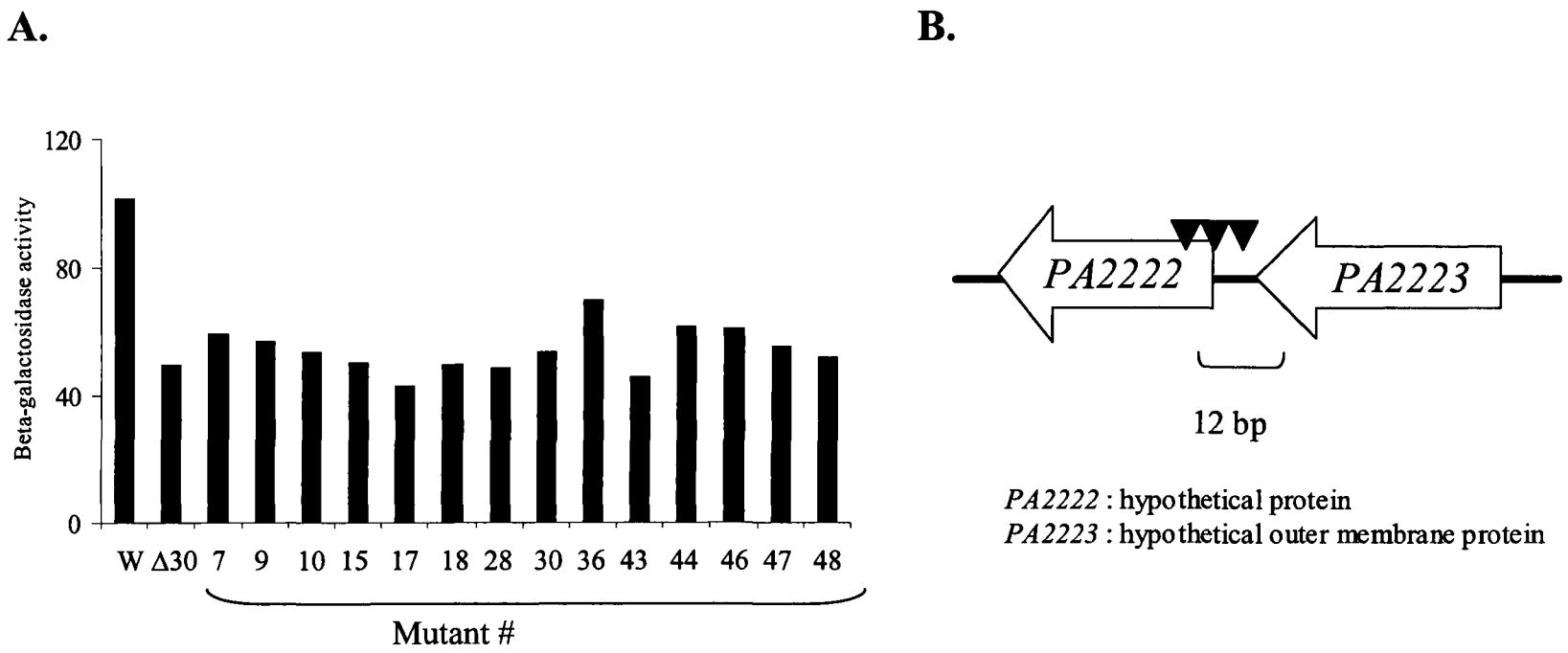
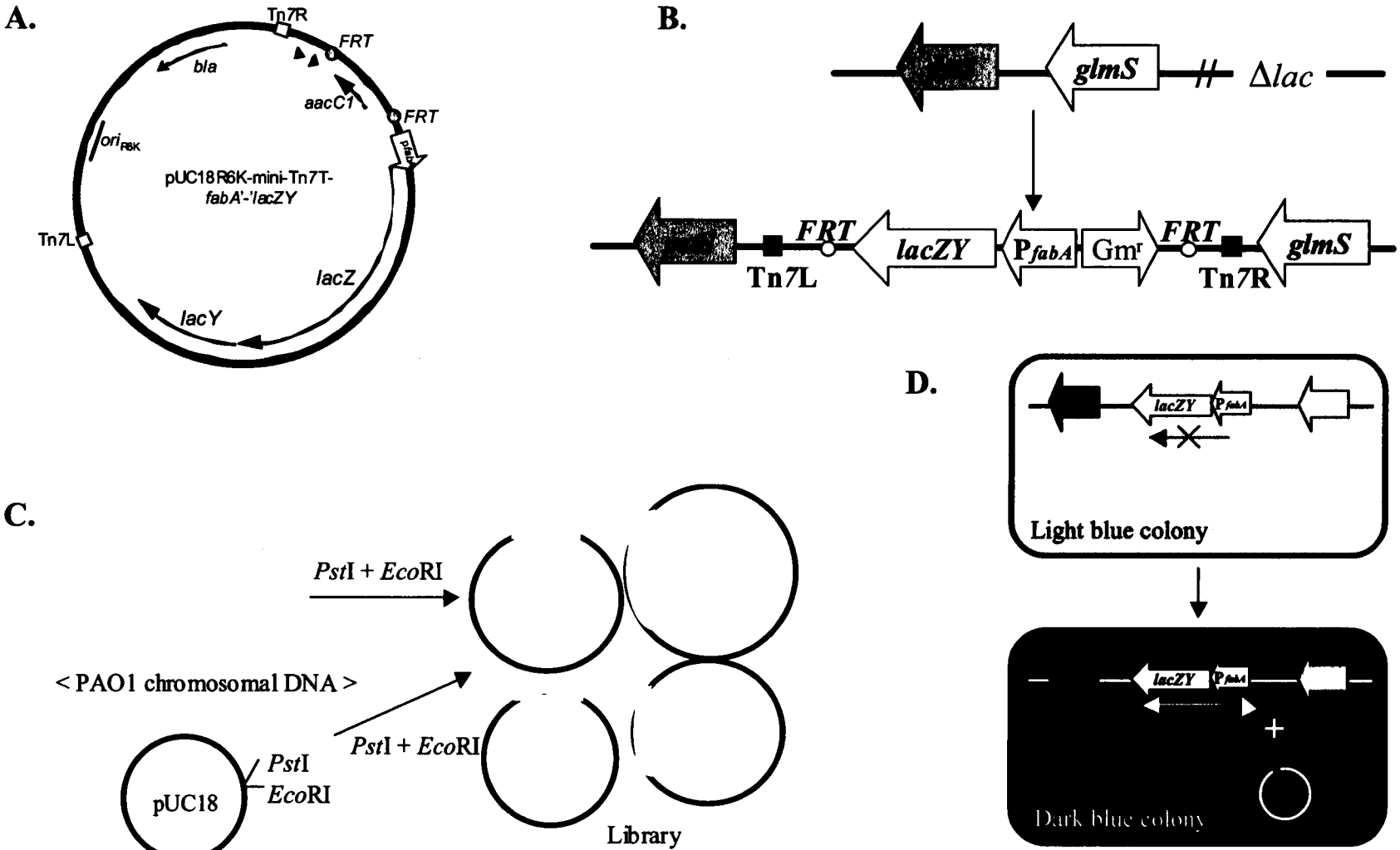


