DISSERTATION

AUTOINDUCER-2-BASED QUORUM SENSING OF FOODBORNE PATHOGENIC BACTERIA UNDER FOOD RELATED CONDITIONS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2006

COLORADO STATE UNIVERSITY

September 7, 2006

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY YOHAN YOON ENTITLED AUTOINDUCER-2-BASED QUORUM SENSING OF FOODBORNE PATHOGENIC BACTERIA UNDER FOOD RELATED CONDITIONS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

AUTOINDUCER-2-BASED QUORUM SENSING OF FOODBORNE PATHOGENIC BACTERIA UNDER FOOD RELATED CONDITIONS

The objective of these studies was to evaluate potential involvement of autoinducer (AI)-2-based quorum sensing (quorum sensing: cell density dependent cell-to-cell signaling) of foodborne pathogenic bacteria in food environments. Under the conditions of these studies, AI-2 activity of *Salmonella* and *Escherichia coli* O157:H7 was not associated with rate of bacterial growth. Production of AI-2 was increased in the presence of glucose (0.5%), and AI-2 activity of *E. coli* O157:H7 was not very high in beef purge containing high levels of natural flora. *Salmonella* and *E. coli* O157:H7 did not form more ($P \geq 0.05$) biofilm on food contact surfaces in the presence of AI-2 activity compared to absence of AI-2 activity, suggesting that AI-2-based quorum sensing may not be involved in biofilm formation by *Salmonella* and *E. coli* O157:H7 on food contact surfaces. AI-2-like activity increased ($P<0.05$) in fresh beef samples inoculated with *E. coli* O157:H7, containing lower levels of natural flora, and stored at higher temperature (25°C) and under aerobic conditions. Under heat (55°C) and acid stress (pH 3.0 or 3.5), surviving cell counts of *Salmonella* were not different ($P \geq 0.05$) in the presence and absence of AI-2 activity, indicating that AI-2-based quorum sensing of *Salmonella* may not be involved in heat and acid stress response. Surviving cell counts of *E. coli* O157:H7 were higher ($P<0.05$) in the presence than in the absence of AI-2 activity under both heat (52 or 55°C) and acid

(pH 3.0) stress, indicating that AI-2-based quorum sensing of *E. coli* O157:H7 may be partially involved in mechanisms of heat and acid resistance. The results of these studies may be potentially helpful in developing novel approaches for improving food safety, while research on quorum sensing should be continued with the goal of finding targets for pathogen control in foods.

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ACKNOWLEDGEMENTS

"The Lord is my shepherd, I shall not want; he makes me lie down in green pastures. He leads me besides still waters; he restores my soul. He leads me in paths of righteousness for his name's sake. Even though I walk through the valley of the shadow of death, I fear no evil; for thou art with me; thy rod and thy staff, they comfort me" Psalms 23:1-4.

I would like to express my deepest appreciation to those individuals who contributed to this research effort. To advisor Dr. John Sofos, I really appreciate his immense patience and helpful guidance. To co-advisor Dr. Patricia Kendall, and my committee members Drs. Robert Ellis and John Scanga, I really appreciate your help and guidance. This research project would not have been possible without their patience, professionalism and guidance. Also I would like to express my appreciation to the research associate Dr. Ifigenia Geornaras for her friendship, patience and professionalism, and to all of the laboratory staff, graduate students and post-doctoral researchers at the Center for Red Meat Safety who were so generous with their help, advice and friendship. To my grandmother, father, mother, and brother, I really appreciate for their praying for me at every dawn, and encouragement. I can never thank you enough and realize how much they love me. Finally, to my wife Kyoung-Hee Choi, I appreciate so much her limitless support, encouragement, and prayers. I am so glad you are my wife. Again, I would like say that this degree of doctor of philosophy would not be possible without those individuals who are listed above.

"The Lord is your keeper; the Lord is your shade on your right hand. The sun shall not smite you by day, nor the moon by night. The Lord will keep you from all evil;

he will keep your life. The Lord will keep your going out and your coming in from this time forth and for evermore" Psalms 121:5-8.

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CHAPTER I

INTRODUCTION

Various bacteria possess cell-to-cell communication systems, called quorum sensing, which are cell density-dependent and use low-molecular-weight signaling compounds (autoinducers) (Smith et al., 2004). Bacteria sense concentrations of the autoinducers and change gene expressions (Waters and Bassler, 2005). Quorum sensing has been observed in various bacterial groups, and quorum sensing molecules have been categorized into at least four groups (reviewed by Smith et al., 2004): (i) *N*-acylhomoserine lactone (AHL) produced by Gram-negative bacteria mostly for interspecies communication (Miller and Bassler, 2001; Whitehead et al., 2001); (ii) a furanosyl borate diester [autoinducer (AI)-2] formed by both Gram-negative and Gram-positive bacteria for intraspecies and interspecies communication (Chen et al., 2002; Schauder and Bassler, 2001; Xavier and Bassler, 2005a); (iii) AI-3 (unknown structure) for cross-communication with mammalian epinephrine cells of host signaling system (Sperandio et al., 2003); and, (iv) oligopeptides in only Gram positive bacteria (Miller and Bassler, 2001; Whitehead et al., 2001). In addition, there are other existing quorum sensing molecules which are found in only few organisms, including diketopiperazines (Degrassi et al., 2002; Holden et al., 1999) and 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal) (Holden et al., 2000; Pesci et al., 1999) in bacteria, and 3,7,11-trimethyl-2,6,10-dodecatrienoate (farnesoic acid)

(Oh et al., 2001), farnesol (Hornby et al., 2001) and tyrosol (Chen et al., 2004) in yeast.

Quorum sensing of bacteria may be involved in virulence (von Bodman et al., 1998), plasmid conjugation (Lithgow et al., 2001), bioluminescence (Cao and Meighen, 1989), protein production (DeLisa et al., 2001c), and biofilm formation (Davies et al., 1998). Based on these observations, quorum sensing of bacteria which survive in food environments has been presented as a potential novel target to improve food safety, and especially food spoilage. Jay et al. (2003) suggested that quorum sensing of dominant bacteria may induce spoilage and biofilm formation in fresh meat at low storage temperature (5-7°C). Christensen et al. (2003) and Rasch et al. (2005) reported that quorum sensing may be involved in spoilage of various foods.

The quorum sensing system of *Pseudomonas aeruginosa* has been reported as involved in the regulation of its pathogenic mechanisms such as expression of virulence factors, biofilm formation, adhesion (Singh et al., 2000; Smith and Iglewski, 2003), and twitching and swarming motility (Glessner et al., 1999; Kohler et al., 2000) in the lung. It was found that some bacteria use quorum sensing for competence development (the ability to internalize exogenous DNA). *Bacillus subtilis* requires a transcription factor ComK (Hahn et al., 1996; van Sinderen et al., 1995), and a small protein ComS (D'Souza et al., 1994; Hamoen et al., 1995), which are components of quorum sensing system for competence development. *Streptococcus* spp. also use quorum sensing to induce competence development (Li et al., 2001a, b; Peterson et al., 2004a, b; Steinmoen et al., 2002). *Agrobacterium tumefaciens* uses AHL, a quorum sensing signal, to regulate a conjugal transfer of Ti plasmid and other Ti plasmid-borne genes among bacteria (Piper et al., 1993). In addition, *Erwinia chrysanthemi* regulates the global system of virulence by AHL (Pirhonen et al., 1993).

Major Gram-negative foodborne pathogenic bacteria such as *Escherichia coli* O157:H7, *Salmonella*, and *Campylobacter* spp. have been reported to synthesize the AI-2 molecule (Cloak et al., 2002; Elvers and Park, 2002; Surette and Bassler, 1998). Because the AI-2 molecule can be used for gene expressions in various Gram-negative and Gram-positive bacteria, it is designated as a universal autoinducer (Lu et al., 2004). In AI-2 biosynthesis, AI-2 is derived from *S*-adenosylmethionine via three enzymatic steps (Chen et al., 2002). Factors promoting AI-2 production in *E. coli* and *Salmonella* Typhimurium include rapid logarithmic growth, presence of a preferred carbon source such as glucose, low pH, and high osmolarity (Surette and Bassler, 1999). *S.* Typhimurium, *E. coli* O157:H7, and *Campylobacter* spp. were found to synthesize the AI-2 molecule in foods such as milk, chicken broth, and brucella broth at different storage temperatures (Cloak et al., 2002). AI-2-like activity was observed in fresh and processed foods such as frozen fish, tomatoes, cantaloupe, carrots, tofu, and milk (Lu et al., 2004). Whereas other products, including turkey patties, chicken breast, homemade cheeses, beef steak, and beef patties appeared to possess an inhibitory effect against AI-2 production. Food additives such as sodium propionate, sodium benzoate, and sodium acetate have inhibitory activity against AI-2 production by bacteria (Lu et al., 2004). Novak and Fratamico (2004) reported that sodium ascorbate and a nonacidic salt of ascorbic acid also inhibited AI-2 activity.

Based on the results of many studies, it has been shown that quorum sensing is signaling by small molecules among bacteria, and it is feasible that autoinducers may be used for regulation of bacterial growth, survival, and virulence in food environments (Cloak et al., 2002). Although major foodborne pathogenic bacteria possess quorum sensing systems which produce AI-2 or AI-3 molecules (Cloak et al., 2002; Elvers and Park, 2002, Kaper and Sperandio, 2005; Sperandio et al., 2003;

Surette et al., 1999), the role of their quorum sensing and the factors affecting quorum sensing molecule production have not been studied in complex food environments. Therefore, it is important to examine the potential role of quorum sensing in major foodborne pathogenic bacteria and the environmental factors that may affect the production of quorum sensing molecules by important bacteria in food environments or related conditions.

OBJECTIVES OF DISSERTATION

The objectives of this dissertation were as follows:

1. To compare growth and AI-2 production by wild-type and mutant strains of *Salmonella* and *E. coli* O157:H7, and to evaluate whether AI-2-based quorum sensing of *Salmonella* and *E. coli* O157:H7 influences their growth.
2. To find optimum incubation time for preparation of preconditioned (PC) media.
3. To evaluate whether *E. coli* O157:H7 can produce AI-2 in liquid food environments such as beef purge.
4. To evaluate whether AI-2 activity is involved in biofilm formation on food contact surfaces by *Salmonella* and *E. coli* O157:H7.
5. To determine whether AI-2-based quorum sensing of *E. coli* O157:H7 can be active in fresh beef, and to examine factors that may affect AI-2-like activity in *E. coli* O157:H7 inoculated beef.
6. To evaluate the potential relationship between AI-2 activity and resistance of *Salmonella* to heat and acid.
7. To evaluate whether AI-2-based quorum sensing may be involved in the heat and acid resistance mechanisms of *E. coli* O157:H7.

CHAPTER II

LITERATURE REVIEW

INTRODUCTION

Various bacterial groups possess cell-to-cell, density-dependent, signaling systems, termed quorum sensing, which use low-molecular-weight signaling compounds, called autoinducers or pheromones (Smith et al., 2004). Bacteria sense concentrations of these low-molecular-weight signaling molecules and change gene expressions (Waters and Bassler, 2005). Interestingly, pathogenic bacteria do not use quorum sensing at low levels of cell density in order to avoid detection by the host immune system; however, as they reach a sufficient level to overcome the host immune system, they use quorum sensing (Bauer and Robinson, 2002). Quorum sensing was first found in *Vibrio harveyi* and *Vibrio fischeri*, which are bioluminescent marine bacteria. Initially, most quorum sensing research focused on *V. fischeri*, which usually colonizes a variety of fishes and squids to produce light (Nealson and Hastings, 1979; Ruby, 1996). Besides the quorum sensing system in *Vibrio* spp., quorum sensing systems were found in *Pseudomonas aeruginosa*, *Erwinia carotovora*, and *Agrobacterium tumefaciens* (Bainton et al., 1992; Fuqua et al., 1994; Gambello and Iglewski, 1991; Jones et al., 1993; Pearson et al., 1994; Piper et al., 1993; Zhang et al., 1993).

After quorum sensing was found to be present in various bacteria, many scientists have confirmed the existence of quorum sensing molecules and such molecules have been categorized into four groups (reviewed by Smith et al., 2004): (i) *N*-acylhomoserine lactone (AHL; autoinducer [AI]-1) used primarily for interspecies communication in Gram-negative bacteria (Miller and Bassler, 2001; Whitehead et al., 2001). The molecules differ in their acyl side chain moieties and have homoserine lactone in common (Bassler, 1999; de Kievit and Iglewski, 2000); (ii) a furanosyl borate diester, called AI-2, for intraspecies and interspecies communication in both Gram-negative and Gram-positive bacteria (Chen et al., 2002; Schauder and Bassler, 2001; Xavier and Bassler, 2005a); (iii) an unknown structure, called AI-3, involved in cross-communication with the mammalian epinephrine cells of host signaling system (Sperandio et al., 2003); and, (iv) oligopeptides present in only Gram-positive bacteria (Miller and Bassler, 2001; Whitehead et al., 2001). Other quorum sensing molecules include diketopiperazines (DKPs) (Degrassi et al., 2002; Holden et al., 1999) and 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal; PQS) (Holden et al., 2000; Pesci et al., 1999) in bacteria. In yeast, 3,7,11-trimethyl-2,6,10-dodecatrienoate (farnesoic acid) (Oh et al., 2001), farnesol (Hornby et al., 2001), and tyrosol (Chen et al., 2004) have been identified as quorum sensing molecules.

Since existence of quorum sensing was first observed (Eberhard, 1972; Greenberg et al., 1979; Nealson, 1977; Nealson and Hastings, 1979), many scientists have studied roles of quorum sensing in bacteria. Many studies have shown that bacteria may control virulence (von Bodman et al., 1998), plasmid conjugation (Lithgow et al., 2001), bioluminescence (Cao and Meighen, 1989), protein production (DeLisa et al., 2001c), and biofilm formation (Davies et al., 1998) using quorum sensing. Recently, food microbiologists have started studying quorum sensing as a

novel approach that may be useful in improving food safety, and especially controlling food spoilage. Jay et al. (2003) found that spoilage and biofilm formation in fresh meat may be related to quorum sensing of dominant bacteria at low storage temperature (5-7°C). In addition, Christensen et al. (2003) reported that quorum sensing may play a role in milk spoilage. Rasch et al. (2005) also reported that bacterial spoilage of some foods may be caused by quorum sensing.

P. aeruginosa uses a quorum sensing system to regulate its pathogenesis mechanism through virulence factor expression, biofilm formation, adhesion (Singh et al., 2000; Smith and Iglewski, 2003), and twitching and swarming motility (Glessner et al., 1999; Kohler et al., 2000) in the lung. *A. tumefaciens* regulates the conjugal transfer of the tumor-inducing (Ti) plasmid and other Ti plasmid-borne genes between bacteria via AHL (Piper et al., 1993). In addition, *Erwinia chrysanthemi* controls the global system of virulence by AHL (Pirhonen et al., 1993). To develop competence (the ability to internalize exogenous DNA), *Bacillus subtilis* requires a transcription factor ComK (Hahn et al., 1996; van Sinderen et al., 1995), and a small protein, ComS (D'Souza et al., 1994; Hamoen et al., 1995), which are components of its quorum sensing system. *Streptococcus* spp. also use quorum sensing to internalize exogenous DNA (Li et al., 2001a, b; Peterson et al., 2004a, b; Steinmoen et al., 2002).

Major Gram-negative foodborne pathogenic bacteria such as *Escherichia coli* O157:H7, *Salmonella*, and *Campylobacter* spp. produce AI-2 (Cloak et al., 2002; Elvers and Park, 2002; Surette and Bassler, 1998). AI-2 is recognized as a universal autoinducer because it has been reported as involved in gene expression in various Gram-negative and Gram-positive bacteria (Lu et al., 2004). AI-2 production is influenced by various factors; in *E. coli* and *Salmonella* Typhimurium, rapid logarithmic growth, preferred carbon source (glucose), low pH, and high osmolarity

increase AI-2 production (Surette and Bassler, 1999). Furthermore, *S.* Typhimurium, *E. coli* O157:H7, and *Campylobacter* spp. were found to produce AI-2 in foods such as milk, chicken broth, and brucella broth at different storage temperatures (Cloak et al., 2002). Interestingly, Lu et al. (2004) showed that some fresh and processed foods, such as frozen fish, tomatoes, cantaloupe, carrots, tofu, and milk, also showed natural AI-2-like activity. However, other products including turkey patties, chicken breast, homemade cheese, beef steak, and beef patties showed an inhibitory effect against AI-2 production (Lu et al., 2004). Interestingly, food additives, such as sodium propionate, sodium benzoate and sodium acetate, also have inhibitory activity against AI-2 in bacteria (Lu et al., 2004). Sodium ascorbate and a nonacidic salt of ascorbic acid also inhibited AI-2 activity (Novak and Fratamico, 2004).

In general, quorum sensing has been shown to be associated with small signaling molecules being transferred among bacteria. Thus, it is feasible that autoinducers may be involved in bacterial growth and survival in food environments, and virulence expression (Cloak et al., 2002). Even though major foodborne pathogens have been shown to possess a quorum sensing system using AI-2 or AI-3 activities (Cloak et al., 2002; Elvers and Park, 2002; Sperandio et al., 2003; Surette et al., 1999), the roles of their quorum sensing and the factors affecting quorum sensing molecule production in complex food environment are not clear. It is important to study the role of AI-2-based quorum sensing of foodborne pathogens in food environments and related conditions. Thus, objective of this review was to overview quorum sensing of bacteria related to foods and to understand roles of quorum sensing in food environments.

OVERVIEW OF QUORUM SENSING IN REPORTER STRAINS

N-acylhomoserine Lactone (AHL) in *Agrobacterium tumefaciens* and *Chromobacterium violaceum*

N-acylhomoserine lactone (AHL), a signaling molecule, has the characteristic of amphipathy which allows it to pass through the phospholipid bilayer of cell membranes, and aqueous intracellular and extracellular environments (Fuqua et al., 2001). All AHL molecules have side chains of 4 to 14 carbons in length, increasing by two carbon units (e.g., C_4, C_6, C_8), and conferring signal specificity. The length of the side chain and chemical modification at the β-position determines the quorum sensing system in various Gram-negative bacteria (Fuqua et al., 2001). Yates et al. (2002) revealed that AHL should contain an *N*-acyl side chain with at least four carbons and that the AHL molecules become more stable with longer acyl side chains. Based on previous findings, sequential procedures to synthesize AHL by LuxI homologues were proposed by Parsek et al. (1999) and Schaefer et al. (1996). *S*-adenosylmethionine (SAM) binds to an active site of the enzyme LuxI. This complex obtains the acyl group that is transferred from a charged acyl carrier protein (ACP). Then, the acyl group builds up an amide bond with the amino group of SAM. Lactonisation subsequently results in the AHL and by-product, 5′-methylthioadenosine (Whitehead et al., 2001). Moreover, on the basis of the previous finding, Whitehead et al. (2001) suggested that the fatty acid chains of AHL are derived from acylated ACP substrates other than acylated CoA substrates.

Quorum Sensing of *Agrobacterium tumefaciens* as an Indicator for AHL Including Long Acyl Side Chain

A. tumefaciens has been used as an indicator bacterium to detect AHL molecules produced by other Gram-negative bacteria in foods for bacterial spoilage study (Bruhn et al., 2004; Christensen et al., 2003; Rasch et al., 2005); a specific strain (NT 1) of *A. tumefaciens* was developed by Cha et al. (1998). The strain induces blue color in well diffusion assays in response to the presence of AHL molecules (Ravn et al., 2001).

A. tumefaciens transfers and integrates its Ti plasmid into the chromosome of plant cells, causing crown gall tumors (Waters and Bassler, 2005; Zhu et al., 2000). The tumors produce metabolites called opines, a food source (carbon and nitrogen) for the invading pathogen (Dessaux et al., 1992; Waters and Bassler, 2005). *A. tumefaciens* regulates the conjugal transfer of the Ti plasmid and other Ti plasmid-borne genes between bacteria via TraR, LuxR homologue, and its AHL ligand, *A. tumefaciens* autoinducer *N*-(3-oxooctanoyl) homoserine lactone (3O-C_8-HSL) (Piper et al., 1993). This molecule is produced intracellularly (Fuqua and Winans, 1994; Hwang et al., 1994), and transferred out of the cell by diffusion (Kaplan and Greenberg, 1985) or active efflux modules (Evans et al., 1998; Pearson et al., 1999).

The autoinduction enhances tumorigenesis of plants through an increase in Ti plasmid copy number (Pappas and Winans, 2003). The quorum sensing circuit of *A. tumefaciens* is composed of TraI and TraR, the LuxI and LuxR homologues of *A. tumefaciens*. These regulatory components are located on the Ti plasmid (Fuqua and Winans, 1994; Hwang et al., 1994; Miller and Bassler, 2001; Zhang et al., 1993). Like other AHLs, TraI that is encoded by a gene on the Ti plasmid synthesizes 3O-C_8-HSL (Dessaux et al., 1992; Miller and Bassler, 2001; Moré et al., 1996; Zhang et al., 1993).

3O-C_8-HSL accumulates at high population density, and TraR-3O-C_8-HSL complex promotes the transcription of *tra* and *trb* genes in a cell population density-dependent manner (Fuqua and Winans, 1996a). TraR has also been shown to sense several DKPs (Holden et al., 1999). Production of TraR is indirectly induced by opine-specific regulators (Fuqua and Winans, 1996b; Miller and Bassler, 2001) and directly regulated by OccR, modulating the expression of the octopine catabolism genes (Fuqua and Winans, 1994; Habeeb, et al., 1991; Wang et al., 1992; Whitehead et al., 2001). Interestingly, recent finding show that TraR of *A. tumefaciens* can be fused to the eukaryotic activation domain of NF-κB p65, creating a new chimaeric transcriptional activator (Neddermann et al., 2003). The result suggests that quorum sensing of *A. tumefaciens* may be used to communicate with eukaryotic cells and tissues.

The other LuxR homologue, known as TrlR (Oger et al., 1998; Whitehead et al., 2001) or TraS (Whitehead et al., 2001; Zhu and Winans, 1998); previously TrlR was designated as TraS, are located on octopine Ti plasmids. The TrlR is a truncated homologue of TraR. This response regulator has high sequence similarity with TraR but lacks a DNA-binding module. However, TrlR negatively influences on the activity of TraR-TrlR heterodimers (Oger et al., 1998; Whitehead et al., 2001; Zhu and Winans, 1998). Interestingly, the expression of TrlR is initiated in the presence of mannopine but interfered by favored catabolites such as succinate, glutamine, and tryptone (Oger et al., 1998; Whitehead et al., 2001; Zhu and Winans, 1998), whereas the expression of TraR is not (Zhu and Winans, 1999).

Quorum Sensing of *Chromobacterium violaceum* for detecting AHL containing Short Acyl Side Chain

McClean et al. (1997) isolated and characterized *N*-hexanoyl homoserine lactone (C_6-HSL) from cell-free supernatants of *Chromobacterium violaceum* ATCC 31532 that is commonly found in soil and water, and developed a procedure to detect AHL compounds containing *N*-acyl chains only from C_4 to C_6 using *C. violaceum* CV026, a mini-Tn*5* mutant of ATCC 31532. *C. violaceum* CV026 was able to induce violacein (purple pigment) production in response to the presence of AHL molecules (McClean et al., 1997). Therefore, this strain has been used to detect AHL molecules in food spoilage studies (Bruhn et al., 2004; Christensen et al., 2003; Gram et al., 1999; Rasch et al., 2005).

In *C. violaceum*, *cviI* and *cviR*, *luxI* and *luxR* homologues, were found by the Brazilian National Genome Project Consortium (2003). Interestingly, Martinelli et al. (2004) showed that some furanones such as emoxyfurane stimulate AHL activity and induce violacein formation. *C. violaceum* uses quorum sensing to regulate chitinolytic activity (Chernin et al., 1998).

Quorum Sensing of *Vibrio harveyi* for Detection of AI-2

Schauder et al. (2001) found that *E. coli*, *S.* Typhimurium, *V. harveyi*, *Vibrio cholerae*, and *Enterococcus faecalis* have an identical biosynthetic procedure and biochemical intermediates in AI-2 biosynthesis, indicating that AI-2 is an interspecies communication signal. Recently, Xavier and Bassler (2005a) also showed interspecies communication between *V. harveyi* and *E. coli* via AI-2. In signal processing, AHL and AI-2 act synergistically (Mok et al., 2003). In AI-2 biosynthesis, LuxS acts as an AI-2 synthase, and AI-2 is derived from SAM through three enzymatic steps (Chen et

al., 2002; Schauder et al., 2001). Methyltransferase enzymes catalyze the transfer of the methyl group from SAM to substrates, and *S*-adenosylhomocysteine (SAH) is derived in this process (Schauder et al., 2001). Since SAH acts as an inhibitor to SAM-dependent methyltransferases, bacteria eliminate adenine from SAH by Pfs (nucleosidase) to produce *S*-ribosylhomocysteine (SRH) (Beeston and Surette, 2002; Schauder et al., 2001). SRH is the substrate for LuxS; SRH is cleaved into AI-2 and homocysteine (Schauder et al., 2001). However, recently, Semmelhack et al. (2004) revealed that the boron complex of 4,5-dihydroxy-2,3-pentanedion (DPD) is *V. harveyi* AI-2; DPD has a high affinity for borate complexation. Chen et al. (2002) suggested that DPD may be rearranged to produce AI-2 (furanosyl borate diester), and revealed the structure of AI-2. Also, Semmelhack et al (2004) revealed that an affinity column with a borate resin is efficient to concentrate and purify *V. harveyi* AI-2 from the biosynthetic product.

Quorum sensing was first observed in *V. fischeri*, a symbiotic bioluminescent bacterium using AHL-based quorum sensing (Nealson and Hastings, 1979). However, reporter strains of *Vibrio* have been developed in *V. harveyi*, which possesses two independent quorum sensing mechanisms (Whitehead et al., 2001). These mechanisms use two different quorum sensing molecules; *N*-(3-hydroxybutanoyl) homoserine lactone (3-hydroxy-C_4-HSL), and AI-2. Two genes, *luxL* and *luxM* (specific to this species), are required for 3-hydroxy-C_4-HSL synthesis, and 3-hydroxy-C_4-HSL is used for bioluminescence (Bassler et al., 1993; Cao and Meighen, 1989; Whitehead et al., 2001). The second signaling molecule, AI-2, is synthesized by LuxS, and it is also used for bioluminescence (Chen et al., 2002; Surette et al., 1999; Xavier and Bassler, 2003).

In *V. harveyi* quorum sensing system, LuxN (sensor kinase protein) is responsible for 3-hydroxy-C_4-HSL (Bassler et al., 1993; Taga and Bassler, 2003), and LuxP (receptor) and LuxQ (sensor kinase protein) are responsible for AI-2 (Bassler et al., 1994; Taga and Bassler, 2003). Signal from both LuxN and LuxQ is transferred to LuxU (Freeman et al., 2000, Taga and Bassler, 2003). When cell density is low, LuxN and LuxQ function as a histidine kinase to autophosphorylate an aspartate residue of the response regulator domains. This phosphate is transferred to a histidine residue of LuxU (phosphotransferase protein), then to the aspartic acid residue of the LuxO (response regulator protein) (Freeman and Bassler, 1999; Ulrich et al., 2005). When cell density is high, LuxO becomes inactive; dephosphorylated, allowing *luxR* translation by small RNA dissociation to produce LuxR protein. The LuxR protein activates *luxCDABE* gene expression to produce light (Mok et al., 2003). Based on this mechanism, *V. harveyi* strains were engineered to detect only AHL or AI-2 (Bassler et al., 1993) and these strains have been widely used to quantify autoinducers. Especially, *V. harveyi* strain BB170 was engineered to detect only AI-2 and the strain has been used to enumerate AI-2 activity (Bassler et al., 1994). *V. harveyi* strain BB120 that synthesizes both AHL and AI-2 (Bassler et al., 1997), and *V. harveyi* strain BB152 that synthesizes only AI-2 (Bassler et al., 1993) have been engineered and used in many studies as positive strains. For growth of the *Vibrio* strains, an autoinducer bioassay (AB) medium was developed by Bassler et al. (1993).

Quorum sensing of *V. harveyi* represses type III secretion system (secretion apparatus) (Henke and Bassler, 2004b), and controls function of siderophores (Lilley and Bassler, 2000), polysaccharides (Lilley and Bassler, 2000) and metalloprotease production (Mok et al., 2003). Recently, a third *V. harveyi* quorum sensing system, composed of *cqsA* (*cholerae* quorum sensing autoinducer) and *cqsS* (*cholerae* quorum

sensing sensor), was identified in genetic and phenotypic analysis by Henke and Bassler (2004a). Together, the three parallel quorum sensing systems [System 1 (3-hydroxy-C_4-HSL), System 2 (AI-2), and System 3 (CqsA and CqsS)] regulate the genes responsible for bioluminescence, type III secretion system, and metalloprotease production (Henke and Bassler, 2004a).

ROLES OF QUORUM SENSING IN GRAM-NEGATIVE FOOD RELATED BACTERIA

Vibrio cholerae

V. cholerae is pathogenic to humans, and it causes life-threatening diarrhea after oral ingestion through consumption of contaminated food and water (Kovacikova and Skorupski, 2002). *V. cholerae* possesses two significant genetic elements; the *Vibrio* pathogenicity island (VPI) (Karaolis et al., 1998, 1999) and the lysogenic CTX phage (Waldor and Mekalanos, 1996). VPI genes are related to synthesis and assembly of toxin-co-regulated pilus (TCP) (Taylor et al., 1987), while CTX encodes cholera toxin (CT) (Kaper et al., 1995). After oral ingestion of *V. cholerae*, the bacterium encounters unknown signals that induce the expression of TCP and CT (Champion et al., 1997; DiRita et al., 1991; Yu and DiRita, 2002). Recently, Zhu et al. (2002) found that quorum sensing is negatively related to virulence gene expressions in *V. cholerae* by repression of HapR, a LuxR homologue. HapR increases the expression of haemagglutinin (H/A protease) that is a part of a family of zinc metalloproteases (Häse and Finkelstein, 1990; Jobling and Holmes, 1997). LuxO (response regulator) in association with σ^{54} regulates *hapR* expression via phosphorylation in response to population density (Lenz et al., 2004; Miller et al., 2002; Zhu et al., 2002). In *V.*

cholerae, HapR automatically represses a single binding site of the *hapR* promoter (Lin et al., 2005).

The HapR affects the virulence cascade by repressing *aphA* expression and overexpression of HapR also decreases *aphA* expression (Kovacikova and Skorupski, 2002); AphA and AphB synergistically act to activate *tcpPH* transcription (Kovacikova and Skorupski, 2000). TcpPH is encoded in the VPI (Carroll et al., 1997; Häse and Mekalanos, 1998). Therefore, the function of the HapR at *aphA* is important for virulence gene regulation in a population density-dependent manner (Kovacikova and Skorupski, 2002). Furthermore, AphB has been shown as the LysR-type regulator (Kovacikova et al., 2004), and AphA is also regulated by quorum sensing in some strains of *V. cholerae* (Kovacikova and Skorupski, 2002; Miller et al., 2002; Zhu et al., 2002). In addition, AphA represses the gene involved in the biosynthesis of acetoin that plays a role in preventing intracellular acidification, essential for viability of *V. cholerae* in the presence of glucose (Kovacikova et al., 2005). Miller et al. (2002) predicted a third sensory system in *V. cholerae* that functions through LuxO, but independently from LuxU.

Even though *V. cholerae* is closely related to *V. harveyi*, they inhabit in different environments (Miller et al., 2002). *V. harveyi* is not pathogenic to humans (Liu et al., 1996; Manefield et al., 2000), while *V. cholerae* is pathogenic to humans (Faruque et al., 1998). Furthermore, *V. harveyi* produces light (Bassler et al., 1993; Cao and Meighen, 1989; Whitehead et al., 2001), while pathogenic *V. cholerae* is not bioluminescent; however, some nonpathogenic *V. cholerae* are bioluminescent (Palmer and Colwell, 1991). Interestingly, the various roles of quorum sensing in *V. cholerae* are not of the same functions as those controlled by quorum sensing of *V. harveyi*, such as bioluminescence and type III secretion system. The role of quorum

sensing in *V. cholerae* is associated with virulence determinants (Henke and Bassler, 2004b).

V. cholerae synthesizes AI-2 via the LuxS quorum sensing system (Bassler et al., 1997; Miller et al., 2002). The quorum sensing system of *V. cholerae* contains at least three parallel signaling channels that control virulence (Miller et al., 2002). *V. cholerae* Hfq which controls interaction between small regulatory RNAs (sRNAs) and specific mRNA, regulates the destabilization of the mRNA encoding the regulator HapR. Also, Hfq produces a very sensitive regulatory switch that mediates the critical transition into the high cell density, quorum sensing mode (Lenz et al., 2004).

Vibrio vulnificus

Vibrio vulnificus has been shown as a fatal pathogenic bacterium because it causes septicemia and necrotizing wound infections (Kim et al., 2003). A major vector for septicemia is shellfish contaminated with *V. vulnificus* (Kim et al., 2003; Park et al., 1991; Tacket et al., 1984). Kim et al. (2003) reviewed that for septicemia, several virulence factors have been identified in pathogenesis; cytolysin (Gray and Kreger, 1985), protease (Kothary and Kreger, 1987), capsular polysaccharide (Simpson et al., 1987), and siderophores (Litwin et al., 1996). Recently, Kim et al. (2003) and McDougald et al. (2001) identified a *luxS* homologue in *V. vulnificus*. The *luxS* mutant significantly delayed protease production and increased hemolysin production (Kim et al., 2003). Thus, Kim et al. (2003) suggested that *V. vulnificus* uses the LuxS quorum sensing circuit to express virulence factors. Quorum sensing of *V. vulnificus* plays a role in a metalloprotease, fimbriae production, motility, biofilm formation, and starvation adaptation by SmcR, a LuxR homologue (McDougald et al., 2001). Therefore, the SmcR seems to act as an activator and a repressor, and plays a role in

the repression of stationary phase phenotypes (McDougald et al., 2001). McDougald et al. (2000) identified a *luxR* homologue (*smcR*) in *V. vulnificus* and the *smcR* showed more than 75% identity with *luxR* of *V. harveyi*. An AI-2 system was also identified in *V. vulnificus* (McDougald et al., 2000, 2001). In addition, McDougald et al. (2001), and Shao and Hor (2001) reported that SmcR is involved in the negative regulation of the cytolysin gene expression. As mentioned above, septicemia is induced by various virulence factors (reviewed by Kim et al., 2003), including cytolysin (Gray and Kreger, 1985), protease (Kothary and Kreger, 1987), capsular polysaccharide (Simpson et al., 1987), and siderophores (Litwin et al., 1996). These virulence factors may activate tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6), and nitric oxide (NO), which are the production of proinflammatory mediators (Shin et al., 2004). Mutation in *luxS* and *smcR* retarded the transcription of TNFα, IL-1β, and IL-6 encoding genes, while the parent strain induced significantly higher levels of both TNFα and NO. The results suggest that quorum sensing in *V. vulnificus* may be involved in regulating virulence factors and facilitating the host's mortality (Shin et al., 2004).

The *smcR* is related to starvation survival and expression of stationary phase phenotypes (McDougald et al., 2001). The AI-2 synthase gene (*luxS*) was identified (Kim et al., 2003; McDougald et al., 2001), and *V. vulnificus* produced more AI-2 in the presence of glucose (0.5%) and at low pH (pH 6.0) (Kim et al., 2003).

McDougald et al. (2003) showed interspecies communication and regulation in *V. vulnificus* and *Vibrio angustum* by AI-2 signaling system. Production of the protease by *V. vulnificus* is increased at high level of iron (Watanabe et al., 2004). Metalloprotease production is closely related to the concentration of ferric iron (Kawase et al., 2004). Kawase et al. (2004) and Kim et al. (2003) found that the

expression of metalloprotease of *V. vulnificus* was related to the expression of the *luxS* gene. These results may suggest that metalloprotease production of *V. vulnificus* is mediated by AI-2 (Kawase et al., 2004). Taken together, AI-2 activity may affect iron concentration, thus influencing metalloprotease production.

A *luxS* mutant of *V. vulnificus* showed decreased hemolysin production, lethality to mice, and cytotoxic activity of the organism against HeLa cells, indicating that the *V. vulnificus* LuxS quorum sensing system plays a role in the expression of virulence factors (Kim et al., 2003).

Vibrio parahaemolyticus

Vibrio parahaemolyticus is one of the pathogens causing gastroenteritis through ingestion of contaminated seafood world-wide (Mead et al., 1999; Oliver and Kaper, 1997; Wong et al., 2000). A type III secretion system was identified in *V. parahaemolyticus* (Makino et al., 2003). Henke and Bassler (2004b) showed that the *V. parahaemolyticus* type III secretion system functions at low population density and gene expression of the secretion system is repressed at high population density. Thus, Henke and Bassler (2004b) suggested that quorum sensing is negatively related to type III secretion system of *V. parahaemolyticus*. Miyamoto et al. (2003) identified the quorum sensing regulator OpaR in *V. parahaemolyticus*, a LuxR homologue. McCarter (1998) found that OpaR regulates colony opacity in *V. parahaemolyticus*.

There are not many studies on *V. parahaemolyticus* quorum sensing. However, cell-free cultures of *V. parahaemolyticus* induced luminescence in *V. harveyi* reporter strain BB120 (sensor 1^+, sensor 2^+), BB886 (sensor 1^+, sensor 2^-), and BB170 (sensor 1^-, sensor 2^+) (Bassler et al., 1997). This result indicated that *V. parahaemolyticus* may synthesize AHL and AI-2.

***Pseudomonas* spp.**

Pseudomonas spp. are known as involved in food spoilage (Deza et al., 2005). *P. aeruginosa* has been reported to possess quorum sensing involving in their pathogenesis mechanisms to regulate expression of virulence factors, biofilm formation, adhesion (Davies et al., 1998; Singh et al., 2000; Smith and Iglewski, 2003), and twitching and swarming motility (Glessner et al., 1999; Kohler et al., 2000). Moreover, *P. aeruginosa* adapts to restricted nutrition environments via quorum sensing under extreme environmental conditions (Van Delden et al., 2001), and the quorum sensing also affects the *xcp* secretion modules (Chapon-Hervé et al., 1997); the *xcp* genes are involved in the secretion of most extracellular proteins in *P. aeruginosa* (Bally et al., 1992).

P. aeruginosa possesses two AHL-signaling systems, LasR (cognate receptor) and LasI (LuxI homologue), and RhlR (cognate receptor) and RhlI (LuxI homologue) (Gambello and Iglewski, 1991; Latifi et al., 1995; Passador et al., 1993; Pearson et al., 1994; Waters and Bassler, 2005; Yates et al., 2002). LasI and RhlI produce quorum sensing signals (AHL), *N*-(3-oxododecanoyl) homoserine lactones (3O-C_{12}-HSL) and *N*-butanoyl homoserine lactone (C_4-HSL), respectively (Pearson et al., 1994; Winson et al., 1995; Yates et al., 2002). Chen et al. (2005) developed models by simple first-order, exponential decay kinetic to describe C_4-HSL degradation and synthesis, produced by *rhlR* mutant and wild-type strains of *P. aeruginosa* during the stationary phase. The C_4-HSL concentration in *rhlR* mutant was similar to wild-type showing an increase followed by a decreasing profile. Heurlier et al. (2005) found that the *lasR* mutant of *P. aeruginosa* was more resistant to cell lysis and death than the wild-type strain.

In *P. aeruginosa*, AHL molecules are transported via various ways. 3O-C_{12}-HSL diffuses in and out of cells by active efflux, but C_4-HSL moves into cells by free diffusion (Pearson et al., 1999). An increase in LasI synthesis enhances the formation of LasR-autoinducer complex (Seed et al., 1995; Whitehead et al., 2001). *lasR* expression is also dependent on population density like *lasI* expression (Albus et al., 1997; Pesci et al., 1997; Whitehead et al., 2001). Recently, in an agreement with Albus et al. (1997), Donabedian (2003) reported that both RhlR-C_4-HSL complex and LasR-3O-C_{12}-HSL complex influence the production of *N*-butyryl homoserine lactone. The production of 3O-C_{12}-HSL is also affected by both regulatory cascades (Donabedian, 2003).

The LasR- and RhlR-autoinducer complexes activate various target genes, the expression of which regulates multiple structural, and regulatory genes of *P. aeruginosa* (Latifi et al., 1996; Seed et al., 1995). The LasRI system plays a role in the regulation of the expression of LasA protease, LasA elastase, LasB elastase, exotoxin A, and alkaline protease (Gambello et al., 1993; Gambello and Iglewski, 1991; Jones et al., 1993; Passador et al., 1993; Toder et al., 1991; Whitehead et al., 2001). While the RhlRI system activates the expression of *rhlA* and *rhlB*, encoding a rhamnosyl transferase (Ochsner, 1994; Whitehead et al., 2001). Chen et al. (2005) showed that two mutants of *P. aeruginosa*, which are deficient of synthesizing C_4-HSL and RhlR, produced small amount of rhamnolipids compared to the wild-type strains. Thus, they suggested that the RhlR-C_4-HSL complex is required to synthesize rhamnolipids.

Interestingly, the RhlRI system is dependent on the LasRI system; the activation of the LasRI system subsequently activates the RhlRI system (Latifi et al., 1996; Pesci et al., 1997; Whitehead et al., 2001). In *P. aeruginosa*, AHL molecules

from two major quorum sensing systems are structurally different and interaction between the two systems is presumably limited (Pearson et al., 1994, 1995). In general, these systems are coupled and regulated by the global regulatory proteins, Vfr (cyclic AMP-binding protein; CRP homologue) (Albus et al., 1997; Whitehead et al., 2001), GacA (Reimmann et al., 1997), RsmA (Pessi et al., 2001), RsaL (de Kievit et al., 1999), QscR (Chugani et al., 2001), MvfR (Cao et al., 2001), MvaT (Diggle et al., 2002), VqsR (Juhas et al., 2004), PprB (Dong et al., 2005), and AmpR (Kong et al., 2005), besides RpoS and RpoN (Heurlier et al., 2003; Whiteley et al., 2000). These complex regulatory networks may help *P. aeruginosa* adapt to environmental conditions (Dong et al., 2005). A global regulatory system, which is dependent on cyclic AMP and Vfr, influences the activation of the type III secretion regulon (Wolfgang et al., 2003). Bleves et al. (2005) demonstrated that the type III secretion system of *P. aeruginosa* is under the quorum sensing control. The two-component system is a major set of hierarchy in the response to environmental conditions (Hoch and Varughese, 2001). In *P. aeruginosa*, GacA-GacS (Gac; global activator) is a two-component system which is involved in regulation of quorum sensing-dependent genes (Reimmann et al., 1997). GacA directly or indirectly activates the expression of *rhlR* (Reimmann et al., 1997; Whitehead et al., 2001).

Recently, Dong et al. (2005) demonstrated that PprB is a novel quorum sensing regulator; a disrupted *pprB* resulted in a quorum sensing molecule production-deficient phenotype. In addition, approximately 175 genes of *P. aeruginosa* are modulated by the PprB (Dong et al., 2005).

It was suggested that activated RhlR may interact with regulation of RpoS expression (Latifi et al., 1996; Whitehead et al., 2001). It is feasible that RpoS regulates the transcription of the genes, the expression of which might be regulated by

quorum sensing (Whitehead et al., 2001). While Suh et al. (1999) and Winzer et al. (2000) demonstrated that functional RpoS protein is a crucial factor for the expression of the genes, controlled by the Las and Rhl regulons (lectin, catalase, pyoverdine activity, and exotoxin A).

It was suggested that interspecies communication of *P. aeruginosa* controlled by AHL can occur because of the conserved autoinducer binding site in regulatory proteins and the similar structure of the signaling molecules (Fuqua et al., 1996; Lewenza et al., 2002; Whitehead et al., 2001). Thus, these results indicate that *P. aeruginosa* may use AHL produced by natural flora in food environments. In addition, the potential effect of *Pseudomonas* quorum sensing on food spoilage and behavior of foodborne pathogenic bacteria in food environment should be studied.

Erwinia spp.

Erwinia spp. causing soft-rot in plants, include *Erwinia chrysanthemi*, and *Erwinia carotovora* subspecies *carotovora* (*Ecc*), *atroseptica* (*Eca*) and *betavascolorum* (*Ecb*). They synthesize exoenzymes in abundance such as pectin methylesterases, pectate lyases, pectin lyases, polygalacturonases, cellulases, and proteases (Thomson et al., 1999; Whitehead et al., 2001). These enzymes are critical factors for successful pathogenesis in plants (Mae et al., 1995; Maritis et al., 1999), and could be related to the shelf-life of produce.

In *Ecc*, production of exoenzymes is related to the synthesis of *N*-(3-oxohexanoyl) homoserine lactone (3O-C_6-HSL) by CraI, a homologue of LuxI (Jone et al., 1993; Pirhonen et al., 1993). 3O-C_6-HSL is degraded by nonenzymatic reaction during stationary phase and can be also unstable in the pH range of pH 7 to 8 (Byers et al., 2002). 3O-C_6-HSL of *Ecc* controls the global system of virulence (Pirhonen et

al., 1993). The 3O-C_6-HSL binds to CarR, a LuxR homologue, and then promotes transcription of the *car* genes (McGowan et al., 1995, 1996). The exoenzymes are secreted to the external environment by a single-step (type I) secretory pathway and a two-step (type II) pathway for proteases, and pectate lyases and cellulases, respectively (Salmond, 1994; Whitehead et al., 2001).

Eca also transcribes virulence genes via 3O-C_6-HSL (Burr et al., 2006). The quorum sensing molecule of *Eca* regulates the type II secretary system for virulence factors other than the pectinase and cellulase (Corbett al., 2005). AHL production of *Ecc* is dependent on temperature; AHL levels were responsible for the higher extracellular protein production at 34.5°C than at 28°C (Hasegawa et al., 2005). Recently, Brader et al. (2005) showed that different strains of *E. carotovora* utilize different quorum sensing molecules having various acyl side chains of AHL.

In *Erwinia stewartii* and *Erwinia amylovora*, the synthesis of extracellular polysaccharides (EPS) is regulated by the *cps* gene cluster and EPS is considered as a primary virulence factor (Coplin et al., 1990; von Bodman et al., 2003). The EPS synthesis is regulated by the EsaR/EsaI quorum sensing circuit. EsaI, a homologue of LuxI, synthesizes 3O-C_6-HSL and insignificant amounts of 3O-C_8-HSL (von Bodman and Farrand, 1995; von Bodman et al., 2003), and has a remarkable structural similarity to the *N*-acetyltransferases (Watson et al., 2002). In some cases, homologues of LuxR play the role of transcriptional repressors (Andersson et al., 2000; von Bodman et al., 1998; von Bodman and Farrand, 1995). The EsaR of *E. stewartti* is a repressor of the gene expression of synthesis of exopolysaccharide by repressing transcription of *rcsA* encoding an essential coactivator of *cps* genes (Minogue et al., 2002, 2005). Venturi et al. (2004) demonstrated that *E. amylovora* produces either a 3O-C_6-HSL or a *N*-(3-hydroxyhexanoyl) homoserine lactone (3-

hydroxy-C_6-HSL). Since they found the isolates producing 3O-C_6-HSL in infected tissues in plants, they suggested that 3O-C_6-HSL could play a role in pathogenesis in plant tissue.

Erwinia herbicola and *E. chrysanthemi* produce different AHL molecules (Bainton et al., 1992; Cha et al., 1998). Especially, *E. chrysanthemi* synthesizes three AHL molecules; 3O-C_6-HSL, C_6-HSL and *N*-decanoyl homoserine lactone (C_{10}-HSL), and the *expR-expI* locus partially contributes to the complex antiregulatory system that controls quorum sensing (Nasser et al., 1998; von Bodman et al., 2003). The *expI* gene product is required to produce 3O-C_6-HSL and C_6-HSL (Reverchon et al., 1998). The ExpR protein of *E. chrysanthemi* binds to various potential target promoters in the absence of its cognate AHL (Fuqua et al., 2001; Nasser et al., 1998). It was suggested that ExpR controls negatively its own gene expression and functions as an activator of *expI* gene expression (Reverchon et al., 1998).

Serratia spp.

Serratia spp. cause food spoilage (Borch et al., 1996; Bruhn et al., 2004). *Serratia proteamaculans* B5a was shown to cause milk spoilage using quorum sensing (Christensen et al., 2003). *Serratia liquefaciens*, which can be isolated from various environments such as water, soil, and animal intestines, is also an opportunistic pathogen of animals (Grimont and Grimont, 1978; Whitehead et al., 2001).

Eberl et al. (1996) detected C_4-HSL and C_6-HSL in cell-free culture supernatants produced by *S. liquefaciens* MG1. *swrI*, a homologue of *luxI*, is responsible for the synthesis of AHL, while *swrR*, a homologue of *luxR*, is located downstream of *swrI* (Givskov et al., 1997). Malfunction of *swrI* sensitized the pathogen to extracellular proteolytic activity but did not affect growth rate and

swimming motility of cells (Eberl et al., 1996). Thomson et al. (2000) found that *smaR* and *smaI* encode LuxI and LuxR homologues, respectively, and SmaI synthesizes AHL molecules in *Serratia* ATCC 39006 (Thomson et al., 2000; Whitehead et al., 2001). *Serratia* ATCC 39006 synthesizes the antibiotic carbapenem via quorum sensing (Thomson et al., 2000; Whitehead et al., 2001) and uses the quorum sensing system to produce the red pigment (prodigiosin; 2-methyl-3-pentyl-6-methoxyprodigiosin) (Thomson et al., 2000). The product, which is also generated by other species of *Serratia*, exerts antimicrobial and immunosuppressive activity (Tsuji et al., 1990, 1992; Williams and Quadri, 1980). When exogenous C_4-HSL and C_6-HSL were added in a *smaI* mutant strain of *Serratia* ATCC 39006, transcription of prodigiosin-encoding gene was induced (Thomson et al., 2000).

Although *Serratia* has been shown to synthesize carbapenem (Cox et al., 1998), red pigment (Thomson et al., 2000), and prodigiosin via AHL, *Serratia* also has LuxS and synthesizes AI-2, which acts as a second quorum sensing system regulating many of the identical phenotypes as the LuxR/AHL systems (Coulthurst et al., 2004; Wei and Lai, 2006). In *Serratia marcescens*, the *luxS* mutant decreased prodigiosin, carbapenem and secreted hemolysin production, and thus decreased virulence in a *Caenorhabditis elegans* model, indicating that the *luxS* controls antibiotic production and virulence in *S. marcescens* (Coulthurst et al., 2004).

Yersinia spp.

Yersinia enterocolitica is a human enteropathogenic bacterium causing enteritis, enterocolitis, mesenteric lymphadenitis, or terminal ileitis when ingested with contaminated food (Bottone, 1997).

Throup et al. (1995) identified AHL molecules from *Yersinia* spp. using a bioluminescent AHL reporter system. *Y. enterocolitica* possesses a LuxI homologue YenI and a LuxR homologue YenR, and produces C_6-HSL and 3O-C_6-HSL, while YenI is required for the expression of other proteins (Throup et al., 1995; Whitehead et al., 2001). *Y. enterocolitica* strains showed different protein profiles between the parent and the *yenI* mutant at both 28°C and 37°C (Throup et al., 1995). *Y. enterocolitica* uses quorum sensing for swimming and swarming motility (Atkinson et al., 2006a, b).

Escherichia coli

E. coli produced a small soluble heat labile organic molecule (AI-2) in Luria-Bertani (LB) broth containing glucose (DeLisa et al., 2001b; Hardie et al., 2003; Surette and Bassler, 1998), with no production of the molecule in LB broth containing no glucose (Surette and Bassler, 1998). *E. coli* O157:H7 is able to produce AI-2 in various foods such as milk, chicken broth, and brucella broth under different storage temperatures (Cloak et al., 2002). Glycerol, maltose, galactose, L-arabinose, and ribose could be used as substitutes of glucose in AI-2 production by *E. coli*, but lactose, D-arabinose, citrate, acetate, and pyruvate could not (Hardie et al., 2003). The AI-2 production by *E. coli* strain K-12 is promoted by Fe(III), NaCl, and dithiothreitol, but reduced by aerobiosis, amino acid starvation, and isopropyl-β-D-thiogalactopyranoside-induced expression of human interleukin-2 (hIL-2) (DeLisa et al., 2001b, c). The accumulation of other recombinant proteins [CAT (bacterial protein) and VP5 (viral protein)] also attenuated AI-2 signaling of the *E. coli* K-12 (DeLisa et al., 2001c).

E. coli uses AI-2 to communicate cell density and metabolic potential in the surroundings (Surette and Bassler, 1998). Interestingly, 242 genes of *E. coli* MDAI2

genome showed significant transcriptional changes to a 300-fold AI-2 signaling differential. Of the 242 genes, *ygeV* (putative σ^{54}-dependent transcriptional activator) and *yhbH* (σ^{54} modulating protein) were significantly induced, indicating that σ^{54} may be implicated in *E. coli* quorum sensing (DeLisa et al., 2001a). AI-2 signaling may be important for eukaryotes to protect themselves from pathogenic bacteria (Xavier and Bassler, 2005a). Gene array of *E. coli* O157:H7 showed that AI-2 is involved in the gene expression of flagella, motility and chemotaxis, and *stx* gene is encoded by an SOS response that is regulated by quorum sensing. The results indicate that quorum sensing of *E. coli* is a global regulatory system (Sperandio et al., 2001). Interestingly, *P. aeruginosa* can remove AI-2 activity in mixture of *P. aeruginosa* and *in vitro*-synthesized AI-2, implying that the AI-2 molecule can be used as a nutrient (Winzer et al., 2002).

SdiA (suppressor of cell division inhibitor) has been shown as a LuxR homologue in *E. coli* and *S.* Typhimurium (Michael et al., 2001; Rahmati et al., 2002). Interestingly, SdiA detects AHLs produced by *Y. enterocolitica* (Smith and Ahmer, 2003). This suggests that *E. coli* and *S.* Typhimurium may use AHL molecules produced by other Gram-negative bacteria in the environment. SdiA is also required to regulate the expression of the *ftsQAZ* gene cluster responsible for cell division (Wang et al., 1991). This indicates that *E. coli* and *S.* Typhimurium quorum sensing (SdiA) may be related to cell division.

As in *V. harveyi*, the LuxS of *E. coli* MG1655 acts as AI-2 synthase, which cleaves SRH into AI-2 and homocysteine, and AI-2 is derived from SAM through three enzymatic steps (Schauder et al., 2001; Winzer et al., 2002). Winzer et al. (2002) suggested that the LuxS protein plays the critical role in the recycling of SAH that is a precursor in AI-2 synthesis.

Production of AI-2 molecules reached maximum in midexponential phase and degraded during the stationary phase (Surette and Bassler, 1998). Surette and Bassler (1998, 1999) suggested that the degradation of extracellular AI-2 molecule was caused by the depletion of glucose during stationary phase.

Xavier and Bassler (2005b) discussed degradation of extracellular AI-2 molecule, based on their recent research. In the presence of glucose, the *lsr* (*luxS* regulated) operon cannot be transcribed because of catabolite repression; *S.* Typhimurium uses an ABC (ATP binding cassette) transporter designated as the Lsr transporter to transfer AI-2 into the cells (Taga et al., 2001; Xavier and Bassler, 2005b). Hence, AI-2 cannot be internalized, and it accumulates in cell culture fluids. While in the absence of glucose, AI-2 is produced; however, its presence is very transitory because AI-2 is rapidly internalized into cells by the Lsr transporter. Specially, the cAMP (cyclic AMP)-CRP (cAMP receptor protein) complex initiates the expression of the *lsr* operon by directly binding to the upstream region of its promoter, then works with the LsrR repressor to mediate AI-2 uptake (Wang et al., 2005b). To support these results, Wang et al. (2005a) showed that *lsrRK* is positively mediated by CRP, but negatively controlled by LsrR. In the presence of glucose, 23 genes were influenced by *luxS* deletion, while in the absence of glucose, 63 genes were affected by *luxS* deletion; the *metE* gene, the *lsrACDBFG* operon, and the flanking genes of the *lsr* operon (*lsrR*, *lsrK*, *tam*, and *yneE*) were most significantly induced by *luxS* in absence of glucose; most of highly induced genes are associated with AI-2 production and transport (Wang et al., 2005a).

Quorum sensing in colonization of *E. coli*

AI-2 of enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) is responsible for the expression of the genes encoding the type III secretion system, the

Tir (translocated intimin receptor), and intimin (adhesin) intestinal colonization (Sperandio et al., 1999). In addition, it was found that the *LEE* (locus of enterocyte effacement) pathogenicity island (Mellies et al., 1999) operons were induced by AI-2 of *E. coli* K-12 and *E. coli* O157:H7, suggesting that low levels of *E. coli* O157:H7 colonize in intestine by quorum sensing signals produced by nonpathogenic *E. coli* (Sperandio et al., 1999). Kanamaru et al. (2000) also showed evidence for *E. coli* O157:H7 colonization using quorum sensing; SdiA, an *E. coli* LuxR homologue, negatively controlled the expression of virulence factor EspD (which sets off signal transduction systems in the host cell, forming the actin based pedestal) and intimin in *E. coli* O157:H7. *LEE2* and *LEE3* operons of EPEC were activated by Ler (*LEE*-encoded regulator) (Sperandio et al., 2000); the *LEE* operons were regulated by AI-2 via Ler (Sperandio et al., 1999).

Sperandio et al. (2002a) identified the *qseBC* (quorum sensing *E. coli* regulator B and C; homology with *S.* Typhimurium *pmrAB*) genes in the *E. coli* K-12 chromosome, which encode the response regulator and the sensor kinase, and *qseBC* is involved in the transcriptional regulator of flagella genes and motility of *E. coli* K-12. QseA that is encoded by open reading frame *b3243* via quorum sensing and homologous to AphB (*V. cholerae*) and PtxR (*P. aeruginosa*) regulators activated transcription of *ler* and therefore of the other *LEE* genes in EHEC and EPEC, indicating that the QseA plays a role in the part of regulation of EHEC and EPEC virulence gene by quorum sensing (Sperandio et al., 2002b).

Recently, Kaper and Sperandio (2005) reviewed the unpublished results from their research team for three additional factors (QseD, QseE, and QseF) recently identified. QseD is a new regulator of the LysR family and controls the expression of

LEE and flagellum genes, and *qseE* and *qseF* encode another two-component system, controlling the expression of the *LEE* genes (reviewed by Kaper and Sperandio, 2005).

Autoinducer-3: novel quorum sensing molecule

Sperandio et al. (2003) proved that the LuxS enzyme of *E. coli* O157:H7 is related to the synthesis of another autoinducer called autoinducer-3 [AI-3; also called bacterial aromatic autoinducer (Walters and Sperandio, 2006)]. It was found that *LEE* genes were regulated by the *luxS*/AI-2 quorum sensing system (Sperandio et al., 1999, 2003). However, additional work using purified AI-2 showed that AI-2 is not involved in the activation of the *LEE* genes of *E. coli* O157:H7 (Sperandio et al., 2003). They isolated two fractions from the freeze-dried *E. coli* O157:H7 culture dissolved in buffer solution via C-18 columns; the fraction containing AI-2 (eluted with buffer solution) or AI-3 (eluted with methanol). Interestingly, both the fraction having AI-2 and *in vitro*-synthesized AI-2 stimulated luminescence of *V. harveyi* but failed to initiate the transcription of *LEE1*. However, the fraction containing AI-3 activated *LEE1* transcription and flagella genes, but did not activate luminescence in *V. harveyi*.

Interestingly, *E. coli* O157:H7 detected the epinephrine (Epi) and norepinephrine (NE) signal (mammalian hormones) and used them to activate the expression of its virulence genes including genes encoding type III secretion system (Sperandio et al., 2003). In addition, Sperandio et al. (2003) suggested that AI-3 may cross-talk with the mammalian hormone Epi, since the virulence phenotypes of *luxS* mutant are restored by exogenous AI-3 and/or Epi; however, the signaling is blocked by α-and β-adrenergic antagonists. The flagella regulon QseC that autophosphorylates in response to Epi and AI-3 transfers phosphate to the response regulator QseB, activating transcription of the flagella genes (Sperandio et al., 2002a; Walters and Sperandio, 2006).

Recently, AI-3 activity was observed in *Lactobacillus reuteri* 100-23 and a *luxS* mutant of this organism was not able to produce AI-3 (Tannock et al., 2005). *Salmonella*, *Shigella flexneri*, and *Yersinia* spp. also produce AI-3 (Kaper and Sperandio, 2005). In addition, the AI-3/Epi/NE-dependent signaling system is present in *Actinobacillus pleuropneumoniae*, *C. violaceum*, *Coxiella burnetti*, *E. carotovora*, *Francisella tularensis*, *Haemophilus influenzae*, *Pasteurella multocida*, *Ralstonia solacearum*, *Salmonella*, *Shigella*, and *Yersinia* (Walters and Sperandio, 2006).

Salmonella

AI-2 activity was observed in *S.* Typhimurium with reporter strain *V. harveyi* BB170 (Surette and Bassler, 1998, 1999). The *luxS*, a gene involved in autoinducer production, was also identified in *S.* Typhimurium (Joyce et al., 2000; Surette et al., 1999). Maximum activity of AI-2 was observed during mid to late exponential phase, in a preferred carbohydrate such as glucose, rapid and nutrient-rich growth, high osmolarity (0.4 M NaCl), and low pH (pH 5.0) environments (Surette and Bassler, 1998). Like *E. coli* O157:H7, *S.* Typhimurium also produced AI-2 in foods such as milk, chicken broth, and brucella broth under at different storage temperatures (4, 25, and 37°C) (Cloak et al., 2002). Extracellular AI-2 of *S.* Typhimurium is degraded during stationary phase (Surette and Bassler, 1998, 1999). AI-2 produced by *S.* Typhimurium is identical to that of *V. harveyi* and *E. coli* (Schauder et al., 2001; Surette et al., 1999).

Taga et al. (2001) found that LuxS and AI-2 mediated the expression of the *lsr* operon encoding an ABC-type transporter that was homologous to the ribose transporter of *E. coli.* The Lsr apparatus plays the role in extracellular AI-2 transport into the cells, and the *lsr* operon is composed of seven genes (*lsrACDBFGE*) and the

operon is regulated by AI-2 (Taga et al., 2001). The first four genes *lsrACDB* encode the elements of the AI-2 importer and *lsrE* encodes a protein that is homologous to ribulose phosphate epimerase (Sprenger, 1995) of *E. coli*, but roles of *lsrFG* have been unknown (Taga et al., 2001). Recently, Taga et al. (2003) identified a new protein LsrK that is necessary for the regulation of the *lsr* operon and the AI-2 uptake process, and is a kinase that phosphorylates AI-2 allowing its internalization into the cell; phosphorylated AI-2 inactivates LsrR repressor, inducing transcription of *lsr* operon (Taga et al., 2003). The crystal structure of LsrB (a second AI-2 binding protein), which binds a chemically different form of the AI-2 signal, was identified in *S.* Typhimurium by Miller et al. (2004). Also, *E. coli* has an *lsr* operon related to detection and uptake of AI-2 (Walters and Sperandio, 2006).

Like in *E. coli*, SdiA was identified as a homologue of LuxR in *S.* Typhimurium (Ahmer et al., 1998). Even though *Salmonella* does not possess an AHL synthase, it has an AHL receptor (named SdiA) detecting the AHL from other species (Michael et al., 2001; Smith and Ahmer, 2003). *sdiA*, regulated by iron and culture density-derived signals, may play a role in *S.* Typhimurium virulence regulation (Volf et al., 2002). Thus, further research is necessary to evaluate whether the behavior of *Salmonella* is affected by AHL.

Campylobacter jejuni

Campylobacter jejuni synthesizes functional AI-2 and contains an orthologue gene of *luxS* (Elvers and Park, 2002; Parkhill et al., 2000). Quorum sensing of *C. jejuni* is not involved in bacterial growth and resistance to oxidative stress (Elvers and Park, 2002).

Jeon et al. (2003) suggested that AI-2 is involved in the regulation of motility and surface properties of *C. jejuni*; *luxS* mutation resulted in reduced transcription of

flaA (a major flagellin gene), motility, and agglutination capability on the surface. In addition, *luxS* plays a role in the regulation of *cdt* (*cdt*: cytolethal distending toxin) genes in *C. jejuni* (Jeon et al. 2005).

PCR and DNA sequencing showed that *C. jejuni* and *Campylobacter coli* had *luxS*, and the deduced amino acid sequences of LuxS between these two bacteria were 90% identical (Cloak et al., 2002). These two organisms also can produce AI-2 in aliquots of foods such as milk, chicken broth, and brucella broth under different conditions (Cloak et al., 2002).

Quorum sensing in other bacteria related to foods

Acinetobacter strains were identified as a producer of two to four quorum sensing molecules with various chromatographic patterns. In *Acinetobacter calcoaceticus* BD413, maximum activity of AHL was time- and medium composition-dependent (Gonzalez et al., 2001).

luxRI homologues were cloned from *Aeromonas hydrophila* and *Aeromonas salmonicida*, and termed *ahyRI* and *asaRI*, respectively (Swift et al., 1997). *Aeromonas* spp. have been shown to produce C_4-HSL and C_6-HSL (Bruhn et al., 2005; Swift et al., 1997), and quorum sensing system of *A. hydrophila* is related to protease activity (Swift et al., 1999; Vivas et al., 2004). AhyI is detected in the exponential phase, but is degraded in stationary phase and *ahyI* expression in *A. hydrophila* is increased by C_4-HSL; while AhyR regulates *ahyI* in a positive and negative manner (Kirke et al., 2004). Sha et al. (2005) showed that there is a correlation between the type III secretion system translocon and *act* (encoding a type II-secreted cytotoxic enterotoxin), and quorum sensing.

Day and Maurelli (2001) proved that *S. flexneri* produces AI-2 which is responsible for *virB* expression; VirB is one of transcription factors, which are required for the expression of the invasive phenotype (Dorman and Porter, 1998).

OVERVIEW OF QUORUM SENSING IN GRAM-POSITIVE BACTERIA INVOLVED IN FOOD SAFETY

Bacillus anthracis

Anthrax is caused by *Bacillus anthracis* (Schoeni and Wong, 2005). Gastrointestinal anthrax can be caused by the ingestion of contaminated foods or drinks (Erickson and Kornacki, 2003; Inglesby et al., 1999). *B. anthracis* synthesizes AI-2 (Jones and Blaser, 2003). The cell-free culture of *B. anthracis* containing an open reading frame (*BA5047*) induced luminescence in *V. harveyi* BB170, indicating that *B. anthracis* produces AI-2 (Jones and Blaser, 2003). In additional experiments, Jones and Blaser (2003) proved that *BA5047* is a functional *luxS* ortholog, which is required for growth-phase-specific AI-2 expression. Only few studies have examined quorum sensing in *B. anthracis*. Thus, the relationship between quorum sensing of *Bacillus* spp. and their pathogenesis should be elucidated.

Bacillus cereus and *Bacillus thuringiensis*

Bacillus thuringiensis may cause foodborne disease (Jackson et al., 1995; Schoeni and Wong, 2005) and infections in humans (Damgaard et al., 1997; Hernandez et al., 1998; Schoeni and Wong, 2005), while *Bacillus cereus* is involved in food poisoning (Drobniewski, 1993; Schoeni and Wong, 2005). A transcriptional regulator PlcR was identified in *B. thuringiensis* (Lereclus et al., 1996), and a quorum sensing peptide

PapR was found in *B. cereus* (Slamti and Lereclus, 2002). PapR is secreted by bacteria, processed as a presumable pentapeptide, and reimported into the cell via oligopeptide permease Opp to interact with its DNA targets (Slamti and Lereclus, 2002). Slamti and Lereclus (2005) identified four PapR pentapeptide classes based on their peptide sequences and found that PapR classes correlate with PlcR clusters. These results show the polymorphism of the PlcR-PapR quorum sensing system in *B. cereus* and *B. thuringiensis*.

