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ORIGINAL ARTICLE

Indole cell signaling occurs primarily at low temperatures in *Escherichia coli*

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We have shown that the quorum-sensing signals acylhomoserine lactones, autoinducer-2 (AI-2) and indole influence the biofilm formation of *Escherichia coli*. Here, we investigate how the environment, that is, temperature, affects indole and AI-2 signaling in *E. coli*. We show in biofilms that indole addition leads to more extensive differential gene expression at 30 °C (186 genes) than at 37 °C (59 genes), that indole reduces biofilm formation (without affecting growth) more significantly at 25 and 30 °C than at 37 °C and that the effect is associated with the quorum-sensing protein SdiA. The addition of indole at 30 °C compared to 37 °C most significantly repressed genes involved in uridine monophosphate (UMP) biosynthesis (*carAB*, *pyrLBI*, *pyrC*, *pyrD*, *pyrF* and *upp*) and uracil transport (*uraA*). These uracil-related genes are also repressed at 30 °C by SdiA, which confirms SdiA is involved in indole signaling. Also, compared to 37 °C, indole more significantly decreased flagella-related *qseB*, *flhD* and *fliA* promoter activity, enhanced antibiotic resistance and inhibited cell division at 30 °C. In contrast to indole and SdiA, the addition of (*S*)-4,5-dihydroxy-2,3-pentanedione (the AI-2 precursor) leads to more extensive differential gene expression at 37 °C (63 genes) than at 30 °C (11 genes), and, rather than repressing UMP synthesis genes, AI-2 induces them at 37 °C (but not at 30 °C). Also, the addition of AI-2 induces the transcription of virulence genes in enterohemorrhagic *E. coli* O157:H7 at 37 °C but not at 30 °C. Hence, cell signals cause diverse responses at different temperatures, and indole- and AI-2-based signaling are intertwined.

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Introduction

Adaptation to diverse environmental conditions is central to the survival of bacteria (Hall-Stoodley *et al.*, 2004). Biofilm formation is one survival strategy against nutrient limitation, antibiotics, numerous stresses (temperature, pH, oxygen, osmosis and heavy metals) and other microbial species (Davies *et al.*, 1998; Hall-Stoodley *et al.*, 2004; Moons *et al.*, 2006), and stress itself increases biofilm formation (Zhang *et al.*, 2007). Due to their resistance to antibiotics, biofilms of pathogens are a major problem for human health (for example, lung infections, dental diseases and urinary tract infections) (Potera, 1999).

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Cell signaling plays a role in the formation of some biofilms (Davies *et al.*, 1998; Stanley and Lazazzera, 2004). For example in *Escherichia coli*, acylhomoserine lactones (AHLs) from other bacteria are sensed through SdiA by *E. coli* that does not synthesize this signal (Michael *et al.*, 2001; Van Houdt *et al.*, 2006), and exogenous AHLs reduce its biofilm formation (Moons *et al.*, 2006; Lee *et al.*, 2007b). In contrast, the addition of purified autoinducer-2 (AI-2) increases *E. coli* biofilm formation and motility (González Barrios *et al.*, 2006; Herzberg *et al.*, 2006). Indole and hydroxyindoles have also been shown to influence biofilm formation of both nonpathogenic *E. coli* (Di Martino *et al.*, 2003; Domka *et al.*, 2006; Lee *et al.*, 2007b) as well as enterohemorrhagic *E. coli* O157:H7 (EHEC) (Bansal *et al.*, 2007; Lee *et al.*, 2007a) by influencing motility, acid resistance, chemotaxis and adherence to HeLa cells. It was first reported that indole stimulates biofilm formation for *E. coli* S17-1 (Di Martino *et al.*, 2003). However, we have shown that indole significantly decreases the biofilm formation of nine nonpathogenic *E. coli* strains (Domka *et al.*,

2006; Zhang *et al.*, 2007; Lee *et al.*, 2007b) as well as decreases the biofilm formation of pathogenic *E. coli* O157:H7 (Lee *et al.*, 2007a).