Fig. 5.10. Identification of the *PA2222-PA2223* putative *fabAB* regulatory genes by *mariner*-based random mutagenesis of PAO1-*fabA*(Ts) containing a chromosomally integrated *fabA'-lacZ* fusion. **A)** β–galactosidase activities in 14 mutants among 62 oleate auxotrophs grown in the presence of OA. Wild-type p*fabA'-lacZ* (W) and p*fabAΔ30'-lacZ* (Δ30) fusions were included for comparisons. **B)** Locations of *mariner* transposon insertions at the *PA2222-PA2223* locus in mutants #17, #18 and #43. The *PA2222* and *PA2223* genes are separated by a 12 bp intergenic region.

mutants could be employed to further study the role of these genes in *fabAB* regulation.

3) Use of a surrogate host strain to identify potential *fabAB* regulators. The complex regulation of *fabAB* operon expression in *P. aeruginosa* may hamper the identification of directly acting *fabAB* activator(s) by genetic means. To avoid this complexity and interference with other factors, an attempt was made to identify activating factors in a Δ*lac E. coli* strain containing a chromosomally-integrated *fabA'-'lacZY* translational fusion. This experimental approach is illustrated in Fig. 5.11. A genomic library of *P. aeruginosa* PAO1 was then constructed and transformed into the *E. coli* strain with the *fabA'-'lacZY* gene fusion. Potential activators would stimulate *fabA'-'lacZ* expression and transformants encoding such factors were therefore selected as dark blue colonies on X-Gal indicator medium. Linkage of this phenotype in transformants to plasmids encoding activating factors was confirmed by re-transforming the plasmids into the same *E. coli* host cells. Twenty-one isolates exhibited a blue colony phenotype. Restriction analyses of plasmids isolated from these transformants indicated the presence of the same *Pst*I-*Eco*RI fragment, but different flanking fragments. Besides the 2.9 kb fragment of pUC18 vector, the restriction enzymes released 0.4 kb, 1.2 kb, and 2.5 kb fragments. Among these, the 0.4 kb and 1.2 kb fragment were present in all plasmids, indicating that this region is responsible for activating *fabA* expression. These plasmids were classified into two groups and one plasmid from each group was selected for sequencing by using M13 forward and reverse primers. Two types of inserts were identified, those shown in the upper part of Fig. 5.12 (50%) and those shown in the lower part of the figure (50%). The only intact gene contained on both types of plasmids was *anr* (anaerobic NO_3^- regulator), which presumably activated *fabA'-*