PlcR activates the expression of extracellular virulence factors such as phospholipase C (Lereclus et al., 1996) and hemolytic enterotoxin protease (Økstad et al., 1999). In addition, PlcR regulates the genes encoding degradative enzymes such as cell wall proteins and enterotoxins (Agaisse et al., 1999). The mutant that is defective of the *papR* gene showed reduction in the virulence in *B. thuringiensis* and the *papR* gene product stimulated expression of the PlcR regulon (Slamti and Lereclus, 2002). Callegan et al. (2005) also showed that the nonmotile/quorum sensing mutants of *B. thuringiensis* had attenuated virulence compared to wild-type strains.

Staphylococcus aureus

Staphylococcus aureus is involved in a wide range of diseases such as food poisoning, cutaneous infections, endocarditis, septic arthritis, pneumonia, and osteomyelitis (Le Loir et al, 2003; Mandal et al. 2002; Tseng and Stewart, 2005). Agr system (Agr; accessory gene regulator) is a quorum sensing system of *S. aureus* (Tseng and Stewart, 2005). The system is encoded by the *agrA*, *B*, *C* and *D* genes, and RNAIII (regulatory RNA molecule), an effector of virulence gene regulation (Jazon and Arvidson, 1990; Morfeldt et al., 1995, 1996; Novick et al., 1993). The expression of *agr* locus is activated by the peptide produced by *S. aureus* (Ji et al., 1995).

Autoinducing peptides (AIP) bind to a putative hydrophobic pocket of the receptor AgrC (sensor; histidine kinase) (Lyon et al., 2002; Wright et al., 2004). The peptide triggers the AgrC (sensor)/AgrA (activator) two-component regulatory system that sequentially stimulates transcription of the *agrBDCA* and RNAIII (Ji et al., 1995, 1997; Otto et al., 1999). The structure of AIPs was hypothesized to be an octapeptide in *S. aureus* (Ji et al., 1997). While Kalkum et al. (2003) showed that the native group-III AIP is a heptapeptide via the analysis of multistage mass spectrometry. Interestingly, they found that the homologous peptide from *Staphylococcus intermedius* is a nonapeptide, containing a lactone ring.

The *Agr* system response involves the production of most toxins, other exoproteins such as toxic shock syndrome toxin-1 (*tst*), α-hemolysin, β-hemolysin, δ-hemolysin and serine protease, and reciprocal repression of the synthesis of surface proteins, which are coagulase, protein A, and fibronectin-binding protein (Chien et al., 1999; Lindberg et al., 1990; Novick et al., 1993). In *S. aureus*, the enterotoxin D (*sed*) determinant is positively controlled by the Agr quorum sensing system, and Agr control of *sed* promoter activity depends on the presence of a functional repressor of toxin (Rot) (Tseng et al., 2004). Agr control of enterotoxin B (*seb*) promoter activity also depends on the existence of Rot protein, and Rot is able to bind to the *seb* promoter region. Hence, the Agr-regulated post-exponential phase augment in *seb* transcription results from inactivating the Agr system by Rot repressor (Tseng and Stewart, 2005). According to the *agr* sequences and certain recognition between the AIP and AgrC, four groups of *S. aureus* strains have been identified (Jarraud et al., 2000; Ji et al., 1997; McDowell et al., 2001; Zhang and Ji, 2004).

S. aureus enters mammalian cells and induces apoptosis via *agr*- and *sar* (global regulatory loci such as *agr* and *rot*)-dependent factors, which are mediated by

quorum sensing (Wesson et al., 1998). Both *agr* and *sar* are coregulators involved in protein A synthesis in *S. aureus* (Cheung et al., 1992, 1997; Chien et al., 1999), and the *sar* locus probably regulates hemolysin production through an *agr*-mediated pathway. Protein A is a main surface protein in *S. aureus* strains (Cheung et al., 1997; Easmon and Adlam, 1983; Waldvogel, 1985).

Global regulatory elements *sarA* (Cheung et al., 1992; Sifri et al., 2003) and *sae* (Giraudo et al., 1997) also regulate exoprotein synthesis in *S. aureus*. The *sae* is downstream of *agr* under the pathway for exoprotein activation, and coordinates the effects of environmental signals by means of the *agr* quorum sensing system. Hence, it is a major regulator in the overall regulatory scheme by which *S. aureus* senses and acts in response to its environment (Novick and Jiang, 2003).

SarR is homologous to SarA with 51% similarity and 28% identity, and it was proposed that SarR down-regulates SarA protein expression in *S. aureus* (Manna and Cheung, 2001). Cheung et al. (2001) found and characterized SarS, a homologue of SarA. *agr* locus may control *spa* (protein A gene) repression via repressing the transcription of *sarS* (Cheung et al., 2001). SarT was identified as a homologue of SarA, and it is down-regulated by *sarA* and *agr*, and represses *hla* (α-hemolysin gene) expression (Schmidt et al., 2001). Recently, SarU, which is a member of the SarA family proteins, was found, and *sarT* may negatively mediate *agr* RNAIII expression via repressing *sarU*, a positive regulator of *agr* expression (Manna and Cheung, 2003).

RNAIII activation protein (RAP; quorum sensing activator) is constantly secreted by *S. aureus* and is also expressed in other bacteria such as *Staphylococcus epidermidis*, *Staphylococcus xylosus*, and *E. coli* (Korem et al., 2003). RAP is a protein with putative 277 amino acids and activates RNAIII production, resulting in the induction of *S. aureus* pathogenesis (Korem et al., 2003). Gov et al. (2004) found

that the target of RAP (TRAP) was very conserved in staphylococci and included three completely conserved histidine residues (His-66, His-79, and His-54), which were phosphorylated and necessary for its activity. Taken together, RAP may induce pathogenesis in *S. aureus* via phosphorylation on three conserved histidine residues of TRAP. Korem et al. (2005) showed that the phosphorylation of TRAP is required for gene regulation which is related to staphylococcal pathogenesis, and TRAP positively mediates the *agr* locus. In addition, Yang et al. (2006) suggested that the RAP quorum sensing system is an appropriate target to prevent staphylococcal intoxications.

Interestingly, Muscholl-Silberhorn et al. (1997) suggested that *S. aureus* can initiate gene transfer from *E. faecalis* via the production of a peptide, which acts as sex pheromone. *S. aureus* produces a peptide *staph*-cAM373, which is very similar to the sex pheromone pAM373 produced by *E. faecalis* (Nakayama et al., 1996).

Enterococcus faecalis

Enterococci are detected naturally in foods and may cause human illness (Cocconcelli et al., 2003). Nakayama et al. (2001) found that *E. faecalis* starts gelatinase (virulence factor) production at late log phase and reaches to a maximum level in early stationary phase, which is an indicator of quorum sensing. *E. faecalis* secretes a gelatinase biosynthesis-activating pheromone (GBAP) (Nakayama et al., 2001). Nakayama et al. (2003) suggested that the three-component regulatory system, composed of autoinducing peptides (AIP), sensor kinase and response regulator, most likely constitutes the quorum sensing system for *E. faecalis*. In addition, *E. faecalis* produces AI-2 identical with *E. coli*, *S.* Typhimurium, *V. harveyi*, and *V. cholerae*

(Schauder et al., 2001). Thus, further research is necessary to find the role of AI-2 in pathogenesis of *E. faecalis*.

***Clostridium* spp.**

Clostridium perfringens is an endospore-forming bacterium that causes gastrointestinal and histotoxic intoxication in humans and animals (Chakrabarti et al., 2003; García-Alvarado et al., 1992; Hall et al., 1963; McClane, 2001; Philippe et al., 2006). Intoxication with *C. perfringens* is caused by consumption of food containing enterotoxigenic strains (Aguilera et al., 2005; Fach and Popoff, 1997; Shandera et al., 1983).

In *C. perfringens*, *luxS* homologue was identified via complete genome sequence (Shimizu et al., 2002), and the cell-free culture supernatant of *C. perfringens* significantly activated the luminescence of *V. harveyi* BB170, but a *luxS* mutant of *C. perfringens* did not stimulate luminescence in *V. harveyi* BB170. These results indicate that *C. perfringens* produces AI-2 via *luxS* gene (Ohtani et al., 2002). Ohtani et al. (2002) examined if AI-2 plays a role in the toxin production in *C. perfringens*. In a *luxS* mutant of *C. perfringens*, production of alpha (*plc*)-, kappa (*colA*)-, and theta (*pfoA*)-toxins was decreased, but the production of toxin in the *luxS* mutant was increased in the presence of exogenous AI-2; however, the genes such as *metB* and *cysK* located upstream of *luxS* are not related to the regulation of toxin production. These results indicate that AI-2 of *C. perfringens* plays a critical role in the regulation of toxin production (Ohtani et al., 2002).

Clostridium botulinum is an anaerobic spore-forming bacterium (Lindström et al., 2006; Sharma et al., 2006). Foodborne botulism can be caused by ingestion of foods contaminated with botulinum toxin (Sobel, 2005). *C. botulinum* showed

positive bioluminescence in an AI-2-based quorum sensing system, but not in an AHL-based quorum sensing system (Zhao et al., 2006). Quorum sensing of *C. botulinum* may influence spore germination of *C. botulinum* (Zhao et al., 2006).

Listeria monocytogenes

Even though *Listeria monocytogenes* is a lethal foodborne pathogenic bacterium, which may be present in ready-to-eat meat products, only few studies have been published on its quorum sensing. Recently, Challan Belval et al. (2006), and Turovskiy and Chikindas (2006) detected AI-2 activity in *L. monocytogenes* strains ScottA and EGD-e, respectively; however, the AI-2 activity produced by *L. monocytogenes* strain ScottA was transient. While Challan Belval et al. (2006) suggested that the role of AI-2 may not be involved in cell-to-cell signaling in *L. monocytogenes*. Ermolaeva et al. (2004) found that a diffusible autorepressor is produced in *L. monocytogenes* and it negatively controls virulence factor PrfA, and suggested that the autorepressor regulates the quorum sensing.

QUORUM SENSING IN BACTERIOCIN PRODUCTION

For the last 50 years, bacteriocins have been researched and suggested as a solution to a myriad of food contamination problems (Cotter et al., 2005). Bacteriocin production in lactobacilli is controlled by a short, strain-specific peptide pheromone which is a quorum sensing molecule (Brurberg et al., 1997).

Lactobacillus plantarum produces plantaricin A (PlnA) possessing the characteristics of bacteriocin and pheromone activities (Hauge et al., 1998). Open reading frame of *L. plantarum* is composed of *plnA* encoding a precursor peptide of plantaricin A, *plnB* encoding histidine protein kinase, and *plnC* and *plnD* encoding

response regulators (Diep et al., 1994). Bacteriocin synthesis is controlled by the response regulators PlnC and PlnD, which are activated by peptide pheromones in *L. plantarum* (Risøen et al., 1998).

To produce bacteriocins of *Streptococcus mutans* strains, competence simulating peptide (CSP)-induced quorum sensing is essential (van der Ploeg, 2005; Yonezawa and Kuramitsu, 2005).

QUORUM SENSING IN BIOFILM FORMATION

Bacterial biofilms are complex cell structures embedded in a matrix produced by microorganisms on inert or living surfaces (Costerton et al., 1999; Van Houdt et al., 2004). Biofilm formation on food contact surfaces is especially important because it has the potential to cause food spoilage and transmission of disease, and cells in biofilm are more resistant to cleaning and sanitation than planktonic cells (Bower and Daeschel, 1999; Joseph et al., 2001; Stepanović et al., 2004).

Many scientists have studied whether biofilm formation of bacteria is regulated by quorum sensing. So far, in certain cases, quorum sensing has not been found to be related to biofilm formation. However, other studies have found quorum sensing to influence biofilm formation (Parsek and Greenberg, 2005). Biofilm formation involves attachment of a bacterium on surface, maturation of the biofilm community, and aggregation and dissolution or dispersal (reviewed by Parsek and Greenberg, 2005).

P. aeruginosa uses flagella and motility to initiate cell-to-surface interactions (O'Toole and Kolter, 1998). The production of mucoid exopolysaccharide is then increased following adherence (Hoyle et al., 1993). To form mature biofilms, quorum sensing is required in *P. aeruginosa* (Davies et al., 1998; Rashid et al., 2000).

Intriguingly, polyphosphate kinase is also involved in biofilm maturation in *P. aeruginosa*, since a strain containing a mutation of the gene encoding polyphosphate kinase had reduced quorum sensing activity (Rashid et al., 2000); this finding supports the concept that quorum sensing is related to biofilm maturation. Thus, Rashid et al. (2000) suggested that polyphosphate kinase may be a desirable target to control biofilms. Bollinger et al. (2001), however, demonstrated that quorum sensing may negatively regulate biofilm formation of *P. aeruginosa.* In *P. aeruginosa*, quorum sensing-controlled functions and surface motility are critical factors in microbial competition (An et al., 2006). Recently, Palmer et al. (2005), and Spoering and Gilmore (2006) found that a novel quorum sensing signal (PQS) is also involved in biofilm formation in *P. aeruginosa*, which uses quorum sensing to resist protozoan grazing during late biofilm formation (Matz et al, 2004).

lasI and *lasI rhlI* deletion mutants of *P. aeruginosa* formed packed biofilms, but little polysaccharide was observed, compared to the wild-type strain which produce AHL; *P. aeruginosa* has LasRI and RhlRI quorum sensing systems (Gambello and Iglewski, 1991; Latifi et al., 1995; Passador et al., 1993; Pearson et al., 1994; Waters and Bassler, 2005; Yates et al., 2002). The biofilms formed by the *P. aeruginosa* mutant strains (Δ*rhlI*, Δ*lasI*, Δ*lasI*Δ*rhlI*) were more susceptible to antibiotics (kanamycin) than the wild-type strain (Shih and Huang, 2002).

George et al. (2005) added two types of *P. aeruginosa* AHL and furanone to wild-type and mutant type of *P. aeruginosa*, *S. aureus*, and *S. epidermidis* cultures to evaluate effect of extracellular AHL and furanone on growth and adhesion to contact lenses. The results showed that exogenous AHL and the furanone insignificantly affected on initial adhesion of *P. aeruginosa* to surfaces, while the furanone

diminished staphylococci growth and elastase production in wild-type *P. aeruginosa*, but promoted growth and elastase production of a quorum sensing mutant strain.

Arevalo-Ferro et al. (2005) evaluated the influence of quorum sensing and biofilm on the protein profile of surface-controlled proteins of *Pseudomonas putida*. The proteomics results showed that quorum sensing system mediated expression of 75% of identified surface-controlled proteins.

Like general influence of *P. aeruginosa* quorum sensing on biofilm formation, *Burkholderia cepacia* may form biofilm via quorum sensing. Crystal violet staining of the biomass revealed that attachment of the *cepI*, *cepR*, *cciR*, and *cepR cciIR* mutants of *B. cepacia* was deficient due to absence of components of the quorum sensing system, and the quorum sensing mutant formed structurally deficient biofilm (Tomlin et al., 2005). Quorum sensing is not related to the regulation of initial cell attachment of *B. cepacia*, but rather regulates the biofilm maturation (Huber et al., 2001). Conway et al. (2002) revealed that *B. cepacia* uses quorum sensing in pathogenesis, but its quorum sensing may not modulate biofilm formation under all conditions. However, Huber et al. (2002) also suggested that the quorum sensing system of *B. cepacia* is a major factor in biofilm formation.

In *Agrobacterium*, the transition to quorum sensing requires higher threshold cell populations in liquid media than in biofilm (Goryachev et al., 2005). Based on this result, they speculated that quorum sensing of *Agrobacterium* acts as the detector of biofilm formation. AHL plays a role in biofilm maturation of *S. liquefaciens* (Labbate et al., 2004). In *S. liquefaciens*, the *swrI* mutant that is defective of producing AHL synthase, attenuates biofilm formation (Labbate et al., 2004). At high cell density, SwrR-C_4-HSL complex of *S. liquefaciens* promotes expression of target genes such as *swrA* required for swarming (Eberl et al., 1999).

In *Vibrio anguillarum*, *vanT*, encoding a LuxR-like transcriptional regulator of *V. harveyi*, positively controlled biofilm production (Croxatto et al., 2002). LuxO, a quorum sensing regulator, upregulated biofilm formation in *V. cholerae* (Vance et al., 2003). Biofilm formation increases in *hapR* mutants (HapR; quorum sensing regulator) of *V. cholerae* via the overexpression of *vps* operons (*vps*; *Vibrio* polysaccharide synthesis) (Zhu and Mekalanos, 2003). A constitutively active LuxO variant of *V. cholerae* also increases biofilm formation (Raychaudhuri et al., 2006). In agreement, Zhu et al. (2002) proved that LuxO and HapR controlled biofilm formation. Interestingly, *Vibrio* biofilms are more resistant to acid than planktonic cells, while the biofilm, which is formed by the quorum sensing-deficient strain of *V. cholerae*, has lower colonization ability than those of wild-type biofilms. This result indicates that quorum sensing may improve cellular exit from the biofilm when the organisms have navigated the acidic environment of the stomach (Zhu and Mekalanos, 2003).

Since AI-2-like activity was observed on Roma tomatoes during storage at 4°C for 9 days, as the level of heterotrophic bacterial populations increased, Lu et al. (2005b) used the rinses from Roma tomato surfaces inoculated with a *luxS* mutant of *E. coli* O157:H7 to evaluate the effect of AI-2-like activity of Roma tomatoes on biofilm formation of *E. coli* O157:H7, and they suggested that AI-2-like activity of Roma tomatoes has the potential to enhance bacterial biofilm formation. The *mqsR* (motility quorum sensing regulator gene) was found in *E. coli* by González Barrios et al. (2006), and they found that AI-2 induced biofilm formation of *E. coli* via MqsR, modulating QseBC (two component regulatory system) and motility.

In *L. monocytogenes*, SRH (a precursor of AI-2) is involved in biofilm formation (Challan Belval et al., 2006). Van Houdt et al. (2004) investigated the

correlation between quorum sensing and biofilm formation capacity in 68 Gram-negative strains isolated from food production environments, which included 26 isolates producing AI-2 and five isolates producing AHL molecules; however, no correlation was observed between *in vitro* biofilm formation and quorum sensing.

A *luxS* mutant of *S.* Typhimurium was defective of biofilm formation on the surfaces of gallstones, indicating that quorum sensing was required to induce a full biofilm formation (Prouty et al., 2002). In agreement, De Keersmaecker et al. (2005) reported that *luxS* mutant of *S.* Typhimurium had impaired biofilm formation on polystyrene pegs. The aggregate formation by *S.* Thompson parental strain was significantly higher than that by its *luxS* mutant in intestine, spleen, and droppings of chicks. In contrast, no significant difference was observed for the aggregate formation on cilantro leaf surfaces between a parental strain and a *luxS* mutant (Brandl et al., 2005).

In *S. mutans*, a peptide pheromone system is composed of *comC*, *comD*, and *comE* which encode quorum sensing essential for cell density-dependent genetic competence, and it functions properly once the cells are living under actively growing biofilms (Li et al., 2001b). A *S. mutans* mutant that is defective in *comC*, *comD*, *comE*, as well as *comCDE*, formed abnormal biofilm compared to wild-type strain; biofilm was observed by scanning electron microscopy and confocal scanning laser microscopy (Li et al., 2002). Merritt et al. (2003) demonstrated that AI-2 is responsible for biofilm formation by *S. mutans*; the *luxS* mutant strain of *S. mutans* formed a much more granular biofilm, rather than the comparatively smooth confluent layer, which is normally observed in the wild-type. Similarly, the *luxS* mutant strain of *S. mutans* formed loose and hive-like biofilm in medium with sucrose, while the wild-type strain had smooth and confluent biofilm. AI-2 of *S. mutans* mediates the

glucosyltransferase gene expression, which is essential for sucrose-dependent biofilm formation (Yoshida et al., 2005). Quorum sensing of *Streptococcus gordonii* is also involved in biofilm formation (Loo et al., 2000). In *Streptococcus intermedius*, a synthetic CSP (competence simulating peptide) improves biofilm formation without affecting the rate of culture growth (Petersen et al., 2004a).

In *S. aureus*, 105 strains were examined for a correlation between the *agr* quorum sensing system (quorum sensing system of *S. aureus*) and adherence ability of *S. aureus* on polystyrene. The result showed that 78% of *agr*-negative and only 6% *agr*-positive strains formed a biofilm, proving negative impact of *agr* quorum sensing system on biofilm formation (Vuong et al., 2000). The *agr* defective mutant of *S. epidermidis* also showed increased biofilm formation and primary attachment (Vuong et al., 2003). Vuong et al. (2004) showed that an isogenic *agr* mutant of *S. epidermidis* augmented biofilm formation and colonization in a rabbit model, indicating that inhibition of the *agr* quorum sensing system probably increases the infection of *S. epidermidis* on surfaces. However, Yarwood et al. (2004) suggested that in *S. aureus*, the role of *agr* expression in biofilm formation is dependent on environmental conditions, because *agr* defective strain formed more biofilms than the parent strain under a static biofilm system, whereas no significant difference in the structure or thickness of the biofilm was observed between the parent and *agr* defective strain in flow cells. Intriguingly, the *luxS* defective strain of *S. epidermidis* enhanced biofilm formation, proving that *luxS* negatively controls biofilm formation by a cell-to-cell signaling mechanism using AI-2. This result is similar to that of the *agr* quorum sensing system (Xu et al., 2006).

The *fsr* quorum sensing system of *E. faecalis* contributes to biofilm formation (Pillai et al., 2004). An *A. hydrophila* strain containing a mutation of *ahyI*, *luxI*

homologue, did not form matured biofilm. Moreover, the bacterial populations in the biofilm, but not in the planktonic phase, were significantly lower than that of the wild-type strain or a strain containing a mutation of *ahyR*, *luxR* homologue; *ahyI* and *ahyR* are involved in the quorum sensing system (Lynch et al., 2002). A *luxS* mutant of *L. reuteri* formed thicker biofilm than the wild-type strain on both plastic surfaces, as well as epithelial surfaces of the forestomach of mice (Tannock et al., 2005). A *luxS* isogenic mutant of *Klebsiella pneumoniae* was examined to determine whether quorum sensing is related to biofilm formation. The results of this study showed that a LuxS-dependent quorum sensing was associated with biofilm formation in the early stage, prior to maturation (Balestrino et al., 2005).

QUORUM SENSING SIGNAL MIMICS AND NOVEL QUORUM SENSING SIGNALS

Lu et al. (2005b) examined the potential presence of AI-2-like activity in various vegetables and fruit products, including eggplant, squash, Roma tomatoes, green peppers, peaches, cucumbers, potatoes, carrots, oranges, apples, bananas and strawberries, and they showed that all samples but strawberries showed various levels of AI-2-like activity.

Novel quorum sensing molecules were identified and named DKP (Holden et al., 1999). DKPs such as cyclo (ΔAla-L-Val) and cyclo (L-Pro-L-Tyr) are found in the cell-free supernatants from *P. aeruginosa*, *Proteus mirabilis*, *Citrobacter freundii*, and *Enterobacter agglomerans* (only cyclo [ΔAla-L-Val]), while *Pseudomonas fluorescens* and *Pseudomonas alcaligenes* synthesize only cyclo (L-Phe-L-Pro). Importantly, DKP activates LuxR-based AHL biosensor in a concentration-dependent manner, and antagonizes the 3O-C_6-HSL-mediated quorum sensing system and LuxR-

based quorum sensing system, but the structure of the DKP was not AHL in mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy analysis (Holden et al., 1999). *P. putida* produces four DKPs; cyclo (L-Pro-L-Tyr), cyclo (L-Pro-L-Leu), cyclo (L-Phe-L-Pro), and cyclo (L-Val-L-Leu). The high performance liquid chromatography (HPLC) fractions of the DKP are also able to activate *C. violaceum* and *A. tumefaciens* biosensors (Degrassi et al., 2002).

Pesci et al. (1999) found that *P. aeruginosa* synthesizes another quorum sensing molecule, designated as PQS (2-heptyl-3-hydroxy-4-quinolone), originally described as a modulator of LasB (elastin-hydrolyzing protease). The PQS is considered as a novel component of quorum sensing originating from anthranilate (Calfee et al., 2001) and synthesized at the end of the exponential phase in *P. aeruginosa* (Diggle et al., 2003). Recently, PQS has been isolated from cystic fibrosis patient lungs contaminated with *P. aeruginosa* (Collier et al., 2002; Guina et al., 2003). Moreover, Collier et al. (2002) revealed that the PQS molecule *in vivo* may be a factor in development of a chronic state of cystic fibrosis. This molecule production is correlated with the Las and Rhl quorum sensing system (Fuqua et al., 2001; Gallagher et al., 2002; Pesci et al., 1999). It is hypothesized that PQS increases the expression of RhlR-dependent genes during slow growth or starvation (Fuqua et al., 2001). LasR has been proved to control PQS production, and the provision of exogenous PQS activity stimulates expression of *rhlI*, *rhlR*, *rpoS* and *lasB* (Diggle et al., 2003; McKnight et al., 2000; Pesci et al., 1999), indicating that PQS is composed of a regulatory linkage between the *las* and *rhl* quorum sensing. Diggle et al. (2003) engineered *pqsE* and *pqsR* double mutant of *P. aeruginosa* to investigate the role of PQS. This mutant decreased the levels of exoproducts; in contrast, the PQS, in wild-type strain, showed a significant increase in PA-IL lectin (cytotoxic lectin/adhesion),

pyocyanin, biofilm formation, and elastase production during early stationary phase. These results indicate that further study needs to be done to find whether PQS influences bacterial responses or activities in biofilm formation, antibiotic resistance, and resistance to stresses.

Quorum sensing molecules are also found in yeast. In *Candida albicans*, the autoregulatory molecule was identified as 3,7,11-trimethyl-2,6,10-dodecatrienoate (farnesoic acid) by NMR and mass spectrometry; this molecule does not restrain yeast cell growth, but blocks filamentous growth (Oh et al., 2001).

Hornby et al. (2001) found and identified another quorum sensing molecule in *C. albicans*. The molecule is farnesol ($C_{15}H_{26}O$), which is extracellular and is synthesized constantly during growth and over a temperature range from 23 to 43°C. Furthermore, production of farnesol does not depend on carbon or nitrogen sources (Hornby et al., 2001). Farnesol inhibits hyphal growth (Chen et al., 2004; Enjalbelt and Whiteway, 2005; Hornby et al., 2001), but a high concentration (>30μM) of farnesol did not suppress fully hyphal formation (Enjalbert and Whiteway, 2005). Also, farnesol influences positively chlamydospore production in *C. albicans* at 37°C (Martin et al., 2005). Like quorum sensing system in other microorganisms, quorum sensing activity of *C. albicans* is cell density-dependent (Hornby et al., 2004).

The farnesol-dependent quorum sensing system of *C. albicans* is regulated by a two-component pathway (Kruppa et al., 2004). Farnesyl pyrophosphate is converted to farnesol by specific enzyme activity (Hornby et al., 2003). There are also farnesol analogs showing quorum sensing molecule activity (Shchepin et al., 2003). The farnesol analogs can be a useful method to detect farnesol binding proteins (Shchepin et al., 2005). In *C. albicans*, high farnesol concentrations (>10μg/ml) eradicates the amino acid incorporation which is enhanced by farnesol, and the level of the increased

incorporation depends on cell-surface hydrophobicity (Braun, 2005). The production of farnesol is increased by treatment with zaragozic acid B, antifungal antibiotics (Hornby et al., 2003).

Semighini et al. (2006) examined the effect of farnesol on *Aspergillus nidulans*. The study showed that exogenous farnesol does not influence hyphal morphogenesis; however, this molecule stimulates morphological feature characteristic of apoptosis. In addition, the study revealed that mitochondria and reactive oxygen species are involved in farnesol-induced apoptosis, and the effects of farnesol are likely regulated by the FadA heterotrimeric G protein complex. These results suggest that *C. albicans* uses quorum sensing to be competitive to other microorganisms. Recently, the active component (tyrosol) was found in a conditioned medium, which was produced continuously during the growth. Tyrosol triggers the formation of filamentous protrusions (Chen et al., 2004).

In other yeast, Mergler and Klinner (2001) suggested pyruvate decarboxylase as a putative quorum sensing signal of *Pichia stipitis*, since pyruvate decarboxylase was activated in a cell density-dependent manner under the aerobic batch cultivation. Ren et al. (2004a) found that the supernatant from *E. coli* DH5α (no AI-2 production) stationary culture depressed *E. coli* K-12 concentrations by 4.8-fold ± 0.4-fold, suggesting that *E. coli* contains an additional quorum sensing system.

QUORUM SENSING IN STRESS RESPONSE

Various quorum sensing systems of microorganisms are involved in stress responses to survive under stressful environments. *P. aeruginosa* may use quorum sensing to resist to oxidative response. The *P. aeruginosa* mutants with deletion of *lasI* and *rhlI* were more susceptible to hydrogen peroxide than the wild-type (Huang and Shih,

2000). Stress responses based *rhlI* quorum sensing system may be related to *rpoS* of *P. aeruginosa.* Latifi et al. (1996) demonstrated that the *rhl* quorum sensing system triggered the transcription of *rpoS*. In contrast, Whiteley et al. (2000) proved that the *rpoS* transcription is not mediated by quorum sensing. RpoS protein of *P. aeruginosa* influences stationary-phase stress tolerance (Jørgensen et al., 1999). Furthermore, the *rpoS* mutant strain of *P. aeruginosa* was more susceptible to heat shock and NaCl (Suh et al., 1999).

A *smcR* (*V. harveyi luxR* homologue) defective strain of *V. vulnificus* was more susceptible to oxidative stress than the wild-type strain (McDougald et al., 2003). In addition, a *smcR* mutant of the *Vibrio* spp. was more sensitive to starvation than the wild-type strain (McDougald et al., 2001). AI-2 signaling in *V. vulnificus* and *V. angustum*, initiates the core response to starvation adaptation and stress resistance (McDougald et al., 2003).

In *S. mutans*, a *luxS* mutant strain became more resistant to hydrogen peroxide (58.8 mM), showed increased sensitivity to acid, and could still go through acid adaptation (Wen and Burne, 2004). It was proved that the level of bacterial populations and biofilm growth mode regulate acid adaptation in *S. mutans*, indicating that appropriate development of acid adaptation involves both low pH induction and cell-to-cell communication (Li et al., 2001a). Expression data indicate that the *agr* quorum sensing system of *S. epidermidis* controls oxidative stress-response factors such as detoxifying enzymes of reactive oxygen species (mechanism in host defense), indicating that quorum sensing of *S. epidermidis* plays a role in protection in human innate host defense mechanism (Yao et al., 2006). *S. epidermidis* mediates oxidative stress-response factors via the *agr* quorum sensing system, which involves enzymes detoxifying reactive oxygen (Yao et al., 2006).

DeLisa et al. (2001b) identified the transcription of the four genes mediated by quorum sensing and 20 stress genes for *E. coli* K-12 during the transitory response to induced expression of hIL-2 and the results showed that a critical overlap was observed among known quorum sensing genes and starvation genes and several other stress genes. *E. coli* K-12 uses AI-2 to communicate the stress or metabolic burden related to overall accumulation of foreign protein (DeLisa et al., 2001c). In addition, when the *E. coli* culture was exposed to a subtherapeutic concentration (2 μg/ml) of chlortetracycline, AI-2 activity was promoted by increasing levels of tetracycline (Lu et al., 2005a). This indicates that *E. coli* may use AI-2 to be resistant to antibiotics.

The transcription of the *comQXP* quorum sensing locus in *B. subtilis* can be inhibited by the superoxide stress, resulting in the reduced expression of *sfrA*, which is required for the development of genetic competence; overexpression of the superoxide decreased the transcription of *comQXP* quorum sensing locus (Ohsawa et al., 2006). The quorum sensing signal degradation system of *A. tumefaciens* is collaborated with and controlled by the generic (p)ppGpp stress response machinery. *A. tumefaciens* breaks off the quorum sensing-dependent and energy consuming conjugation development under starvation stress (Zhang et al., 2004). Farnesol, which is one of the quorum sensing molecules produced by *C. albicans*, plays a role in protecting *C. albicans* cells against hydrogen peroxide toxicity (Westwater et al., 2005).

QUORUM SENSING IN FOOD SPOILAGE

Microbial food spoilage may be associated with bacterial quorum sensing of natural flora. Gram et al. (1999) evaluated the relationship between AHL of *Enterobacteriaceae*, which are members of food spoilage flora (Borch et al., 1996;

Gram et al., 1999; Jiménez et al., 1997), and food spoilage. In this study, AHL was detected in foods naturally contaminated with many strains of *Enterobacteriaceae* and in samples inoculated with *Enterobacteriaceae* once the levels of *Enterobacteriaceae* reached 10^5 to 10^7 CFU/g. The results suggested that AHL may play an important role in food spoilage and thus, AHL can be used as an indicator of food quality. Several hydrolytic enzymes, which are related to food spoilage, are controlled by quorum sensing, and quorum sensing is also related to the production of spoilage characteristics *in situ* in food products (Christensen et al., 2003). In fresh ground beef, which was stored aerobically at 5 to 7°C for up to 28 days, *Pseudomonas* spp. were determined as dominant organism in the spoilage of samples. Based on this result, Jay et al. (2003) speculated that *Pseudomonas* spp. become dominant during spoilage by biofilm formation via quorum sensing. Recently, Rasch et al. (2005) demonstrated that quorum sensing-regulated phenotypes influence the bacterial spoilage in some of food products.

However, AHL may not be related to the spoilage of vacuum-packaged roasts of beef, while AHL-producing *Hafnia alvei* may affect food spoilage when *Enterobacteriaceae* are involved in the spoilage process (Bruhn et al., 2004).

INHIBITION AND DEGRADATION OF QUORUM SENSING

Since many microorganisms use quorum sensing systems in physiological behaviors such as virulence, stress response, biofilm formation, competence, and food spoilage, quorum sensing systems could be a potential target for microbial control.

Garlic inhibited the production of quorum sensing molecules but not antibiotics (Bjarnsholt et al., 2005; Persson et al., 2005). Rasmussen et al. (2005b) examined the anti-quorum sensing effect of a garlic extract and 4-nitro-pyridine-*N*-

oxide (4-NPO). The results revealed that the garlic extract and 4-NPO regulated quorum sensing-dependent gene expression in *P. aeruginosa*, and reduced biofilm tolerance to tobramycin and bacterial virulence to *C. elegance* (Bjarnsholt et al., 2005; Rasmussen et al., 2005b). In addition, the garlic extracts decreased biofilm tolerance of *P. aeruginosa* to the cytokines such as granulocyte colony-stimulating factor and macrophage inflammatory protein-2 (Bjarnsholt et al., 2005). Polyphenolic compounds, which are widely distributed in the plant kingdom, were also reported as able to block the bacterial quorum sensing (Huber et al., 2003). Exogenous furanone was not effective for initial *P. aeruginosa* attachment, but diminished elastase production of wild-type *P. aeruginosa* (George et al., 2005). Hentzer et al. (2003) showed that furanone application to biofilm makes *P. aeruginosa* more sensitive against the antibiotic tobramycin and sodium dodecyl sulfate, and in a mouse pulmonary infection model, the furanone drug inhibited quorum sensing formed by infecting bacteria. Furanone interferes with quorum sensing in biofilm-forming cells by penetrating microcolonies; it disrupts the architecture of biofilm rather than initial attachment to an abiotic substratum (Hentzer et al., 2002).

Furanone at 60 μg/ml decreased biofilm formation of *E. coli* by 55%, suggesting that natural furanone can be useful to regulate bacterial biofilm, and furanone at 10 μg/ml also inhibited the quorum sensing of *V. harveyi* by 33,000-fold (AHL) and 5,500-fold (AI-2) as well as the quorum sensing of *E. coli* (AI-2) by 26,600-fold (Ren et al., 2001). Ren et al. (2004a) found that in *E. coli* K-12, 166 genes were differentially expressed by AI-2, but 90 genes were differentially depressed by furanone. Interestingly, 79% of the genes repressed by furanone were induced by AI-2, indicating that AI-2 activity is inhibited by furanone; the majority of these genes were related to chemotaxis, motility, and flagellar synthesis. They also

found that the AI-2 induced aerotaxis genes (*aer* and *tsr*), but expression of these genes was inhibited by furanone. Furanone blocked AI-2 production in ground beef inoculated with *C. perfringens*, and ascorbic acid interfered with AI-2 production of *C. perfringens* (Novak and Fratamico, 2004).

Growth of *B. anthracis* Sterne strain was decreased in the presence of furanone. As furanones were added to midlog-phase cultures of *B. anthracis*, growth and the expression of the virulence genes such as *pagA*, *lef*, and *cya* (toxin components) were inhibited, suggesting that quorum sensing inhibitors (furanones) could be used as novel therapies for anthrax (Jones et al., 2005). Interestingly, carbapenem production of *E. carotovora*, which is related to quorum sensing, was inhibited by the algal metabolite (halogenated furanone) (Manefield et al., 2001). Manefield et al. (2001) also suggested that the furanone-dependent inhibition of carbapenem production resulted from the interruption in the 3O-C_6-HSL-dependent expression of the *carABCEFGH* operon.

L. plantarum cultures and filtrates inhibit AHL production of *P. aeruginosa*, suggesting that *L. plantarum* can be a useful potential therapeutic agent against *P. aeruginosa* (Valdez et al., 2005). Fusaric acid (5-butylpicolinic acid), produced by *Fusarium* spp. (Bacon et al., 1996; Notz et al., 2002; Schouten et al., 2004), suppressed the synthesis of C_6-HSL by *Pseudomonas chlororaphis* (van Rij et al., 2004). A fish symbiont, *Rhodococcus erythropolis* W2, inhibited transfer of pathogenicity in *A. tumefaciens* and interfered with violacein production of *C. violaceum* (Uroz et al., 2003). Interestingly, a synthetic *S. epidermidis* pheromone was able to inhibit the *S. aureus agr* quorum sensing system. Thus, Otto et al. (1999) suggested that effective quorum sensing blockers to inhibit virulence factors in *S. aureus* can be designed based on the *S. epidermidis* pheromone. Since the blockade of

quorum sensing can be a novel strategy to attenuate infection of multiple antibiotic-resistant staphylococci, McDowell et al. (2001) looked for insights into the structure, activity, and turnover of the staphylococcal pheromones. Thus, they found that the exogenous synthetic autoinducing peptides prevent the production of toxic shock syndrome toxin and enterotoxin C3 by *S. aureus*, proving the likely blockade of quorum sensing as a novel therapeutic strategy.

Intriguingly, the supernatants of *P. aeruginosa* containing 3O-C_{12}-HSL and C_4-HSL inhibited *agr* expression in *S. aureus*; long chain AHL (3O-C_{12}-HSL) is able to interact with the *S. aureus* cytoplasmic membrane with a saturable, specific process and down-regulates exotoxin production, and both *sarA* and *agr* expression at sub-growth-inhibitory concentration (Qazi et al., 2006).

A. tumefaciens produces AHL lactonase, which has similar structure of the AHL lactonase of *B. cereus* (Dong et al., 2000, 2001; von Bodman et al., 2003; Zhang et al., 2002). The enzyme (AHL lactonase) hydrolyzes the homoserine lactone ring of 3O-C_8-HSL (Zhang et al., 2002). Thus, the AHL lactonases produced by *A. tumefaciens* and *B. cereus* could be used to destroy AHL molecules produced by other bacteria. Genetically engineered plants producing AHL lactonase significantly increased the resistance to *E. carotovora* infection (Dong et al., 2001; Fray, 2002). This finding is important as it relates to retardation of produce spoilage in foods.

AHL lactonase, an enzyme found in *Bacillus* spp. (Dong et al., 2000), hydrolyzes the lactone bond of AHL reducing AHL activity (Dong et al., 2001). AHL lactonase encoded by the *aiiA* gene (*aii*; autoinducer inactivation) of *Bacillus* spp. reduced the accumulation of AHL in the culture supernatant of *B. cepacia*, and lactonase expression in *B. cepacia* decreased swarming motility, protease production, biofilm formation, and *C. elegans* killing efficiency (Wang et al., 2004; Wopperer et

al., 2006). The subspecies of *B. thuringiensis* having an *aiiA* gene denatured AHL, indicating that these strains may compete with Gram-negative bacteria in the natural environment using AHL-degrading activity (Lee et al., 2002). *B. cereus* and *Bacillus mycoides* also express AHL-inactivating enzymes (Dong et al., 2002; Hu et al., 2003). Kim et al. (2005a) purified AHL lactonase from *B. thuringiensis*, and the zinc ion-bound from AHL lactonases of *B. thuringiensis* hydrolyzes AHL (Kim et al., 2005b; Liu et al., 2005; Thomas et al., 2005). These results indicate that lactonase may be useful molecule in the control of quorum sensing of pathogenic bacteria. Transgenic tobacco plants generating 3O-C_6-HSL were less infected by *E. carotovora*, and exogenous addition of AHL to the wild-type plants also enhanced their resistance to *E. carotovora* (Mae et al., 2001; Newton and Fray, 2004). Toth et al. (2004) showed that transgenic potato producing AHL had increased susceptibility to *E. carotovora* infection. These results may lead hypothesis that quorum sensing based physiological activities of some bacteria may be used to regulate quorum sensing of other pathogenic bacteria.

In addition, Park et al. (2003) revealed that the *ahlD* gene product from *Arthrobacter* IBN110 degraded AHL molecules via enzymatic catalysis (hydrolyzing lactone ring of 3O-C_6-HSL), indicating that AhlD is an AHLase. Also, they suggested that AhlD-like AHL lactonase may exist in many other bacteria. Thus, this molecule can also be used to control quorum sensing of bacteria that are involved in food spoilage and in foodborne illness. Interestingly, *Acinetobacter* is able to degrade AHL produced by *P. chlororaphis* O6 and *Burkholderia glumae* (Kang et al., 2004). Hence, *Acinetobacter* should be tested to determine whether the bacteria can also degrade the AHL molecules, produced by other bacteria. Thus, Dong et al. (2000, 2004) suggested

that signal-degrading enzymes of one bacterium may disrupt cell signaling among other bacteria and may be useful for disease prevention.

A recent study suggested that starvation of either carbon or nitrogen induces 3O-C_8-HSL degradation in *A. tumefaciens* (Zhang et al., 2004). Hence, nutrients can be one of the factors to control quorum sensing of bacteria in food environments. In a screening of 100 extracts from 50 *Penicillium* spp., 33 extracts had quorum sensing inhibition ability. Especially, patulin increased sensitivity of *P. aeruginosa* biofilm to tobramycin treatment (Rasmussen et al., 2005a). Recently, farnesol showed an inhibitory effect on mycelial development of *Ceratocystis ulmi* at up to 100μM of farnesol (Hornby et al., 2004), biofilm formation by *C. albicans* (Cao et al., 2005), and biofilm formation by the crepe, concentric, and crater phenotypes of *Candida parapsilosis* (Laffey and Butler, 2005). Very recently, a computer-aided design has been applied to design novel compounds to inhibit AHL-dependent communication systems (Riedel et al., 2006).

CONCLUSIONS AND FUTURE DIRECTIONS

Since many researchers have shown the effect of quorum sensing on physiological activities such as biofilm formation, colonization competence, stress response, antimicrobial production, and antimicrobial resistance in many bacteria, disruption of quorum sensing systems might be useful as a novel strategy to control pathogenic and food spoilage bacteria. Novel strategies, which target quorum sensing systems of the bacteria, may improve food safety and quality. Further studies should be performed to elucidate the role of quorum sensing of bacteria related to food safety and quality, and to find potential synergistic effects of quorum sensing with processing treatments used by the industry. In addition, the antagonistic effect of quorum sensing molecules

among bacteria should be clarified to improve food safety and quality using antagonistic relationships. Thus, this review could be useful for further research on quorum sensing in food related conditions.

CHAPTER III

QUORUM SENSING SIGNAL PRODUCTION BY *SALMONELLA* THOMPSON AND *ESCHERICHIA COLI* O157:H7 UNDER VARIOUS CONDITIONS

ABSTRACT

These studies compared growth and autoinducer (AI)-2 production by wild-type and AI-2-negative mutant strains of *Salmonella* Thompson and *Escherichia coli* O157:H7 in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth, evaluated the role of AI-2 in bacterial growth, and determined optimum conditions (ingredients in culture media and incubation time) for preparation of "preconditioned" (PC) media (cell-free supernatants with adjusted pH, depending on target pH) containing high AI-2 activity, and evaluated activity of AI-2-based quorum sensing of *E. coli* O157:H7 in beef purge. To compare cell growth/density (i.e., optical density; OD_{600}), and AI-2 production, *S.* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative), and *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) were incubated in LB broth at 35°C for 14 h, and the samples were analyzed every 2 h for cell counts [enumerated on tryptic soy agar (TSA)], cell density (OD_{600}), and AI-2 activity. In addition, to find the appropriate incubation time for preparation

of PC media containing AI-2 activity, AI-2-producing strains *S.* Thompson RM1987N and *E. coli* O157:H7 86-24 were incubated in LB + 0.5% glucose (LBG) broth for 8 h (*S.* Thompson strain RM1987N) and 14 h (*E. coli* O157:H7 strain 86-24) at 35°C. The samples were analyzed every 1 h (*S.* Thompson strain RM1987N) and every 2 h (*E. coli* O157:H7 strain 86-24) for cell density and AI-2 activity. To evaluate activity of AI-2-based quorum sensing of *E. coli* O157:H7 strain ATCC 43895 in beef purge, inoculated samples were incubated at 4, 10, and 25°C for 21, 18, and 9 days, respectively. The samples were analyzed every 7 days, 6 days, and 3 days during storage at 4, 10, and 25°C, respectively. Total bacterial populations were enumerated on TSA and *E. coli* O157:H7 populations were enumerated on sorbitol McConkey agar supplemented with cefixime and potassium tellurite. AI-2 activity was determined in cell-free supernatants of the samples, using the luminescence-based reporter *Vibrio harveyi* strain BB170. No difference ($P \geq 0.05$) in bacterial populations and cell density was observed between the AI-2-positive and -negative strains. AI-2 activity became constant in LBG after 4 h (*S.* Thompson strain RM1987N) and 8 h (*E. coli* O157:H7 strain 86-24) of incubation. These results indicate that mutation for AI-2 production does not affect bacterial growth of *S.* Thompson and *E. coli* O157:H7 at 35°C; AI-2-based quorum sensing of *S.* Thompson and *E. coli* O157:H7 is not involved in bacterial growth, and glucose should be contained in growth media to prepare PC media for higher AI-2 activity. In preparation of PC media, optimal time to obtain cell-free supernatants should be during the stationary phase of growth and the time is dependent on bacterial species. In beef purge inoculated with *E. coli* O157:H7, significant AI-2-like activity was not detected. This finding indicates that AI-2-based quorum sensing of *E. coli* O157:H7 may not be of significance in foods containing high levels of natural flora.

INTRODUCTION

Various bacteria possess cell-to-cell signaling systems, called quorum sensing, and use low-molecular-weight signaling compounds (autoinducers or pheromones) (Smith et al., 2004). Bacteria detect the level of these signaling molecules in their environment and they change gene expression by synthesizing the signaling molecules as bacterial populations reach a threshold (Waters and Bassler, 2005). When the cell populations are low, bacteria do not produce quorum sensing molecules. Bauer and Robinson (2002) suggested that pathogenic bacteria do not produce quorum sensing molecules at low cell levels in order to not be detected by the host immune system; however, when pathogenic bacteria reach populations sufficient to overcome the host immune system, they activate their quorum sensing system.

Bacteria synthesize various quorum sensing molecules (reviewed by Smith et al., 2004), including: (i) *N*-acylhomoserine lactone [AHL; autoinducer (AI)-1] in Gram-negative bacteria (Miller and Bassler, 2001; Whitehead et al., 2001); (ii) a furanosyl borate diester (AI-2) in both Gram-negative and Gram-positive bacteria (Chen et al., 2002; Schauder and Bassler, 2001; Xavier and Bassler, 2005a); (iii) AI-3, related to cross-communication with the mammalian epinephrine cell of host signaling system (Sperandio et al., 2003); and, (iv) oligopeptides in Gram-positive bacteria (Miller and Bassler, 2001; Whitehead et al., 2001). Diketopiperazines (Degrassi et al., 2002; Holden et al., 1999) and 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal) (Holden et al., 2000; Pesci et al., 1999) were also found in bacteria, and 3,7,11-trimethyl-2,6,10-dodecatrienoate (farnesoic acid) (Oh et al., 2001), farnesol (Hornby et al., 2001) and tyrosol (Chen et al., 2004) have been identified in yeast.

Gram-negative foodborne pathogenic bacteria such as *Escherichia coli* O157:H7, *Salmonella*, and *Campylobacter* spp. synthesize AI-2 (Cloak et al., 2002; Elvers and Park, 2002; Surette and Bassler, 1998). Recently, Turovskiy and Chikindas (2006) showed that *Listeria monocytogenes* Scott A also produced AI-2. In the process of AI-2 biosynthesis, LuxS acts as the AI-2 synthase and AI-2 is derived from *S*-adenosylmethionine via several enzymatic steps (Chen et al., 2002; Schauder et al., 2001). *S*-ribosylhomocysteine (SRH) is the substrate for LuxS; SRH is degraded to AI-2 and homocysteine (Schauder et al., 2001). *E. coli*, *Salmonella* Typhimurium, *Vibrio harveyi*, *Vibrio cholerae*, and *Enterococcus faecalis* possess an identical AI-2 biosynthetic pathway and biochemical intermediates, indicating that AI-2 is a signal used to communicate between species (Schauder et al., 2001). Recently, Xavier and Bassler (2005a) also showed interspecies communication between *V. harveyi* and *E. coli* via AI-2.

Since both Gram-negative and Gram-positive bacteria use the AI-2 signal for their gene expression, AI-2 is considered as a universal autoinducer (Lu et al., 2004). In enterohemorrhagic *E. coli* and enteropathogenic *E. coli*, AI-2 is responsible for the genes encoding the type III secretion system, the Tir (translocated intimin receptor), and intimin (adhesin) intestinal colonization (Sperandio et al., 1999). Furthermore, AI-2 was found to induce the *LEE* [locus of enterocyte effacement; pathogenicity island (Mellies et al., 1999)] operons in *E. coli* K-12 and *E. coli* O157:H7, suggesting that *E. coli* O157:H7 can colonize in the intestine through a quorum sensing molecule which is synthesized by nonpathogenic *E. coli* in the intestine (Sperandio et al., 1999), while additional work using purified AI-2 showed that AI-2 does not activate the *LEE* genes of *E. coli* O157:H7 (Sperandio et al., 2003). Kanamaru et al. (2000) proved that *E. coli* O157:H7 colonizes in the intestine via quorum sensing; SdiA (suppressor of

cell division inhibitor), a LuxR homologue of *E. coli* O157:H7, negatively regulates the expression of EspD, which is involved in the formation actin-based pedestal and intimin in *E. coli* O157:H7. Xavier and Bassler (2005a) also suggested that AI-2 signaling may be important for eukaryotes to protect themselves from pathogenic bacteria.

Production of AI-2 by *E. coli* and *S.* Typhimurium can be improved by rapid logarithmic growth, sufficient glucose level (0.5%), low pH (5.0), and high osmolarity (0.4 M NaCl) (Surette and Bassler, 1999). *Vibrio vulnificus* synthesizes more AI-2 in the presence of glucose (0.5%) and at low pH (pH 6.0) (Kim et al., 2003). *E. coli* produced AI-2 in Luria-Bertani medium (LB) containing glucose (DeLisa et al., 2001b; Hardie et al., 2003; Surette and Bassler, 1998), but no production of the molecule in LB containing no glucose was detected (Surette and Bassler, 1998). Like glucose, glycerol, maltose, galactose, L-arabinose, and ribose increased AI-2 production of *E. coli*, but lactose, D-arabinose, citrate, acetate, and pyruvate did not increase AI-2 production (Hardie et al., 2003). The degradation of extracellular AI-2 molecule was observed during stationary phase due to the depletion of glucose (Surette and Bassler, 1998, 1999). In *S.* Typhimurium, disappearance of AI-2 was caused by an ATP binding cassette (ABC) transporter designated the Lsr (*luxS* regulated) transporter (Taga et al., 2001; Xavier and Bassler, 2005b). Xavier and Bassler (2005b) elucidated the degradation of extracellular AI-2 molecule during stationary phase. In the presence of glucose, the *lsr* operon cannot be transcribed because of catabolite repression. Hence, AI-2 cannot be internalized by the Lsr transporter, and it accumulates in the environment around the cells. In the absence of glucose, AI-2 is produced, but AI-2 is rapidly internalized by the Lsr transporter because the *lsr* operon is not depressed. More specifically, the cyclic AMP-cAMP

receptor protein (cAMP-CRP) complex initiates the expression of the *lsr* operon by directly binding to the upstream region of its promoter, then it acts with the LsrR repressor to mediate AI-2 uptake (Wang et al., 2005b). Thus, it appears that there is no AI-2 production during growth of cultures containing low concentrations of glucose. Wang et al. (2005a) showed that *lsrRK* is positively controlled by CRP, but negatively regulated by LsrR. In the presence of glucose, *luxS* deletion influenced 23 genes, while 63 genes were influenced by *luxS* deletion in the absence of glucose; the *metE* gene, the *lsrACDBFG* operon, and the flanking genes of the *lsr* operon (*lsrR*, *lsrK*, *tam*, and *yneE*) were obviously induced by *luxS* in the absence of glucose; most of the highly induced genes are associated with AI-2 production and transport (Wang et al., 2005a).

Salmonella and *E. coli* O157:H7 strains, which synthesize AI-2 (wild-type strain) or no AI-2 (mutant strain) have been genetically engineered. Comparing the wild-type and mutant strains for cell growth and AI-2 production may be useful in future studies to determine the potential role of the AI-2 in foodborne pathogens. Even though positive effect of glucose (0.5%) was shown on AI-2 production (Kim et al., 2003; Surette and Bassler, 1999) and the concentration of glucose was used in preparation of "preconditioned" (PC) media (Sperandio et al., 1999), studying bacterial growth rates and changes in AI-2 production during incubation could be also useful in preparation of PC media (Sperandio et al., 1999) containing high AI-2 activity or no AI-2 activity; the PC media can be used to provide exogenous AI-2 to bacterial cultures. Thus, the objectives of this study were to compare bacterial growth and AI-2 production by wild-type and AI-2-negative mutant strains of *Salmonella* and *E. coli* O157:H7, to evaluate the role of AI-2 in growth of *Salmonella* and *E. coli* O157:H7, and to determine optimum incubation time for preparation of PC media.

Also, this study evaluated whether *E. coli* O157:H7 can produce AI-2 activity in liquid type food systems such as beef purge.

MATERIALS AND METHODS

Preparation of inoculum. Individual colonies of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) maintained in Luria-Bertani (LB; Difco, Becton Dickinson and Company, Sparks, MD) agar + nalidixic acid (25 μg/ml; Sigma-Aldrich, St. Louis, MO), and LB agar + nalidixic acid (25 μg/ml) and kanamycin (30 μg/ml; Sigma-Aldrich), respectively, were activated and subcultured (0.1 ml) in 10 ml of LB broth at 35°C for 24 h; AI-2 production phenotype of the *Salmonella* strains was not changed in LB without appropriate antibiotics during incubation at 35°C. *S.* Thompson strains RM1987N and RM1987NLUX were kindly provided by Dr. Maria T. Brandl (USDA/ARS, WRRC, Produce Safety and Microbiology Research Unit, Albany, CA). Individual colonies of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative), maintained on sorbitol McConkey agar (Difco) supplemented with cefixime and potassium tellurite (Dynal, Oslo, Norway) (SMAC-CT), were activated and subcultured according to the procedure for *S.* Thompson strains. *E. coli* O157:H7 strains 86-24 and VS94 were kindly provided by Dr. Vanessa Sperandio (University of Texas Southwestern Medical Center, Dallas, TX). In addition, a loopful of *E. coli* O157:H7 strain ATCC 43895 frozen culture maintained in glycerol supplemented (10%) LB broth at -70°C was activated and subcultured (0.1 ml) in 10 ml of LB broth at 35°C for 24 h. Stationary phase cells of all strains were harvested by centrifugation (4,629×*g*, 15 min, 4°C) and washed with phosphate buffered saline (pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 liter of distilled water;

PBS). The cell pellets were resuspended and diluted with PBS to yield approximately 4.5 log CFU/ml for *S.* Thompson strains RM1987N and RM1987NLUX, and *E. coli* O157:H7 strains 86-24 and VS94. Inoculum of *E. coli* O157:H7 strain ATCC 43895 was prepared as above at levels of 4 and 8 log CFU/ml.

Inoculation procedure. To compare growth, as cell populations and cell density (i.e., optical density; OD_{600}), and AI-2 activity between the AI-2-positive and -negative strains, a volume of 0.1 ml of the inoculum (*S.* Thompson strains RM1987N and RM1987NLUX, and *E. coli* O157:H7 strains 86-24 and VS94) was inoculated in tubes containing 10 ml of sterile LB broth and the samples were statically incubated at 35°C for 14 h. To evaluate optimum conditions for the preparation of PC medium containing high AI-2 activity (Sperandio et al., 1999), activated cultures of AI-2-producing strain *S.* Thompson RM1987N and *E. coli* O157:H7 86-24 were inoculated in tubes containing sterile LB + 0.5% glucose (Fisher Scientific, Fair Lawn, NJ) (LBG) with the 1:100 dilution, and the samples were statically incubated for 8 h (*S.* Thompson strain RM1987N) and 14 h (*E. coli* O157:H7 strain 86-24) at 35°C; since the mutant strains showed no AI-2 production during incubation in LB, only the wild-type strains were evaluated for AI-2 production in LBG broth. To evaluate activity of AI-2-based quorum sensing of *E. coli* O157:H7 in beef purge, a volume of 0.1 ml of the inoculum (*E. coli* O157:H7 strain ATCC 43895) was inoculated in tubes containing 5 ml of beef purge (passed through sterile cheese cloth, followed by five-fold dilution with sterile distilled water) and the beef purge samples were statically stored at 4, 10, and 25°C for 21, 18, and 9 days, respectively.

Microbial analysis. For comparison of growth as cell counts and cell density (OD_{600}) of the AI-2-positive and -negative strains of *S.* Thompson and *E. coli* O157:H7 in LB broth, inoculated and incubated samples were analyzed every 2 h for 14 h. To

enumerate cell counts in LB broth, serial dilutions of each sample were made in 0.1% buffered peptone-water (Difco) and 0.1 ml portions were plated onto tryptic soy agar (Difco; TSA) plates. OD_{600} values of the samples in LBG broth were measured every hour for 8 h (*S.* Thompson strain RM1987N) and every 2 h for 14 h (*E. coli* O157:H7 strain 86-24); cell counts in LBG broth were not enumerated because OD_{600} values can be rapidly used to determine the time to obtain cell-free supernatants in process for preparation of PC media during incubation. A portion of 1 ml of the culture was used for OD_{600} measurement with a spectrophotometer (Spectronic® 1001, Bausch & Lomb, Rochester, NY). The beef purge samples stored at 4, 10, and 25°C were analyzed every 7, 6, and 3 days for 21, 18, and 9 days, respectively. One ml portions of beef purge samples were diluted in 0.1% sterile buffered peptone water (BPW, Difco). After serially diluting each sample in BPW, 0.1 ml portions were plated onto each of duplicate agar plate; total bacterial populations of beef purge were enumerated on TSA, while sorbitol McConkey agar (Difco) supplemented with cefixime and potassium tellurite (Dynal, Oslo, Norway) (SMAC-CT) was used as a selective medium for *E. coli* O157:H7 strain ATCC 43895 in beef purge. Plates were incubated at 35°C for 48 h. The beef purge samples used for microbial analyses were also used to determine pH values using a pH meter with a glass pH electrode (Denver Instrument, Arvada, CO).

Autoinducer-2 bioassay. To determine AI-2 activity of the samples, the bioluminescence response of *Vibrio harveyi* strain BB170 (sensor1$^-$/sensor2$^+$) sensing only AI-2 molecule was detected (Surette and Bassler, 1998, 1999; Surette et al., 1999). Cell-free supernatants of *V. harveyi* strains BB120 (AI-1$^+$ AI-2$^+$) and BB152 (AI-1$^-$ AI-2$^+$) were used as positive controls in the AI-2 bioassay (Surette and Bassler, 1998). All *V. harveyi* strains were kindly provided by Dr. Bonnie L. Bassler

(Princeton University, Princeton, NY). Negative control was sterile autoinducer bioassay (AB) medium (Bassler et al., 1993). A 1 ml portion of the samples was centrifuged at 15,600×*g* (4°C) for 5 min in a microcentrifuge and the supernatant was subsequently passed through a filter (0.2μm, 25 mm; Nalgene, Rochester, NY) to prepare cell-free supernatants (Surette and Bassler, 1998, 1999; Surette et al., 1999). The cell-free supernatants were assayed for AI-2 activity by induction of bioluminescence in *V. harveyi* strain BB170 with a procedure similar to that described by Surette and Bassler (1998, 1999) and Surette et al. (1999). Specifically, *V. harveyi* BB170 (0.1 ml) maintained in glycerol supplemented (10 %) LB + 1.5% NaCl broth at -70°C was activated (12 h) and subcultured (7.5 h) in 10 ml of LB + 1.5% NaCl broth at 30°C with shaking (200 rpm), and diluted 1:5,000 into AB medium. The mixtures (1:9 of cell-free supernatant : diluted *V. harveyi* BB170) were incubated at 30°C for 3.5 h. AI-2 activity was measured at the same time intervals as microbial analysis. To the measure AI-2 activity of the mixture, bioluminescence was measured with a luminometer (Model TD-20e luminometer, Turner Designs, Inc., Mt. View, CA). AI-2 activity is presented as relative AI-2 activity (RAI) and was calculated as the ratio of bioluminescence of the test and negative control samples (Lu et al., 2004). The RAI was converted to log RAI/ml; RAI is referred to as AI-2 activity or AI-2-like activity (beef purge). Lu et al. (2005b) referred to AI-2 activity detected in food samples inoculated with *E. coli* O157:H7, as "AI-2-like activity".

Statistical analysis and curve fitting. The study was repeated two times with two samples for each replication. Bacterial populations and AI-2 activity data were converted to log CFU/ml and log RAI/ml, respectively, before statistical analysis. Cell counts, cell density (OD_{600}), and AI-2 activity data for the strains and incubation time, or inoculation level and storage time for beef purge were analyzed by the mixed

model procedure of SAS® (SAS institute Inc., 2002-2003). All mean comparisons were performed with the Tukey's multiple comparison and a *P*-value of 0.05 was used for statistical significance. Cell count data (log CFU/ml) were fitted with the Baranyi model (Baranyi and Roberts, 1994) using the in-house program DMFit (Institute of Food Research, Norwich, UK) to calculate exponential growth rate (EGR; log CFU/ml/h) and lag phase duration (h).

RESULTS AND DISCUSSION

Comparison of cell counts and cell density, and AI-2 production of *S.* Thompson strains RM1987N and RM1987NLUX. Initial cell counts were 2.8 and 2.9 log CFU/ml for *S.* Thompson strains RM1987N and RM1987NLUX, respectively, and increased significantly ($P<0.05$) during the 14 h at 35°C (Figure III-1). The EGR values of *S.* Thompson strains RM1987N and RM1987NLUX were 0.93 log CFU/ml/h ($R^2=0.999$) and 0.91 log CFU/ml/h ($R^2=0.998$), respectively. This indicates that AI-2-based quorum sensing of *S.* Thompson is not associated with growth at 35°C. The EGR values can be used to estimate the cell counts of *S.* Thompson strains RM1987N and RM1987NLUX in other studies. Estimated lag phase durations (LPD) were also not different (approximately 1.3 h) and stationary phase started after 8 h of incubation (Figure III-1). In agreement with these findings, the mutation of *swrI* (*luxI* homologue) in *Serratia liquefaciens* did not affect growth rate (Eberl et al., 1996).

The OD_{600} values of *S.* Thompson strains RM1987N and RM1987NLUX were not significantly different ($P\geq0.05$), and they did not increase significantly during the first 4 h of incubation (bacterial populations of *S.* Thompson at 4 h of incubation: 5.3 to 5.4 log CFU/ml, regardless of strain), which is a longer time than the estimated lag phase time (approximately 1.3 h) (Figure III-2). This indicates that OD_{600} values may

not be appropriate for evaluation of the concentration of bacteria during the early incubation period. OD_{600} values also showed that stationary phase started after 8 h of incubation (Figure III-2).

AI-2 activity of *S.* Thompson strain RM1987N in LB broth increased significantly ($P<0.05$) by 3.1 log RAI/ml (OD_{600} = 0.624) during the exponential phase, but it decreased rapidly ($P<0.05$) immediately after stationary phase started (Figure III-2). As expected, *S.* Thompson strain RM1987NLUX did not produce AI-2 during the incubation (Figure III-2). These results suggest that use of LB broth to prepare PC media containing AI-2 molecules may not be appropriate due to the rapid decrease of AI-2 activity during the stationary phase of growth.

Since higher AI-2 activity was detected at higher glucose concentrations (DeLisa et al., 2001b; Hardie et al., 2003; Kim et al., 2003; Surette and Bassler, 1998), we examined AI-2 production by *S.* Thompson strain RM1987N in LBG broth during 8 h of incubation. To set up the simple and rapid procedure for the preparation of the PC media, a portion of the activated culture of *S.* Thompson strain RM1987N in LB broth was inoculated into LBG broth at the ratio of 100-fold dilution. AI-2 activity of *S.* Thompson strain RM1987N reached a maximum after 4 h of incubation at 35°C in LBG, which was the beginning of stationary phase and the AI-2 activity was not significantly ($P\geq0.05$) changed during stationary phase (Figure III-3). After 4 h of incubation of *S.* Thompson strain RM1987N, AI-2 activity ranged from 3.3 to 3.8 log RAI/ml ($P\geq0.05$) and OD_{600} values were within 0.656 to 0.771 ($P\geq0.05$). Unlike AI-2 production by *S.* Thompson strain RM1987N in LB broth, AI-2 activity stayed constant in LBG broth during the stationary phase (Figure III-3). These results suggest that the optimum incubation time should be more than 4 h and $OD_{600} \geq 0.65$ in LBG broth for PC media preparation. In agreement, *Vibrio vulnificus* also produced

more AI-2 activity in the presence of glucose (0.5%) and at lower pH (pH 6.0) (Kim et al., 2003). Maximum AI-2 activity in LB broth (3.1 log RAI/ml) was slightly lower than the value in LBG (3.8 log RAI/ml) broth. During stationary phase, OD_{600} values were higher in LBG (0.656 to 0.771) than in LB (0.615 to 0.645) (Figures III-2 and III-3). Production of AI-2 by *S.* Typhimurium was increased by various extracellular factors such as rapid logarithmic growth, preferred carbon source, low pH, and high osmolarity (Surette and Bassler, 1999).

