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## The Role of Autoinducer-2 in Escherichia coli Biofilm Formation and the Discovery of a Plant-derived Quorum Sensing Inhibitor

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THE ROLE OF AUTOINDUCER-2 IN *ESCHERICHIA COLI* BIOFILM FORMATION  
AND THE DISCOVERY OF A PLANT-DERIVED QUORUM SENSING INHIBITOR

by

CHEN NIU

Under the direction of Eric Gilbert

ABSTRACT

The objectives of this work are: 1) to determine whether plant essential oil components influence the ability of *Escherichia coli* and several *Pseudomonas* species to form biofilms, and inhibit bacterial quorum sensing; 2) to understand the role of autoinducer-2 (AI-2) in biofilm formation by *E. coli* W3110. The biofilm formation assays determined that cinnamon, cassia and citronella oils differentially affected growth-normalized biofilm formation by *E. coli*. Cinnamaldehyde (CA) also inhibited the swimming motility of *E. coli*. Subinhibitory concentrations of CA were effective at inhibiting two types of acyl homoserine lactone (HSL) mediated quorum sensing (QS), and also AI-2 mediated QS. Because CA is widely used in the food and flavor industries, its potential to affect bacterial QS regulated processes should be recognized. The role of AI-2 mediated QS expression in physiology of *E. coli* W3110 was pleiotropic, including carbon utilization, fimbriae production, and the biofilm development. Overall, the research presented in this dissertation supported the concept that QS, biofilm formation, and cell adhesion may be broadly correlated. The anti-biofilm and anti-QS capability of

CA implies that plant essential oil components might be promising for preventing the formation of detrimental biofilms.

INDEX WORDS: Biofilm, *Escherichia coli*, Cinnamaldehyde, Quorum sensing, Autoinducer-2, Fimbriation, Microbial ecology

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CHEN NIU

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2006

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May 2006

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## List of Abbreviations

Autoinducer .....	AI
Autoinducer-bioassay .....	AB
Cinnamaldehyde .....	CA
Colony forming units .....	CFU
Confocal laser scanning microscopy .....	CLSM
Crystal violet .....	CV
Enterohaemorrhagic <i>E. coli</i> .....	EHEC
Exopolysaccharide .....	EPS
Flame ionization detection gas chromatography .....	GC-FID
Gastrointestinal .....	GI
Green fluorescent protein .....	GFP
acyl- homoserine lactone .....	HSL
Luria-Bertani .....	LB
Optical density .....	OD
Quorum sensing .....	QS
Specific biofilm formation .....	SBF
Specific cell adhesion .....	SCA
Specific fluorescence .....	SFL
Transmission electron microscopy .....	TEM
Uropathogenic <i>Escherichia coli</i> .....	UPEC

## CHAPTER 1 INTRODUCTION

### 1.1 MICROBIAL BIOFILMS

#### **1.1.1 What is a microbial biofilm?**

A biofilm is a sessile microbial community consisting of cells that are irreversibly attached to a substratum and embedded in an extracellular polymeric matrix (Donlan & Costerton, 2002). There is a generalized model for biofilm formation. First, microorganisms colonize and attach to an abiotic or biotic surface; second, microorganisms transform from planktonic status to immobilized status; finally, the microcolony is formed and continuous proliferation of microorganisms will build a three-dimensional architecture of biofilm, which is considered as mature biofilm (Branda *et al.*, 2005; Watnick & Kolter, 2000).

The most apparent difference between biofilm and planktonic styles of microorganisms is the mobility of the microorganisms. The architecture of a biofilm has distinct characteristics (Stoodley *et al.*, 2002), such as water channels which allow the diffusion of necessary nutrients, including oxygen and carbon source, for cells to grow. Some researchers have reported differential gene expression between biofilm and planktonic cells (Ren *et al.*, 2004a; Resch *et al.*, 2005; Schembri *et al.*, 2003b).

### **1.1.2 The advantages of biofilms for microorganisms**

In the environment, many bacteria exist in biofilms rather than as free-living planktonic cells (Costerton *et al.*, 1995; Watnick & Kolter, 2000). Biofilms are found in diverse locations, including human dental plaque (Marsh, 2004), gastrointestinal tract (Probert & Gibson, 2002); leaves and root surfaces of plants (Morris & Monier, 2003; Ramey *et al.*, 2004); biocorrosion and biofouling of industrial materials (Judd, 2004; Videla & Herrera, 2005). There are several advantages for living in biofilms, as follows: (1) cells may survive with limited nutrient resources, due to decreased bacterial growth rates in biofilms (Stewart, 1994); (2) cells may have better survival ability under harsh conditions, especially exposure to antibiotics and other antimicrobial agents, due to the barrier effects of biofilms (Mah & O'Toole, 2001); (3) for pathogenic microorganisms, cells have a higher gene transfer rate than planktonic cells (Hausner & Wuertz, 1999).

### **1.1.3 Economic significance of microbial biofilms**

Microbial biofilms may have both beneficial and detrimental characteristics from a human perspective. On one hand, microbial biofilms can be useful in bioremediation, including sewage wastewater treatment (Yu *et al.*, 2003), biodegradation of insecticides (Gilbert *et al.*, 2003), and biofiltration of air pollutants (Miller & Allen, 2005). On the other hand, microbially influenced corrosion is a serious concern in industry (Beech *et al.*, 2005; Videla & Herrera, 2005). For example, water distribution systems are usually accompanied with biofilm formation (Coetser & Cloete, 2005; Morton & Surman, 1994).

Disagreeable air quality in some automobiles was connected to mixed biofilms in automobile air conditioning systems (Simmons *et al.*, 1999).

One of the biggest concerns over biofilms is their ability to harm human health. Persistent infections are usually caused or related to bacterial biofilms (Costerton, 2001). For example, cystic fibrosis is difficult to be treated by using antibiotics due to the accompanying *Pseudomonas aeruginosa* biofilms. Many other diseases, such as native valve endocarditis, otitis media, chronic bacterial prostatitis, periodontitis, and dental plaques are linked to bacterial biofilms (Donlan & Costerton, 2002; Parsek & Singh, 2003). The establishment of biofilms on indwelling implants (e.g. catheters) can lead to treatment failure because biofilms are difficult to treat by common antimicrobial therapy (Vinh & Embil, 2005). These industrial and medical impacts have made biofilms a significant research topic in biology.

#### **1.1.4 Strategies to prevent biofilm formation**

Many approaches have been proposed to inhibit bacterial biofilm formation, including several major directions. The most widely studied way is material surface coating. An example is a photocatalytic titanium dioxide (TiO<sub>2</sub>) surface, which has potential as a self-cleaning technology to reduce the adhesion of *Deinococcus geothermalis* cells in warm water-using industries (Raulio *et al.*, 2005). Antibiotic coating systems are sometimes effective in preventing biofilm formation, such as the application of ciprofloxacin-releasing bioabsorbable polymer in fighting *Staphylococcus epidermidis* bio films (Niemela *et al.*, 2006). Application of antimicrobial compounds has

also been examined. For example, hydrogen peroxide-based disinfectants were proven effective for inhibiting biofilm formation in dental unit waterlines (Tuttlebee *et al.*, 2002). Physical methods showed that ultrasound improved the transport of gentamicin through colony biofilms of *P. aeruginosa* and *Escherichia coli* (Carmen *et al.*, 2004). Recently a research report showed that an efficient way to prevent *P. aeruginosa* biofilm formation is by releasing ciprofloxacin from self-assembled coatings by ultrasonic control (Norris *et al.*, 2005). Other novel approaches are also being examined, such as utilizing bacteriophage to degrade exopolysaccharide (EPS) (Hughes *et al.*, 1998), and the application of cell-to-cell signaling inhibitors (discussed in section 1.4 below).

## 1.2 QUORUM SENSING (QS): MICROBIAL CELL-TO-CELL SIGNALING

### 1.2.1 QS systems involve different AI molecules

QS, or cell-density dependent regulation of gene expression, controls a wide variety of prokaryotic phenotypes including enzyme secretion, virulence factor production, bioluminescence and biofilm development in some cases (Bassler, 1999; Whitehead *et al.*, 2001). QS occurs widely in both Gram-positive and Gram-negative bacteria, and in both groups, autoinducer (AI) molecules are the extracellular signals produced during the growth of bacteria and detected by themselves or other microorganisms to sense cell population density. Two main types of AIs have been classified: AI-1 and AI-2 (**Fig 1.1**). AI-1 molecules are acyl homoserine lactones (HSLs) and have been proposed to function as intra-species signals (Miller & Bassler, 2001; Withers *et al.*, 2001). AI-1 synthesis is dependent on LuxI-like synthases, and a LuxR-



like transcriptional regulator protein combines the cognate HSL. Then, the LuxR-HSL complex stimulates the transcription of the target gene (**Fig 1.2a**) and related physiological functions are expressed (Withers *et al.*, 2001). AI-2 is a DPD-derived molecule synthesized by LuxS-like synthases and has a role in inter-species communication (Camilli & Bassler, 2006; Xavier & Bassler, 2003). LuxP and LuxQ recognize AI-2, and then a series of phosphorylation relays transmits the signal and activates the transcription of target gene (**Fig 1.2c**) and consequent physiological behaviors of many Gram-negative and Gram-positive bacteria (Xavier & Bassler, 2003).

### **1.2.2 *Pseudomonas* quinolone signal (PQS)**

For *P. aeruginosa*, a third intercellular signal, PQS (2-heptyl-3-hydroxy-4-quinolone) regulates the expression of *lasB*, encoding the virulence factor, LasB elastase (Pesci *et al.*, 1999). PQS is considered as an integral component of the QS circuitry in *P. aeruginosa* (Diggle *et al.*, 2003). PQS synthesis is regulated by the expression of several operons, including *pqsABCDE* (Wade *et al.*, 2005).

### **1.2.3 QS in Gram positive bacteria**

Gram positive bacteria utilize peptide signaling molecules instead of HSLs as QS signals and the signals are transported through a two-component phosphorelay mechanism (**Fig 1.2b**) (Kleerebezem *et al.*, 1997). This QS system regulates several significant bacterial functions, including virulence in *S. aureus*, genetic competence in

*Bacillus subtilis*, and the production of bacteriocins in lactic acid bacteria (Bassler, 1999; Sturme *et al.*, 2002).

#### **1.2.4 AI-2 mediated QS and correlated regulating functions**

AI-2 mediated QS is widely distributed among both Gram-positive and Gram-negative bacteria (Xavier & Bassler, 2003). Bioluminescence of *Vibrio harveyi* is the first reported bacterial function controlled by AI-2 mediated QS (Bassler *et al.*, 1997). AI-2 may be used by several strains to mediate the virulence factors expression, such as cholera toxin in *Vibrio cholerae* (Zhu *et al.*, 2002); protease hemolysin in *Streptococcus pyogenes* (Lyon *et al.*, 2001); type III secretion in enterohaemorrhagic *E. coli* (EHEC) *E. coli* (Sperandio *et al.*, 1999); alpha, kappa, and theta toxins of *Clostridium perfringens* (Ohtani *et al.*, 2002) and transcriptional virulence gene expression of *Clostridium difficile* (Lee & Song, 2005); carbapenem antibiotic, prodigiosin and secreted haemolysin production in *Serratia marcescens* (Coulthurst *et al.*, 2004). Biofilm formation of several kinds of bacteria is also regulated through AI-2 mediated QS. For some bacteria, a *luxS* mutation causes either decreased amount or altered structure of biofilm development compared to correlated wild type strains, including *Streptococcus mutans* (Merritt *et al.*, 2003; Yoshida *et al.*, 2005), *Salmonella typhi* (Prouty *et al.*, 2002), and *Klebsiella pneumoniae* (Balestrino *et al.*, 2005). However, the lack of AI-2 may lead to enhanced virulence or/and increased biofilm formation in some cases. There are several recent reports on *luxS* mutants of various bacteria, including *Lactobacillus reuteri* (Tannock *et al.*, 2005), *Haemophilus influenzae* (Daines *et al.*, 2005), and *Staphylococcus epidermidis*

(Xu *et al.*, 2006). As a universal signal, AI-2 might be used by more untested microorganisms for regulation of different functions.

### **1.2.5 The significance of *E. coli* biofilms and factors affecting *E. coli* biofilm development**

Biofilm formation by *E. coli* on catheters is most commonly associated with urinary tract infections, which happens to almost all patients undergoing long-term catheterization (Donlan & Costerton, 2002). Several potential mechanisms have been proposed to mediate *E. coli* biofilm formation. First, initial biofilm formation might be regulated by motility and type 4 pili (Pratt & Kolter, 1998). Second, EPS production was considered necessary for the development of *E. coli* K-12 biofilms (Danese *et al.*, 2000). Also, the global regulator CsrA was found to be capable of regulating carbon flow during biofilm formation and controlled dispersal of an *E. coli* K-12 strain (Jackson *et al.*, 2002). RpoS was also found to have important effects on the initiation of *E. coli* biofilm formation and the amount of biomass that accumulated (Corona-Izquierdo & Membrillo-Hernandez, 2002).

### **1.2.6 Known factors about AI-2 mediated QS in *E. coli***

*E. coli* synthesizes the QS signal molecule AI-2 through the *luxS* gene. It has been confirmed that *E. coli* has a system for take-up of AI-2 signaling molecules (Xavier & Bassler, 2005). It has also been reported that extracellular AI-2 reaches its peak activity in mid-late exponential phase and decreases quickly when cells enter stationary phase

(Surette & Bassler, 1998). Based on the experimental results from DNA microarray studies, AI-2 mediated QS might regulate many functions of *E. coli* including cell division, DNA processing (DeLisa *et al.*, 2001); virulence factors production (Sperandio *et al.*, 1999), flagella synthesis and motility in EHEC (Sperandio *et al.*, 2002).

### 1.3 THE EFFECT OF PLANT-DERIVED CHEMICALS ON BIOFILM FORMATION AND QS

#### 1.3.1 The first reported QS modulating compound: furanone

The first discovered natural QS modulating chemical is furanone, which is extracted from a marine alga, *Delisea pulchra*. Furanones probably help to reduce the biofouling of seaweeds, such as *D. pulchra* (Steinberg *et al.*, 1997). Furanone is capable of competitively inhibiting the binding of HSL compounds to their intracellular receptors (Manefield *et al.*, 1999). Several more artificially compounds have been synthesized to interrupt QS expression by mimicking HSL molecules, such as an agonist to AI-1, 3-oxo-C12-(2-aminocyclohexanol) (Smith *et al.*, 2003). It is interesting that some higher plants can also synthesize AI mimics to interrupt colonization by bacteria (Bauer & Robinson, 2002). This function may contribute to self-defense mechanisms of plants, to regulate bacterial cell density.

#### 1.3.2 Practical application of QS modulating compounds

Since biofilm formation has been demonstrated harmful in many industrial and healthcare related issues and traditional antibiotics are not very effective for treating

bacterial infections caused by biofilms, chemicals capable of inhibiting biofilm formation could be significant. A novel approach to treat biofilm-related bacterial infections is to aim at QS targets (Hentzer *et al.*, 2003a). Some progress has been made in seeking useful agents inhibiting AI-1 mediated QS, such as several modified furanone-like compounds (Hentzer *et al.*, 2003b; Smith *et al.*, 2003). Derivatives of furanone compounds were used as QS inhibitors and have potential applications, including: (1) reducing exoenzyme virulence factor production in *Erwinia caratovora* (Manefield *et al.*, 2001), (2) reduce the thickness of *E. coli* biofilms to 55 percent less than the non-furanone control (Ren *et al.*, 2001), (3) beneficial in conjunction with antibiotics for treating *P. aeruginosa* biofilm infections in a mouse pulmonary infection model (Hentzer *et al.*, 2002; Wu *et al.*, 2004).

### **1.3.3 Anti-microbial, and potential anti-QS/biofilm effects of nature products from plants**

Plant-derived compounds inhibit peptidoglycan synthesis (Ogunlana *et al.*, 1987), damage microbial membrane structures (Cox *et al.*, 2000), modify bacterial membrane surface hydrophobicity (Turi *et al.*, 1997), and modulate QS (Gao *et al.*, 2003; Lu *et al.*, 2005), all of which could influence biofilm formation. However, many plant essential oils, which are mixtures of numerous organic chemicals, contain compounds that inhibit microbial growth (Baratta *et al.*, 1998; Cowan, 1999; Shelef, 1983). Thus, a screening procedure to identify phytochemicals with specific anti-biofilm activity must take into account the cytotoxicity of plant essential oils.

## 1.4 METHODS FOR RESEARCHING THE EFFECT OF PLANT-DERIVED CHEMICALS ON BIOFILMS AND QS

### **1.4.1 Specific biofilm formation (SBF) assay and its usage in this research**

Crystal violet (CV) staining, a colorimetric method, has been used widely to measure biofilm formation in part because of its amenability to large screening procedures (O'Toole & Kolter, 1998; Pitts *et al.*, 2003). For many applications, particularly screening assays for surface-adhesion deficient mutants (O'Toole & Kolter, 1998; Pratt & Kolter, 1998), measuring the absolute amount of biofilm formed by CV staining is suitable. To search for anti-biofilm compounds within growth-inhibitory plant essential oils, the CV assay must be modified to measure the amount of biofilm formed relative to overall growth. Also, it is generally assumed that CV binds proportionally to biomass, although there are multiple physical, chemical and biological factors that could influence the binding of CV to biofilms. These factors include (a) structural factors that affect dye diffusion; (b) morphological and physiological differences in individual cells that influence dye binding; and (c) chemical interactions between plant essential oil components and CV. A CV assay that measures growth-normalized biofilm accumulation, referred to as the specific biofilm formation (SBF), was used for locating plant essential oil components that affect biofilm formation. The SBF method is a modification of a technique employed by Pratt and Kolter (Pratt & Kolter, 1998), and like all CV staining methods, is an indirect determination of biofilm formation.

To evaluate the relevance of the SBF assay, two microscopic techniques were employed. First, biofilms cultivated in flow cells were imaged by confocal laser scanning

microscopy (CLSM) and quantitatively analyzed using the software package COMSTAT (Heydorn *et al.*, 2000). Second, adhesion was investigated by direct microscopy using the specific cell adhesion (SCA) assay, also introduced in this work. Using the SBF assay, three essential oils with different effects on biofilm formation by *E. coli* were compared, and the influences of their principal chemical components on biofilm structure, adhesion, cell morphology, viability and swimming motility were measured. Lastly, the SBF assay was used to investigate biofilm formation by three species of *Pseudomonas* following exposure to plant essential oil components.

#### **1.4.2 The application of green fluorescent protein (GFP) to investigate modulating effects of CA on QS**

GFP is a protein originally observed in the jellyfish *Aequorea* (Heim *et al.*, 1994). The gene for GFP was isolated and has been widely used in biological research, because its intrinsic fluorescence can be visualized in living cells. A bioreporter may be constructed by linking the gene of interest to the GFP structural gene. Bioreporters can be useful tools in QS research field because the sensitivity and easy measurable property of GFP (Leveau & Lindow, 2001). The fluorescence could be easily measured through a fluorometer with FITC filter sets.

## 1.5. HYPOTHESES

### **1.5.1 Statements of hypotheses regarding essential oil principal components**

#### **1.5.1.1 Rationale**

Plant essential oil components might be expected to inhibit biofilm formation and/or QS because: (1) many compounds secreted from different portions of various plants have modulating effects on bacterial QS (Bauer & Robinson, 2002; Teplitski *et al.*, 2000); (2) terrestrial plants support populations of surface-attached bacteria (Beattie & Lindow, 1999; Morris & Monier, 2003) and could potentially produce phytochemicals that attenuate biofilm development through specific mechanisms.