Bacterial biofilm formation varies based on environmental conditions (Hall-Stoodley *et al.*, 2004). We observed that several *E. coli* strains showed different biofilm architecture due to the different growth conditions (Wood *et al.*, 2006). The effect of temperature is of interest in this study as *E. coli* encounters a temperature shift (for example, inside vs outside the human body). Van Houdt *et al.* (2006) reported that the responsiveness of AHLs in *E. coli* was temperature dependent showing a strong response at 30 °C, but only a weak response at 37 °C. We hypothesize here that the functions of AI-2 and indole cell signaling molecules may be affected by environmental temperature. Evidence provided by seven sets of whole-transcriptome studies, biofilm assays, promoter transcriptional assays, quantitative reverse transcription-PCR (qRT-PCR), antibiotic resistance assays and cell division were used to investigate the effect of temperature on *E. coli* cell signaling by indole and AI-2. Furthermore, we show that SdiA is the regulator through which indole signaling is mediated and report for the first time the genes controlled by SdiA at the temperature where it is most active, 30 °C.

Materials and methods

Bacterial strains, materials and growth rate measurements

The strains used are listed in Table 1. To eliminate the kanamycin-resistance gene in the *tnaA* deletion mutant, pCP20 (Cherepanov and Wackernagel, 1995) expressing the FLP recombinase protein was used as the kanamycin-resistance gene is flanked by an FLP recognition target that is excised by FLP recombinase (Baba *et al.*, 2006). LB (Sambrook *et al.*, 1989) was used to preculture all the *E. coli* cells. Indole was purchased from Fisher Scientific Co. (Pittsburg, PA, USA), and (*S*)-4,5-dihydroxypentane-2,3-dione (DPD that forms AI-2) was purchased from Omm Scientific (Dallas, TX, USA). To determine the toxicity of indole, the specific growth rates of the indole-deficient mutant *E. coli* K-12 BW25113 *tnaA* in the presence of indole (1 mM) were determined in LB medium at 30 and 37 °C by using the linear portion of the logarithm of absorbance vs time (absorbance from 0.2 to 0.7 at 600 nm); indole was dissolved in dimethylformamide (dimethylformamide alone was used as a control), and each experiment was performed with two independent cultures.

Crystal violet biofilm assay

This assay was adapted (Pratt and Kolter, 1998); overnight cultures of *E. coli* were diluted to an absorbance of 0.05 at 600 nm and were incubated in polystyrene 96-well plates at 25, 30 or 37 °C for 16 h without shaking in LB medium. The crystal violet dye staining the biofilms (air–liquid interface biofilm as well as bottom liquid–solid biofilm) was dissolved in 95% ethanol, and an absorbance at 540 nm was measured to quantify the total biofilm mass. Each data point was averaged from more than 12 replicate wells (six wells from two independent cultures).

AI-2 uptake assay

Overnight cultures of the AI-2-deficient mutant BW25113 *luxS* were diluted in LB to an absorbance of 0.05 at 600 nm. The cells were grown to an absorbance of approximately 0.5 at 600 nm at 30 or 37 °C before adding 100 μM AI-2 (DPD). To determine the amount of extracellular AI-2 remaining at each time point, culture supernatants were collected and

Table 1 Strains and plasmids used in this study

Strain or plasmid	*Genotype*	*Source*
Strains		
E. coli K-12 BW25113	$lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$, $\Delta rhaBAD_{LD78}$	(Baba *et al.*, 2006)
E. coli K-12 BW25113 *luxS*	K-12 BW25113 Δ*luxS* Ω Km^R	(Baba *et al.*, 2006)
E. coli K-12 BW25113 *sdiA*	K-12 BW25113 Δ*sdiA* Ω Km^R	(Baba *et al.*, 2006)
E. coli K-12 BW25113 *tnaA*	K-12 BW25113 Δ*tnaA* Ω Km^R	(Baba *et al.*, 2006)
E. coli K-12 BW25113 *yceK*	K-12 BW25113 Δ*yceK* Ω Km^R	(Baba *et al.*, 2006)
E. coli K-12 BW25113 *tnaA* Δ*Km*	K-12 BW25113 Δ*tnaA* Δ*Km*	This study
E. coli O157:H7 *luxS*	$Stx2^+$ EHEC VS94 Δ*luxS* Ω Tet^R	(Sperandio *et al.*, 2001)
Plasmids		
pCP20	Amp^R, Cm^R plasmid with temperature-sensitive replication and thermal induction of FLP recombinase	(Cherepanov and Wackernagel, 1995)
pVS159	Amp^R, *qseB*::*lacZ* in pRS551	(Sperandio *et al.*, 2002)
pVS182	Amp^R, *flhD*::*lacZ* in pRS551	(Sperandio *et al.*, 2002)
pVS183	Amp^R, *fliAehK-12*::*lacZ* in pRS551	(Sperandio *et al.*, 2002)