Comparison of bacterial populations and OD_{600}, and AI-2 production by *E. coli* O157:H7 strains 86-24 and VS94. Cell counts were 2.3 log CFU/ml at 0 h for both strains of *E. coli* O157:H7 (Figure III-4). Like the *S.* Thompson strains, no variations of cell counts of *E. coli* O157:H7 strains 86-24 and VS94 were observed ($P \geq 0.05$) during incubation in LB broth at 35°C for 14 h (Figure III-4). Furthermore, EGRs were also not different between the two strains of *E. coli* O157:H7 (*E. coli* O157:H7 strain 86-24: 0.90 log CFU/ml/h, R^2=0.947; *E. coli* O157:H7 strain VS94: 0.89 log CFU/ml/h, R^2=0.951) (Figure III-4). These EGRs can be also applied to estimate the cell counts of *E. coli* O157:H7 strains 86-24 and VS94 in other studies under the same conditions. Estimated lag phase durations (LPD) were approximately 1.7 h and 1.6 h for *E. coli* O157:H7 strains 86-24 and VS94, respectively. Like the *S.* Thompson strains, stationary phase of the *E. coli* O157:H7 strains started after 8 h of incubation (Figure III-4). These results indicate that the mutation in AI-2 production of *E. coli* O157:H7 does not affect cell growth; in other words, AI-2-based quorum sensing of *E. coli* O157:H7 is not related to growth at 35°C. Estimated lag phase durations of *E. coli* O157:H7 strains are slightly longer than those of the *S.* Thompson strains.

The OD_{600} values of *E. coli* O157:H7 strain 86-24 were not significantly ($P \geq 0.05$) different than the values of *E. coli* O157:H7 strain VS94 in LB broth (Figure

III-5). Interestingly, OD_{600} values of both strains of *E. coli* O157:H7 in LB broth were not changed by 6 h of incubation, which is a longer time than the estimated lag phase time of both strains of *E. coli* O157:H7 (Figure III-5). OD_{600} values showed that stationary phase started after 10 h of incubation in LB broth (Figure III-5). After 6 h of incubation in LB broth, AI-2 activity of *E. coli* O157:H7 strain 86-24 significantly ($P<0.05$) increased by 3.7 log RAI/ml (Figure III-5).

Unlike the *S.* Thompson strain RM1987N, the AI-2 activity (3.2 to 3.7 log RAI/ml) of *E. coli* O157:H7 strain 86-24 did not decrease rapidly ($P\geq0.05$) in LB broth during stationary phase (Figure III-5). As expected, *E. coli* O157:H7 strain VS94 did not produce AI-2 during incubation (Figure III-5). Because of the same reason for the *S.* Thompson strain RM1987N, we evaluated the AI-2 production by *E. coli* O157:H7 strain 86-24 in LBG broth for the 14 h of incubation. A portion of the activated culture of *E. coli* O157:H7 strain 86-24 in LB broth was inoculated into LBG broth at the ratio of 100-fold dilution. AI-2 activity of *E. coli* O157:H7 strain 86-24 reached a maximum (4.1 log RAI/ml) after 6 h of incubation at 35°C in LBG broth, which was the early stationary phase and the AI-2 activities stayed constant (3.9 to 4.1 log RAI/ml; $P\geq0.05$) during stationary phase (Figure III-6). Maximum AI-2 activity of *E. coli* O157:H7 was slightly higher in LBG (4.1 log RAI/ml) than in LB broth (3.7 log RAI/ml) and OD_{600} values were higher in LBG broth (1.076 to 1.159) than in LB broth (0.777 to 0.855) during stationary phase (Figures III-5 and III-6). These results suggest that optimum incubation time should be more than 6 h and $OD_{600} \geq 1.0$ in LBG broth for PC media preparation of *E. coli* O157:H7.

DeLisa et al. (2001b), Hardie et al. (2003), and Surette and Bassler (1998) also proved that *E. coli* produces AI-2 activity in LB broth containing glucose. Surette and Bassler (1998) showed no production of the molecule by *E. coli* and *Salmonella* in LB

broth containing no glucose. In other studies, degradation of extracellular AI-2 molecule of *E. coli* and *Salmonella* was observed during stationary phase (Surette and Bassler, 1998, 1999). They suggested that this was the result of the depletion of glucose during stationary phase. Hardie et al. (2003) suggested that modulation of LuxS does not regulate alteration in extracellular AI-2 levels in response to glucose. Xavier and Bassler (2005b) explained differently the degradation of extracellular AI-2 molecules during stationary phase. In the presence of glucose, AI-2 cannot be internalized by Lsr (*luxS* regulated) transporter because glucose depresses the *lsr* operon, resulting in the accumulation of AI-2 molecule in the extracellular environment. However, even though AI-2 molecules are produced, AI-2 is rapidly internalized by the Lsr transporter in the absence of glucose. Thus, presence of AI-2 activity seems very transitory. The cAMP-CRP complex directly binds to the upstream region of the *lsr* operon promoter for the expression of the *lsr* operon, controlling AI-2 uptake (Wang et al., 2005b). In our study, higher concentration of glucose (0.5%) also resulted in higher OD_{600} values with constant AI-2 activity during stationary phase.

Activity of AI-2-based quorum sensing of *E. coli* O157:H7 in beef purge. Lu et al. (2005b) used the term "AI-2-like activity" instead of "AI-2 molecules" in their study because they were not sure that the response observed in the AI-2 bioassay with food samples was caused only by AI-2 molecules. They detected AI-2-like activity in food samples such as tomatoes, carrots, cantaloupes, fish, eggplant, squash, green peppers, peaches, cucumbers, potatoes, oranges, apples, and bananas (Lu et al., 2004, 2005a). Therefore, we also called the AI-2 activity detected in *E. coli* O157:H7 strain ATCC 43895 cultures inoculated beef purge sample as AI-2-like activity.

The pH values of the beef purge samples were 5.48 to 5.49 at day 0. In general, the pH values of the samples ranged from 4.46 to 6.46 during storage regardless of storage temperature, storage time, and inoculation level (Table III-1).

At day 0, total bacterial populations were 4.9, 4.9, and 6.3 log CFU/ml in the samples inoculated with no, 2, and 6 log CFU/ml of *E. coli* O157:H7 strain ATCC 43895, respectively (Figures III-7 to III-9). During storage, total bacterial populations significantly ($P<0.05$) increased, regardless of storage temperature and inoculation level (Figures III-7 to III-9). The cell counts of *E. coli* O157:H7 strain ATCC 43895 decreased during storage at 4°C (Figure III-7), did not change dramatically at 10°C (Figure III-8), and increased ($P<0.05$) during storage at 25°C (Figure III-9). Although growth of *E. coli* O157:H7 strain ATCC 43895 was variable in the samples, depending on storage temperature, significant AI-2-like activity was not observed at all storage temperatures and inoculation levels (Figures III-10 to III-12). These results indicate that AI-2-based quorum sensing of *E. coli* O157:H7 may not be active in beef purge. While Cloak et al. (2002) showed that *E. coli* O157:H7, *Salmonella*, and *Campylobacter* spp. produced AI-2 in milk, chicken broth, and brucella broth. Therefore, we assume that *E. coli* O157:H7 may not produce AI-2 in the environment with high levels of natural flora. Cloak et al. (2002) suggested that production of autoinducer could be influenced by environmental factors such as the type of food or other sample matrix, incubation temperature, and atmospheric conditions.

For further research to evaluate the roles of AI-2 in food safety, AI-2 activity should be evaluated in rumen microbial communities, and the human stomach and intestine environments. AI-2 activity may be related to *E. coli* O157:H7 survival in rumen microbial communities of cattle, and the human stomach and intestine environments. Mitsumori et al. (2003) found that the quorum sensing system

regulated by AI-2 is present in the rumen microbial community. AI-2 of enterohemorrhagic *E. coli* and enteropathogenic *E. coli* is responsible for the genes encoding the type III secretion system, the Tir, and intimin for intestinal colonization (Sperandio et al., 1999). In addition, the *LEE* operons are induced by AI-2 of *E. coli* K-12 and *E. coli* O157:H7, suggesting that *E. coli* O157:H7 colonizes in intestine by quorum sensing signal produced by nonpathogenic *E. coli* (Sperandio et al., 1999). *LEE2* and *LEE3* operons of enteropathogenic *E. coli* are activated by Ler (*LEE*-encoded regulator) (Sperandio et al., 2000); *LEE* operon is regulated by AI-2 via Ler (Sperandio et al., 1999). Xavier and Bassler (2005a) suggested that AI-2 signaling may be important methods to protect eukaryotes from pathogenic bacteria. While in additional work, Sperandio et al. (2003) showed that AI-2 does not activate the *LEE* genes of *E. coli* O157:H7. They obtained two fractions from the freeze-dried culture of *E. coli* O157:H7 dissolved in cold buffer solution via C-18 columns; the fraction containing AI-2 (eluted with buffer solution) or AI-3 (eluted with methanol). Interestingly, the fraction containing AI-2 did fail to initiate the transcription of *LEE1*, but the fraction containing AI-3 activated *LEE1* transcription and flagella genes. *E. coli* O157:H7 may use AI-3 to communicate with the host (Sperandio et al., 2003). Thus, the role of AI-3 in food safety should be further studied.

CONCLUSIONS

In conclusion, the present study has demonstrated that mutation for AI-2 production of *S.* Thompson and *E. coli* O157:H7 does not affect bacterial growth; AI-2-based quorum sensing is not involved in growth of *S.* Thompson and *E. coli* O157:H7 at 35°C. Therefore, the strains of *S.* Thompson and *E. coli* O157:H7 can be used in other studies to examine the potential roles of AI-2 of these bacteria in food safety and

spoilage. For preparation of PC media, 0.5% of glucose should be used in growth media, and incubation time is dependent on the bacteria; cell-free supernatants from growth can be used during stationary phase to prepare PC media.

AI-2-based quorum sensing of *E. coli* O157:H7 may not be active in beef purge containing high levels of natural flora. Thus, AI-2-based quorum sensing of *E. coli* O157:H7 may not be of significance in foods containing high level of natural flora. Further research on the role of AI-2 produced by *E. coli* O157:H7 in other foods is necessary because a clear understanding of the roles of AI-2 produced by bacteria in foods could be potentially be helpful in improving food safety.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. Pina M. Fratamico, Dr. John S. Novak, Dr. Bonnie L. Bassler, Dr. Vanessa Sperandio, and Dr. Maria T. Brandl for their friendly and critical advice, and the strains used in this study. This work was funded by the Colorado State University Agricultural Experiment Station.

Table III-1. pH values (mean ± standard error) of beef purge, uninoculated or inoculated (2 or 6 log CFU/ml) with *Escherichia coli* O157:H7 strain ATCC 43895, during storage at 4, 10, and 25°C for 21, 18, and 9 days, respectively

Temperature (°C)	Storage time (days)	Inoculation level		
		No inoculation	2 log CFU/ml	6 log CFU/ml
4	0	5.48±0.00 ^{Aabc}	5.48±0.01 ^{Aa}	5.49±0.01 ^{Aab}
	7	4.97±0.14 ^{Abcd}	4.90±0.21 ^{Aa}	4.89±0.18 ^{Aab}
	14	4.76±0.04 ^{Ad}	4.78±0.02 ^{Aa}	5.70±0.53 ^{Aab}
	21	4.87±0.05 ^{Acd}	4.84±0.01 ^{Aa}	5.93±0.44 ^{Aa}
10	0	5.48±0.00 ^{Aabc}	5.48±0.01 ^{Aa}	5.49±0.01 ^{Aab}
	6	5.01±0.37 ^{Abcd}	4.73±0.12 ^{Aa}	4.65±0.03 ^{Ab}
	12	5.72±0.51 ^{Aab}	4.71±0.03 ^{Aa}	4.97±0.11 ^{Aab}
	18	6.46±0.47 ^{Aa}	4.88±0.02 ^{Ba}	5.44±0.56 ^{Bab}
25	0	5.48±0.00 ^{Aabc}	5.48±0.01 ^{Aa}	5.49±0.01 ^{Aab}
	3	4.64±0.04 ^{Ad}	4.87±0.20 ^{Aa}	4.83±0.11 ^{Aab}
	6	5.13±0.16 ^{Abcd}	4.93±0.05 ^{Aa}	5.03±0.03 ^{Aab}
	9	5.21±0.03 ^{Abcd}	5.09±0.08 ^{Aa}	5.34±0.11 ^{Aab}

^{AB}: means within the same row with different superscript letters are different ($P<0.05$).

^{abcd}: means within the same column with different superscript letters are different ($P<0.05$).

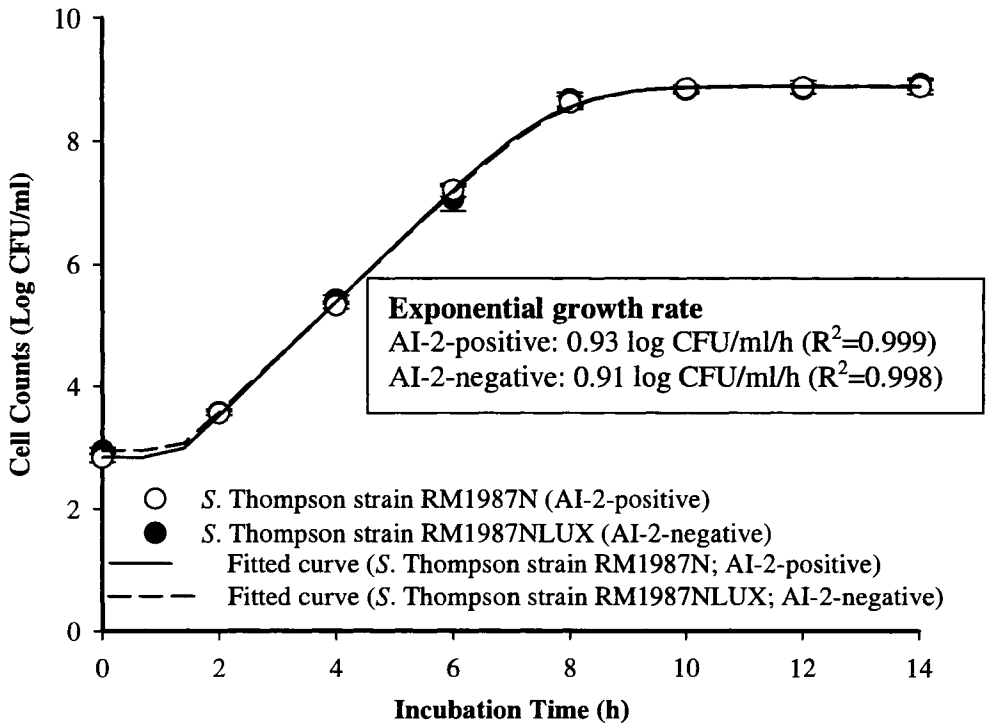

Figure III-1. Cell counts of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative), recovered with tryptic soy agar, during incubation at 35°C for 14 h in Luria-Bertani broth; fitted curves and exponential growth rates (log CFU/ml/h) were generated by the Baranyi model (Baranyi and Roberts, 1994) (Data in APPENDIX Table 1).

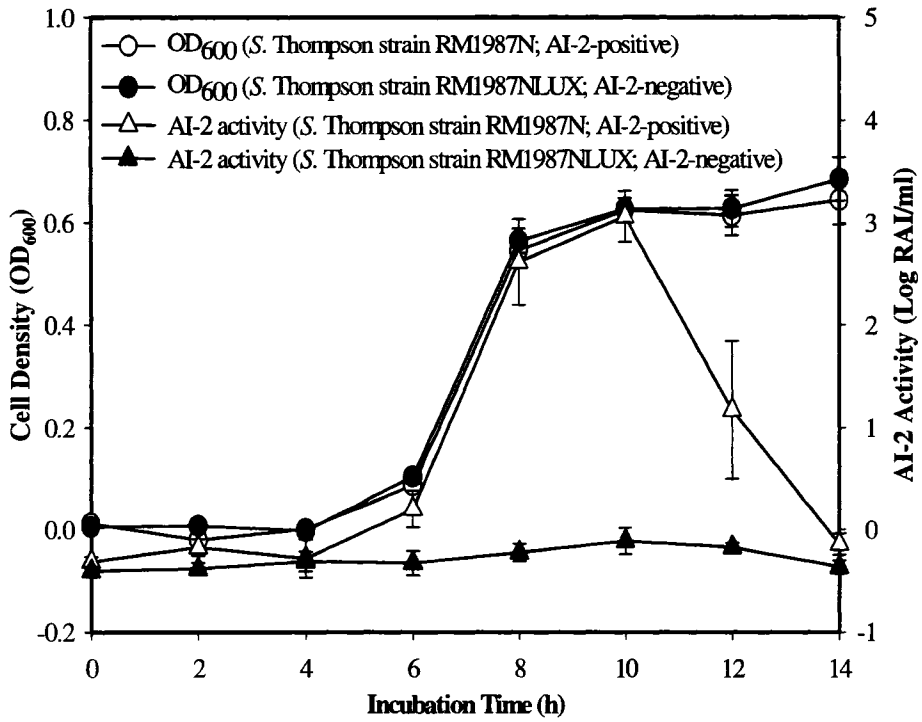

Figure III-2. Cell density and AI-2 activity of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) during incubation at 35°C for 14 h in Luria-Bertani broth (Data in APPENDIX Table 2).

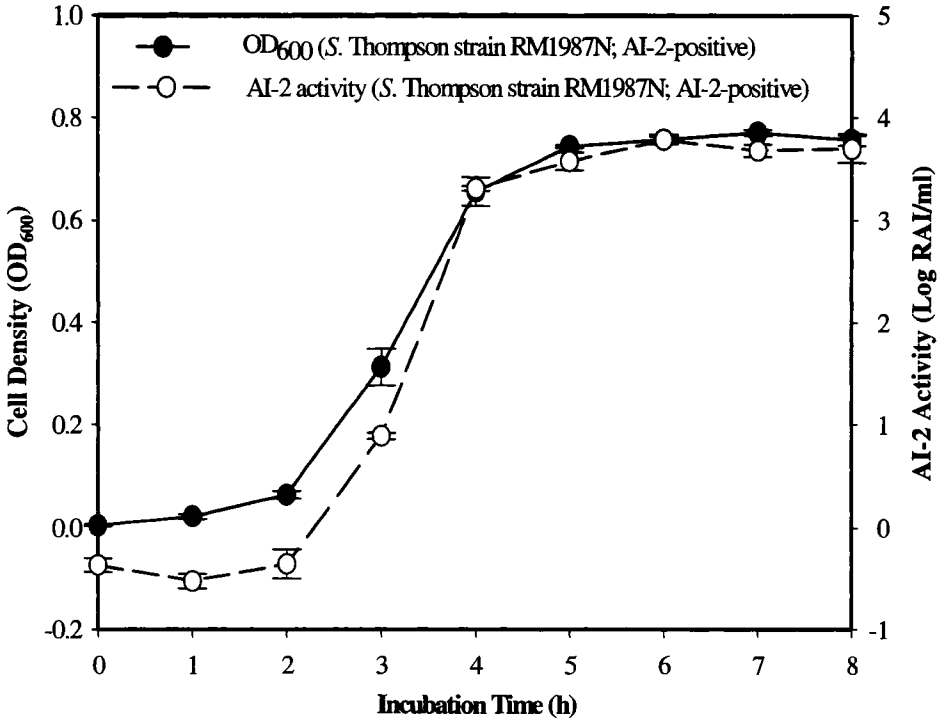

Figure III-3. Cell density and AI-2 activity of *Salmonella* Thompson strain RM1987N (AI-2-positive) during incubation at 35°C for 8 h in Luria-Bertani + 0.5% glucose broth (Data in APPENDIX Table 3).

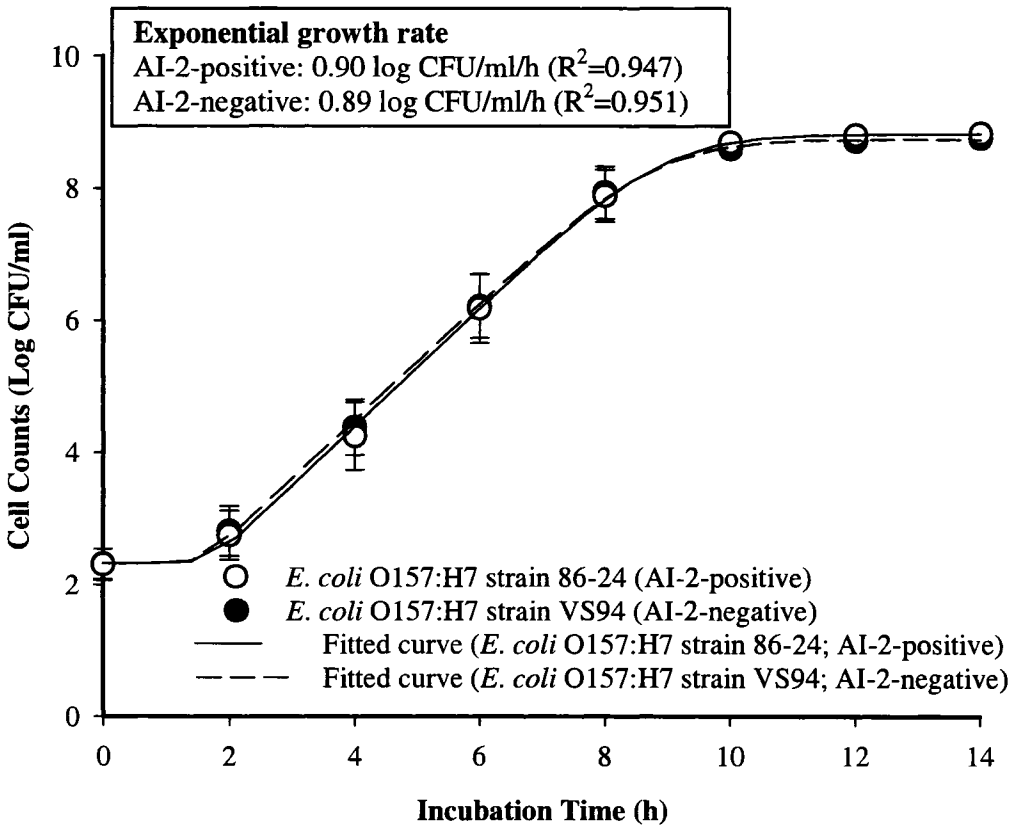

Figure III-4. Cell counts of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative), recovered with tryptic soy agar, during incubation at 35°C for 14 h in Luria-Bertani broth; fitted curve lines and exponential growth rates (log CFU/ml/h) were generated by the Baranyi model (Baranyi and Roberts, 1994) (Data in APPENDIX Table 1).

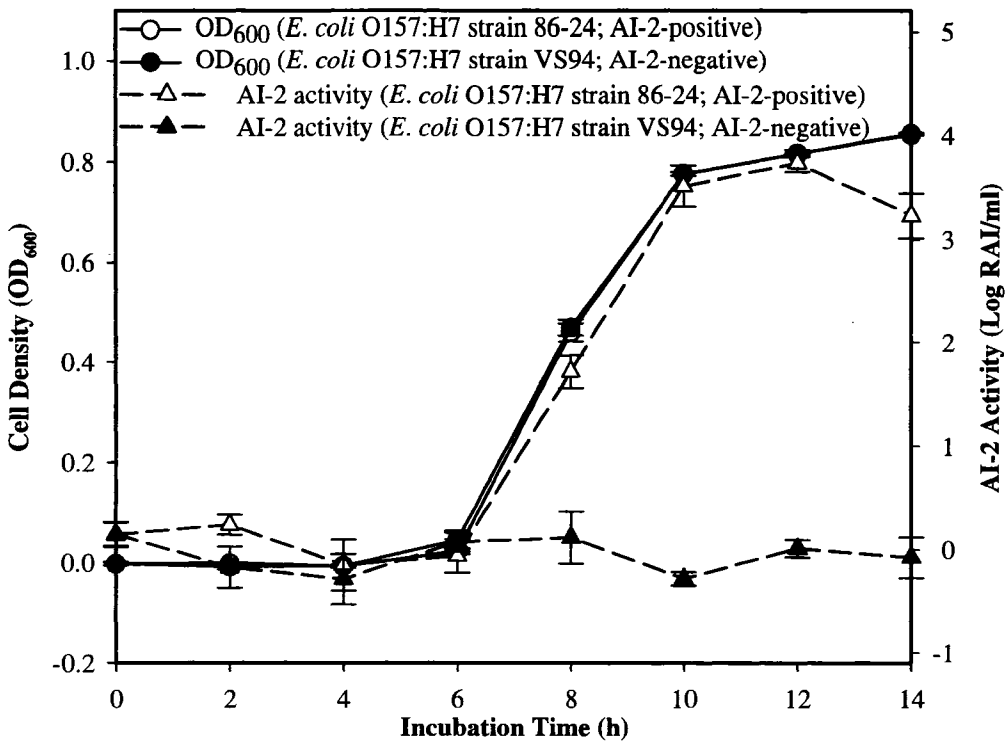

Figure III-5. Cell density and AI-2 activity of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) during incubation at 35°C for 14 h in Luria-Bertani broth (Data in APPENDIX Table 4).

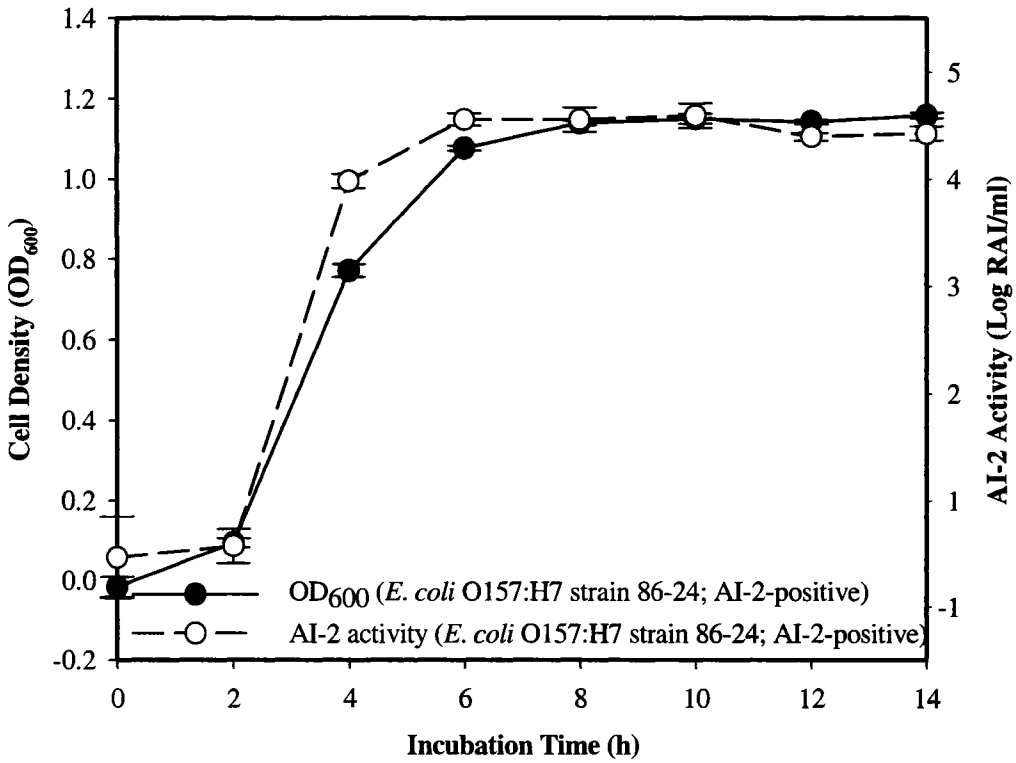

Figure III-6. Cell density and AI-2 activity of *Escherichia coli* O157:H7 strain 86-24 (AI-2-positive) during incubation at 35°C for 14 h in Luria-Bertani + 0.5% glucose broth (Data in APPENDIX Table 5).

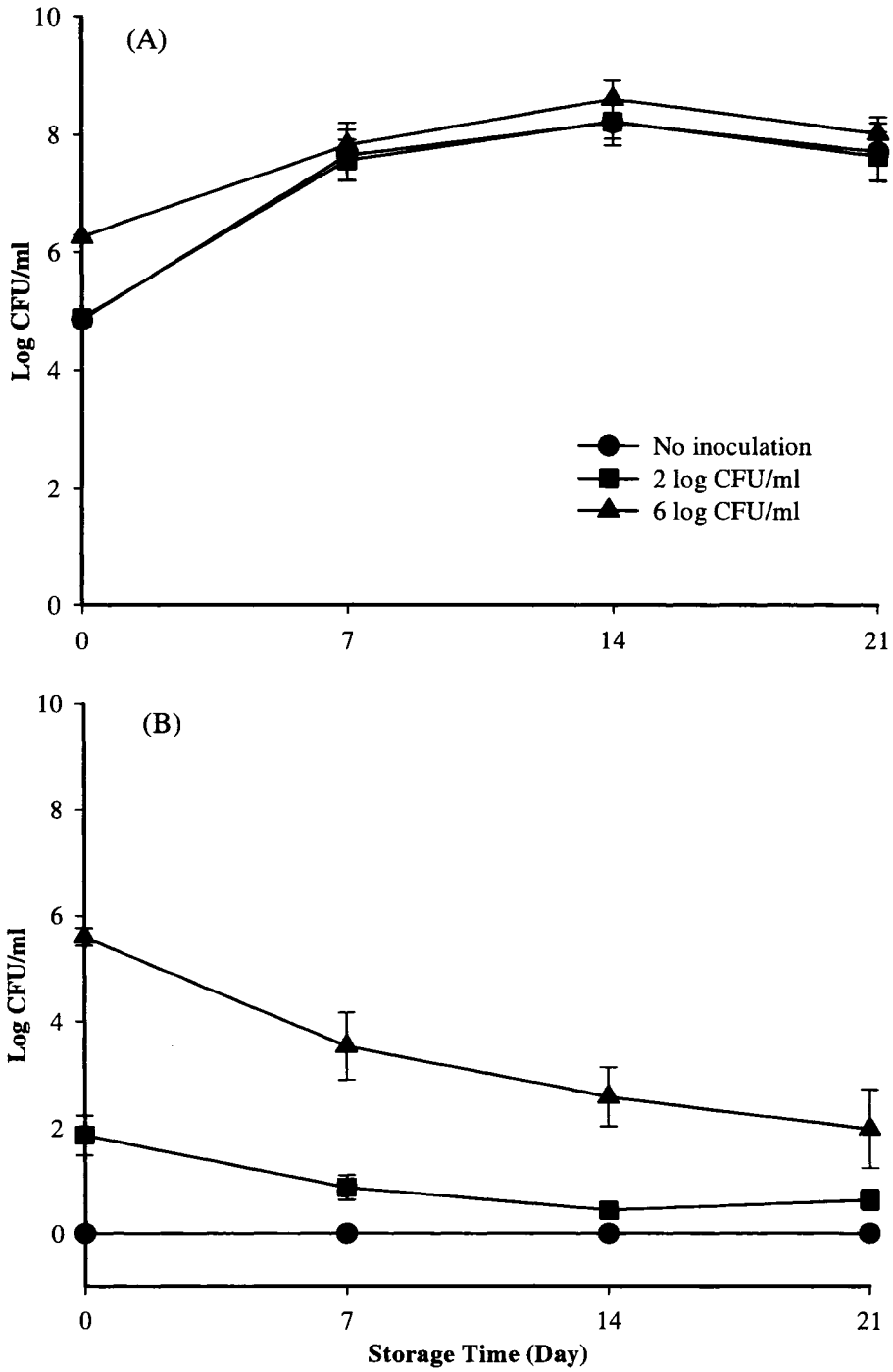

Figure III-7. Total bacterial populations recovered with tryptic soy agar (TSA; A) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (SMAC-CT; B) in beef purge, inoculated with no, 2, or 6 log CFU/ml of *E. coli* O157:H7 strain ATCC 43895, during storage at 4°C for 21 days (Data in APPENDIX Table 6).

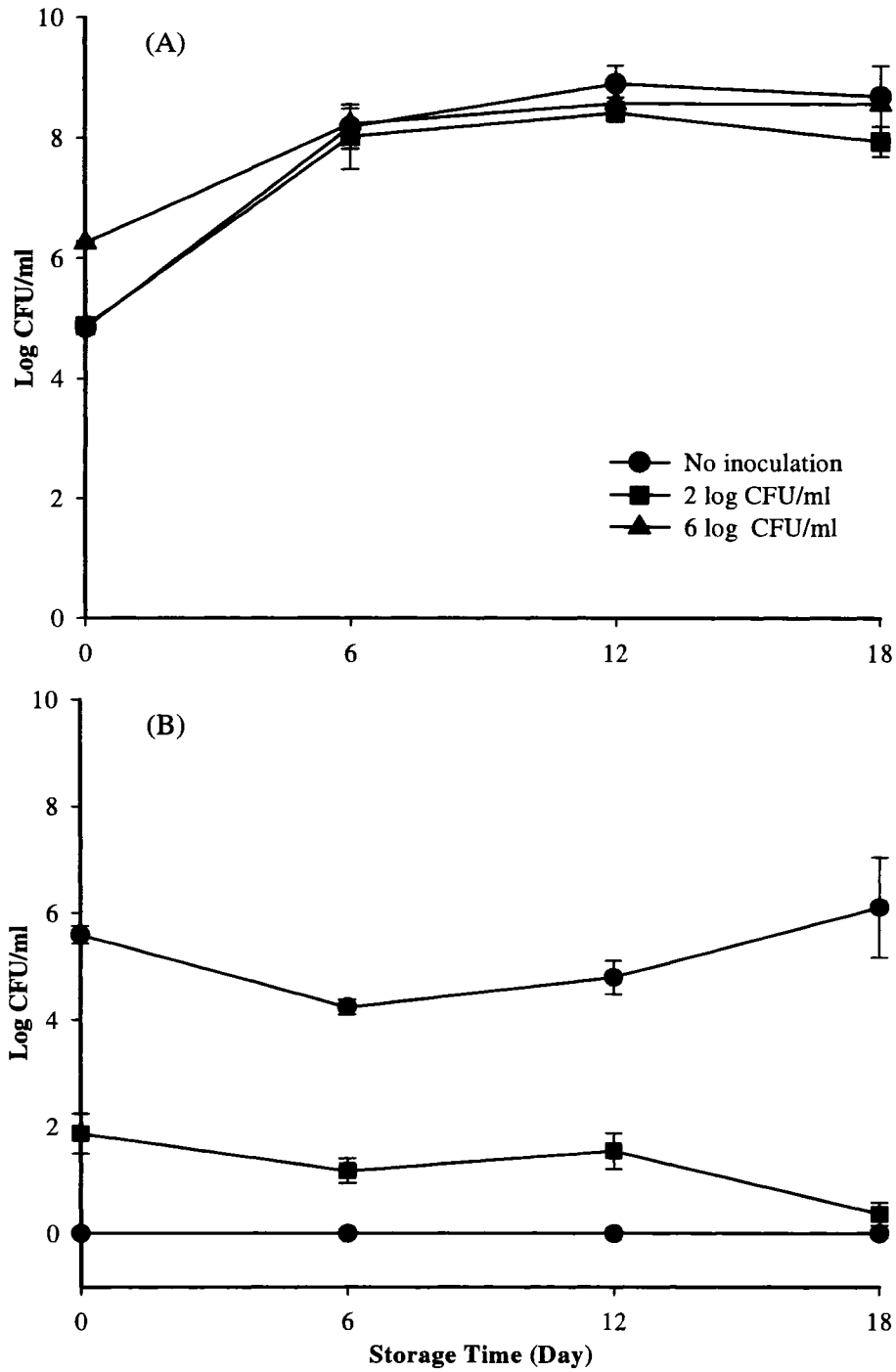

Figure III-8. Total bacterial populations recovered with tryptic soy agar (TSA; A) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (SMAC-CT; B) in beef purge, inoculated with no, 2, or 6 log CFU/ml of *E. coli* O157:H7 strain ATCC 43895, during storage at 10°C for 18 days. (Data in APPENDIX Table 7).

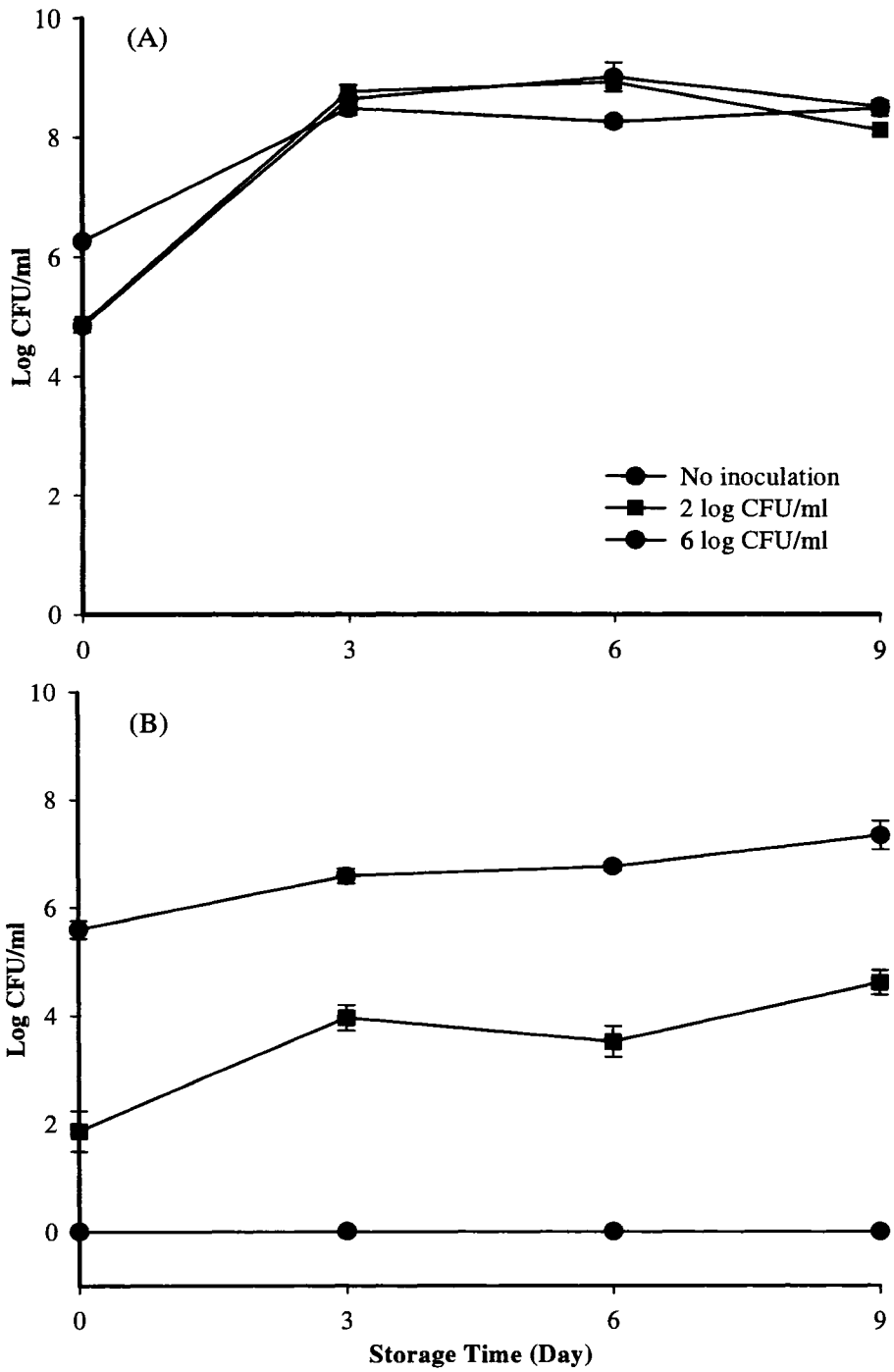

Figure III-9. Total bacterial populations recovered with tryptic soy agar (TSA; A) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (SMAC-CT; B) in beef purge, inoculated with no, 2, or 6 log CFU/ml of *E. coli* O157:H7 strain ATCC 43895, during storage at 25°C for 9 days (Data in APPENDIX Table 8).

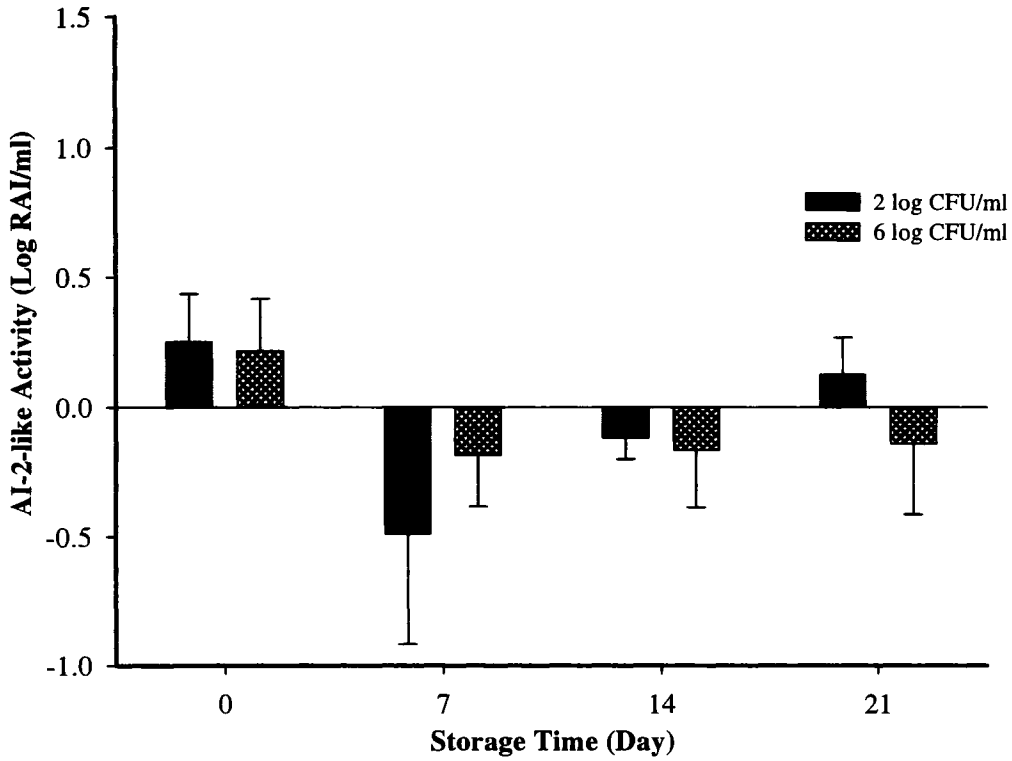

Figure III-10. AI-2-like activity in beef purge inoculated (2 or 6 log CFU/ml) with *Escherichia coli* O157:H7 strain ATCC 43895 during storage at 4°C for 21 days. Data presented in the figures show differences in AI-2-like activity between *E. coli* O157:H7 strain ATCC 43895 inoculated and uninoculated samples (Data in APPENDIX Table 9).

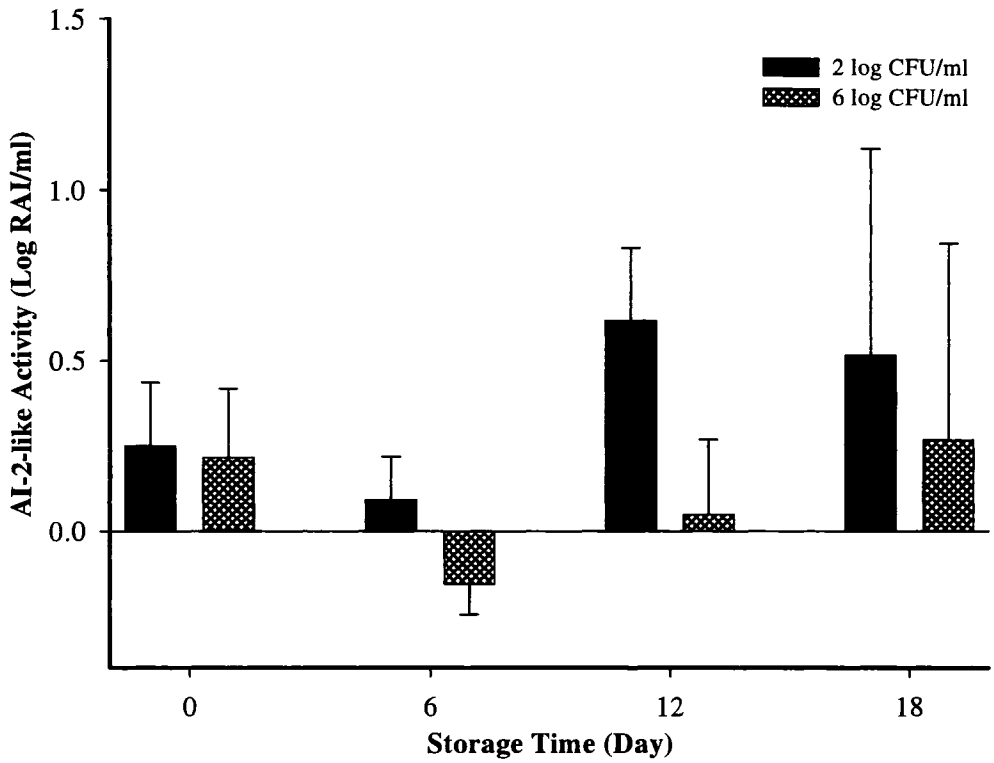

Figure III-11. AI-2-like activity in beef purge inoculated (2 or 6 log CFU/ml) with *Escherichia coli* O157:H7 strain ATCC 43895 during storage at 10°C for 18 days. Data presented in the figures show differences in AI-2-like activity between *E. coli* O157:H7 strain ATCC 43895 inoculated and uninoculated samples (Data in APPENDIX Table 10).

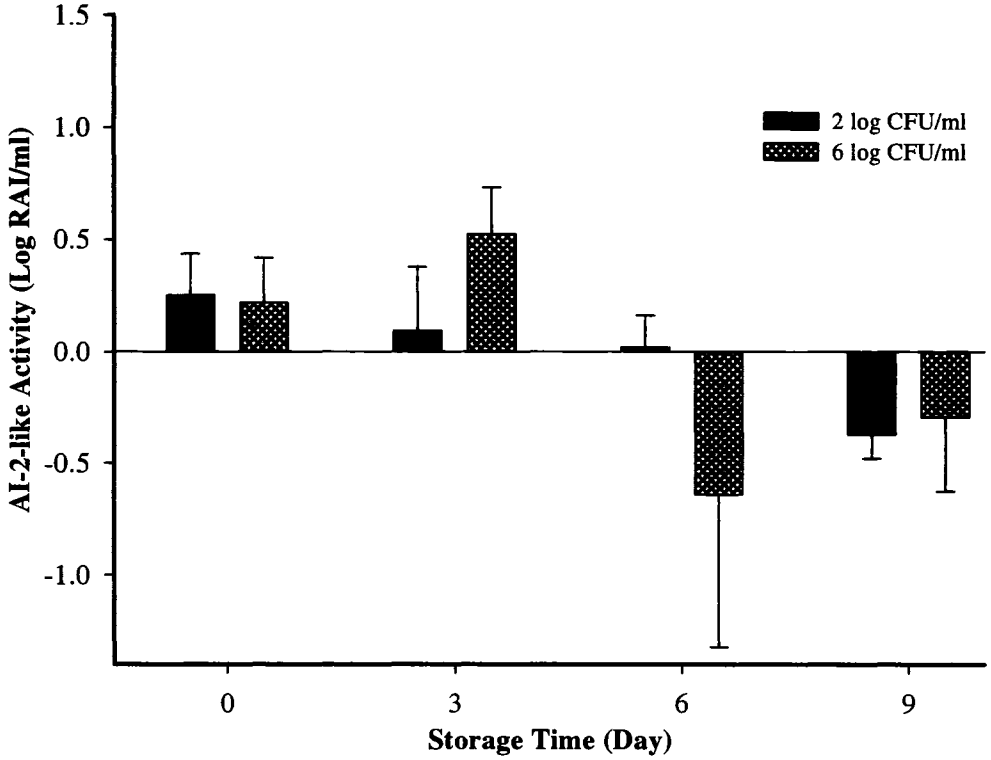

Figure III-12. AI-2-like activity in beef purge inoculated (2 or 6 log CFU/ml) with *Escherichia coli* O157:H7 strain ATCC 43895 during storage at 25°C for 9 days. Data presented in the figures show differences in AI-2-like activity between *E. coli* O157:H7 strain ATCC 43895 inoculated and uninoculated samples (Data in APPENDIX Table 11).

CHAPTER IV

AUTOINDUCER-2 ACTIVITY BY GRAM-NEGATIVE BACTERIA PATHOGENS AND ITS INFLUENCE ON BIOFILM FORMATION

ABSTRACT

Bacteria possess cell-to-cell density-dependent signaling systems, called quorum sensing, which have been proposed to be associated with cellular activities including biofilm formation. The potential role of quorum sensing in biofilm formation on food contact surfaces is unknown. This study evaluated whether autoinducer-2 (AI-2) activity, involved in quorum sensing of Gram-negative bacteria, is involved in biofilm formation on food contact surfaces by *Salmonella* and *Escherichia coli* O157:H7. Study I: *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP and *E. coli* O157:H7 strain ATCC 43895/ISEHGFP were individually inoculated into 50 ml of Luria-Bertani (LB) and LB + 0.5% glucose (LBG) broth, without or with stainless steel or polypropylene (only *Salmonella*) coupons (2 × 5 cm). At 0, 14 (only *Salmonella*), 24, 48 and 72 h of storage (25°C), samples of cell suspension and detached cells from the coupons obtained by 2 min-vortexing were plated on tryptic soy agar and incubated at 35°C for 48 h. Study II: *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative), and *E. coli* O157:H7

strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) were inoculated into LB broth with stainless steel coupons. Cell counts were enumerated as in Study I. In both studies, AI-2 activity was determined in cell-free supernatants, using the luminescence-based reporter strain *Vibrio harveyi* strain BB170. The studies were replicated twice. *Salmonella* formed more ($P<0.05$) biofilm in LB than LBG, but *E. coli* O157:H7 showed no difference ($P\geq0.05$) in biofilm formation between LB and LBG. Both *Salmonella* and *E. coli* O157:H7 produced higher ($P<0.05$) AI-2 activity in LBG than LB cell suspensions (Study I). Populations of the AI-2-positive and -negative *Salmonella* and *E. coli* O157:H7 strains were not different ($P\geq0.05$) within suspension or coupons (Study II). The results indicated no difference in biofilm density with variable AI-2 activity, and thus, quorum sensing may not be of significance in food equipment sanitation.

INTRODUCTION

Bacteria may use cell-to-cell density-dependent signaling systems (quorum sensing), based on low-molecular-weight signaling compounds called autoinducers or pheromones (Smith et al., 2004). As the low-molecular-weight signaling molecules reach threshold concentrations around bacteria, cells sense the accumulation of the molecules and change gene expression (Waters and Bassler, 2005). Quorum sensing signals can be categorized into four groups (reviewed by Smith et al., 2004): (i) *N*-acylhomoserine lactone (AHL) in Gram-negative bacteria (Miller and Bassler, 2001; Whitehead et al., 2001); (ii) a furanosyl borate diester called autoinducer (AI)-2 in both Gram-negative and Gram-positive bacteria (Chen et al., 2002; Schauder and Bassler, 2001; Xavier and Bassler, 2005a); (iii) AI-3, involved in cross-communication with the mammalian epinephrine cell of host signaling system

(Sperandio et al., 2003); and, (iv) oligopeptides in Gram-positive bacteria (Miller and Bassler, 2001; Whitehead et al., 2001). In addition, diketopiperazines (Degrassi et al., 2002; Holden et al., 1999) and 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal) (Holden et al., 2000; Pesci et al., 1999) were revealed in bacteria, and 3,7,11-trimethyl-2,6,10-dodecatrienoate (farnesoic acid) (Oh et al., 2001), farnesol (Hornby et al., 2001) and tyrosol (Chen et al., 2004) have been identified in yeast.

Bacteria use quorum sensing to regulate behaviors or properties such as virulence (von Bodman et al., 1998), plasmid conjugation (Lithgow et al., 2001), bioluminescence (Cao and Meighen, 1989), protein production (DeLisa et al., 2001c), the global system of virulence (Pirhonen et al., 1993), competence development (the ability to internalize exogenous DNA) (D'Souza et al., 1994; Hahn et al., 1996; Hamoen et al., 1995; Li et al., 2001a, b; Peterson et al., 2004a, b; Steinmoen et al., 2002; van Sinderen et al., 1995), and biofilm formation (Davies et al., 1998). *Pseudomonas aeruginosa* has been reported to use the quorum sensing system to regulate its pathogenesis mechanisms such as virulence factor expression, biofilm formation, adhesion (Singh et al., 2000; Smith and Iglewski, 2003), as well as twitching and swarming motility in the lung (Glessner et al., 1999; Kohler et al., 2000).

Major Gram-negative foodborne pathogenic bacteria such as *Escherichia coli* O157:H7, *Salmonella*, and *Campylobacter* spp. produce AI-2 (Cloak et al., 2002; Elvers and Park, 2002; Surette and Bassler, 1998), which is used for gene expression in various Gram-negative and Gram-positive bacteria. Thus, AI-2 is regarded as a universal autoinducer (Lu et al., 2004). Chen et al. (2002) revealed that AI-2 is formed from *S*-adenosylmethionine via at least three enzymatic steps. In *E. coli* and

Salmonella Typhimurium, the production of AI-2 can be increased by various exogenous conditions such as rapid logarithmic growth, preferred carbon source, high osmolarity, and low pH (Surette and Bassler, 1999). In addition, *S.* Typhimurium, *E. coli* O157:H7, and *Campylobacter* spp. are able to synthesize AI-2 in foods such as milk, chicken broth, and brucella broth at various storage temperatures (4, 25, and 37°C) (Cloak et al., 2002). AI-2-like activity was observed in some uninoculated fresh and processed foods such as frozen fish, tomatoes, cantaloupe, carrots, tofu, and milk. Other products, however, such as turkey patties, chicken breast, homemade cheeses, beef steak, and beef patties have shown an inhibitory effect against AI-2 production (Lu et al., 2004). Some commercial food additives were found to possess inhibitory activity against AI-2 formation by bacteria; they include sodium ascorbate, nonacidic salt of ascorbic acid (Novak and Fratamico, 2004), sodium propionate, sodium benzoate, and sodium acetate (Lu et al., 2004).

Biofilm formation by bacteria has been discussed in many studies, and numerous methods have been proposed as able to prevent and remove biofilms, especially those formed by foodborne pathogenic bacteria. To find more effective methods to control biofilm formation, scientists have studied whether quorum sensing is related to biofilm formation. In certain bacteria, quorum sensing has appeared to influence biofilm formation (Parsek and Greenberg, 2005), and especially, it is related to biofilm maturation in *P. aeruginosa* (Davies et al., 1998; de Kievit et al., 2001; Rashid et al., 2000). In addition, AHL was found to influence biofilm maturation of *Serratia liquefaciens* (Labbate et al., 2004). Balestrino et al. (2005) showed that LuxS-dependent quorum sensing (LuxS; AI-2 synthase) of *Klebsiella pneumoniae* was associated with the early stages of biofilm formation. Quorum sensing may also play a role in biofilm formation by *Vibrio cholerae* (Raychaudhuri et al., 2006; Vance

et al., 2003; Zhu et al., 2002). Interestingly, AI-2-like activity in Roma tomatoes has been shown to potentially enhance bacterial biofilm formation (Lu et al., 2005b). AI-2 induced biofilm formation of *E. coli* K-12 via MqsR (quorum sensing regulator) by modulating *qseBC* (two-component motility regulatory system) and motility (González Barrios et al., 2006). In *Listeria monocytogenes*, *S*-ribosylhomocysteine, the precursor of AI-2, was involved in biofilm formation (Challan Belval et al., 2006). *S.* Typhimurium may use quorum sensing to induce biofilm formation in gallstones (Prouty et al., 2002). In agreement, De Keersmaecker et al. (2005) reported that a *luxS* mutant of *S.* Typhimurium had impaired biofilm formation on polystyrene pegs. In contrast, no significant difference was observed for cell aggregate formation on cilantro leaf surfaces between a parental strain and a *luxS* mutant of *Salmonella* Thompson (Brandl et al., 2005).

Van Houdt et al. (2004) investigated the correlation between quorum sensing and biofilm formation capacity in 68 Gram-negative strains isolated from a food production environment; no correlation was observed between *in vitro* biofilm formation and quorum sensing. Bollinger et al. (2001) demonstrated that quorum sensing may regulate negatively biofilm formation in the biofilm mode of growth. Even though some studies showed a relationship between biofilm formation and quorum sensing of *Salmonella* and *E. coli*, the role of AI-2 in biofilm formation on food contact surfaces has not been studied. Therefore, the objective of this study was to evaluate whether AI-2 activity, involved in quorum sensing of Gram-negative bacteria, is involved in biofilm formation on food contact surfaces by *Salmonella* and *E. coli* O157:H7.

MATERIALS AND METHODS

Preparation of inoculum. In Study I, a loopful of *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP (Noah et al., 2005) and *E. coli* O157:H7 strain ATCC 43895/ISEHGFP (Noah et al., 2005) frozen culture maintained in glycerol supplemented (10%) Luria-Bertani (LB; Difco, Becton Dickinson and Company, Sparks, MD) broth at -70°C was activated and subcultured (0.1 ml) in 10 ml of LB broth at 35°C for 24 h. In Study II, a colony of *S.* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) maintained in LB agar + nalidixic acid (25 μg/ml; Sigma-Aldrich, St. Louis, MO), and LB agar + nalidixic acid (25 μg/ml) and kanamycin (30 μg/ml; Sigma-Aldrich), respectively, was activated and subcultured (0.1 ml) in 10 ml of LB broth at 35°C for 24 h; in a preliminary study, no antibiotic addition into LB broth had no effect on characteristics of AI-2 production by the strains during incubation in LB broth at 35°C, and thus, antibiotics were not added in LB broth. *S.* Thompson strains RM1987N and RM1987NLUX cultures were kindly provided by Dr. Maria T. Brandl (USDA/ARS, WRRC, Produce Safety and Microbiology Research Unit, Albany, CA). In addition, a colony of each *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative), maintained on sorbitol McConkey agar (Difco) supplemented with cefixime and potassium tellurite (Dynal, Olso, Norway) (SMAC-CT), were activated and subcultured according to the method for *S.* Thompson strains. *E. coli* O157:H7 strains 86-24 and VS94 cultures were kindly provided by Dr. Vanessa Sperandio (University of Texas Southwestern Medical Center, Dallas, TX). In both studies, stationary phase cells were harvested by centrifugation (4,629×*g*, 15 min, 4°C) and washed once with sterile phosphate buffered saline (pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 7H_2O$, 8.0 g of NaCl, and

0.2 g of KCl in 1 liter of distilled water; PBS). The resulting cell pellets were resuspended and diluted with PBS to yield approximately 4.5 log CFU/ml.

Inoculation and biofilm formation. Study I: a volume of 0.1 ml of the inoculum was inoculated in tubes (85 ml) containing 50 ml of sterile LB broth or LB + 0.5% glucose (Fisher Scientific, Fair Lawn, NJ) (LBG), individually, without or with stainless steel (type 304, #2b finish) or polypropylene (applicable for *Salmonella*, Type D, Density 920 kg/m^3) coupons (2 × 5 cm). Since more AI-2 activity has been detected in higher glucose concentrations (DeLisa et al., 2001b; Hardie et al., 2003; Kim et al., 2003; Surette and Bassler, 1998), two different glucose levels were used to have different levels of AI-2 activity.

Study II: a volume (0.1 ml) of the inoculum composed of AI-2-positive and – negative strain of *S.* Thompson or *E. coli* O157:H7 was inoculated in tubes (85 ml) containing 50 ml of LB broth with stainless steel coupons. Inoculated tubes were stored statically at 25°C for 72 h for biofilm formation.

Microbial analysis. The stored samples were analyzed at 0, 14 (only Study I), 24, 48, and 72 h. To enumerate planktonic cell populations in suspension, serial dilutions of each sample were made in 0.1% buffered peptone-water (Difco) and 0.1 ml portions were plated in duplicate onto tryptic soy agar (Difco) plates (TSA). To enumerate attached cell populations (biofilm) on coupons, each coupon was taken out of the tube, loose cells on the coupons were removed with a 5 sec-spray application of sterile distilled water, and coupons were then transferred into new tubes containing 50 ml of maximum recovery diluent (MRD; 0.1 % peptone and 0.85 % NaCl) and 10 glass beads. The tubes were vortexed for 2 min to detach the cells from the coupons (Stopforth et al., 2002). Serial dilutions of each sample were made in 0.1% buffered peptone-water (Difco), and 0.1 ml portions were plated in duplicate onto TSA plates.

All plates were incubated at 35°C for 48 h. Samples used for microbial analyses were also used to determine pH values using a pH meter with a glass pH electrode (Denver Instrument, Arvada, CO).

Autoinducer-2 bioassay. To determine AI-2 activity in cell-free culture suspension and biofilm supernatant, the bioluminescence response of *Vibrio harveyi* strain BB170 (sensor1$^-$ sensor2$^+$) was detected (Surette and Bassler, 1998, 1999; Surette et al., 1999). Cell-fee supernatants of *V. harveyi* strain BB170 responds only to AI-2 molecules, while the *V. harveyi* strains BB120 (AI-1$^+$ AI-2$^+$) and BB152 (AI-1$^-$ AI-2$^+$) were used as positive controls in the AI-2 bioassay (Surette and Bassler, 1998). All *V. harveyi* strains were kindly provided by Dr. Bonnie L. Bassler (Princeton University, Princeton, NJ). Sterile autoinducer bioassay (AB) medium was used as a negative control (Bassler et al., 1993). A 1 ml portion of the samples of suspension of the culture or biofilm supernatant (MRD containing detached cells from coupons by 2 min-vortex with 10 glass beads) was centrifuged at 15,600×*g* (4°C) for 5 min in a microcentrifuge, and the supernatant was subsequently passed through a filter (0.2μm, 25 mm; Nalgene, Rochester, NY) to prepare cell-free supernatants (Surette and Bassler, 1998, 1999; Surette et al., 1999). The cell-free supernatants were assayed for AI-2 activity by induction of bioluminescence in *V. harveyi* strain BB170 with a procedure similar to that described by Surette and Bassler (1998, 1999) and Surette et al. (1999). Specifically, *V. harveyi* BB170 (0.1 ml) maintained in glycerol supplemented (10 %) LB + 1.5% NaCl broth at -70°C was activated (12 h) and subcultured (7.5 h) in 10 ml of LB + 1.5 % NaCl broth at 30°C with shaking (200 rpm), and diluted 1:5,000 into AB medium. The mixtures (1:9 of cell-free supernatant : diluted *V. harveyi* BB170) were incubated at 30°C for 3.5 h.

In Study I, AI-2 activity was measured at 0, 14, 24, 48, and 72 h, while in Study II, AI-2 activity was measured at 0 and 72 h. To measure AI-2 activity of the mixture, bioluminescence was measured with a luminometer (Model TD-20e luminometer, Turner Designs, Inc., Mt. View, CA). AI-2 activity was described as relative AI-2 activity (RAI) and was calculated as the ratio of bioluminescence of the test and -negative control samples (Lu et al., 2004). The relative AI-2 activity was converted to log RAI/cm^2. RAI activity is referred to as AI-2 activity.

Statistical analysis. The study was repeated two times with two samples for each replication. Microbiological and AI-2 activity data were converted to log CFU/cm^2 and log CFU/ml, and log RAI/cm^2 and log RAI/ml, respectively before statistical analysis. Microbiological and AI-2 activity data for type of coupon, media and storage time (Study I), and the strains and storage time (Study II) were analyzed for suspension and biofilm separately. The data were analyzed by the mixed model procedure of SAS® (SAS institute Inc., 2002-2003). All mean comparisons were performed by the Tukey's multiple comparison and a *P*-value of 0.05 was used for statistical significance.

RESULTS AND DISCUSSION

Changes in pH during storage. In Study I, the pH values of cell suspensions in LB inoculated with *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP and *E. coli* O157:H7 strain ATCC 43895/ISEHGFP did not change dramatically during storage (pH 6.08 to 6.96), while the pH values in LBG suspensions with *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP and *E. coli* O157:H7 strain ATCC 43895/ISEHGFP decreased (pH 4.41 to 4.84) during incubation at 25°C for 72 h, regardless of the type of coupon (Tables IV-1 and IV-2). As expected, the pH values

of MRD containing detached cells (biofilm) were not different ($P \geq 0.05$) between LB broth (pH 6.55 to 6.78) and LBG broth (pH 6.51 to 6.83) during incubation, irrespective of type of coupon (Tables IV-1 and IV-2). In Study II, no difference in pH values was observed between AI-2-positive strains (pH 6.02 to 6.98) and AI-2-negative strains (pH 6.13 to 6.96) for both, cell suspensions, and MRD containing detached cells, of either *S.* Thompson strains RM1987N and RM1987NLUX, or *E. coli* O157:H7 strains 86-24 and VS94 (Tables IV-3 and IV-4).

AI-2 may be negatively related to biofilm formation in *S.* Typhimurium (Study I). No differences ($P \geq 0.05$) in *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP populations were observed between LB and LBG suspensions. However, higher ($P < 0.05$) AI-2 activity, produced by *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP, was detected in LBG broth than LB broth, regardless of type of coupon. No AI-2 activity was detected in LB suspensions during storage at 25°C for 72 h (Figures IV-1 and IV-2). These results indicate that glucose may affect AI-2 production by bacteria, and AI-2 production by *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP may be transient in LB broth. Xavier and Bassler (2005b) suggested that AI-2 can be produced even in the absence of glucose, but its presence is very transitory, since AI-2 is rapidly internalized by the Lsr (*luxS* regulated) transporter. Since there was no difference ($P \geq 0.05$) in AI-2 activity at 0 h between LB and LBG broth, glucose contains no AI-2-like activity (Figures IV-1 and IV-2). *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP formed more ($P < 0.05$) biofilm in LB than LBG broth, but in general, no difference in AI-2 activity was detected in biofilm cells, irrespective of the type of coupon during storage at 25°C for 72 h (Figure IV-3). These results suggest that *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP forms more biofilm in limited nutrients and may secrete AI-2

molecule to the exogenous environment rather than retain AI-2 in the biofilm for more efficient communication. As *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP formed more biofilm in LB than LBG broth, AI-2 activity was lower in LB than LBG broth (Figures IV-2 and IV-3). This result indicates that AI-2 of *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP may be negatively related to its biofilm formation.

The *luxS* defective strain of *Staphylococcus epidermidis* increased biofilm formation, suggesting that *luxS* negatively controlled biofilm formation by a cell-to-cell signaling mechanism using AI-2 (Xu et al., 2006). In *Staphylococcus aureus*, 105 strains were examined for a correlation between the *agr* (*agr*; accessory gene regulator) quorum sensing system and adherence ability of *S. aureus* to polystyrene. The result showed that some 78% of *agr*-negative and only 6% *agr*-positive strains formed a biofilm, proving a negative impact of *agr* quorum sensing system on biofilm formation (Vuong et al., 2000). The *agr* defective mutant of *S. epidermidis* also showed increased biofilm formation and primary attachment (Vuong et al., 2003). Vuong et al. (2004) showed that an isogenic *agr* mutant of *S. epidermidis* augmented biofilm formation and colonization in a rabbit model. While a *luxS* mutant of *S.* Typhimurium was defective in biofilm formation on the surfaces of gallstones, indicating that quorum sensing is required to induce full biofilm formation (Prouty et al., 2002). In addition, De Keersmaecker et al. (2005) reported that a *luxS* mutant of *S.* Typhimurium was impaired in biofilm formation on polystyrene pegs. Aggregate formation by a *S.* Thompson parental strain was significantly higher than by a *luxS* mutant in intestine and spleen as well as droppings of chicks (Brandl et al., 2005). This study was conducted on simulated food contact surfaces such as polypropylene and stainless steel to find if AI-2 was related to biofilm formation of *Salmonella* on

food contact surface. However, other studies were not conducted with food contact surfaces, and this difference may influence the results. Thus, to study the role of AI-2 in biofilm formation on food contact surfaces, surfaces which are used in food industry were used.