#### **1.5.1.2 Statements of hypotheses**

The objective of this work is to determine whether plant essential oil components influence the ability of *E. coli* and several *Pseudomonas* species to form biofilms, and inhibit bacterial quorum sensing. This effort may be useful for evaluating the effects of promising natural products on QS and biofilm formation of microorganisms.

### **1.5.2 Statements of hypotheses regarding AI-2 mediated QS in *E. coli* biofilm development**

#### **1.5.2.1 Rationale**

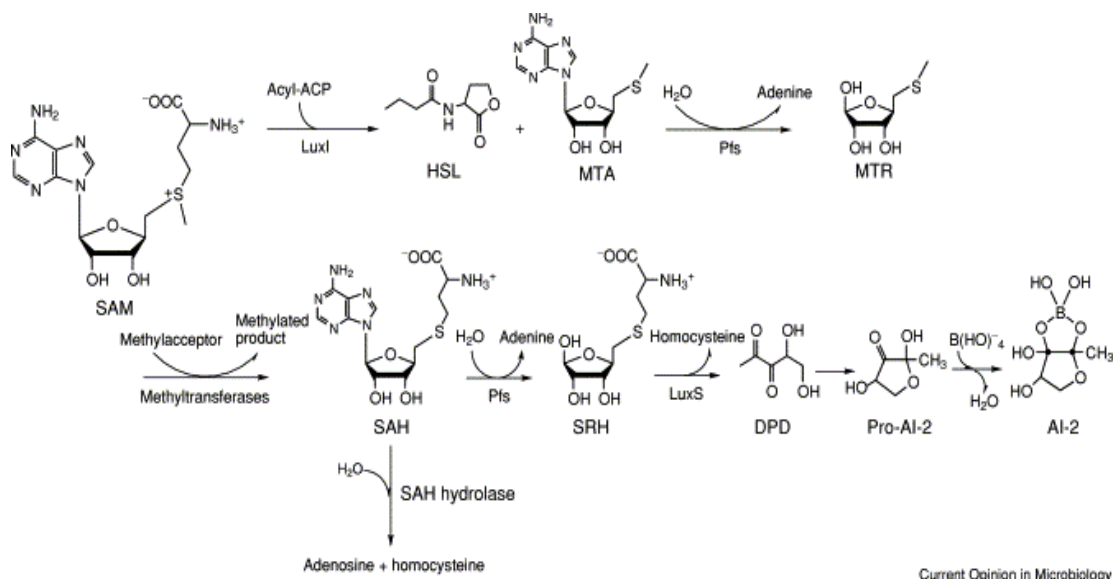
Some debate exists over whether AI-2 plays a role in *E. coli* biofilm formation. Reisner reported that there was no connection of AI-2 to biofilm differentiation by *E. coli* containing conjugative IncF plasmids (Reisner *et al.*, 2003). However, in a recent report,



AI-2 has been shown to control *E. coli* biofilm formation by regulating motility (Gonzalez Barrios *et al.*, 2006). In 2001, DeLisa *et al.* investigated the differential gene expression of wild type *E. coli* W3110 and a *luxS* deletion mutant (strain MDAI2) and found that 5.6% of the genome exhibited significant transcriptional changes upon the induction of cell-free supernatants containing AI-2 (DeLisa *et al.*, 2001). Several of the upregulated genes that were identified could potentially influence cell physiology, including putative adhesion or fimbrial-like protein (*yadK*, *yadN*), carbon storage regulator (*csrA*), and lipopolysaccharide core biosynthesis (*rfaJ*, *rfaY*). These factors have been shown in other research to modulate *E. coli* adhesion or biofilm formation (Blumer *et al.*, 2005; Genevoux *et al.*, 1999; Jackson *et al.*, 2002). In a continuation of this research, it was reported that deletion of *luxS* had no effect on growth, motility, or biofilm formation for *E. coli* W3110 (Wang *et al.*, 2005).

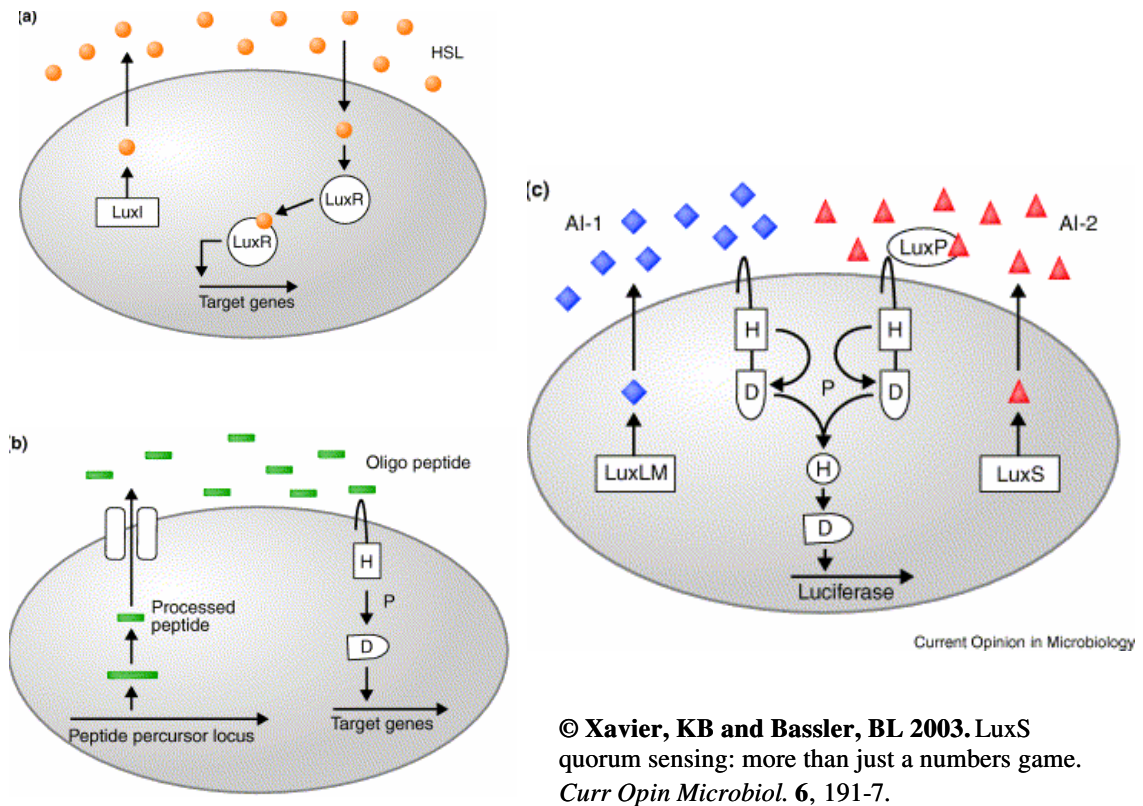
### 1.5.2 Statement of hypothesis

*E. coli* biofilm structure could be affected by AI-2 mediated QS because AI-2 production by *E. coli* W3110 in batch culture was growth phase dependent, and AI-2 has been illustrated as an important mediating factor in the biofilm development of *S. mutans* (Merritt *et al.*, 2003) and *K. pneumoniae* (Balestrino *et al.*, 2005). Thus, to understand the putative role of AI-2 in biofilm formation by strain W3110, phenotypes expressed by the wild type strain, its *luxS* deletion mutant MDAI2 which cannot synthesize AI-2, and a complemented variant of MDAI2 that can overexpress AI-2 will be compared.



© Xavier, KB and Bassler, BL 2003. LuxS quorum sensing: more than just a numbers game. *Curr Opin Microbiol.* 6, 191-7.

**Fig. 1.1** Biosynthesis of AI-1 and AI-2. SAM, S-adenosylmethionine; HSL, homoserine lactone; MTA, methylthioadenosine; MTR, methylthioribose; SAH, S-adenosylhomocysteine; SRH, S-ribosylhomocysteine; DPD, 4,5-dihydroxy 2,3-pentanedione.



**Fig. 1.2** Several typical QS systems among bacteria. **(a)** typical QS system in many Gram-negative bacteria; LuxI, HSL synthase; LuxR, transcriptional regulator protein; **(b)** representative QS circuit in Gram-positive bacteria; H (histidine) and D (aspartate), the conserved phosphorylation sites on the two-component proteins; P, phosphorylation; **(c)** *V. harveyi* QS circuit, LuxLM, AI-1 synthase; LuxS, enzyme for production of AI-2.

## CHAPTER 2

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### A COLORIMETRIC METHOD FOR IDENTIFYING PLANT ESSENTIAL OIL COMPONENTS THAT AFFECT BIOFILM FORMATION AND STRUCTURE

C. Niu and E. S. Gilbert

#### 2.1 ABSTRACT

The specific biofilm formation (SBF) assay, a technique based on crystal violet staining, was developed to locate plant essential oils and their components that affect biofilm formation. SBF analysis determined that cinnamon, cassia and citronella oils differentially affected growth-normalized biofilm formation by *Escherichia coli*. Examination of the corresponding essential oil principal components by the SBF assay found that cinnamaldehyde decreased biofilm formation in comparison to biofilms grown in Luria-Bertani broth, eugenol did not effect a change, and citronellol increased the SBF. To evaluate these results, two microscopy-based assays were employed. First, confocal laser scanning microscopy (CLSM) was used to image *E. coli* biofilms cultivated in flow cells, which were quantitatively analyzed by COMSTAT, an image analysis program. The overall trend for five parameters characterizing biofilm development corroborated the findings of the SBF assay. Second, an assay measuring growth-normalized adhesion by direct microscopy concurred with the results of the SBF assay and CLSM imaging. Viability staining indicated that there was reduced toxicity of the essential oil

components to cells in biofilms in comparison to planktonic cells, but revealed morphological damage to *E. coli* after cinnamaldehyde exposure. Cinnamaldehyde also inhibited the swimming motility of *E. coli*. SBF analysis of three *Pseudomonas* species exposed to cinnamaldehyde, eugenol or citronellol generated diverse responses. The SBF assay could be useful as an initial step for finding plant essential oils and their components that affect biofilm formation and structure.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Strains and culture conditions

The following strains were used in this study: *E. coli* ATCC 33456 (Wang & Shen, 1995), *Pseudomonas aeruginosa* PAO1 (Pseudomonas Genetic Stock Center, North Carolina, USA), *P. putida* KT2440 (Ramos-Diaz & Ramos, 1998), and *P. fluorescens* pSMC21 (Bloemberg *et al.*, 1997). All strains were cultured in Luria-Bertani (LB) broth. *E. coli* ATCC 33456 and *P. aeruginosa* PAO1 were grown at 37°C; *P. putida* KT2440 and *P. fluorescens* pSMC21 were grown at 30°C.

### 2.2.2 Chemicals

Cinnamaldehyde and citronellol were obtained from Aldrich (Milwaukee, WI); eugenol was purchased from Acros Organics (New Jersey, USA). All solvents were of analytical grade. All plant essential oils were obtained from Aura Cacia (Weaverville, CA). Crystal violet solution was obtained from Becton Dickinson (Sparks, MD).

### 2.2.3 Toxicity analyses

17 × 100 mm (14 mL) capped polystyrene culture tubes (Fisher Scientific, Pittsburgh, PA) containing 3 mL LB medium and plant essential oils of varying concentrations were inoculated with 30 µL of cells in log phase growth. For each concentration tested, tubes were prepared in triplicate and incubated in an orbital shaker (200 rpm). Growth was determined turbidimetrically (600 nm) at the initiation of the experiment and after 1.5 h (*E. coli*) or 2.5 h (*Pseudomonas* spp.). The essential oils that were tested were *Cinnamomum camphora* (camphor), *Cinnamomum cassia* (cassia), *Cinnamomum zeylanicum* (cinnamon), *Melaleuca alternifolia* (tea tree), *Cymbopogon nardus* (citronella) and *Zingiber officinale* (ginger). Plant essential oils were diluted in methanol (20% v/v) prior to use; cinnamaldehyde, eugenol and citronellol stock solutions were diluted in methanol (4% v/v).

### 2.2.4 Specific biofilm formation (SBF) assay

Bacteria were grown in 14 mL polystyrene culture tubes containing 2 mL LB and varying concentrations of essential oils or individual chemicals. Compounds to be tested were dissolved in methanol as described above, and the total volume added never exceeded 1.5 percent of the culture volume (methanol only added in this amount had no observable effect on growth). Nine identically prepared tubes were used for each concentration. Three of the nine tubes were used to measure growth in suspended culture (G tubes), three more tubes were used to measure biofilm growth (B tubes), and three tubes served as controls for abiotic factors (NC tubes).

Inocula were grown to late log phase. Subsequently, the B and G tubes received 20  $\mu\text{L}$  of inoculum, and all (B, G and NC) tubes were incubated in an orbital cabinet shaker for  $17 \pm 1$  h. Following incubation, cells in the G tubes were mixed well, and the optical density of the cultures was measured (600 nm;  $\text{OD}_{600}$ ). The B and NC tubes each received 125  $\mu\text{L}$  of a 4% solution of CV. After 15 minutes, the suspended culture was poured out, and the tubes were rinsed well with distilled deionized water (6 rinses of approximately 4 mL per rinse). Any remaining crystal violet was dissolved in 2 mL of an ethanol-acetone (80:20) solution, and the absorbances of the resultant solutions were measured spectrophotometrically (570 nm).

Biofilm accumulation was normalized with respect to growth, yielding the specific biofilm formation (SBF). The SBF was determined using the following formula:  $\text{SBF} = (\text{B} - \text{NC})/\text{G}$ , where B was the amount of biofilm formed, NC was the amount of CV that adhered to the polystyrene tubes due to abiotic factors, and G was the optical density of cells grown in suspended culture. At least two replicate experiments were performed for each concentration of chemical that was tested.

### **2.2.5 Flow cell determination of specific cell adhesion (SCA)**

Bench-scale parallel plate flow cells (Gilbert & Keasling, 2004) were used to quantify the effect of essential oil components on the initial adhesion of *E. coli* ATCC 33456 to a glass surface. Briefly, media reservoirs supplemented with essential oil components were incubated at 37°C in a water bath, and filter-sterilized air was pumped into the growth medium at a flow rate of 8.0 mL min<sup>-1</sup>. The aerated media were

inoculated with *E. coli* ATCC 33456 ( $OD_{600}$ , 0.03) and recirculated through the flow cell at a flow rate of  $0.84 \text{ mL min}^{-1}$ . After 2 h, the optical density of the suspended cells in the media reservoir was measured, the flow cell apparatus was reconfigured to a continuous flow arrangement, and the channels were rinsed for 20 min with 50 mM phosphate buffer. After rinsing, the flow cells were imaged with a Nikon Eclipse E600 microscope equipped with differential interference contrast optics, and four fields were collected from randomly selected positions along the length of the channel using a 40X Plan Fluor objective. To determine the specific cell adhesion (SCA), the number of cells per field was counted, multiplied to give the number of cells attached per square millimeter, and divided by the optical density of the suspended cells that grew concurrently in the medium reservoir. At least eight fields per tested concentration were obtained to estimate the SCA.

### **2.2.6 Cultivation of biofilms for viability staining and structure analysis**

Biofilms of *E. coli* ATCC 33456 were cultivated using the flow cell technique described above, with the following changes: (1) after 2 h, media bottles were replaced and the flow cell apparatus was reconfigured to a continuous flow arrangement with a flow rate of  $0.35 \text{ mL min}^{-1}$  for an 18 h period; (2) after 18 h, biofilms were rinsed for 20 min with sterile 50 mM KCl/NaCl solution (pH=7.0). After rinsing, 1 mL 1:1000 diluted Live/Dead® dye solution (Molecular Probes; Eugene, OR) was pumped into the flow cell apparatus. After 15 minutes, the flow cells were rinsed for another 5 minutes and



subsequently imaged by CLSM. Experiments were conducted in triplicate, and at least ten fields per tested condition were collected.

### **2.2.7 Quantitative analysis of biofilm structure**

Image stacks collected by CLSM were evaluated using the digital image analysis program COMSTAT (Heydorn *et al.*, 2000), designed for quantifying features of biofilm structure. Parameters analyzed by COMSTAT included maximum thickness, total biomass, substratum coverage, biomass, average diffusion distance, roughness coefficient and surface to volume ratio. COMSTAT analysis was performed on data collected in the green channel (SYTO 9 stained cells).

### **2.2.8 Statistical analysis**

Differences in biofilm structure measured by COMSTAT were tested for significance using Student's t-test. Error bars in all graphs indicate the standard error of the mean.

### **2.2.9 Swimming motility assay**

The swimming motility of *E. coli* ATCC 33456 was tested using a modification of a previously published technique (Burkart *et al.*, 1998). 100 × 15 mm petri dishes (Fisher Scientific, Pittsburgh, PA) containing 20 mL LB broth and 0.3% Bacto agar (Difco, Detroit, MI) were used. Cinnamaldehyde, eugenol, and citronellol were diluted in methanol (4% v/v) and added at concentrations that effected 60% inhibition of planktonic

cell growth in the toxicity analyses. Plates were inoculated using sterilized toothpicks and incubated for  $17 \pm 1$  h in a  $30^{\circ}\text{C}$  incubator and the diameter of each motility halo was measured. Six replicates were conducted for each treatment.

### 2.2.10 Gas chromatography

Analysis of the components of *C. cassia*, *C. zeylanicum* and *C. nardus* oils was conducted by flame ionization detection gas chromatography (GC-FID). Analyses were performed with a PerkinElmer Autosystem XL gas chromatograph (PerkinElmer, Wellesley, MA), using a Supelco SPB-20 column (length, 30 m, i.d. 0.32 mm; Supelco, Bellefonte, PA). The injector temperature was  $250^{\circ}\text{C}$ , and the detector temperature was  $300^{\circ}\text{C}$ . The carrier gas was helium ( $1.3 \text{ mL min}^{-1}$ ), and detector gases were hydrogen ( $45 \text{ mL min}^{-1}$ ) and air ( $450 \text{ mL min}^{-1}$ ). Oils of *C. cassia* and *C. zeylanicum* were analyzed with the following temperature program: initial column temperature was  $150^{\circ}\text{C}$ , oven temperature was increased by  $10^{\circ}\text{C min}^{-1}$  until  $260^{\circ}\text{C}$ ; temperature was held for 7 min. *C. nardus* oil was analyzed with the following temperature program: initial column temperature was  $210^{\circ}\text{C}$  and was held for 2 min; subsequently, oven temperature was increased by  $5^{\circ}\text{C min}^{-1}$  until  $260^{\circ}\text{C}$ .

## 2.3 RESULTS

### 2.3.1 Effect of plant essential oils on growth and biofilm formation

Six plant essential oils were screened for their ability to inhibit the growth of *E. coli* ATCC 33456 (**Fig. 2.1a**). *Z. officinale* essential oil had no effect on growth, and

essential oils derived from *C. camphora*, and *M. alternifolia* slightly inhibited the growth of *E. coli* ATCC 33456. *C. zeylanicum* and *C. cassia* essential oils substantially reduced the growth rate of *E. coli* ATCC 33456. *C. nardus* had a minimal effect on growth after 2 h, but growth inhibition was evident after 15 h (**Fig. 2.1b**). *C. zeylanicum*, *C. cassia* and *C. nardus* were selected for further study.

The effects of *C. zeylanicum*, *C. cassia*, and *C. nardus* essential oils on biofilm formation by *E. coli* ATCC 33456 were evaluated by the SBF assay. After 17 h, *C. cassia* reduced the extent of biofilm formation as a function of increasing essential oil concentration, whereas the SBF increased in response to *C. zeylanicum* and *C. nardus* essential oils. Representative changes in SBF values relative to controls were: *C. cassia*,  $-100 \pm 22$  percent; *C. zeylanicum*,  $241 \pm 38$  percent; and *C. nardus*,  $586 \pm 54$  percent, following exposure to essential oil concentrations resulting in  $73 \pm 4$  percent inhibition of planktonic growth. Corresponding OD<sub>600</sub> values in suspended culture ranged from  $0.72 \pm .07$  for cells exposed to *C. nardus* to  $0.92 \pm .20$  for cells exposed to *C. cassia*.