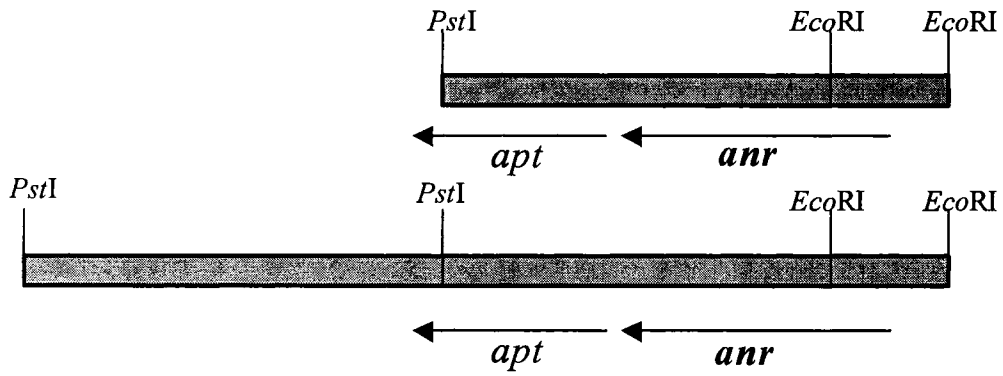


Fig. 5.12. DNA fragments present in plasmids that stimulate *fabA*'-'*lacZY* transcription in *E. coli*. The restriction maps shown are from chromosomal DNA fragments isolated from blue, putative activator-expressing colonies. Plasmids isolated from ten blue colonies were sequenced by using M13 forward and reverse primers to verify the presence of the indicated genes.

'lacZY transcription. The *anr* gene product, which senses low oxygen, supports anaerobic growth by activating numerous genes. *P. aeruginosa* is able to survive in an anaerobic environment. Since *P. aeruginosa* grows as a biofilm-type in the anaerobic CF lung mucus, anaerobic conditions may be an important factor to regulate metabolic pathways for robust growth and establishment of persistent infections. The anaerobic survival mode is supported by denitrification of nitrate or nitrite (Carlson et al., 1983, Davies et al., 1989, Zumft, 1997). In addition, the arginine deaminase (ADI) pathway plays a key role in catabolizing L-arginine to L-ornithine, with the formation of ATP from ADP, resulting in the growth of *P. aeruginosa* under anaerobic condition in the absence of terminal electron acceptors such as molecular oxygen or nitrate (reviewed in (Lu et al., 1999)). To assess a possible role of Anr in regulation of *fabAB* expression in *P. aeruginosa*, the *anr* gene was deleted from PAO1 containing a chromosomal p*fabA'-lacZ* fusion and βGal activities were measured in wild-type PAO1 and its Δ*anr* mutant. The presence of the *anr* deletion reduced *lacZ* expression only by ~25% compared to the wild-type (Fig. 5.13). In addition, this effect could only be complemented when anr^+ was present on the high-copy number plasmid pUCP20, but not on the low-copy number plasmid pVLT35, even with IPTG induction (Fig. 5.13). Since Anr globally regulates expression of many genes, *P. aeruginosa fabAB* regulation via Anr may be indirect via other Anr-dependent factors. An alternate explanation might be that Anr activity may be inhibited by another factor(s) which may be highly expressed under aerobic growth, but Anr expression from a high-copy number plasmid may be able to saturate this "anti-Anr" factor and thus complement the deletion mutant. Since Δ*anr* mutants are unable to grow anaerobically, additional experiments should be performed with cells grown under microaerophilic conditions to further assess the potential role of

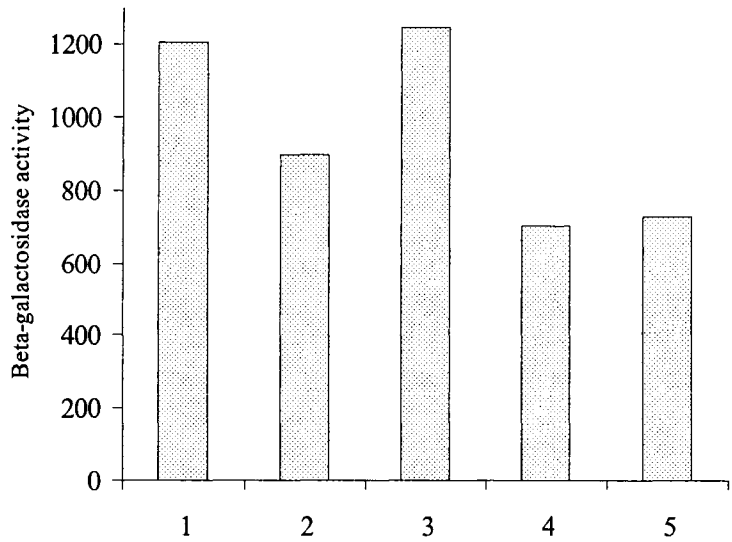


Fig. 5.13. Effects of *anr* on *fabA* expression in *P. aeruginosa*. β-galactosidase activities were measured in wild-type and Δ*anr* PAO mutants containing a chromosomally integrated p*fabA'-lacZ* fusion. Columns: 1, PAO1 (wild-type) ; 2, PAO1010 (PAO1 Δ*anr*); 3, PAO1010 with pPS1684 (pUCP20 with *anr*+); 4, PAO1010 with pPS1682 (pVLT35 with *anr*+) in the absence of IPTG; 5, PAO1010 with pPS1682 in the presence of IPTG.