***E. coli* O157:H7 may not use AI-2 in the formation of biofilm on food contact surfaces (Study I).** Cell populations of *E. coli* O157:H7 strain ATCC 43895/ISEHGFP were not different ($P \geq 0.05$) between LB and LBG suspensions (Figures IV-4 and IV-5), but in general, higher AI-2 activity was observed in suspensions of LBG than in LB broth. Obvious AI-2 activity of *E. coli* O157:H7 strain ATCC 43895/ISEHGFP was detected even in LB suspension (Figures IV-4 and IV-5). These results suggest *E. coli* O157:H7 strain ATCC 43895/ISEHGFP may also use glucose to produce more AI-2. *Vibrio vulnificus* produced more AI-2 in the presence of glucose (0.5%) and at low pH (pH 6.0) (Kim et al., 2003). In addition, *E. coli* produced a small soluble heat labile organic molecule (AI-2) in LB containing glucose (DeLisa et al., 2001b; Hardie et al., 2003; Surette and Bassler, 1998), but the molecule was not produced in LB without glucose (Surette and Bassler, 1998). Surette and Bassler (1998, 1999) suggested that the degradation of extracellular AI-2 molecule is caused by depletion of glucose during stationary phase. Xavier and Bassler (2005b) elucidated the degradation of extracellular AI-2 molecule during stationary phase. In the presence of glucose, catabolites repress the *lsr* operon, and it cannot be transcribed. Hence, AI-2 cannot be internalized by Lsr transporter, and AI-2 accumulates in cell culture fluids. In the absence of glucose, AI-2 is produced, but its presence is very transitory because it is rapidly internalized by the Lsr transporter; transcription of *lsr* operon is not repressed by the absence of glucose (DeLisa et al., 2001a; Xavier and Bassler, 2005b). The cAMP (cyclic AMP)-CRP (cAMP receptor

protein) complex initiates the expression of the *lsr* operon by directly binding to the upstream region of its promoter in absence of glucose, and then works with the LsrR repressor to mediate AI-2 uptake (Wang et al., 2005b). In the presence of glucose, 23 genes were influenced by *luxS* deletion, while in the absence of glucose, 63 genes were affected by *luxS* deletion; the *metE* gene, the *lsrACDBFG* operon, and the flanking genes of the *lsr* operon (*lsrR*, *lsrK*, *tam*, and *yneE*) were most significantly induced by *luxS* in absence of glucose. In this result, most of highly induced genes are associated with AI-2 production and transport (Wang et al., 2005a). While Hardie et al. (2003) showed that the modulation of LuxS is not related to this alteration of extracellular AI-2 levels in response to glucose.

Unlike *Salmonella*, *E. coli* O157:H7 strain ATCC 43895/ISEHGFP populations in biofilm were not different ($P \geq 0.05$) between the stainless steel coupons stored in LB broth and the stainless steel coupons stored in LBG broth at 25°C for 72 h (Figure IV-6). No obvious AI-2 activity was observed in biofilm formed in both LB and LBG broth inoculated with *E. coli* O157:H7 strain ATCC 43895/ISEHGFP (Figure IV-6). These results indicate that the AI-2-based quorum sensing of *E. coli* O157:H7 may not be related to biofilm formation, and glucose concentration does not influence the biofilm formation ability of *E. coli* O157:H7.

A preliminary study in our laboratory showed that AI-2 may not be involved in biofilm formation of *L. monocytogenes* on stainless steel coupons. Very similar bacterial populations of *L. monocytogenes* were observed between stainless steel coupons stored in AI-2-positive and on stainless steel coupons stored in AI-2-negative preconditioned (PC) media (Sperandio et al., 1999) containing AI-2 activity or no AI-2 activity stored at 25°C for 96 h; PC media were derived from the cell-free supernatants derived from *E. coli* O157:H7 strains 86-24 and VS94 cultures in LBG

broth followed by pH adjustment to pH 7.0. PC media can be applied in studies to evaluate the role of exogenous AI-2. In the *luxS* mutant of *C. perfringens*, the production of alpha (*plc*)-, kappa (*colA*)-, and theta (*pfoA*)-toxins was decreased, but the production of toxin in the *luxS* mutant was increased by exogenous AI-2 (Ohtani et al., 2002). Biofilm formation by *E. coli* K-12 was increased by exogenous AI-2 (González Barrios et al., 2006).

L. monocytogenes produces AI-2 molecules (Challan Belval et al., 2006; Turovskiy and Chikindas, 2006). For further study, the roles of AI-2-based quorum sensing of *L. monocytogenes* should be studied on survival in ready-to-eat meat products, and then the effect of the ingredients in ready-to-eat meat products on the AI-2 production of *L. monocytogenes* needs to be studied.

Difference between AI-2-positive and -negative strains in biofilm formation (Study II). Populations of *S.* Thompson strains RM1987N and RM1987NLUX in suspension of LB broth were not different ($P \geq 0.05$) during incubation at 25°C for 72 h (Figure IV-7). Bacterial attachment was not different on stainless steel coupons placed in LB broth at 25°C for 72 h between *S.* Thompson strains RM1987N and RM1987NLUX (Figure IV-8). This result was different compared to bacterial attachments of *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP of Study I in presence or absence of AI-2 activity because different serotype may induce difference in bacterial attachments. Brandl et al. (2005) reported no significant differences in cell aggregates formed on cilantro leaf surfaces by *S.* Thompson strains RM1987N and RM1987NLUX. Like the *S.* Thompson strains, cell populations of *E. coli* O157:H7 strains 86-24 and VS94 were not different ($P \geq 0.05$) in suspensions of LB and on biofilms formed in LB during storage at 25°C for 72 h (Figures IV-9 and IV-10). At 0 h, no noticeable AI-2 activity of *S.* Thompson was detected in suspensions of LB (-0.3

to -0.5 log RAI/ml) and on biofilm cells (0.2 to 0.5 log RAI/cm^2), and at the end of storage (72 h), no apparent AI-2 activity was observed in suspension of LB (-0.1 to -0.3 log RAI/ml) and biofilms (-0.2 to -0.3 log RAI/cm^2), regardless of strain. As shown in Study I, the transiently produced AI-2 activity in LB containing a low glucose level may cause this result. Low AI-2 activity was observed in suspension of LB and biofilm for both *E. coli* O157:H7 strains at 0 h, but higher AI-2 activity was detected in the suspension of LB inoculated with *E. coli* O157:H7 strain 86-24 (2.4 log RAI/ml) than the suspension of LB inoculated with *E. coli* O157:H7 strain VS94 (-0.4 log RAI/ml) at 72 h. Like AI-2 activity of the *S.* Thompson strains in biofilm, low AI-2 activity was detected in biofilm (0.3 to 0.6 log RAI/cm^2), regardless of strain. These results indicate that AI-2-based quorum sensing of *S.* Thompson and *E. coli* O157:H7 may not be involved in biofilm formation on food contact surfaces.

In agreement with these results, Van Houdt et al. (2004) studied the relationship between quorum sensing and biofilm formation in 68 Gram-negative strains isolated from a food production environment, including AI-2-positive strains and AHL-positive strains. No correlation was found between *in vitro* biofilm formation and quorum sensing. In addition, even though biofilm formation of *E. coli* K-12 was inhibited by ursolic acid, AI-2-based quorum sensing of *E. coli* K-12 was not affected. This result indicates that AI-2-based quorum sensing of *E. coli* K-12 is not involved in biofilm formation (Ren et al., 2005). The *luxS* defective strain of *S. epidermidis* had enhanced biofilm formation, proving that *luxS* negatively controlled biofilm formation by a cell-to-cell signaling mechanism using AI-2 (Xu et al., 2006). This agrees with results of biofilm formation by *Salmonella* in Study I. Some studies showed relationship between AI-2-based quorum sensing and biofilm formation. González Barrios et al. (2006) reported that AI-2 induced biofilm formation of *E. coli*

K-12 via MqsR, modulating *qseBC* and motility. A *luxS* isogenic mutant of *K. pneumoniae* was examined whether its quorum sensing influenced biofilm formation. The results showed that a LuxS-dependent quorum sensing was associated with biofilm formation in early stages, prior to maturation (Balestrino et al., 2005). Merritt et al. (2003) demonstrated that AI-2 was responsible for biofilm formation by *Streptococcus mutans*; the *luxS* mutant of *S. mutans* formed a much more granular biofilm, rather than the comparatively smooth confluent layer, which is normally observed in the wild type. Lu et al. (2005b) used the washings from Roma tomato surfaces, followed by inoculation with *luxS* mutant of *E. coli* O157:H7, to evaluate the effect of AI-2-like activity of Roma tomatoes on biofilm formation of *E. coli* O157:H7, and they found that AI-2-like activity of Roma tomatoes has the potential to enhance bacterial biofilm formation. Exogenous AI-2 increased biofilm formation of *E. coli* K-12 (González Barrios et al., 2006).

For further research, studies to determine the roles of AHL on biofilm formation of *Salmonella* and *E. coli* on food contact surfaces is necessary because SdiA (suppressor of cell division inhibitor) has been shown as LuxR homologue in *E. coli* and *S.* Typhimurium (Michael et al., 2001; Rahmati et al., 2002). SdiA responded to AHL molecules produced by *Yersinia enterocolitica* (Smith and Ahmer, 2003). These findings suggest that *E. coli* and *S.* Typhimurium may use AHL molecules produced by other Gram-negative bacteria in the environment. In addition, AHL could be a nutrient source during process of biofilm formation of *Salmonella* and *E. coli* O157:H7. *Arthrobacter* VAI-A can use exogenous AHL as a nitrogen source, but as a carbon and energy source (Flagan et al., 2003: Yang et al., 2006).

Furthermore, the relationship between different quorum sensing systems should be studied for biofilm formation. Recently, novel quorum sensing regulatory

genes, *raiI* and *raiR*, were identified in *Rhizobium leguminosarum* (Wisniewki-Dyé et al., 2002). Interestingly, in this study, *raiR*-dependent *raiI* expression was strongly induced by the product of CinI, LuxI homologue, indicating that the *raiI* expression could be initiated by other quorum sensing molecules. Mok et al. (2003) showed that in signal processing, AHL and AI-2 act synergistically. The third *V. harveyi* quorum sensing system composed of CqsA (*cholerae* quorum sensing autoinducer) and CqsS (*cholerae* quorum sensing sensor) acts in parallel to AHL and AI-2-based quorum sensing system for bioluminescence, type III secretion, and metalloprotease production (Henke and Bassler, 2004a).

Also, the effect of environmental conditions combined with quorum sensing should be studied in biofilm formation of *Salmonella* and *E. coli* O157:H7. Yarwood et al. (2004) suggested that in *S. aureus*, the role of *agr* expression in biofilm formation is dependent on environmental conditions, because an *agr* defective strain formed more biofilms than the parent strain did under a static biofilm system, whereas no significant difference in the structure or thickness of the biofilm was observed for the parent and the *agr* defective strain in flow cell.

CONCLUSIONS

The results indicated no difference or negative influence in biofilm density with variable AI-2 activity, and thus, quorum sensing may not be of significance in food equipment sanitation. However, since *Salmonella* and *E. coli* O157:H7 may use AHL produced by natural flora and the bacteria may synergistically increase the efficiency of their quorum sensing system with other quorum sensing systems, studies to find whether AI-2-based quorum sensing systems of *Salmonella* and *E. coli* O157:H7 are influenced by other quorum sensing systems and molecules are necessary. In addition,

the effect of quorum sensing of *Salmonella* and *E. coli* O157:H7 should be studied in flow cells and dried surfaces because flowing liquid condition and dried surfaces are usually found in food industries.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. Pina M. Fratamico, Dr. John S. Novak, Dr. Maria T. Brandl, Dr. Vanessa Sperandio, and Dr. Bonnie L. Bassler for their friendly and critical advice, and cultures. This work was funded by the Colorado State University Agricultural Experiment Station.

Table IV-1. pH values (mean ± standard error) of both cell suspensions and detached biofilm cells, suspended in maximum recovery diluent, of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP during incubation of the strains in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth containing none, or polypropylene and stainless steel coupons at 25°C for 72 h

			Storage time (h)				
Cond	Coup	Media	0	14	24	48	72
Sus	None	LB	6.83±0.02 ^{Aa}	6.61±0.01 ^{ABa}	6.45±0.01 ^{Ba}	6.61±0.02 ^{ABa}	6.37±0.04 ^{Ba}
		LBG	6.75±0.01 ^{Aa}	6.65±0.01 ^{Aa}	4.81±0.05 ^{Bb}	4.55±0.03 ^{BCb}	4.43±0.04 ^{Cb}
	PP	LB	6.86±0.04 ^{Aa}	6.64±0.01 ^{ABa}	6.48±0.01 ^{Ba}	6.66±0.02 ^{ABa}	6.45±0.05 ^{Ba}
		LBG	6.75±0.00 ^{Aa}	6.66±0.02 ^{Aa}	4.82±0.03 ^{Bb}	4.54±0.03 ^{BCb}	4.42±0.03 ^{Cb}
	ST	LB	6.84±0.02 ^{Aa}	6.64±0.02 ^{Aa}	6.48±0.01 ^{Aa}	6.67±0.02 ^{Aa}	6.64±0.05 ^{Aa}
		LBG	6.72±0.02 ^{Aa}	6.66±0.01 ^{Aa}	4.82±0.04 ^{Bb}	4.56±0.03 ^{BCb}	4.41±0.03 ^{Cb}
Bio	PP	LB	6.71±0.04 ^{Aa}	6.78±0.06 ^{Aa}	6.70±0.03 ^{Aa}	6.71±0.03 ^{Aa}	6.67±0.07 ^{Aa}
		LBG	6.73±0.04 ^{Aa}	6.83±0.07 ^{Aa}	6.58±0.04 ^{Aa}	6.62±0.07 ^{Aa}	6.52±0.07 ^{Aa}
	ST	LB	6.67±0.06 ^{Aa}	6.73±0.06 ^{Aa}	6.75±0.06 ^{Aa}	6.77±0.02 ^{Aa}	6.68±0.12 ^{Aa}
		LBG	6.70±0.05 ^{Aa}	6.75±0.07 ^{Aa}	6.76±0.02 ^{Aa}	6.69±0.03 ^{Aa}	6.65±0.05 ^{Aa}

Cond: cell growth conditions; Coup: coupon; Sus: suspension; Bio: detached biofilm cells suspended in maximum recovery diluent.

PP: polypropylene; ST: stainless steel.

^{ABC}: means within the same row with different superscript letters are different (P<0.05).

^{ab}: means within the same column with different superscript letters are different (P<0.05).

Table IV-2. pH values (mean ± standard error) of both cell suspensions and detached biofilm cells suspended in maximum recovery diluent of *Escherichia coli* O157:H7 strain ATCC 43895/ISEHGFP during incubation of the strains in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth containing none and stainless steel coupons at 25°C for 72 h

			Storage time (h)			
Cond	Coup	Media	0	24	48	72
Sus	None	LB	6.96±0.05 ^{Aa}	6.08±0.01 ^{Bb}	6.23±0.04 ^{Bb}	6.21±0.00 ^{Bb}
		LBG	6.87±0.01 ^{Aab}	4.84±0.03 ^{Bc}	4.69±0.02 ^{Bc}	4.66±0.01 ^{Bc}
	ST	LB	6.95±0.05 ^{Aa}	6.09±0.02 ^{Bb}	6.27±0.02 ^{Bb}	6.24±0.02 ^{Bb}
		LBG	6.86±0.01 ^{Aab}	4.84±0.02 ^{Bc}	4.69±0.00 ^{Bc}	4.58±0.03 ^{Bc}
Bio	ST	LB	6.60±0.08 ^{Ab}	6.55±0.03 ^{Aa}	6.62±0.03 ^{Aa}	6.65±0.04 ^{Aa}
		LBG	6.61±0.05 ^{Ab}	6.51±0.05 ^{Aa}	6.61±0.04 ^{Aa}	6.52±0.05 ^{Aa}

Cond: cell growth conditions; Coup: coupon; Sus: suspension; Bio: detached biofilm cells suspended in maximum recovery diluent.

ST: stainless steel.

^{AB}: means within the same row with different superscript letters are different (P<0.05).

^{abc}: means within the same column with different superscript letters are different (P<0.05).

Table IV-3. pH values (mean ± standard error) of both cell suspensions and detached biofilm cells suspended in maximum recovery diluent of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) during incubation of the strains in Luria-Bertani (LB) broth containing stainless steel coupons at 25°C for 72 h

		Storage time (h)			
Cond	Strain	0	24	48	72
Sus	RM1987N	6.82±0.01 ^{Aa}	6.33±0.03 ^{Ba}	6.56±0.02 ^{ABa}	6.55±0.01 ^{ABa}
	RM1987NLUX	6.87±0.03 ^{Aa}	6.33±0.04 ^{Ba}	6.61±0.03 ^{ABa}	6.62±0.07 ^{ABa}
Bio	RM1987N	6.52±0.04 ^{Aa}	6.48±0.06 ^{Aa}	6.59±0.06 ^{Aa}	6.55±0.03 ^{Aa}
	RM1987NLUX	6.60±0.06 ^{Aa}	6.53±0.04 ^{Aa}	6.57±0.05 ^{Aa}	6.50±0.09 ^{Aa}

Cond: cell growth conditions; Sus: suspension.

Bio: detached biofilm cells suspended in maximum recovery diluent.

^{AB}: means within the same row with different superscript letters are different ($P<0.05$).

^{a}: means within the same column are not different ($P\geq0.05$).

Table IV-4. pH values (mean ± standard error) of both cell suspensions and detached biofilm cells suspended in maximum recovery diluent of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) during incubation of the strains in Luria-Bertani broth containing stainless steel coupons at 25°C for 72 h

		Storage time (h)			
Cond	Strain	0	24	48	72
Sus	86-24	6.98±0.02 ^{Aa}	6.02±0.10 ^{Ca}	6.36±0.01 ^{BCa}	6.41±0.04 ^{Ba}
	VS94	6.96±0.04 ^{Aa}	6.13±0.02 ^{Ba}	6.34±0.01 ^{Ba}	6.41±0.01 ^{Ba}
Bio	86-24	6.45±0.04 ^{Ab}	6.26±0.02 ^{Aa}	6.28±0.01 ^{Aa}	6.40±0.06 ^{Aa}
	VS94	6.41±0.02 ^{Ab}	6.34±0.03 ^{Aa}	6.33±0.04 ^{Aa}	6.36±0.06 ^{Aa}

Cond: cell growth conditions; Sus: suspension.

Bio: detached biofilm cells suspended in maximum recovery diluent.

^{ABC}: means within the same row with different superscript letters are different (P<0.05).

^{ab}: means within the same column with different superscript letters are different (P<0.05).

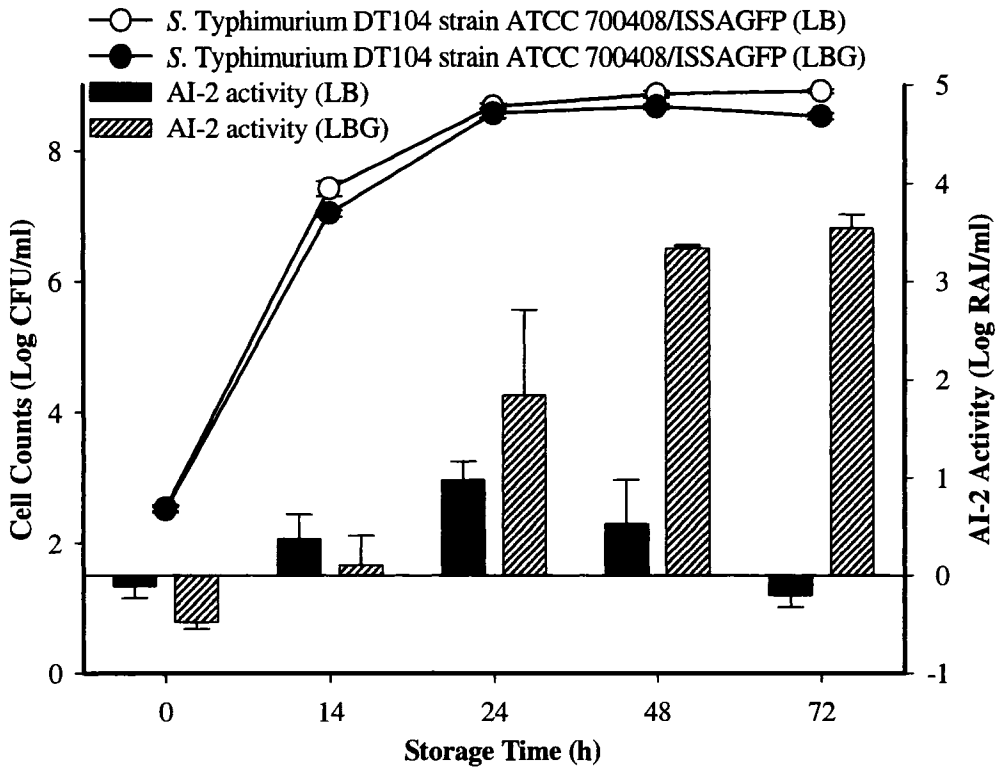

Figure IV-1. Cell counts and AI-2 activity of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP, enumerated on tryptic soy agar, in cell suspensions during incubation of the strains in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth containing no coupons stored at 25°C for 72 h (Data in APPENDIX Tables 12 and 13).

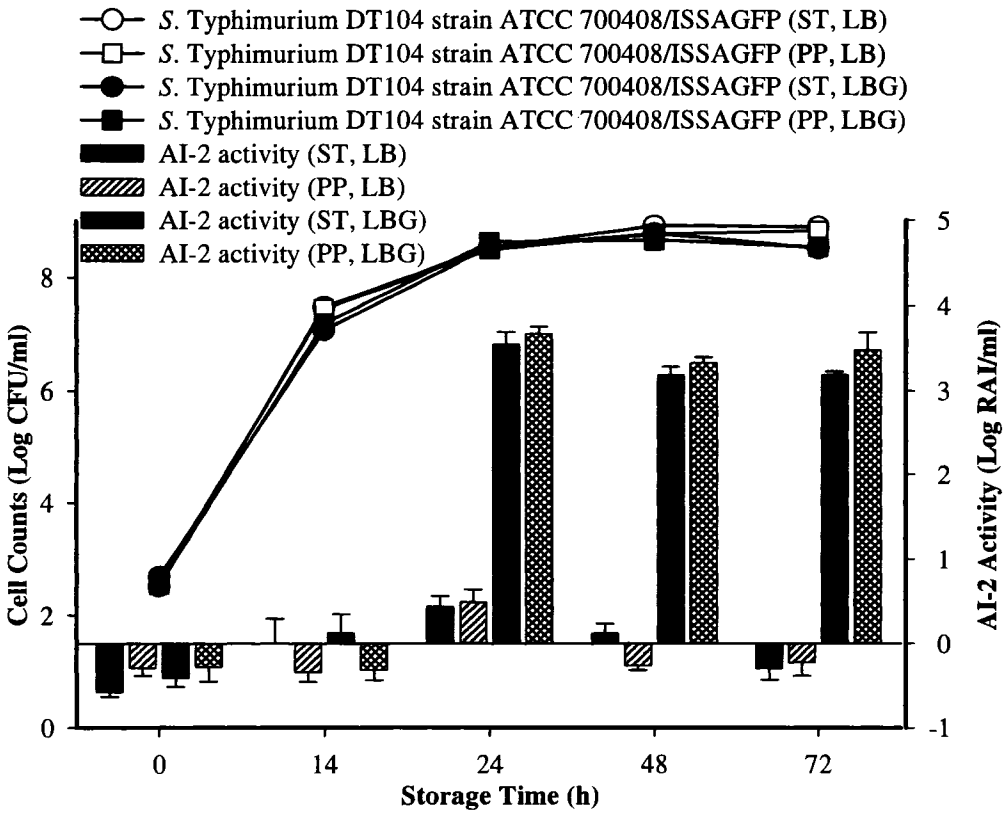

Figure IV-2. Cell counts and AI-2 activity of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP, enumerated on tryptic soy agar, in cell suspensions during incubation of the strains in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth containing stainless steel (ST) and polypropylene (PP) coupons stored at 25°C for 72 h (Data in APPENDIX Tables 12 and 13).

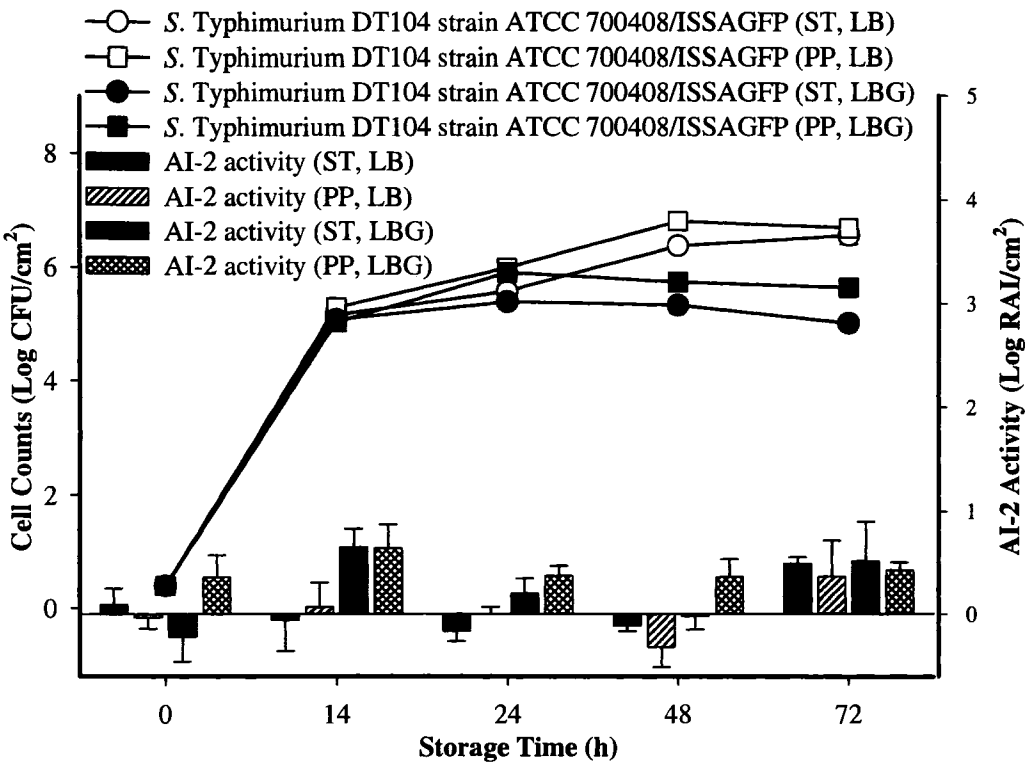

Figure IV-3. Cell counts, enumerated on tryptic soy agar, and AI-2 activity of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP in detached biofilm cells, suspended in maximum recovery diluent, formed on stainless steel (ST) and polypropylene (PP) coupons during incubation of the strains in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth at 25°C for 72 h (Data in APPENDIX Tables 14 and 15).

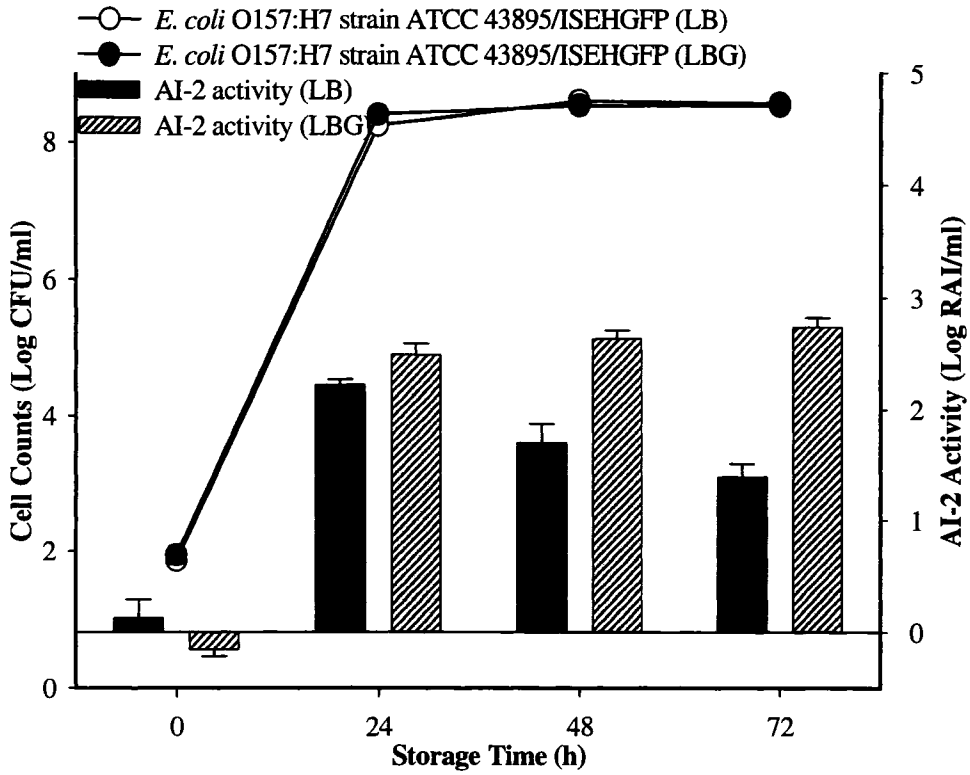

Figure IV-4. Cell counts, enumerated on tryptic soy agar, and AI-2 activity of *Escherichia coli* O157:H7 strain ATCC 43895/ISEHGFP in cell suspensions during incubation of the strains in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth containing no coupons stored at 25°C for 72 h (Data in APPENDIX Tables 16 and 17).

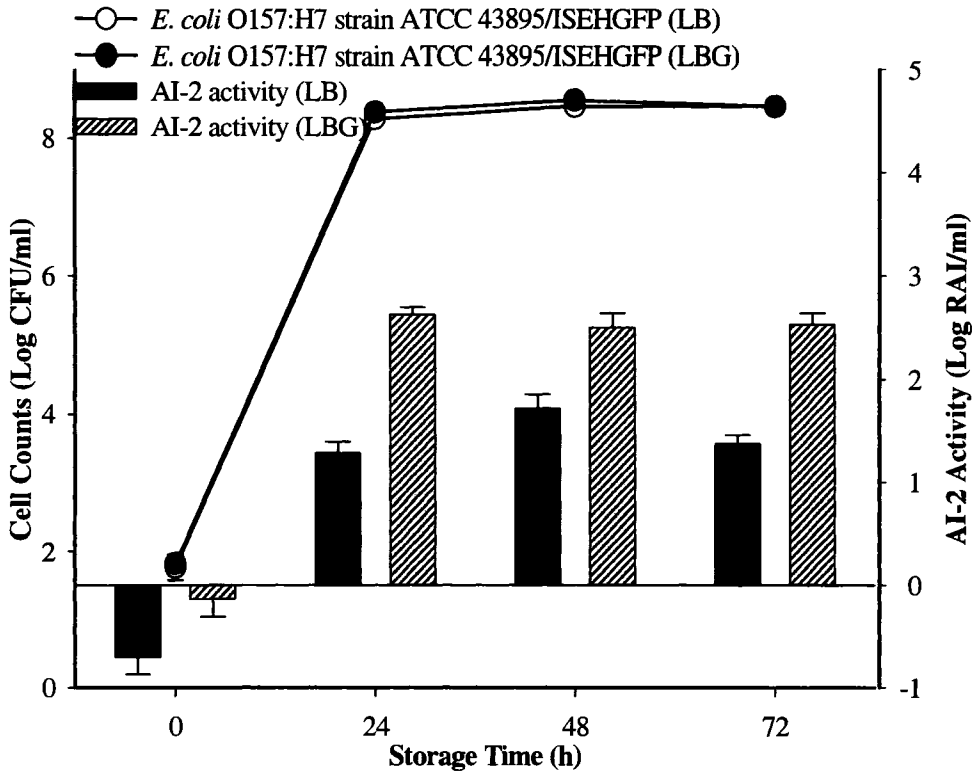

Figure IV-5. Cell counts, enumerated on tryptic soy agar, and AI-2 activity of *Escherichia coli* O157:H7 strain ATCC 43895/ISEHGFP in cell suspensions during incubation of the strains in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth containing stainless steel coupons stored at 25°C for 72 h (Data in APPENDIX Tables 16 and 17).

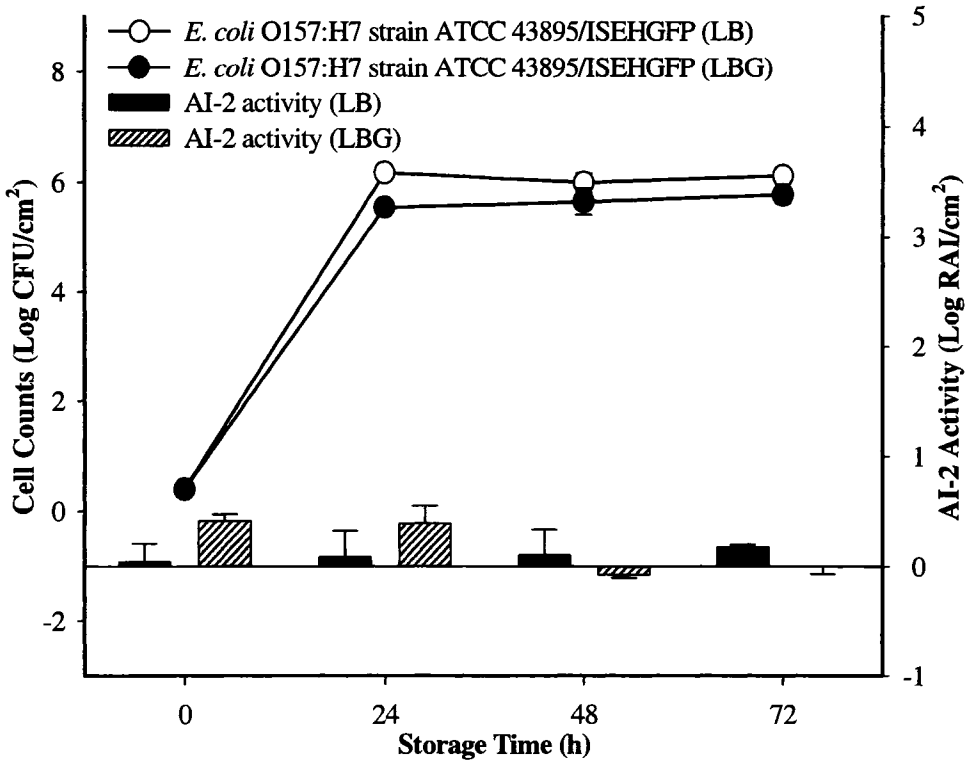

Figure IV-6. Cell counts, enumerated on tryptic soy agar, and AI-2 activity of *Escherichia coli* O157:H7 strain ATCC 43895/ISEHGFP in detached biofilm cells, suspended in maximum recovery diluent, formed on stainless steel coupons during incubation of the strains in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth at 25°C for 72 h (Data in APPENDIX Tables 16 and 17).

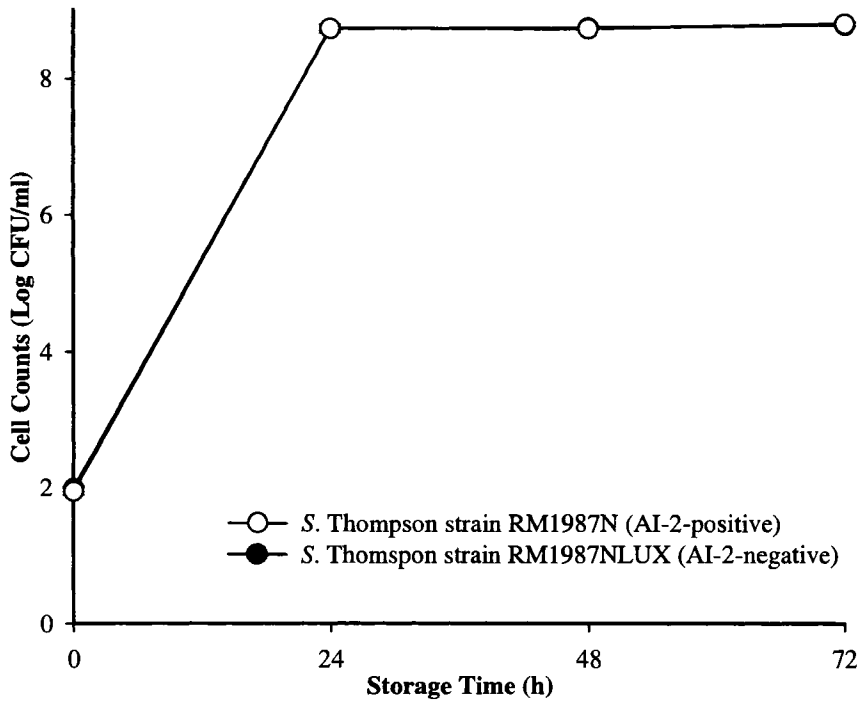

Figure IV-7. Cell counts, enumerated on tryptic soy agar, of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) in cell suspensions during incubation of the strains in Luria-Bertani broth containing stainless steel coupons at 25°C for 72 h (Data in APPENDIX Table 18).

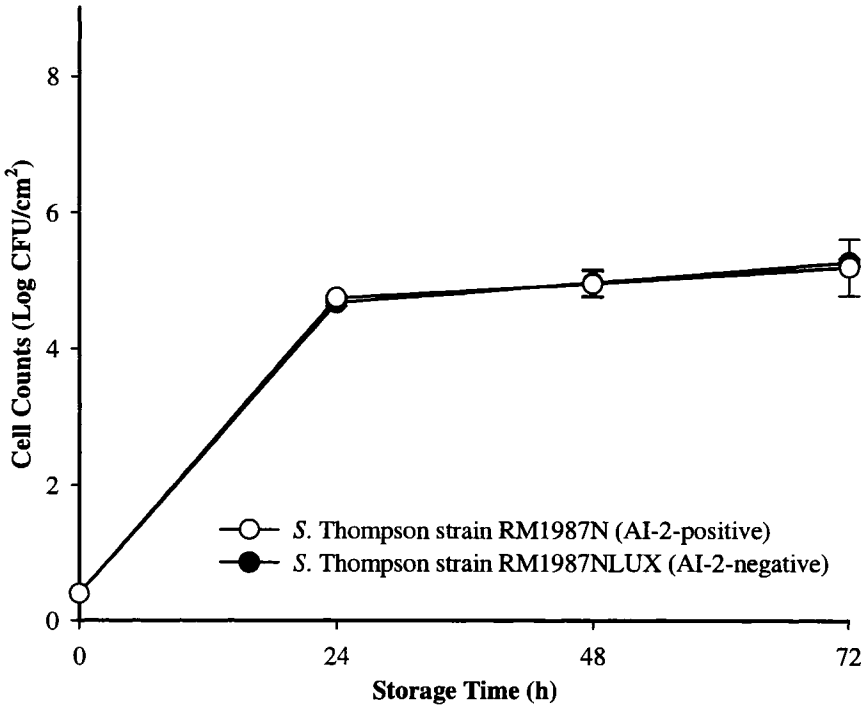

Figure IV-8. Cell counts, enumerated on tryptic soy agar, of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) in detached biofilm cells formed on stainless steel coupons, suspended in maximum recovery diluent during incubation of the strains in Luria-Bertani broth containing stainless steel coupons at 25°C for 72 h (Data in APPENDIX Table 18).

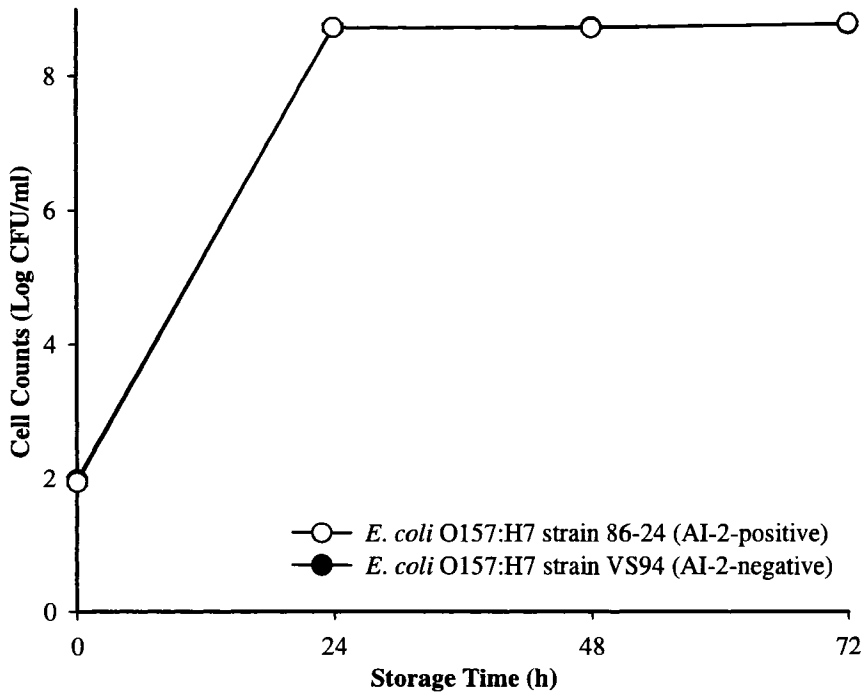

Figure IV-9. Cell counts, enumerated on tryptic soy agar, of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) in cell suspensions during incubation of the strains in Luria-Bertani broth containing stainless steel coupons at 25°C for 72 h (Data in APPENDIX Table 19).

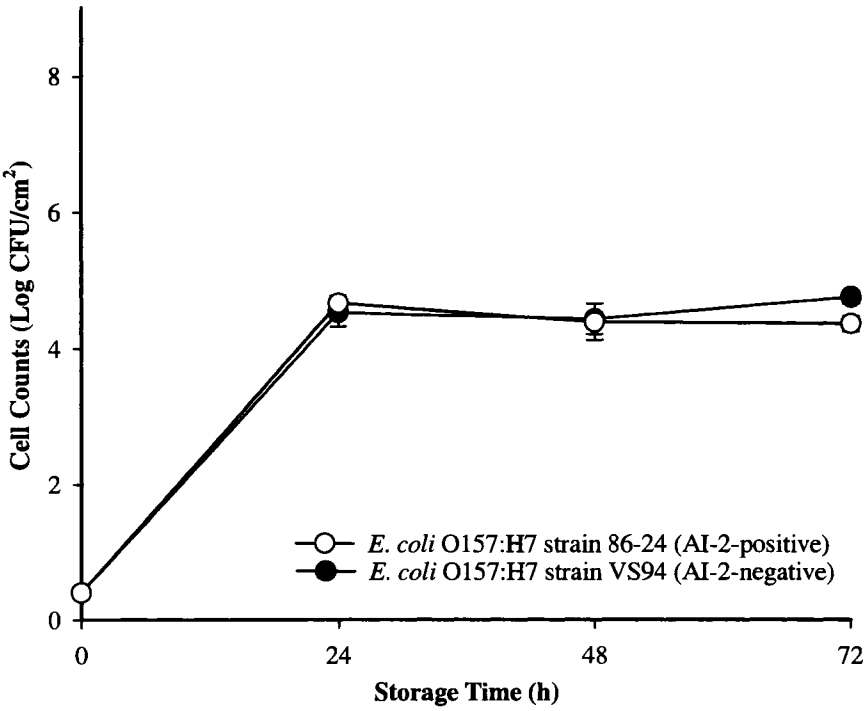

Figure IV-10. Cell counts, enumerated on tryptic soy agar, of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) in detached biofilm cells formed on stainless steel coupons, suspended in maximum recovery diluent, during incubation of the strains in Luria-Bertani broth containing stainless steel coupons at 25°C for 72 h (Data in APPENDIX Table 19).

CHAPTER V

AUTOINDUCER-2-BASED QUORUM SENSING IN *ESCHERICHIA COLI* O157:H7 INOCULATED FRESH BEEF AND INTERACTION WITH LEVEL OF NATURAL MICROBIAL FLORA

ABSTRACT

This study examined factors that may affect autoinducer-2 (furanosyl borate diester; AI-2)-based quorum sensing of *Escherichia coli* O157:H7 inoculated on fresh meat. Fresh beef slices were prepared to contain low (LNB; 0.9 log CFU/cm^2; cut after immersing beef inside rounds in 85°C water for 15 min) or high (HNB; 2.9 log CFU/cm^2; no immersion) initial levels of natural flora. Two levels (2 or 6 log CFU/cm^2) of *E. coli* O157:H7 strain ATCC 43895 were inoculated on beef slices (4 × 4 × 1 cm) with each level of natural flora, and were then stored aerobically or in vacuum packages at 4, 10, or 25°C for 21, 18, and 9 days, respectively. Relative AI-2-like activity (referred as AI-2-like activity), as a potential indicator of quorum sensing, was determined using the luminescence-based reporter strain *Vibrio harveyi* strain BB170, and total bacterial populations and *E. coli* O157:H7 strain ATCC 43895 cell counts were determined on tryptic soy agar and sorbitol McConkey agar supplemented with cefixime and potassium tellurite, respectively during storage. Total bacterial populations increased significantly (P<0.05), regardless of storage

temperature, inoculation level, and level of natural flora. *E. coli* O157:H7 strain ATCC 43895 showed less growth in HNB than LNB at 10°C; at 25°C, there was rapid growth of both inoculation levels during the early stages of storage, irrespective of level of natural flora. In general, AI-2-like activity was higher ($P<0.05$) in LNB than in HNB at 25°C, while levels of AI-2-like activity were minimal at 4 and 10°C. Aerobically stored beef slices had higher ($P<0.05$) AI-2-like activity than those stored in vacuum packages at 25°C. The results of this study indicated that AI-2-like activity in *E. coli* O157:H7 strain ATCC 43895 inoculated fresh beef depended on level of natural flora, presence of oxygen, and storage temperature; it decreased with higher initial level of natural flora, absence of oxygen, and lower storage temperature.

INTRODUCTION

Various bacterial groups possess cell-to-cell density-dependent signaling systems, known as quorum sensing, which use low-molecular-weight signaling compounds, called autoinducers or pheromones (Smith et al., 2004). Since quorum sensing was first observed in bacteria, studies have shown that it may be associated with bacterial properties (reviewed by Ren et al., 2004b) such as virulence (von Bodman et al., 1998), plasmid conjugation (Lithgow et al., 2001), bioluminescence (Cao and Meighen, 1989), protein production (DeLisa et al., 2001c), and biofilm formation (Davies et al., 1998). Recently, food microbiologists have also speculated on the possibility of quorum sensing being associated with processes involved in bacterial food quality and safety (Christensen et al., 2003; Jay et al., 2003; Rasch et al., 2005).

Quorum sensing molecules can be categorized into four groups (reviewed by Smith et al., 2004): (i) *N*-acylhomoserine lactone used primarily for interspecies communication by Gram-negative bacteria [AHL; autoinducer (AI)-1] (Miller and

Bassler, 2001; Whitehead et al., 2001); (ii) a furanosyl borate diester (AI-2) for intraspecies and interspecies communication in both Gram-negative and Gram-positive bacteria (Chen et al., 2002; Schauder and Bassler, 2001); (iii) an unknown structure, called autoinducer-3 (AI-3), involved in cross-communication with the mammalian epinephrine cells of host systems (Sperandio et al., 2003); and, (iv) oligopeptides, active only in Gram-positive bacteria (Miller and Bassler, 2001; Whitehead et al., 2001). Other potential quorum sensing molecules may include diketopiperazines (Degrassi et al., 2002; Holden et al., 1999), 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal) (Holden et al., 2000; Pesci et al., 1999), 3,7,11-trimethyl-2,6,10-dodecatrienoate (farnesoic acid) (Oh et al., 2001), farnesol (Hornby et al., 2001), and tyrosol (Chen et al., 2004).

Presence of AI-2 activity has been detected in *Escherichia coli* O157:H7 (Cloak et al., 2002; Surette et al., 1999), *Salmonella* Typhimurium (Surette and Bassler, 1998) and *Listeria monocytogenes* Scott A (Turovskiy and Chikindas, 2006), and the molecule is considered as a universal autoinducer because it is involved in gene expression in various Gram-negative and Gram-positive bacteria (Lu et al., 2004). The production of AI-2 was reviewed by Chen et al. (2002); *S*-adenosylmethionine is converted to *S*-adenosylhomocysteine by methyltransferases, and *S*-adenosylhomocysteine is hydrolyzed by Pfs (nucleosidase), producing adenine and *S*-ribosylhomocysteine (Cornell et al, 1996; Della Ragione et al., 1985). Subsequently, *S*-ribosylhomocysteine is changed to 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine (Miller and Duerre, 1968) via LuxS (Lewis et al., 2001; Schauder et al., 2001). DPD is then possibly further processed to yield AI-2 (Schauder et al., 2001). Recently, Chen et al. (2002) proposed an AI-2 structure involving two fused five-

membered rings; the rings are stabilized in the LuxP binding site by polar forces and the authors suggested that the atom bridging the diester is boron.

Factors that have increased AI-2 production by *S.* Typhimurium include rapid, nutrient-rich growth, presence of glucose, acidic environment (pH 5.0), and high osmolarity (0.4M NaCl) (Surette and Bassler, 1999). These factors may also affect quorum sensing activity of other AI-2 producing bacteria. Furthermore, AI-2 has been produced in milk, chicken broth, and brucella broth by *Campylobacter* spp., *S.* Typhimurium, and *E. coli* O157:H7 at different storage temperatures (Cloak et al., 2002). Interestingly, some fresh and processed foods also showed indigenous AI-2-like activity. They included frozen fish, tomatoes, cantaloupe, carrots, tofu, and milk. Other products, including turkey patties, chicken breast, homemade cheeses, beef steak, and beef patties showed an inhibitory effect against AI-2 production or probably detection (Lu et al., 2004). Food additives, such as sodium propionate, sodium benzoate and sodium acetate have been found to inhibit AI-2 activity in bacteria (Lu et al., 2004). Sodium ascorbate and a nonacidic salt of ascorbic acid also inhibited AI-2 activity (Novak and Fratamico, 2004).

Cloak et al. (2002) suggested that autoinducers may be involved in growth, survival, and virulence of bacteria. Even though *E. coli* O157:H7 is known to possess quorum sensing systems based on AI-2 (Cloak et al., 2002; Surette et al., 1999) and AI-3 (Sperandio et al., 2003) activities, the role of its quorum sensing activity and the factors affecting quorum sensing molecule production in foods are not clear. Thus, it is important to examine AI-2-based quorum sensing in foods. The objective of this study was to examine AI-2-based quorum sensing of *E. coli* O157:H7 in raw meat and factors that may affect activity of AI-2-based quorum sensing of *E. coli* O157:H7 in beef.

MATERIALS AND METHODS

Preparation of inoculum. A 0.1 ml portion of *E. coli* O157:H7 strain ATCC 43895 maintained in glycerol supplemented (10%) Luria-Bertani (LB; Difco, Becton Dickinson and Company, Sparks, MD) broth at -70°C was activated and subcultured in 10 ml of LB broth at 35°C for 24 h. Stationary phase cells were harvested by centrifugation (4,629×*g*, 15 min, 4°C) and washed with phosphate buffered saline (pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 liter of distilled water; PBS). The resulting cell pellets were resuspended and diluted with PBS to yield approximately 7 and 3 log CFU/ml.

Preparation of beef slices. For samples containing low levels of natural flora (LNB; 0.9 log CFU/cm^2), beef inside round was immersed in hot water (85°C) for 15 min. The surface was then removed with a flame-sterilized knife (0.5 to 1.0 cm deep), and the remaining product was cut aseptically into 4 × 4 × 1 cm slices. For the samples containing higher levels of natural flora (HNB; 2.9 log CFU/cm^2), beef inside rounds were cut into 4 × 4 × 1 cm slices without immersion in hot water.

Inoculation procedure. A volume of 0.1 ml of the *E. coli* O157:H7 strain ATCC 43895 inoculum was applied to one flat surface of beef slices and spread with a flame-sterilized glass rod. Beef slices inoculated with *E. coli* O157:H7 strain ATCC 43895 were kept at 4°C for 15 min to allow for attachment, and were flipped over to be inoculated on the other flat side according to the same procedure. The *E. coli* O157:H7 strain ATCC 43895 inoculation levels evaluated were no inoculation, 2, and 6 log CFU/cm^2 of beef. Inoculated LNB and HNB samples were packaged aerobically (samples were placed on retail foam trays, 7.5 by 12.5 cm; Pactiv, Lake Forest, IL) by covering with air-permeable film (Omni-film, Pliant Corporation, Uniontown, OH), or in vacuum bags (15 by 22 cm, 3 mil standard barrier, nylon/PE vacuum pouch,

Koch, Kansas City, MO) evacuated and sealed with a vacuum packager (Hollymatic Corp., Countryside, IL), and stored at 4, 10, and 25°C for 21, 18, and 9 days, respectively.

Microbial analysis. Samples stored at 4, 10, and 25°C were analyzed every 7, 6, and 3 days, respectively. Two samples from LNB or HNB were individually transferred into 24-oz Whirl-Pak (Nasco, Modesto, CA) bags and 20 ml of maximum recovery diluent (MRD; 0.1 % peptone and 0. 85 % NaCl) was added to each sample bag prior to pummeling (Masticator, IUL Instruments, Barcelona, Spain) for 2 min (120 strokes per min) at ambient (25°C) temperature. After serially diluting each sample in 0.1% buffered peptone-water (Difco), 0.1 ml portions were plated onto each of duplicate agar plates. Total bacterial populations were enumerated on tryptic soy agar (Difco) (TSA), while sorbitol McConkey agar (Difco) supplemented with cefixime and potassium tellurite (Dynal, Oslo, Norway) (SMAC-CT) was used as a selective medium for *E. coli* O157:H7. All plates were incubated at 35°C for 48 h. The enumeration detection limit was –0.2 log CFU/cm^2. Samples used for microbial analyses were also used to determine pH values using a pH meter with a glass pH electrode (Denver Instrument, Arvada, CO).

Autoinducer-2 bioassay. To determine AI-2-like activity in beef samples inoculated with *E. coli* O157:H7 strain ATCC 43895, the bioluminescence response of *Vibrio harveyi* strain BB170 (sensor1$^-$ sensor2$^+$) was measured (Surette and Bassler, 1998, 1999; Surette et al., 1999). The strain is engineered to respond only to AI-2 molecules, while cell-free supernatants of the *V. harveyi* strains BB120 (AI-1$^+$ AI-2$^+$) and BB152 (AI-1$^-$ AI-2$^+$) were used as positive controls in the AI-2 bioassay (Surette and Bassler, 1998). All *V. harveyi* strains were kindly provided by Dr. Bonnie L. Bassler (Princeton University, Princeton, NJ). Sterile autoinducer bioassay (AB) medium

(Bassler et al., 1993) was used as a negative control. Two beef slices (one slice per sample) per treatment were aseptically transferred into 18-oz Whirl-Pak bags containing 20 ml of AB medium and then vertically shaken 30 times for 30 s. A 1 ml portion of the beef sample that was shaken in AB medium was centrifuged at 15,600×*g* (4°C) for 5 min in a microcentrifuge and the supernatant was subsequently passed through a filter (0.2 μm, 25 mm; Nalgene, Rochester, NY) to prepare cell-free supernatants. The cell-free supernatants were assayed for AI-2-like activity by induction of bioluminescence in *V. harveyi* strain BB170 with a procedure similar to that described by Surette and Bassler (1998, 1999) and Surette et al. (1999). Specifically, *V. harveyi* BB170 (0.1 ml) maintained in glycerol supplemented (10 %) LB + 1.5% NaCl broth at -70°C was activated (15 h) and subcultured (7.5 h) in 10 ml of LB + 1.5% NaCl broth at 30°C with shaking (200 rpm), and diluted 1:5,000 into AB medium. The mixtures (1:9 of cell-free supernatant : diluted *V. harveyi* BB170) were incubated at 30°C for 3.5 h. To measure AI-2-like activity of the mixture, bioluminescence was measured with a luminometer (Model TD-20e luminometer, Turner Designs, Inc., Mt. View, CA). AI-2-like activity was described as relative AI-2-like activity (RAI) and calculated as the ratio of bioluminescence of the test and negative control samples (Lu et al., 2004). The RAI was converted to log RAI/cm^2. To obtain AI-2-like activity (log RAI/cm^2) of only *E. coli* O157:H7 strain ATCC 43895, the difference of AI-2-like activity between *E. coli* O157:H7 strain ATCC 43895 inoculated samples and noninoculated samples were calculated; relative AI-2-like activity is referred to as AI-2-like activity in this study.

Statistical analysis. The study was repeated two times with two samples for each replication. Microbiological and AI-2-like activity data were converted to log CFU/cm^2 and log RAI/cm^2, respectively, before being analyzed statistically.

Microbiological and AI-2-like activity data for treatments and storage temperatures were analyzed by the mixed model procedure of SAS® (SAS institute Inc., 2002-2003). The treatments and storage temperatures were compared by the pairwise t-test and a *P*-value of 0.05 was used for statistical significance.

RESULTS AND DISCUSSION

In general, initial pH values of samples were between 5.46 and 5.67, and their range changed to 5.09 and 7.75 after storage (Tables V-1 to V-3). At the end of storage, the pH values of beef samples stored under aerobic conditions significantly ($P<0.05$) increased (5.33-7.75) compared to those of samples stored in vacuum packages (5.09-5.45) (Tables V-1 to V-3). Under aerobic conditions, HNB samples had higher ($P<0.05$) pH values (7.19 to 7.75) than LNB samples (5.33 to 7.24), while pH values were very similar between HNB samples (5.09 to 5.44) and LNB samples (5.11 to 5.45) in vacuum packages. However, pH value ranges were not different in samples with different inoculation levels (no inoculation [5.22 to 7.62]; 2 log [5.09 to 7.75]; 6 log [5.11 to 7.55]), and storage temperatures (4°C [5.30 to 7.34]; 10°C [5.12 to 7.62]; 25°C [5.09 to 7.75]) (Tables V-1 to V-3). These results confirm that during aerobic storage, increases in the pH of beef samples may be the result of growth of aerobic natural flora which are able to grow over a broad temperature range. Jay et al. (2003) reported that *Pseudomonas* spp. were dominant in the spoilage of ground beef stored under aerobic conditions at 5-7°C. In general, the high oxidation-reduction potential of beef may contribute to dominance of *Pseudomonas* spp. under these conditions. Thus, the higher pH values of beef samples stored under aerobic conditions may have been caused by *Pseudomonas* spp. growth.

Uninoculated samples showed very low AI-2-like activity (day 0: 1.3 to 1.6 log RAI/cm^2, storage period: -1.6 to 0.9 log RAI/cm^2) regardless of storage condition, level of natural flora, and storage temperature (data not shown). These results indicate that in this study, fresh beef and its natural flora did not show major amounts of AI-2-like activity. Therefore, data presented in the figures and discussed in this paper show differences in AI-2-like activity between *E. coli* O157:H7 strain ATCC 43895 inoculated and uninoculated samples.

Lower storage temperatures, as expected, and higher levels of natural flora retarded growth of *E. coli* O157:H7 strain ATCC 43895 on beef samples (Figures V-1 to V-6). This finding is in agreement with results of Vold et al. (2000) who reported that high levels of background flora inhibited growth of *E. coli* O157:H7 in ground beef. Regardless of inoculation level and atmospheric condition, obvious AI-2-like activity was not detected on *E. coli* O157:H7 strain ATCC 43895 inoculated LNB and HNB beef samples stored at lower temperatures (4 and 10°C) (Figures V-7 to V-10). In general, total bacterial populations increased significantly (P<0.05) during storage, regardless of storage temperature, inoculation level, and level of natural flora (Figures V-1 to V-6). *E. coli* O157:H7 strain ATCC 43895 declined slightly or showed no growth in both LNB and HNB at 4°C (Figures V-1 and V-2), and at 10 °C, HNB limited the growth of *E. coli* O157:H7 more than LNB (Figures V-3 and V-4). However, *E. coli* O157:H7 strain ATCC 43895 showed rapid growth (Figures V-5 and V-6) and high AI-2-like activity was detected (Figures V-11 and V-12), especially in LNB than HNB samples stored at 25°C. These results indicate that AI-2-based quorum sensing of *E. coli* O157:H7 may be active in beef only during extensive and vigorous growth. In other words, even though *E. coli* O157:H7 cell densities are high enough to be associated with high AI-2-like activity, AI-2-based quorum sensing

may not be continuously active if there is no active growth of *E. coli* O157:H7 in beef. Cloak et al. (2002) also showed that *E. coli* O157:H7, *Salmonella*, and *Campylobacter* spp. inoculated in milk, chicken broth and brucella broth, and incubated at 4, 25 and 37°C generally produced more AI-2 at higher incubation temperatures. This result could be caused by an active growth of the bacteria at optimum temperatures. The results of our research also suggest that high levels of natural flora in the early stages of storage may retard AI-2-based quorum sensing of *E. coli* O157:H7. This finding (higher AI-2-like activity in LNB than HNB) also indicates that *E. coli* O157:H7 may not use AI-2-based quorum sensing to compete against the natural microbial flora. However, *E. coli* O157:H7 has receptor and regulator for the AHL produced by other Gram-negative bacteria (Ahmer et al., 1998). Thus, the effect of exogenous AHL on *E. coli* O157:H7 needs to be studied relative to varying levels of natural microbial flora.

Furthermore, more AI-2-like activity was detected on aerobically stored beef slices inoculated with *E. coli* O157:H7 strain ATCC 43895 than on those stored in vacuum packages at 25°C ($P<0.05$) (Figures V-11 to V-12), indicating that AI-2-based quorums sensing of *E. coli* O157:H7 strain ATCC 43895 is affected by atmospheric conditions. Fong et al. (2003) reported that *luxS*, being responsible for the production of AI-2, might act to control aerobic growth of *Actinobacillus actinomycetemcomitans* JP2 and its adaptation to iron-limiting conditions.

In general, total bacterial populations and *E. coli* O157:H7 strain ATCC 43895 populations on aerobically stored beef slices were higher ($P<0.05$) than those on product stored in vacuum packages, regardless of storage temperature (Figures V-1 to V-6). Irrespective of storage condition, total bacterial populations on LNB and HNB samples were greatly ($P<0.05$) increased during the early stages of storage, and then, they remained unchanged during the rest of the storage period at 10 and 25°C (Figures

V-3 to V-6). Regardless of inoculation level, *E. coli* O157:H7 strain ATCC 43895 populations on both aerobically stored LNB and HNB samples increased dramatically ($P<0.05$) during the early stage of storage at 25°C (Figures V-5 and V-6).

Unpublished data from our laboratory showed that in similar studies with beef purge, instead of meat, AI-2-like activity associated with *E. coli* O157:H7 strain ATCC 43895 was detected earlier and at higher levels in inoculated purge compared to results of this study with beef tissue (Figures V-7 to V-12). This indicates that AI-2-based quorum sensing of *E. coli* O157:H7 may also depend on factors such as the physical properties of the substrate. Surette and Bassler (1998) showed that production of AI-2 by *S.* Typhimurium and *E. coli* depended on, not only cell densities, but also on growth conditions. Cloak et al. (2002) suggested that production of autoinducer could be influenced by environmental factors such as the type of food or other sample matrices, incubation temperature, and atmospheric conditions.

In this study, the finding that AI-2-like activity was not from beef or natural flora is supported by the following: (i) beef products such as beef steak and beef patty rinsates showed very low AI-2-like activity in the presence of high indigenous bacterial populations (6.4-7.4 log CFU/ml) in the Lu et al. (2004) study; and (ii) in the current study, there was no significant ($P\geq0.05$) increase in AI-2-like activity during the early stages of storage, while AI-2-like activity increased as *E. coli* O157:H7 strain ATCC 43895 populations increased on beef. Recently, we found that higher AI-2-like activity was detected in *E. coli* O157:H7 strain ATCC 43895 inoculated beef purge samples containing low levels of natural flora than in samples containing higher levels of natural flora (unpublished). This finding is in agreement with results of this study indicating that higher AI-2-like activity was detected on LNB than on HNB. Interestingly, unlike beef samples stored at 4°C, an increase of AI-2-like activity was

observed in beef purge samples with low levels of natural flora at 4°C, even though *E. coli* O157:H7 strain ATCC 43895 levels were not increased (unpublished). Cloak et al. (2002) reported that AI-2 activity was detected in different liquid type foods inoculated with *E. coli* and *S.* Typhimurium, and then stored at 4°C. Thus, *E. coli* O157:H7 may produce AI-2 even at a low (4°C) temperature if they are suspended in a liquid substrate. Lu et al. (2005) showed that AI-2-like activity on tomato surfaces did not increase as the heterotrophic bacterial populations increased during storage at 4°C; they prefer using the term "AI-2-like activity" to "AI-2 molecules" because they were not sure that the response observed in the AI-2 bioassay used in their study was caused only by AI-2 molecules.

CONCLUSIONS

The present study has demonstrated that AI-2-based quorum sensing of *E. coli* O157:H7 could be active in fresh beef. In addition, activity of AI-2-based quorum sensing of *E. coli* O157:H7 depended not only on the level of natural flora but also on *E. coli* O157:H7 growth and cell density in beef. Moreover, presence of oxygen and higher storage temperature may influence AI-2-based quorum sensing of *E. coli* O157:H7 in beef. Further research on the role of AI-2-based quorum sensing of *E. coli* O157:H7 in foods is necessary because a clear understanding of the role of AI-2 produced by bacteria in foods could potentially be helpful in developing approaches for improving food safety.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. Pina M. Fratamico, Dr. John S. Novak and Dr. Bonnie L. Bassler for their friendly and critical advice, and cultures. This work was funded by the Colorado State University Agricultural Experiment Station.

Table V-1. pH values (mean ± standard error) of fresh beef which contained low (LNB) or high levels of natural flora (HNB) and was not inoculated or it was inoculated (2 or 6 log CFU/cm^2) with *Escherichia coli* O157:H7 strain ATCC 43895, during aerobic and vacuum storage for 21 days at 4°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	7	14	21
Aerobic	LNB	No	5.48±0.01 ^{Ba}	5.49±0.01 ^{Ba}	5.90±0.17 ^{Ab}	6.26±0.40 ^{Ab}
		2	5.53±0.04 ^{Ba}	5.48±0.02 ^{Ba}	6.09±0.02 ^{Ab}	6.44±0.19 ^{Ab}
		6	5.48±0.02 ^{Ca}	5.50±0.01 ^{Ca}	6.21±0.21 ^{Bb}	7.02±0.13 ^{Aa}
	HNB	No	5.54±0.05 ^{Ca}	5.74±0.14 ^{Ca}	6.61±0.17 ^{Ba}	7.19±0.11 ^{Aa}
		2	5.48±0.01 ^{Ca}	5.72±0.08 ^{Ca}	6.64±0.14 ^{Ba}	7.34±0.09 ^{Aa}
		6	5.46±0.01 ^{Ca}	5.65±0.06 ^{Ca}	6.78±0.16 ^{Ba}	7.26±0.07 ^{Aa}
Vacuum	LNB	No	5.49±0.01 ^{Aa}	5.50±0.02 ^{Aa}	5.49±0.03 ^{Ac}	5.45±0.03 ^{Ac}
		2	5.52±0.03 ^{Aa}	5.52±0.01 ^{Aa}	5.48±0.04 ^{Ac}	5.44±0.11 ^{Ac}
		6	5.48±0.02 ^{Aa}	5.47±0.03 ^{Aa}	5.48±0.02 ^{Ac}	5.44±0.02 ^{Ac}
	HNB	No	5.67±0.12 ^{Aa}	5.44±0.01 ^{Aa}	5.41±0.01 ^{Ac}	5.44±0.07 ^{Ac}
		2	5.48±0.02 ^{Aa}	5.49±0.02 ^{Aa}	5.40±0.02 ^{Ac}	5.33±0.01 ^{Ac}
		6	5.49±0.04 ^{Aa}	5.45±0.03 ^{Aa}	5.34±0.03 ^{Ac}	5.30±0.07 ^{Ac}

Inoc: level of inoculation.