### **2.3.2 Effect of essential oil principal components on growth**

The principal chemical components of the three essential oils were quantified by GC-FID. The major components of *C. zeylanicum* and *C. cassia* were cinnamaldehyde and eugenol, comprising 2.1 and 53.4 percent of *C. zeylanicum* extract and 55.3 and 0.4 percent of *C. cassia* extract respectively. The major component of *C. nardus* was citronellol, comprising 48.8 percent of the total compounds present. The identities of the

principal essential oil components were confirmed by comparison with retention times of cinnamaldehyde, eugenol and citronellol standards.

The effect of cinnamaldehyde, eugenol and citronellol on the growth of *E. coli* ATCC 33456, *P. aeruginosa* PA01, *P. putida* KT2440 and *P. fluorescens* pSMC21 was evaluated (**Fig. 2.2**). Cinnamaldehyde significantly inhibited the growth of all four species that were tested. Although eugenol strongly inhibited the growth of *E. coli* ATCC 33456, the growth of the three *Pseudomonas* spp. was only reduced by  $20 \pm 5$  percent at a eugenol concentration of  $2600 \mu\text{M}$  (425 ppm), the maximum concentration tested. The growth of all four species was moderately inhibited by citronellol after 2 h. Similarly to *C. nardus* essential oil, a delay in toxicity was observed when *E. coli* ATCC 33456 was treated with citronellol. By 18 h,  $1850 \mu\text{M}$  (290 ppm) citronellol inhibited *E. coli* growth by 60 percent relative to controls, and  $2650 \mu\text{M}$  (415 ppm) inhibited growth by 81 percent.

### **2.3.3 Effect of cinnamaldehyde, eugenol and citronellol on *E. coli* ATCC 33456 biofilm formation and structure, viability, and swimming motility**

The SBF and SCA assays indicated the same general trends for cinnamaldehyde, eugenol and citronellol in response to increasing concentrations (**Fig. 2.3**). For example, the SBF after exposure to  $2010 \mu\text{M}$  (265 ppm) cinnamaldehyde was reduced by  $46 \pm 8$  percent relative to the SBF measured in the control treatment. Similarly, representative SCA values were  $100239 \pm 18809 \text{ cells mm}^{-2} \text{ OD}_{600} \text{ unit}^{-1}$  for cells grown in LB only,

and  $50600 \pm 19448$  cells  $\text{mm}^{-2}$   $\text{OD}_{600}$  unit $^{-1}$  for cells grown in 2120  $\mu\text{M}$  (280 ppm) cinnamaldehyde, a reduction of  $50 \pm 10$  percent ( $p < 0.001$ ).

To measure the effects of cinnamaldehyde, eugenol and citronellol on *E. coli* ATCC 33456 biofilm structure and viability, flow cell-grown biofilms were investigated by CLSM. Biofilms were grown for 18 h, similar to the growth period of the SBF assay. Essential oil components were added at concentrations causing 60 percent inhibition of the planktonic cell growth (cinnamaldehyde, 2170  $\mu\text{M}$  [285 ppm]; eugenol, 1690  $\mu\text{M}$  [275 ppm]; citronellol, 1850  $\mu\text{M}$  [290 ppm]). Quantitative analysis of biofilm structure using COMSTAT indicated several significant differences resulting from exposure to plant essential oil components (**Table 2.1**).

Viability staining indicated that both live and dead cells were present in all the tested biofilms (**Fig. 2.4**). No more than 30 percent of the total population was killed by the chemical treatments, although the concentrations of essential oil components used reduced planktonic growth by approximately 60 percent. Cinnamaldehyde-treated biofilms had a significantly higher ( $p < 0.001$ ) percentage of dead cells in comparison to the other treatments and many cells were abnormally long and appeared stressed. Cinnamaldehyde reduced the swimming motility of *E. coli* ATCC 33456 by  $60 \pm 8$  percent relative to controls ( $p < 0.001$ ). In contrast, eugenol ( $p < 0.29$ ) and citronellol ( $p < 0.26$ ) had no significant effect.

### **2.3.4 Effect of cinnamaldehyde, eugenol and citronellol on biofilm formation by *Pseudomonas* spp.**

To determine whether the SBF assay could identify differences in biofilm formation by other bacteria, the effects of cinnamaldehyde, eugenol and citronellol on biofilm formation by three *Pseudomonas* species were investigated (**Fig. 2.5**). For *P. aeruginosa* PA01, biofilm development was significantly inhibited by both eugenol and citronellol. In contrast, the specific biofilm formation of *P. aeruginosa* PA01 decreased initially, but subsequently increased at cinnamaldehyde concentrations greater than 2000  $\mu\text{M}$  (265 ppm). Biofilm development of *P. putida* KT2440 was reduced by increasing concentrations of all three chemicals. The SBF of *P. fluorescens* pSMC21 was not substantially inhibited at any concentration of cinnamaldehyde or citronellol. On the other hand, biofilm development was significantly inhibited by eugenol (no biofilm formed at any concentration higher than 700  $\mu\text{M}$  [115 ppm]).

## **2.4. DISCUSSION**

CV staining has been widely adopted by microbiologists to investigate mutants in adhesion or biofilm formation (Favre-Bonte *et al.*, 2003; Genevaux *et al.*, 1996; Jackson *et al.*, 2002; O'Toole & Kolter, 1998; Pratt & Kolter, 1998), attachment to diverse surfaces (Blackman & Frank, 1996; Merritt *et al.*, 2000), and compare biofilm development among pathogens (Jin *et al.*, 2003; Li *et al.*, 2003). Its greatest features are that it is inexpensive, relatively quick and adaptable for use in high-throughput screening using microtiter plates (Djordjevic *et al.*, 2002; O'Toole & Kolter, 1998; Pitts *et al.*,

2003). Adhesion assays have also been developed based on staining with safranin (Christensen *et al.*, 1982; Romeo *et al.*, 1993) and trypan blue (Christensen *et al.*, 1982). The SBF assay was conceived as a simple CV-based approach to find plant essential oils with anti-biofilm properties. The assay is significant because it incorporates growth and biofilm accumulation in a single parameter, illustrating the relative tendency of cells in a population to attach to surfaces in response to changing essential oil concentrations. The SBF assay differs in this regard from other CV-based studies of biofilms that have measured the corresponding growth of cells (Djordjevic *et al.*, 2002) or cellular activity (Pitts *et al.*, 2003), and could benefit investigations into the influence of chemicals on biofilm development.

The divergent responses of *E. coli* ATCC 33456 to cinnamaldehyde and citronellol measured by the SBF assay were unexpected. To establish whether the observed trends in SBF concurred with the microscopy-based direct assays of biofilm development, three data analyses were considered. First, the effects of cinnamaldehyde, eugenol and citronellol on biofilm formation by *E. coli* ATCC 33456 were compared to each other (**Table 2.1**). Five parameters measuring biomass calculated by COMSTAT (total biomass, maximum biofilm thickness, substratum coverage, surface-to-biovolume ratio and average diffusion distance) were significantly greater for biofilms exposed to citronellol than for biofilms exposed to cinnamaldehyde. This result strongly agreed with the trend determined by the SBF assay. In contrast, the COMSTAT parameters for eugenol were not significantly different than for citronellol, and were only significantly different from cinnamaldehyde for one parameter (substratum coverage), a finding that

was analogous to the intermediate response of eugenol seen in the SBF assay. Second, the changes in COMSTAT parameters for treated biofilms relative to untreated controls were compared. For cinnamaldehyde, a decrease in three of five parameters characterizing biomass relative to LB-only grown biofilms was measured, with an increase in one. For eugenol, there was an increase in three parameters, and a decrease in two. For citronellol, there was an increase in three parameters, and a decrease in one. In general, these results followed the trend that was observed in the SBF assay. Third, the SCA assay, measuring growth-normalized adhesion by direct microscopy, showed similar tendencies to the SBF assay in response to increasing concentrations of each of the tested plant essential oil components (**Fig. 2.3**). On the whole, the results of the microscopy-based analyses corroborated the overall trend in biofilm accumulation determined by the SBF assay.

Swimming motility has been linked to biofilm formation in several kinds of bacteria (Soutourina & Bertin, 2003), is mediated by flagella (Harshey, 2003), and initiates cell-to-surface contact. In some cases, flagellar motility has been found to be essential for normal biofilm formation (Pratt & Kolter, 1998). However, flagellar motility was not required for initial adhesion and biofilm formation by curli-producing strains of *E. coli* (Prigent-Combaret *et al.*, 2000), or for biofilm formation by *E. coli* strains carrying conjugative plasmids (Reisner *et al.*, 2003). In this work, cinnamaldehyde significantly reduced the swimming motility of *E. coli* ATCC 33456. We hypothesize that cinnamaldehyde may have reduced biofilm formation by *E. coli* ATCC 33456 in part by interfering with its ability to reach the substratum, a finding consistent with the results of the SCA assay.



Several patterns of SBF were observed in response to increasing concentrations of the three essential oil components tested in this research, suggesting that the compounds may interact with microorganisms through different mechanisms. For example, the SBF for *E. coli* in response to cinnamaldehyde decreased gradually, but declined rapidly at concentrations greater than 1750  $\mu\text{M}$ , possibly due to accumulated cell membrane damage, which was detected during viability staining. In contrast, over a similar concentration range, the SBF of *P. aeruginosa* first decreased and then subsequently increased to a level higher than in the absence of cinnamaldehyde. This behavior suggested the activation of a stress-induced response that led to increased CV binding, possibly by increased EPS production, seen elsewhere in response to toxicity (Aquino & Stuckey, 2004; Fang *et al.*, 2002). Similarly, citronellol substantially increased the SBF of *E. coli* ATCC 33456, possibly by acting as a poly-L-lysine-like adhesive (Cowan *et al.*, 2001), but decreased the SBFs of *P. aeruginosa* and *P. putida* over equivalent concentration ranges, indicating a different interaction with these microorganisms. By characterizing SBF patterns for chemicals with well-understood mechanisms of activity, the SBF assay could provide insight into the mode of action of new compounds. However, direct microscopy would ultimately be required to evaluate their effect on biofilm formation and structure.

**TABLE 2.1** COMSTAT analysis of *E. coli* ATCC 33456 biofilm structure <sup>a</sup>

Compounds tested	Parameters <sup>b</sup>					
	Total biomass ( $\mu\text{m}^3/\mu\text{m}^2$ )	Maximum thickness ( $\mu\text{m}$ )	Substratum coverage, percent	Roughness coefficient	Average diffusion distance ( $\mu\text{m}$ )	Surface to biovolume ratio ( $\mu\text{m}^2/\mu\text{m}^3$ )
LB	$1.5 \pm 0.9$	$46.2 \pm 5.7$	$34 \pm 19$	$1.7 \pm 0.2$	$0.02 \pm 0.02$	$4.1 \pm 0.7$
Cinnamaldehyde	$1.6 \pm 0.7$ <sup>f</sup>	$18.3 \pm 1.8$ <sub>df</sub>	$16 \pm 5$ <sub>df</sub>	$1.5 \pm 0.2$ <sub>d,e</sub>	$0.05 \pm 0.03$ <sub>c,f</sub>	$2.0 \pm 0.2$ <sub>df</sub>
Eugenol	$4.1 \pm 2.8$ <sup>c</sup>	$22.2 \pm 6.1$ <sub>d</sub>	$57 \pm 19$ <sup>c</sup>	$1.1 \pm 0.5$ <sup>d</sup>	$0.18 \pm 0.18$ <sup>c</sup>	$2.8 \pm 1.0$ <sup>d</sup>
Citronellol	$4.8 \pm 2.0$ <sup>c</sup>	$28.5 \pm 10.0$ <sub>d</sub>	$52 \pm 18$ <sup>c</sup>	$0.9 \pm 0.3$ <sup>d</sup>	$0.12 \pm 0.09$ <sup>c</sup>	$3.5 \pm 0.5$

<sup>a</sup> Biofilms grown for 18 h in flow cells. Essential oil components were added at concentrations causing 60 percent inhibition of the planktonic growth rate.

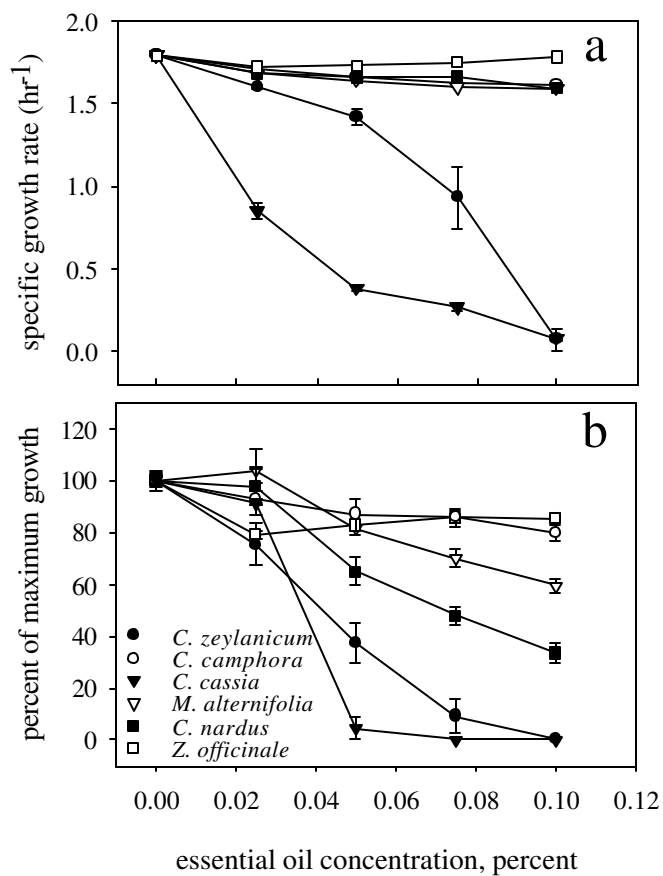
<sup>b</sup> All values represent means  $\pm$  standard deviations.

<sup>c</sup> Significantly larger ( $p < 0.05$ ) than corresponding values in LB control.

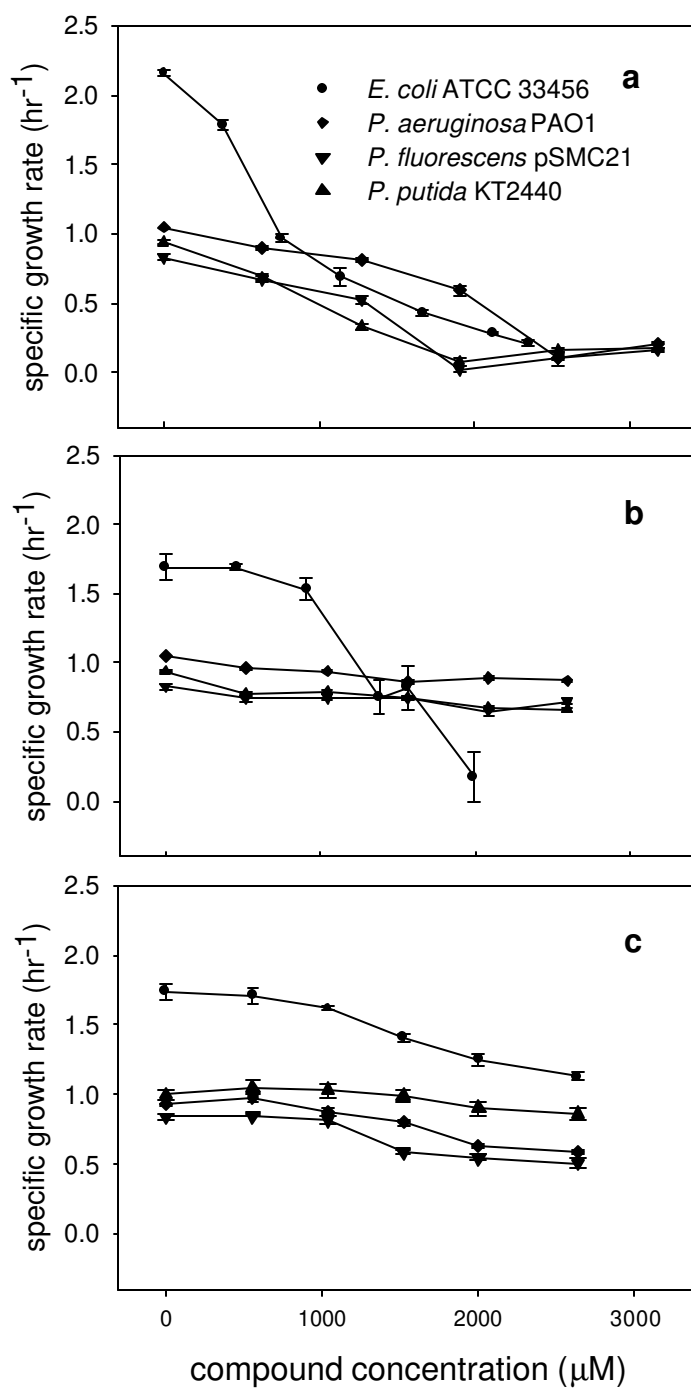
<sup>d</sup> Significantly smaller ( $p < 0.05$ ) than corresponding values in LB control.

<sup>e</sup> Significantly larger ( $p < 0.05$ ) than corresponding values in citronellol.

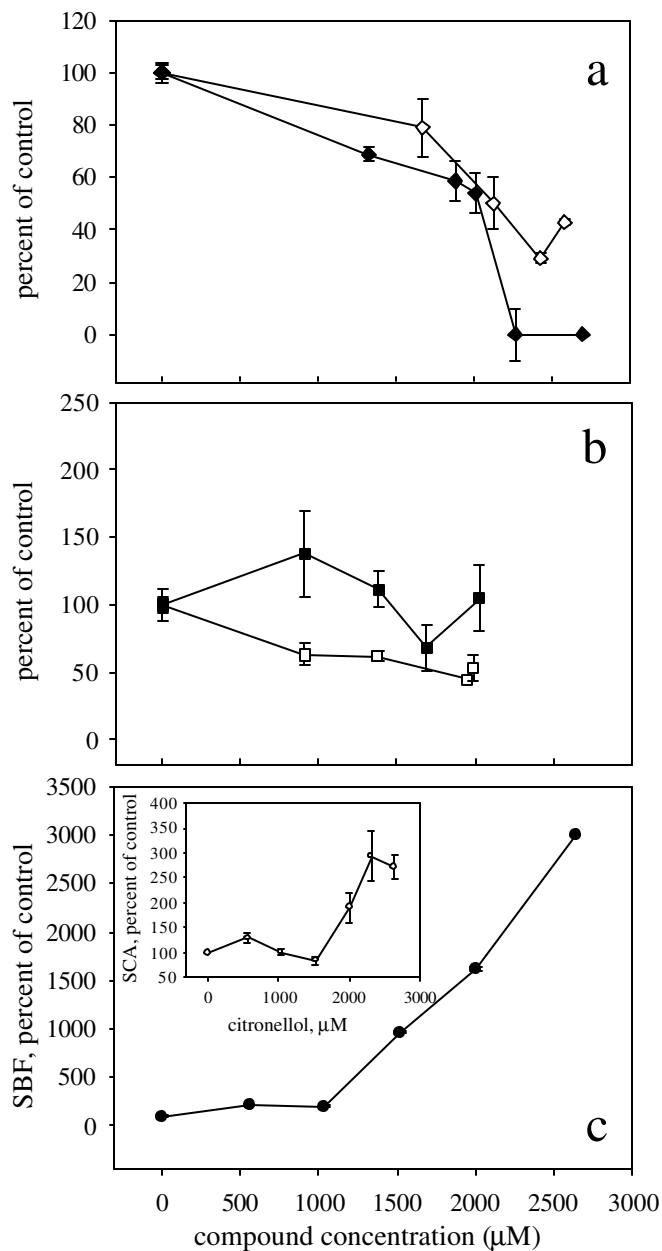
<sup>f</sup> Significantly smaller ( $p < 0.05$ ) than corresponding values in citronellol.



