Signaling in *Escherichia coli* Biofilms

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**Abstract**

Cell signaling in *Escherichia coli* biofilms is more important than originally known, in that autoinducer two (AI-2), AI-1 (N-acylhomoserine lactones), indole, hydroxylated indoles, norepinephrine, and epinephrine are all functional signals in this organism. This gives the bacterium the ability to monitor not only the presence of cells of its own species (through AI-2 and indole) but also the activity of the human host (through norepinephrine and epinephrine) as well as that of the other commensal bacteria (through hydroxylated indoles).

We have also found that *E. coli* monitors the presence of bacteria that produce acylhomoserine lactones as these signals influence formation of its biofilms through the LuxR homolog SdiA. Hence, *E. coli* monitors its surroundings through signals it synthesizes as well as those synthesized by both prokaryotes and eukaryotes. In this chapter, we present recent findings on the role of these different signals as well as provide a detailed account of the complex features of uptake and regulation of the AI-2 signal in *E. coli*.

**AI-2 signaling through the motility quorum sensing regulator, MqsR**

Cell signaling (quorum sensing) is established for biofilm formation in bacteria. For example, it controls the production and secretion of exopolysaccharides for *Vibrio cholerae* biofilms (Hammer and Bassler, 2003). This signaling may be complex as *V. harveyi* uses three quorum sensing signals including N-(3-hydroxybutanoyl) homoserine lactone (AI-1), furanosyl borate diester or related compounds (AI-2) (Lombardia *et al.*, 2006), and a signal, synthesized by CqsA, whose structure is unknown (Henke and Bassler, 2004). Other examples include the quorum signal N-(3-oxododecanoyl)-L-homoserine lactone which controls biofilm formation in *P. aeruginosa* (Davies *et al.*, 1998), and the quorum signal N-butanoyl-L-homoserine lactone which controls biofilm formation in *Serratia liquefaciens* MG1 (Labbate *et al.*, 2004).

AI-2 is produced by the enzymes Pfs (nucleosidase) and LuxS (terminal synthase) from S-adenosylhomocysteine and is a species non-specific signal used by both Gram-negative and Gram-positive bacteria (Surette *et al.*, 1999) which has been found in at least 55 strains (Bassler *et al.*, 1997). We have focused our AI-2 based studies on *E. coli* biofilms since *E. coli* is the most thoroughly studied bacterium (Blattner *et al.*, 1997), its genome is sequenced...
(Blattner et al., 1997), microarrays are available (Selinger et al., 2000), the function of many of its proteins have been elucidated (Riley and Labedan, 1996), and many isogenic mutants are available (Kang et al., 2004). Also, our group has experience in determining the genetic basis of *E. coli* biofilm formation and its inhibition with natural, plant-derived antagonists such as furanone and ursolic acid (Ren et al., 2001; Ren et al., 2004). For *E. coli*, three groups have used DNA microarrays to show that AI-2 controls 166 to 404 genes including those for chemotaxis, flagellar synthesis, motility, and virulence factors (Beloin et al., 2004; Ren et al., 2004a; Schembri et al., 2003). Remarkably, 80% of these same phenotypes were found to be regulated by the quorum sensing inhibitor furanone in an opposite manner (Ren et al., 2004b); addition of furanone reduces biofilm formation for *E. coli* (Ren et al., 2001; Ren et al., 2004b). This shows the importance of AI-2 as a quorum signal in *E. coli*. A recent report (Walters et al., 2006) has attributed the function of AI-2 as more growth-related in *E. coli* because its deletion affects homocysteine synthesis; however, the authors used a non-robust phenotype method (Biolog) to survey the impact of a *luxS* mutation so that their results were restricted to changes in these growth phenotypes, rather than being able to address the regulatory functions of AI-2.