^{ABC}: means within the same row with different superscript letters are different (P<0.05).

^{abc}: means within the same column with different superscript letters are different (P<0.05).

Table V-2. pH values (mean ± standard error) of fresh beef which contained low (LNB) or high levels of natural flora (HNB) and was not inoculated or it was inoculated (2 or 6 log CFU/cm^2) with *Escherichia coli* O157:H7 strain ATCC 43895, during aerobic and vacuum storage for 18 days at 10°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	6	12	18
Aerobic	LNB	No	5.48±0.01 ^{Ba}	5.76±0.11 ^{ABcd}	6.14±0.15 ^{Ac}	6.23±0.35 ^{Ab}
		2	5.53±0.04 ^{Ba}	6.10±0.26 ^{ABbc}	6.53±0.19 ^{Abc}	6.58±0.21 ^{Ab}
		6	5.48±0.02 ^{Ca}	6.40±0.36 ^{Bab}	5.99±0.06 ^{BCcd}	7.24±0.37 ^{Aa}
	HNB	No	5.54±0.05 ^{Ca}	6.99±0.19 ^{Ba}	7.03±0.11 ^{ABab}	7.62±0.08 ^{Aa}
		2	5.48±0.01 ^{Ca}	6.81±0.36 ^{Ba}	7.13±0.09 ^{ABa}	7.61±0.09 ^{Aa}
		6	5.46±0.01 ^{Ba}	6.94±0.32 ^{Aa}	6.94±0.04 ^{Aab}	7.55±0.19 ^{Aa}
Vacuum	LNB	No	5.49±0.01 ^{Aa}	5.55±0.03 ^{Acd}	5.39±0.08 ^{Ade}	5.22±0.10 ^{Ac}
		2	5.52±0.03 ^{Aa}	5.58±0.04 ^{Acd}	5.30±0.07 ^{Ae}	5.24±0.09 ^{Ac}
		6	5.48±0.02 ^{Aa}	5.54±0.04 ^{Acd}	5.31±0.04 ^{Ae}	5.17±0.03 ^{Ac}
	HNB	No	5.67±0.12 ^{Aa}	5.51±0.02 ^{Acd}	5.29±0.08 ^{Ae}	5.31±0.11 ^{Ac}
		2	5.48±0.02 ^{Aa}	5.41±0.06 ^{Ad}	5.10±0.08 ^{Ae}	5.12±0.04 ^{Ac}
		6	5.49±0.04 ^{Aa}	5.35±0.09 ^{Ad}	5.15±0.09 ^{Ae}	5.20±0.09 ^{Ac}

Inoc: level of inoculation.

^{ABC}: means within the same row with different superscript letters are different (P<0.05).

^{abcde}: means within the same column with different superscript letters are different (P<0.05).

Table V-3. pH values (mean ± standard error) of fresh beef which contained low (LNB) or high levels of natural flora (HNB) and was not inoculated or it was inoculated (2 or 6 log CFU/cm^2) with *Escherichia coli* O157:H7 strain ATCC 43895, during aerobic and vacuum storage for 9 days at 25°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	3	6	9
Aerobic	LNB	No	5.48±0.01 ^{Ba}	5.91±0.05 ^{Bbc}	6.95±0.18 ^{Ac}	6.94±0.49 ^{Ab}
		2	5.53±0.04 ^{Ba}	5.48±0.14 ^{Bcd}	6.31±0.18 ^{Ad}	6.67±0.42 ^{Ab}
		6	5.48±0.02 ^{Aa}	5.37±0.06 ^{Ad}	5.37±0.04 ^{Ae}	5.33±0.08 ^{Ac}
	HNB	No	5.54±0.05 ^{Ca}	6.57±0.09 ^{Ba}	7.55±0.14 ^{Aab}	7.54±0.14 ^{Aa}
		2	5.48±0.01 ^{Ca}	6.32±0.16 ^{Bab}	7.56±0.16 ^{Aa}	7.75±0.06 ^{Aa}
		6	5.46±0.01 ^{Ba}	5.90±0.08 ^{Bbc}	7.08±0.05 ^{Abc}	7.44±0.09 ^{Aa}
Vacuum	LNB	No	5.49±0.01 ^{Aa}	5.45±0.05 ^{Acd}	5.36±0.12 ^{Ae}	5.36±0.24 ^{Ac}
		2	5.52±0.03 ^{Aa}	5.37±0.05 ^{Ad}	5.26±0.10 ^{Af}	5.30±0.16 ^{Ac}
		6	5.48±0.02 ^{Aa}	5.45±0.03 ^{Acd}	5.30±0.08 ^{Ae}	5.11±0.02 ^{Ac}
	HNB	No	5.67±0.12 ^{Aa}	5.22±0.15 ^{Ad}	5.23±0.16 ^{Ag}	5.26±0.13 ^{Ac}
		2	5.48±0.02 ^{Aa}	5.17±0.03 ^{ABd}	4.97±0.01 ^{Bg}	5.09±0.07 ^{ABc}
		6	5.49±0.04 ^{Aa}	5.22±0.06 ^{Ad}	5.14±0.02 ^{Ag}	5.13±0.05 ^{Ac}

Inoc: level of inoculation.

^{ABC}: means within the same row with different superscript letters are different (P<0.05).

^{abcdefg}: means within the same column with different superscript letters are different (P<0.05).

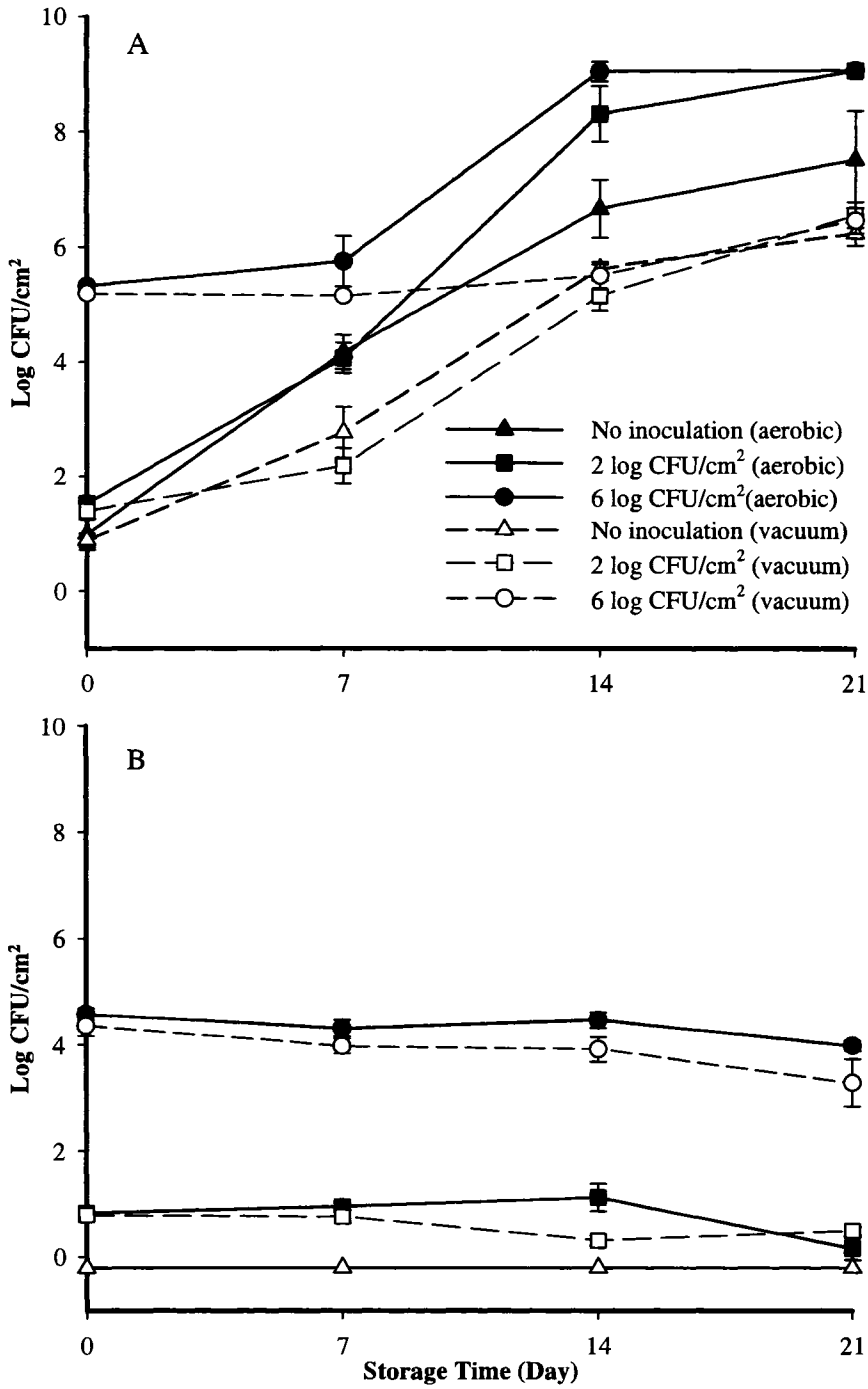

Figure V-1. Total bacterial populations recovered with tryptic soy agar (A) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (B) in *E. coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low levels of natural flora (LNB) during aerobic and vacuum storage for 21 days at 4°C (Data in APPENDIX Tables 20 and 21).

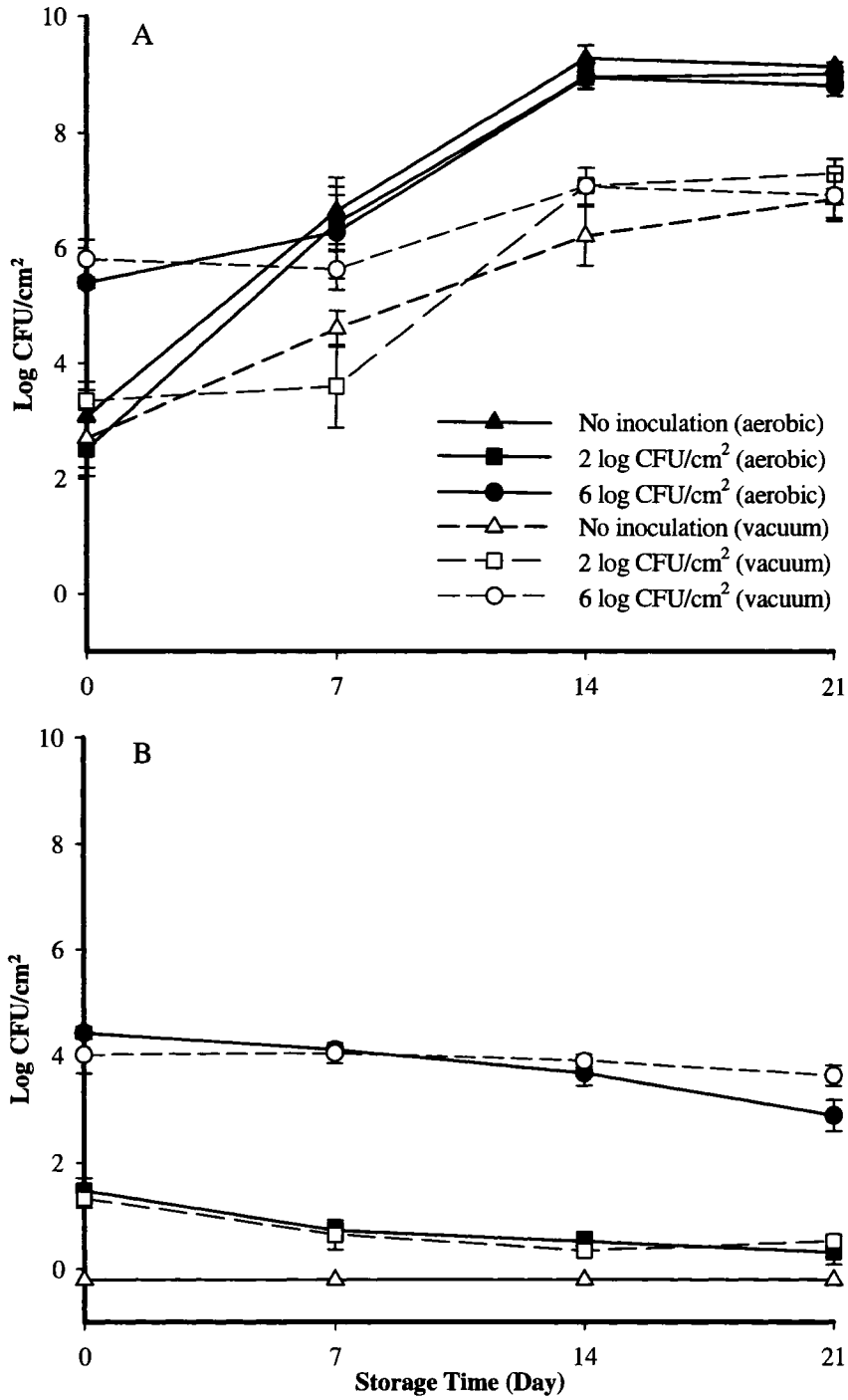

Figure V-2. Total bacterial populations recovered with tryptic soy agar (A) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (B) in *E. coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing high levels of natural flora (HNB) during aerobic and vacuum storage for 21 days at 4°C (Data in APPENDIX Tables 20 and 21).

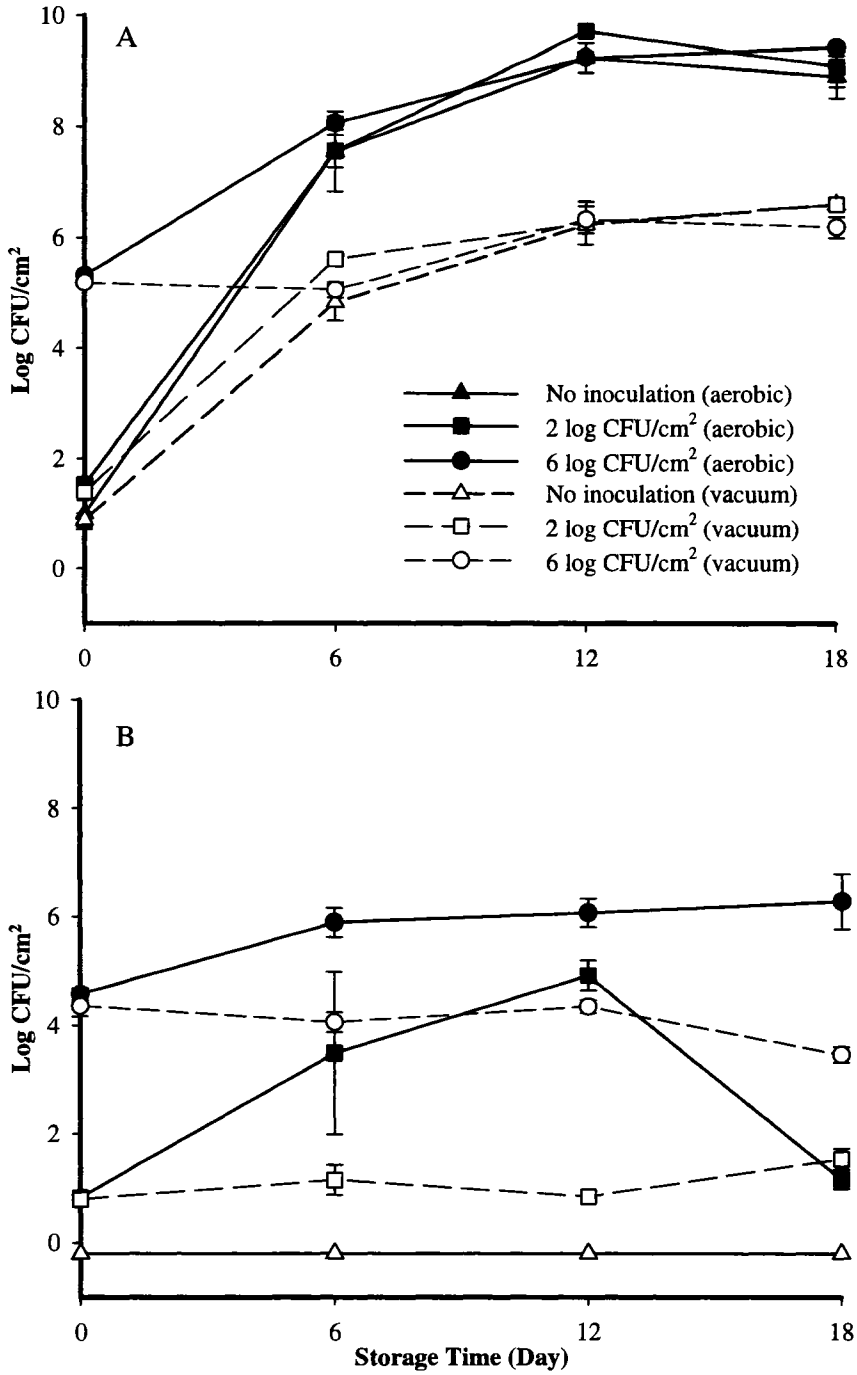

Figure V-3. Total bacterial populations recovered with tryptic soy agar (A) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (B) in *E. coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low levels of natural flora (LNB) during aerobic and vacuum storage for 18 days at 10°C (Data in APPENDIX Tables 22 and 23).

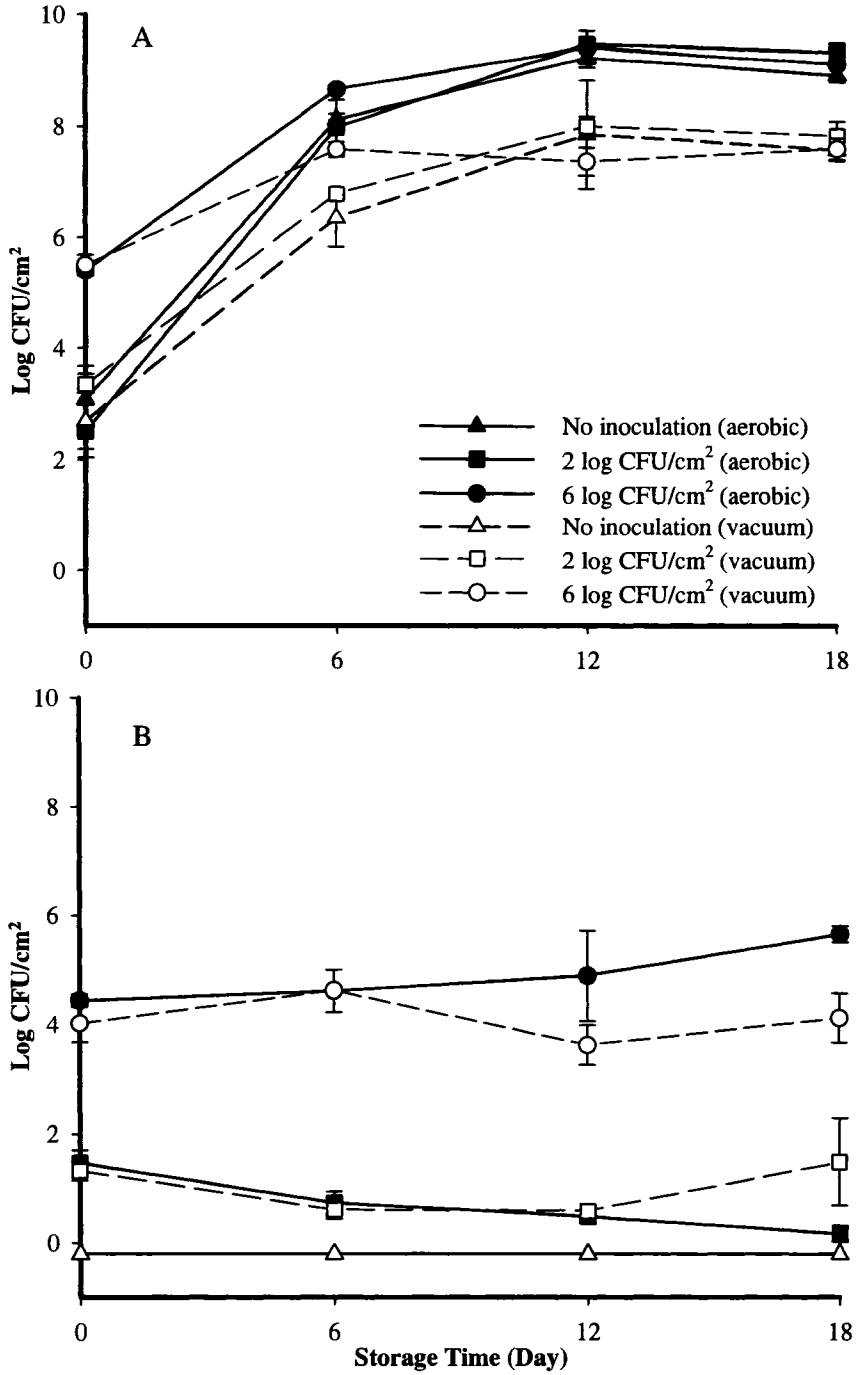

Figure V-4. Total bacterial populations recovered with tryptic soy agar (A) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (B) in *E. coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing high levels of natural flora (HNB) during aerobic and vacuum storage for 18 days at 10°C (Data in APPENDIX Tables 22 and 23).

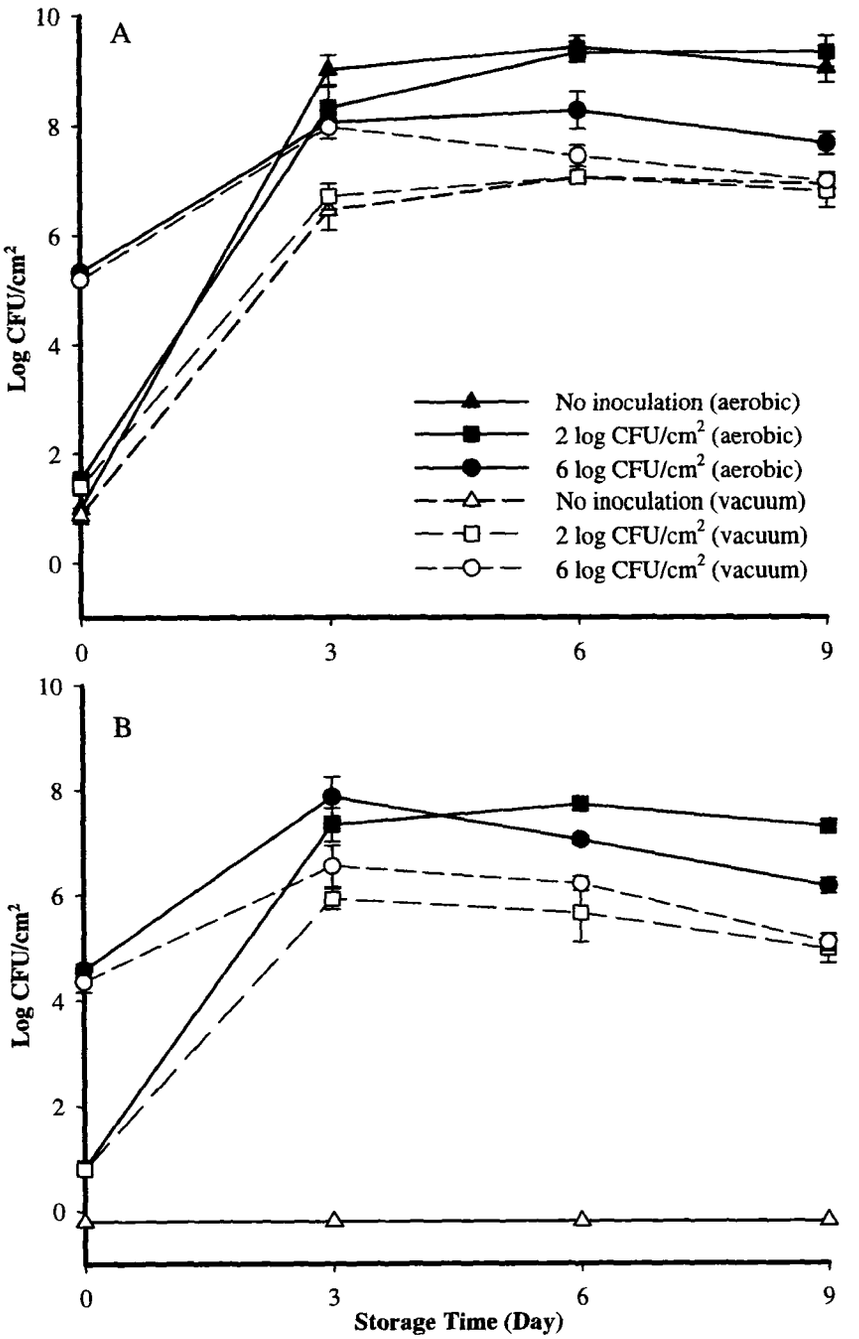

Figure V-5. Total bacterial populations recovered with tryptic soy agar (A) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (B) in *E. coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low levels of natural flora (LNB) during aerobic and vacuum storage for 9 days at 25°C (Data in APPENDIX Tables 24 and 25).

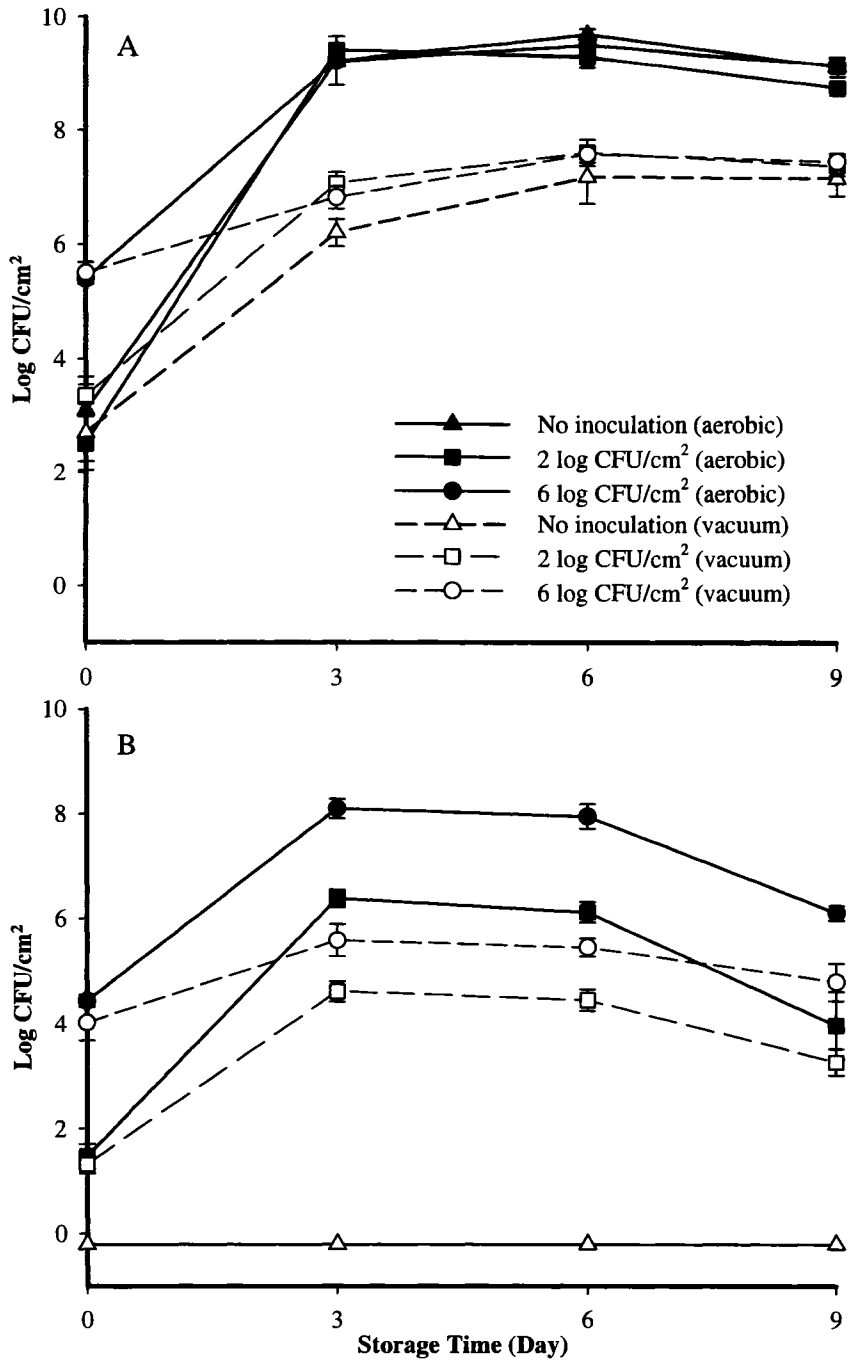

Figure V-6. Total bacterial populations recovered with tryptic soy agar (A) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (B) in *E. coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing high levels of natural flora (HNB) during aerobic and vacuum storage for 9 days at 25°C (Data in APPENDIX Tables 24 and 25).

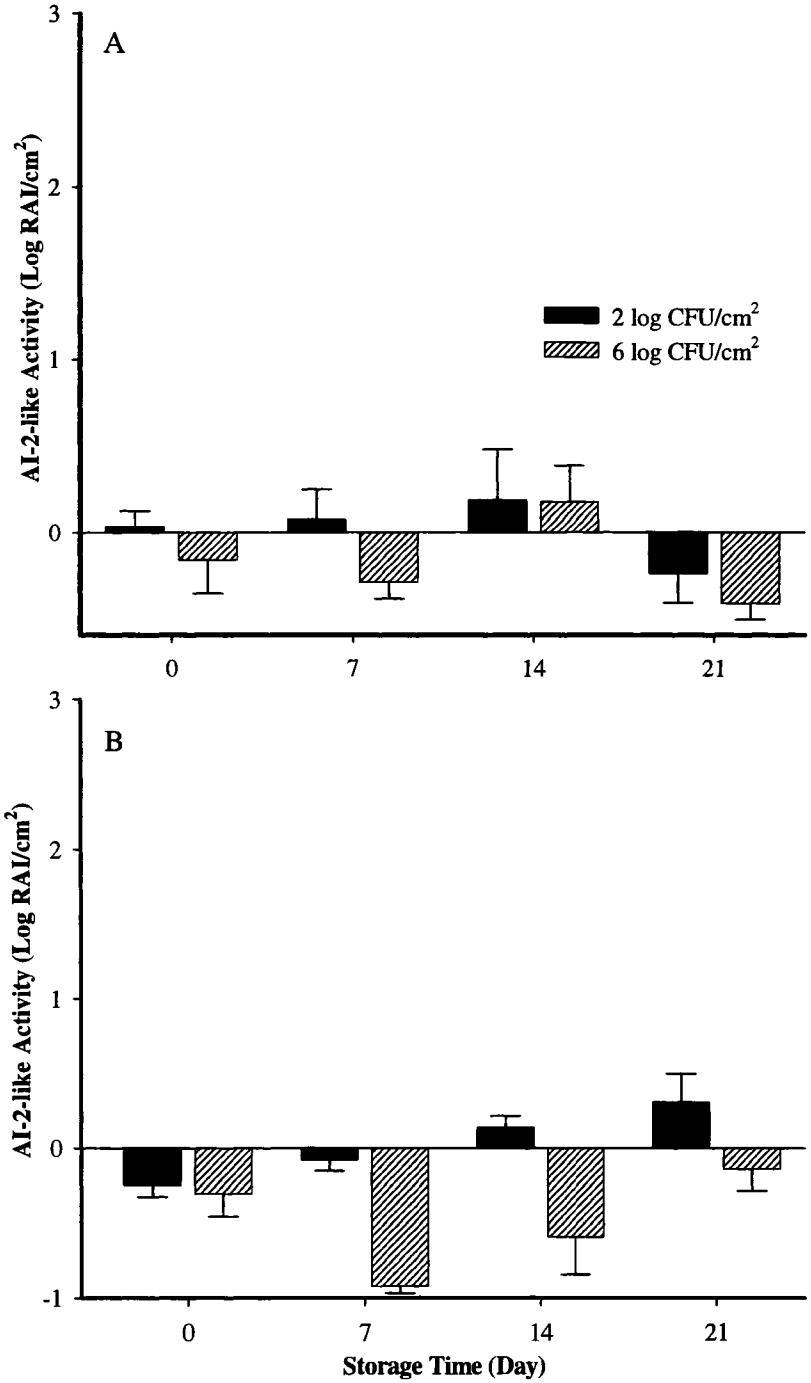

Figure V-7. AI-2-like activity in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low levels of natural flora (LNB) under aerobic (A) and vacuum packaged (B) storage conditions for 21 days at 4°C (Data in APPENDIX Table 26).

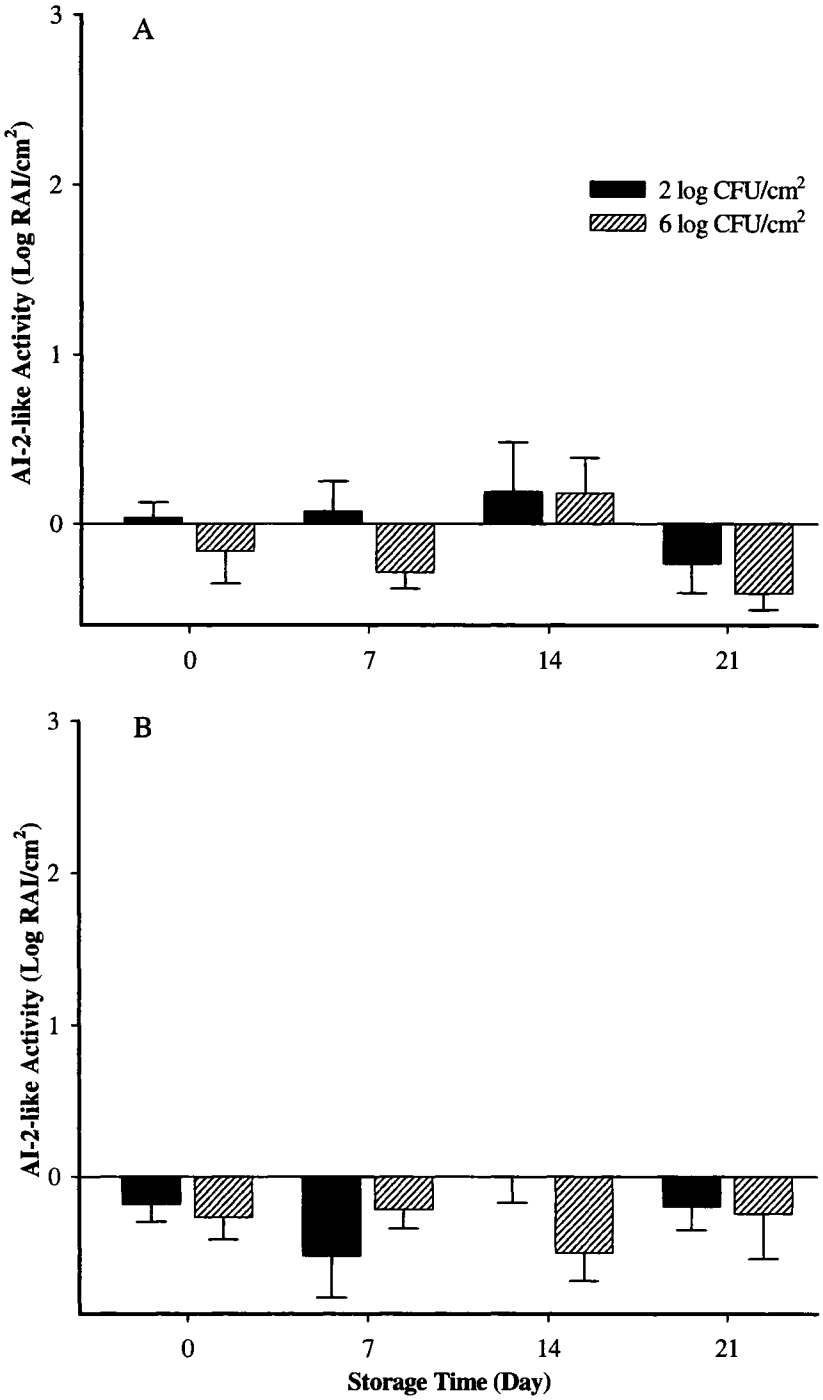

Figure V-8. AI-2-like activity in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing high levels of natural flora (HNB) under aerobic (A) and vacuum packaged (B) storage conditions for 21 days at 4°C (Data in APPENDIX Table 26).

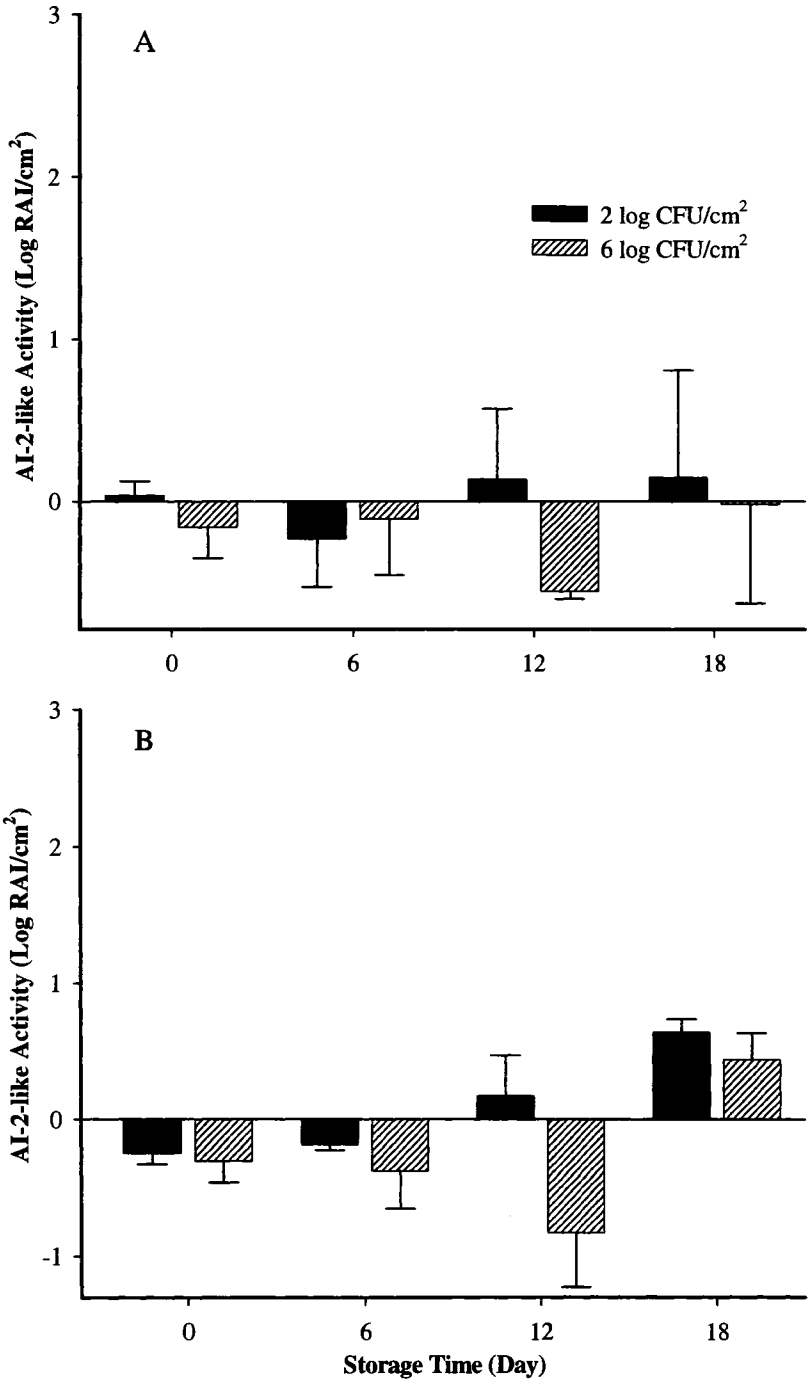

Figure V-9. AI-2-like activity in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low levels of natural flora (LNB) under aerobic (A) and vacuum packaged (B) storage conditions for 18 days at 10°C (Data in APPENDIX Table 27).

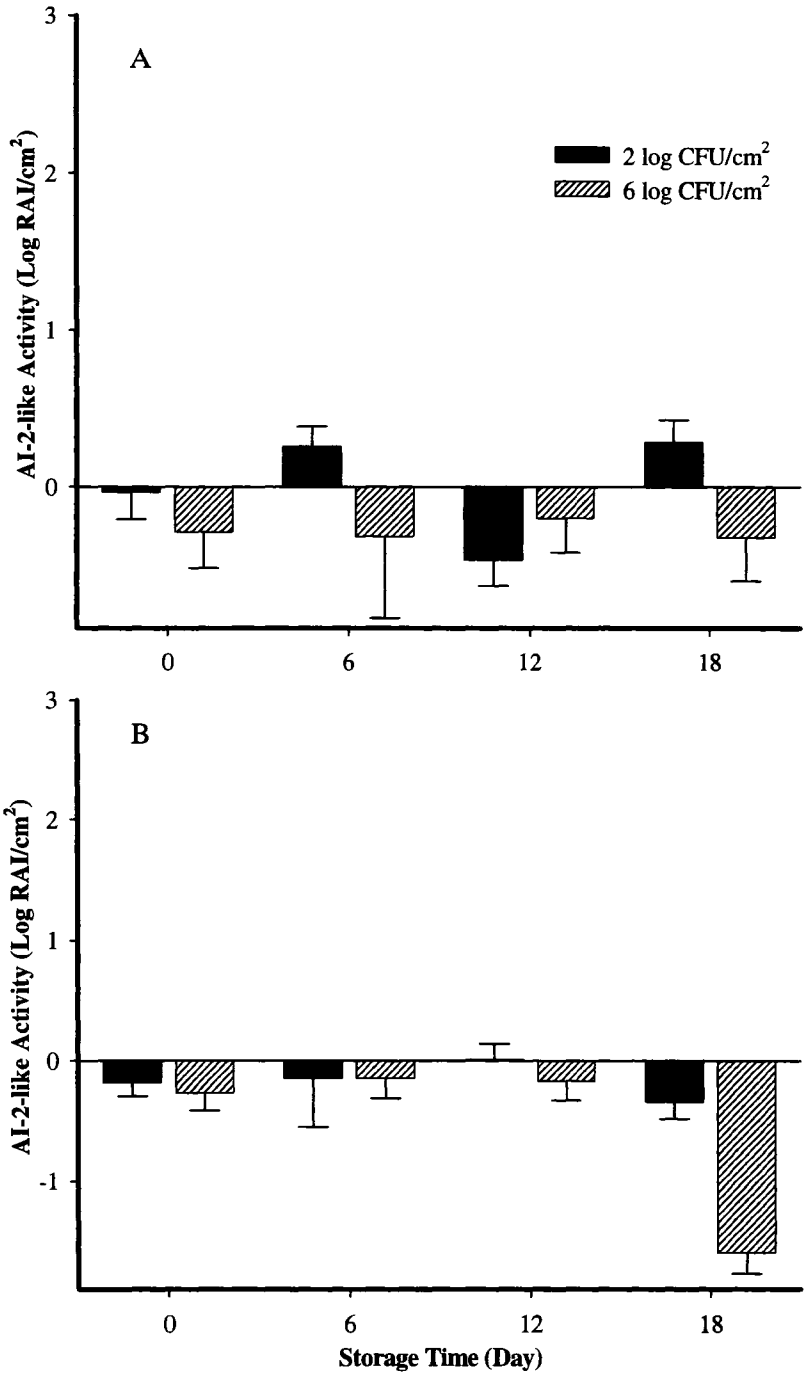

Figure V-10. AI-2-like activity in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing high levels of natural flora (HNB) under aerobic (A) and vacuum packaged (B) storage conditions for 18 days at 10°C (Data in APPENDIX Table 27).

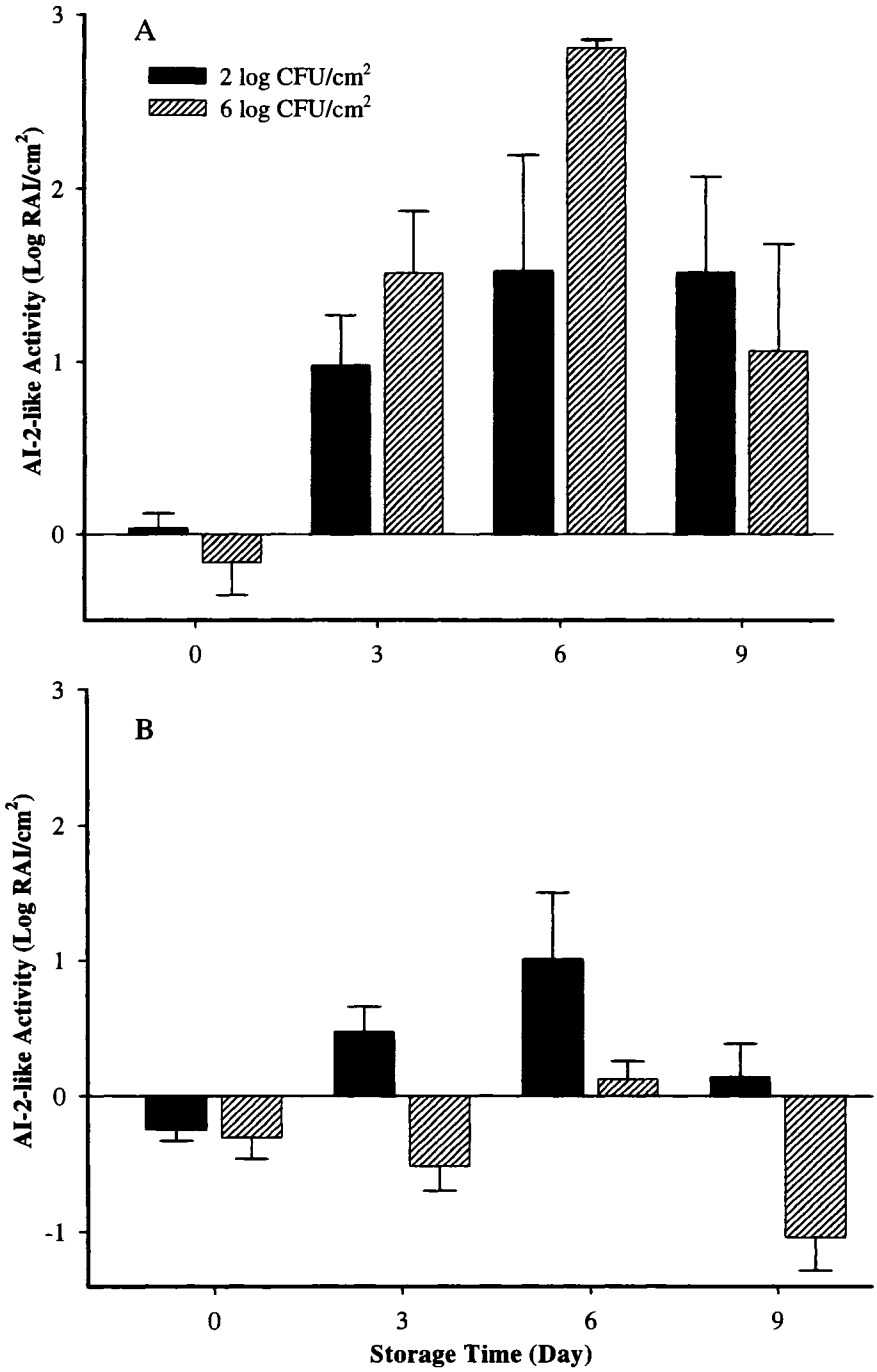

Figure V-11. AI-2-like activity in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low levels of natural flora (LNB) under aerobic (A) and vacuum packaged (B) storage conditions for 9 days at 25°C (Data in APPENDIX Table 28).

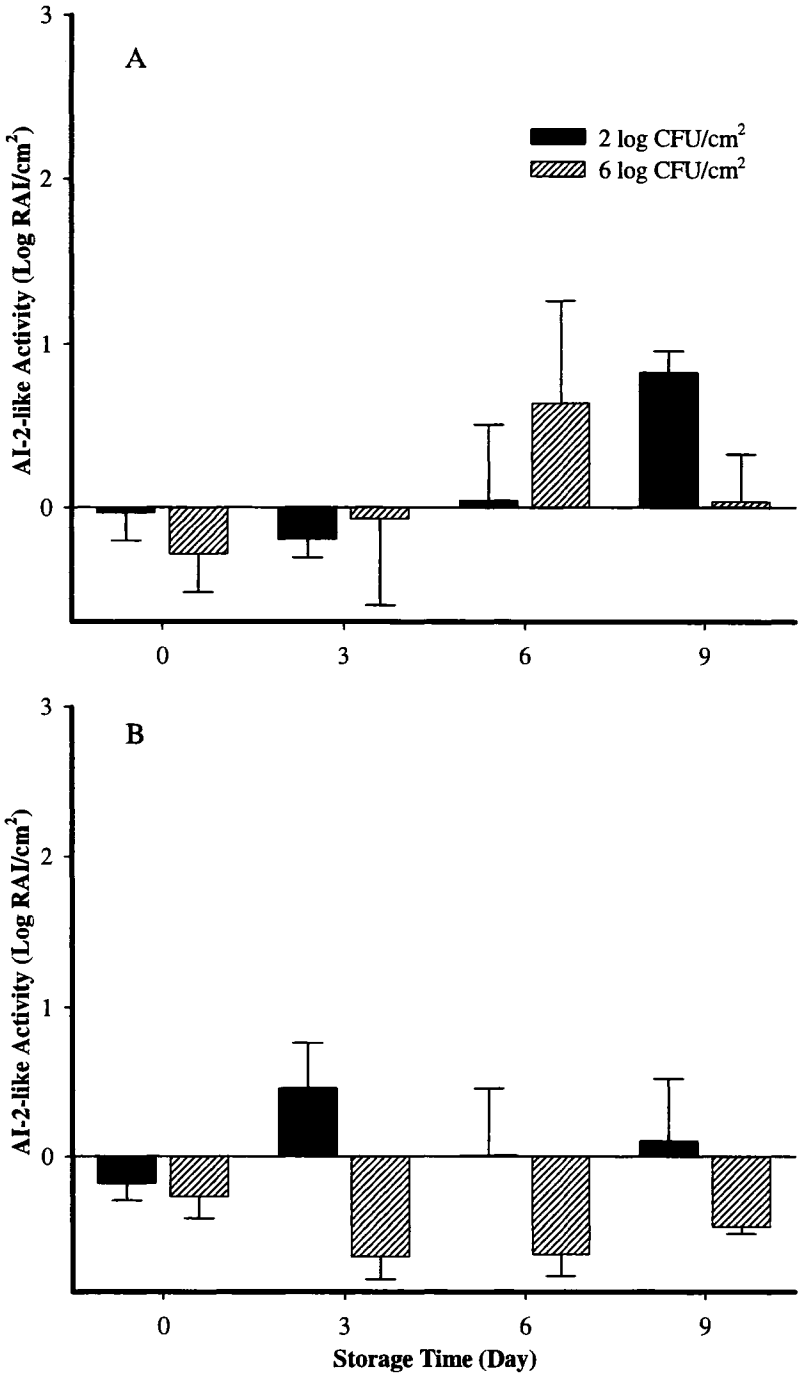

Figure V-12. AI-2-like activity in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing high levels of natural flora (HNB) under aerobic (A) and vacuum packaged (B) storage conditions for 9 days at 25°C (Data in APPENDIX Table 28).

CHAPTER VI

QUORUM SENSING AND STRESS RESISTANCE RELATIONSHIP IN *SALMONELLA*

ABSTRACT

The objective of this study was to evaluate the potential relationship between autoinducer-2 (AI-2) activity and resistance of *Salmonella* to heat and acid. Filter (0.22 μm) sterilized supernatants (pH 3.5 and pH 7.0; adjusted with HCl and NaOH, respectively) of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative; *luxS* mutant of *S.* Thompson strain RM1987N), or *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative; *luxS* mutant of *E. coli* O157:H7 strain 86-24) cultured in Luria-Bertani (LB) + 0.5% glucose broth, were prepared as preconditioned culture (PC) media. *S.* Thompson strains RM1987N and RM1987NLUX were exposed to 55°C (6 h) in LB, and *S.* Thompson strain RM1987NLUX and *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP (AI-2-positive) were exposed to 55°C in the above AI-2-positive and -negative PC media. In addition, *S.* Thompson strain RM1987NLUX was acid challenged in the PC media (pH 3.5; 35°C), and acid adapted [tryptic soy broth (TSB) with 1% glucose] and nonacid adapted (TSB without glucose) cultures of *S.* Thompson strains RM1987N and RM1987NLUX were challenged in LB (pH 3.0; 35°C). At hourly intervals, samples were plated on tryptic soy agar. The Baranyi

model (heat) and linear regression (acid) were used to obtain death rates of the pathogen. During heat challenge, surviving counts of *S.* Thompson strains RM1987N and RM1987NLUX were not different ($P \geq 0.05$) in LB, and survivors of *S.* Thompson strain RM1987NLUX and *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP were also not different ($P \geq 0.05$) in various PC media preparations. In addition to survival, death rates (-3.17 to -3.90 log CFU/ml/h) were similar in the presence or absence of AI-2 activity. During acid challenge, survivor counts of *S.* Thompson strain RM1987NLUX were similar ($P \geq 0.05$) in AI-2-positive and -negative PC media, and survivor counts of acid adapted and nonacid adapted *S.* Thompson strains RM1987N and RM1987NLUX were not different ($P \geq 0.05$) in LB. In general, death rates of *Salmonella* showed no differences (≤ 0.08 log CFU/ml/h) in media with or without AI-2 activity. These results suggest that AI-2-based quorum sensing may not play a major role in heat and acid resistance of *Salmonella.*

INTRODUCTION

Many groups of bacteria possess cell-to-cell density-dependent signaling systems, called quorum sensing using low-molecular-weight signaling molecules (Smith et al., 2004). Bacteria produce the quorum sensing signaling molecules when cell populations reach thresholds and they sense the level of these signaling molecules around them; this results in change of gene expression (Waters and Bassler, 2005). Quorum sensing was first observed in *Vibrio harveyi* and *Vibrio fischeri* that are bioluminescent marine bacteria (Nealson and Hastings, 1979; Ruby, 1996). After quorum sensing was found in these bacteria, many scientists have found quorum sensing molecules in various other bacteria and the quorum sensing molecules have been categorized into four groups (reviewed by Smith et al., 2004), *N*-acylhomoserine

lactone [AHL or autoinducer (AI)-1] in Gram-negative bacteria (Miller and Bassler, 2001; Whitehead et al., 2001), AI-2 (furanosyl borate diester) in both Gram-negative and Gram-positive bacteria (Chen et al., 2002; Schauder and Bassler, 2001; Xavier and Bassler, 2005a), AI-3 (cross-communication with the mammalian epinephrine cell of host signaling system; Sperandio et al., 2003), and oligopeptides in Gram-positive bacteria (Miller and Bassler, 2001; Whitehead et al., 2001). In addition, certain quorum sensing molecules such as diketopiperazines (Degrassi et al., 2002; Holden et al., 1999), 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal) (Holden et al., 2000; Pesci et al., 1999), 3,7,11-trimethyl-2,6,10-dodecatrienoate (farnesoic acid) (Oh et al., 2001), farnesol (Hornby et al., 2001), and tyrosol (Chen et al., 2004) have been identified only in some organisms. Of the quorum sensing molecules, Gram-negative foodborne pathogenic bacteria such as *Escherichia coli* O157:H7, *Salmonella*, and *Campylobacter* spp. produce AI-2 (Cloak et al., 2002; Elvers and Park, 2002; Surette and Bassler, 1998). Interestingly, *Listeria monocytogenes* Scott A also produces AI-2 (Turovskiy and Chikindas, 2006). In the AI-2 biosynthesis, LuxS works as the AI-2 synthase and AI-2 is derived from *S*-adenosylmethionine (SAM) via three enzymatic steps (Chen et al., 2002; Schauder et al., 2001).

In bacteria, quorum sensing may regulate properties or functions such as virulence (von Bodman et al., 1998), plasmid conjugation (Lithgow et al., 2001), bioluminescence (Cao and Meighen, 1989), protein production (DeLisa et al., 2001c), and biofilm formation (Davies et al., 1998). Recently, food microbiologists have studied quorum sensing as a potential novel target that could be used to improve food safety or quality. Jay et al. (2003) suggested that spoilage and biofilm formation in fresh meat at low storage temperatures may be related to quorum sensing. Christensen

et al. (2003) reported that quorum sensing may be involved in milk spoilage, and Rasch et al. (2005) also suggested that bacterial spoilage of some foods may be associated with quorum sensing. AI-2 was presented as related to biofilm formation of *E. coli* K-12 (González Barrios et al., 2006), and *S*-ribosylhomocysteine, the precursor of AI-2, was also found to be involved in biofilm formation of *L. monocytogenes* (Challan Belval et al., 2006). *Salmonella* Typhimurium has also been reported as using quorum sensing to induce biofilm formation (De Keersmaecker et al., 2005; Prouty et al., 2002). In contrast, Brandl et al. (2005) found no relationship between quorum sensing of *Salmonella* Thompson and biofilm formation on cilantro leaf surfaces.

In enterohemorrhagic and enteropathogenic *E. coli*, AI-2 has been related to the type III secretion system, the Tir (translocated intimin receptor), and intimin intestinal colonization (Sperandio et al., 1999). Kanamaru et al. (2000) suggested that *E. coli* O157:H7 colonizes the intestine via quorum sensing. In addition, AI-2 was found to induce the *LEE* [locus of enterocyte effacement; pathogenicity island (Mellies et al., 1999)] operons in *E. coli* K-12 and *E. coli* O157:H7 (Sperandio et al., 1999). However, additional work using an AI-2 fraction showed that AI-2 does not activate the *LEE* genes of *E. coli* O157:H7 (Sperandio et al., 2003). AI-2 signaling may be necessary to protect eukaryotes from pathogenic bacteria (Xavier and Bassler, 2005a).

In *E. coli* K-12, DeLisa et al. (2001b) found the obvious overlap present among known quorum sensing, starvation genes, and several stress genes. In addition, *E. coli* K-12 utilizes AI-2 to communicate the stress or metabolic burden related to overall accumulation of foreign protein (DeLisa et al., 2001c). *Pseudomonas aeruginosa* may use quorum sensing to resist the oxidative stress response; *P. aeruginosa* mutants with deletion of *lasI* and *rhlI* were more susceptible to hydrogen

peroxide than the wild type (Huang and Shih, 2000). This stress response based *rhlI* quorum sensing system may be related to *rpoS* of *P. aeruginosa*. Latifi et al. (1996) suggested that the *rhl* quorum sensing system initiates the transcription of *rpoS*. RopS protein of *P. aeruginosa* affects the stationary phase stress tolerance (Jørgensen et al., 1999). The *rpoS* mutant strains of *P. aeruginosa* were more susceptible to heat shock and NaCl stress compared to the wild-type strain (Suh et al., 1999). According to these results, *rhl* quorum sensing of *P. aeruginosa* may be involved in resistance to heat and NaCl. In disagreement with these findings, Whiteley et al. (2000) proved that the *rpoS* transcription is not related to quorum sensing. In *Vibrio vulnificus*, the *smcR* (*V. harveyi luxR* homologue; transcriptional regulator in quorum sensing) defective strain was more susceptible to oxidative stress and starvation than the wild-type strain (McDougald et al., 2001, 2003). In *V. vulnificus* and *Vibrio angustum*, AI-2-like signaling induced the response to starvation adaptation and stress resistance (McDougald et al., 2003). Farnesol, the quorum sensing molecule synthesized by *Candida albicans*, protected *C. albicans* cells from hydrogen peroxide toxicity (Westwater et al., 2005). The *luxS* mutant strain of *Streptococcus mutans* was more resistant to hydrogen peroxide (58.8 mM) and the strains showed increased susceptibility to acid, but could still go through acid adaptation (Wen and Burne, 2004). *Staphylococcus epidermidis* regulates general and oxidative stress-response factors with *agr* quorum sensing system, which contains detoxifying enzymes of reactive oxygen (Yao et al., 2006).

S. Typhimurium also uses *rpoS* to be resistant to acid stress. Acid sensitive *S.* Typhimurium showed no induction or a reduced level of RpoS (Berk et al., 2005; Jørgensen et al., 2000). Many studies also showed that *S.* Typhimurium can be heat resistant (Bunning et al., 1990; Foster and Spector, 1995; Mackey and Derrick, 1986,

1990). Under heat stress, *S.* Typhimurium increases levels of heat shock chaperones such as DnaK and GroEL (Berk et al., 2005). Acid stressed cells increase cross-protection to heat, oxidative stress and osmotic stress, and this cross protection is dependant on RpoS (Foster and Spector, 1995; Leyer and Johnson, 1993). Like *P. aeruginosa*, these resistance mechanisms may be related to quorum sensing. Thus, the objective of this study was to evaluate the potential relationship between AI-2 activity and resistance of *Salmonella* to heat and acid.

MATERIALS AND METHODS

Preparation of inoculum. Colonies of *S.* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative; *luxS* mutant of strain RM1987N) maintained in Luria-Bertani (LB; Difco, Becton Dickinson and Company, Sparks, MD) agar containing nalidixic acid (25 μg/ml; Sigma-Aldrich, St. Louis, MO), and LB agar containing nalidixic acid (25 μg/ml) and kanamycin (30 μg/ml; Sigma-Aldrich), respectively, were individually activated and subcultured (0.1 ml) in 10 ml of LB broth at 35°C for 24 h. Preliminary studies showed that no antibiotic addition into LB broth did not influence AI-2 production of the strains during incubation in LB broth at 35°C. Thus, antibiotics were not added to LB broth. To obtain acid adapted and nonacid adapted cells, a 0.1 ml portion of activated cells was subcultured in tryptic soy broth without glucose (Difco) + 1% glucose (Fisher Scientific, Fair Lawn, NJ) (acid adaptation) or no glucose (nonacid adaptation) at 35°C for 24 h. A colony of *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP (Noah et al., 1995), maintained in xylose lysine desoxycholate (Difco) agar, was also activated and subcultured (0.1 ml) in 10 ml of LB broth at 35°C for 24 h. Stationary phase cells were harvested by centrifugation (4,629×*g*, 15 min, 4°C) and washed with phosphate

buffered saline (pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 liter of distilled water; PBS). The cell pellets were resuspended with PBS to yield approximately 8 log CFU/ml. *S.* Thompson strains RM1987N and RM1987NLUX cultures were kindly provided by Dr. Maria T. Brandl (USDA/ARS, WRRC, Produce Safety and Microbiology Research Unit, Albany, CA).

Preparation of preconditioned (PC) media. A similar method to that described by Sperandio et al. (1999) was applied in the preparation of preconditioned (PC) media with and without AI-2 activity. A colony of *S.* Thompson strains RM1987N (AI-2-positive; for AI-2-positive PC medium) or RM1987NLUX (AI-2-negative; for AI-2-negative PC medium) from cultures maintained in LB agar containing nalidixic acid (25 μg/ml), and LB agar containing nalidixic acid (25 μg/ml) and kanamycin (30 μg/ml), respectively, was activated in 10 ml of sterile LB broth at 35°C for 24 h. Then 0.5 ml portion of the cultures was inoculated into 50 ml of sterile LB + 0.5% glucose (Fisher Scientific, Fair Lawn, NJ) (LBG) broth and incubated at 35°C for 6 h ($OD_{600} \geq$ 0.7). The cultures were centrifuged (10,000×*g*, 10 min, 4°C) and supernatants were collected. The pH of the supernatant was adjusted to pH 7.0 with 1N NaOH for heat challenge or pH 3.5 with 1N HCl for acid challenge, followed by filter-sterilization (0.22μm, Nalgene, Rochester, NY). To evaluate whether the source of the PC media influenced the response of *Salmonella* to heat stress, PC media from *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) or VS94 (AI-2-negative) were also prepared. A colony of *E. coli* O157:H7 strains 86-24 (AI-2-positive; for AI-2-positive PC medium) and VS94 (AI-2-negative; isogenic mutant of 86-24; for AI-2-negative PC medium), maintained on sorbitol McConkey agar (Difco) supplemented with cefixime and potassium tellurite (Dynal, Oslo, Norway) (SMAC-CT), was activated according to the procedure for the *S.* Thompson strains. Then, 0.5 ml portion of the activated

cultures was inoculated into 50 ml of LBG broth and incubated at 35°C for 13 h (OD_{600} ≈ 1.0). The cultures were centrifuged (10,000×*g*, 10 min, 4°C), and supernatants were collected. The pH of the supernatants was adjusted to pH 7.0 with 1N NaOH and to pH 3.5 with 1N HCl for heat challenge and acid challenge, respectively. Then, AI-2 activity of PC media was measured at 0 h. *E. coli* O157:H7 strains 86-24 and VS94 were kindly provided by Dr. Vanessa Sperandio (University of Texas Southwestern Medical Center, Dallas, TX).

Heat challenge. Heat challenge studies were conducted in a 55°C water bath. To compare cell survival of *S.* Thompson strains RM1987N and RM1987NLUX during heat stress, a 1 ml portion of the cultures composed of *S.* Thompson strains RM1987N or RM1987NLUX was inoculated in 29 ml of sterile LB broth (pH 7.0; 55°C), while for evaluation of whether *S.* Thompson could use exogenous AI-2 molecules, a 1 ml portion of *S.* Thompson strain RM1987NLUX culture was inoculated into 29 ml of PC media, derived from *S.* Thompson strains RM1987N and RM1987NLUX (pH 7.0; 55°C). Since PC media lacked nutrients, a 0.75 ml portion of sterile 20×LB broth was added to the PC media to give a 0.5% final concentration of LB broth. A 1 ml portion of *S.* Thompson strain RM1987NLUX culture was inoculated into 29 ml of PC media (pH 7.0; 55°C) derived from *E. coli* O157:H7 strains 86-24 and VS94 to determine whether the exogenous AI-2 activity of *E. coli* O157:H7 could influence the survivors of *S.* Thompson strain RM1987NLUX under heat stress. To evaluate whether exogenous AI-2 molecules produced by other AI-2-positive *Salmonella* strain can influence resistance to heat and whether exogenous AI-2 molecules from different bacteria affects the survivors of *Salmonella* under heat stress, a 1 ml portion of *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP culture was inoculated into the 29 ml of PC media (pH 7.0; 55°C) derived from *S.* Thompson strains RM1987N and

RM1987NLUX, and the 29 ml of PC media (pH 7.0; 55°C) derived from *E. coli* O157:H7 strains 86-24 and VS94.