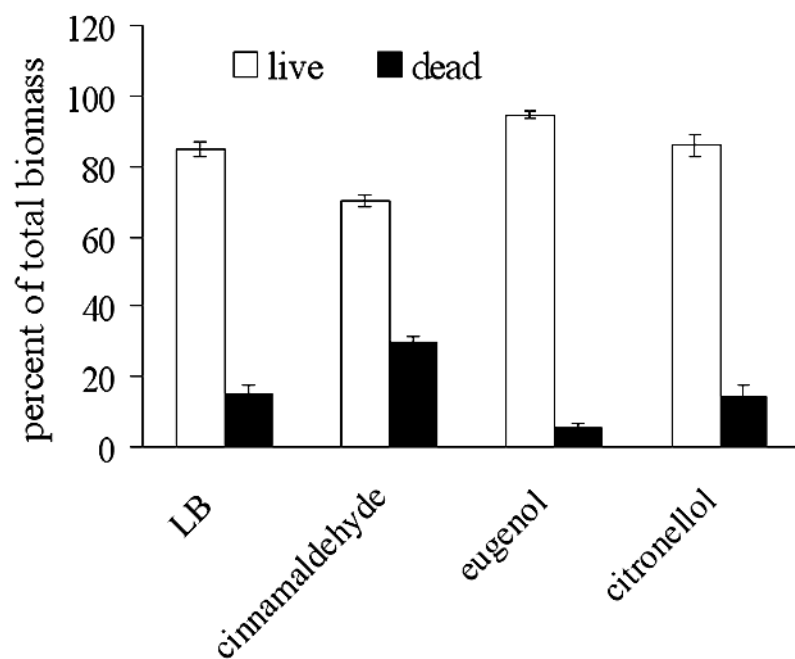
**Fig. 2.1** Plant essential oil toxicity to *E. coli* ATCC 33456. (a) Toxicity measured as specific growth rate. (b) Toxicity reported as percent growth relative to growth in LB medium after 15 h.



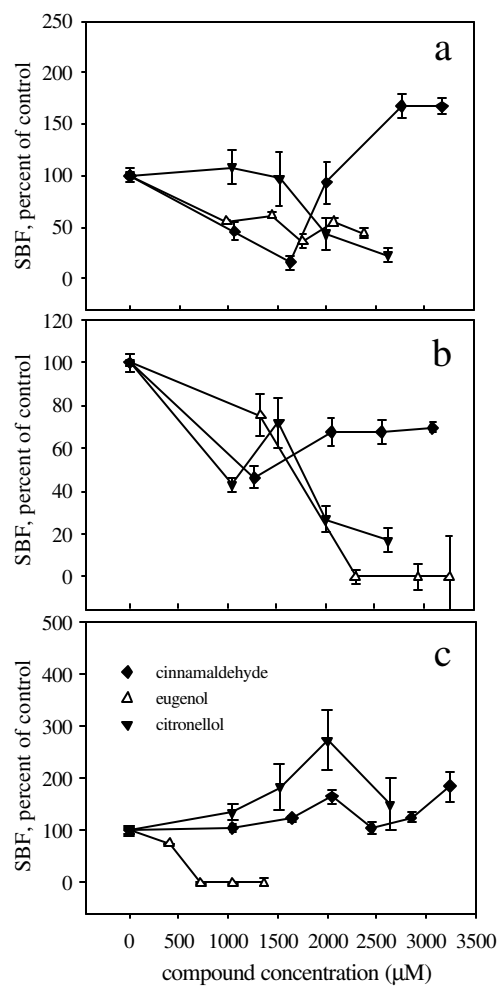
**Fig. 2.2** Effects of (a) cinnamaldehyde, (b) eugenol and (c) citronellol on the specific growth rate of *E. coli* ATCC 33456 and selected *Pseudomonas* species.



**Fig. 2.3** Comparison of SBF and SCA assays as a function of increasing plant essential oil component concentration. Reported activities are relative to the activity of *E. coli* ATCC 33456 in LB medium. (a) Cinnamaldehyde, (b) eugenol, (c) citronellol. Inset, SCA assay using citronellol. Closed symbols, SBF assay; open symbols, SCA assay.



**Fig. 2.4** Cell viability in *E. coli* ATCC 33456 biofilms after 18 h growth in the presence of plant essential oil components. White bar: live cells, black bar: dead cells.



**Fig. 2.5** Effects of cinnamaldehyde, eugenol, or citronellol on specific biofilm formation (SBF) by *Pseudomonas* species. Diamond, cinnamaldehyde; triangle up, eugenol; triangle down, citronellol. (a) *P. aeruginosa* PAO1, (b) *P. putida* KT2440, (c) *P. fluorescens* pSMC21.

## CHAPTER 3

### INTERFERENCE WITH QUORUM SENSING BY SUBINHIBITORY CONCENTRATIONS OF CINNAMALDEHYDE

C. Niu, S. Afre and E. S. Gilbert

#### 3.1 ABSTRACT

Species of the genus *Cinnamomum* are broadleafed trees that widely grow in tropical and subtropical areas of Asia. The essential oils of this genus are widely used in the perfume and flavor industry (Zhu *et al.*, 1994). The principal chemical component of the volatile oils from the leaves of *C. zeylanicum* is cinnamaldehyde (CA) (Chalchat & Valade, 2000). *Trans*-CA is an antimicrobial compound existing in many kinds of commercial cinnamon-containing foods; the concentration of CA usually ranges from 100 to 300 ppm (Friedman *et al.*, 2000). The aim of this study was to investigate the effect of CA on quorum sensing (QS). The action of CA on QS was assayed using two *Escherichia coli* green fluorescent protein (GFP) based bioreporters (one inducible and the other constitutive) and two *Vibrio harveyi* bioluminescent reporter strains. LuxR mediated QS, which is induced by 3-oxo-C6-HSL, was reduced by 70 percent following exposure to 200  $\mu$ M CA (26 ppm). The bioluminescence of *V. harveyi* BB886, which is mediated by 3-hydroxy-C4-HSL, was reduced by 55 percent after exposure to 60  $\mu$ M CA (8 ppm), and 100  $\mu$ M CA (13 ppm) inhibited the bioluminescence of the AI-2 responsive reporter strain *V. harveyi* BB170 by nearly 60 percent. CA did not inhibit the growth of the bioreporter strains at these concentrations. CA had a minimal effect on LasR



mediated QS, induced by and 3-oxo-C12-HSL. Because CA is widely used in the food and flavor industries, its potential to affect bacterial QS regulated processes should be recognized.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Strains, plasmids and media

The strains and plasmids used are reported in **Table 3.1**. All strains were cultured in Luria-Bertani (LB) broth, except for *V. harveyi* BB886 and BB170, which were grown in autoinducer-bioassay (AB) medium (Greenberg *et al.*, 1979). Media used for culturing *E. coli* ATCC 33456 pJBA89, *E. coli* ATCC 33456 pJBA113, and *E. coli* ATCC 33456 pMHLAS were supplemented with 100  $\mu\text{g mL}^{-1}$  ampicillin. All strains were grown at 30°C for fluorescence or bioluminescence measurements.

### 3.2.2 Chemicals

CA was obtained from Aldrich (Milwaukee, WI); *N*-3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HSL) was obtained from Quorum Sciences, Inc. (Coralville, IA). *N*-Dodecanoyl-DL-homoserine lactone (3-oxo-C12-HSL) was obtained from Fluka (Buchs, Switzerland). Structures of these compounds are illustrated in Fig. 3.1. CA was diluted in methanol (1% v/v) prior to use; 3-oxo-C6-HSL and 3-oxo-C12-HSL were diluted in ethanol to make 20  $\mu\text{M}$  and 200  $\mu\text{M}$  stock solutions, respectively.

### 3.2.3 Effect of CA on 3-oxo-C6- and 3-oxo-C12-HSL mediated cell signaling

The activity of CA towards 3-oxo-C6-HSL (LuxR) mediated cell signaling was measured using the bioreporters *E. coli* ATCC 33456 pJBA89 and *E. coli* ATCC 33456 pJBA113. The plasmids pJBA89 and pJBA113 are identical except that pJBA89 has the 3-oxo-C6-HSL inducible promoter  $P_{luxI}$  (Andersen *et al.*, 2001) which was cloned from *V. fischeri*, and pJBA113 has the constitutive promoter  $P_{AI/04/03}$  (Andersen *et al.*, 1998). The ability of CA to modulate 3-oxo-C12-HSL mediated cell signaling was also investigated using *E. coli* ATCC 33456 pMHLAS, a  $P_{lasR-gfp}$ (ASV) bioreporter cloned from *Pseudomonas aeruginosa* (Hentzer *et al.*, 2002). In either case, 3-oxo-C6-HSL or 3-oxo-C12-HSL were used to activate a population of bioreporter cells, followed by spiking a subset of the induced cells with CA after a short period of time. Assay protocols are described in detail elsewhere (Andersen *et al.*, 2001; Hentzer *et al.*, 2002). Briefly, three replicate tubes containing 4 mL of *E. coli* ATCC 33456 pJBA89 or *E. coli* ATCC 33456 pJBA113 were induced with 100 nM 3-oxo-C6-HSL, and *E. coli* ATCC 33456 pMHLAS was induced with 1  $\mu$ M 3-oxo-C12-HSL. After 20 min (*E. coli* ATCC 33456 pJBA89 or *E. coli* ATCC 33456 pJBA113) or 120 min (*E. coli* ATCC 33456 pMHLAS), tubes were spiked with selected concentrations of CA. The specific fluorescence (measured fluorescence/OD<sub>450</sub>) was determined for the LuxR-*gfp* bioreporters at 20 and 80 minutes. Specific fluorescence of the LasR-*gfp* bioreporter was measured at 120 and 240 minutes to accommodate its slower maturation rate (Leveau & Lindow, 2001). Fluorescence was measured with a Bio-Rad VersaFluor<sup>TM</sup> fluorometer (Bio-Rad, Hercules, CA), using excitation and emission filters of 490 nm and 510 nm, respectively. All reported values

are means  $\pm$  standard errors, and statistical significance was determined by Student's t-test.

### **3.2.4 Effect of CA on 3-hydroxy-C4-HSL and AI-2 mediated cell signaling**

The effect of CA on 3-hydroxy-C4-HSL (Cao & Meighen, 1989) and AI-2 mediated cell signaling was determined using *V. harveyi* BB886 and BB170, two bioluminescent reporter strains (Bassler *et al.*, 1997), based on a modification of a method published by Ren *et al.* (Ren *et al.*, 2001) Briefly, an overnight culture of strain *V. harveyi* BB886 or strain *V. harveyi* BB170 was diluted 1:5000 into fresh AB medium, and tubes were shaking incubated at 30°C. Additionally, 10% (v/v) cell-free *V. harveyi* BB170 supernatant was added as an external source of AI-2 for *V. harveyi* BB170. Bioluminescence was measured hourly with a Uni-lite XCEL luminometer (BioTrace; NJ, USA). When bioluminescence reached a minimum value (<500 RLU), CA was added to the culture. The final bioluminescence was measured 1.5 h following the addition of CA, and the activity of CA treated samples was compared to *V. harveyi* BB886 or *V. harveyi* BB170 controls not receiving CA.

### **3.2.5 Effect of CA on bioreporter growth**

Cell viability assays were performed concurrently with the bioreporter assays. Cell densities were measured at both the initial and final time points by serial dilution and plate counts. To determine whether growth occurred over the course of each assay, final colony forming units (CFU) values for treated samples were compared with initial CFU

measurements. To determine whether the added CA inhibited growth, final CFU values for LB-methanol only grown controls were compared with final values of CA treated samples.

### 3.3 RESULTS

#### 3.3.1 Effect of CA on *E. coli* LuxR bioreporter growth

Concentrations up to 200  $\mu\text{M}$  (26 ppm) CA had no inhibitory effect on the growth of *E. coli* ATCC 33456 pJBA89 or *E. coli* ATCC 33456 pJBA113 (**Figs. 3.2 & 3.3**). For all samples, the initial cell density ranged from 4.7 to  $8.1 \times 10^6$  CFU/mL, and the final cell density was between  $1.5$  to  $2.0 \times 10^7$  CFU/mL. These results indicate that the cell density increased by approximately 2.5 fold over the course of the 80 minute assay.

#### 3.3.2 Effect of CA on LuxR-mediated QS

CA reduced LuxR (3-oxo-C6-HSL inducible) mediated cell signaling relative to control cells (**Fig. 3.2**). At the same concentrations, CA did not inhibit the constitutive production of GFP by the control strain *E. coli* ATCC 33456 pJBA113 (**Fig. 3.3**); thus, no non-specific inhibition of GFP fluorescence was detected following CA exposure. Statistically significant reductions in GFP fluorescence were evident in response to incremental additions of 50  $\mu\text{M}$  CA (6.6 ppm), indicating that the inducible bioreporter present on plasmid pJBA89 had a high degree of sensitivity. CA was not capable of upregulating GFP expression by the biosensor strains.

### 3.3.3 Effect of CA on LasR-mediated QS

The effects of CA on the growth and the specific fluorescence of the  $P_{lasR}$ -*gfp* bioreporter, *E. coli* ATCC 33456 pMHLAS, were investigated. The bioreporter growth was not inhibited by 200  $\mu$ M CA ( $99 \pm 15$  percent of measured growth compared to the LB-only control). The specific fluorescence of the bioreporter was slightly reduced at 200  $\mu$ M CA ( $8 \pm 3$  percent relative to LB control;  $p < 0.03$ ).

### 3.3.4 Effects of CA on 3-hydroxy-C4-HSL and AI-2 mediated QS and *Vibrio* bioreporter growth

60  $\mu$ M CA (7.9 ppm) generated a  $54 \pm 3$  percent reduction ( $p < 0.008$ ) in the activity of the 3-hydroxy-C4-HSL bioreporter *V. harveyi* BB886 (**Fig. 3.4**). The initial cell density was approximately  $1 \times 10^4$  CFU mL<sup>-1</sup>, and increased to  $4.4 \pm 2.2 \times 10^4$  CFU mL<sup>-1</sup> during the course of the assay. No significant difference in cell density was measured between treated and untreated samples at the end of the experiment ( $p < 0.39$ ). The effect of CA on bioreporter growth was also measured turbidimetrically in an overnight assay. After 20 hr, the OD<sub>600</sub> of *V. harveyi* BB886 growing in LB broth plus 60  $\mu$ M CA was 0.89, which was  $94 \pm 5$  percent of the control and was not significantly different from it ( $p < 0.26$ ). 100  $\mu$ M CA (13.2 ppm) generated a  $62 \pm 2$  percent reduction ( $p < 0.003$ ) in the activity of the AI-2 bioreporter *V. harveyi* BB170. The OD<sub>600</sub> of *V. harveyi* BB170 in LB broth plus 100  $\mu$ M CA was 1.09, which was  $101 \pm 1$  percent of the control and was not significantly different from it ( $p < 0.28$ ).

### 3.4 DISCUSSION

Quorum sensing signal pathways frequently have multiple steps, and there are several potential interactions that CA could have that would interfere with signaling. Manefield *et al.* (2002) determined that low concentrations of furanones ( $=100\ \mu\text{M}$ ) caused an accelerated turnover of the LuxR receptor protein through an unidentified mechanism. For cyclic dipeptides that inhibited quorum sensing, competitive inhibition was found to be the most probable mechanism (Holden *et al.*, 1999). The concentrations of cyclic dipeptides that were required to inhibit quorum sensing were substantially higher than those required for furanone activity. The mechanism of action of the cyclic dipeptides was called “crosstalk”, to indicate that it was most likely nonspecific.

In the work presented herein, concentrations of CA of  $100\ \mu\text{M}$  or less significantly reduced both 3-hydroxy-C4- and 3-oxo-C6-HSL mediated signaling, similar to previously reported concentrations required for furanone to inhibit QS (Manefield *et al.*, 1999). We speculate that the three carbon aliphatic side chain of CA may have interfered with the binding of the smaller C4- and 3-oxo-C6-HSLs to their cognate receptors, but was not sufficiently long enough to substantially reduce the binding of 3-oxo-C12-HSL to LasR. We also observed that  $100\ \mu\text{M}$  CA significantly reduced AI-2 mediated signaling. There is little structural similarity between AI-2 and CA, and it is unlikely that CA acts as an antagonist to AI-2 receptor binding. It is more likely that CA or one of its metabolites interferes with the AI-2 synthetic pathway, which involves small molecules derived from S-adenosylmethionine (Chen *et al.*, 2002). Ren *et al.* (2004) have

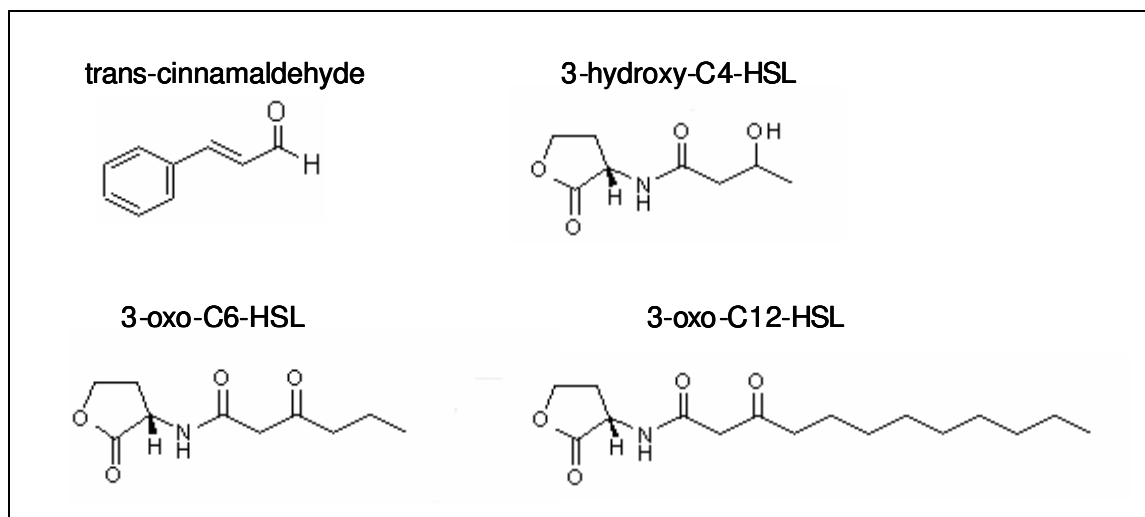
proposed that furanone compounds may interfere with AI-2 regulated signaling posttranslationally.

CA, which is a component of cinnamon and cassia essential oils, is used as a food and flavoring agent as well as a fungicide (USEPA, 2005). The potential influence of CA on C4- and 3-oxo-C6-HSL mediated quorum sensing could affect bacterial activity, and consequently is relevant to food safety.