Anr in *fabAB* gene expression. In conclusion, use of gene fusion technology revealed Anr as an activator of *fabA'-lacZ* expression in *E. coli*, but it played only a minor and probably indirect role in *P. aeruginosa.*

5.4.2.3 Biochemical attempts aimed at identification of *fabAB* regulatory proteins. Since previous experimentation suggested that the 30 bp sequence within the *fabA* upstream region has a regulatory function in *fabAB* expression, fragments containing this sequence were used for identification of DNA binding proteins. Unfortunately, none of the strategies was successful in identifying regulatory proteins, indicating that the experimental conditions employed in these studies were possibly not correct. As mentioned above, *fabA* expression is highly upregulated in biofilm-type grown cells, suggesting that anaerobic growth or biofilm-type growth might be proper conditions for identification of *fabAB* regulatory proteins. Although less likely, low abundancy but tight-binding DNA proteins may have been lost during extract preparation. This possibility could be eliminated in future experimentation by treating crude cell lysates with DNase or high salt buffer to dissociate proteins from DNA, and thus avoid possible sedimentation and loss in centrifugation steps.

Alternatively, *fabAB* expression may be regulated not via protein-binding, but via a yet-to-be discovered mechanism. One possibility lies in small regulatory RNA (srRNA)-mediated regulation of *fabAB* expression. srRNA can regulate gene expression at the transcriptional and translational levels. Intracellular metabolites such as amino acids, sugars and nucleotides can bind to *cis*-acting metabolite-sensing regulatory RNA elements and control gene expression, which are called riboswitches (reviewed in (Cromie et al., 2006)). Various types of riboswitches are present in bacteria. Generally, non-coding regulatory RNA elements are found in the 5'-

untranslated region (5'-UTR) and can give rise to three-dimensional conformational alternate changes in response to changes intracellular metabolite signals. Recently, it was found that besides metabolites, metals such as intracellular Mg^{2+} can regulate the Mg^{2+} transporter MgtA of *Salmonella enterica* serovar Typhimurium by the metal-sensing 5'-UTR of the *mgtA* gene (Cromie et al., 2006). Furthermore, changes in environmental conditions such as temperature can result in conformational change of regulatory RNA, thus functioning as a "thermometer". For example, virulence genes are highly expressed at 37°C by an PrfA transcriptional activator, which is thermally regulated by the 5'UTR of mRNAs of *prfA* in *Listeria monocytogenes* (Johansson et al., 2002). In addition, the ROSE (repressor of heat-shock gene expression) element placed in the 5'UTR region of heat-shock genes in many Gram-negative bacteria senses temperature changes in order to control their gene expression (Chowdhury et al., 2006). *E. coli rpoS* mRNA translation is regulated by the DsrA small RNA which disrupts a sequestering helix of a ribosome binding site during temperature downshift (Repoila et al., 2003).

P. aeruginosa may employ a regulatory RNA for controlling anaerobic UFA synthesis. One probable pathway is that a metabolite-sensing non-coding RNA may recognize intracellular or exogenous UFA and change its structure, resulting in *fabAB* gene repression. This is consistent with the observation that OA supplementation repressed *fabA* expression up to 50% (Fig. 5.5). Another possible explanation is that low growth temperature might be an important controlling factor. Since cells should maintain the membrane fluidity for normal function required for transport and movement, they adapt to changes in environmental conditions, especially temperature. Upon exposure to low temperatures, cell membranes become solid-state, but they preserve their fluidity by increasing UFA levels. As shown in Fig. 3.8, *fabA*

expression is highly up-regulated in cells grown at 16°C compared to cells grown at at 37°C, suggesting positive regulation by low temperature. Therefore, the 5'-UTR of *fabA* may sense low temperature and coordinate an adaptation process caused by a temperature downshift. However, having said all of this, searches of the *fabAB* upstream sequences have yet to reveal possible srRNAs.