As the first clear example of the impact of AI-2 and biofilm formation and of the importance of AI-2 on phenotypes of a bacterium other than *Vibrio*, we demonstrated that *in vitro* synthesized AI-2 from purified Pfs and LuxS directly stimulates *E. coli* biofilm formation by 30-fold in the absence of a conjugation plasmid (González Barrios et al., 2006). Note that in the absence of AI-2, *E. coli* BW25113 ΔluxS made 50% less biofilm compared to the isogenic wild-type strain, which indicates again that AI-2 stimulates biofilm formation in *E. coli* since LuxS forms AI-2 (González Barrios et al., 2006). Biofilm could be restored by complementing *luxS* in *trans* using plasmid pCA24N *luxS* + (46% of the wild-type biofilm was formed at 0 mM IPTG and 110% of the wild-type biofilm was formed at 0.25 mM IPTG in LB medium). To confirm that AI-2 is the cause of the increase in biofilm formation, we measured (González Barrios et al., 2006) biofilm stimulation with the isogenic MG1655 *lsrK* mutant because this mutation dramatically impairs the AI-2 uptake compared with other mutations in the *lsr* system (Wang et al., 2005a; Xavier and Bassler, 2005a). AI-2 phosphokinase, LsrK, is one of the essential components of the LuxS regulated AI-2 uptake operon, *lsrADCBFG* (Wang et al., 2005a). As expected, AI-2 was not able to induce biofilm formation of the *lsrK* mutant at 6.4 μM; hence, AI-2 induces biofilm formation through the LsrK transport pathway (Wang et al., 2005a). Note that the presence of a conjugation plasmid alters dramatically how *E. coli* forms a biofilm (Ghigo, 2001; Reisner et al., 2003). Showing that purified AI-2 controls biofilm formation is significant, as other groups have relied on the use of conditioned medium or *luxS* mutations (Balestrino et al., 2005; Cole et al., 2004; Labbate et al., 2004; Li et al., 2001; McNab et al., 2003), and this result demonstrates that AI-2 regulates more than the *lsr* operon (Walters et al., 2006).

We then set about to determine how AI-2 controls biofilms by studying motility since this trait has been linked to biofilm formation in the absence of a conjugation plasmid (Pratt and Kolter, 1998) and since our microarrays (Ren et al., 2004a) indicated that motility genes were induced by AI-2 (determined using a *luxS* mutant). It was found that the motility of several *E. coli* K-12 strains (ATCC 25404, MG1655, DH5α, and BW25113)
increased by about 30–80% upon addition of 0.8 to 3.2 μM AI-2 (González Barrios et al., 2006).

To discern the genetic basis of this increase in motility upon AI-2 addition, we probed the ability of AI-2 to induce the promoters of motility genes qseB, flhD, fliA, fliC, and motA (González Barrios et al., 2006). Upon addition of 6.4 μM AI-2, the quorum-sensing flagella regulon qseB (Sperandio et al., 2002) was induced 8-fold (González Barrios et al., 2006). These results corroborate the ones reported by Sperandio et al. (2002) who previously found qseB to be induced 17-fold compared with the luxS mutant through DNA microarray studies using E. coli O157:H7 and its isogenic luxS mutant. The induction of qseB here led to a 4.0 fold increase in transcription of flhD (master controller of the flagella regulon), 2.6 fold increase of fliA (sigma factor S₂₈), 3.6-fold of fliC (flagellin), and 6-fold increase of motA (proton conductor for flagella movement) (González Barrios et al., 2006).

Given that the purified AI-2 controlled motility (González Barrios et al., 2006), that b3022 is induced 8-fold in biofilms (Ren et al., 2004a) and is nearby qseB (b3025) which is clearly induced by the addition of purified AI-2, we hypothesized that B3022 may interact with AI-2 and control biofilm formation. B3022 is a conserved regulator protein (98 amino acids) since it has more than 50% identity with hypothetical proteins from Yersinia pseudotuberculosis, Y. pestis, Cupriavidus oxalaticus, Bordetella bronchiseptica, P. fluorescens, and Bordetella pertussis (Chain et al., 2004; Parkhill et al., 2003; Paulsen et al., 2005; Song et al., 2004; Toussaint et al., 2003). As part of the 300-gene, quorum-sensing regulon in E. coli (Beloin et al., 2004; Ren et al., 2004a; Schembri et al., 2003), qseBC (b3025, b3026) are organized in an operon in the E. coli chromosome with QseB playing a role as a response regulator and QseC, the sensor kinase (Sperandio et al., 2002). Flagella expression is temporally regulated, and the operons are divided into early, middle, and late genes. QseBC regulates transcription of the master regulon flhDC and therefore expression of the middle operon (e.g. fliA encoding sigma factor S₂₈) and late operon (e.g. fliC encoding flagellin, motA encoding the proton exchange conductor for flagella movement) (Chilcott and Hughes, 2000).