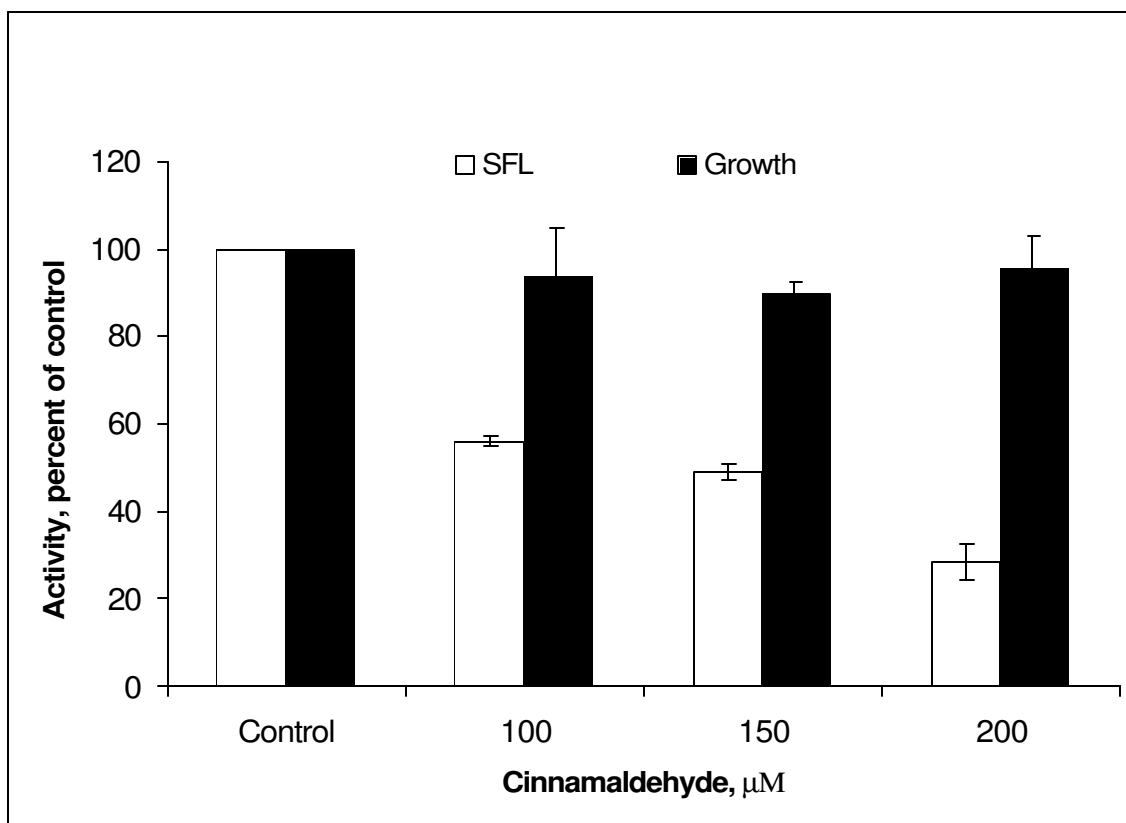
**TABLE 3.1** Strains and plasmids used in this study.

Strains and plasmids		Description	Reference or source
Strains	<i>E. coli</i> ATCC 33456	wild type	(Wang & Shen, 1995)
	<i>V. harveyi</i> BB886	autoinducer-1 biosensor, synthesizes 3-hydroxy-C4-HSL	(Bassler <i>et al.</i> , 1997)
	<i>V. harveyi</i> BB170	autoinducer-2 biosensor	(Bassler <i>et al.</i> , 1997)
Plasmids	pJBA89	<i>luxR</i> biosensor with destabilized GFP, 3-oxo-C6-HSL inducible	(Andersen <i>et al.</i> , 2001)
	pJBA113	pUC18-based vector carrying destabilized GFP, constitutive expression	(Andersen <i>et al.</i> , 1998)
	pMHLAS	<i>lasR</i> biosensor with destabilized GFP, 3-oxo-C12-HSL inducible	(Hentzer <i>et al.</i> , 2002)

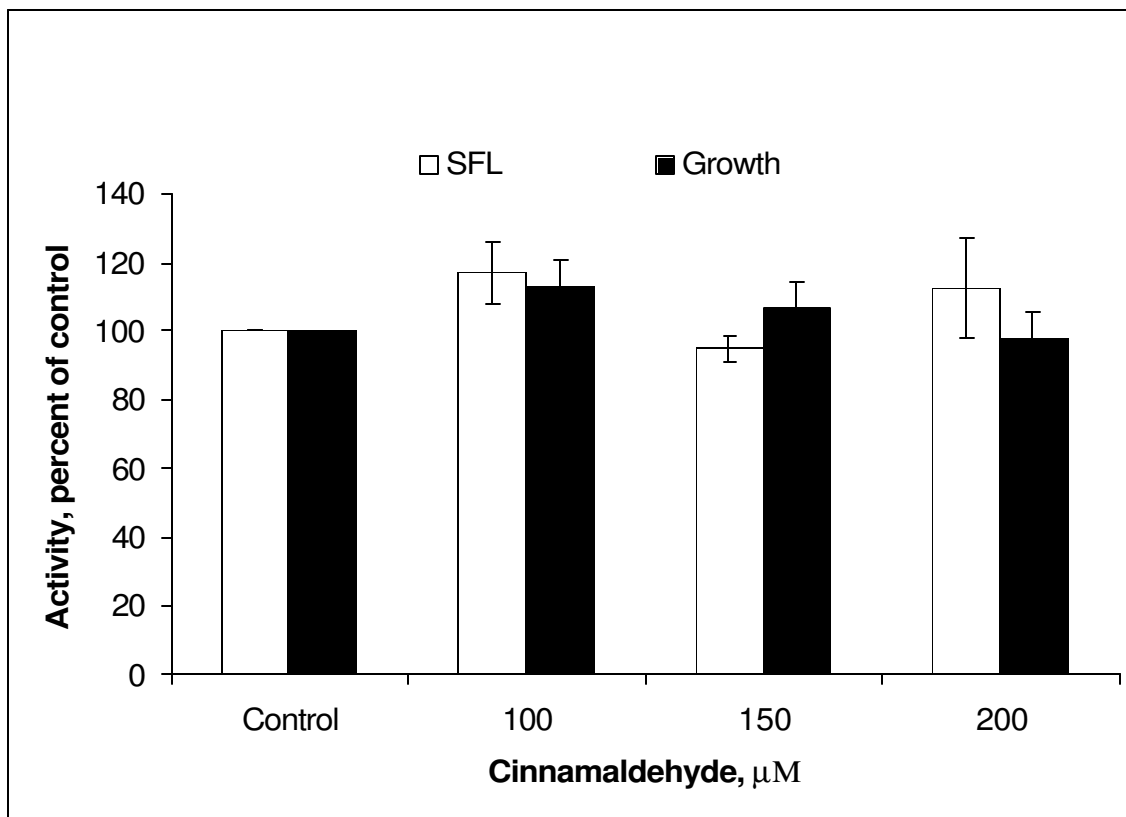




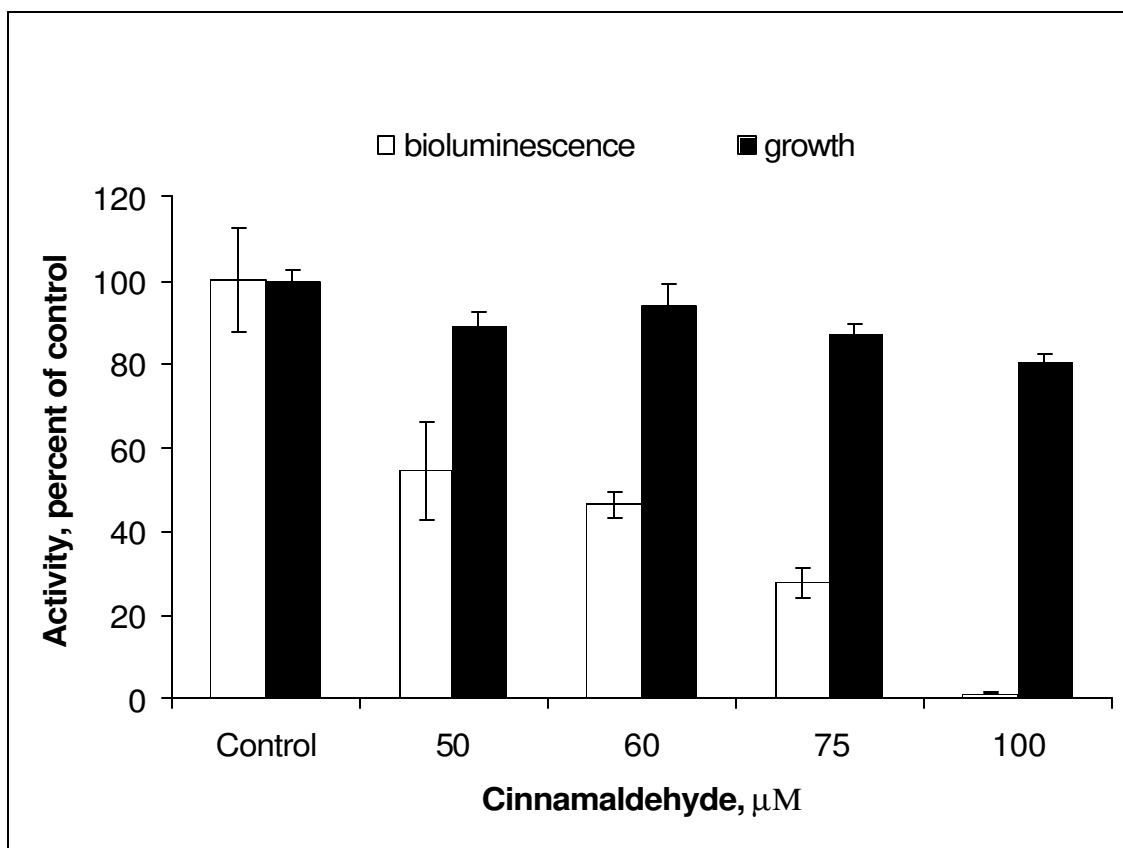
**Fig. 3.1** Structures of compounds used in this study.



**Fig. 3.2** Effect of cinnamaldehyde on the growth and bioluminescence of the 3-oxo-C6-HSL inducible bioreporter *E. coli* pJBA89. Open bars, specific fluorescence (SFL). Closed bars, growth.



**Fig. 3.3** Effect of cinnamaldehyde on the growth and bioluminescence of the constitutive bioreporter *E. coli* pJBA113. Open bars, specific fluorescence (SFL). Closed bars, growth.



**Fig. 3.4** Effect of cinnamaldehyde on the growth and bioluminescence of the 3-hydroxy-C4-HSL responsive bioreporter *V. harveyi* BB886. Open bars, bioluminescence. Closed bars, growth.

## CHAPTER 4

### THE ROLE OF AI-2 MEDIATED QUORUM SENSING IN BIOFILM FORMATION OF NON-MOTILE *ESCHERICHIA COLI* W3110

C. Niu, R. B. Simmons and E. S. Gilbert

#### 4.1 OBJECTIVE

Controlling biofilm formation by regulating QS has led to promising approaches in treating *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections (Balaban *et al.*, 2005; Wu *et al.*, 2004), and could help control urogenital infections originating from *E. coli* biofilms (Ren *et al.*, 2001; Ren *et al.*, 2004b). Some debate exists over whether AI-2 regulates *E. coli* biofilm formation. Reisner (2004) reported no connection of AI-2 to biofilm formation by *E. coli* containing conjugative IncF plasmids. However, AI-2 has been shown to control *E. coli* biofilm formation through a motility regulatory mechanism in a recent report (Gonzalez Barrios *et al.*, 2006). DeLisa (DeLisa *et al.*, 2001) investigated differential gene expression in wild type *E. coli* W3110 and a *luxS* deletion mutant (strain MDAI2) after adding AI-2-containing supernatant and found significant transcriptional changes in putative adhesion or fimbrial-like proteins (*yadK*, *yadN*), carbon storage regulator (*csrA*), and lipopolysaccharide core biosynthesis (*rfaJ*, *rfaY*), which contribute to *E. coli* adhesion or biofilm formation (Genevaux *et al.*, 1999; Jackson *et al.*, 2002; Torres *et al.*, 2005). In a recent work with *E. coli* W3110 and strain MDAI2, it was reported that deletion of *luxS* had no effect on growth, motility, or biofilm formation (Wang *et al.*, 2005).

In preliminary research, we determined that AI-2 production by *E. coli* W3110 in batch culture was growth phase dependent. During early log phase as well as stationary phase, AI-2 levels were low, but during mid- and late-log phase AI-2 levels were substantial. Thus, to investigate the putative role of AI-2 in *E. coli* W3110 biofilm formation, it was necessary to consider mutant strains which either under- or over-produced AI-2. To this end, we investigated phenotypes expressed by wild type *E. coli* W3110, its *luxS* deletion mutant strain MDAI2 which cannot synthesize AI-2, and a complemented variant of MDAI2 that carried *luxS* on a plasmid under the control of an arabinose-inducible promoter and could overexpress AI-2. Using these three strains, we investigated the role of AI-2 in regulating physiological factors that could affect biofilm formation by *E. coli* W3110, including AI-2 activity assay, carbon utilization, uronic acid production, transmission electron microscopy (TEM) imaging of batch culture and biofilm samples, autoaggregation assay, analysis of antibiotic sensitivity, sand column adhesion assay, the specific biofilm formation (SBF) assay, examination of biofilms through DIC-light microscopy and confocal laser scanning microscopy (CLSM) imaging.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Bacterial strains, plasmid, and media

The following strains were used in this study: *E. coli* W3110 (wild type), *E. coli* MDAI2 (*luxS* mutant) (DeLisa *et al.*, 2001), and *Vibrio harveyi* BB170 (Bassler *et al.*, 1997). The plasmid pMS234 (a pBAD18 vector containing the *E. coli luxS* gene) (Schauder *et al.*, 2001) was transformed into *E. coli* MDAI2 (*luxS* mutant) through

electroporation using a MicroPulser (Bio-Rad). The transformation was confirmed by plasmid extraction and gel electrophoresis, as well as by the AI-2 activity assay (**Table 4.1**).

*E. coli* strains were grown in Luria-Bertani (LB) medium at 37 °C, and *V. harveyi* BB170 was cultivated in autoinducer bioassay (AB) medium (Greenberg *et al.*, 1979) at 30 °C. Media for *E. coli* W3110 pMS234 was supplemented with 0.1% w/v filter-sterilized arabinose. For ease of communication, the strains *E. coli* W3110, *E. coli* MDAI2 and *E. coli* W3110 pMS234 are collectively referred to as ‘the W3110 set’ throughout this chapter.

#### **4.2.2 Using bioluminescence assay to test AI-2 activity of the W3110 set**

Supernatants were collected at 2, 4.5, 8, 14 and 20 h from batch cultures of *E. coli* W3110, *E. coli* MDAI2, and *E. coli* MDAI2 pMS234, and AI-2 activity was measured using the bioreporter *V. harveyi* BB170 (Bassler *et al.*, 1997; Ren *et al.*, 2001) (the details of this technique was given in section **3.2.4**).

#### **4.2.3 SBF assay and motility assay**

All three strains were cultivated in LB broth at 200 rpm shaking in a 37 °C incubator overnight for  $19 \pm 1$  h. Growth normalized biofilm formation was determined colorimetrically using the SBF assay. Swimming motility of the W3110 set was determined using the motility assay (details were given in section **2.2**).

#### **4.2.4 Flow cell cultivation, DIC-light microscopy, and CLSM imaging**

Biofilm cultivation using flow cell, DIC-light and CLSM were carried out using previously described techniques (details were demonstrated in section 2.2) except that Cyto 59, a nucleic acid stain, was used for staining and 0.3% agarose was used to stabilize biofilms prior to CLSM.

#### **4.2.5 Carbon utilization assay**

The W3110 set was streaked onto LB plates with or without 0.1% w/v arabinose and incubated overnight at 37 °C. Cells were transferred from a plate to a glass tube containing GN/GP-IF inoculating fluid to reach a turbidity of  $70 \pm 3$  % transmittance. GN2 MicroPlate was (Biolog, Hayward, CA) inoculated by putting 150  $\mu$ L cell suspensions into each well. All MicroPlates were incubated at 37 °C for  $24 \pm 1$  h. Tetrazolium violet in wells colorimetrically indicated the utilization of carbon sources. MicroPlates were scanned using a Biolog MicroPlate Reader and absorbance data were analyzed in Microsoft Excel. Four replications were performed for each strain.

#### **4.2.6 Uronic acids production assay**

All three strains were cultivated overnight in AB medium at 200 rpm, 37 °C. 1 mL culture of each strain was centrifuged at 13,000 rpm for 5 min. 200  $\mu$ L supernatant was analyzed and OD<sub>520</sub> values were measured using a spectrophotometer after a series of chemical treatments (Blumenkrantz & Asboe-Hansen, 1973).



#### **4.2.7 TEM imaging of batch and flow cell cultivated samples**

In general, samples were prepared for TEM by negative staining on Formvar-coated grids, using 0.5% phosphotungstic acid at pH 6.9. A sample of batch culture (2 mL) or the biomass from flow cell channels (about 0.5 mL) was centrifuged at 10,000 rpm for 1.5 min and the cell pellet was resuspended in 1 mL 50 mM  $\text{KH}_2\text{PO}_4$  buffer. 10  $\mu\text{L}$  cell suspensions were applied to cover the grid surface and sedimented for 2 minutes. 5  $\mu\text{L}$  of 0.5% PTA was applied onto each grid and staining for 1 min. Excess liquid was removed by using filter paper. Grids were examined in a LEO906e transmission electron microscope at a voltage of KV80.

#### **4.2.8 Autoaggregation assay**

Autoaggregation was determined as per Schembri (Schembri *et al.*, 2001). All three strains were cultivated in LB at 200 rpm shaking in a 37 °C incubator overnight for 17 h. 4 mL overnight culture of each strain was centrifugated at 10K rpm for 2 min then resuspended in the same amount of 50 mM  $\text{KH}_2\text{PO}_4$  buffer. The resuspension was incubated statically at 37 °C for 4 h. A positive reaction was indicated by precipitation at the bottom and transparent upper portion of the culture. Turbid culture was considered a negative reaction.

#### **4.2.9 Antibiotic sensitivity assay**

Cells were incubated at 37 °C with shaking in LB broth till the growth status reached exponential phase. Cells were spread onto surface of LB plates using sterile

swabs. 12 various BBL antibiotic disks (3 disks per plate) were applied. All plates were incubated at 37 °C overnight. The diameters of “zones of inhibition” were measured and compared to the standard chart to determine the antibiotic sensitivity according to manufacturer’s instructions. Duplicate tests were performed for each strain.

#### **4.2.10 Sand column adhesion assay**

All three strains were cultivated in LB medium at 200 rpm, 37 °C overnight. 1 mL culture was centrifuged at 10,000 rpm for 1.5 min and pellet was resuspended in 10 mL 50 mM KH<sub>2</sub>PO<sub>4</sub> buffer. The resuspension was diffused through a sterile polystyrene column filled with 10 g sterilized sand. OD<sub>600</sub> values were measured using a spectrophotometer before and after sand column treatment.

### **4.3. RESULTS**

#### **4.3.1 AI-2 activity of the W3110 set**

AI-2 production by *E. coli* W3110 growing in batch culture peaked by 4.5 h, and returned to background levels by 20 h (**Table 4.1**). AI-2 production by *E. coli* MDAI2 pMS234 increased during the first 8 h and did not decline below 70 times controls levels for at least 20 h. *E. coli* MDAI2 produced minimal amounts of AI-2. The *luxS* complemented strain overproduces AI-2 compared to the natural cycle of *E. coli* W3110. After 24 h, AI-2 production in biofilms by *E. coli* W3110 increased 10-fold relative to initial levels, and stabilized for the following 48 h (data was not shown).