5.5 CONCLUSIONS

Previous work demonstrated that UFA biosynthesis in *P. aeruginosa* is governed by two pathways, depending on the oxygen availability: 1) two aerobic fatty acid desaturase pathways consisting of DesA and DesBC, and 2) the anaerobic FabAB pathway. The *fabAB*-encoded proteins play an essential and dominant role in UFA biosynthesis under both aerobic and anaerobic conditions. It was previously shown that the expression of this operon was up-regulated in biofilms and repressed by exogenous UFAs, in particular OA, demonstrating the existence of *fabAB* regulation. In addition, the discovery of a 30 nt *fabA* upstream sequence, which is conserved in three *Pseudomonas* spp., suggests positive regulation by an unknown regulatory mechanism. RT-PCR analyses indicated that the main *fabAB* promoter may lie far upstream of the *fabAB* operon. This probably explains why identification of transcription start sites using primers with binding sites near the 5' end of *fabA* failed in primer extension experiments. Therefore, precise localization of the *fabA* promoter will necessitate alternative methods such as Northern blot analyses or RNase protection assays. In chapter 4, *PA4890* encoding the DesT repressor was shown to function as a repressor of *desBC* operon expression (Zhu et al., 2006). Two lines of evidence suggest a role, albeit modest, of DesT in *fabAB* operon expression: 1) DesT was shown to bind to its putative binding site located upstream of *fabA*

(Yong-Mei Zhang, unpublished data); and 2) *fabA* transcription was less repressible in a Δ*desT* mutant in the presence of OA. However, in microarray experiments a *desT* mutant did not show any significant changes in *fabAB* expression (Zhu et al., 2006).

The identification of a *fabAB* activator was attempted using various genetic and biochemical approaches. However, neither of the suspected transcriptional regulators played a role in *fabAB* regulation. In addition, generation of unconditional *fabA* mutants by *mariner*-based random mutagenesis demonstrated that the genes identified in this screen all seemed to play indirect roles in *fabAB* regulation. Furthermore, although use of gene fusion technology in *E. coli* revealed *anr* as a weak *fabAB* activator, follow up experiments showed that Anr does not directly control *fabAB* expression in *P. aeruginosa*. Moreover, failure of regulatory protein identification by biochemical means using the conserved 30 bp sequence as a bait may tell us that we should not exclude the possibility that *fabAB* expression is regulated not via protein-binding, but via unknown mechanism such as a riboregulatory element.

5.6 ACKNOWLEDGMENTS

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CHAPTER 6

6.1 CONCLUSIONS AND FUTURE DIRECTIONS

The completion of genome sequences for many bacteria now facilitates high-throughput functional genomic studies, including DNA microarray and proteomics experiments. By comparison, the genetic methods required for the manipulation of bacterial genomes are much less well developed and, for the most part, not amenable to high-throughput. Furthermore, many of the bacteria for which genome sequences are available are recalcitrant to genetic manipulation and/or live in environments where traditional, plasmid-based methods are not applicable because of the requirement for continued antibiotic selection. I therefore wanted to develop a system that is very efficient, easy to use and applicable to many different bacteria and environments. Because they enable chromosomal insertion without the need for continued antibiotic selection, transposon-based systems have been used in the past for such purposes. However, with the exception of a few rather narrow-host-range systems, most gene delivery methods rely on transposons that generate random insertions, most often within coding sequences. To avoid the many potential problems associated with random insertions, as detailed in chapter 2, a Tn*7*-based gene integration system was developed. Unlike most other transposons, Tn*7* inserts site-specifically downstream of the essential *glmS* gene at a naturally evolved *att*Tn*7* site. Insertion events, therefore, have little or no effect on bacterial fitness and can be reproduced in many different genetic backgrounds and even across species boundaries. Since all bacteria possess a *glmS* gene, *att*Tn*7* sites are conserved in all bacteria and Tn*7* transposition has subsequently been demonstrated not only in *P. aeruginosa*

(Choi et al., 2005, 2006a), but also in *Yersinia pestis*, *Burkholderia thailandensis*, *B. mallei* (Choi et al., 2006b), *B. pseudomallei* (Ifor Beacham, Griffin University, Gold Coast, Australia, pers. commun.) and *Proteus mirabilis* (Choi et al., 2000c).

Rapid Tn*7* delivery was made possible by development of a rapid, yet highly efficient transformation procedure, which can also be used for delivery of other DNA elements, such as replicative and non-replicative plasmids, and other suicide transposon-delivery vectors (chapter 3). The efficiency of the transformation procedure, combined with Gateway cloning, also allowed establishment of a high-throughput, cost-effective gene deletion method. In this study, I used this method to delete the structural genes for 25 unidentified transcriptional regulators of GntR family in *P. aeruginosa* (chapter 3), as well as many other genes.