We found that deletion of b3022 abolished motility which was restored by expressing b3022 in trans (González Barrios et al., 2006). Deletion of b3022 also decreased biofilm formation significantly relative to the wild-type strain in three media (46–74%) in 96 well plates as well as decreased biomass (8-fold) and substratum coverage (19-fold) in continuous flow cells with minimal medium (growth rate not altered and biofilm restored by expressing b3022 in trans). Deleting b3022 changed the wild-type biofilm architecture from a thick (54-μm), complex structure to one that contained only a few microcolonies (González Barrios et al., 2006). B3022 positively regulates expression of qseBC, flhD, fliA, and motA since deleting b3022 decreased their transcription by 61-, 25-, 2.4-, and 18-fold, respectively (González Barrios et al., 2006). Transcriptome analysis also revealed that B3022 induces crl (26-fold) and flhCD (8 to 27-fold). Adding AI-2 (6.4 μM) increased biofilm formation of wild-type K12 MG1655 but not that of the isogenic b3022, qseBC, fliA, and motA mutants. Adding AI-2 also increased motA transcription for the wild-type strain but did not stimulate motA transcription for the b3022 and qseB mutants; therefore, the induction of motility is likely mediated by both MqsR and QseBC.
Evidence that mqsR is first in the cascade was provided by measuring the transcription of qseB with the wild-type strain and the b3022 mutant upon addition of AI-2. If B3022 is first in the cascade and necessary for the transduction of the AI-2 signal, then the addition of AI-2 should only increase qseB transcription when b3022 is present (González Barrios et al., 2006). We found that adding AI-2 at 6.4 μM induced the expression of qseB 3.2 fold in the wild-type strain in M9C glu but did not induce the qseB in the b3022 mutants. As expected, the wild-type strain responded to AI-2 addition in a dose-dependent manner. To show further that MqsR is first in the cascade, the expression of qseB from pVS159 was also measured while inducing B3022 expression in trans in the mqsR mutant by adding IPTG to strains harboring pVLT31 b3022+. As expected if B3022 is required for signal transduction to QseB, expression of qseB was induced 4-fold in a dose-dependent manner in M9C glu (see Figure 7.6B). Together, these results indicate that AI-2 induces biofilm formation in E. coli through B3022 (either directly or indirectly), which then regulates QseBC and motility; hence, b3022 has been re-named motility quorum sensing regulator (mqsR), and B3022 is the master regulator of motility (Figure 7.1).

**AI-2 import through LsrACDB and export through the quorum-sensing transport protein TqsA**

Since AI-2 is highly polar, we reasoned that it must be actively transported from the cell. It has been previously established that internalization of AI-2 requires an ABC transporter. Moreover, we have hypothesized that the formation of AI-2 from its precursor, 4,5-dihydro-
droxy-2,3-pentanedione (DPD) may require biological assistance. However, the means by which AI-2 is exported has not been identified.

AI-2 induces transcription of the \textit{lsrACDBFGE} operon of \textit{Salmonella typhimurium} in which the first four genes encode an ATP binding cassette-type import protein with high homology to the ribose transporter (Taga et al., 2001; Taga et al., 2003). In \textit{E. coli}, the proteins for AI-2 transport are encoded by \textit{lsrACDBFG} (Wang et al., 2005a; Wang et al., 2005b). AI-2 is phosphorylated inside the cell by LsrK, and the phosphorylated AI-2 induces inactivation of the \textit{lsr} operon repressor LsrR (Taga et al., 2003; Wang et al., 2005a); hence, AI-2 uptake is enhanced. Surprisingly, expression of \textit{lsrR}, \textit{lsrK}, \textit{tam}, and \textit{yneE}, which flank the \textit{lsrACDBFG} operon, were significantly induced by \textit{luxS} (2.2, 5.5, 2.8 and 3.5-fold respectively (Wang et al., 2005b)). We investigated their regulation in detail and found that deletion of \textit{lsrR} significantly increased \textit{lsrR} expression, indicating that the \textit{lsrR} transcription was autorepressed. Additionally, the intergenic region between the \textit{lsrR} and \textit{lsr} operon, which are divergently transcribed, revealed a CRP binding site which has a typical 6-bp spacer between two conserved motifs. Gel mobility shifts were used to demonstrate CRP binding to the intergenic region containing this site. Moreover, we discovered that \textit{lsrR} and \textit{lsrK} are transcribed into one mRNA, forming an \textit{lsrRK} operon, which is repressed by LsrR and activated by CRP. Interestingly, the \textit{tam} gene was also found to be a component of the \textit{lsr} operon. This gene encodes an \textit{S}-adenosyl-\textit{L}-methionine-dependent methyltransferase, which catalyzes the methyl esterification of trans-aconitate. The trans-aconitate appears to be formed spontaneously from the citric acid cycle intermediate cis-aconitate. The benefit of methylation of the trans-aconitate to the \textit{E. coli} cells is not clear.