Acid challenge. Acid challenge was performed in a 35°C water bath to evaluate whether *S.* Thompson can use exogenous AI-2 molecules to be resistant to acid. A 1 ml portion of *S.* Thompson RM1987NLUX culture was inoculated into 29 ml of PC media (pH 3.5; 35°C), which was derived from *S.* Thompson strains RM1987N and RM1987NLUX. Unlike PC media for heat challenge, PC media for acid challenge did not contain 0.75 ml of 20×LB broth for providing nutrients. To examine whether AI-2-based quorum sensing of *S.* Thompson influences acid adaptation, the 1 ml portions of acid adapted and nonacid adapted cells of *S.* Thompson strains RM1987N and RM1987NLUX were inoculated into the 29 ml of LB broth (pH 3.0 adjusted with 1N HCl, 35°C); acid adaptation of cells was proven with results showing difference in survivor counts of *S.* Thompson strains in acidified LB broth (pH 3.0).

Analysis. To enumerate surviving cells every 1 h for 6 h, serial dilutions of each sample were made in 0.1% buffered peptone-water (Difco), and 0.1 ml portions were plated in duplicate onto tryptic soy agar (Difco) plates (TSA). All plates were incubated at 35°C for 48 h. The samples used for microbial analyses at 0 and 6 h were also used to determine pH values using a pH meter with a glass pH electrode (Denver Instrument, Arvada, CO). To determine AI-2 activity of the samples at 0 and 6 h of heat challenge, the bioluminescence response of *Vibrio harveyi* strain BB170 (sensor1$^-$ sensor2$^+$) was measured (Surette and Bassler, 1998, 1999; Surette et al., 1999); *V. harveyi* strain BB170 reacts only with AI-2 molecules. Cell-free supernatants of the *V. harveyi* strains BB120 (AI-1$^+$ AI-2$^+$) and BB152 (AI-1$^-$ AI-2$^+$) were used as positive controls (Surette and Bassler, 1998) and the negative control was the sterile autoinducer bioassay (AB) medium (Bassler et al., 1993). The *Vibrio*

strains were kindly provided by Dr. Bonnie L. Bassler (Princeton University, Princeton, NY). A 1 ml portion of the samples was centrifuged at 15,600×*g* (4°C) for 5 min in a microcentrifuge and then the supernatant was filtered (0.2μm, 25 mm; Nalgene, Rochester, NY) to prepare cell-free supernatants (Surette and Bassler, 1998, 1999; Surette et al., 1999). The cell-free supernatants were assayed for AI-2 activity by induction of bioluminescence in *V. harveyi* strain BB170 with a procedure similar to that described by Surette and Bassler (1998, 1999), and Surette et al. (1999). Specifically, *V. harveyi* BB170 (0.1 ml) maintained in glycerol supplemented (10 %) LB + 1.5% NaCl broth at -70°C was activated (12 h) and subcultured (7.5 h) in 10 ml of LB + 1.5% NaCl broth at 30°C with shaking (200 rpm), and diluted 1:5,000 into AB medium. The mixtures (1:9 of cell-free supernatant : diluted *V. harveyi* BB170) were incubated at 30°C for 3.5 h. AI-2 activity of the samples from the acid challenge was not measured because in preliminary studies, we found that the low pH of the sample caused unstable measurement. In our preliminary study, when pH of the sample containing AI-2 activity decreased to pH 3.0, no AI-2 activity was observed; however, when pH of the sample increased to pH 7.0, AI-2 activity was observed. To measure AI-2 activity of the mixture, bioluminescence was measured with a luminometer (Model TD-20e luminometer, Turner Designs, Inc., Mt. View, CA). AI-2 activity was described as relative AI-2 activity (RAI), and was calculated as the ratio of bioluminescence of the test and -negative control samples (Lu et al., 2004). The RAI activity was converted to log RAI/ml. RAI activity is referred to as AI-2 activity.

Statistical analysis and curve fitting. The study was repeated two times with three samples for each replication; the experiment for acid challenge of *S.* Thompson strain RM1987NLUX in AI-2-positive and -negative PC media was repeated three times.

Counts of surviving bacteria were converted to log CFU/ml before statistical analysis. Bacterial survival counts for the strains and time, PC media and time, and the strains and acid adaptation were analyzed by the mixed model procedure of SAS® (SAS institute Inc., 2002-2003). All mean comparisons were performed with the Tukey's multiple comparison and a *P*-value of 0.05 was used for statistical significance. Survivor data (log CFU/ml) of heat challenge were fitted with the Baranyi model (Baranyi and Roberts, 1994) using the in-house program DMFit (Institute of Food Research, Norwich, UK) to calculate death rates (log CFU/ml/h), and survivors data (log CFU/ml) of acid challenge were fitted with linear regression [Survivors (log CFU/ml) = death rate (log CFU/ml/h) × time (h) + initial level (log CFU/ml)] to calculate the death rates (log CFU/ml/h).

RESULTS AND DISCUSSION

The AI-2-based quorum sensing system of *S.* Thompson may not be involved in heat resistance. To evaluate the effect of AI-2 on the heat resistance of *Salmonella*, the AI-2-positive strain (*S.* Thompson strain RM1987N) and AI-2-negative strain (*S.* Thompson strain RM1987NLUX) were challenged at 55°C in LB broth for 6 h. No difference ($P \geq 0.05$) in surviving cell counts of *S.* Thompson strains RM1987N and RM1987NLUX was observed during heat challenge (Figure VI-1). Interestingly, survivor counts of the *S.* Thompson strains were gradually increased after 3 h of the heat challenge (Figures VI-1 to VI-3). This observation may indicate recovery of injured cells of *S.* Thompson. AI-2 activity of the AI-2-positive *S.* Thompson strain RM1987N was 0.2 log RAI/ml at 0 h and -0.2 log RAI/ml at 6 h of the heat challenge (Table VI-1). Since during preparation of inoculum, *S.* Thompson strain RM1987N culture was centrifuged followed by washing of the pellet with PBS, AI-2 activity of

S. Thompson strain RM1987N was not detected in samples at 0 h. AI-2 of *S.* Thompson strain RM1987N may not be produced due to rapidly decreased bacterial populations at high temperature (55°C), or may be lasted transient due to low glucose level of LB broth. *E. coli* produced a small soluble heat labile organic molecule in Luria-Bertani medium (LB) containing glucose (DeLisa et al., 2001b; Hardie et al., 2003; Surette and Bassler, 1998), but the molecule was not produced in LB containing no glucose (Surette and Bassler, 1998). *V. vulnificus* also produced more AI-2 in the presence of glucose (0.5%) (Kim et al., 2003). Our preliminary study also showed that AI-2 activity of *S.* Thompson strain RM1987N lasted for a short time (approximately 2 h) in LB broth during incubation at 35°C. These results suggest that the heat resistance mechanism of *S.* Thompson may not be correlated with the AI-2-based quorum sensing system.

Since no AI-2 activity was detected in LB broth at 0 and 6 h of heat challenge, it was proved that LB broth contained no AI-2-like activity (Table VI-1).

***S.* Thompson may not use exogenous AI-2 molecules of *S.* Thompson for increased heat resistance**. Since the results suggested that *S.* Thompson may not use its own AI-2-based quorum sensing system for heat resistance, we examined whether *S.* Thompson can be heat resistant by using exogenous AI-2 molecules produced by *S.* Thompson. This study also hypothesized that potentially, when bacteria are in environments where they cannot produce quorum sensing molecules, they may be able to use exogenous quorum sensing molecules from natural flora and become resistant to stress. Exogenous AI-2 molecules restored virulence of mutant strains (*luxS*, *luxP* or *luxO*) of *V. harveyi* toward *Artemia* (Defoirdt et al., 2005).

Lu et al. (2005b) found potential AI-2-like activity in various vegetable and fruit products; eggplant, squash, Roma tomatoes, green peppers, peaches, cucumbers,

potatoes, carrots, oranges, apples, and bananas. Lu et al. (2005b) used rinses from Roma tomato surfaces inoculated with *luxS* mutant of *E. coli* O157:H7 to evaluate the effect of AI-2-like activity of Roma tomatoes on biofilm formation by *E. coli* O157:H7, and they found that AI-2-like activity of Roma tomatoes had the potential to enhance bacterial biofilm formation. Surviving cell counts of *S.* Thompson strain RM1987NLUX in AI-2-positive PC medium were not different ($P \geq 0.05$), than those of the strain in AI-2-negative PC medium during heat challenge at 55°C for 6 h (Figure VI-2). This result showed that *S.* Thompson may not use exogenous AI-2 molecule to be heat resistant. AI-2 activity of the PC media was 2.9 log RAI/ml and -0.3 log RAI/ml for AI-2-positive and -negative PC media, respectively; however, after 6 h of the heat challenge, AI-2 activity decreased to -0.1 log RAI/ml and 0.0 log RAI/ml for AI-2-positive and -negative PC media, respectively (Table VI-1). This suggests that AI-2 activity is susceptible to high temperature (55°C). Thus, *S.* Thompson may not be able to use exogenous AI-2 molecules. While Surette and Bassler (1998) showed that AI-2 activity of *Salmonella* and *E. coli* resisted to heat at 80°C but not 100°C in their preliminary study. However, stability of the AI-2 molecule to heat should be dependent on exposure time; no information of exposure time was available in the study.

Source of PC media does not affect heat resistance of *S.* Thompson. To evaluate whether different source of PC media may affect the response of *S.* Thompson to heat stress, *S.* Thompson strain RM1987NLUX was challenged in AI-2-positive and -negative PC media, which were derived from *E. coli* O157:H7 strains 86-24 and VS94 for AI-2-positive and -negative PC media, respectively. Cell counts of *S.* Thompson strain RM1987NLUX in the AI-2-positive PC medium were not different ($P \geq 0.05$) compared to those of the same strain in the AI-2-negative PC medium

(Figure VI-3). This indicated that a different source of exogenous PC media did not affect the heat resistance of *S.* Thompson. In addition, AI-2 activity of the PC media was 3.0 log RAI/ml, and 0.2 log RAI/ml for AI-2-positive and -negative PC media, respectively at 0 h of heat challenge, but AI-2 activity decreased to 0.0 log RAI/ml for the both AI-2-positive and -negative PC media after 6 h of heat challenge at 55°C (Table VI-1). These results also show that the AI-2 molecule may be degraded at 55°C.

Different serotypes of *Salmonella* showed similar resistance to heat stress. *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP (AI-2-positive) was used to evaluate whether different serotypes of *Salmonella* had similar surviving bacterial counts in identical conditions as those used for *S.* Thompson strain RM1987NLUX. Cell counts of *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP in AI-2-positive PC medium were similar ($P \geq 0.05$) with those bacterial survivors of *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP in AI-2-negative PC medium, regardless of source of the PC media (Figures VI-4 and VI-5). These show that *Salmonella* may not use AI-2 to survive when exposed to heat stress, regardless of serotype of *Salmonella.* AI-2 activity of the PC media was 2.9 to 3.0 log RAI/ml and 0.2 to 0.6 log RAI/ml for AI-2-positive and -negative PC media, respectively at 0 h of heat challenge, but AI-2 activity was 0.4 to 0.5 log RAI/ml and 0.0 to 0.4 log RAI/ml for AI-2-positive and -negative PC media, respectively, after 6 h of heat challenge, regardless of sources of PC media (Table VI-1). This also shows that AI-2 activity decreased by heat. The pH values of the samples were not changed during heat challenge (pH 6.66 to 7.13) (Table VI-2).

Death rates of *Salmonella* under heat stress. To compare the AI-2-positive and -negative effects on *Salmonella* heat resistance using mathematical method, death rates

(log CFU/ml/h) of *Salmonella* were calculated with the Baranyi model (Baranyi and Roberts, 1994) using the DMFit. To show data distribution, raw data points which were accounted in the Baranyi model were presented in figures. In general, death rates of *Salmonella* were not different under AI-2-positive and -negative conditions; AI-2-positive treatment: -3.17 to -3.88 log CFU/ml/h, AI-2-negative treatment: -3.35 to -3.90 log CFU/ml/h (Figures VI-6 to VI-10). This also shows that presence of AI-2 may not affect heat resistance of *Salmonella*. Interestingly, the death rates of *S.* Typhimurium DT104 strain ATCC 700408/ISSAGFP were higher (-3.88 to -3.90 log CFU/ml/h) in PC media derived from *E. coli* O157:H7 than death rates in other treatments (-3.17 to -3.48 log CFU/ml/h), regardless of AI-2 treatment (Figures VI-6 to VI-10). The R^2 values from fitting raw data with the Baranyi model (Baranyi and Roberts, 1994) were ranged 0.684 to 0.945. In general, survivors of *Salmonella* dramatically decreased by 1 h of heat challenge, followed by a tail of survivors without initial shoulder periods (initial period of time during heat challenge resulting in no significant decreases in bacterial counts) (Figures VI-6 to VI-10). Since similar tailing of *Salmonella* survivors were observed in both AI-2-positive and -negative treatments, it is assumed that AI-2 did not affect tailing effect of *Salmonella* under heat stress.

AI-2-based quorum sensing of *Salmonella* may not be involved in acid resistance mechanisms. We evaluated whether *S.* Thompson could become acid resistant in the presence of exogenous AI-2 molecule derived from *S.* Thompson strains. *S.* Thompson strain RM1987NLUX cells were challenged in AI-2-positive and -negative PC media (pH 3.5, 35°C) derived from *S.* Thompson strains RM1987N and RM1987NLUX. No difference ($P \geq 0.05$) of survivors of *S.* Thompson was observed in AI-2-positive and -negative PC media (Figure VI-11). To evaluate whether acid

adaptation of *S.* Thompson is related to AI-2, and the AI-2-positive and -negative strains of *S.* Thompson show difference in acid stress, acid adapted and nonacid adapted cultures of *S.* Thompson strains RM1987N and RM1987NLUX were acid challenged in LB (pH 3.0, 35°C) for 6 h. Surviving cell counts of acid adapted *S.* Thompson strain RM1987N were not different ($P \geq 0.05$) than those of acid adapted *S.* Thompson strain RM1987NLUX, and no differences ($P \geq 0.05$) in cells of nonacid adapted *S.* Thompson strains RM1987N and RM1987NLUX were observed (Figure VI-12). These results indicate that acid adaptation may not be related to AI-2-based quorum sensing of *S.* Thompson. Acid adapted cells were more resistant than nonacid adapted cells in acid, regardless of the *S.* Thompson strain, and surviving cell counts of *S.* Thompson strain RM1987N were not different ($P \geq 0.05$) than those of *S.* Thompson strain RM1987NLUX, regardless of acid adaptation (Figure VI-12).

The pH of the LB broth and PC media were not changed during the acid challenge; PC media (pH 3.49 to 3.55) and LB broth (pH 3.00 to 3.05) (Table VI-3). Since *S.* Thompson strains RM1987N and RM1987NLUX were very sensitive in pH 3.0 PC media, we increased the pH of PC media to pH 3.50.

Death rates of *Salmonella* in acid stress. Unlike counts of surviving bacteria exposed to heat challenge, *S.* Thompson populations from acid challenge decreased linearly (Figures VI-13 to VI-15). Thus, linear regression was used to calculate death rates (log CFU/ml/h) of *S.* Thompson during acid challenge. The death rates of *S.* Thompson strain RM1987NLUX were not different in AI-2-positive PC medium compared to AI-2-negative PC medium; AI-2-positive PC medium: -0.97 log CFU/ml/h, AI-2-negative PC media: -0.89 log CFU/ml/h (Figure VI-13). No differences in death rates were observed between acid adapted *S.* Thompson strains RM1987N (-0.41 log CFU/ml/h) and RM1987NLUX (-0.38 log CFU/ml/h), and in

nonacid adapted *S.* Thompson strains RM1987N (-0.52 log CFU/ml/h) and RM1987NLUX (-0.48 log CFU/ml/h) (Figures VI-14 and VI-15). These results also show that presence of AI-2 may not affect acid resistance and acid adaptation of *Salmonella.* Unlike survivor data from heat challenge, a tailing effect of *Salmonella* was not observed in acid challenge. The R^2 values from fitting of raw data with linear regression were >0.800.

It has been shown that quorum sensing of certain bacteria may be related to stress response. *P. aeruginosa* mutants with deletion of LuxI homologues (*lasI* and *rhlI*) were more susceptible to hydrogen peroxide than the wild-type culture (Huang and Shih, 2000). This stress response based *rhlI* quorum sensing system may be related to *rpoS* of *P. aeruginosa.* Latifi et al. (1996) proved that the *rhl* quorum sensing system initiated transcription of *rpoS.* Furthermore, cells of the *rpoS* mutant strain of *P. aeruginosa* were more susceptible to heat shock and NaCl (Suh et al., 1999). Taken together, the *rhl* quorum sensing system may be related to resistance to heat and NaCl. However, Whiteley et al. (2000) showed that the *rpoS* transcription is not related to quorum sensing. In *V. vulnificus* and *V. angustum,* AI-2-like signaling triggered the stress response to starvation adaptation and oxidative stress (McDougald et al., 2003). *Agrobacterium tumefaciens* breaks off the quorum sensing-dependent and energy consuming conjugation development under starvation stress (Zhang et al., 2004). In *S. mutans, luxS* mutant cells were more resistant to hydrogen peroxide (58.8 mM) than wild-type strain cells (Wen and Burne, 2004). *S. epidermidis* regulates general and oxidative stress-response factors through the *agr* quorum sensing system, which involves detoxifying enzymes of reactive oxygen (Yao et al., 2006). Farnesol, which is one of the quorum sensing molecules produced by *C. albicans,* plays a role in protecting *C. albicans* cells against hydrogen peroxide toxicity (Westwater et al.,

2005). DeLisa et al. (2001b) showed that significant overlap was observed among quorum sensing genes and starvation genes and several stress genes of *E. coli* K-12. *E. coli* K-12 communicates the stress or metabolic burden related to overall accumulation of foreign protein via AI-2 (DeLisa et al., 2001c). When the *E. coli* culture was exposed to low concentration (2 μg/ml) of chlortetracycline, AI-2 activity was increased by increasing levels of tetracycline (Lu et al., 2005a). This indicates that *E. coli* may use AI-2 to be resistant to antibiotics. The *smcR* (*luxR* homologue) mutant strain of *V. vulnificus* was more sensitive to oxidative stress and starvation, compared to wild-type strain cells (McDougald et al., 2001, 2003). While the overexpression of the superoxide decreased the transcription of *comQXP* quorum sensing locus in *Bacillus subtilis* (Ohsawa et al., 2006). This indicates that quorum sensing of *B. subtilis* may not be involved in stress resistance to superoxide.

CONCLUSIONS

AI-2-based quorum sensing of *Salmonella* may not play a major role in its heat and acid resistance mechanisms and it was not related to the tailing effect of *Salmonella* during heating, which is a concern in food processing. In addition, AI-2 molecules of *Salmonella* may not be used to induce acid adaptation. Thus, AI-2-based quorum sensing of *Salmonella* may not be of significance in the application of heat and acid treatments to foods.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. Pina M. Fratamico, Dr. John S. Novak, Dr. Maria T. Brandl, Dr. Vanessa Sperandio, and Dr. Bonnie L. Bassler for their friendly and critical advice, and cultures. This work was funded by the Colorado State University Agricultural Experiment Station.

Table VI-1. AI-2 activity [mean (log RAI/ml) ± standard error] of *Salmonella* during heat challenge at 55°C for 6 h in Luria-Bertani (LB) broth and preconditioned (PC) media under various conditions

				Time (h)	
Figure	Challenged strain	AI-2 activity	Media	0	6
VI-1	RM1987N	+	LB	0.2±0.1	-0.2±0.1
	RM1987NLUX	-	LB	0.0±0.1	-0.2±0.1
VI-2	RM1987NLUX	-	AI-2-positive PC_{SA}	2.9±0.2	-0.1±0.1
	RM1987NLUX	-	AI-2-negative PC_{SA}	-0.3±0.1	0.0±0.1
VI-3	RM1987NLUX	-	AI-2-positive PC_{EC}	3.0±0.2	0.0±0.1
	RM1987NLUX	-	AI-2-negative PC_{EC}	0.2±0.0	0.0±0.0
VI-4	DT104	+	AI-2-positive PC_{SA}	2.9±0.1	0.5±0.2
	DT104	+	AI-2-negative PC_{SA}	0.6±0.2	0.4±0.2
VI-5	DT104	+	AI-2-positive PC_{EC}	3.0±0.2	0.4±0.3
	DT104	+	AI-2-negative PC_{EC}	0.2±0.3	0.0±0.1

PC_{SA}: preconditioned media derived from the cell-free supernatants of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative).

PC_{EC}: preconditioned media derived from the cell-free supernatants of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative).

Table VI-2. pH values (mean ± standard error) of Luria-Bertani (LB) broth and preconditioned (PC) media inoculated with *Salmonella* during heat challenge at 55°C for 6 h under various conditions

Figure	Challenged strain	AI-2 activity	Media	Time (h) 0	Time (h) 6
VI-1	RM1987N	+	LB	6.80±0.01	6.66±0.01
	RM1987NLUX	-	LB	6.71±0.04	6.68±0.02
VI-2	RM1987NLUX	-	AI-2-positive PC_{SA}	6.93±0.00	6.78±0.01
	RM1987NLUX	-	AI-2-negative PC_{SA}	6.87±0.01	6.73±0.02
VI-3	RM1987NLUX	-	AI-2-positive PC_{EC}	7.11±0.02	7.13±0.01
	RM1987NLUX	-	AI-2-negative PC_{EC}	7.07±0.01	7.04±0.03
VI-4	DT104	+	AI-2-positive PC_{SA}	6.94±0.01	6.87±0.01
	DT104	+	AI-2-negative PC_{SA}	6.88±0.03	6.81±0.03
VI-5	DT104	+	AI-2-positive PC_{EC}	6.96±0.03	6.96±0.01
	DT104	+	AI-2-negative PC_{EC}	6.91±0.01	6.87±0.02

PC_{SA}: preconditioned media derived from the cell-free supernatants of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative).

PC_{EC}: preconditioned media derived from the cell-free supernatants of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative).

Table VI-3. pH values (mean ± standard error) of Luria-Bertani (LB) broth and preconditioned (PC) media inoculated with *Salmonella* during acid challenge at 35°C for 6 h under various conditions

Figure	Challenged strain	AI-2 activity	Acid adaptation	Media	Time (h) 0	Time (h) 6
VI-11	RM1987NLUX	-	NA	AI-2-positive PC	3.49±0.01	3.54±0.01
	RM1987NLUX	-	NA	AI-2-negative PC	3.52±0.01	3.55±0.00
VI-12	RM1987N	+	+	LB	3.00±0.02	3.04±0.01
	RM1987N	+	-	LB	3.01±0.01	3.03±0.01
	RM1987NLUX	-	+	LB	3.02±0.00	3.05±0.01
	RM1987NLUX	-	-	LB	3.02±0.00	3.03±0.01

NA: not applicable.

PC: preconditioned media (pH 3.5) derived from the cell-free supernatants of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative).

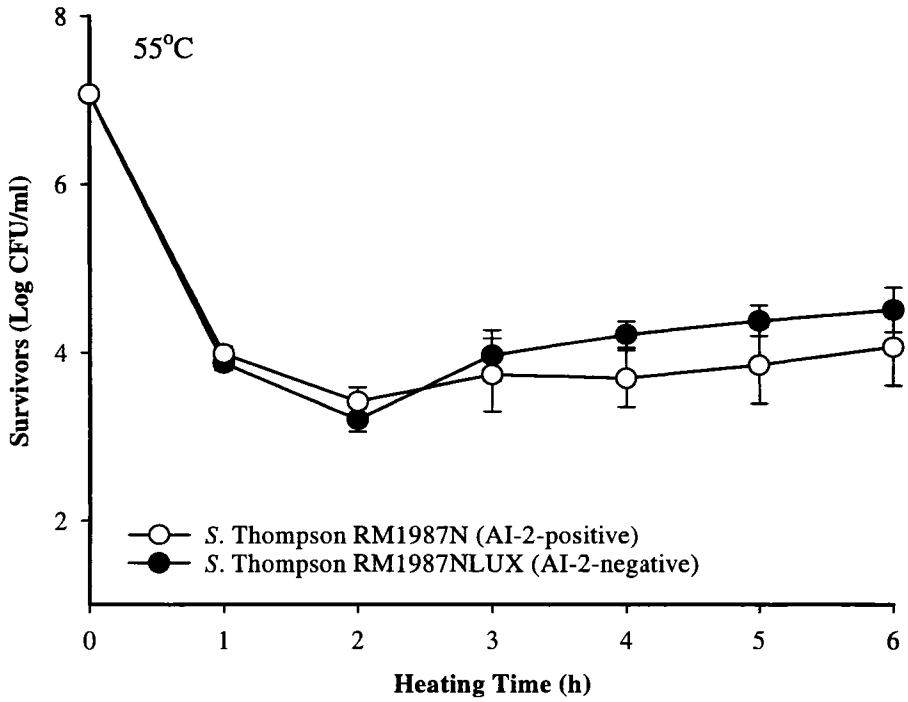

Figure VI-1. *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) survivors, recovered with tryptic soy agar, during heat challenge at 55°C for 6 h in Luria-Bertani broth (pH 7.0) (Data in APPENDIX Table 29).

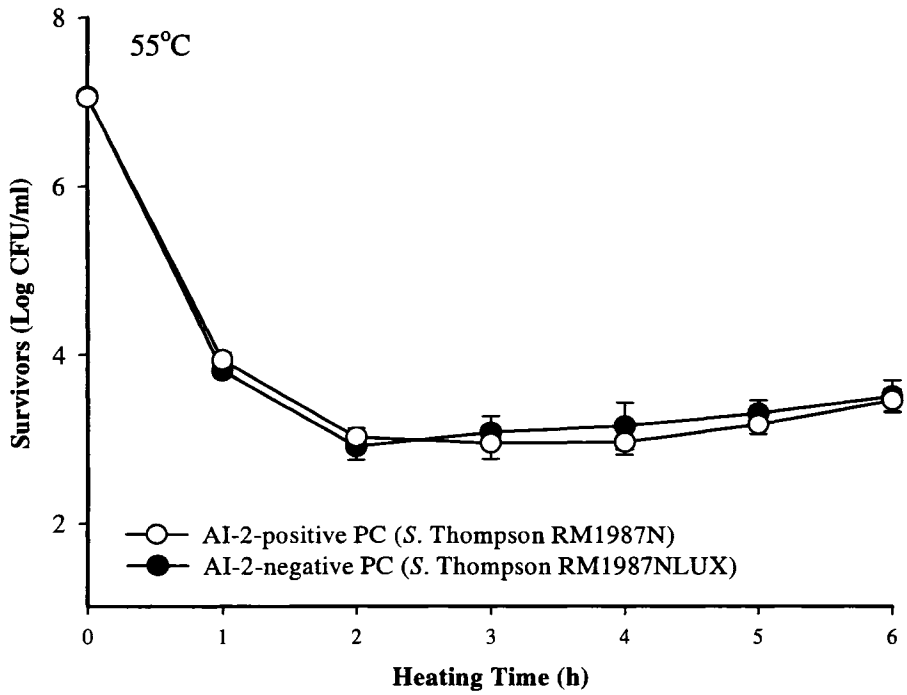

Figure VI-2. *Salmonella* Thompson strain RM1987NLUX (AI-2-negative) survivors, recovered with tryptic soy agar, during heat challenge at 55°C for 6 h in AI-2-positive (pH 7.0) and -negative preconditioned (PC) media (pH 7.0); PC media were derived from the cell-free supernatants of *S.* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 30).

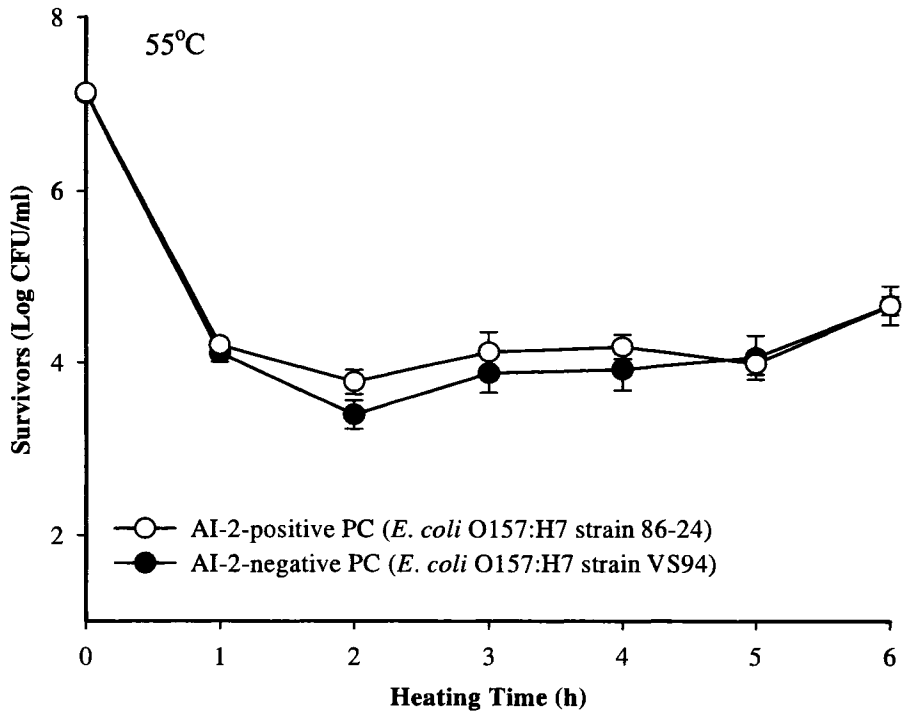

Figure VI-3. *Salmonella* Thompson strain RM1987NLUX (AI-2-negative) survivors, recovered with tryptic soy agar, during heat challenge at 55°C for 6 h in AI-2-positive (pH 7.0) and -negative preconditioned (PC) media (pH 7.0); PC media were prepared from the cell-free supernatants of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 31).

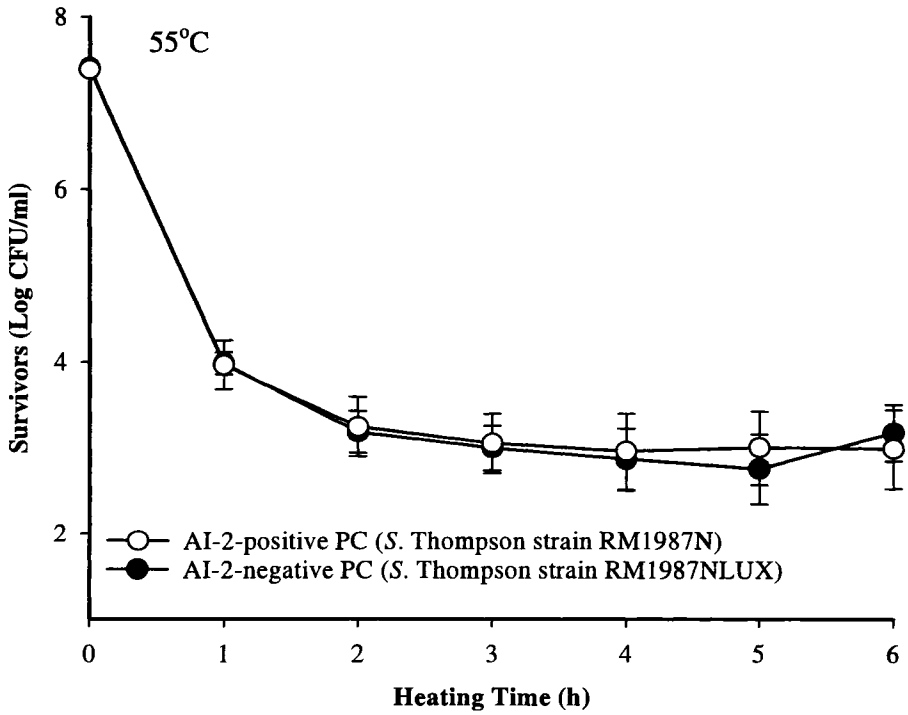

Figure VI-4. *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP survivors, recovered with tryptic soy agar, during heat challenge at 55°C for 6 h in AI-2-positive and -negative preconditioned (PC) media (pH 7.0); PC media were derived from the cell-free supernatants of *S.* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 32).

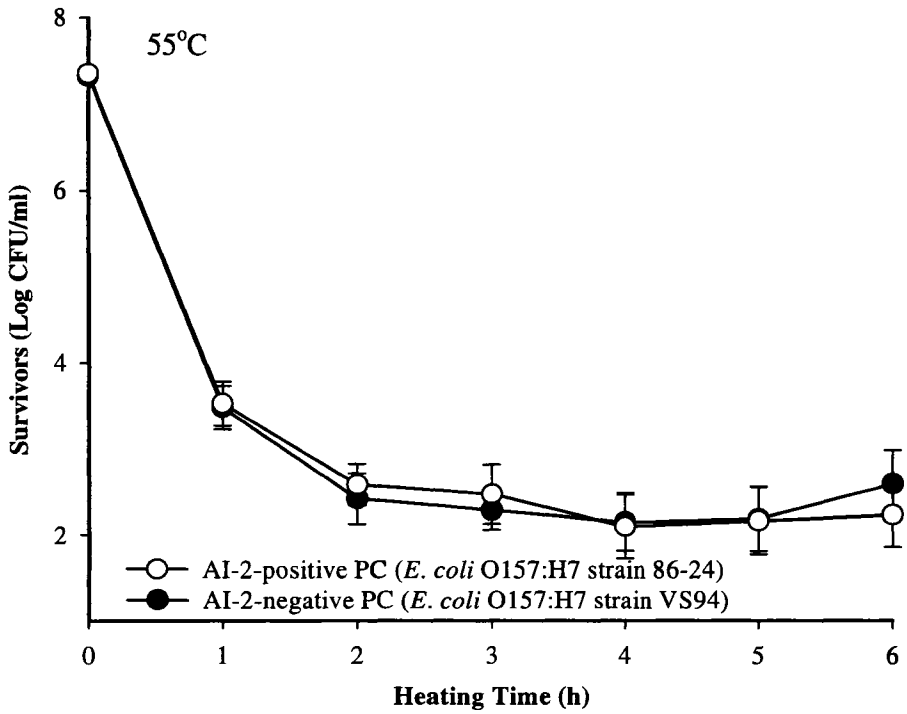

Figure VI-5. *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP survivors, recovered with tryptic soy agar, during heat challenge at 55°C for 6 h in AI-2-positive (pH 7.0) and -negative preconditioned (PC) media (pH 7.0); PC media were prepared from the cell-free supernatants of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data APPENDIX Table 33).

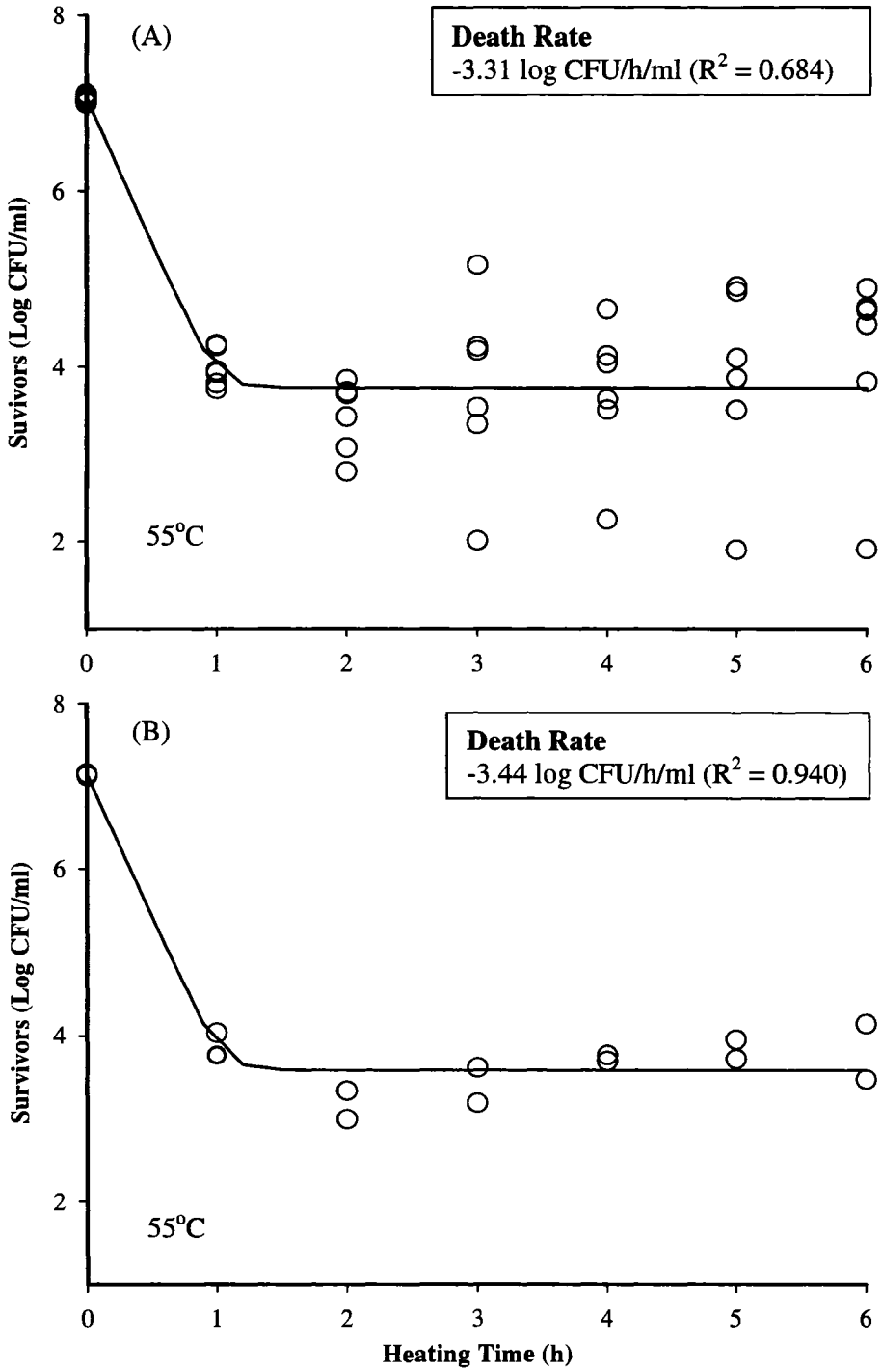

Figure VI-6. Raw data points (survivors) of *Salmonella* Thompson strains RM1987N (AI-2-positive; A) and RM1987NLUX (AI-2-negative; B), recovered with tryptic soy agar, during heat challenge at 55°C for 6 h in Luria-Bertani broth (pH 7.0), and fitted curves and death rates that were generated from fitting the raw data with the Baranyi model (Baranyi and Roberts, 1994) (Data in APPENDIX Table 34).

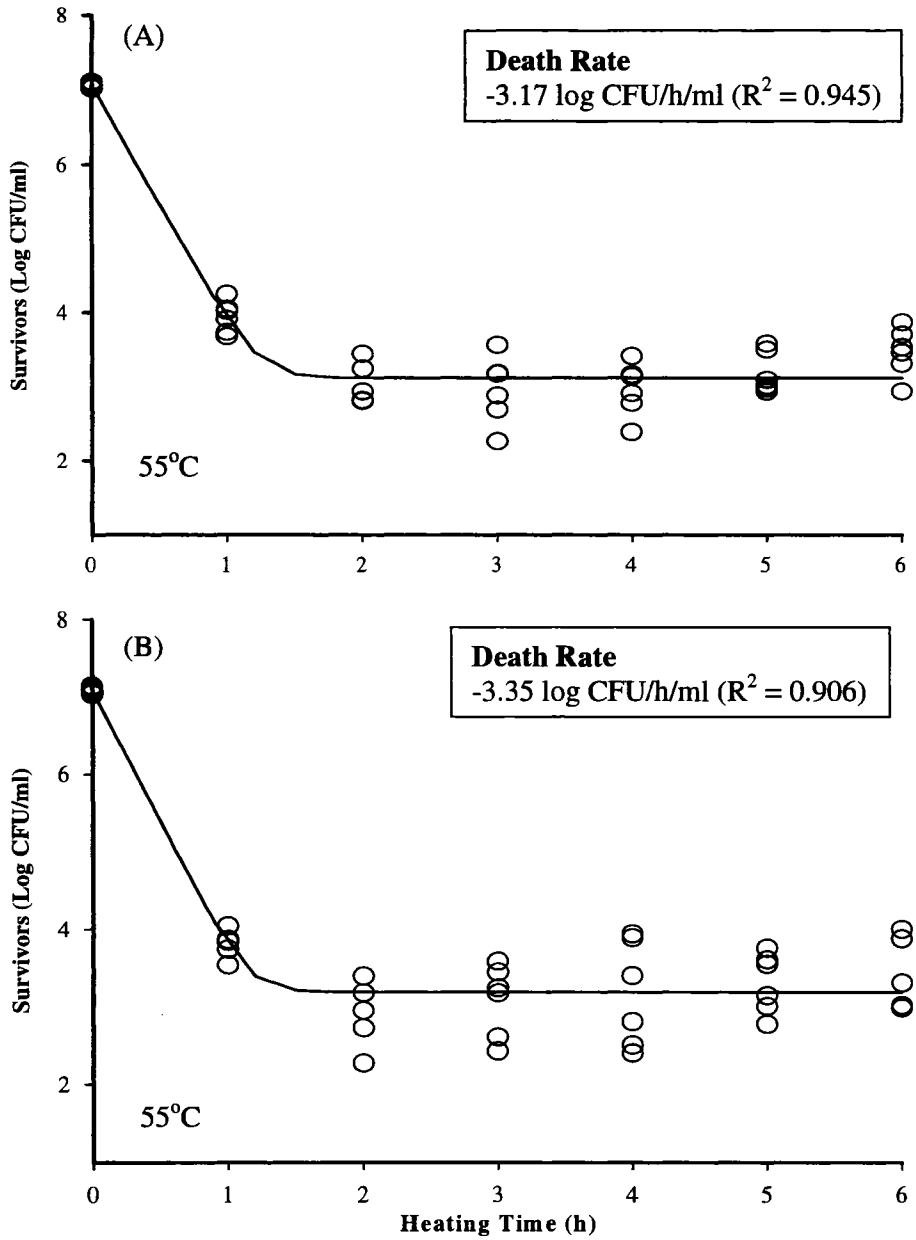

Figure VI-7. Raw data points (survivors) of *Salmonella* Thompson strain RM1987NLUX (AI-2-negative), recovered with tryptic soy agar, during heat challenge at 55°C for 6 h in AI-2-positive (pH 7.0; A) and -negative preconditioned (PC) media (pH 7.0; B), and fitted curves and death rates that were generated from fitting the raw data with the Baranyi model (Baranyi and Roberts, 1994); PC media were derived from the cell-free supernatants of *S.* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 34).

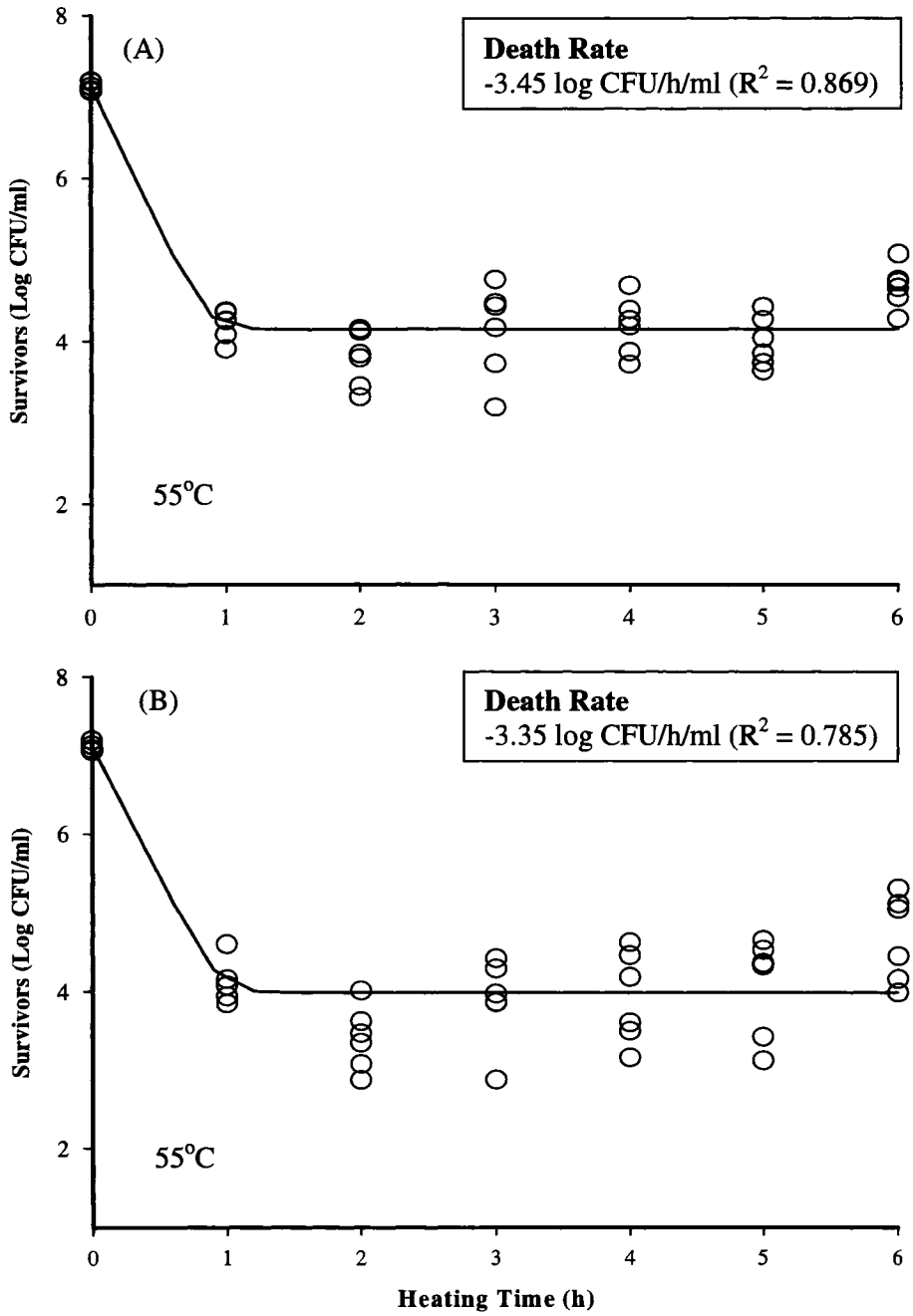

Figure VI-8. Raw data (survivors) of *Salmonella* Thompson strain RM1987NLUX (AI-2-negative), recovered with tryptic soy agar, during heat challenge at 55°C for 6 h in AI-2-positive (pH 7.0; A) and -negative preconditioned (PC) media (pH 7.0; B), and fitted curves and death rates that were generated from fitting the raw data with the Baranyi model (Baranyi and Roberts, 1994); PC media were prepared from the cell-free supernatants of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 34).

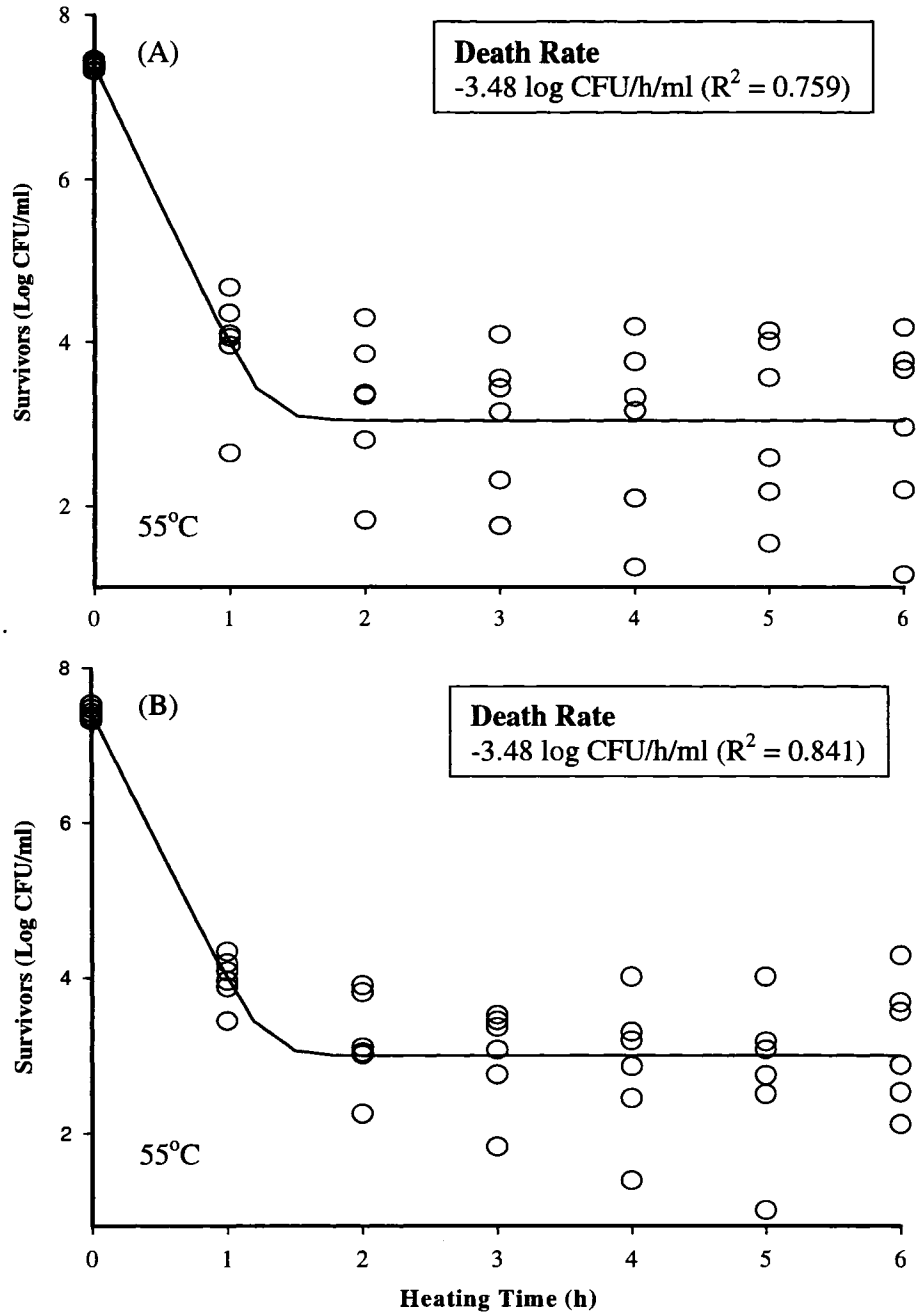

Figure VI-9. Raw data points (survivors) of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP, recovered with tryptic soy agar, during heat challenge at 55°C for 6 h in AI-2-positive (pH 7.0; A) and -negative preconditioned (PC) media (pH 7.0; B), and fitted curves and death rates from fitting the raw data with the Baranyi model (Baranyi and Roberts, 1994); PC media were derived from the cell-free supernatants of *S.* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 34).

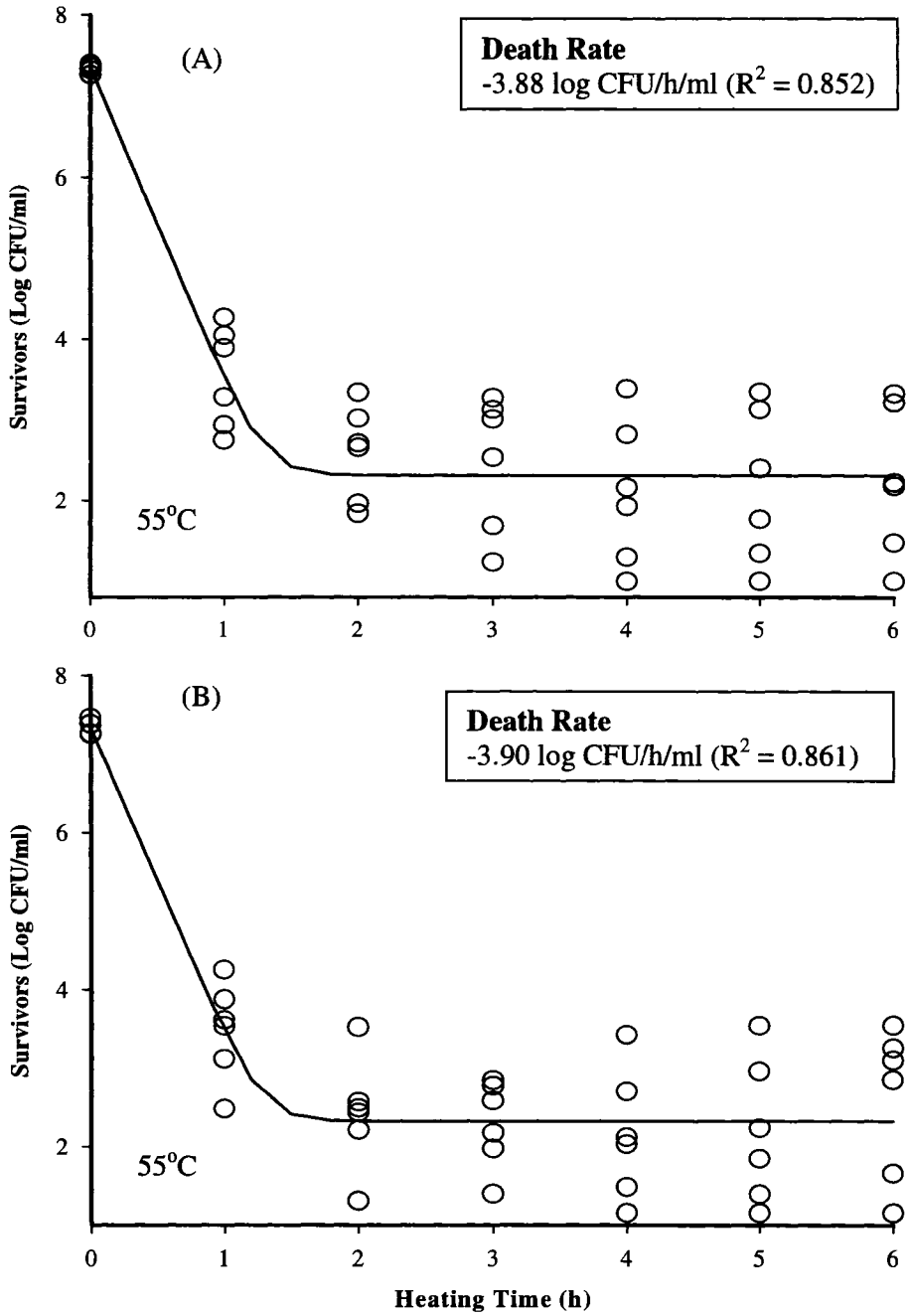

Figure VI-10. Raw data points (survivors) of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP, recovered with tryptic soy agar, during heat challenge at 55°C for 6 h in AI-2-positive (pH 7.0; A) and -negative preconditioned (PC) media (pH 7.0; B), and fitted curves and death rates from fitting the raw data with the Baranyi model (Baranyi and Roberts, 1994); PC media were prepared from the cell-free supernatants of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 34).

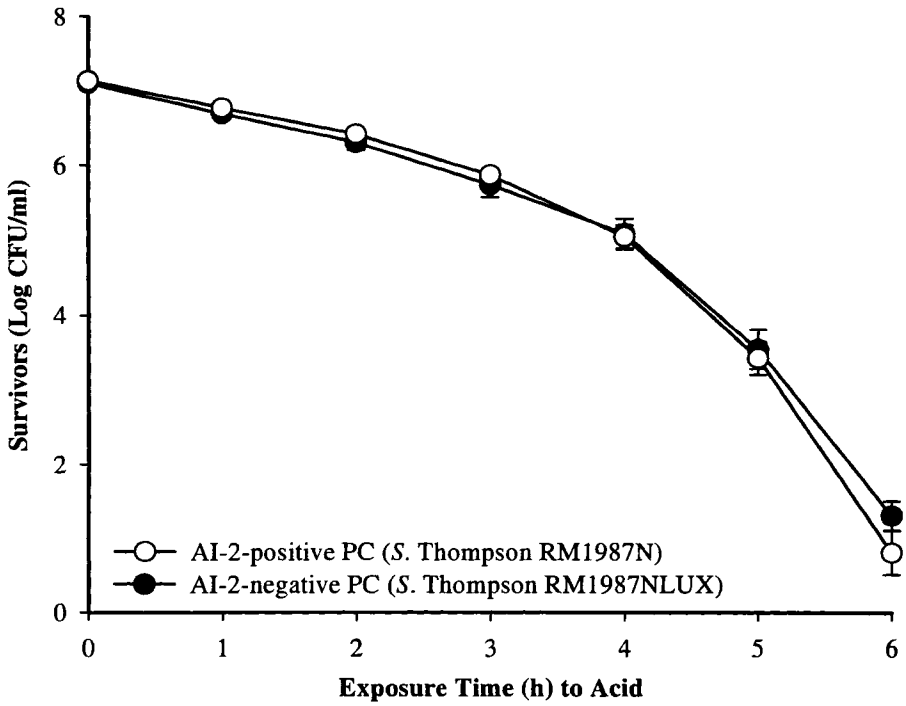

Figure VI-11. *Salmonella* Thompson strain RM1987NLUX (AI-2-negative) survivors, recovered with tryptic soy agar, during acid challenge at 35°C for 6 h in AI-2-positive (pH 3.5) and -negative preconditioned (PC) media (pH 3.5); PC media were derived from the cell-free supernatants of *S.* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 35).

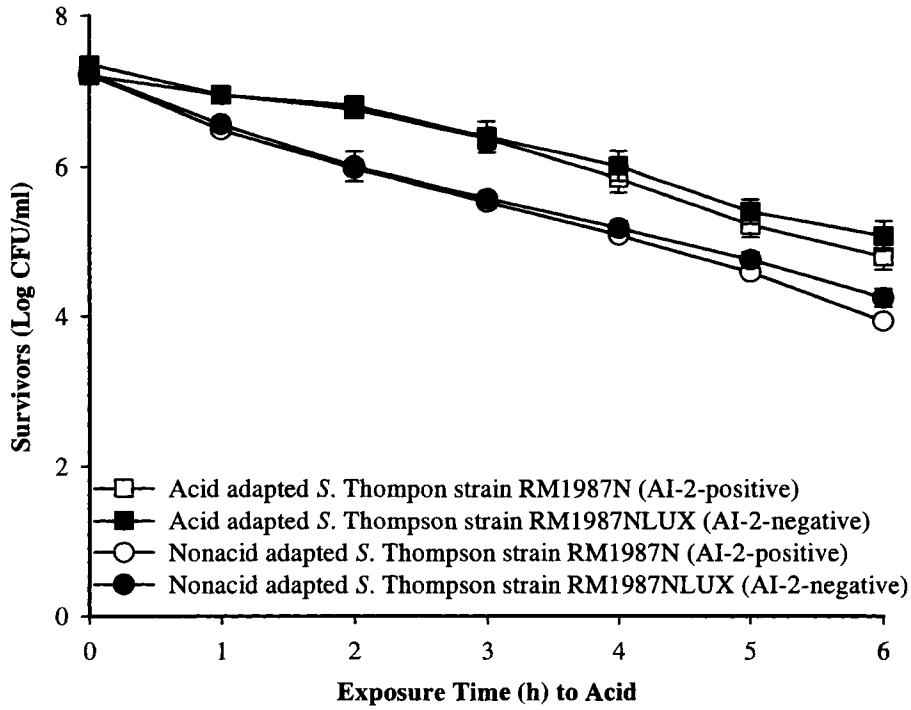

Figure VI-12. Acid adapted and nonacid adapted *Salmonella* Thompson strain RM1987N (AI-2-positive), and acid adapted and nonacid adapted *S.* Thompson strain RM1987NLUX (AI-2-negative) survivors, recovered with tryptic soy agar, during acid challenge at 35°C for 6 h in Luria-Bertani broth (pH 3.0) (Data in APPENDIX Table 36).

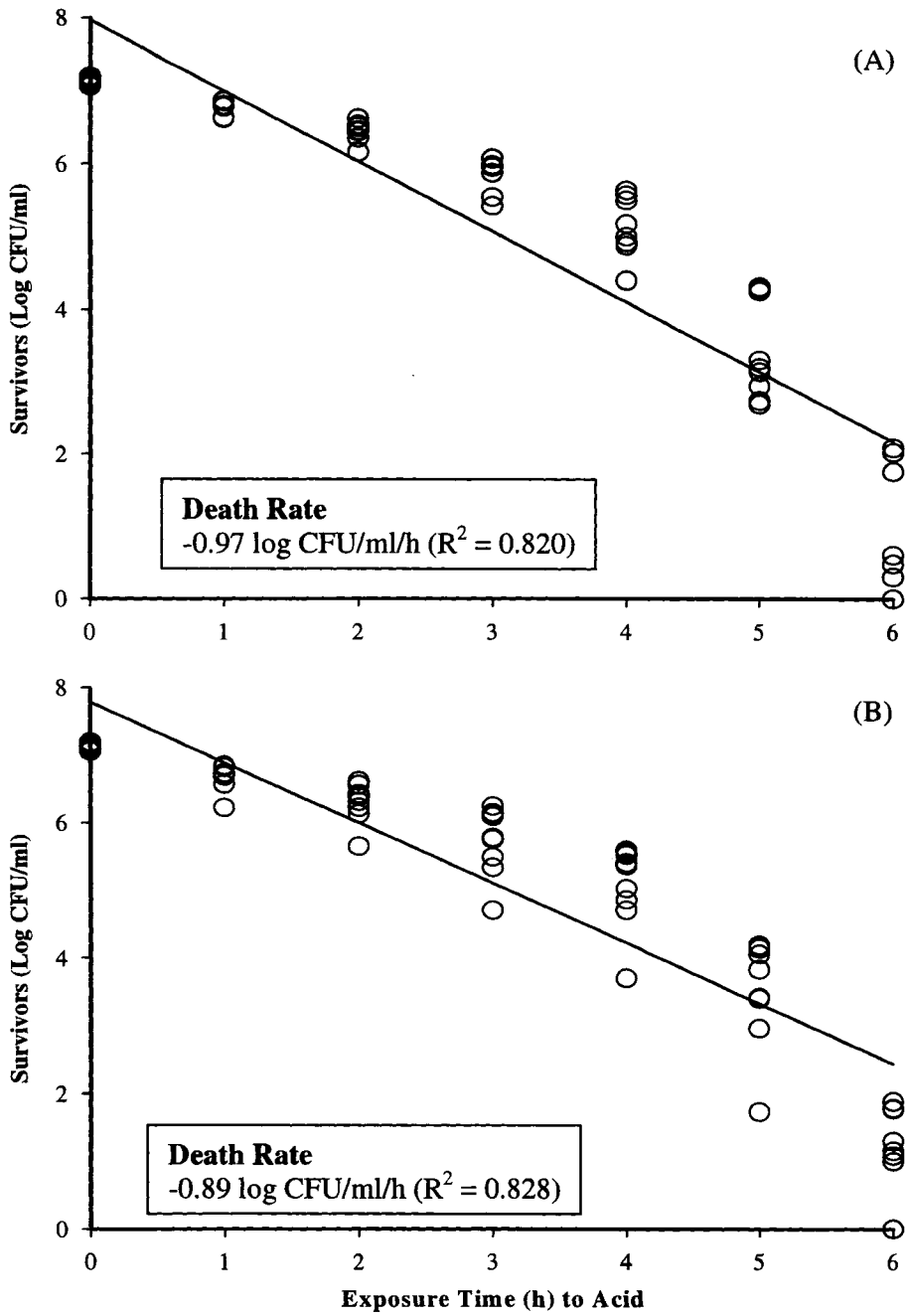

Figure VI-13. Raw data points (survivors) of *Salmonella* Thompson strain RM1987NLUX (AI-2-negative), recovered with tryptic soy agar, during acid challenge at 35°C for 6 h in AI-2-positive (pH 3.5; A) and -negative preconditioned (PC) media (pH 3.5; B), and fitted lines and death rates were generated from fitting the raw data with linear regression; PC media were derived from the cell-free supernatants of *S.* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 37).

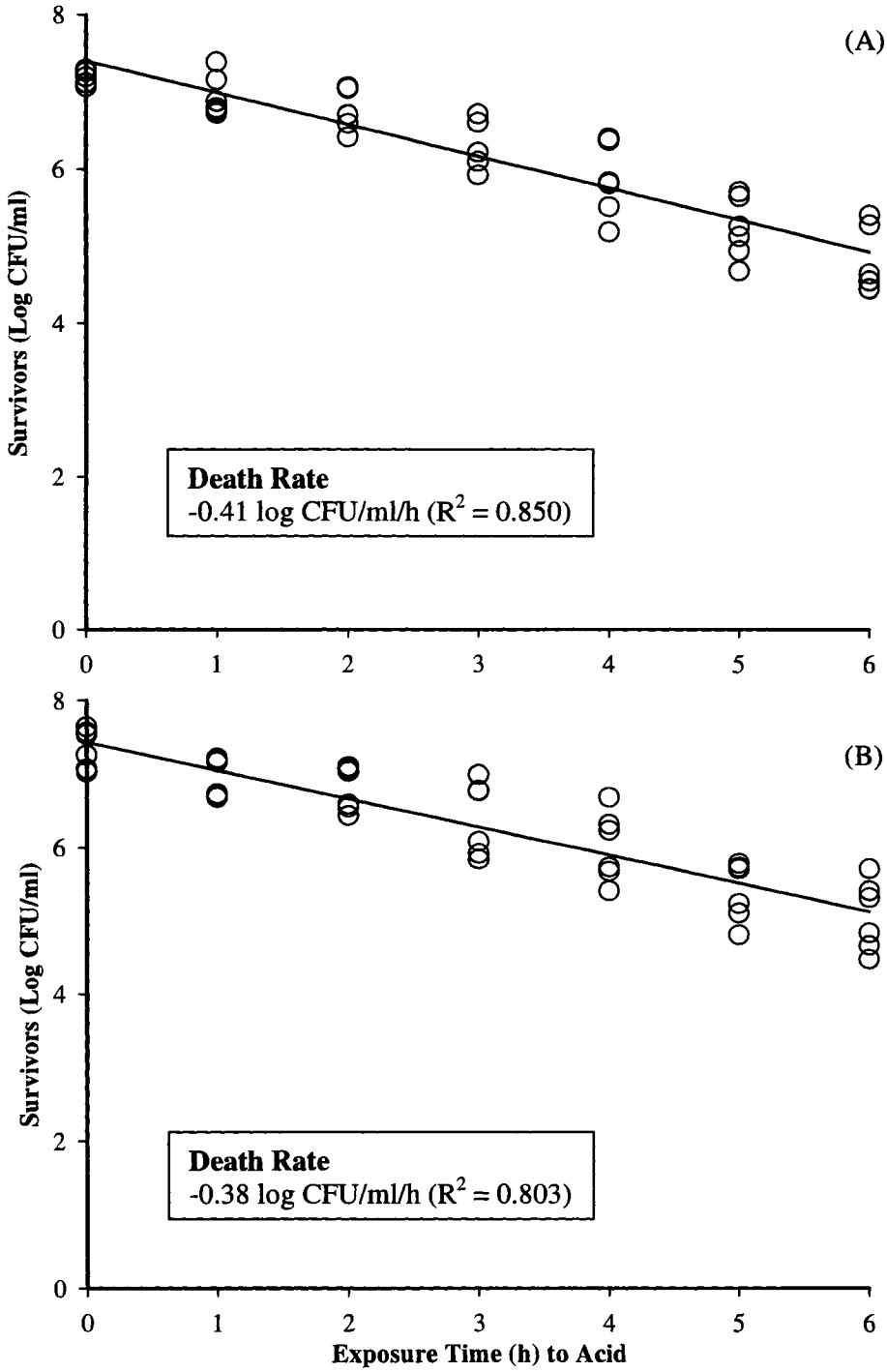

Figure VI-14. Raw data points (survivors) of acid adapted *Salmonella* Thompson strains RM1987N (AI-2-positive; A) and RM1987NLUX (AI-2-negative; B), recovered with tryptic soy agar, during acid challenge at 35°C for 6 h in Luria-Bertani broth (pH 3.0), and fitted lines and death rates were generated from fitting the raw data with linear regression (Data in APPENDIX Table 37).