Using these improved methods, studies were initiated to characterize the pathways for and genetic regulation of UFA synthesis in *P. aeruginosa*. These studies established that, unlike any other bacterium studied to date, *P. aeruginosa* possesses a combination of aerobic and anaerobic pathways for UFA synthesis (chapter 4). The two aerobic pathways are catalyzed by the DesA and DesBC enzyme systems, which are oxygen-dependent fatty acyl desaturases normally found in *Bacillus spp.*, mycobacteria, yeasts and plants. Although the two desaturase systems share many commonalities such as being oxygen-dependent, membrane-bound and containing conserved histidine clusters, they utilize different substrates. While DesA introduces a double bond into a fatty acyl group placed in the *sn*-2 position of membrane phospholipids, DesB desaturates saturated fatty acyl-CoAs derived from fatty acid transport and metabolism with the aid of the DesC oxidoreductase, which presumably channels electrons from the electron transport chain. The expression of the oxygen-dependent *desBC* operon is negatively regulated by a repressor encoded

by the upstream *desT* gene. Anaerobic UFA synthesis requires the FabA and FabB proteins, which are responsible for maintaining the double bond in a growing fatty acyl chain produced by a type II fatty acid biosynthetic pathway and elongating the resulting UFA by two-carbons every synthesis cycle until a full-length UFA is synthesized. It was suggested that UFAs are produced predominantly by this mechanism under both aerobic and anaerobic conditions. While DesA supports UFA production in the absence of FabA, DesBC is not able to introduce the double bond either into fatty acyl groups of phospholipids or acyl-ACPs, resulting in cell death unless exogenous saturated fatty acids are present.

While the existence of and the mechanisms of UFA synthesis via the anaerobic pathway were known at the onset of the studies presented in this dissertation, little was known about the regulation of expression of the *fabA* and *fabB* genes. It was known that, unlike *E. coli* where *fabA* and *fabB* map to two distinct locations on the chromosome, these two genes form an operon in *P. aeruginosa*. It was also known that expression of this operon was up-regulated in biofilm-grown cells and repressed by addition of fatty acids, especially oleic acid, to the growth medium. While the studies presented in chapter 5 did not reveal any specific regulatory protein(s) involved in regulation of *fabAB* operon expression or the exact molecular mechanisms governing regulation of expression of this operon, they revealed some previously unknown findings. First, RT-PCR analyses indicated that the *fabAB* operon is transcribed from at least two promoters. It is co-transcribed with the upstream, seemingly unrelated *PA1612-PA1611* operon, but an additional promoter located in the *PA1611-fabA* intergenic region which also contributes to *fabAB* expression. Second, the DesT (FabR) repressor binds to a palindromic sequence in the *PA1611-fabA* intergenic region, but seems to play only a modest role

in *fabAB* operon expression. Third, a 30 bp sequence present in the *PA1611-fabA* intergenic region is a regulatory element involving the positive regulation of *fabA*. The position of this sequence at an appropriate position upstream of a putative -10 promoter consensus sequence indicates that it may be an activator-binding site. Fourth, while *E. coli* FadR belongs to the GntR family of transcriptional regulators, deletion of none of the 25 genes encoding the GntR family of regulators in *P. aeruginosa* affected *fabA* transcription. Therefore, the *P. aeruginosa* FadR-like activator, if it exists, must belong to a different family of regulators. Additionally, deletion of the immediate upstream gene *PA1611* encoding a hybrid sensor kinase/response regulator protein did not adversely affect *fabA* transcription, at least not under the conditions employed in the present studies. Fifth, numerous other cellular factors including RpoN, DesA and Anr seem to play at least minor roles in regulation of *fabAB* transcription, possibly through modulation of intracellular fatty acid levels or other metabolites. Sixth, DINAMelt analysis (http://www.bioinfo.rpi.edu/applications/hybrid/hybrid2.php) of the *fabA* 5' untranslated region (UTR) revealed that this region can assume a number of stable secondary structures. Two of these structures are shown in Fig. 6.1. These structures exhibit some of the criteria of riboregulatory elements (Winkler et al., 2005): i) they are found in the 5' UTR; ii) they contain a number of stem-loop structures, one of which is conserved for ligand binding and the others are variable. It is noteworthy that the structure shown in Fig. 6.1A contains a stem-loop followed by a stretch of U residues, a situation found in typical *rho*-independent transcriptional terminators. This stem-loop structure is not present in the second structure shown in Fig. 6.1B.

While none of these findings does yet provide a clear picture of the molecular mechanisms governing transcription of the *fabAB* operon, they indicate that regulation