The addition of sugars that are part of the phospho-transferase system increase AI-2 extracellular activity to its maximum level in the mid to late exponential phase (Surette and Bassler, 1998) through a decrease of adenosine 3’,5’-cyclic monophosphate (cAMP) concentration (Wang et al., 2005a). Once glucose is depleted, cAMP concentrations increase, the \textit{lsr} operon is activated by cAMP, and AI-2 is internalized by cells (Surette and Bassler, 1998). While it is tempting to speculate that the enormous increase in extracellular AI-2 in the presence of PTS sugar, glucose, is due to the tight restriction of its uptake, we have performed stochastic model analysis of the synthesis/uptake mechanism in \textit{E. coli} and predicted that the rate of DPD to AI-2 conversion requires either (i) additional biological steps or (ii) fueling from another reaction pathway (non LuxS-mediated). While the details of this analysis are found elsewhere (Li et al., 2006), we have shown that the addition of adenosine, the product of eukaryotic SAH hydrolase activity, to extracts of \textit{luxS} and \textit{pfs} mutants leads to AI-2 activity.

It is also important to note that dihydroxyacetone phosphate (DHAP) represses the \textit{lsr} operon (Xavier and Bassler, 2005a). Hence, mutations leading to changes in DHAP synthesis affect AI-2 concentrations. For example, both the \textit{gatC} and \textit{agaY} mutations increase intracellular AI-2 concentrations and biofilms (Domka et al., 2007).

While studying another one of the genes identified by us as induced in biofilms through DNA microarrays (Ren et al., 2004a), we found that the uncharacterized protein YdgG is involved in export of AI-2 (Herzberg et al., 2006). The evidence for this discovery is that deleting \textit{ydgG} decreased extracellular and increased intracellular concentrations of AI-2 by 13-fold and 16-fold, respectively (Herzberg et al., 2006). Consistent with this hypothesis,
deleting ydgG resulted in a 7000-fold increase in biofilm thickness and 574-fold increase in biomass in flow cells (Herzberg et al., 2006). Also consistent with the hypothesis, deleting ydgG increased cell motility by increasing transcription of flagella genes (genes induced by AI-2). By expressing ydgG in trans, the wild-type phenotypes for extracellular AI-2 activity, motility, and biofilm were restored. YdgG is predicted to be a membrane-spanning protein that is conserved in many bacteria (Figure 7.2), and it influences resistance against several antimicrobials including crystal violet and streptomycin (this phenotype could also be complemented). Deleting ydgG also caused 31% of the bacterial chromosome to be differentially expressed in biofilms as expected since AI-2 affects hundreds of genes (Beloin et al., 2004; Ren et al., 2004a; Schembri et al., 2003). YdgG was found to negatively modulate expression of flagella- and motility-related genes as well as other known products essential for biofilm formation including operons for type 1 fimbriae, the autotransporter protein Ag43, curli production, colanic acid production, and production of polysaccharide adhesin. Eighty genes not previously related to biofilm formation were also identified including those that encode transport proteins (yihN and yihP), polysialic acid production (gutM and gutQ), CP4-57 prophage functions (yR and alpA), methionine biosynthesis (metR), biotin and thiamine biosynthesis (bioF and thiDFH), anaerobic metabolism (focB, byfACDR, ttdA, and fumB), and genes with unknown function (ybsG, yceO, yjhQ, and yjbE); ten of these genes were verified through mutation to decrease biofilm formation by 40% or more (yR, bioF, yccW, yjbE, yceO, ttdA, fumB, yjiP, gutQ, and yihR). Hence, the gene that encodes YdgG was renamed tqsA (for transport of the quorum sensing signal AI-2 (Herzberg et al., 2006)).