#### 4.3.2. SBF assay and microscopic observation of three strains in the W3110 set

In the SBF assay, the *luxS* mutant had similar amount of biomass ( $114 \pm 12$  percent) relative to wild type, whereas the complement formed  $26 \pm 9$  percent less biofilm (**Fig. 4.1**). DIC microscopy of biofilms illustrated that *E. coli* MDAI2 cells arranged in clusters whereas *E. coli* MDAI2 pMS234 formed flat biofilm and occupied bigger surface area than *E. coli* MDAI2 (**Fig. 4.2**). The biofilm architecture of *E. coli* W3110 was a combination of the *luxS* mutant and the complement strain. CLSM images indicated that *E. coli* MDAI2 formed rough biofilms with low biomass, and *E. coli* W3110 and *E. coli* MDAI2 pMS234 developed dense biofilm structures (**Fig. 4.3, Table 4.2**).

#### 4.3.3. TEM imaging and autoaggregation assay of the W3110 set

In batch culture, TEM imaging determined that fimbriae were present after 3.5 h and 14 h cultivation for *E. coli* MDAI2, whereas no fimbriation occurred at either time point for *E. coli* MDAI2 pMS234 (**Fig. 4.4a**). No fimbriae were detected at 3.5 h but were present at 14 h for *E. coli* W3110. In biofilm-grown cells, fimbriae were evident on *E. coli* MDAI2, but almost none were observed on *E. coli* MDAI2 pMS234, similar to batch culture. Fimbriae were occasionally seen on biofilm cells of *E. coli* W3110 (**Fig. 4.4b**). *E. coli* MDAI2 pMS234 autoaggregated, but not *E. coli* W3110 or strain MDAI2; and this autoaggregation was not caused by arabinose in culture (**Fig. 4.5**).

#### **4.3.4. Carbon utilization assay, uronic acids production, adhesion to sand, and antibiotic sensitivity test**

As shown in **Table 4.3**, utilization of 6 out of 96 tested carbon sources was controlled by *luxS* expression, especially D-Galactonic acid lactone and D-Glucuronic acid. Data in **Table 4.4** indicated that none of the tested strains synthesized significant amount of uronic acids, which are components of colonic acid, one kind of EPS. All three strains had a similar sand adhesion (45 to 50 percent). As shown in **Table 4.5**, *E. coli* W3110 and *E. coli* MDAI2 have similar antibiotic sensitivity. The resistance of *E. coli* MDAI2 pMS234 to several antibiotics may be due to the ampicillin resistant marker the plasmid pMS234 carries.

### **4.4 DISCUSSION**

#### **4.4.1 The role of AI-2 in fimbriation and biofilm formation of *E. coli* W3110**

Two corroborating pieces of information indicated that production of fimbriae by *E. coli* W3110 is AI-2 dependent. First, TEM images from both planktonically grown cells and flow cell biofilms (**Fig. 4.4**) detected fimbriae when AI-2 levels were low and an absence of fimbriae when AI-2 levels were high. Second, the *luxS* complemented strain MDAI2 pMS234 autoaggregated whereas the *luxS* mutant MDAI2 did not, in agreement with the work of Hasman (1999), who reported that autoaggregation is inhibited by fimbriation (Hasman *et al.*, 1999). We propose the following model that integrates these observations with the observed differences in biofilm structure and growth phase dependent synthesis of AI-2. When *E. coli* W3110 initially colonizes a new

surface, its cell density is low, and consequently AI-2 concentrations are low. Under these conditions, fimbriae are actively synthesized, similar to strain MDAI2. The production of fimbriae may facilitate surface colonization and other aspects of initial biofilm formation by *E. coli* (Moreira *et al.*, 2003; Ottoa *et al.*, 1999; Pratt & Kolter, 1998). As the population of surface-attached cells increases, the AI-2 concentration in their vicinity also increases. This reduces fimbriae production and allows autoaggregation to occur, possibly mediated by antigen 43 or other adhesins (Schembri *et al.*, 2003a; Sherlock *et al.*, 2005), as was observed with strain MDAI2 pSM34. In turn, this produces a densely packed biofilm (**Fig. 4.2 & 4.3**). This form of biofilm could potentially assist in resisting shear stress.

For several *E. coli* strains, AI-2 is important for biofilm development through a motility regulatory mechanism (Gonzalez Barrios *et al.*, 2006). Our result is likely different because (a) *E. coli* W3110 is nonmotile, and (b) both fimbriation and autoaggregation are considered facilitating factors for cell attachment to surfaces (Branda *et al.*, 2005; Sherlock *et al.*, 2005). Thus, regardless of AI-2 levels, biofilm formation by strain W3110 would still be expected. Differences in biofilm structure resulting from different types of adhesion would however be anticipated. Altered biofilm structure was also reported in *luxS* mutants of *Streptococcus mutans* (Merritt *et al.*, 2003) and *Klebsiella pneumoniae* (Balestrino *et al.*, 2005). Type 1 pili (e.g. fimbriae) are significant virulence factors associated with uropathogenic *Escherichia coli* (UPEC) (Mulvey *et al.*, 1998; Snyder *et al.*, 2005), and Type 1 fimbriation is controlled by more than one mechanism. Fimbriation is phase variation-controlled, and recent research found that

phase-locked off mutants which produced little or no fimbriae did not colonize the murine bladder efficiently (Gunther *et al.*, 2002; Snyder *et al.*, 2006). Besides, fimbriation is repressed by RpoS stationary phase specific sigma factor (Dove *et al.*, 1997). In this study, AI-2 was found regulating fimbriation as well. Consequently, interfering with AI-2 mediated fimbriae production could possibly aid in treating urinary tract infections caused by UPEC.

#### **4.4.2 The role of AI-2 in adhesion of *E. coli* W3110**

Adhesion to sand surface by *E. coli* W3110 is not affected by AI-2 mediated QS. Since sand particle has similar chemical structure ( $\text{SiO}_2$ ) and electron charge to glass, it was speculated that AI-2 mediated QS does not affect *E. coli* cells attaching to the surface of glass, which is the substratum of flow cell channels, through a physical adhesion mechanism. The overexpression of *luxS* reduced biofilm development in batch culture (**Fig. 4.1**) but increased biofilm density in flow cell cultivation (**Table 4.2**). This controversy may be due to the hydrodynamics. In batch culture, the force of shaking might facilitate the attachment by fimbriae whereas the shear force of flow could favor the attachment through adhesins. This observation also indicated that different experimental conditions may affect experimental results in biofilm research.

#### **4.4.3 The role of AI-2 in *E. coli* ecology**

It was reported that AI-2 affects the metabolism of *E. coli* (Wang *et al.*, 2005). Similarly, our data showed that the utilization of several carbon sources was regulated by

*luxS* expression, including D-glucuronic acid. However, no considerable amount of uronic acids was produced by either strain. Therefore, substrate utilization regulated by *luxS* expression may go into central metabolism instead of EPS synthesis. Enhanced glucuronic acid metabolism may reflect an adaptation to utilize this substrate, which is abundant in the animal gastrointestinal tract (Brandl *et al.*, 2005). With a steady supply of nutrients, as may be the case in the mammalian gut, the developing biofilm will continue to produce steady elevated levels of AI-2 and will remain in a state with little fimbriae.

The natural expression of *luxS* is important in *E. coli* biofilm formation, especially for *E. coli* W3110 which is originally *luxS* positive. It is important to recognize that QS is a complex system which is also regulated by global networking and may be affected by factors including nutrient availability, cell growth phase, and environmental stresses. Therefore, the QS system may coordinate with other global systems including cell growth, metabolism, chemotaxis, motility; together these systems regulate the development of *E. coli* biofilms.

In conclusion, *luxS* expression is important in carbon utilization, fimbriae production, and the biofilm development of *E. coli* W3110. The role of AI-2 mediated QS in microbial physiology of *E. coli* W3110 is pleiotropic. Overall, this work supports the hypothesis that AI-2 plays a significant role in the ecology of *E. coli* biofilm formation.

**TABLE 4.1** AI-2 production during growth in batch culture

Strain	Time point (h)				
	2	4.5	8	14	20
<i>E. coli</i> W3110	2.9 ± 0.8 <sup>a</sup>	545.6 ± 72.3	512.6 ± 76.6	36.1 ± 7.0	1.0 ± 0.0
<i>E. coli</i> MDAI2 pMS234	5.2 ± 2.7	407.7 ± 58.6	923.5 ± 32.7 <sup>b</sup>	78.9 ± 6.3 <sup>b</sup>	72.7 ± 24.6 <sup>b</sup>
<i>E. coli</i> MDAI2	0.2 ± 0.1 <sup>c</sup>	0.7 ± 0.0 <sup>c</sup>	NT	NT	1.3 ± 0.3

<sup>a</sup> The data are presented as the fold values relative to the endogenous level of bioluminescence by *V. harveyi* BB170 not receiving AI-2-containing supernatant (Surette & Bassler, 1998). All values represent means ± standard errors. NT, not tested.

<sup>b</sup> AI-2 production of *E. coli* MDAI2 pMS234 (complement) is significantly greater ( $p < 0.05$ ) than corresponding values of *E. coli* W3110 (wild type).

<sup>c</sup> AI-2 production of *E. coli* MDAI2 (mutant) is significantly less ( $p < 0.05$ ) than corresponding values of *E. coli* W3110 (wild type).



**TABLE 4.2** COMSTAT analysis of biofilm structure <sup>a</sup>

Strains tested	Parameters <sup>b</sup>			
	Total biomass ( $\mu\text{m}^3/\mu\text{m}^2$ )	Maximum thickness ( $\mu\text{m}$ )	Substratum coverage, percent	Roughness coefficient
<i>E. coli</i> W3110	26.4 ± 3.2 <sup>c</sup>	47.3 ± 0.8 <sup>c</sup>	73.2 ± 9.7 <sup>c, e</sup>	0.2 ± 0.0 <sup>d</sup>
<i>E. coli</i> MDAI2	10.8 ± 2.0	39.3 ± 0.5	36.2 ± 8.5	0.6 ± 0.1
<i>E. coli</i> MDpMS234	24.0 ± 4.4 <sup>c</sup>	48.0 ± 0.0 <sup>c</sup>	43.4 ± 8.9	0.2 ± 0.0 <sup>d</sup>

<sup>a</sup> Biofilms grown for 24 ± 1 h in flow cells. 8 image stacks were analyzed for each strain.

<sup>b</sup> All values represent means ± standard errors.

<sup>c</sup> Significantly larger (p < 0.05) than corresponding values in the *luxS* mutant.

<sup>d</sup> Significantly smaller (p < 0.05) than corresponding values in the *luxS* mutant.

<sup>e</sup> Significantly larger (p < 0.05) than corresponding values in the complement.

**TABLE 4.3** Differential utilization of carbon sources by the W3110 set

Compounds	Mutant / parental <sup>a</sup>	Complemented / parental <sup>a</sup>
D-Melibiose	0.71 ± 0.10	0.95 ± 0.12
Pyruvic acid methyl ester	0.80 ± 0.08	1.12 ± 0.08
D-Galactonic acid lactone	0.57 ± 0.10	1.30 ± 0.03
D-Glucuronic acid	0.85 ± 0.18	1.77 ± 0.23
Propionic acid <sup>b</sup>	0.71 ± 0.07	1.18 ± 0.45
L-Proline <sup>b</sup>	0.54 ± 0.20	1.52 ± 0.72

<sup>a</sup> Parental strain, *E. coli* W3110; mutant strain, *E. coli* MDAI2; complemented strain, *E. coli* MDAI2 pMS234.

<sup>b</sup> Not all three strains gave positive reactions.

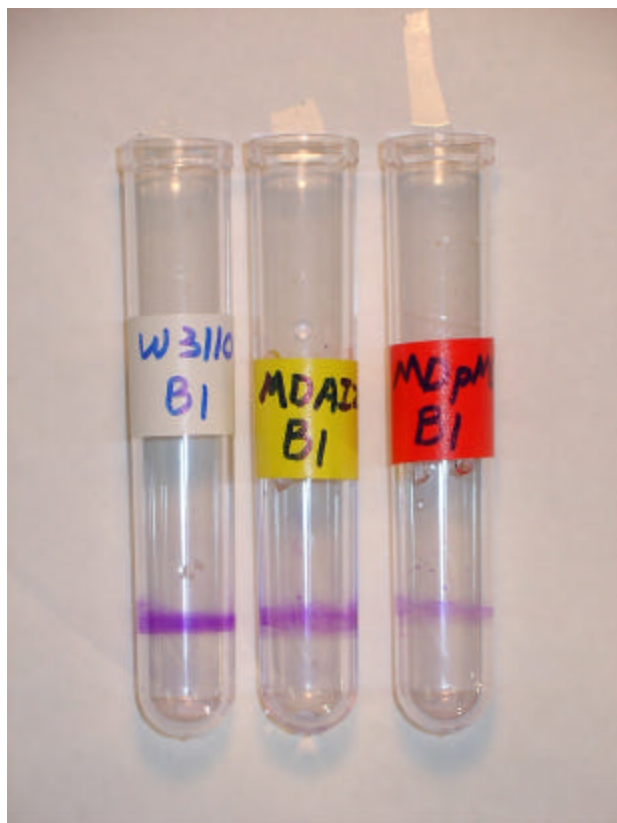
**TABLE 4.4** Uronic acids production by three strains cultivated in AB medium

Temperature (°C) / OD <sub>520</sub>	No cell control (medium only)	<i>E. coli</i>		
		<i>E. coli</i> W3110	<i>E. coli</i> MDAI2	<i>E. coli</i> MDAI2 pMS234
30	0.020	0	0.009	0.020
37	0.020	0.003	0.003	0.031

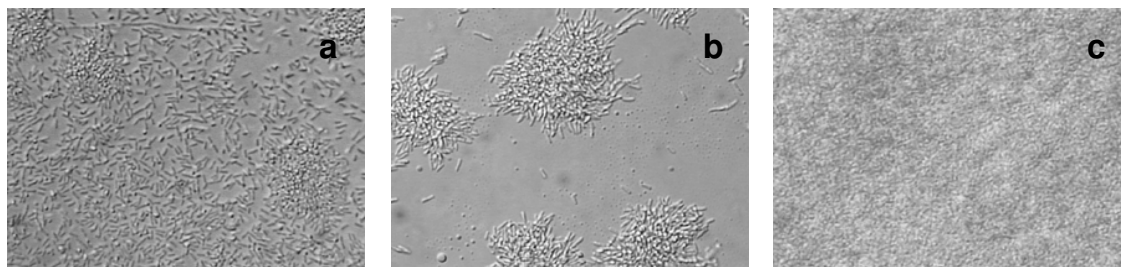
**TABLE 4.5** Antimicrobial sensitivity test

Antibiotics ( $\mu\text{g/mL}$ ) / strains	Zone of inhibition, average diameter (mm) / Sensitivity		
	<i>E. coli</i> W3110	<i>E. coli</i> MDAI2	<i>E. coli</i> MDAI2
	(wild type)	( <i>luxS</i> mutant)	pMS234 (complement)
Ampicillin (10)	18 / S <sup>a</sup>	20 / S	0 / R
Carbenicillin (100)	24 / S	27 / S	13 / R
Aztreonam (30)	27 / S	25 / S	27 / S
Erythromycin (15)	11 / R	6 / R	0 / R
Streptomycin (10)	20 / S	21 / S	14 / I
Neomycin (30)	16 / S	19 / S	13 / I
Kanamycin (30)	21 / S	23 / S	17 / I
Gentamycin (10)	19 / S	20 / S	14 / S
Totramycin (10)	19 / S	20 / S	14 / I
Amikacin (30)	20 / S	23 / S	16 / I
Imipenem (10)	29 / S	28 / S	21 / S
Cefotaxime (30)	26 / S	29 / S	25 / S

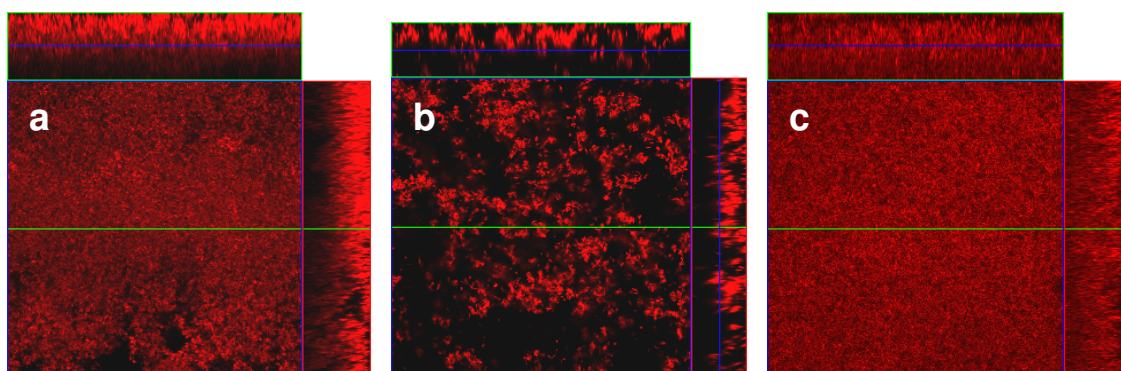
<sup>a</sup> S, susceptible; R, resistant; I, intermediate.



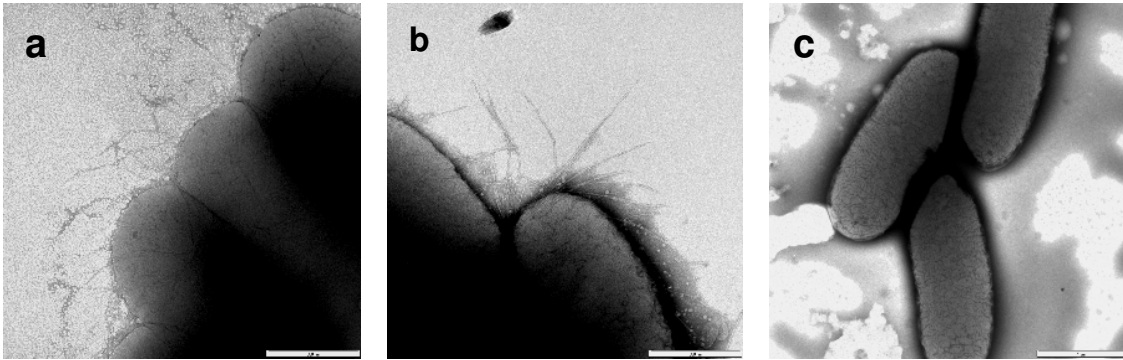
**Fig. 4.1** SBF assay of the strains in the W3110 set after  $19 \pm 1$  h shaking incubation.



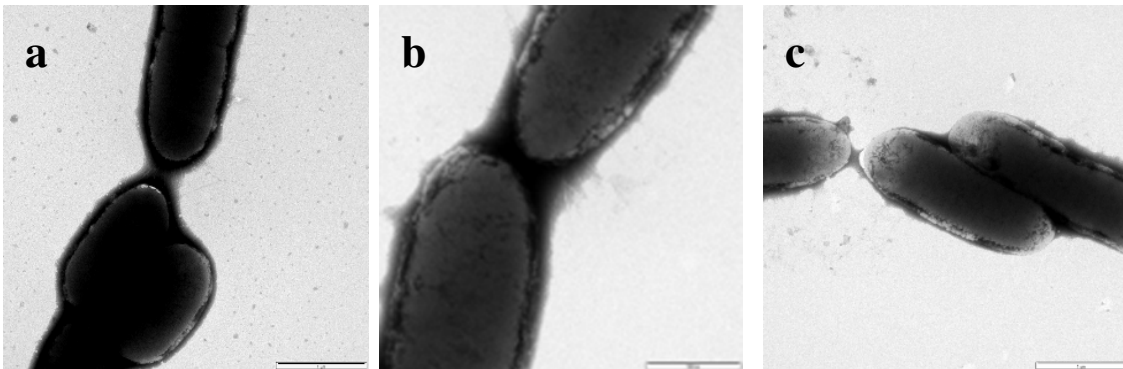
**Fig. 4.2** DIC-light microscopy images of 24 h biofilms by the W3110 set; a, *E. coli* W3110; b, *E. coli* MDAI2; c, *E. coli* MDAI2 pMS234. Magnification, 400 X.