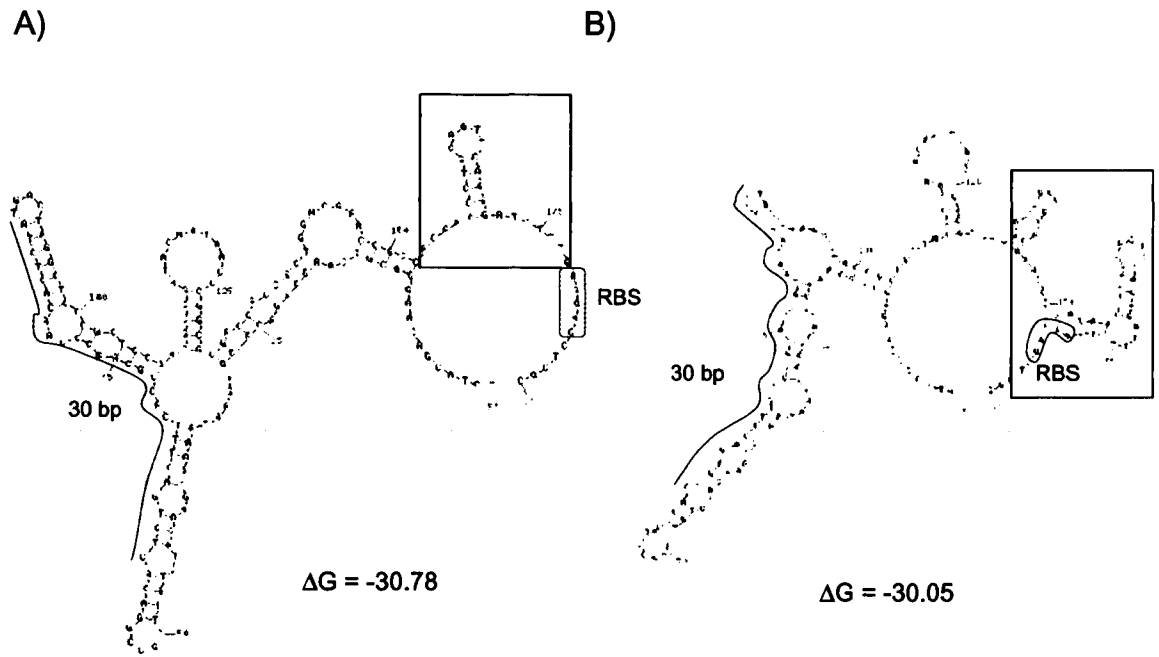


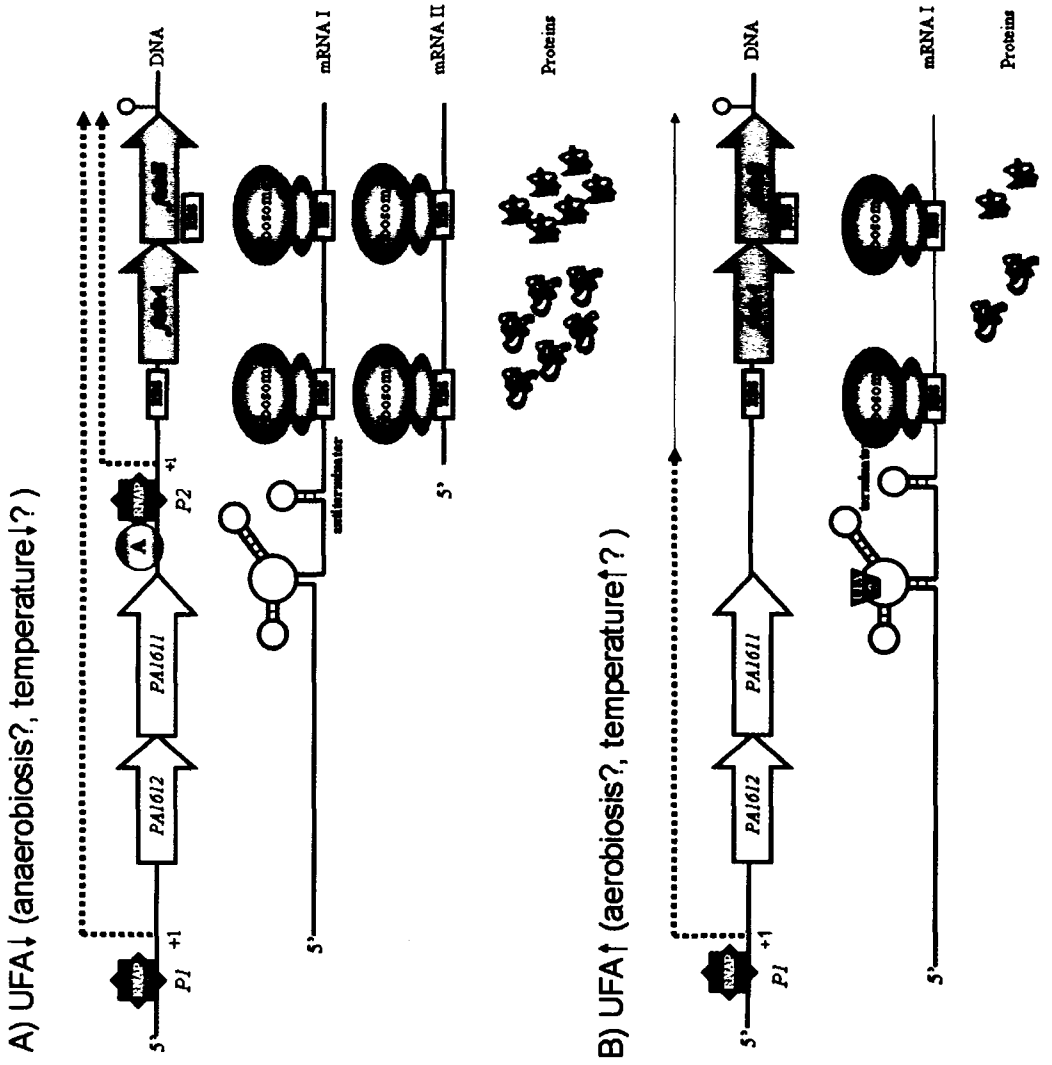
Fig. 6.1. Possible RNA secondary structures formed on transcripts containing the *PA1611-fabA* intergenic region. DINAMelt analysis (http://www.bioinfo.rpi.edu/applications/hybrid/hybrid2.php) suggests the possible formation of two principal RNA structures by the 5' UTR *fabA* mRNA. The structure in **A)** contains a stem-loop followed by a stretch of T's which is the signature of a *rho*-independent transcriptional terminator (boxed area). This stem-loop is missing from the structure shown in **B)** where it is replaced by a number of other stem-loop structures that could possibly function as antiterminator elements. In both structures, the sequence encompassing the 30 bp is marked with a red line and the putative *fabA* ribosome-binding site (RBS) is marked with a green box.