Figure 7.2 Schematic of YdgG combining data predicted by different bioinformatics programs.
AI-3 signaling

Enterohemorrhagic *E. coli* 0157:H7 and several other bacteria (e.g. *Shigella* sp.) also have another signal known as AI-3 whose synthesis depends on LuxS (Sperandio et al., 2003). The composition of AI-3 is not known but it appears to require tyrosine for its synthesis (Walters et al., 2006). Furthermore, it remains to be shown that AI-3 is a quorum sensing signal in that the cellular response has not been shown to extend beyond the metabolism required to generate it (Winzer et al., 2002). The importance of AI-3 lies in its ability to activate transcription of pathogenicity genes found in the locus of enterocyte effacement (Sperandio et al., 2003). Both AI-3 (4 μM) (Sperandio et al., 2003) and AI-2 (6.4 μM) (González Barrios et al., 2006) have been shown to control motility directly by inducing transcription of *qseBC*.

*N-Acylhomoserine lactone signaling through SdiA*

The physiological role of SdiA has been unclear in *E. coli* (Lindsay and Ahmer, 2005). SdiA is a LuxR homologue that is a quorum-sensing-regulated transcription factor in *E. coli* (Rahmati et al., 2002); in other bacteria, LuxR systems control density-dependent gene regulation through acyl homoserine lactones (AI-1) but *E. coli* does not have an AI-1 synthase. In *E. coli* O157-H7, SdiA has been shown to regulate virulence factors (Kanamaru et al., 2000), and SdiA has been shown (by overexpressing SdiA from a plasmid) to inhibit chemotaxis and motility genes in *E. coli* K-12, to repress *tnaA* (encodes tryptophanase), as well as to induce indole export via AcrEF (Wei et al., 2001).

Recently, it has been determined that purified SdiA responds to three different AI-1 signals (Yao et al., 2006) and that SdiA controls acid resistance via a synthetic AI-1 (van Houdt et al., 2006). Since AI-1 controls biofilms in other strains (Davies et al., 1998), and since we found indole controls biofilms through SdiA (below) (Lee et al., 2006), we investigated if AI-1 signals can control biofilm formation in *E. coli* (Lee et al., 2006). Adding three naturally occurring AI-1 signals (*N*-butyryl-DL-homoserine lactone produced by *Pseudomonas aeruginosa* (Pesci et al., 1999), *N*-hexanoyl-DL-homoserine lactone produced by *P. syringae* (Elasri et al., 2001), and *N*-octanoyl-DL-homoserine lactone produced by *P. fluorescens* (Elasri et al., 2001)) inhibited K-12 biofilm formation in LB medium in a dose-dependent manner without inhibiting growth by 25%, 27%, and 18%, respectively; however, the isogenic *sdiA* mutant does not respond to the AI-1 signals (see Figure 7.3 for *N*-butyryl-DL-homoserine lactone). Hence, *E. coli* responds to AI-1 signals (signals that are produced by neighboring bacteria but not by itself) by altering its own biofilm formation and it does so through SdiA (Lee et al., 2006).

Indole signaling through SdiA

Indole is an extracellular signal in *E. coli* as it has been shown to regulate expression of *astD* (encodes arginine succinyltransferase), *tnaB* (encodes a low-affinity tryptophan permease), and *gabT* (encodes glutamate:succinate semialdehyde aminotransferase) in the stationary phase for suspension cells (Wang et al., 2001). Indole has also been shown to control multidrug exporters in *E. coli* (Hirakawa et al., 2005) as well as to regulate the pathogenicity island of pathogenic *E. coli* (Anyanful et al., 2005) (note tryptophanase activity has also been linked to killing of nematodes by *E. coli* but indole is not directly responsible for this
effect (Anyanful et al., 2005). Using DNA microarrays, we discovered that genes for the synthesis of indole (tnaAL) were induced by a stationary phase signal (Ren et al., 2004c) and that the gene encoding tryptophanase, tnaA, was repressed 13-fold in 6-day-old E. coli biofilms in complex medium (Ren et al., 2004a). These results implied that indole plays a role in biofilm formation since biofilm cells most closely resemble stationary-phase cells (Beloin and Ghigo, 2005; Lazazzera, 2005). Using two E. coli mutants yliH and yceP, which are involved in regulating biofilm formation via both AI-2 and indole, we found that indole probably inhibits biofilm formation since these two mutations lead to biofilms with lower intracellular indole concentrations which leads to dramatic increases in biofilm formation and since the addition of extracellular indole reduced biofilm formation for these mutants (Domka et al., 2006). In contrast, others have reported that indole induces biofilm formation in E. coli as the tnaA deletion decreased biofilm formation and the addition of indole restored it (Di Martino et al., 2003).