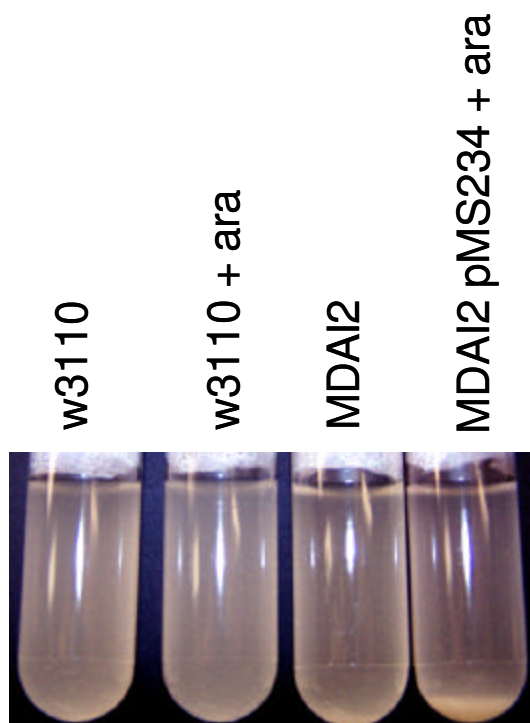
**Fig. 4.3** Vertical and horizontal sections of 24 h flow cell cultivated biofilms by the W3110 set imaged by CLSM; a, *E. coli* W3110; b, *E. coli* MDAI2; c, *E. coli* MDAI2 pMS234. Magnification, 400 X.



**Fig. 4.4a** TEM images of fimbriae formed by 14 h batch cultures of the W3110 set; a, *E. coli* W3110; b, *E. coli* MDAI2; c, *E. coli* MDAI2 pMS234.



**Fig. 4.4b** TEM images of samples from 20h biofilms by the W3110 set; a, *E. coli* W3110; b, *E. coli* MDAI2; c, *E. coli* MDAI2 pMS234.



**Fig. 4.5** Representative photo of autoaggregation by the W3110 set; ara, 0.1% w/v arabinose

## CHAPTER 5 CONCLUSION

### 5.1 THE IMPROVEMENT ON TWO WIDELY USED TECHNIQUES

Two techniques were optimized in this research. The SBF assay is a modification of Pratt and Kolter's CV staining method (Pratt & Kolter, 1998), adapted for use with growth-inhibiting compounds such as plant essential oils. In the SBF assay, the factor of growth is included in the analysis, and it benefits the examination of biofilm formation. The addition of Tween 80 in the swarming plate assay (details in Appendix A) significantly increases motility haloes in some cases and makes the assay better for screening motility-impaired mutants.

### 5.2 THE NOVELTY AND SIGNIFICANCE OF EACH CHAPTER IN THIS WORK

As demonstrated in Chapter 2, plant essential oil components may be useful in biofilm prevention. The anti-microbial effects of plant essential oils have been examined and applied in food industry for many years. However, the importance and application of plant essential oils components or natural products on biofilm prevention just started to receive attention from scientists. The work described in this dissertation has recently been supported by several publications: (1) Alviano et al. reported that essential oil components could be helpful to control the formation of microbial biofilms on fixed orthodontic appliances (Alviano *et al.*, 2005), and (2) cranberry juice was found capable of inhibiting glucan-mediated biofilm development and acid production of *S. mutans* (Koo *et al.*, 2006). As natural products, plant essential oils have been widely used in



traditional medicine (Cowan, 1999). Over 60 percent of infections in the developed world are related to biofilms (Costerton, 2001), and antibiotics may be limited in their effectiveness for treatment to infections caused by biofilms (Mah & O'Toole, 2001). Therefore, the application of plant essential oil components may be a promising approach in future after further scientific research on mechanisms, side effects and validation has been conducted. Since mixed-species biofilms exist in the normal microflora of humans, it will also be necessary to evaluate the effects of plant-derived compounds on commensal bacteria species in these environments.

In Chapter 3, we proposed that food products consisting of plant essential oil derivatives could affect QS. There is a normal microflora in many organs of humans, including mouth, respiratory tract, and gastrointestinal (GI) tract. Oral flora and GI tract each consist of more than 300 different culturable species (Probert & Gibson, 2002; Wilson *et al.*, 1997). Effects of plant essential oil derivatives or even antibiotics on QS deserve more attention because QS possibly mediates the physiology of normal flora and the interaction between bacteria and human cells (Shiner *et al.*, 2005).

In Chapter 4, the role of AI-2 mediated QS in *E. coli* biofilm formation was analyzed. This is the first report about the role of *luxS* in fimbriation and biofilm development of a non-motile *E. coli* strain. Since many factors are involved in microbial biofilm development, such as adhesins, growth phase, motility, and EPS formation (Kjelleberg & Molin, 2002), the role of QS in biofilm development appears to be varied in different microorganisms.

Overall, the research presented in this dissertation supported the concept that QS, biofilm formation, and cell adhesion may be broadly correlated. The anti-biofilm and anti-QS capability of CA implies that plant essential oil components might be promising for preventing the formation of detrimental biofilms.

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## APPENDIX A

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### ENHANCED SWARMING OF BACTERIA ON AGAR PLATES CONTAINING THE SURFACTANT TWEEN 80

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#### A.1 ABSTRACT

A widely used method for quantifying swarming motility is the swarm plate assay. A significant increase in the motility halo size formed by *Escherichia coli* or *Azospirillum brasilense* was measured on Tween 80-containing agar relative to untreated agar. This improvement could benefit the identification of mutants in swarming motility.

#### A.2 TEXT

Swarming motility is a type of flagella-mediated movement that facilitates the colonization of surfaces, and consequently is relevant to biofilm formation and pathogenesis (Harshey, 2003; Kirov, 2003; Mobley & Belas, 1995; Rozalski *et al.*, 1997). Swarming motility is often measured using the swarm plate assay (Burkart *et al.*, 1998; Harshey & Matsuyama, 1994; Ren *et al.*, 2001), where cells are spotted onto the surface of an agar plate and the diameter of the resultant “motility halo” is measured after a defined period of time. A factor that affects the motility halo size is the consistency of the agar. Harshey (1994) reported enhanced swarming when bacteria were grown on solid

media containing Eiken agar (Eiken Chemical Company, Tokyo, Japan) relative to swarming on Bacto Agar (Difco, Detroit, MI), and attributed the effect to increased surface wettability (Harshey & Matsuyama, 1994). Adding surfactin, a biosurfactant synthesized by *Bacillus subtilis*, also improved swarming by *Escherichia coli* and *Salmonella enterica*, presumably by lowering the surface tension and improving the wettability of the agar (Toguchi *et al.*, 2000). An advantage of incorporating surfactant into swarm plates is that the resultant motility halo can be large, making it easier to identify environmental and genetic factors that influence the swarming process. We investigated the possibility of using Tween 80, a commercially available anionic surfactant, to enhance the swarm plate assay. The cost of Tween 80 is substantially less than that of surfactin, which could make this assay more accessible.

The following strains were used: *Azospirillum brasilense* Sp7 (ATCC 29145; wild type), *A. brasilense* GA38 (non-motile mutant), *Escherichia coli* RP437 (Parkinson, 1978), and *E. coli* RP3098 (non-motile mutant) (Smith & Parkinson, 1980). Prior to experiments, *E. coli* was cultured at 37°C, and *A. brasilense* was cultured at 30°C. Tween 80 was obtained from Mallinckrodt Baker (Phillipsburg, NJ). To prepare swarm plates, Eiken or Bacto agar was added to 250 mL Erlenmeyer flasks each containing 40 mL Luria-Bertani (LB) broth, resulting in the concentrations listed in Tables 1 and 2. The contents were stirred and brought to a boil using a heated magnetic stir plate prior to autoclaving. After autoclaving, 8 µL of Tween 80 was gently mixed into the agar, resulting in a final surfactant concentration of 0.02%. A 50 mL sterile conical tube was used to aliquot 20 mL of agar into petri dishes, which were then solidified with lids on

and placed flat in a 30°C, non-vented incubator for 2 h prior to inoculation. Freshly prepared swarm plates were inoculated from cultures grown overnight on solid medium using sterilized toothpicks (Ren *et al.*, 2001). Plates were incubated for  $24 \pm 1$  h face up at 30°C; then motility halo diameters were measured (longest diameter for oblong halos). At least twelve replicates were conducted per treatment.

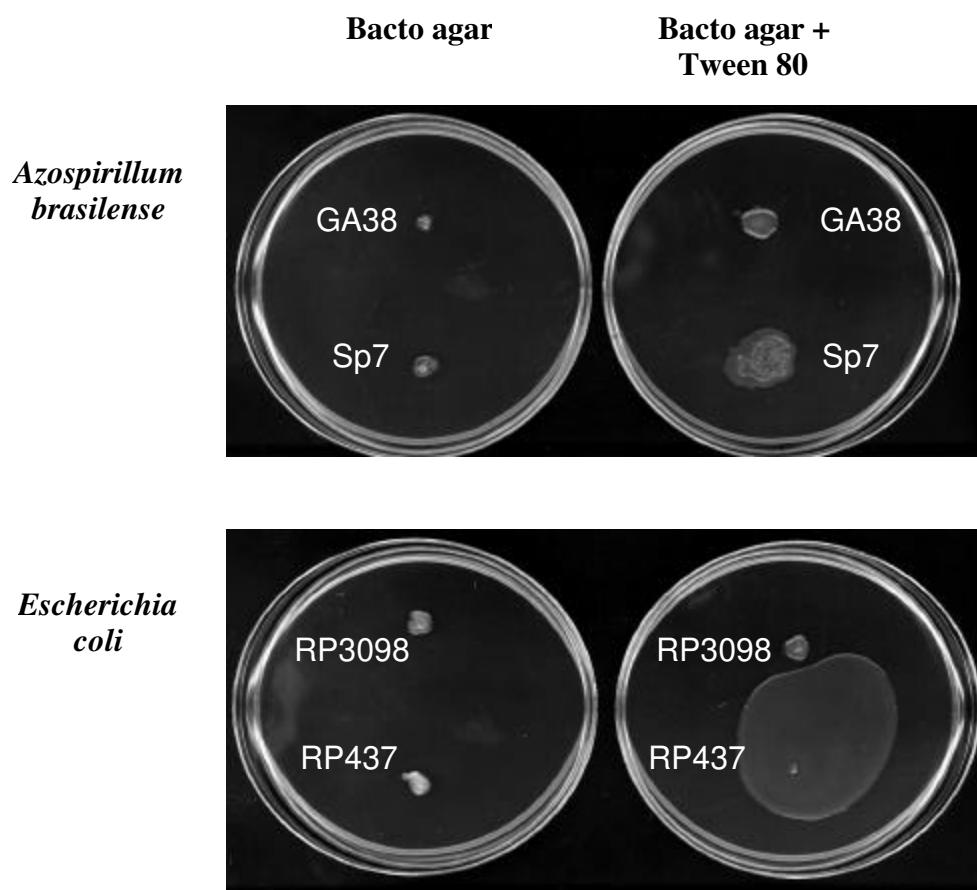
To improve the quality of the swarm plates, the following steps were taken. First, if the autoclaved agar was permitted to solidify in the Erlenmeyer flasks for storage, it was heated but not boiled before pouring. Second, the agar was not heated after the addition of Tween 80 to minimize chemical changes to the surfactant. Third, freshly poured plates were not incubated for more than two hours before inoculation. Fourth, swarm plates were used on the day that they were prepared; storing them in the refrigerator led to erroneous results. Differences in swarming motility were tested for significance using Student's t-test.

Tween 80 significantly increased the average motility halo diameters of the wild type strains (**Table A.1, Fig. A.1**). To determine whether the increase in the motility halo diameter was due to non-specific factors, swarming by motile and non-motile strains was compared. In all cases, the average motility halo diameter of wild type *E. coli* was significantly larger than its corresponding non-motile mutant on Tween-containing agar (**Table A.1**), and for *A. brasilense*, at agar concentrations of 0.7% and 0.8% (**Table A.2**). There was no evidence of Tween 80 toxicity under the conditions that were examined. The effect of Eiken agar on the motility halo diameter size of *E. coli* and *A. brasilense* was evaluated. Modest increases were observed at select agar concentrations (**Table A.1**,

**Table A.2).** To determine the relative benefit of Tween 80 on swarming, the ratios of the average motility halo diameters of Tween-containing Bacto agar to unamended Bacto agar were calculated (**Table A.1, Table A.2**). For wild type *E. coli*, ratios ranged from 2.8 to 7.2 for agar concentrations between 0.6% and 0.8%. These ratios were notably larger than those of the non-motile mutant. For *A. brasilense*, there were no major differences in the ratios between the wild type and non-motile strains. Ratios ranging from 1.2 to 2.3 were observed for the non-motile strains, indicating that Tween 80 contributed to flagella-independent translocation, such as sliding or spreading (Kinsinger *et al.*, 2003; Matsuyama *et al.*, 1995).

There are several benefits that emerged from adding Tween 80 to swarm plates. First, Tween 80 significantly increased the extent of *E. coli* swarming. Advantages of a large motility halo include ease of measurement and reduced relative error. The motility halos that formed on agar containing Tween 80 were similar in size to halos measured on swarm plates containing surfactin (Toguchi *et al.*, 2000). Second, the large motility halos resulting from the addition of Tween 80 improved the sensitivity of the swarm plate assay, which could benefit the identification of swarming mutants. Third, larger motility halos were formed at higher agar concentrations. Consequently, swarming could be measured on more rigid agar surfaces, reducing the likelihood of the agar not solidifying uniformly, tearing, or undesired swimming motility that can occur at low agar concentrations. The conditions required for optimal swarming may be strain specific. Others have reported that Tween 80 can promote bacterial motility (Van Der Drift & De Jong, 1974) or inhibit it (Henrichsen, 1975; Matthews *et al.*, 1978). Consequently, an

initial screening would be recommended to assess the utility of the technique described in this paper.



**Figure A.1** Effect of Tween 80 on the motility halo size of *A. brasilense* and *E. coli*. *E. coli* RP437: wild type; *E. coli* RP3098: non-motile mutant. *A. brasilense* Sp7: wild type; *A. brasilense* GA38: non-motile mutant. Left, swarm plates prepared with unamended 0.6% Bacto agar. Right, swarm plates containing 0.6% Bacto agar plus 0.02% Tween 80.

**TABLE A.1** Swarming analysis of *E. coli* RP437 and *E. coli* RP3098 <sup>a</sup>

Strains	Agar type	Agar concentration			
		0.5%	0.6%	0.7%	0.8%
<i>E. coli</i> RP437 (motile)	Eiken	8 ± 2 (12)	7 ± 2 (16)	7 ± 3 (16) <sup>c</sup>	7 ± 1 (16)
	Bacto only (B)	25 ± 14 (16) <sup>b</sup>	6 ± 1 (16)	6 ± 2 (16)	10 ± 9 (14) <sup>b</sup>
	Bacto + Tween 80 (T80)	33 ± 4 (8) <sup>e</sup>	43 ± 24 (11) <sup>d,e</sup>	30 ± 21 (16) <sup>d,e</sup>	28 ± 20 (14) <sup>d,e</sup>
	Ratio of T80 to B	1.3	7.2	5.0	2.8
<i>E. coli</i> RP3098 (non- motile)	Eiken	9 ± 3 (12)	8 ± 3 (18)	8 ± 2 (20)	8 ± 2 (20)
	Bacto only	7 ± 2 (20)	10 ± 10 (20)	7 ± 2 (20)	7 ± 2 (20)
	Bacto + Tween 80	16 ± 11 (11) <sup>d</sup>	13 ± 9 (18)	10 ± 4 (20) <sup>d</sup>	12 ± 11 (20) <sup>d</sup>
	Ratio of T80 to B	2.3	1.3	1.4	1.7

<sup>a</sup> The number of samples is given in parentheses. All values represent means ± standard deviations of swarming halo diameters (mm).

<sup>b</sup> Diameter of halo on Bacto agar is significantly larger ( $p < 0.05$ ) than corresponding values on Eiken agar.

<sup>c</sup> Diameter of halo on Eiken agar is significantly larger ( $p < 0.05$ ) than corresponding values on Bacto agar.

<sup>d</sup> Diameter of halo on Bacto agar + Tween 80 is significantly larger ( $p < 0.05$ ) than corresponding values on Bacto agar.

<sup>e</sup> Halo diameter of motile strain is significantly larger ( $p < 0.05$ ) than corresponding values for non-motile mutant.

**TABLE A.2** Swarming analysis of *A. brasilense* SP7 and *A. brasilense* GA38 <sup>a</sup>

Strains	Agar type	Agar concentration			
		0.5%	0.6%	0.7%	0.8%
<i>A. brasilense</i> SP7 (motile)	Eiken	9 ± 4 (32)	11 ± 11(32) <sup>c</sup>	9 ± 4 (32)	10 ± 4 (32)
	Bacto only (B)	8 ± 5 (32)	8 ± 3 (32)	8 ± 3 (32)	10 ± 4 (32)
	Bacto + Tween 80 (T80)	16 ± 8 (22) <sup>d</sup>	13 ± 5 (32) <sup>d</sup>	15 ± 9 (32) <sup>d,e</sup>	13 ± 8 (28) <sup>d,e</sup>
	Ratio of T80 to B	2.0	1.6	1.9	1.3
<i>A. brasilense</i> GA38 (non-motile)	Eiken	6 ± 1 (20)	6 ± 1 (20)	6 ± 1 (16)	6 ± 1 (20)
	Bacto only	9 ± 5 (20)	6 ± 1 (20)	6 ± 2 (20)	6 ± 1 (20)
	Bacto + Tween 80	19 ± 14 (16) <sup>d</sup>	10 ± 4 (20) <sup>d</sup>	9 ± 3 (20) <sup>d</sup>	7 ± 1 (20) <sup>d</sup>
	Ratio of T80 to B	2.1	1.7	1.5	1.2

<sup>a</sup> The number of samples is given in parentheses. All values represent means ± standard deviations of swarming halo diameters (mm).

<sup>b</sup> Diameter of halo on Bacto agar is significantly larger ( $p < 0.05$ ) than corresponding values on Eiken agar.

<sup>c</sup> Diameter of halo on Eiken agar is significantly larger ( $p < 0.05$ ) than corresponding values on Bacto agar.

<sup>d</sup> Diameter of halo on Bacto agar + Tween 80 is significantly larger ( $p < 0.05$ ) than corresponding values on Bacto agar.

<sup>e</sup> Halo diameter of motile strain is significantly larger ( $p < 0.05$ ) than corresponding values for non-motile mutant.