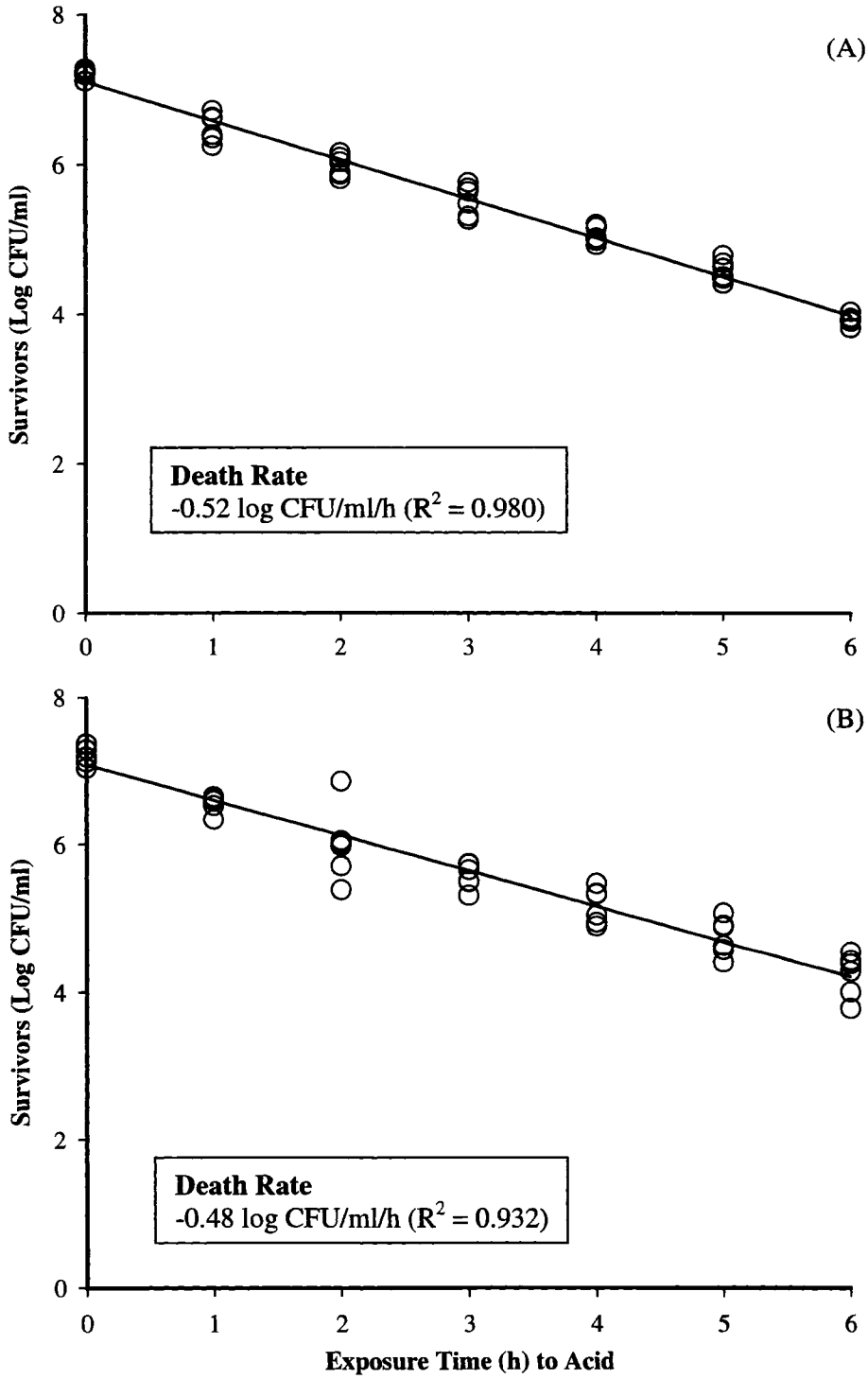

Figure VI-15. Raw data points (survivors) of nonacid adapted *Salmonella* Thompson strains RM1987N (AI-2-positive; A) and RM1987NLUX (AI-2-negative; B), recovered with tryptic soy agar, during acid challenge at 35°C for 6 h in Luria-Bertani broth (pH 3.0), and fitted lines and death rates were generated from fitting the raw data with linear regression (Data in APPENDIX Table 37).

CHAPTER VII

ASSOCIATION OF AUTOINDUCER-2 ACTIVITY WITH HEAT AND ACID RESISTANCE OF *ESCHERICHIA COLI* O157:H7

ABSTRACT

This study evaluated whether autoinducer-2 (AI-2; quorum sensing molecule) may be involved in the heat and acid resistance of *Escherichia coli* O157:H7. AI-2-positive or -negative preconditioned (PC) media (pH 7.0) were prepared from cell-free supernatants of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative; *luxS* mutant of *E. coli* O157:H7 strain 86-24) in Luria-Bertani (LB) + 0.5% glucose broth. Heat challenge of the pathogens was conducted as follows: (i) *E. coli* O157:H7 strain ATCC 43895/ISEHGFP (AI-2-positive; 7.0 log CFU/ml) was inoculated and heated (55°C, 4 h) in AI-2-positive and -negative PC media, and (ii) *E. coli* O157:H7 strains 86-24 and VS94 were inoculated and heated in LB broth (52°C, 6 h). Acid resistance of *E. coli* O157:H7 strains ATCC 43895/ISEHGFP or VS94 was determined in AI-2-positive and -negative PC media, adjusted to pH 3.0 (35°C, 6 h). Survivors were determined by plating on tryptic soy agar and incubating at 35°C for 48 h. Death rates and fitted lines were obtained with the Baranyi model (heat) or linear regression (acid). Survivors of *E. coli* O157:H7 strain ATCC 43895/ISEHGFP during heating was higher ($P<0.05$) in AI-2-positive than -negative PC media, and survivors of *E. coli* O157:H7 strain 86-24 were higher ($P<0.05$) than those of *E. coli*

O157:H7 strain VS94 in LB. While average thermal death rates from the heat challenge were not different in the presence (-1.51 to -1.55 log CFU/ml/h) or absence (-1.51 to -1.65 log CFU/ml/h) of AI-2 activity. Fitted curves of *E. coli* O157:H7 strains ATCC 43895/ISEHGFP and 86-24 in AI-2-positive PC medium showed a more obvious tailing effect compared to those of the *E. coli* O157:H7 strains in AI-2-negative PC medium. During acid challenge, *E. coli* O157:H7 strains ATCC 43895/ISEHGFP and VS94 had higher ($P<0.05$) survivors in AI-2-positive than in AI-2-negative PC media. Acid death rates of *E. coli* O157:H7 strains were also slightly lower by 0.2 log CFU/ml/h in the presence of AI-2-based quorum sensing. These results indicate that AI-2 activity may be partially involved in heat and acid resistance mechanisms in *E. coli* O157:H7.

INTRODUCTION

Once the levels of bacteria increase, they release small signaling molecules that process quorum sensing activity and provide signals to neighboring bacterial cells about cell density (Brandl et al., 2005). These quorum sensing molecules have been categorized into at least four groups (reviewed by Smith et al., 2004), *N*-acylhomoserine lactone (AHL) in Gram-negative bacteria (Miller and Bassler, 2001; Whitehead et al., 2001), autoinducer (AI)-2 (furanosyl borate diester) in both Gram-negative and Gram-positive bacteria (Chen et al., 2002; Schauder and Bassler, 2001; Xavier and Bassler, 2005a), AI-3 (unknown structure) in *Escherichia coli* O157:H7 (Sperandio et al., 2003), and oligopeptides in Gram-positive bacteria (Miller and Bassler, 2001; Whitehead et al., 2001). Of these quorum sensing molecules, foodborne pathogenic bacteria such as *E. coli* O157:H7, *Salmonella*, and *Campylobacter* spp. synthesize AI-2 (Cloak et al., 2002; Elvers and Park, 2002;

Surette and Bassler, 1998). In addition, unclassified quorum sensing molecules have been also found, and include diketopiperazines (Degrassi et al., 2002; Holden et al., 1999) and 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal) (Holden et al., 2000; Pesci et al., 1999) in bacteria, while farnesoic acid (Oh et al., 2001), farnesol (Hornby et al., 2001), and tyrosol (Chen et al., 2004) have been observed in yeast.

Bacteria use quorum sensing systems to regulate behaviors such as virulence (von Bodman et al., 1998), plasmid conjugation (Lithgow et al., 2001), bioluminescence (Cao and Meighen, 1989), protein production (DeLisa et al., 2001c), and biofilm formation (Davies et al., 1998). However, scientists have recently found that quorum sensing can be blocked by several chemicals (Bjarnsholt et al., 2005; Hentzer et al., 2002; Huber et al., 2003; Novak and Fratamico, 2004). Furanone blocked quorum sensing in most biofilm cells via penetrating microcolonies; it affected the structure of biofilm rather than initial attachment to the abiotic substratum (Hentzer et al., 2002). For food safety, furanone inhibited AI-2 production by *Clostridium perfringens*, which was inoculated in ground beef, while ascorbic acid also interfered with AI-2 production by *C. perfringens* (Novak and Fratamico, 2004). Garlic, a natural product, interfered with production of quorum sensing signals, but it did not influence microbial growth (Bjarnsholt et al., 2005; Persson et al., 2005). Garlic also reduced biofilm resistance of *Pseudomonas aeruginosa* to tobramycin (Bjarnsholt et al., 2005). Polyphenolic compounds, which are widely distributed in the plant kingdom, can also block bacterial quorum sensing (Huber et al., 2003).

Even though many treatments have been developed and applied to foods to improve food safety, we still have food safety issues. Thus, the necessity for quorum sensing studies has been raised by food microbiologists in order to find novel targets

for improvement of food safety. First, food microbiologists have studied the potential relationship between quorum sensing and food spoilage. Quorum sensing may be involved in spoilage of milk (Christensen et al., 2003) and other foods (Rasch et al., 2005). Jay et al. (2003) suggested that quorum sensing of dominant bacteria may be involved in spoilage and biofilm formation at low storage temperature of fresh meat. Second, scientists have researched whether quorum sensing is related to biofilm formation. Recently, González Barrios et al. (2006) proved that AI-2 is involved in biofilm formation of *E. coli* K-12, and Challan Belval et al. (2006) demonstrated that *S*-ribosylhomocysteine, the precursor of AI-2, is related to biofilm formation of *Listeria monocytogenes*. In addition, *Salmonella* Typhimurium was reported to regulate biofilm formation via quorum sensing (De Keersmaecker et al., 2005; Prouty et al., 2002). In disagreement with these studies, Brandl et al. (2005) found no relationship between quorum sensing of *Salmonella* Thompson and biofilm formation on cilantro leaf surfaces. Third, some scientists have researched the roles of quorum sensing in pathogenesis of foodborne pathogenic bacteria in the host. In enterohaemorrhagic *E. coli* and enteropathogenic *E. coli*, AI-2 was related to the type III secretion system, the Tir (translocated intimin receptor), and intimin intestinal colonization (Sperandio et al., 1999). *E. coli* O157:H7 is believed to colonize the intestine using quorum sensing (Kanamaru et al., 2000). The AI-2 molecule was found to induce the *LEE* [locus of enterocyte effacement; pathogenicity island (Mellies et al., 1999)] operons in *E. coli* K-12 and *E. coli* O157:H7 (Sperandio et al., 1999, 2003). However, Sperandio et al. (2003) showed that AI-2 does not activate the *LEE* genes of *E. coli* O157:H7 in additional work using purified AI-2. AI-2 may be used to protect eukaryotes from pathogenic bacteria (Xavier and Bassler, 2005a). Interestingly, *E. coli* uses the AI-2 molecule for interspecies communication with *V.*

harveyi (Xavier and Bassler, 2005a), and to communicate cell density and metabolic potential in the surroundings (Surette and Bassler, 1998).

In addition, studies have been conducted to examine whether quorum sensing is related to bacterial resistance to stress. DeLisa et al. (2001b) demonstrated that a clear overlap was found among known quorum sensing, starvation genes, and several stress genes in *E. coli* K-12. Interestingly, *E. coli* K-12 can communicate the stress or metabolic burden related to overall accumulation of foreign protein via AI-2 (DeLisa et al., 2001c). *P. aeruginosa* may be able to resist oxidative stress through use of quorum sensing. *lasI* and *rhlI* (quorum sensing system) mutants of *P. aeruginosa* were more sensitive to hydrogen peroxide compared to wild-type (Huang and Shih, 2000). This stress response which was involved in the *rhlI* quorum sensing system may be related to *rpoS* of *P. aeruginosa* because the *rhl* quorum sensing system triggered the transcription of *rpoS* (Latifi et al., 1996). RopS protein influences stress tolerance of *P. aeruginosa* during stationary phase (Jørgensen et al., 1999) and affects the resistance of *P. aeruginosa* to heat shock and NaCl stress (Suh et al., 1999). Whiteley et al. (2000) demonstrated that the *rpoS* gene transcription is not involved in quorum sensing. *E. coli* O157:H7 also uses *rpoS* regulation of acid and heat resistance (Cheville et al., 1996). *Vibrio* spp. use quorum sensing for stress resistance (McDougald et al., 2001, 2003). The quorum sensing of *Streptococcus mutans* is involved in acid resistance (Wen and Burne, 2004).

Acid adapted cells of *E. coli* O157:H7 and *Salmonella* in tryptic soy broth (TSB) containing glucose were more heat resistant than nonacid adapted cells incubated in TSB containing no glucose (Sharma et al., 2005). Bacon et al. (2003) showed that heat resistance of *Salmonella* strains increased as the level (0 to 1%) of glucose increased in TSB supplemented with 0.6% yeast extract. *E. coli* O157:H7

possesses acid and heat resistance (Bacon et al., 2003; Cheville et al., 1996; Samelis et al., 2004; Sharma et al., 2005; Tosun and Gonul, 2005). Therefore, it was hypothesized that *E. coli* O157:H7 may use its AI-2-based quorum sensing system to be resistant to heat and acid. If this is the case, blocking the quorum sensing of *E. coli* O157:H7 could be an effective approach to control the pathogen. Thus, this study evaluated whether AI-2-based quorum sensing may be involved in the heat and acid resistance mechanisms of *E. coli* O157:H7.

MATERIALS AND METHODS

Preparation of inoculum. Colonies of *E. coli* O157:H7 strains ATCC 43895/ISEHGFP (Noah et al., 2005) (AI-2-positive strain), 86-24 (AI-2-positive), and VS94 (AI-2-negative; *luxS* mutant of *E. coli* O157:H7 strain 86-24) maintained on sorbitol McConkey agar (Difco, Becton Dickinson and Company, Sparks, MD) supplemented with cefixime and potassium tellurite (Dynal, Oslo, Norway) (SMAC-CT), were individually activated in 10 ml of Luria-Bertani (Difco; LB) broth and subcultured (0.1 ml) in 10 ml of LB broth at 35°C for 24 h. Stationary phase cells of each strain were harvested by centrifugation (4,629×*g*, 15 min, 4°C) and washed with phosphate buffered saline (pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 liter of distilled water; PBS). The cell pellets were resuspended with PBS to yield approximately 8 log CFU/ml. *E. coli* O157:H7 strains 86-24 and VS94 were kindly provided by Dr. Vanessa Sperandio (University of Texas Southwestern Medical Center, Dallas, TX).

Preparation of preconditioned (PC) media. A similar method to that described by Sperandio et al. (1999) was applied in the preparation of PC media. A colony of *E. coli* O157:H7 86-24 (for AI-2-positive medium) and VS94 (for AI-2-negative

medium), maintained on SMAC-CT, was activated in 10 ml of LB broth at 35°C for 24 h. A 0.5 ml portion of each culture was inoculated into 50 ml of LB + 0.5% glucose (Fisher Scientific, Fair Lawn, NJ) (LBG) broth and incubated at 35°C (OD_{600} ≈ 1.0). The cells were centrifuged (10,000×*g*, 10 min, 4°C) and supernatants were collected. The pH (approximately 4.50 to 4.80) of the supernatants were adjusted to 7.0 with 1N NaOH for heat challenge, or to pH 3.0 with 1N HCl for acid challenge, followed by filter-sterilizing (0.22μm, Nalgene, Rochester, NY). 29 ml portions of each PC medium from each strain were distributed in tubes (85 ml).

Heat challenge. All samples were placed in a water bath for heat challenge. To evaluate the heat resistance of AI-2 producing *E. coli* O157:H7 strain ATCC 43895/ISEHGFP and whether exogenous AI-2 molecules from a different strain (*E. coli* O157:H7 strain 86-24) affected survival of *E. coli* O157:H7 strain ATCC 43895/ISEHGFP under heat stress, 1 ml portions of *E. coli* O157:H7 strain ATCC 43895/ISEHGFP inoculum were inoculated into 29.75 ml of PC media [29 ml of PC media + 0.75 ml of 20×LB (to supply media nutrients); pH 7.0, 55°C], which were derived from *E. coli* O157:H7 strains 86-24 or VS94 for AI-2-positive and -negative PC media, respectively. The inoculated samples were heat challenged at 55°C for 4 h. To compare the resistance of *E. coli* O157:H7 strains 86-24 and VS94 to heat stress, 1 ml portions of the *E. coli* O157:H7 strains 86-24 or VS94 inoculum prepared as previously described were inoculated in 29 ml of sterile LB broth (pH 7.0; 52°C). The inoculated samples were exposed to heat (52°C) for 6 h.

Acid challenge. Samples were placed in a water bath (35°C) during acid challenge. To evaluate whether *E. coli* O157:H7 strain ATCC 43895/ISEHGFP (AI-2-positive) can use exogenous AI-2 molecules from a different strain (*E. coli* O157:H7 strain 86-24) to be resistant to acid, 1 ml portions of *E. coli* O157:H7 strain ATCC

43895/ISEHGFP inoculum were inoculated into 29 ml of PC media (pH 3.0; 35°C), which were derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 and VS94 for AI-2-positive and -negative PC media, respectively. The cells were exposed to acid (pH 3.0; 35°C) for 6 h. 0.75 ml of 20×LB broth was not added to PC media for acid challenge. To evaluate whether an AI-2-negative strain (*E. coli* O157:H7 strain VS94) could use exogenous AI-2 molecules in PC media to be resistant to acid (pH 3.0), 1 ml portions of the *E. coli* O157:H7 strain VS94 inoculum were inoculated into 29 ml of PC media (pH 3.0; 35°C), which were derived from *E. coli* O157:H7 strains 86-24 and VS94 for AI-2-positive and -negative PC media, respectively. The cells were exposed to acid (pH 3.0; 35°C) for 6 h.

Analysis. The samples were analyzed microbiologically every 1 h during heat and acid challenge. To enumerate bacterial survivors, serial dilutions of each sample were made in 0.1% buffered peptone-water (Difco) and 0.1 ml portions were plated in duplicate on tryptic soy agar (Difco) plates (TSA). All plates were incubated at 35°C for 48 h. Samples used for microbial analyses at 0 and 4 or 6 h were also used to determine pH values using a pH meter with a glass pH electrode (Denver Instrument, Arvada, CO).

To determine AI-2 activity of the samples for 0 and 4 or 6 h of the heat challenge, the bioluminescence response of *Vibrio harveyi* strain BB170 (sensor 1^- sensor 2^+) was measured (Surette and Bassler, 1998, 1999; Surette et al., 1999); *V. harveyi* strain BB170 reacts only with AI-2 molecules. Cell-free supernatants of the *V. harveyi* strains BB120 (AI-1^+ AI-2^+) and BB152 (AI-1^- AI-2^+) were used as positive controls (Surette and Bassler, 1998), while the negative control was sterile autoinducer bioassay (AB) medium (Bassler et al., 1993). The *Vibrio* strains were kindly provided by Dr. Bonnie L. Bassler (Princeton University, Princeton, NY). A 1

ml portion of the samples was centrifuged at 15,600×*g* (4°C) for 5 min in a microcentrifuge, and then the supernatant was filtered (0.2μm, 25 mm; Nalgene, Rochester, NY) to prepare cell-free supernatants (Surette and Bassler, 1998, 1999; Surette et al., 1999). The cell-free supernatants were assayed for AI-2 activity by induction of bioluminescence in *V. harveyi* strain BB170 with a procedure similar to that described by Surette and Bassler (1998, 1999) and Surette et al. (1999). Specifically, *V. harveyi* BB170 (0.1 ml) maintained in glycerol supplemented (10 %) LB + 1.5% NaCl broth at -70°C was activated (12 h) and subcultured (7.5 h) in 10 ml of LB + 1.5% NaCl broth at 30°C with shaking (200 rpm), and diluted 1:5,000 into AB medium. The mixtures (1:9 of cell-free supernatant : diluted *V. harveyi* BB170) were incubated at 30°C for 3.5 h. AI-2 activity of the samples from the acid challenge was not measured because the low pH of the samples caused unstable AI-2-activity measurement. In a preliminary study, when the pH of the samples containing AI-2 activity decreased to pH 3.0, no AI-2 activity was observed. Interestingly, when the pH of the same samples was increased to pH 7.0, AI-2 activity was observed. To measure AI-2 activity, bioluminescence of the mixture was measured with a luminometer (Model TD-20e luminometer, Turner Designs, Inc., Mt. View, CA). AI-2 activity was described as relative AI-2 activity (RAI) and was calculated as the ratio of bioluminescence of the test and negative control samples (Lu et al., 2004). The RAI was converted to log RAI/ml. RAI is referred as AI-2 activity.

Statistical analysis and calculation of death rates. The study was repeated three times with three samples for each replication. Bacterial survivors were converted to log CFU/ml before statistical analysis. Bacterial survivor data for each strain and exposure time, and for PC media and exposure time were analyzed by the mixed model procedure of SAS® (SAS institute Inc., 2002-2003). All mean comparisons

were performed by the Tukey's multiple comparison and a *P*-value of 0.05 was used for statistical significance. Survivor data (log CFU/ml) during heat challenge were fitted with the Baranyi model (Baranyi and Roberts, 1994) using the in-house program DMFit (Institute of Food Research, Norwich, UK) to calculate death rates (log CFU/ml/h), and survivor data (log CFU/ml) of acid challenge were fitted with linear regression [Bacterial survivor (log CFU/ml) = death rate (log CFU/ml/h) × time (h) + initial level (log CFU/ml)] to calculate the death rates.

RESULTS AND DISCUSSION

The AI-2-based quorum sensing system of *E. coli* O157:H7 may be partially involved in the mechanism of heat resistance. To evaluate the potential effect of AI-2 in the heat resistance of *E. coli* O157:H7, cells of *E. coli* O157:H7 strain ATCC 43895/ISEHGFP were exposed to 55°C in AI-2-positive and -negative PC media for 4 h. After 4 h of heat challenge, surviving *E. coli* O157:H7 strain ATCC 43895/ISEHGFP counts were higher by 1.1 log CFU/ml ($P<0.05$) in AI-2-positive than in AI-2-negative PC media (Figure VII-1). The higher heat resistance of *E. coli* O157:H7 strain ATCC 43895/ISEHGFP observed in AI-2-positive PC media, resulted in an obvious tailing effect, which was not obvious in the AI-2-negative PC medium (Figure VII-1). AI-2 activities of AI-2-positive and -negative PC media were 3.2 and -0.2 log RAI/ml, respectively at 0 h of heating, while after 4 h of heating, AI-2 activities decreased to 0.0 and -0.2 log RAI/ml for AI-2-positive and -negative PC media, respectively (Table VII-1). This indicates that AI-2 biosynthesis by *E. coli* O157:H7 strain ATCC 43895/ISEHGFP may be inhibited by rapidly decreasing bacterial populations due to high temperature (55°C), and that existing AI-2 molecules may be degraded by high temperature (55°C). A preliminary study of Surette and

Bassler (1998) showed that AI-2 activity of *Salmonella* and *E. coli* was heat resistant to 80°C but not 100°C. Exposure time of AI-2 to heat could be a major factor in determining the temperature stability of the AI-2 molecule; no information of exposure time was available in the study of Surette and Bassler (1998). In addition, it is hypothesized that exogenous AI-2 molecules of AI-2-positive PC media may be internalized into the cells under heat stress, and the molecules may be in part involved in the heat resistance mechanism of *E. coli* O157:H7. To prove these hypotheses, the AI-2-activity of uninoculated AI-2-positive PC medium was determined at 0 h (3.5 log RAI/ml) and 4 h (0.3 log RAI/ml) of heat challenge at 55°C; no AI-2 activity was detected at 4 h of heat challenge. This indicates that exogenous AI-2 was destroyed by heat (55°C). However, it may be possible that internalization of AI-2 by *E. coli* O157:H7 was also involved in the decrease of AI-2 activity in AI-2-positive PC medium.

Based on these results, it may also be assumed that AI-2 molecules may be partially involved in the process to protect *E. coli* O157:H7 under heat stress. In *P. aeruginosa*, RpoS protein influences the stationary-phase stress tolerance (Jørgensen et al., 1999), and the *rpoS* gene is positively related to heat shock and NaCl (Suh et al., 1999). Latifi et al. (1996) demonstrated that the *rhl* quorum sensing system of *P. aeruginosa* triggers the transcription of *rpoS*. Gawande and Griffiths (2005) showed that *rpoS* promoter is upregulated by stresses commonly encountered during food processing, especially osmotic stress. Also, *E. coli* O157:H7 regulates acid and heat resistance using *rpoS* (Cheville et al., 1996). Taken together, it could be assumed that quorum sensing of *E. coli* O157:H7 may be involved in regulation of heat resistance.

Since AI-2-positive PC medium was prepared by culturing *E. coli* O157:H7 strain 86-24 in LB broth with glucose, the resulting AI-2-positive PC medium may

contain low glucose concentration. Low levels of glucose in AI-2-positive PC medium may be partially related to decreased AI-2 activity in AI-2-positive PC medium by increasing the internalization of AI-2 molecules. *E. coli* produced a small soluble, heat labile molecule (AI-2) in LB containing glucose (DeLisa et al., 2001b; Hardie et al., 2003; Surette and Bassler, 1998), but no production of the molecule was observed in LB containing no glucose (Surette and Bassler, 1998), and the molecule was degraded during the stationary phase (Surette and Bassler, 1998). Surette and Bassler (1998, 1999) suggested that the degradation of extracellular AI-2 molecule is due to the lack of glucose. In the presence of glucose, the *luxS* regulated (*lsr*) operon is repressed and not transcribed. Thus, AI-2 molecules cannot be internalized by Lsr transporter; the *lsr* operon transcribes the Lsr transporter. However, in the absence of glucose, AI-2 is rapidly internalized by the Lsr transporter (Xavier and Bassler, 2005b). Specifically, cAMP (cyclic AMP)-CRP (cAMP receptor protein) complex triggers the expression of the *lsr* operon and works with the LsrR repressor to mediate AI-2 uptake by cells (Wang et al., 2005b). Wang et al. (2005a) also showed that in the presence of glucose, 23 genes were influenced by *luxS* deletion, while in the absence of glucose, 63 genes were affected by *luxS* deletion; the genes were most significantly induced by *luxS* in absence of glucose, and most of highly induced genes are associated with AI-2 production and transport (Wang et al., 2005a). Taken together, it can be suggested that the internalization of AI-2 molecules might be due to Lsr transporter of *E. coli* O157:H7 in AI-2-positive PC medium, which partially contributed to the decrease of AI-2 activity.

To evaluate whether the AI-2-positive strain was more heat resistant than the AI-2-negative strain, survivor counts of *E. coli* O157:H7 strain 86-24 (AI-2-positive) were compared to those of *E. coli* O157:H7 strain VS94 (AI-2-negative) in LB broth

at 52°C; a temperature of 52°C was used to see clear difference in survivor counts between AI-2-positive and –negative strains. In general, survivors of *E. coli* O157:H7 strain 86-24 were significantly higher ($P<0.05$) than those of *E. coli* O157:H7 VS94 after 3 h (0.9 log CFU/ml) of heat challenge until the end (6 h) of heat challenge (1 log CFU/ml) (Figure VII-2). AI-2 activity of both strains was not detected in LB broth at 0 and 6 h of heat challenge (Table VII-1). These results suggest that the AI-2-based quorum sensing of *E. coli* O157:H7 may be partially involved in the mechanisms of heat resistance. AI-2 molecules may be produced by *E. coli* O157:H7, but AI-2 molecules may not be released to the extracellular environment for rapid use by heat resistance mechanism. The pH values of the samples used for heat challenge were not changed (pH 6.74 to 6.93), regardless of the presence of AI-2 and media (Table VII-2).

Thermal rates of *E. coli* O157:H7 in heat stress. To compare the effects of AI-2-positive and -negative treatments on *E. coli* O157:H7 under heat stress mathematically, death rates (log CFU/ml/h) of *E. coli* O157:H7 were calculated with the Baranyi model (Baranyi and Roberts, 1994) using the in-house program DMFit. To show data distribution, raw data points which were used in the Baranyi model (Baranyi and Roberts, 1994) are presented in Figures VII-3 and VII-4. Unlike results presented in Figure VII-1, death rates of *E. coli* O157:H7 strain ATCC 43895/ISEHGFP were similar between AI-2-positive and -negative PC media (Figure VII-3); death rates in AI-2-positive and -negative PC media were -1.51 (R^2=0.868) and -1.65 log CFU/ml/h (R^2=0.793), respectively (Figure VII-3). Even thermal death rates of *E. coli* O157:H7 strain 86-24 (AI-2-positive) and VS94 (AI-2-negative) in LB broth (52°C) were not different. *E. coli* O157:H7 strain 86-24 in LB broth: -1.55 log CFU/ml/h (R^2=0.691), *E. coli* O157:H7 strain VS94: -1.51 log CFU/ml/h (R^2=0.754)

(Figure VII-4). Difference in cell survivors between AI-2-positive and -negative treatments was observed only after 3 or 4 h of heat challenge (Figures VII-1 and VII-2), and the fitted curves by the Baranyi model (Baranyi and Roberts, 1994) showed higher levels of predicted bacterial populations in presence of AI-2 activity than in absence of AI-2 activity during the period (tailing effect) (Figures VII-3 and VII-4). However, the curves fitted through raw data points of bacterial survivors showed a more obvious and longer tailing effect in the presence of AI-2 activity (Figures VII-3 and VII-4). This suggests that AI-2 molecule may be only partially associated with the tailing effect of *E. coli* O157:H7 under heat stress.

AI-2-based quorum sensing system of *E. coli* O157:H7 may partially contribute to the acid resistance mechanism. To evaluate the potential effect of AI-2-based quorum sensing on the acid resistance of *E. coli* O157:H7, cells of *E. coli* O157:H7 strain ATCC 43895/ISEHGFP were exposed to low pH (pH 3.0, 35°C) in AI-2-positive and -negative PC media for 6 h. Differences in survivor counts increased between AI-2-positive and -negative PC media after 2 h of acid challenge (Figure VII-5). After 6 h of the acid challenge, survivors of *E. coli* O157:H7 strain ATCC 43895/ISEHGFP were higher (0.8 log CFU/ml; $P<0.05$) in AI-2-positive than in AI-2-negative PC media (Figure VII-5). This shows that AI-2-based quorum sensing of *E. coli* O157:H7 may be associated with its acid resistance mechanism. In both the heat and acid challenge experiment of this study, differences in surviving bacterial populations between presence and absence of AI-2 activity are obvious but not major. This indicates that the AI-2-based quorum sensing may be one of several factors that may be related to resistance mechanisms against heat and acid stress. It is rare that only one factor would be involved in the physiological mechanisms of bacteria

against stressful conditions. Individual stress response systems are incorporated into a multidefense strategy (Foster and Spector et al., 1995).

AI-2-positive and –negative PC media derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 and VS94 were used to examine whether the AI-2-negative strain (*E. coli* O157:H7 strain VS94) can utilize exogenous AI-2 molecules under acid stress. In general, surviving cell counts of *E. coli* O157:H7 strain VS94 were higher ($P<0.05$) in AI-2-positive than in AI-2-negative PC media (pH 3.0). After 6 h of the acid challenge, survivor counts of *E. coli* O157:H7 strain VS94 were higher by 0.8 log CFU/ml ($P<0.05$) in AI-2-positive than in AI-2-negative PC media (Figure VII-6). This indicates that *E. coli* O157:H7 may take up exogenous AI-2 molecules to contribute to its acid resistance mechanism. AI-2 molecules can be taken up by LsrR transporter (Wang et al., 2005b). It was also suggested that cell-to-cell communication may be related to develop appropriate acid adaptation in *S. mutans* (Li et al., 2001a). *E. coli* K-12 uses AI-2-based quorum sensing to communicate the stress or metabolic burden related to overall accumulation of foreign protein (DeLisa et al., 2001c). DeLisa et al. (2001b) mapped the four quorum sensing regulated genes and 20 stress genes for *E. coli* K-12 during the transient response to induced expression of hIL-2, and the result showed that significant overlap was observed among quorum sensing genes, starvation genes, and several stress genes. When *E. coli* cells were exposed to low concentration (2 μg/ml) of chlortetracycline, AI-2 activity of *E. coli* was increased by increasing levels of tetracycline (Lu et al., 2005a). This indicates that *E. coli* may use AI-2 to be resistant to antibiotics. Overexpression of the superoxide decreased the transcription of *comQXP* quorum sensing locus in *Bacillus subtilis* (Ohsawa et al., 2006). This indicates that quorum sensing of *B. subtilis* may not be involved in stress resistance to superoxide.

The pH of the PC media was consistent during acid challenge; pH 3.01 to 3.11) (Table VII-3).

Death rates of *E. coli* O157:H7 in acid stress. Unlike survivor data during heat challenge, populations of *E. coli* O157:H7 strains decreased linearly during acid stress (Figures VII-7 and VII-8). Thus, linear regression was applied to calculate death rates (log CFU/ml/h) of *E. coli* O157:H7 strains during acid challenge. The death rates of *E. coli* O157:H7 strain ATCC 43895/ISEHGFP was slightly lower in AI-2-positive (-0.48 log CFU/ml/h; R^2=0.787) than in AI-2-negative PC media (-0.62 log CFU/ml/h; R^2=0.884) (Figure VII-7). In addition, the death rates of *E. coli* O157:H7 strain VS94 were slightly lower in AI-2-positive PC media (-0.29 log CFU/ml/h; R^2=0.604) than in AI-2-negative PC media (-0.45 log CFU/ml/h; R^2=0.650) (Figure VII-8). In agreement with the results of Figures VII-5 and VII-6, these results indicate that AI-2-based quorum sensing may be partially involved in the acid resistance mechanism of *E. coli* O157:H7.

CONCLUSIONS

The quorum sensing mechanism of *E. coli* O157:H7 using the AI-2 signal may be partially involved in its heat and acid resistance mechanism, and it may also be partially related to the tailing effect of *E. coli* O157:H7 under heat stress. Thus, quorum sensing of *E. coli* O157:H7 could be one of the novel targets for improvement of food safety.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. Pina M. Fratamico, Dr. John S. Novak, Dr. Vanessa Sperandio, and Dr. Bonnie L. Bassler for their friendly and critical advice, and cultures. This work was funded by the Colorado State University Agricultural Experiment Station.

Table VII-1. AI-2 activity [mean (log RAI/ml) ± standard error] of *Escherichia coli* O157:H7 in samples used for heat challenge of cells under various conditions

				Time	
Figure	Challenged strain	AI-2 activity	Media	Beginning	End
VII-1	ATCC 43895/ISEHGFP	+	AI-2-positive PC	3.2±0.2	0.0±0.1
	ATCC 43895/ISEHGFP	+	AI-2-negative PC	-0.2±0.1	-0.2±0.1
VII-2	86-24	+	LB	-0.3±0.1	-0.3±0.1
	VS94	-	LB	-0.3±0.1	-0.3±0.1

RAI: relative AI-2 activity; LB: Luria-Bertani broth.

Beginning: 0 h of the heat challenge.

End: 4 h of the heat challenge for the Figure VII-1 and 6 h of the heat challenge for the Figure VII-2.

PC: preconditioned media derived from the cell-free supernatants of *E. coli* O157:H7 86-24 (AI-2-positive) and VS94 (AI-2-negative).

Table VII-2. pH values (mean ± standard error) of samples inoculated with *Escherichia coli* O157:H7 during heat challenge for 6 h under various conditions

				Time	
Figure	Challenged strain	AI-2 activity	Media	Beginning	End
VII-1	ATCC 43895/ISEHGFP	+	AI-2-positive PC	6.91±0.03	6.80±0.04
	ATCC 43895/ISEHGFP	+	AI-2-negative PC	6.93±0.01	6.90±0.04
VII-2	86-24	+	LB	6.83±0.03	6.76±0.03
	VS94	-	LB	6.85±0.04	6.74±0.03

LB: Luria-Bertani broth.

Beginning: 0 h of the heat challenge.

End: 4 h of heat challenge for the Figure VII-1 and 6 h of heat challenge for the Figure VII-2.

PC: preconditioned media derived from the cell-free supernatants of *E. coli* O157:H7 86-24 (AI-2-positive) and VS94 (AI-2-negative).

Table VII-3. pH values (mean ± standard error) of samples inoculated with *Escherichia coli* O157:H7 during acid challenge at 35°C for 6 h under various conditions

Figure	Challenged strain	AI-2 activity	Media	Time (h)	
				0	6
VII-5	ATCC 43895/ISEHGFP	+	AI-2-positive PC	3.03±0.02	3.11±0.01
	ATCC 43895/ISEHGFP	+	AI-2-negative PC	3.03±0.03	3.11±0.02
VII-6	VS94	-	AI-2-positive PC	3.01±0.01	3.07±0.01
	VS94	-	AI-2-negative PC	3.02±0.01	3.07±0.02

PC: preconditioned media derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative).

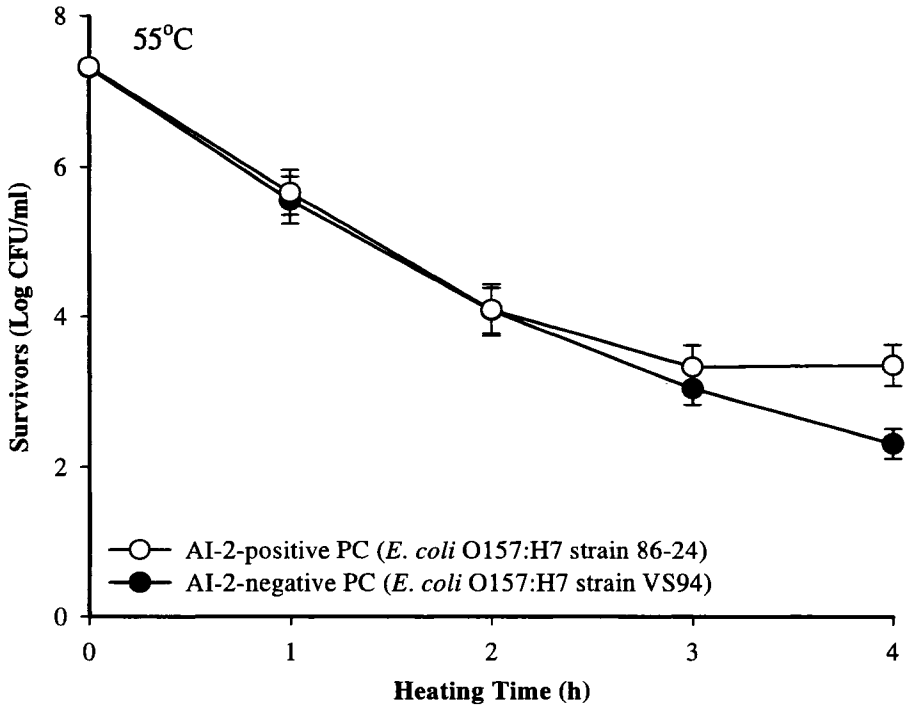

Figure VII-1. *Escherichia coli* O157:H7 strain ATCC 43895/ISEHGFP survivors, recovered with tryptic soy agar, during heating at 55°C for 4 h in AI-2-positive (pH 7.0) and -negative preconditioned (PC) media (pH 7.0); PC media were derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 38).

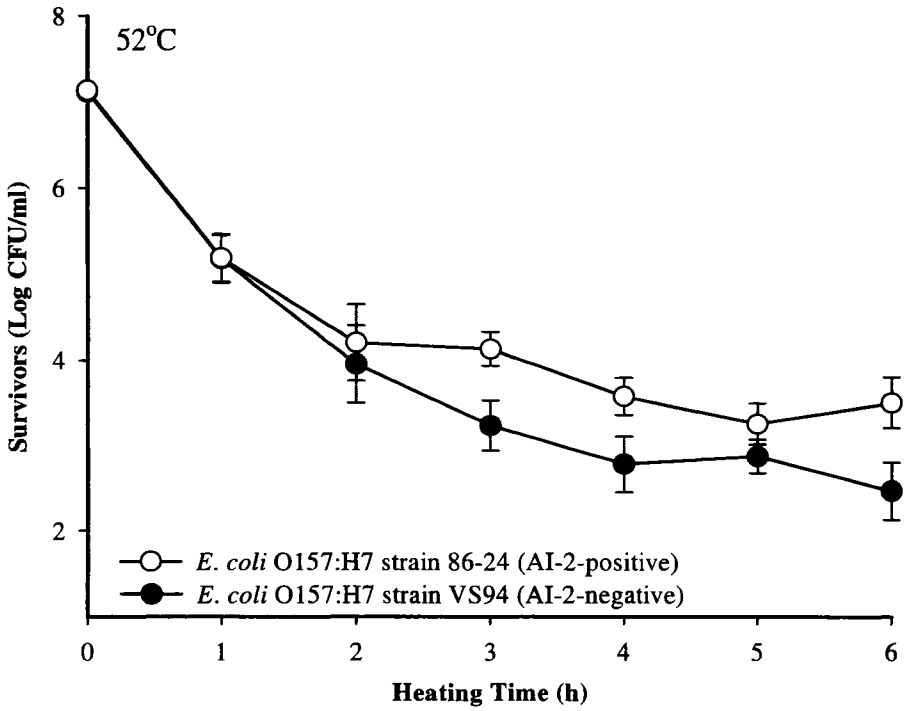

Figure VII-2. *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) survivors, recovered with tryptic soy agar, during heat challenge at 52°C for 6 h in Luria-Bertani broth (pH 7.0) (Data in APPENDIX Table 39).

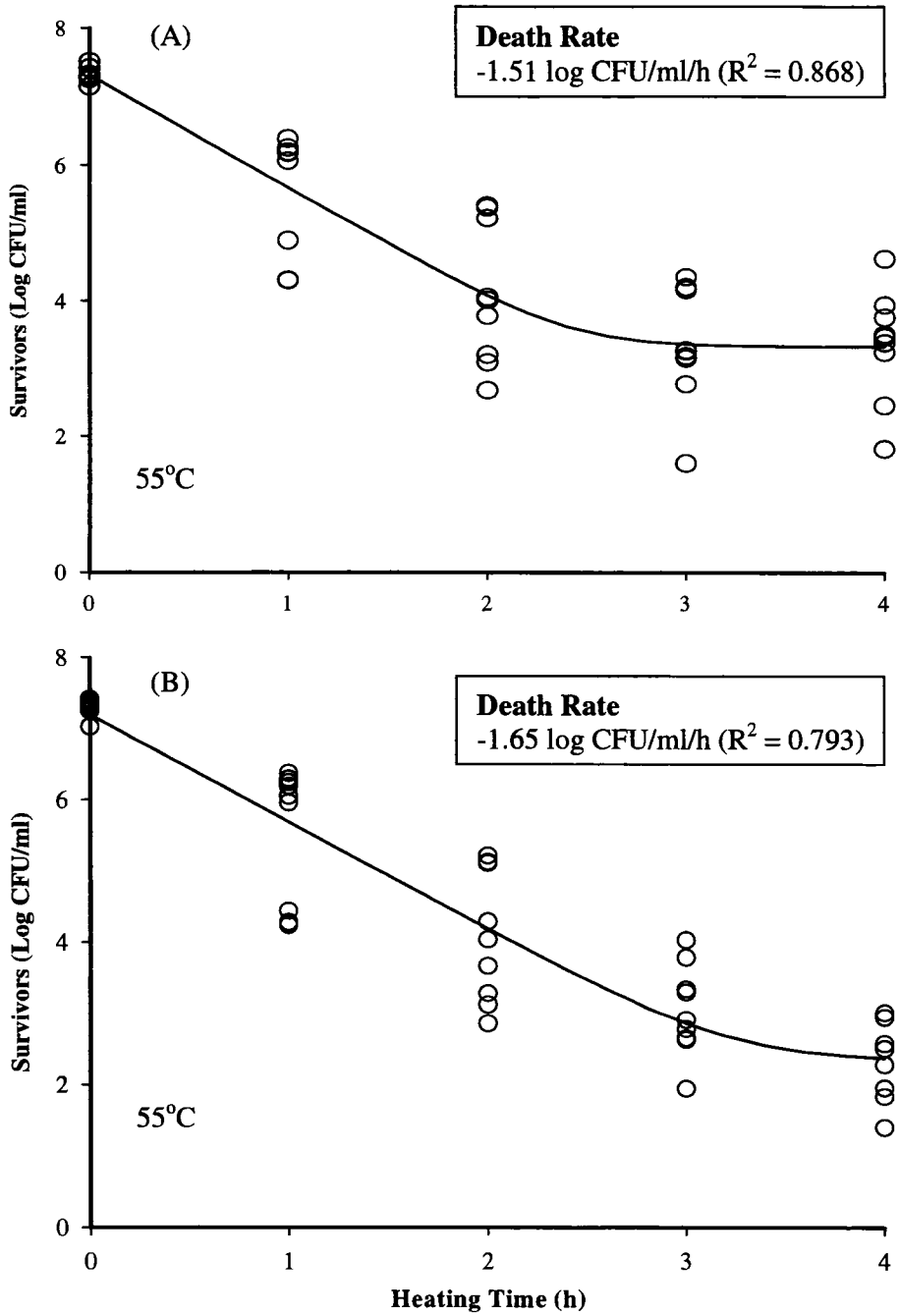

Figure VII-3. Raw data points (survivors) of *Escherichia coli* O157:H7 strain ATCC 43895/ISEHGFP, recovered with tryptic soy agar, during heat challenge at 55°C for 4 h in AI-2-positive (pH 7.0; A) and -negative preconditioned (PC) media (pH 7.0; B), and fitted curves and death rates that were generated from fitting the raw data with the Baranyi model (Baranyi and Roberts, 1994); PC media were derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 40).

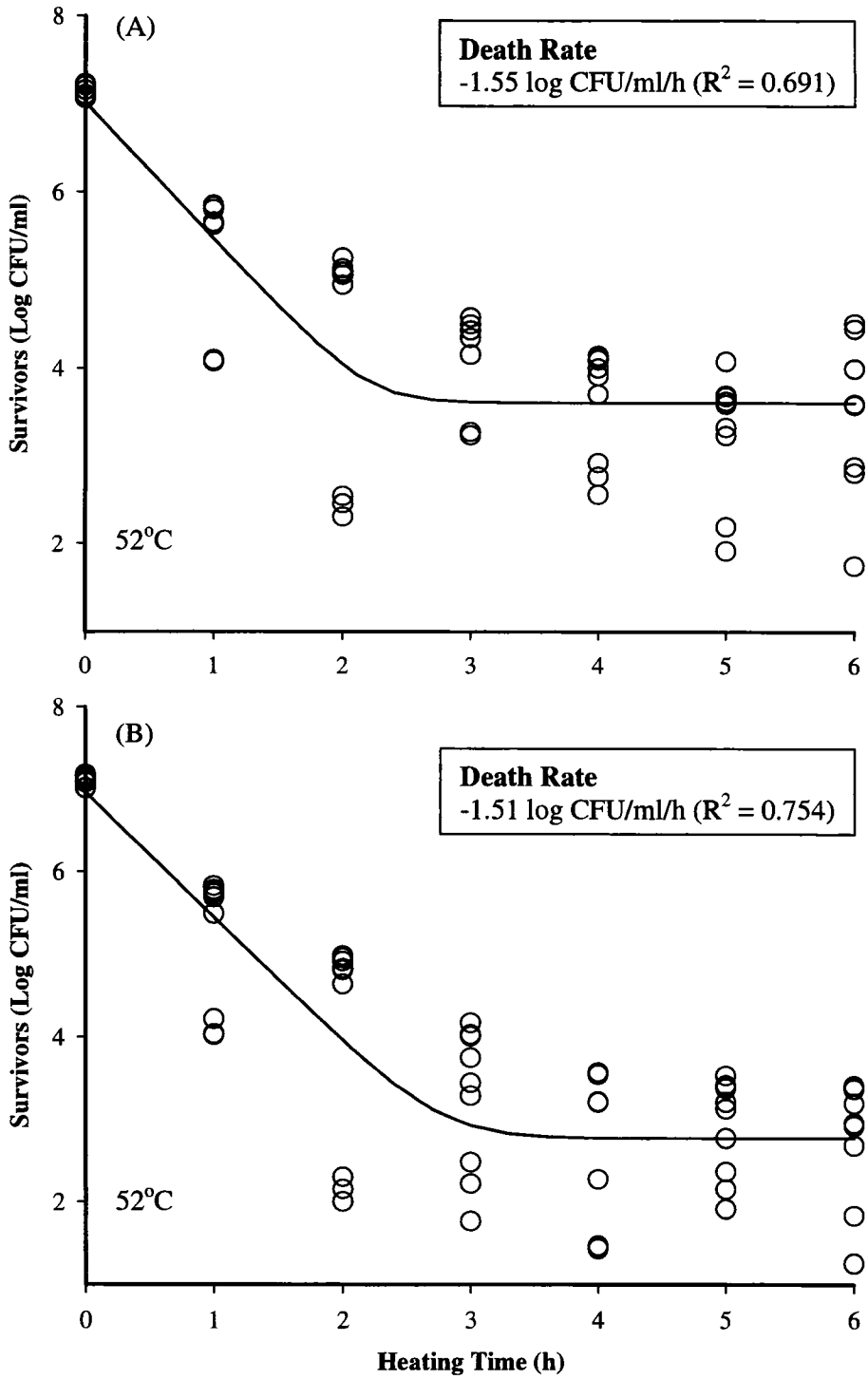

Figure VII-4. Raw data points (survivors) of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive; A) and VS94 (AI-2-negative; B), recovered with tryptic soy agar, during heat challenge at 52°C for 6 h in Luria-Bertani broth (pH 7.0), and fitted curves and death rates that were generated from fitting the raw data with the Baranyi model (Baranyi and Roberts, 1994) (Data in APPENDIX Table 40).

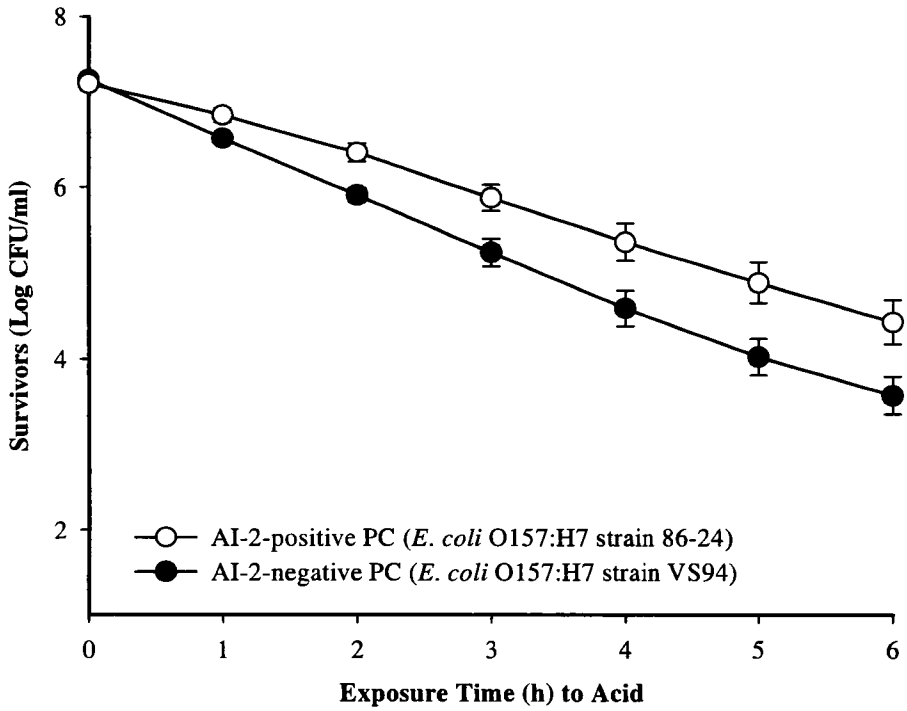

Figure VII-5. *Escherichia coli* O157:H7 strain ATCC 43895/ISEHGFP survivors, recovered with tryptic soy agar, during acid challenge at 35°C for 6 h in AI-2-positive (pH 3.0) and -negative preconditioned (PC) media (pH 3.0); PC media were derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 41).

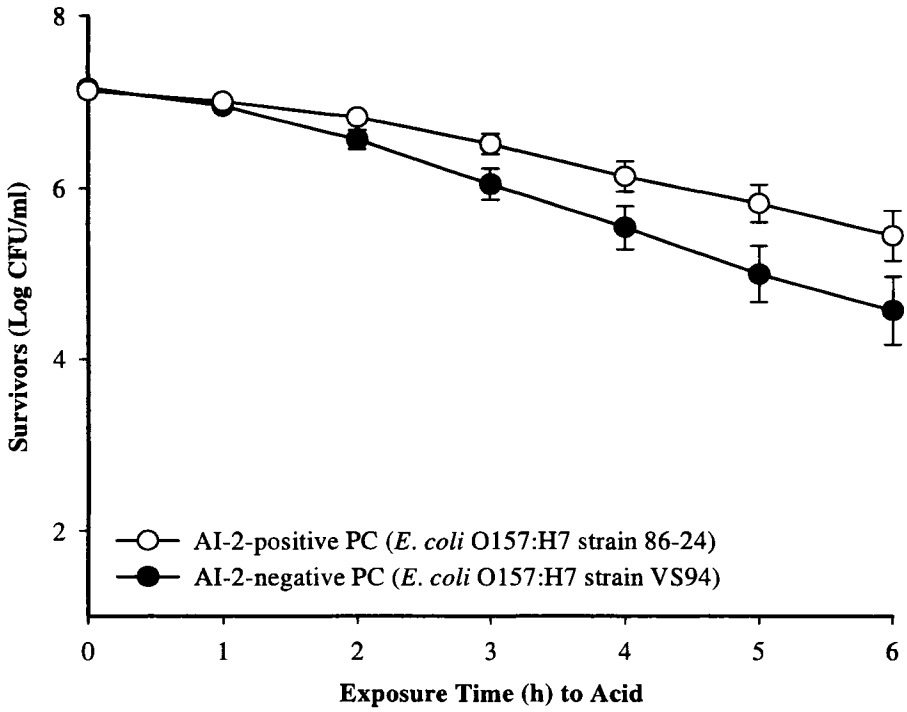

Figure VII-6. *Escherichia coli* O157:H7 strain VS94 (AI-2-negative) survivors, recovered with tryptic soy agar, during acid challenge at 35°C for 6 h in AI-2-positive (pH 3.0) and -negative preconditioned (PC) media (pH 3.0); PC media were derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 42).

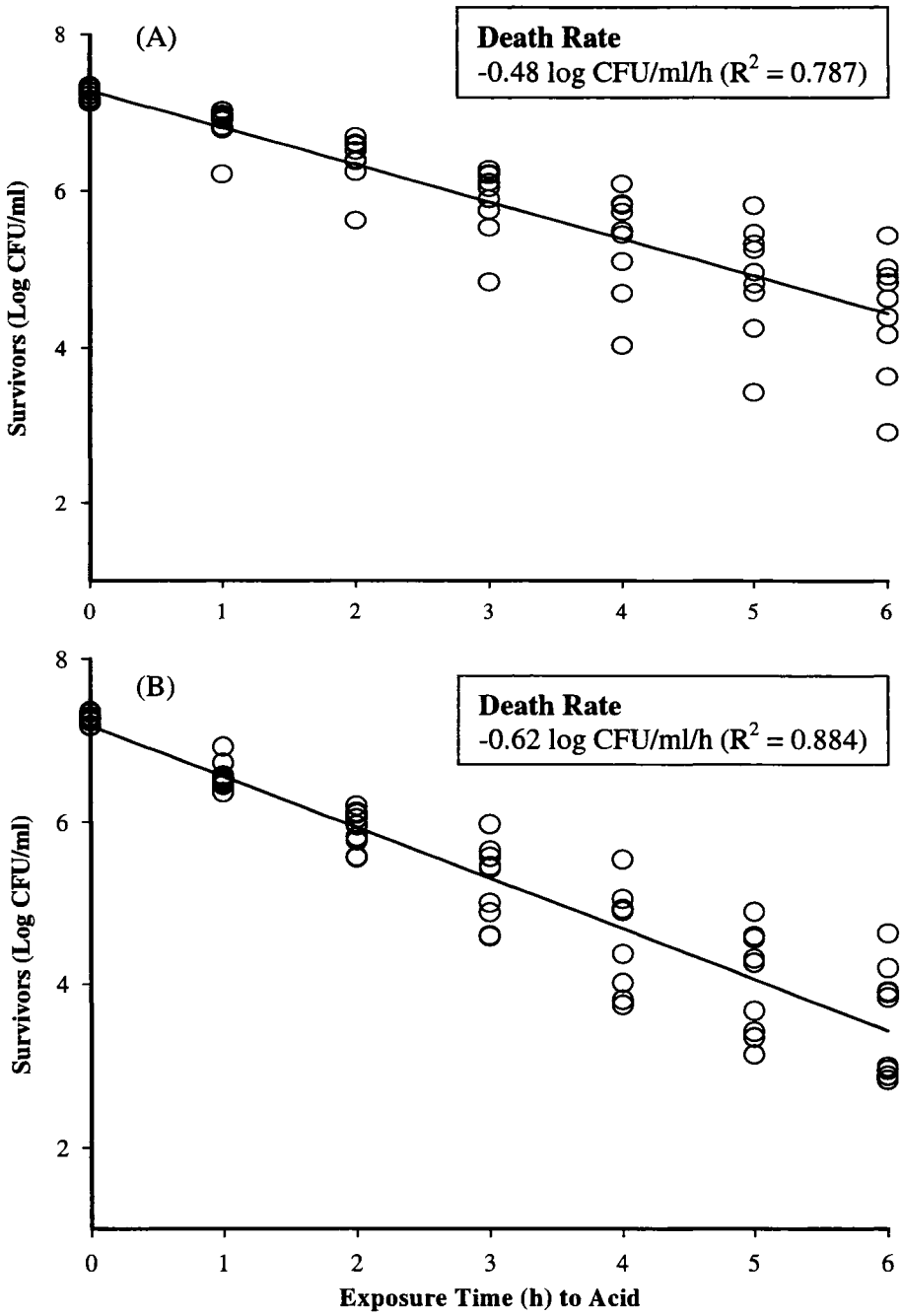

Figure VII-7. Raw data points (survivors) of *Escherichia coli* O157:H7 strain ATCC 43895/ISEHGFP, recovered with tryptic soy agar, during acid challenge at 35°C for 6 h in AI-2-positive (A; pH 3.0) and -negative preconditioned (PC) media (B; pH 3.0), and fitted lines and death rates that were generated from fitting the raw data with linear regression; PC media were derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 43).

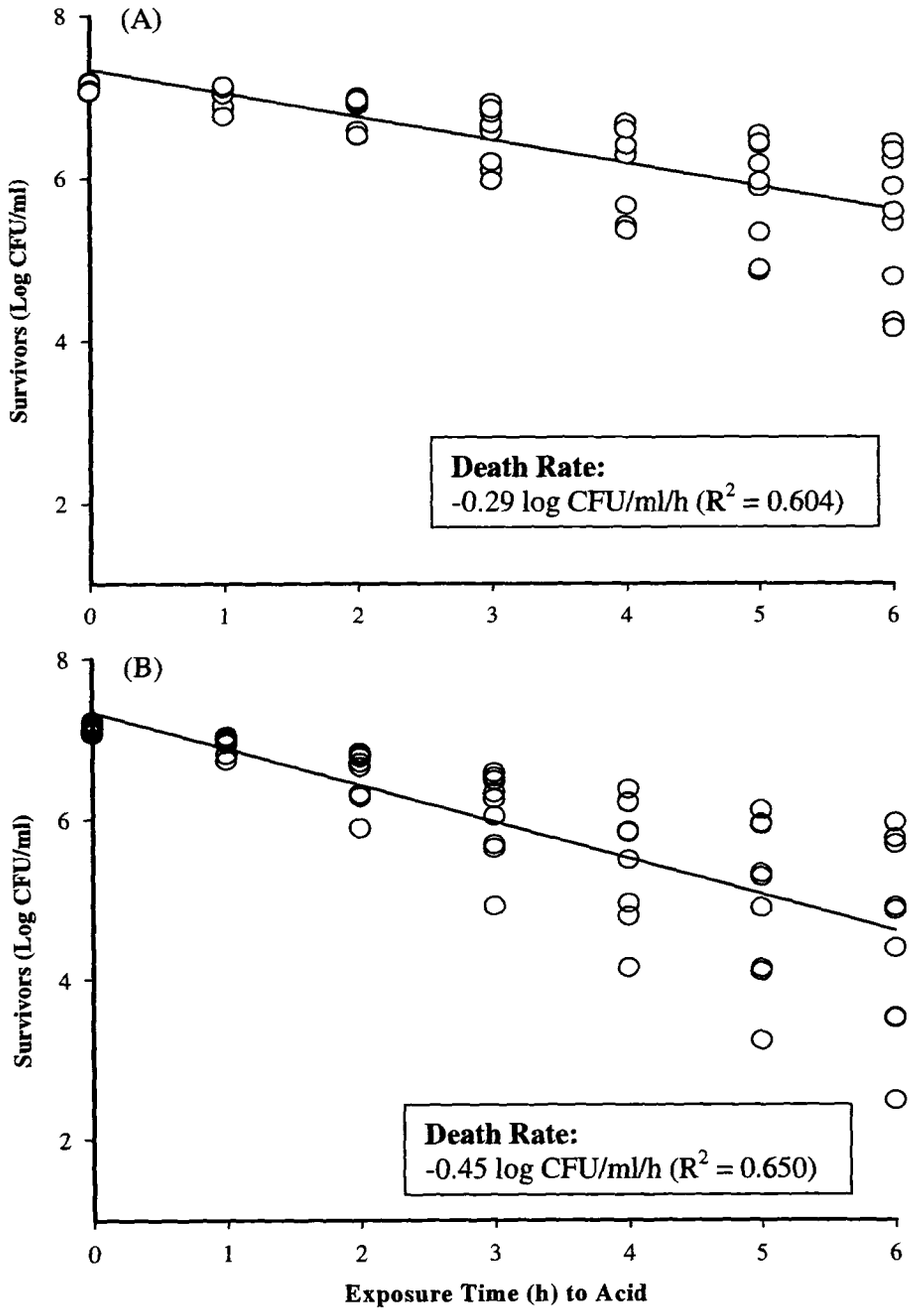

Figure VII-8. Raw data points (survivors) of *Escherichia coli* O157:H7 strain VS94 (AI-2-negative), recovered with tryptic soy agar, during acid challenge at 35°C for 6 h in AI-2-positive (A; pH 3.0) and -negative preconditioned (PC) media (B; pH 3.0), and fitted lines and death rates that were generated from fitting the raw data with linear regression; PC media were derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively (Data in APPENDIX Table 43).

CHAPTER VIII

SUMMARY AND RECOMMENDATIONS

Various bacteria possess quorum sensing systems using low-molecular-weight signaling molecules called autoinducers. Many scientists have examined the roles of quorum sensing in bacteria, and they have found that bacteria use quorum sensing for a variety of physical activities. In food industry, even though many treatments have been developed and applied on foods to improve food safety, we still have food safety problems and concerns. Thus, it could be useful to determine whether autoinducers of foodborne pathogenic bacteria use quorum sensing for regulation of bacterial growth, survival, and virulence in food environments and the host system. Although major foodborne pathogenic bacteria such as *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*, possess quorum sensing systems using autoinducer (AI)-2 or AI-3 activities or both AI-2 and AI-3, depending on type of bacteria, the roles of their quorum sensing and the environmental factors affecting quorum sensing molecule production in the complex food environment are not clear. Hence, it is important to study the roles of AI-2-based quorum sensing of foodborne pathogenic bacteria in food environments.

In *Salmonella* Thompson and *E. coli* O157:H7, the mutation for AI-2 production did not influence bacterial growth; therefore, the mutant and wild-type strains of *S.* Thompson and *E. coli* O157:H7 can be used to study the roles of AI-2

molecules of these bacteria in food environments. In addition, AI-2-based quorum sensing of *Salmonella* and *E. coli* O157:H7 is not involved in bacterial growth. To prepare preconditioned media (cell-free supernatants with adjusted pH) with absent or high AI-2 activity, which can be used to provide exogenous AI-2, glucose should be present in growth media, and the incubation time depends on the type of bacteria; cell-free supernatants with high AI-2 activity should be obtained during the stationary phase.

E. coli O157:H7 may not produce AI-2 molecules in beef purge containing high levels of natural flora. Thus, AI-2-based quorum sensing of *E. coli* O157:H7 may not be of significant concern in liquid type foods containing high level of natural flora. Further research is necessary to find the role of AI-2 produced by *E. coli* O157:H7 in other foods because a clear understanding of the role of AI-2 produced by bacteria in foods could be potentially helpful in improving food safety.

The AI-2-based quorum sensing system of *Salmonella* negatively regulated biofilm formation or was not involved in biofilm formation on food contact surfaces depending on serotype, while the AI-2-based quorum sensing was not involved in *E. coli* O157:H7 biofilm formation on stainless steel surfaces. Thus, the AI-2-based quorum sensing system of *Salmonella* and *E. coli* O157:H7 may not be of significance in food equipment sanitation. Since *Salmonella* and *E. coli* O157:H7 exist with natural flora in food environments, further research is necessary to find whether exogenous quorum sensing molecules from the natural flora influence biofilm formation of *Salmonella* and *E. coli* O157:H7, and whether AI-2 molecules of bacteria upregulate biofilm formation of natural flora, which may help attachment of *Salmonella* and *E. coli* O157:H7 cells on food contact surfaces.

E. coli O157:H7 may produce AI-2 in fresh beef. In fresh beef samples, AI-2-based quorum sensing of *E. coli* O157:H7 depends not only on the level of natural flora, but also on *E. coli* O157:H7 cell density, and presence of oxygen. Higher storage temperature may also increase activity of AI-2-based quorum sensing of *E. coli* O157:H7 in fresh beef samples. Further research on the role of AI-2-based quorum sensing of *E. coli* O157:H7 and other foodborne pathogenic bacteria in a variety of foods is necessary.