To determine the genetic basis of indole regulation of biofilms, we utilized a series of tryptophan mutants (Figure 7.4, tnaA encodes tryptophanase synthase, trpE encodes anthranilate synthase component I, tnaL (tnaC) encodes the tryptophanase leader peptide, and trpL encodes the Trp operon leader peptide (Neidhardt, 1996)) as well as conducted three sets of microarray experiments using RNA extracted from biofilm cells grown on glass wool (Lee et al., 2006): (i) direct addition of 600 μM indole to K-12 yceP in LB glu since this strain has elevated biofilm formation due to low intracellular indole and the biofilm responds to added indole (Domka et al., 2006), (ii) K-12 trpE vs. wild type in LB glu since the trpE cells had significantly reduced indole levels (Figure 7.5) and had elevated biofilm in LB glu at 24 hours, and (iii) K-12 tnaA vs. wild type in LB since the tnaA cells also had little detectable indole in this medium.

Figure 7.3 Effect of N-butyryl-DL-homoserine lactone on biofilm formation of E. coli K-12 BW25113 and the sdiA mutant in LB medium at 30°C. Biomass was measured at 540 nm after 24 hours. The experiments were repeated five times (one representative data set shown), and one standard deviation is shown.
Notably SdiA was one of the most-induced genes (2.9-fold) upon addition of 600 µM indole (Lee et al., 2006). Since SdiA inhibits chemotaxis and motility (Wei et al., 2001), it was expected that a sdiA deletion should lead to enhanced motility and biofilm formation. Corroborating our microarray results, the motility of the isogenic sdiA strain was increased 2-fold and biofilm formation was increased 6.4-fold in LB glu at 37°C at 24 hours and increased 3.5-fold in LB at 30°C at 24 hours. In addition, for short time experiments (8 hours), the sdiA mutation caused a 51-fold increase in biofilm formation at 30°C in LB; however, there was no change in biofilm formation upon deleting sdiA at 37°C with LB (Lee et al., 2006). As expected, the addition of 1000 µM indole to the sdiA mutant in LB glu did not appreciably decrease its elevated biofilm levels. Hence, indole induces expression of SdiA which results in SdiA repressing biofilm formation by decreasing motility which leads to decreased biofilm formation. These are some of the first results with SdiA showing a phenotypic change in E. coli (Walters and Sperandio, 2006). Similarly, the trpE mutation, which diminishes intracellular indole, led to both increased biofilm and motility which indicates again that indole is a biofilm inhibitor which controls biofilms by reducing motility via SdiA (Lee et al., 2006).

In addition, two research groups have found that SdiA induces the multi-drug efflux pump AcrAB of E. coli (Rahmati et al., 2002; Wei et al., 2001), and AcrAB has been hypothesized to control the efflux of quorum signals (Rahmati et al., 2002). Given that we demonstrated that TqsA of E. coli controls the efflux of the quorum signal AI-2 (Herzberg et al., 2006), and others demonstrated that MexAB-OprM of P. aeruginosa controls the efflux of the quorum signal N-(3-oxododecanoyl)-L-homoserine lactone (Evans et al., 1998;
Pearson et al., 1999), it appears that indole via its control of SdiA, may also control the efflux of quorum signals as well as control antibiotic resistance (Hirakawa et al., 2005).

Indole addition also repressed the glutamate decarboxylase acid-resistance genes gad-ABCEX 4-fold (Lee et al., 2006). The genes encoding chaperones to prevent aggregation of periplasmic proteins under extremely acidic conditions (Masuda and Church, 2003), hde-ABD, were also repressed 3- to 5-fold as was a new locus related to acid resistance, ymgABC (Lee et al., 2006). To show clearly that indole is directly related to acid resistance, 2 mM indole was added to the wild type at pH 2.5, and we found that its survival was decreased 350 to 650-fold. In addition, the trpE mutant (which produces 10 times less indole), was 53 times less sensitive to pH 2.5 than the wild type. Hence, indole decreases acid resistance as shown by these two independent experiments (Lee et al., 2006). In addition, isogenic mutants showed these genes are directly related to biofilm formation (6.4-fold increase for sdiA, 6-fold for gadA, 7.3-fold for gadE, 4.3-fold for hdeA, 1.7-fold for hdeB, and 2-fold for hdeD) (Lee et al., 2006). Because indole accumulates extracellularly during a specific phase of growth (stationary) (Baca-Delancey et al., 1999), is recognized by a specific receptor (Mtr) (Wang et al., 2001), and has been shown to control genes beyond those used to synthesize it or detoxify it (e.g. sdiA, gadABCEX, hdeABD, ymgABC), it is likely that it is a true quorum sensing signal (Winzer et al., 2002).