of *P. aeruginosa fabAB* operon expression is very complex and most likely quite different from what has been described in *E. coli*. A working model summarizing my findings is presented in Fig. 6.2.

As mentioned above, the *fabAB* operon is transcribed from at least two promoters, *P1* and *P2*, resulting in transcription of two mRNAs, mRNA I and mRNA II. In the absence of exogenous UFAs and under anaerobic, as well as perhaps low temperature conditions, the *fabAB* operon is transcribed from both of these promoters and the respective transcripts terminate at a single transcriptional terminator which is located immediately downstream of the *fabAB* operon. Transcription from *P2* requires an activator protein which binds to the 30 bp region. Maximal transcription and translation ensures an adequate supply of FabA and FabB proteins for UFA synthesis. At the mRNA level, this situation is characterized by a riboregulatory element configuration on mRNA I containing an antitermination element.

In the presence of exogenous UFAs and/or aerobic or high (37°C) temperature conditions, the proposed activator probably binds an UFA-CoA ligand and the activator-UFA-CoA complex dissociates from the 30 bp activation element resulting in loss of *fabAB* transcription from *P2*. At the mRNA level, this situation is characterized by a riboregulatory element configuration that contains a terminator. The result is low level transcription and translation of *fabA* and *fabB*, a desirable situation when oxygen levels and temperatures are high, exogenous UFAs are available and overall cellular demand for UFAs is either low or can be satisfied by the activities of the aerobic available and the desaturase pathways.

While many of my experimental results support this model, several questions do remain unanswered. For example, why were repeated genetic and biochemical attempts aimed at identification of the proposed activator protein unsuccessful? Why



does purified DesT bind to the *PA1611-fabA* intergenic region, but there is little to no effect on *fabA* expression in *lacZ* fusion or microarray experiments? A possible explanation for this may be that most of the experiments described in this dissertation were performed with cells grown aerobically and at 37°C, possibly conditions during which synthesis and/or activity of an activator protein is repressed or DesT activity is masked. This notion is supported by the fact that *fabA'-lacZ* expression was significantly higher in cells grown at 16°C when compared to cells grown at 37°C. Future experiments should therefore be performed with cells grown under anaerobic or microaerophilic conditions, as well as with cells grown at lower temperatures. Although DINAMelt analysis suggests the possibility that a riboregulatory element may be involved in regulation of *fabAB* operon expression, there is currently no experimental evidence supporting this possibility.

Future efforts should therefore include experiments that would address the existence of various mRNA species and a 5' UTR RNA with a high degree of secondary structure. First, the existence of the mRNA species illustrated in Fig. 6.2 should be demonstrated in Northern blots. Second, site specific mutagenesis should be employed to change residues critical for formation of the stem-loop structures. If these experiments indicate the existence of the proposed mRNA species and show an effect on *fabAB* operon expression, additional experimentaion aimed a specifically demonstrating conformational changes in the mRNA secondary structures would be warranted. Such experiments would entail in-line probing (Soukup et al., 1999, Winkler et al., 2002) and RNase H cleavage assays of RNA-DNA hybrids (Fuchs et al., 2006) in the presence and absence of substrate to determine if the structure not only exists, but also undergoes a conformational change upon addition of substrate or changes in an environmental cue. The nature of the substrate(s) or environmental

cue(s), of course, remains speculative. Substrates could be metabolites such as a UFA-CoAs and environmental cues signals such as low temperature or low oxygen levels.

Studies on anaerobic UFA synthesis by the *P. aeruginosa* FabAB pathway and identification of its regulatory signals are significant because many infections caused by this bacterium, especially cystic fibrosis lung infections, are biofilm-type infections during which this bacterium's physiology is adapted to an anaerobic lifestyle. Such studies have therefore the potential for discovering new targets and drugs for treatment of biofilm-type infections.

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