Under heat stress, the AI-2-based quorum sensing of *Salmonella* may not be related to surviving cells forming a tail, which is of concern in food processing. In addition, AI-2 may not be used to induce heat and acid resistance, and acid adaptation in *Salmonella*. Therefore, the AI-2-based quorum sensing of *Salmonella* may not increase its heat and acid resistance in food environments.

Unlike *Salmonella*, the quorum sensing of *E. coli* O157:H7 using the AI-2 signal may be involved in part of heat and acid resistance mechanisms of *E. coli* O157:H7 and may be partially related to the tailing effect of *E. coli* O157:H7 under heat stressing conditions. Since numerous physiological features are concurrently involved in heat and acid resistance mechanisms of *E. coli* O157:H7, the AI-2-based quorum sensing of *E. coli* O157:H7 could be one of such mechanisms. Thus, quorum sensing of *E. coli* O157:H7 could be one of the novel targets to improve food safety.

Research on quorum sensing should be continued with the goal of finding targets that may improve food quality in food spoilage, affect resistance of foodborne pathogenic bacteria to stressful conditions and antimicrobial treatments, and reduce pathogen survival within the human immune system. In addition, the effect of interspecies communication of foodborne pathogenic bacteria with natural flora using quorum sensing molecules should be examined to find various physiological and

biochemical behaviors of bacteria to survive under stressful conditions. Quorum sensing research could potentially be helpful in developing novel approaches for improving food safety.

CHAPTER IX

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APPENDIX

Appendix Table 1 (for Figures III-1 and III-4). Cell counts [mean (log CFU/ml) ± standard error] of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative), and *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative), recovered with tryptic soy agar, during incubation at 35°C for 14 h in Luria-Bertani broth

Incubation time (h)	*Salmonella**		*E. coli* O157:H7*	
	RM1987N	RM1987NLUX	86-24	VS94
0	2.8±0.0 ^{Ae}	2.9±0.0 ^{Ae}	2.3±0.2 ^{Ac}	2.3±0.2 ^{Ad}
2	3.6±0.0 ^{Ad}	3.6±0.0 ^{Ad}	2.8±0.4 ^{Ac}	2.8±0.4 ^{Ad}
4	5.3±0.0 ^{Ac}	5.4±0.0 ^{Ac}	4.2±0.5 ^{Abc}	4.4±0.4 ^{Acd}
6	7.2±0.1 ^{Ab}	7.1±0.1 ^{Ab}	6.2±0.5 ^{Ab}	6.2±0.5 ^{Abc}
8	8.6±0.1 ^{Aa}	8.7±0.1 ^{Aa}	7.9±0.4 ^{Aab}	7.9±0.4 ^{Aab}
10	8.9±0.0 ^{Aa}	8.8±0.0 ^{Aa}	8.7±0.1 ^{Aa}	8.6±0.1 ^{Aa}
12	8.9±0.1 ^{Aa}	8.8±0.0 ^{Aa}	8.8±0.0 ^{Aa}	8.7±0.0 ^{Aa}
14	8.9±0.1 ^{Aa}	8.9±0.0 ^{Aa}	8.8±0.1 ^{Aa}	8.8±0.1 ^{Aa}

*Statistical analysis was performed separately for different bacteria.

^{A}: means within the same row with same superscript letters are not different ($P \geq 0.05$).

^{abcde}: means within the same column with different superscript letters are different ($P < 0.05$).

Appendix Table 2 (for Figure III-2). Cell density (OD_{600}; mean ± standard error) and AI-2 activity [mean (log RAI/ml) ± standard error] of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) during incubation at 35°C for 14 h in Luria-Bertani broth

Incubation time (h)	OD_{600}*		AI-2 activity*	
	RM1987N	RM1987NLUX	RM1987N	RM1987NLUX
0	0.012±0.003 ^{Ab}	0.006±0.005 ^{Ab}	-0.3±0.1 ^{Ab}	-0.4±0.0 ^{Aa}
2	-0.020±0.027 ^{Ab}	0.008±0.004 ^{Ab}	-0.2±0.1 ^{Ab}	-0.4±0.1 ^{Aa}
4	0.001±0.006 ^{Ab}	-0.002±0.001 ^{Ab}	-0.3±0.2 ^{Ab}	-0.3±0.1 ^{Aa}
6	0.087±0.011 ^{Ab}	0.104±0.004 ^{Ab}	0.2±0.2 ^{Ab}	-0.3±0.1 ^{Aa}
8	0.546±0.030 ^{Aa}	0.565±0.024 ^{Aa}	2.9±0.6 ^{Aa}	0.2±0.1 ^{Ba}
10	0.624±0.011 ^{Aa}	0.627±0.021 ^{Aa}	3.1±0.2 ^{Aa}	-0.1±0.1 ^{Ba}
12	0.615±0.039 ^{Aa}	0.628±0.036 ^{Aa}	1.2±0.7 ^{Aab}	-0.2±0.0 ^{Aa}
14	0.645±0.047 ^{Aa}	0.685±0.042 ^{Aa}	-0.1±0.1 ^{Ab}	-0.4±0.1 ^{Aa}

RAI: relative AI-2 activity.

*Statistical analysis was performed separately for OD_{600} and AI-2 activity.

^{AB}: means within the same row with different superscript letters are different ($P<0.05$).

^{ab}: means within the same column with different superscript letters are different ($P<0.05$).

Appendix Table 3 (for Figure III-3). Mean (standard error) cell density (OD_{600}) and AI-2 activity (log RAI/ml) of *Salmonella* Thompson strain RM1987N (AI-2-positive) during incubation at 35°C for 8 h in Luria-Bertani + 0.5% glucose broth

	Incubation time (h)								
	0	1	2	3	4	5	6	7	8
OD_{600}*	0.005 ^{C} (0.002)	0.002 ^{C} (0.005)	0.064 ^{C} (0.007)	0.313 ^{B} (0.036)	0.656 ^{A} (0.028)	0.744 ^{A} (0.002)	0.757 ^{A} (0.009)	0.771 ^{A} (0.006)	0.757 ^{A} (0.011)
AI-2*	-0.4 ^{C} (0.1)	-0.5 ^{C} (0.1)	-0.4 ^{C} (0.1)	0.9 ^{B} (0.0)	3.3 ^{A} (0.0)	3.6 ^{A} (0.1)	3.8 ^{A} (0.0)	3.7 ^{A} (0.1)	3.7 ^{A} (0.1)

RAI: relative AI-2 activity.

*Statistical analysis was performed separately for OD_{600} and AI-2 activity.

^{ABC}: means within the same row with different superscript letters are different ($P<0.05$).

Appendix Table 4 (for Figure III-5). Cell density (OD_{600}; mean ± standard error) and AI-2 activity [mean (log RAI/ml) ± standard error] of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) during incubation at 35°C for 14 h in Luria-Bertani broth

Incubation time (h)	OD_{600}*		AI-2 activity*	
	86-24	VS94	86-24	VS94
0	-0.002±0.004 ^{Ad}	-0.003±0.003 ^{Ade}	0.1±0.1 ^{Ac}	0.1±0.1 ^{Aa}
2	-0.001±0.001 ^{Ad}	-0.008±0.005 ^{Ae}	0.2±0.1 ^{Ac}	-0.2±0.2 ^{Aa}
4	-0.007±0.003 ^{Ad}	-0.006±0.001 ^{Ade}	-0.2±0.3 ^{Ac}	-0.3±0.2 ^{Aa}
6	0.024±0.006 ^{Ad}	0.046±0.018 ^{Ad}	-0.1±0.2 ^{Ac}	0.1±0.1 ^{Aa}
8	0.459±0.018 ^{Ac}	0.470±0.016 ^{Ac}	1.7±0.2 ^{Ab}	0.1±0.3 ^{Ba}
10	0.777±0.004 ^{Ab}	0.777±0.003 ^{Ab}	3.5±0.2 ^{Aa}	-0.3±0.1 ^{Ba}
12	0.817±0.007 ^{Aab}	0.816±0.001 ^{Aab}	3.7±0.1 ^{Aa}	0.0±0.1 ^{Ba}
14	0.855±0.002 ^{Aa}	0.853±0.001 ^{Aa}	3.2±0.2 ^{Aa}	-0.1±0.2 ^{Ba}

RAI: relative AI-2 activity.

*Statistical analysis was performed separately for OD_{600} and AI-2 activity.

^{AB}: means within the same row with different superscript letters are different ($P<0.05$).

^{abcde}: means within the same column with different superscript letters are different ($P<0.05$).

Appendix Table 5 (for Figure III-6). Mean (standard error) cell density (OD_{600}) and AI-2 activity (log RAI/ml) of *Escherichia coli* O157:H7 strain 86-24 (AI-2-positive) during incubation at 35°C for 14 h in Luria-Bertani + 0.5% glucose broth

	Incubation time (h)							
	0	2	4	6	8	10	12	14
OD_{600}*	-0.017 ^{E} (0.025)	0.093 ^{D} (0.011)	0.771 ^{B} (0.016)	1.076 ^{A} (0.006)	1.139 ^{A} (0.006)	1.150 ^{A} (0.012)	1.143 ^{A} (0.005)	1.159 ^{A} (0.007)
AI-2*	0.0 ^{B} (0.4)	0.1 ^{B} (0.2)	3.5 ^{A} (0.1)	4.1 ^{A} (0.1)	4.1 ^{A} (0.1)	4.1 ^{A} (0.1)	3.9 ^{A} (0.0)	3.9 ^{A} (0.1)

RAI: relative AI-2 activity.

*Statistical analysis was performed separately for OD_{600} and AI-2 activity.

^{ABCDE}: means within the same row with different superscript letters are different ($P<0.05$).

Appendix Table 6 (for Figure III-7). Total bacterial populations [mean (log CFU/ml) ± standard error] recovered with tryptic soy agar (TSA) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts [mean (log CFU/ml) ± standard error] recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (SMAC-CT) in beef purge, inoculated with no, 2, or 6 log CFU/ml of *E. coli* O157:H7 strain ATCC 43895, during the storage at 4°C for 21 days

	TSA*			SMAC-CT*		
Storage time (day)	No inoculation	2 log CFU/ml	6 log CFU/ml	No inoculation	2 log CFU/ml	6 log CFU/ml
0	4.9±0.1 ^{Bb}	4.9±0.1 ^{Bb}	6.3±0.0 ^{Ab}	<0.0±0.0 ^{Ba}	1.9±0.4 ^{Aa}	5.6±0.2 ^{Aa}
7	7.7±0.4 ^{Aa}	7.6±0.3 ^{Aa}	7.8±0.4 ^{Aab}	<0.0±0.0 ^{Ba}	0.9±0.2 ^{Aa}	3.5±0.6 ^{Aab}
14	8.2±0.4 ^{Aa}	8.2±0.3 ^{Aa}	8.6±0.3 ^{Aa}	<0.0±0.0 ^{Ba}	0.4±0.0 ^{Aa}	2.6±0.6 ^{Ab}
21	7.7±0.5 ^{Aa}	7.6±0.4 ^{Aa}	8.0±0.3 ^{Aab}	<0.0±0.0 ^{Ba}	0.6±0.2 ^{Aa}	2.0±0.7 ^{Ab}

*Statistical analysis was performed separately for different media.

^{AB}: means within the same row with different superscript letters are different (P<0.05).

^{ab}: means within the same column with different superscript letters are different (P<0.05).

Appendix Table 7 (for Figure III-8). Total bacterial populations [mean (log CFU/ml) ± standard error] recovered with tryptic soy agar (TSA) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts (mean ± standard error) recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (SMAC-CT) in beef purge, inoculated with no, 2, or 6 log CFU/ml of *E. coli* O157:H7 strain ATCC 43895, during storage at 10°C for 18 days

	TSA*			SMAC-CT*		
Storage time (day)	No inoculation	2 log CFU/ml	6 log CFU/ml	No inoculation	2 log CFU/ml	6 log CFU/ml
0	4.9±0.1 ^{Ab}	4.9±0.1 ^{Ab}	6.3±0.0 ^{Ab}	<0.0±0.0 ^{Ca}	1.9±0.4 ^{Ba}	5.6±0.2 ^{Aa}
6	8.2±0.4 ^{Aa}	8.0±0.5 ^{Aa}	8.2±0.3 ^{Aab}	<0.0±0.0 ^{Ba}	1.2±0.2 ^{Aa}	4.2±0.1 ^{Aa}
12	8.9±0.3 ^{Aa}	8.4±0.1 ^{Aa}	8.6±0.1 ^{Aa}	<0.0±0.0 ^{Ca}	1.5±0.3 ^{Ba}	4.8±0.3 ^{Aa}
18	8.7±0.5 ^{Aa}	7.9±0.3 ^{Aa}	8.6±0.1 ^{Aa}	<0.0±0.0 ^{Ca}	0.4±0.2 ^{Ba}	6.1±0.9 ^{Aa}

*Statistical analysis was performed separately for different media.

^{ABC}: means within the same row with different superscript letters are different ($P<0.05$).

^{ab}: means within the same column with different superscript letters are different ($P<0.05$).

Appendix Table 8 (for Figure III-9). Total bacterial populations [mean (log CFU/ml) ± standard error] recovered with tryptic soy agar (TSA) and *Escherichia coli* O157:H7 strain ATCC 43895 cell counts [mean (log CFU/ml) ± standard error] recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite (SMAC-CT) in beef purge, inoculated with no, 2, or 6 log CFU/ml of *E. coli* O157:H7 strain ATCC 43895, during storage at 25°C for 9 days

	TSA*			SMAC-CT*		
Storage time (day)	No inoculation	2 log CFU/ml	6 log CFU/ml	No inoculation	2 log CFU/ml	6 log CFU/ml
0	4.9±0.1 ^{Bb}	4.9±0.1 ^{Bb}	6.3±0.0 ^{Ab}	<0.0±0.0 ^{Ca}	1.9±0.4 ^{Bb}	5.6±0.2 ^{Ab}
3	8.6±0.2 ^{Aa}	8.8±0.2 ^{Aa}	8.5±0.1 ^{Aa}	<0.0±0.0 ^{Ca}	4.0±0.2 ^{Ba}	6.6±0.1 ^{Aab}
6	9.0±0.2 ^{Aa}	8.9±0.3 ^{Aa}	8.3±0.1 ^{Aa}	<0.0±0.0 ^{Ca}	3.5±0.3 ^{Bab}	6.8±0.1 ^{Aab}
9	8.5±0.1 ^{Aa}	8.1±0.0 ^{Aa}	8.5±0.1 ^{Aa}	<0.0±0.0 ^{Ca}	4.6±0.2 ^{Ba}	7.3±0.3 ^{Aa}

*Statistical analysis was performed separately for different media.

^{ABC}: means within the same row with different superscript letters are different ($P<0.05$).

^{ab}: means within the same column with different superscript letters are different ($P<0.05$).

Appendix Table 9 (for Figure III-10). AI-2-like activity [mean (log RAI/ml) ± standard error] in beef purge inoculated (2 or 6 log CFU/ml) with *Escherichia coli* O157:H7 strain ATCC 43895 during storage at 4°C for 21 days

	Storage time (day)			
Inoculation level	0	7	14	21
2 log CFU/ml	0.3±0.2 ^{Aa}	-0.5±0.4 ^{Aa}	-0.1±0.1 ^{Aa}	0.1±0.1 ^{Aa}
6 log CFU/ml	0.2±0.2 ^{Aa}	-0.2±0.2 ^{Aa}	-0.2±0.2 ^{Aa}	-0.1±0.3 ^{Aa}

RAI: relative AI-2 activity.

^{A}: means within the same row with same superscript letters are not different ($P \geq 0.05$).

^{a}: means within the same column with same superscript letters are not different ($P \geq 0.05$).

Data presented in the table show differences in AI-2-like activity between *E. coli* O157:H7 inoculated and uninoculated samples.

Appendix Table 10 (for Figure III-11). AI-2-like activity [mean (log RAI/ml) ± standard error] in beef purge inoculated (2 or 6 log CFU/ml) with *Escherichia coli* O157:H7 strain ATCC 43895 during storage at 10°C for 18 days

	Storage time (day)			
Inoculation level	0	6	12	18
2 log CFU/ml	0.3±0.2 ^{Aa}	0.1±0.1 ^{Aa}	0.6±0.2 ^{Aa}	0.5±0.6 ^{Aa}
6 log CFU/ml	0.2±0.2 ^{Aa}	-0.2±0.1 ^{Aa}	0.0±0.2 ^{Aa}	0.3±0.6 ^{Aa}

RAI: relative AI-2 activity.

^{A}: means within the same row with same superscript letters are not different ($P \geq 0.05$).

^{a}: means within the same column with same superscript letters are not different ($P \geq 0.05$).

Data presented in the table show differences in AI-2-like activity between *E. coli* O157:H7 inoculated and uninoculated samples.

Appendix Table 11 (for Figure III-12). AI-2-like activity [mean (log RAI/ml) ± standard error] in beef purge inoculated (2 or 6 log CFU/ml) with *Escherichia coli* O157:H7 strain ATCC 43895 during storage at 25°C for 9 days

	Storage time (day)			
Inoculation level	0	3	6	9
2 log CFU/ml	0.3±0.2 ^{Aa}	0.1±0.3 ^{Aa}	0.0±0.1 ^{Aa}	-0.4±0.1 ^{Aa}
6 log CFU/ml	0.2±0.2 ^{Aa}	0.5±0.2 ^{Aa}	-0.6±0.7 ^{Aa}	-0.3±0.3 ^{Aa}

RAI: relative AI-2 activity.

^{A}: means within the same row with same superscript letters are not different ($P \geq 0.05$).

^{a}: means within the same column with same superscript letters are not different ($P \geq 0.05$).

Data presented in the table show differences in AI-2-like activity between *E. coli* O157:H7 inoculated and uninoculated samples.

Appendix Table 12 (for Figures IV-1 and IV-2). Cell counts [mean (log CFU/ml) ± standard error], enumerated on tryptic soy agar, of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP in cell suspensions during incubation of the strains in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth containing none, or polypropylene and stainless steel coupons at 25°C for 72 h

		Storage time (h)				
Coup	Media	0	14	24	48	72
No	LB	2.5±0.0 ^{Ca}	7.4±0.1 ^{Ba}	8.7±0.0 ^{Aa}	8.9±0.0 ^{Aa}	8.9±0.0 ^{Aa}
	LBG	2.5±0.0 ^{Ca}	7.1±0.0 ^{Ba}	8.6±0.1 ^{Aa}	8.7±0.0 ^{Aa}	8.5±0.0 ^{Aa}
PP	LB	2.6±0.0 ^{Ca}	7.4±0.1 ^{Ba}	8.5±0.1 ^{Aa}	8.8±0.0 ^{Aa}	8.8±0.0 ^{Aa}
	LBG	2.5±0.1 ^{Ca}	7.2±0.1 ^{Ba}	8.6±0.0 ^{Aa}	8.6±0.1 ^{Aa}	8.5±0.1 ^{Aa}
ST	LB	2.5±0.0 ^{Ca}	7.5±0.1 ^{Ba}	8.5±0.0 ^{Aa}	8.9±0.1 ^{Aa}	8.9±0.1 ^{Aa}
	LBG	2.7±0.0 ^{Ca}	7.1±0.1 ^{Ba}	8.5±0.1 ^{Aa}	8.8±0.0 ^{Aa}	8.5±0.0 ^{Aa}

Coup: coupon; PP: polypropylene; ST: stainless steel.

^{ABC}: means within the same row with different superscript letters are different ($P<0.05$).

^{a}: means within the same column with same superscript letters are not different ($P\geq0.05$).

Appendix Table 13 (for Figures IV-1 and IV-2). AI-2 activity [mean (log RAI/ml) ± standard error] of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP in cell suspensions during incubation of the strain in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth containing none, or polypropylene and stainless steel coupons at 25°C for 72 h

		Storage time (h)				
Coup	Media	0	14	24	48	72
No	LB	-0.1±0.1 ^{Aa}	0.4±0.2 ^{Aa}	1.0±0.2 ^{Ab}	0.5±0.4 ^{Ab}	-0.2±0.1 ^{Ab}
	LBG	-0.5±0.1 ^{Ba}	0.1±0.3 ^{Ba}	1.8±0.9 ^{ABab}	3.3±0.0 ^{Aa}	3.5±0.1 ^{Aa}
PP	LB	-0.3±0.1 ^{Aa}	-0.3±0.1 ^{Aa}	0.5±0.1 ^{Ab}	-0.3±0.1 ^{Ac}	-0.2±0.2 ^{Ab}
	LBG	-0.3±0.2 ^{Ba}	-0.3±0.1 ^{Ba}	3.7±0.1 ^{Aa}	3.3±0.1 ^{Aa}	3.5±0.2 ^{Aa}
ST	LB	-0.6±0.1 ^{Aa}	0.0±0.3 ^{Aa}	0.4±0.1 ^{Ab}	0.1±0.1 ^{Ac}	-0.3±0.1 ^{Ab}
	LBG	-0.4±0.1 ^{Ba}	0.1±0.2 ^{Ba}	3.5±0.1 ^{Aa}	3.2±0.1 ^{Aa}	3.2±0.0 ^{Aa}

RAI: relative AI-2 activity.

Coup: coupon; PP: polypropylene; ST: stainless.

^{AB}: means within the same row with different superscript letters are different (P<0.05).

^{abc}: means within the same column with different superscript letters are different (P<0.05).

Appendix Table 14 (for Figure IV-3). Cell counts [mean (log CFU/cm^2) ± standard error], enumerated on tryptic soy agar, of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP in detached biofilm cells, suspended in maximum recovery diluent, formed on polypropylene and stainless steel coupons which were placed in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth, and incubated at 25°C for 72 h

		Storage time (h)				
Coup	Media	0	14	24	48	72
PP	LB	<0.4±0.0 ^{Da}	5.3±0.1 ^{Ca}	6.0±0.0 ^{Ba}	6.8±0.1 ^{Aa}	6.7±0.1 ^{Aa}
	LBG	<0.4±0.0 ^{Ca}	5.0±0.1 ^{Ba}	5.9±0.0 ^{Aab}	5.7±0.0 ^{Ab}	5.6±0.0 ^{Ab}
ST	LB	<0.4±0.0 ^{Ca}	5.2±0.1 ^{Ba}	5.6±0.1 ^{Bab}	6.4±0.0 ^{Aa}	6.6±0.2 ^{Aa}
	LBG	<0.4±0.0 ^{Ba}	5.1±0.0 ^{Aa}	5.4±0.0 ^{Ab}	5.3±0.0 ^{Ab}	5.0±0.1 ^{Ac}

Coup: coupon; PP: polypropylene; ST: stainless steel.

^{ABCD}: means within the same row with different superscript letters are different (P<0.05).

^{abc}: means within the same column with different superscript letters are different (P<0.05).

Appendix Table 15 (for Figure IV-3). AI-2 activity [mean (log RAI/cm^2) ± standard error] of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP in detached biofilm cells, suspended in maximum recovery diluent, formed on polypropylene and stainless steel coupons placed in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth containing none, or polypropylene and stainless steel coupons at 25°C for 72 h

		Storage time (h)				
Coup	Media	0	14	24	48	72
PP	LB	0.0±0.1 ^{Aa}	0.1±0.5 ^{Aa}	0.0±0.1 ^{Aa}	-0.3±0.2 ^{Aa}	0.4±0.3 ^{Aa}
	LBG	0.4±0.2 ^{Aa}	0.6±0.2 ^{Aa}	0.4±0.1 ^{Aa}	0.4±0.2 ^{Aa}	0.4±0.1 ^{Aa}
ST	LB	0.1±0.2 ^{Aa}	-0.1±0.3 ^{Aa}	-0.2±0.1 ^{Aa}	-0.1±0.0 ^{Aa}	0.5±0.1 ^{Aa}
	LBG	-0.2±0.2 ^{Aa}	0.7±0.2 ^{Aa}	0.2±0.1 ^{Aa}	0.0±0.1 ^{Aa}	0.5±0.4 ^{Aa}

RAI: relative AI-2 activity.

Coup: coupon; PP: polypropylene; ST: stainless steel.

^{A}: means within the same row with same superscript letters are not different ($P \geq 0.05$).

^{a}: means within the same column with same superscript letters are not different ($P \geq 0.05$).

Appendix Table 16 (for Figures IV-4 to IV-6). Cell counts [mean (log CFU/ml or cm^2) ± standard error], enumerated on tryptic soy agar, of *Escherichia coli* O157:H7 strain ATCC 43895/ISEHGFP in cell suspensions and detached biofilm cells, suspended in maximum recovery diluent, formed on stainless steel coupons during incubation of the strains in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth containing none and stainless steel coupons at 25°C for 72 h

			Storage time (h)			
Cond	Coup	Media	0	24	48	72
Sus	None*	LB	1.9±0.1 ^{Ba}	8.2±0.0 ^{Aa}	8.6±0.0 ^{Aa}	8.6±0.1 ^{Aa}
		LBG	1.9±0.1 ^{Ba}	8.4±0.1 ^{Aa}	8.5±0.0 ^{Aa}	8.5±0.0 ^{Aa}
	ST*	LB	1.8±0.2 ^{Ba}	8.3±0.0 ^{Aa}	8.5±0.0 ^{Aa}	8.5±0.1 ^{Aa}
		LBG	1.8±0.1 ^{Ba}	8.4±0.0 ^{Aa}	8.6±0.0 ^{Aa}	8.5±0.1 ^{Aa}
Bio	ST*	LB	<0.4±0.0 ^{Ba}	6.2±0.1 ^{Aa}	6.0±0.2 ^{Aa}	6.1±0.0 ^{Aa}
		LBG	<0.4±0.0 ^{Ba}	5.5±0.1 ^{Aa}	5.6±0.2 ^{Aa}	5.8±0.1 ^{Aa}

Cond: cell growth condition; Sus: suspension.

Bio: detached biofilm cells suspended in maximum recovery diluent.

Coup: coupon; ST: stainless steel.

^{AB}: means within the same row with different superscript letters are different ($P<0.05$).

^{a}: means within the same column with same superscript letters are not different ($P\geq0.05$).

*Statistical analysis was performed separately for condition and the type of coupon.

Appendix Table 17 (for Figures IV-4 to IV-6). AI-2 activity [mean (log RAI/ml or cm^2) ± standard error] of *Escherichia coli* O157:H7 strain ATCC 43895/ISEHGFP in cell suspensions and in detached biofilm cells, suspended in maximum recovery diluent, formed on stainless steel coupons during incubation of the strains in Luria-Bertani (LB) broth and LB + 0.5% glucose (LBG) broth containing no and stainless steel coupons at 25°C for 72 h

			Storage time (h)			
Cond	Coup	Media	0	24	48	72
Sus	No*	LB	0.1±0.2 ^{Ba}	2.2±0.0 ^{Aa}	1.7±0.2 ^{Aa}	1.4±0.1 ^{Ab}
		LBG	-0.2±0.1 ^{Ba}	2.5±0.1 ^{Aa}	2.6±0.1 ^{Aa}	2.7±0.1 ^{Aa}
	ST*	LB	-0.7±0.2 ^{Ba}	1.3±0.1 ^{Aa}	1.7±0.1 ^{Aa}	1.4±0.1 ^{Ab}
		LBG	-0.1±0.2 ^{Ba}	2.6±0.1 ^{Aa}	2.5±0.1 ^{Aa}	2.5±0.1 ^{Aa}
Bio	ST*	LB	0.0±0.2 ^{Aa}	0.1±0.2 ^{Aa}	0.1±0.2 ^{Aa}	0.2±0.0 ^{Aa}
		LBG	0.4±0.1 ^{Aa}	0.4±0.2 ^{Aa}	-0.1±0.0 ^{Aa}	0.0±0.1 ^{Aa}

RAI: relative AI-2 activity.

Cond: cell growth condition; Sus: suspension; Bio: detached biofilm cells suspended in maximum recovery diluent.

Coup: coupon; ST: stainless steel.

^{AB}: means within the same row with different superscript letters are different ($P<0.05$).

^{ab}: means within the same column with different superscript letters are different ($P<0.05$).

*Statistical analysis was performed separately for condition and the type of coupon.

Appendix Table 18 (for Figures IV-7 and IV-8). Cell counts [mean (log CFU/ml or cm^2) ± standard error], enumerated on tryptic soy agar, of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) in cell suspensions and in detached biofilm cells, suspended in maximum recovery diluent, formed on stainless steel coupons during incubation of the strains in Luria-Bertani (LB) broth containing stainless steel coupons at 25°C for 72 h

		Storage time (h)			
Cond	Strain	0	24	48	72
Sus*	RM1987N	2.2±0.1 ^{Ba}	8.7±0.1 ^{Aa}	8.9±0.1 ^{Aa}	8.9±0.0 ^{Aa}
	RM1987NLUX	2.2±0.0 ^{Ba}	8.7±0.1 ^{Aa}	8.9±0.0 ^{Aa}	8.9±0.0 ^{Aa}
Bio*	RM1987N	<0.4±0.0 ^{Ba}	4.7±0.0 ^{Aa}	5.0±.02 ^{Aa}	5.2±0.4 ^{Aa}
	RM1987NLUX	<0.4±0.0 ^{Ba}	4.7±0.1 ^{Aa}	5.0±0.2 ^{Aa}	5.3±0.1 ^{Aa}

Cond: condition; Sus: suspension.

Bio: detached biofilm cells suspended in maximum recovery diluent.

^{AB}: means within the same row with different superscript letters are different ($P<0.05$).

^{a}: means within the same column with same superscript letters are not different ($P\geq0.05$).

*Statistical analysis was performed separately for condition.

Appendix Table 19 (for Figures IV-9 and IV-10). Cell counts [mean (log CFU/ml or cm^2) ± standard error], enumerated on tryptic soy agar, of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) in cell suspensions and in detached biofilm cells, suspended in maximum recovery diluent, formed on stainless steel coupons during incubation of the strains in Luria-Bertani (LB) broth containing stainless steel coupons at 25°C for 72 h

		Storage time (h)			
Cond	Strain	0	24	48	72
Sus*	86-24	1.9±0.1 ^{Ba}	8.7±0.0 ^{Aa}	8.7±0.0 ^{Aa}	8.8±0.0 ^{Aa}
	VS94	2.0±0.0 ^{Ba}	8.7±0.1 ^{Aa}	8.7±0.0 ^{Aa}	8.8±0.0 ^{Aa}
Bio*	86-24	<0.4±0.0 ^{Ba}	4.7±0.1 ^{Aa}	4.4±0.3 ^{Aa}	4.4±0.1 ^{Aa}
	VS94	<0.4±0.0 ^{Ba}	4.5±0.2 ^{Aa}	4.4±0.2 ^{Aa}	4.8±0.1 ^{Aa}

Cond: condition; Sus: suspension.

Bio: detached biofilm cells suspended in maximum recovery diluent.

^{AB}: means within the same row with different superscript letters are different ($P<0.05$).

^{a}: means within the same column with same superscript letters are not different ($P\geq0.05$).

*Statistical analysis was performed separately for condition.

Appendix Table 20 (for Figures V-1 and V-2). Total bacterial populations [mean (log CFU/cm^2) ± standard error], recovered with tryptic soy agar, in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low (LNB) or high levels of natural flora (HNB) during aerobic and vacuum storage for 21 days at 4°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	7	14	21
Aerobic	LNB	No	1.0±0.3 ^{Cef}	4.2±0.3 ^{Bcdef}	6.7±0.5 ^{Acd}	7.5±0.8 ^{Abc}
		2	1.5±0.1 ^{Cdef}	4.1±0.3 ^{Bdef}	8.3±0.5 ^{Aab}	9.1±0.1 ^{Aa}
		6	5.3±0.0 ^{Ba}	5.7±0.4 ^{Babc}	9.0±0.2 ^{Aa}	9.1±0.1 ^{Aa}
	HNB	No	3.1±0.5 ^{Cbc}	6.7±0.6 ^{Ba}	9.3±0.2 ^{Aa}	9.2±0.1 ^{Aa}
		2	2.5±0.5 ^{Cbcde}	6.4±0.5 ^{Bab}	9.0±0.2 ^{Aa}	9.0±0.1 ^{Aab}
		6	5.4±0.1 ^{Ba}	6.3±0.8 ^{Bab}	9.0±0.2 ^{Aa}	8.8±0.2 ^{Aabc}
Vacuum	LNB	No	0.9±0.1 ^{Cf}	2.8±0.4 ^{Bfg}	5.6±0.1 ^{Acd}	6.2±0.2 ^{Ad}
		2	1.4±0.1 ^{Bdef}	2.2±0.3 ^{Bg}	5.1±0.3 ^{Ad}	6.6±0.2 ^{Ad}
		6	5.2±0.0 ^{Aa}	5.1±0.1 ^{Aabcde}	5.5±0.2 ^{Acd}	6.5±0.2 ^{Ad}
	HNB	No	2.7±0.5 ^{Cbcd}	4.6±0.3 ^{BCbcde}	6.2±0.5 ^{ABcd}	6.9±0.4 ^{Ad}
		2	3.3±0.3 ^{Bb}	3.6±0.7 ^{Befg}	7.1±0.1 ^{Abc}	7.3±0.3 ^{Acd}
		6	5.8±0.3 ^{Aa}	5.6±0.3 ^{Aabcd}	7.1±0.3 ^{Abc}	6.9±0.4 ^{Ad}

Inoc: level of inoculation.

^{ABC}: means within the same row with different superscript letters are different (P<0.05).

^{abcdefg}: means within the same column with different superscript letters are different (P<0.05).

Appendix Table 21 (for Figures V-1 and V-2). Cell counts [mean (log CFU/cm^2) ± standard error] of *Escherichia coli* O157:H7 strain ATCC 43895, recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite, in *E. coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low (LNB) or high levels of natural flora (HNB) during aerobic and vacuum storage for 21 days at 4°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	7	14	21
Aerobic	LNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ae}	<-0.2±0.0 ^{Af}
		2	0.8±0.1 ^{ABb}	0.9±0.1 ^{Ab}	1.1±0.3 ^{Ac}	0.2±0.2 ^{Be}
		6	4.6±0.1 ^{Aa}	4.3±0.2 ^{Aa}	4.5±0.1 ^{Aa}	4.0±0.1 ^{Aa}
	HNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ae}	<-0.2±0.0 ^{Af}
		2	1.5±0.2 ^{Ab}	0.7±0.2 ^{Bb}	0.5±0.2 ^{Bcd}	0.3±0.2 ^{Be}
		6	4.4±0.1 ^{Aa}	4.1±0.1 ^{ABa}	3.7±0.2 ^{Bb}	2.9±0.3 ^{Ccd}
Vacuum	LNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ae}	<-0.2±0.0 ^{Af}
		2	0.8±0.1 ^{Ab}	0.8±0.1 ^{Ab}	0.3±0.1 ^{Ad}	0.5±0.1 ^{Ae}
		6	4.4±0.2 ^{Aa}	4.0±0.1 ^{Aa}	3.9±0.2 ^{ABb}	3.3±0.4 ^{Bbc}
	HNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ae}	<-0.2±0.0 ^{Af}
		2	1.3±0.2 ^{Ab}	0.6±0.3 ^{ABb}	0.3±0.1 ^{Bd}	0.5±0.1 ^{Be}
		6	4.0±0.3 ^{Aa}	4.0±0.2 ^{Aa}	3.9±0.1 ^{Ab}	3.6±0.2 ^{Aab}

Inoc: level of inoculation.

^{ABC}: means within the same row with different superscript letters are different (P<0.05).

^{abcdef}: means within the same column with different superscript letters are different (P<0.05).

Appendix Table 22 (for Figures V-3 and V-4). Total bacterial populations [mean (log CFU/cm^2) ± standard error], recovered with tryptic soy agar, in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low (LNB) or high levels of natural flora (HNB) during aerobic and vacuum storage for 18 days at 10°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	6	12	18
Aerobic	LNB	No	1.0±0.3 ^{Cd}	7.5±0.7 ^{Babcd}	9.2±0.3 ^{Aab}	8.9±0.4 ^{Aabc}
		2	1.5±0.1 ^{Ccd}	7.6±0.3 ^{Babcd}	9.7±0.1 ^{Aa}	9.1±0.4 ^{Aab}
		6	5.3±0.0 ^{Ca}	8.1±0.1 ^{Bab}	9.2±0.1 ^{ABab}	9.4±0.1 ^{Aa}
	HNB	No	3.1±0.5 ^{Bb}	8.1±0.1 ^{Aab}	9.2±0.2 ^{Aab}	8.9±0.1 ^{Aab}
		2	2.5±0.5 ^{Cbc}	8.0±0.5 ^{Bab}	9.5±0.1 ^{Aa}	9.3±0.1 ^{Aa}
		6	5.4±0.1 ^{Ba}	8.7±0.1 ^{Aa}	9.4±0.3 ^{Aa}	9.1±0.3 ^{Aab}
Vacuum	LNB	No	0.9±0.1 ^{Cd}	4.8±0.3 ^{Bf}	6.2±0.2 ^{Ad}	6.6±0.1 ^{Ade}
		2	1.4±0.1 ^{Bcd}	5.6±0.1 ^{Adef}	6.3±0.4 ^{Ad}	6.6±0.1 ^{Ae}
		6	5.2±0.0 ^{Aa}	5.0±0.1 ^{Aef}	6.3±0.2 ^{Ad}	6.2±0.2 ^{Ae}
	HNB	No	2.7±0.5 ^{Cbc}	6.3±0.5 ^{Bcde}	7.9±1.0 ^{Abc}	7.6±0.2 ^{Ad}
		2	3.3±0.3 ^{Bb}	6.8±0.1 ^{Abcd}	8.0±0.2 ^{Abc}	7.8±0.3 ^{Abcd}
		6	5.5±0.2 ^{Ba}	7.6±0.1 ^{Aabc}	7.4±0.2 ^{Acd}	7.6±0.2 ^{Acd}

Inoc: level of inoculation.

^{ABC}: means within the same row with different superscript letters are different (P<0.05).

^{abcdef}: means within the same column with different superscript letters are different (P<0.05).

Appendix Table 23 (for Figures V-3 and V-4). Cell counts [mean (log CFU/cm^2) ± standard error] of *Escherichia coli* O157:H7 strain ATCC 43895, recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite, in *E. coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low (LNB) or high levels of natural flora (HNB) during aerobic and vacuum storage for 18 days at 10°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	6	12	18
Aerobic	LNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ae}	<-0.2±0.0 ^{Ad}	<-0.2±0.0 ^{Ae}
		2	0.8±0.1 ^{Bb}	3.5±1.5 ^{Abc}	4.9±0.3 ^{Aab}	1.2±0.2 ^{Bd}
		6	4.6±0.1 ^{Ba}	5.9±0.3 ^{ABa}	6.1±0.3 ^{ABa}	6.3±0.5 ^{Aa}
	HNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ae}	<-0.2±0.0 ^{Ad}	<-0.2±0.0 ^{Ae}
		2	1.5±0.2 ^{Ab}	0.7±0.2 ^{Ad}	0.5±0.1 ^{Ac}	0.2±0.1 ^{Ad}
		6	4.4±0.1 ^{Aa}	4.6±0.4 ^{Aab}	4.9±0.8 ^{Aab}	5.7±0.1 ^{Aab}
Vacuum	LNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ae}	<-0.2±0.0 ^{Ad}	<-0.2±0.0 ^{Ae}
		2	0.8±0.1 ^{Ab}	1.1±0.3 ^{Acd}	0.8±0.2 ^{Ac}	1.5±0.2 ^{Ad}
		6	4.4±0.2 ^{Aa}	4.1±0.2 ^{Abc}	4.3±0.1 ^{Ab}	3.5±0.1 ^{Ac}
	HNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Ae}	<-0.2±0.0 ^{Ad}	<-0.2±0.0 ^{Ae}
		2	1.3±0.2 ^{Ab}	0.6±0.2 ^{Ad}	0.6±0.1 ^{Ac}	1.5±0.8 ^{Ad}
		6	4.0±0.3 ^{Aa}	4.6±0.0 ^{Aab}	3.6±0.4 ^{Ab}	4.1±0.5 ^{Ab}

Contam: levels of contamination; Inoc: level of inoculation.

^{AB}: means within the same row with different superscript letters are different (P<0.05).

^{abcde}: means within the same column with different superscript letters are different (P<0.05).

Appendix Table 24 (for Figures V-5 and V-6). Total bacterial populations [mean (log CFU/cm^2) ± standard error], recovered with tryptic soy agar, in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low (LNB) or high levels of natural flora (HNB) during aerobic and vacuum storage for 9 days at 25°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	3	6	9
Aerobic	LNB	No	1.0±0.3 ^{Bc}	9.0±0.3 ^{Aab}	9.4±0.2 ^{Aa}	9.0±0.3 ^{Aa}
		2	1.5±0.1 ^{Bbc}	8.3±0.4 ^{Abc}	9.3±0.2 ^{Aa}	9.3±0.3 ^{Aa}
		6	5.3±0.0 ^{Ba}	8.1±0.0 ^{Ac}	8.3±0.3 ^{Abc}	7.7±0.2 ^{Abc}
	HNB	No	3.1±0.5 ^{Bb}	9.2±0.4 ^{Aab}	9.7±0.1 ^{Aa}	9.1±0.2 ^{Aa}
		2	2.5±0.5 ^{Bb}	9.4±0.1 ^{Aa}	9.3±0.2 ^{Aab}	8.7±0.1 ^{Aab}
		6	5.4±0.1 ^{Ba}	9.2±0.1 ^{Aab}	9.5±0.2 ^{Aa}	9.1±0.1 ^{Aa}
Vacuum	LNB	No	0.9±0.1 ^{Bc}	6.5±0.4 ^{Ae}	7.1±0.1 ^{Ad}	6.9±0.2 ^{Ac}
		2	1.4±0.1 ^{Bc}	6.7±0.2 ^{Ae}	7.1±0.1 ^{Ad}	6.8±0.3 ^{Ac}
		6	5.2±0.0 ^{Ba}	8.0±0.2 ^{Acd}	7.4±0.2 ^{Acd}	7.0±0.1 ^{Ac}
	HNB	No	2.7±0.5 ^{Bb}	6.2±0.2 ^{Ae}	7.2±0.5 ^{Ad}	7.1±0.3 ^{Ac}
		2	3.3±0.3 ^{Bb}	7.1±0.2 ^{Ade}	7.6±0.2 ^{Ac}	7.4±0.2 ^{Ac}
		6	5.5±0.2 ^{Ba}	6.8±0.2 ^{Ae}	7.6±0.1 ^{Acd}	7.4±0.1 ^{Ac}

Inoc: level of inoculation.

^{AB}: means within the same row with different superscript letters are different (P<0.05).

^{abcde}: means within the same column with different superscript letters are different (P<0.05).

Appendix Table 25 (for Figures V-5 and V-6). Cell counts [mean (log CFU/cm^2) ± standard error] of *Escherichia coli* O157:H7 strain ATCC 43895, recovered with sorbitol McConkey agar supplemented with cefixime and potassium tellurite, in *E. coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low (LNB) or high levels of natural flora (HNB) during aerobic and vacuum storage for 9 days at 25°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	3	6	9
Aerobic	LNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Af}	<-0.2±0.0 ^{Af}	<-0.2±0.0 ^{Ae}
		2	0.8±0.1 ^{Bb}	7.3±0.3 ^{Aab}	7.7±0.1 ^{Aab}	7.3±0.1 ^{Aa}
		6	4.6±0.1 ^{Ca}	7.9±0.4 ^{Aa}	7.0±0.1 ^{ABbc}	6.2±0.1 ^{Bb}
	HNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Af}	<-0.2±0.0 ^{Af}	<-0.2±0.0 ^{Ae}
		2	1.5±0.2 ^{Cb}	6.4±0.2 ^{Acd}	6.1±0.2 ^{Ad}	4.0±0.6 ^{Bc}
		6	4.4±0.2 ^{Ca}	8.1±0.2 ^{Aa}	7.9±0.2 ^{Aa}	6.1±0.1 ^{Bb}
Vacuum	LNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Af}	<-0.2±0.0 ^{Af}	<-0.2±0.0 ^{Ae}
		2	0.8±0.1 ^{Cb}	5.9±0.2 ^{Acd}	5.6±0.5 ^{ABd}	5.0±0.3 ^{Bc}
		6	4.4±0.1 ^{Ba}	6.5±0.4 ^{Abc}	6.2±0.1 ^{Acd}	5.1±0.2 ^{Bc}
	HNB	No	<-0.2±0.0 ^{Ac}	<-0.2±0.0 ^{Af}	<-0.2±0.0 ^{Af}	<-0.2±0.0 ^{Ae}
		2	1.3±0.2 ^{Cb}	4.6±0.2 ^{Ae}	4.4±0.2 ^{Ae}	3.3±0.2 ^{Bd}
		6	4.0±0.3 ^{Ba}	5.6±0.3 ^{Ad}	5.4±0.2 ^{Ad}	4.8±0.4 ^{ABc}

Inoc: level of inoculation.

^{ABC}: means within the same row with different superscript letters are different (P<0.05).

^{abcdef}: means within the same column with different superscript letters are different (P<0.05).

Appendix Table 26 (for Figures V-7 and V-8). AI-2-like activity [mean (log RAI/cm^2) ± standard error] in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low (LNB) or high levels of natural flora (HNB) under aerobic and vacuum packaged storage conditions for 21 days at 4°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	7	14	21
Aerobic	LNB	2	0.0±0.1 ^{Aa}	0.1±0.2 ^{Aa}	0.2±0.3 ^{Aa}	-0.2±0.2 ^{Aa}
		6	-0.2±0.2 ^{Aa}	-0.3±0.1 ^{Aa}	0.2±0.2 ^{Aa}	-0.4±0.1 ^{Aa}
	HNB	2	0.0±0.2 ^{Aa}	-1.1±0.4 ^{Aa}	0.0±0.3 ^{Aa}	-0.2±0.1 ^{Aa}
		6	-0.3±0.2 ^{Aa}	-0.7±0.7 ^{Aa}	0.0±0.8 ^{Aa}	0.1±0.2 ^{Aa}
Vacuum	LNB	2	-0.2±0.1 ^{Aa}	-0.1±0.1 ^{Aa}	0.1±0.1 ^{Aa}	0.3±0.2 ^{Aa}
		6	-0.3±0.2 ^{Aa}	-0.9±0.0 ^{Aa}	-0.6±0.3 ^{Aa}	-0.1±0.1 ^{Aa}
	HNB	2	-0.2±0.1 ^{Aa}	-0.5±0.3 ^{Aa}	0.0±0.2 ^{Aa}	-0.2±0.2 ^{Aa}
		6	-0.3±0.1 ^{Aa}	-0.2±0.1 ^{Aa}	-0.5±0.2 ^{Aa}	-0.2±0.3 ^{Aa}

RAI: relative AI-2 activity.

Inoc: level of inoculation.

^{A}: means within the same row with same superscript letters are not different ($P \geq 0.05$).

^{a}: means within the same column with same superscript letters are not different ($P \geq 0.05$).

Appendix Table 27 (for Figures V-9 and V-10). AI-2-like activity [mean (log RAI/cm^2) ± standard error] in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low (LNB) or high levels of natural flora (HNB) under aerobic and vacuum packaged storage conditions for 18 days at 10°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	6	12	18
Aerobic	LNB	2	0.0±0.1 ^{Aa}	-0.2±0.3 ^{Aa}	0.1±0.4 ^{Aab}	0.1±0.7 ^{Aa}
		6	-0.2±0.2 ^{Aa}	-0.1±0.4 ^{Aa}	-0.6±0.0 ^{Aab}	0.0±0.6 ^{Aa}
	HNB	2	0.0±0.2 ^{Aa}	0.3±0.1 ^{Aa}	-0.5±0.2 ^{Aab}	0.3±0.1 ^{Aa}
		6	-0.3±0.2 ^{Aa}	-0.3±0.5 ^{Aa}	-0.2±0.2 ^{Aa}	-0.3±0.3 ^{Aa}
Vacuum	LNB	2	-0.2±0.1 ^{Aa}	-0.2±0.0 ^{Aa}	0.2±0.3 ^{Aab}	0.6±0.1 ^{Aa}
		6	-0.3±0.2 ^{ABa}	-0.4±0.3 ^{ABa}	-0.8±0.4 ^{Bab}	0.4±0.2 ^{Aa}
	HNB	2	-0.2±0.1 ^{Aa}	-0.1±0.4 ^{Aa}	0.0±0.1 ^{Aab}	-0.3±0.1 ^{Aa}
		6	-0.3±0.1 ^{Aa}	-0.1±0.2 ^{Aa}	-0.2±0.2 ^{Aab}	-1.6±0.2 ^{Bb}

RAI: relative AI-2 activity.

Inoc: level of inoculation.

^{AB}: means within the same row with different superscript letters are different (P<0.05).

^{ab}: means within the same column with different superscript letters are different (P<0.05).

Appendix Table 28 (for Figures V-11 and V-12). AI-2-like activity [mean (log RAI/cm^2) ± standard error] in *Escherichia coli* O157:H7 strain ATCC 43895 inoculated (2 or 6 log CFU/cm^2) fresh beef containing low (LNB) or high levels of natural flora (HNB) under aerobic and vacuum packaged storage conditions for 9 days at 25°C

			Storage time (days)			
Storage	Natural flora	Inoc	0	3	6	9
Aerobic	LNB	2	0.0±0.1 ^{Aa}	1.0±0.3 ^{Aab}	1.5±0.7 ^{Aab}	1.5±0.6 ^{Aa}
		6	-0.2±0.2 ^{Ca}	1.5±0.4 ^{ABa}	2.8±0.0 ^{Aa}	1.1±0.6 ^{BCab}
	HNB	2	0.0±0.2 ^{Aa}	-0.2±0.1 ^{Abc}	0.0±0.5 ^{Abc}	0.8±0.1 ^{Aabc}
		6	-0.3±0.2 ^{Aa}	-0.1±0.5 ^{Aabc}	0.6±0.6 ^{Abc}	0.0±0.3 ^{Aabc}
Vacuum	LNB	2	-0.2±0.1 ^{Aa}	0.5±0.2 ^{Aabc}	1.0±0.5 ^{Ab}	0.1±0.2 ^{Aabc}
		6	-0.3±0.2 ^{Aa}	-0.5±0.2 ^{Abc}	0.1±0.1 ^{Abc}	-1.0±0.2 ^{Ac}
	HNB	2	-0.2±0.1 ^{Aa}	0.5±0.3 ^{Aabc}	0.0±0.4 ^{Abc}	0.1±0.4 ^{Aabc}
		6	-0.3±0.1 ^{Aa}	-0.7±0.2 ^{Ac}	-0.6±0.1 ^{Ac}	-0.5±0.0 ^{Abc}

RAI: relative AI-2 activity.

Inoc: level of inoculation.

^{ABC}: means within the same row with different superscript letters are different (P<0.05).

^{abc}: means within the same column with different superscript letters are different (P<0.05).

Appendix Table 29 (for Figure VI-1). Survivors [mean (log CFU/ml) ± standard error] of *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative), recovered with tryptic soy agar, in Luria-Bertani broth (pH 7.0) during heat challenge at 55°C for 6 h

	Heating time (55°C, h)						
Strain	0	1	2	3	4	5	6
AI-2	7.1±0.0 ^{Aa}	4.0±0.1 ^{Ba}	3.4±0.2 ^{Ba}	3.7±0.4 ^{Ba}	3.7±0.3 ^{Ba}	3.9±0.5 ^{Ba}	4.1±0.5 ^{Ba}
No AI-2	7.1±0.0 ^{Aa}	3.9±0.1 ^{BCa}	3.2±0.1 ^{Ca}	4.0±0.3 ^{Ba}	4.2±0.2 ^{Ba}	4.4±0.2 ^{Ba}	4.5±0.3 ^{Ba}

^{ABC}: means within the same row with different superscript letters are different ($P<0.05$).

^{a}: means within the same column with same superscript letters are not different ($P\geq0.05$).

Appendix Table 30 (for Figure VI-2). Survivors [mean (log CFU/ml) ± standard error] of *Salmonella* Thompson strain RM1987NLUX (AI-2-negative), recovered with tryptic soy agar, in AI-2-positive (pH 7.0) and -negative preconditioned (PC) media (pH 7.0) during heat challenge at 55°C for 6 h

	Heating time (55°C, h)						
PC medium	0	1	2	3	4	5	6
AI-2	7.1±0.0 ^{Aa}	3.9±0.1 ^{Ba}	3.0±0.1 ^{Ca}	3.0±0.2 ^{Ca}	3.0±0.1 ^{Ca}	3.2±0.1 ^{BCa}	3.5±0.1 ^{BCa}
No AI-2	7.1±0.0 ^{Aa}	3.8±0.1 ^{Ba}	2.9±0.2 ^{Ca}	3.1±0.2 ^{Ca}	3.2±0.3 ^{BCa}	3.3±0.2 ^{BCa}	3.5±0.2 ^{BCa}

PC media were derived from the cell-free supernatants of *S.* Thompson strain RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) for AI-2-positive and -negative PC media, respectively.

^{ABC}: means within the same row with different superscript letters are different ($P<0.05$).

^{a}: means within the same column with same superscript letters are not different ($P\geq0.05$).

Appendix Table 31 (for Figure VI-3). Survivors [mean (log CFU/ml) ± standard error] of *Salmonella* Thompson strain RM1987NLUX (AI-2-negative), recovered with tryptic soy agar, in AI-2-positive (pH 7.0) and -negative preconditioned (PC) media (pH 7.0) during heat challenge at 55°C for 6 h

	Heating time (55°C, h)						
PC medium	0	1	2	3	4	5	6
AI-2	7.1±0.0 ^{Aa}	4.2±0.1 ^{Ba}	3.8±0.1 ^{Ca}	4.1±0.2 ^{BCa}	4.2±0.1 ^{BCa}	4.0±0.1 ^{Ca}	4.7±0.1 ^{Ba}
No AI-2	7.1±0.0 ^{Aa}	4.1±0.1 ^{Ba}	3.4±0.2 ^{Ca}	3.9±0.2 ^{Ca}	3.9±0.2 ^{Ca}	4.1±0.3 ^{BCa}	4.7±0.2 ^{Ba}

PC media were prepared from the cell-free supernatants of *Escherichia coli* O157:H7 strain 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively.

^{ABC}: means within the same row with different superscript letters are different ($P<0.05$).

^{a}: means within the same column with same superscript letters are not different ($P\geq0.05$).

Appendix Table 32 (for Figure VI-4). Survivors [mean (log CFU/ml) ± standard error] of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP, recovered with tryptic soy agar, in AI-2-positive (pH 7.0) and -negative preconditioned (PC) media (pH 7.0) during heat challenge at 55°C for 6 h

	Heating time (55°C, h)						
PC medium	0	1	2	3	4	5	6
AI-2	7.4±0.0 ^{Aa}	4.0±0.3 ^{Ba}	3.2±0.4 ^{Ca}	3.0±0.4 ^{Ca}	3.0±0.4 ^{Ca}	3.0±0.4 ^{Ca}	3.0±0.5 ^{Ca}
No AI-2	7.4±0.0 ^{Aa}	4.0±0.1 ^{Ba}	3.2±0.2 ^{Ca}	3.0±0.3 ^{Ca}	2.9±0.4 ^{Ca}	2.7±0.4 ^{Ca}	3.2±0.3 ^{Ca}

PC media were derived from the cell-free supernatants of *Salmonella* Thompson strain RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) for AI-2-positive and -negative PC media, respectively.

^{ABC}: means within the same row with different superscript letters are different ($P<0.05$).

^{a}: means within the same column with same superscript letters are not different ($P\geq0.05$).

Appendix Table 33 (for Figure VI-5). Survivors [mean (log CFU/ml) ± standard error] of *Salmonella* Typhimurium DT104 strain ATCC 700408/ISSAGFP, recovered with tryptic soy agar, in AI-2-positive (pH 7.0) and -negative preconditioned (PC) media (pH 7.0) during heat challenge at 55°C for 6 h

	Heating time (55°C, h)						
PC medium	0	1	2	3	4	5	6
AI-2	7.4±0.0 ^{Aa}	3.5±0.3 ^{Ba}	2.6±0.2 ^{Ba}	2.5±0.3 ^{Ba}	2.1±0.4 ^{Ba}	2.2±0.4 ^{Ba}	2.2±0.4 ^{Ba}
No AI-2	7.3±0.0 ^{Aa}	3.5±0.3 ^{Ba}	2.4±0.3 ^{Ba}	2.3±0.2 ^{Ba}	2.1±0.3 ^{Ba}	2.2±0.4 ^{Ba}	2.6±0.4 ^{Ba}

PC media were prepared from the cell-free supernatants of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively.

^{AB}: means within the same row with different superscript letters are different ($P<0.05$).

^{a}: means within the same column with same superscript letters are not different ($P\geq0.05$).

Appendix Table 34 (for Figures VI-6 to VI-10). Death kinetics of *Salmonella*, obtained from the Baranyi model (Baranyi and Roberts, 1994), under various conditions during heat challenge at 55°C for 6 h

Figure	Death rate (log CFU/ml/h)	Standard error	y_0[a] (log CFU/ml)	y_{end}[b] (log CFU/ml)	R^2
VI-6 (A)	-3.31	0.74	7.07	3.76	0.684
VI-6 (B)	-3.44	0.47	7.14	3.58	0.940
VI-7 (A)	-3.17	0.21	7.06	3.11	0.945
VI-7 (B)	-3.35	0.29	7.08	3.19	0.906
VI-8 (A)	-3.45	1.04	7.13	4.15	0.869
VI-8 (B)	-3.35	0.78	7.11	3.99	0.785
VI-9 (A)	-3.48	0.54	7.39	3.05	0.759
VI-9 (B)	-3.48	0.42	7.41	2.99	0.841
VI-10 (A)	-3.88	0.45	7.36	2.31	0.852
VI-10 (B)	-3.90	0.43	7.33	2.33	0.861

[a] The upper asymptote of the bacterial death curve.

[b] The low asymptote of the bacterial death curve.

Appendix Table 35 (for Figure VI-11). Survivors [mean (log CFU/ml) ± standard error] of *Salmonella* Thompson strain RM1987NLUX (AI-2-negative), recovered with tryptic soy agar, in AI-2-positive (pH 3.5) and -negative preconditioned (PC) media (pH 3.5) during acid challenge at 35°C for 6 h

	Exposure time to acid (pH 3.5, h)						
PC medium	0	1	2	3	4	5	6
AI-2	7.1±0.0 ^{Aa}	6.8±0.0 ^{Ba}	6.4±0.1 ^{Ba}	5.9±0.1 ^{Ca}	5.0±0.2 ^{Da}	3.4±0.2 ^{Ea}	0.8±0.3 ^{Fa}
No AI-2	7.1±0.0 ^{Aa}	6.7±0.1 ^{Aa}	6.3±0.1 ^{Ba}	5.7±0.2 ^{Ca}	5.1±0.2 ^{Da}	3.5±0.3 ^{Ea}	1.3±0.2 ^{Fa}

PC media were derived from the cell-free supernatants of *S.* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative) for AI-2-positive and -negative PC media, respectively.

^{ABCDEF}: means within the same row with different superscript letters are different ($P<0.05$).

^{a}: means within the same column with same superscript letters are not different ($P\geq0.05$).

Appendix Table 36 (for Figure VI-12). Survivors [mean (log CFU/ml) ± standard error] of acid adapted and nonacid adapted *Salmonella* Thompson strains RM1987N (AI-2-positive) and RM1987NLUX (AI-2-negative), recovered with tryptic soy agar, in Luria-Bertani broth (pH 3.0) during acid challenge at 35°C for 6 h

	AI-2		No AI-2	
Exposure time to acid (pH 3.0, h)	Acid adapted	Nonacid adapted	Acid adapted	Nonacid adapted
0	7.2±0.0 ^{Aa}	7.2±0.0 ^{Aa}	7.3±0.1 ^{Aa}	7.2±0.1 ^{Aa}
1	6.9±0.1 ^{Aab}	6.5±0.1 ^{Ab}	6.9±0.1 ^{Ab}	6.6±0.0 ^{Ab}
2	6.8±0.1 ^{ABb}	6.0±0.1 ^{Cc}	6.8±0.1 ^{Abc}	6.0±0.2 ^{BCc}
3	6.4±0.1 ^{ABc}	5.5±0.1 ^{Cd}	6.4±0.2 ^{Ad}	5.6±0.1 ^{BCd}
4	5.8±0.2 ^{ABd}	5.1±0.0 ^{Ce}	6.0±0.2 ^{Ae}	5.2±0.1 ^{BCe}
5	5.2±0.2 ^{ABe}	4.6±0.1 ^{Cf}	5.4±0.2 ^{Af}	4.8±0.1 ^{BCf}
6	4.8±0.2 ^{ABf}	3.9±0.0 ^{Cg}	5.1±0.2 ^{Af}	4.2±0.1 ^{BCg}

^{ABC}: means within the same row with different superscript letters are different ($P<0.05$).

^{abcdefg}: means within the same column with different superscript letters are different ($P<0.05$).

Appendix Table 37 (for Figures VI-13 to VI-15). Death kinetics of *Salmonella* which were obtained from linear regression under various conditions during acid challenge at 35°C for 6 h

Figure	Death rate (log CFU/ml/h)	Standard error	R^2
VI-13 (A)	-0.97	0.06	0.820
VI-13 (B)	-0.89	0.09	0.828
VI-14 (A)	-0.41	0.03	0.850
VI-14 (B)	-0.38	0.03	0.803
VI-15 (A)	-0.52	0.01	0.980
VI-15 (B)	-0.48	0.02	0.932

Appendix Table 38 (for Figure VII-1). Survivors [mean (log CFU/ml) ± standard error] of *Escherichia coli* O157:H7 ATCC 43895/ISEHGFP, recovered with tryptic soy agar, in AI-2-positive (pH 7.0) and -negative preconditioned (PC) media (pH 7.0) during heat challenge at 55°C for 4 h

	Heating time (55°C, h)				
PC medium	0	1	2	3	4
AI-2	7.3±0.0 ^{Aa}	5.7±0.3 ^{ABa}	4.1±0.3 ^{Ba}	3.3±0.3 ^{Ba}	3.4±0.3 ^{Ba}
No AI-2	7.3±0.0 ^{Aa}	5.6±0.3 ^{ABa}	4.1±0.3 ^{BCa}	3.0±0.2 ^{Ca}	2.3±0.2 ^{Cb}

PC media were derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively.

^{ABC}: means within the same row with different superscript letters are different ($P<0.05$).

^{ab}: means within the same column with different superscript letters are different ($P<0.05$).

Appendix Table 39 (for Figure VII-2). Survivors [mean (log CFU/ml) ± standard error] of *Escherichia coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative), recovered with tryptic soy agar, during heat challenge at 52°C for 6 h in Luria-Bertani broth (pH 7.0)

	Heating time (52°C, h)						
Strain	0	1	2	3	4	5	6
AI-2	7.1±0.0 ^{Aa}	5.2±0.3 ^{Ba}	4.2±0.4 ^{BCa}	4.1±0.2 ^{BCa}	3.6±0.2 ^{BCa}	3.3±0.2 ^{Ca}	3.5±0.3 ^{Ca}
No AI-2	7.1±0.3 ^{Aa}	5.2±0.3 ^{Ba}	4.0±0.5 ^{BCa}	3.2±0.3 ^{Cb}	2.8±0.3 ^{Ca}	2.9±0.2 ^{Ca}	2.5±0.3 ^{Cb}

^{ABC}: means within the same row with different superscript letters are different ($P<0.05$).

^{ab}: means within the same column with different superscript letters are different ($P<0.05$).

Appendix Table 40 (for Figures VII-3 and VII-4). Death kinetics of *Escherichia coli* O157:H7, obtained from the Baranyi model (Baranyi and Roberts, 1994), under various conditions during heat challenge at 55°C and 52°C for 4 h (Figure VII-3) and 6 h (Figure VII-4), respectively

Figure	Death rate (log CFU/ml/h)	Standard error	y_0[a] (log CFU/ml)	y_{end}[b] (log CFU/ml)	R^2
VII-3 (A)	-1.51	0.13	7.20	2.32	0.868
VII-3 (B)	-1.65	0.21	7.32	3.33	0.793
VII-4 (A)	-1.55	0.24	7.01	3.60	0.691
VII-4 (B)	-1.51	0.20	6.96	2.77	0.754

[a] The upper asymptote of the bacterial death curve.

[b] The low asymptote of the bacterial death curve.

Appendix Table 41 (for Figure VII-5). Survivors [mean (log CFU/ml) ± standard error] of *Escherichia coli* O157:H7 strain ATCC 43895/ISEHGFP, recovered with tryptic soy agar, during acid challenge at 35°C for 6 h in AI-2-positive (pH 3.0) and -negative preconditioned (PC) media (pH 3.0)

	Exposure time to acid (pH 3.0, h)						
PC medium	0	1	2	3	4	5	6
AI-2	7.2±0.0 ^{Aa}	6.8±0.1 ^{ABa}	6.4±0.1 ^{BCa}	5.9±0.2 ^{CDa}	5.4±0.2 ^{DEa}	4.9±0.2 ^{EFa}	4.4±0.3 ^{Fa}
No AI-2	7.3±0.1 ^{Aa}	6.6±0.1 ^{ABa}	5.9±0.1 ^{BCa}	5.2±0.2 ^{CDb}	4.6±0.2 ^{DEb}	4.0±0.2 ^{EFb}	3.6±0.2 ^{Fb}

PC media were derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively.

^{ABCDEF}: means within the same row with different superscript letters are different ($P<0.05$).

^{ab}: means within the same column with different superscript letters are different ($P<0.05$).

Appendix Table 42 (for Figure VII-6). Survivors [mean (log CFU/ml) ± standard error] of *Escherichia coli* O157:H7 strain VS94 (AI-2-negative), recovered with tryptic soy agar, during acid challenge at 35°C for 6 h in AI-2-positive (pH 3.0) and -negative preconditioned (PC) media (pH 3.0)

	Exposure time to acid (pH 3.0, h)						
PC medium	0	1	2	3	4	5	6
AI-2	7.1±0.0 ^{Aa}	7.0±0.0 ^{Aa}	6.8±0.1 ^{ABa}	6.5±0.1 ^{ABa}	6.1±0.2 ^{ABa}	5.8±0.2 ^{ABa}	5.4±0.3 ^{Ba}
No AI-2	7.2±0.0 ^{Aa}	6.9±0.0 ^{ABa}	6.6±0.1 ^{ABa}	6.0±0.2 ^{ABCa}	5.5±0.3 ^{BCDb}	5.0±0.3 ^{CDb}	4.6±0.4 ^{Cb}

PC media were derived from the cell-free supernatants of *E. coli* O157:H7 strains 86-24 (AI-2-positive) and VS94 (AI-2-negative) for AI-2-positive and -negative PC media, respectively.

^{ABCD}: means within the same row with different superscript letters are different ($P<0.05$).

^{ab}: means within the same column with different superscript letters are different ($P<0.05$).

Appendix Table 43 (for Figures VII-7 and VII-8). Death kinetics of *Escherichia coli* O157:H7 strains, obtained from linear regression analysis, under various conditions during acid challenge at 35°C for 6 h

Figure	Death rate (log CFU/ml/h)	Standard error	R^2
VII-7 (A)	-0.48	0.03	0.787
VII-7 (B)	-0.62	0.03	0.884
VII-8 (A)	-0.29	0.03	0.604
VII-8 (B)	-0.45	0.04	0.650