Toluene o-monoxygenase (TOM) of the soil bacterium Burkholderia cepacia G4 converts indole into isoindigo (Rui et al., 2005); hence, we hypothesized that if indole represses E. coli biofilm formation, then E. coli would be present in higher numbers in a dual-species biofilm in which TOM was expressed. TOM was integrated into the chromosome of the P. fluorescens strain to diminish the metabolic burden of this locus (Yee et al., 1998) and

Figure 7.5 Intracellular and extracellular indole concentration in LB for the E. coli K-12 strains BW25113, BW25113 ΔtrpE, BW25113 ΔtnaL, BW25113 ΔtnaA, and BW25113 ΔtrpL. Each experiment was performed in duplicate, and one standard deviation is shown.

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because this strain does not produce indole. When the *Pseudomonas* was tagged with red fluorescent protein and *E. coli* with a green fluorescent protein so that both bacteria could be visualized, constitutive expression of TOM led to a 12-fold increase in *E. coli* biofilm after five days in the flow cell (Figure 7.6). By expressing TOM to remove indole, both substratum coverage and mean thickness increased 10-fold. Thus, including an organism that actively expressed TOM increased the amount of *E. coli* biofilm by decreasing the amount of indole by 22-fold (Lee et al., 2006). These results show that indole is an interspecies signal and indicate that it may be manipulated to control biofilms in complex communities (Lee et al., 2006).

Indole is also a signal for pseudomonads since indole at 500 μM increased biofilm formation 1.6-fold and 1000 μM increased biofilm formation 2.5-fold; hence, indole, although not synthesized by *P. aeruginosa* (extracellular concentration was 0 μM), indole is a signal that stimulates biofilm in this strain. In addition, removing the 1000 μM added indole by expressing TOM in *P. fluorescens* 2–79 results in a 5.7-fold reduction in its biofilm again showing that indole stimulates biofilm formation in this pseudomonad. Hence, indole is an

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**Figure 7.6** Biofilm formation in LB medium after 5 days in flow cells for (A) dual species of *E. coli* K-12 XL1-Blue/pCM18 (stained with GFP) and *P. fluorescens* 2-79TOM/pHKT3 expressing toluene o-monoxygenase (TOM) (stained with RFP), and (B) dual species of *E. coli* K-12 XL1-Blue/pCM18 (stained with GFP) and *P. fluorescens* 2-79/pHKT3 (stained with RFP). Scale bar is 10 μm. Both pseudomonads contained RFP via the broad-host-range plasmid pHKT3 (Tomlin et al., 2004) and *E. coli* K-12 XL1-Blue was used with GFP from pCM18 (Hansen et al., 2001); in this way, both bacteria were tagged with a fluorophore. TOM was active in *P. fluorescens* 2-79TOM/pHKT3 (RFP) (0.24 nmol/minute mg protein) based on a naphthalene to naphthol assay and high-pressure liquid chromatography (HPLC) (Tao et al., 2005) (constitutively expressed TOM converts indole to isoindigo (Yee et al., 1998)). See also Plate 7.6.
interspecies signal that may be manipulated by oxygenases of another bacterium (one that does not necessarily synthesize it) to control biofilm formation.

The type of signal manipulation reported above suggests that competition and control for signals are intense in biofilms. Competition for signals for planktonic cells has been demonstrated previously for AI-2 (Xavier and Bassler, 2005b) and for AI-1 (Zhang and Dong, 2004). This competition extends beyond prokaryotes as eukaryotes are well known for manipulating the quorum sensing signals of bacteria, too. For example, algae block bacterial biofilm formation by controlling both AI-1 and AI-2 signaling via furanones (Ren et al., 2001; Ren et al., 2004b), and mammals (including humans) blocking AI-1 signaling via lactonase in sera (Yang et al., 2005). Furthermore, it appears that the mechanism by which prokaryotes (such as the pseudomonads in this study) manipulate the biofilm signal indole is through the relaxed substrate range of many oxygenases found in bacteria that bring about indole hydroxylation (Rui et al., 2005); i.e. we propose that some of the oxygenases bacteria use for catabolism (Fishman et al., 2005) have also evolved to regulate concentrations of the inter-species signal indole by removing it via precipitation: competitors that wish to remove indole simply oxidize it in one step to indigo which is insoluble and hence leaves the system (Lee et al., 2006).