## APPENDIX B

### DYNAMICS OF AUTOINDUCER-2 PRODUCTION BY AN *ESCHERICHIA COLI* BIOFILM

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#### B.1 ABSTRACT

In order to gain insight into the ecology of autoinducer-2 (AI-2) production by *Escherichia coli* in a biofilm setting, biofilms of *E. coli* ATCC 33456 were cultivated in flow cells under continuous flow conditions and AI-2 production was monitored as a function of time. Biofilms were supplied with fresh LB medium over the course of experiments, and AI-2 was sampled periodically from the flow cell effluent during cultivation. Using a bioreporter assay based on *Vibrio harveyi* BB170, the activity of AI-2 could be monitored by measuring bioluminescence. Measurement of AI-2 levels in the effluent indicated that the level of AI-2 activity increased during the first 24 hours and then was maintained at 50 to 100 times the levels observed in controls for at least an additional 72 hours. In contrast, the AI-2 activity in LB-grown batch cultures reached a peak value (2700-fold increase relative to control) during early stationary phase but returned to background levels within 24 h after inoculation. Growth media composition affected AI-2 production by *E. coli*, since cells cultured in glycerol containing autoinducer bioassay (AB) medium maintained a high concentration of AI-2 (3600-fold increase relative to control) in batch culture after 24 h. These observations indicated several potential factors that could influence AI-2 synthesis in planktonic culture by *E.*

*coli*, including nutrient availability and growth phase. In general, the results suggest that *E. coli* living in biofilms can produce AI-2 over extended periods of time, and could potentially influence the activity of neighboring AI-2 responsive bacteria.

## B.2 INTRODUCTION

*E. coli* resides in the human colon within the mucus layer, living in a biofilm-like microflora. *E. coli* synthesizes the QS signal molecule through LuxS. In the past several years, AI-2 has been proposed to be related to biofilm formation in some microorganisms (Merritt *et al.*, 2003; Prouty *et al.*, 2002; Ren *et al.*, 2001). One of the features of the intestinal environment is a relatively continuous flow of nutrients. The objective of this study is to examine the AI-2 levels of *E. coli* ATCC 33456 in batch culture and flow cell cultivated *E. coli* biofilms.

## B.3 MATERIALS AND METHODS

### B.3.1 Strains and media

*Escherichia coli* ATCC 33456 (Wang & Shen, 1995) and *Vibrio harveyi* BB170 (Bassler *et al.*, 1997) were used in this study. *E. coli* ATCC 33456 was cultured in Luria-Bertani (LB) broth at 37°C, and *V. harveyi* BB170 was grown in autoinducer bioassay (AB) medium at 30°C (Greenberg *et al.*, 1979). The principal carbon source in AB medium is 1 % v/v glycerol.

### **B.3.2 Flow cell cultivation of *E. coli* ATCC 33456 biofilm and effluent collection**

Biofilms were cultivated in flow cells (Gilbert & Keasling, 2004) as demonstrated in section 2.2.5. 1 mL effluent was collected into a 1.7 mL sterile centrifuge tube from the tubing on the distal end of the flow cell for per channel at various time points until 120 h. Cell-free supernatants from the flow chamber effluents were prepared by centrifugation at 8,000 rpm for 10 min followed by filter-sterilization. All cell-free supernatants were kept at -20 °C prior to testing.

### **B.3.3 Bioluminescence assay to test AI-2 activity**

The bioluminescence assay that was used is a modification of the AI-2 cell signaling assay (Surette & Bassler, 1998; Ren *et al.*, 2001). The principle behind the assay is that AI-2 can induce bioluminescence expression of reporter strain *V. harveyi* BB170 (AI-1 sensor -, AI-2 sensor +) (Bassler *et al.*, 1993). AI-2 activity was expressed as fold value of endogenous levels of bioluminescence of *V. harveyi* BB170 cells (Surette & Bassler, 1998). The details of this technique were described in section 3.2.4.

## **B.4 RESULTS**

### **B.4.1 AI-2 production by *E. coli* ATCC 33456 batch culture in LB and AB medium**

Levels of AI-2 activity in *E. coli* batch culture were examined as a function of growth media (Fig. B.1). The AI-2 activity in LB-grown batch cultures reached a peak value (2700-fold increase relative to control) during early stationary phase but returned to background levels within 24 hours after inoculation. Growth media composition affected

AI-2 production, since cells cultured in AB medium maintained a high concentration of AI-2 (3600-fold increase relative to control) in batch culture after 24 hours. During the same experiments, growth in LB medium reached a maximum OD<sub>600</sub> of  $2.14 \pm 0.07$ , whereas growth in AB medium reached a maximum OD<sub>600</sub> of  $2.19 \pm 0.30$ .

#### **B.4.2 AI-2 production by *E. coli* ATCC 33456 biofilms**

The AI-2 production in effluents of *E. coli* ATCC 33456 biofilms cultivated in flow cells was measured (**Fig. B.2**). After the 2 h recirculation period, AI-2 activity was approximately 8 times the activity level measured in the control. The system was switched to continuous flow at 2 h. Between 2 and 8 h after the switching from recirculation to continuous cultivation, the level of AI-2 production was less than the level of the control. From 8 h to 20 h, there was an exponential increase of AI-2 activity, and from 20 h to 120 h, AI-2 activity remained between 50 to 100 times the level of the control.

#### **B.4.3 AI-2 production by *E. coli* ATCC 33456 biofilms with varying nutrient supply**

To determine whether a steady supply of nutrients was required to maintain AI-2 production, biofilms were grown in an alternating supply of LB broth and 50 mM KH<sub>2</sub>PO<sub>4</sub> buffer after every 24 h till 72 h. A change in measured AI-2 levels correlated with the switched bottles of LB and KH<sub>2</sub>PO<sub>4</sub> buffer (**Fig. B.3**). The AI-2 level increased in the presence of LB but decreased in the presence of KH<sub>2</sub>PO<sub>4</sub> buffer.

#### **B.4.4 Effect of temperature and exposure time on AI-2 stability of *E. coli* ATCC 33456 supernatants**

Supernatants were collected from 6 h shaking incubated *E. coli* cultures in LB and AB medium. Effects of temperature and exposure time on the AI-2 activity in cell-free supernatants were determined (**Fig. B.4**). AI-2 activity dramatically decreased after 2 h for all three tested temperatures (22.5 °C, 30 °C, and 37 °C). There was approximately 20% activity left compared to the original activity after 8 h exposure for all three temperatures.

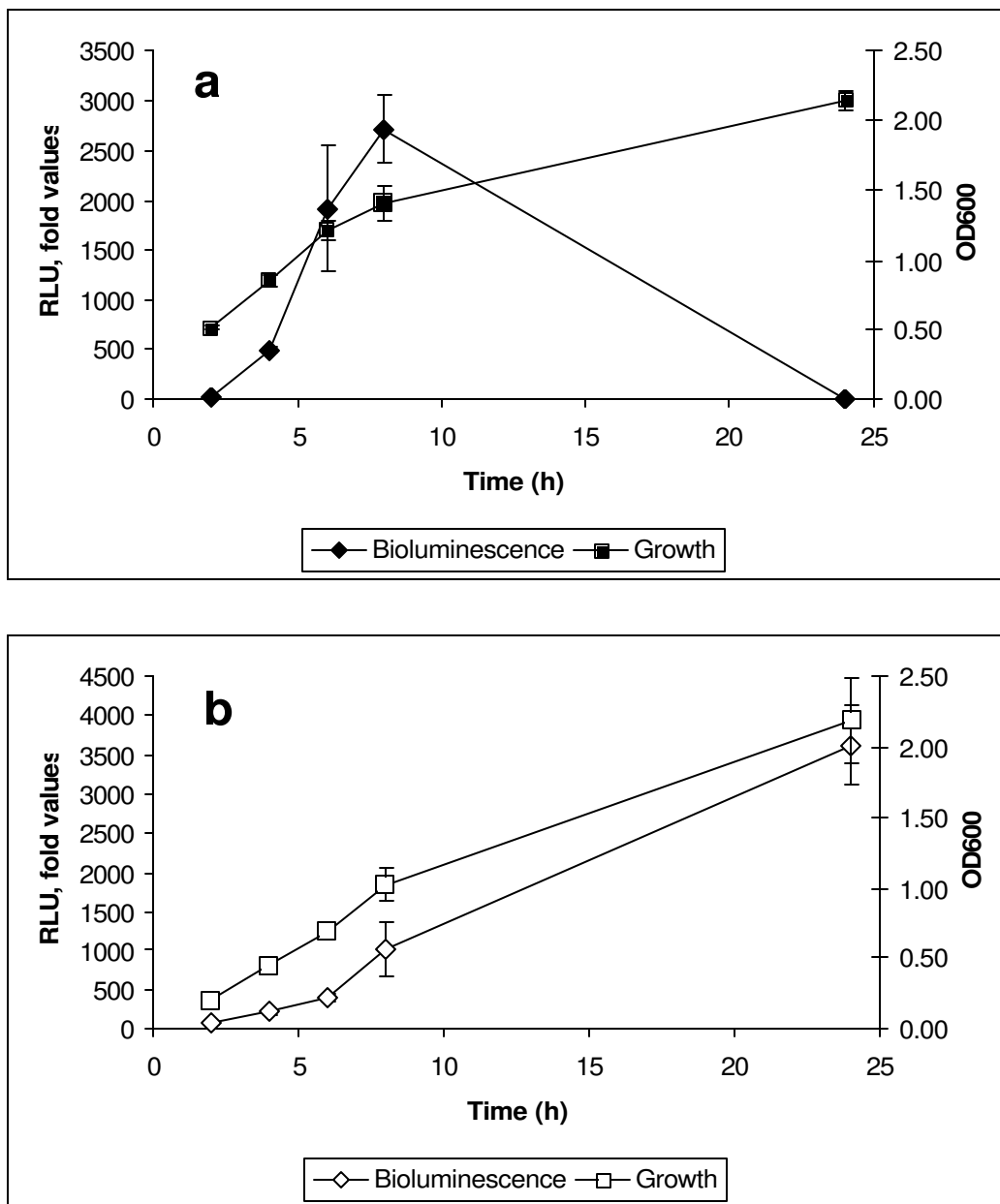
### **B.5 DISCUSSION**

#### **B.5.1 Variation of AI-2 activity in the development of *E. coli* biofilm**

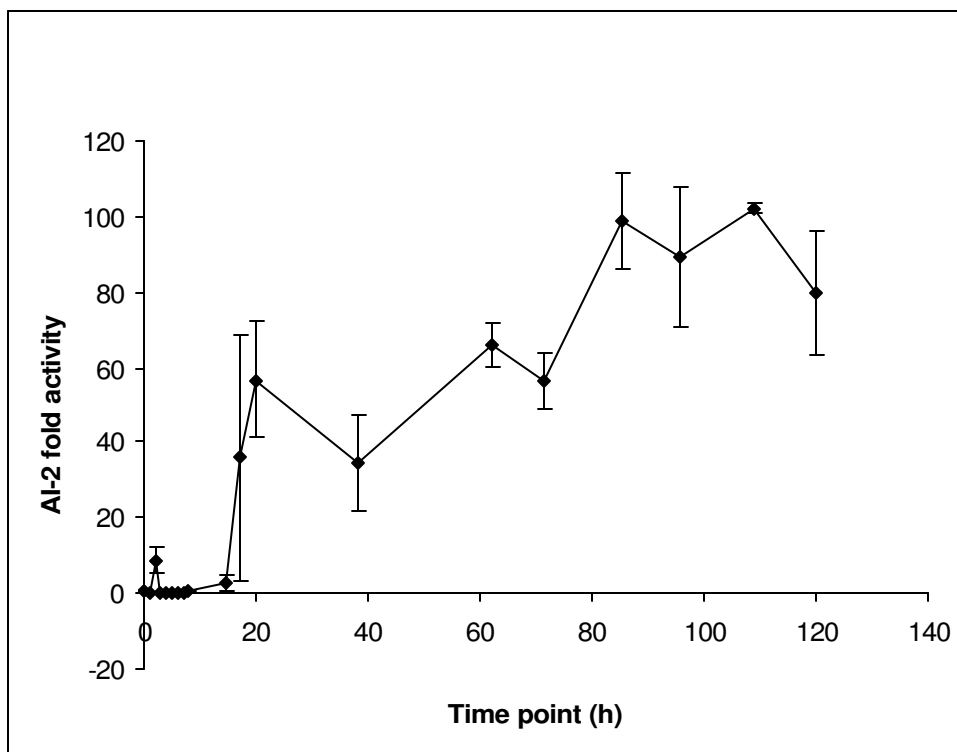
The variation of AI-2 activity in *E. coli* is considered as an indicator of nutrient availability in media and growth potential of the planktonic population (Xavier & Bassler, 2003). In this investigation, AI-2 activity was maintained over the course of biofilm development under continuous cultivation in LB medium (**Fig. B.2**), but dropped quickly in response to a shortage of nutrients (**Fig. B.3**). This work suggests that AI-2 molecules would be present in the gut as long as food was present. AI-2 secreted by *E. coli* could affect other species since many bacteria recognize AI-2 as an interspecies QS signal.

### **B.5.2 Limitation of usage of AI-2 contained supernatant in autoinducer bioassay**

In acyl homoserine lactone (HSL) mediated QS, several stable inducer compounds have been synthesized (Smith *et al.*, 2003) and commercial products (e.g. 3-oxo-C6- and 3-oxo-C12-HSL) are practically applicable in research. There is no commercially synthesized AI-2 at this time, thus AI-2 contained bacterial culture supernatant is widely used in AI-2 mediated QS research. However, it was found in this study that AI-2 activity in cell culture supernatant declines under common experimental temperatures (22.5 °C, 30 °C, and 37 °C) for culturing many bacteria. This is an important consideration for using AI-2 containing bacterial culture supernatant in research. It is recommended that AI-2 containing cell culture supernatants will be most effective for short experiments, up to 3 or 4 h.

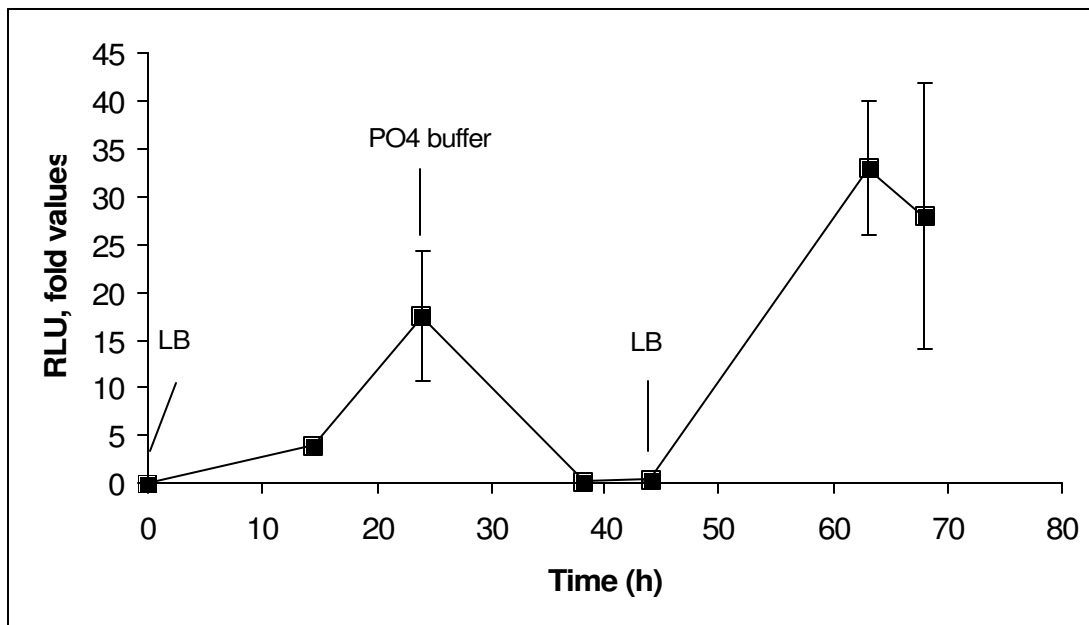


**Fig. B.1** AI-2 production by *E. coli* ATCC 33456 in batch cultures shaking incubated at 37°C. a, LB medium; b, AB medium.

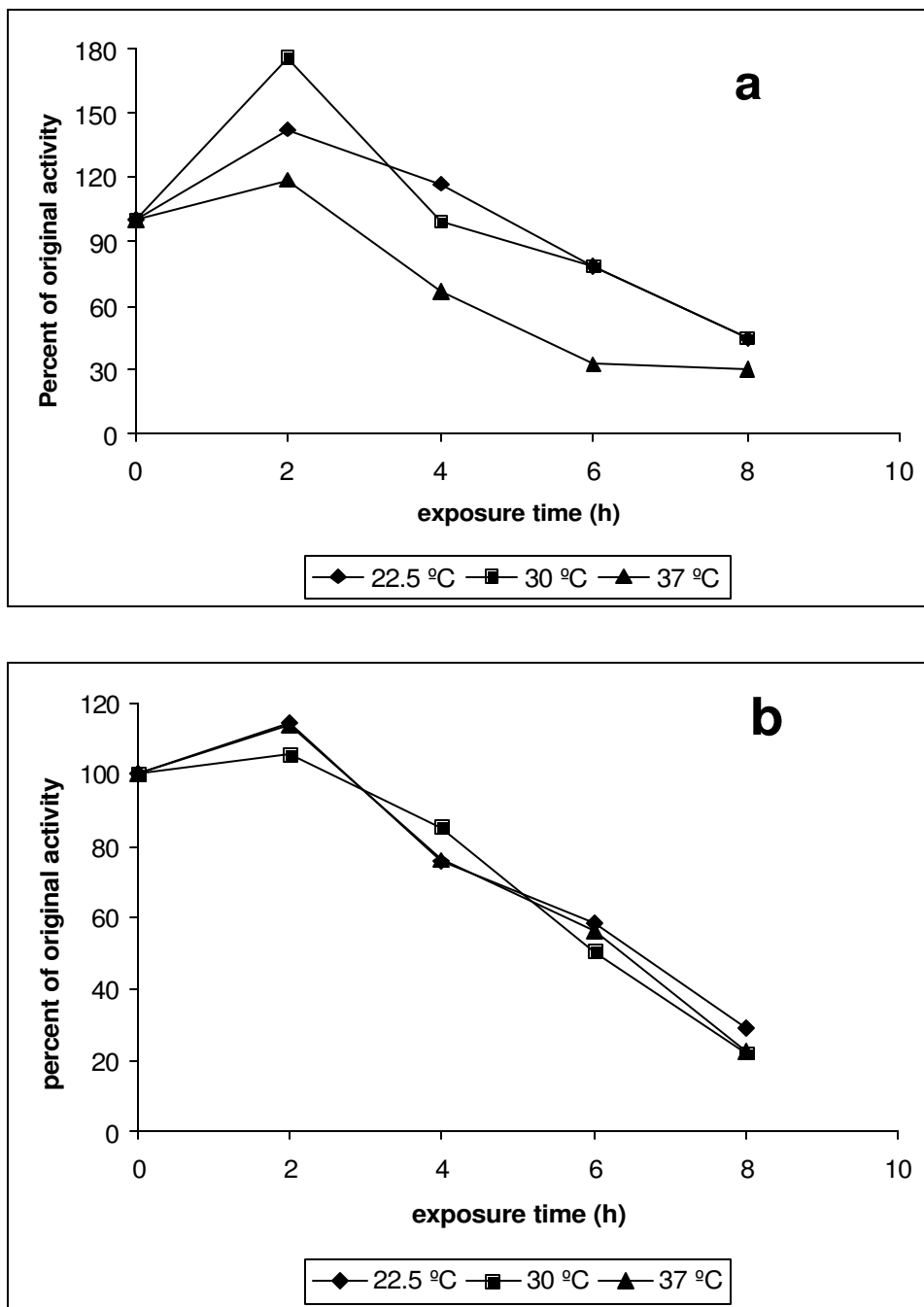


**Fig. B.2** AI-2 activity of *E. coli* ATCC 33456 in chamber effluents during flow cell cultivation.





**Fig. B.3** AI-2 activity of *E. coli* ATCC 33456 in chamber effluents during flow cell cultivation.



**Fig. B.4** Exposure temperature and time on degradation of AI-2 activity of *E. coli* ATCC 33456. Supernatants were collected from a, LB medium or b, AB medium.