5-Hydroxyindole and isatin signaling
Since competition in biofilms for signals is intense (there are as many as 500 to 1000 different bacteria in the gastrointestinal tract) and given that the human host itself makes indole-like hormones, it is expected that the large quantity of indole produced by E. coli would be manipulated by surrounding intestinal bacteria and that the intestinal bacteria would utilize the indole-like compounds epinephrine and norepinephrine produced by the human host. We reasoned that given the high indole concentrations for E. coli in rich medium (Figure 7.5) (Lee et al., 2006) and given the ability of oxygenases to convert indole into insoluble indigoids (Rui et al., 2005), other bacteria would take advantage of this interspecies signal indole and manipulate it. Hence we tested a series of hydroxyindoles for their ability to alter biofilm formation of E. coli O157:H7. We found that the doubly hydroxylated isatin increases the bottom biofilm formation in a 96-well plate by 4-fold whereas 5-hydroxyindole reduces biofilm formation by 6-fold (84%) (unpublished). Hence, hydroxylated indoles affect E. coli in different ways which opens the possibility that this bacterium can discern the presence of other bacteria by detecting the way its signal is manipulated.

Indole, norepinephrine, and epinephrine signaling in EHEC
We also propose that the DNA microarray analyses and results with isogenic mutants provide insight into how the bacteria of the gastrointestinal (GI) tract may help to restrict access to pathogens. Since indole represses the genes for acid resistance (gad, hde, and ymg acid-resistance operons) in E. coli K-12 (Lee et al., 2006), increases in extracellular indole in the GI tract may result in the exclusion of the pathogen E. coli O157:H7 from the GI tract environment by affecting its ability to resist the low-pH environment of the stomach. In addition, since indole represses genes that encode virulence regulators such as gadX (gadX was repressed 2-fold by indole (Lee et al., 2006)), indole may reduce virulence in the duodenum since GadX activates virulence genes there (Moreira et al., 2006). Alternatively,
the repression of motility genes by indole could also lead to increased washout of pathogen from the GI tract and, thereby, decrease their colonization. We speculate that the extracellular levels of indole increase only after the biofilm has reached a certain critical thickness so that indole impacts acid resistance and motility only in the colonizing pathogenic bacteria. The pathogen *E. coli* O157:H7 may use indole as a signal since SdiA represses expression of the virulence factors EspD and intimin (Kanamaru *et al.*, 2000). In addition, indole from bacteria is absorbed into the body (Gillam *et al.*, 2000) so cells of the gastrointestinal tract may also manipulate indole levels to control bacteria.

It is becoming clear that prokaryotes and eukaryotes signal not only themselves but also one another; for example, there appears to be crosstalk between *E. coli* O157:H7 (EHEC) and cells of the gastrointestinal tract through the hormones epinephrine and norepinephrine (catecholamines) (Kaper and Sperandio, 2005). Using microarrays, we have found that the interspecies signal indole represses key virulence genes of EHEC as well as that the human hormones epinephrine and norepinephrine stimulate virulence (unpublished). Other hormones are also present in the gastrointestinal tract including melatonin (Lee and Pang, 1993) and serotonin (Meyer and Brinck, 1997); both are neural hormones which maintain homeostasis and both reduce chlamydial infection (Rahman *et al.*, 2005). In addition, plants use indole 3-acetic acid as their main hormone (for cell growth, division, tissue differentiation, and response to light and gravity), and bacteria are known to interrupt this eukaryotic signaling by using indole 3-acetic acid as a source of carbon, nitrogen, and energy (Leveau and Lindow, 2005). All five hormones have indole-like chemical structures (Figure 7.7); hence, although it is highly speculative, it is intriguing to ponder whether indole that was produced by bacteria was incorporated into the metabolism of eukaryotic hosts (plants and animals) and is the archetypal hormone.

![Chemical structures of indole, melatonin, serotonin, epinephrine, and indole-3-acetic acid.](caister.com/biofilmsbooks)

**Figure 7.7** Chemical structures of indole, melatonin, serotonin, epinephrine, and indole-3-acetic acid. Indole motifs are in bold.
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