Amp^R, Cm^R, Km^R, and Tet^R are the antibiotic resistance genes for ampicillin, chloramphenicol, kanamycin and tetracycline, respectively. Stx is the Shiga toxin.

filter-sterilized every hour for 6 h after addition of AI-2, and the absorbance was measured at the same time point to determine cell growth. The *Vibrio harveyi* autoinducer assay was performed as described previously (Surette and Bassler, 1998) with the supernatants assayed for AI-2 for 4 h at 30 °C with shaking (this corresponds to a minimum in *V. harveyi* AI-2 production). The negative control was supernatant from a bacterial culture without addition of AI-2. Luminescence was measured using a TD-20e luminometer (Turner Biosystems, Sunnyvale, CA, USA). Each experiment was performed with two independent cultures.

Total RNA isolation for DNA microarrays

We performed seven sets of microarray experiments in LB medium (Table 2): (i) biofilm cells of the BW25113 wild-type strain grown for 7 h at 30 vs 37 °C to determine the effect of temperature on *E. coli* biofilm formation (initial absorbance of 0.05), (ii) biofilm cells of the *tnaA* mutant grown for 7 h with 1 mM indole vs no indole at 30 °C to determine the effect of temperature on indole signaling in *E. coli* (initial absorbance of 0.05), (iii) biofilm cells of the *tnaA* mutant grown for 7 h with 1 mM indole vs no indole at 37 °C to determine the effect of temperature on indole signaling in *E. coli*, (iv) suspension cells of the wild-type strain vs the *sdiA* mutant at an absorbance of 4.0 at 600 nm at 30 °C (as the production of indole takes place primarily in the stationary phase) to discern the genes that SdiA controls (initial absorbance of 0.05), (v) biofilm cells of the *sdiA* mutant grown for 7 h with 1 mM indole vs no indole at 30 °C to determine gene expression in response to indole in the absence of SdiA, (vi) suspension cells of the *luxS* mutant with 100 μM AI-2 vs no AI-2 at 30 °C for 3 h to determine the effect of temperature on AI-2 signaling in *E. coli* (initial absorbance of 0.5) and (vii) suspension cells of the *luxS* mutant with 100 μM AI-2 vs no AI-2 at 37 °C for 3 h to determine the effect of temperature on AI-2 signaling in *E. coli*. Ten grams of glass wool (Corning Glass Works, Corning, NY, USA) was used to form large amounts of biofilms (Ren *et al.*, 2004a) in 250 ml LB in 1 l Erlenmeyer shake flasks. RNA was isolated from the suspension and biofilm cells as described previously (Ren *et al.*, 2004a). To obtain biofilm cells, the glass wool was washed twice in 200 ml of 0 °C 0.85% NaCl buffer for 30 s, and the biofilm cells were removed from the glass wool by sonicating at 22 W (FS3 sonicator, Fisher Scientific Co.) in 200 ml of 0 °C 0.85% NaCl buffer. The buffer was centrifuged at 10 000 *g* for 2 min at 4 °C (J2-HS centrifuge, Beckman, Palo Alto, CA, USA).

DNA microarray analysis

The *E. coli* Genechip antisense genome array (Affymetrix, P/N 900381, Santa Clara, CA, USA) and the *E. coli* GeneChip Genome 2.0 array (Affymetrix, P/N 900551) were used to study the differential gene expression profile of the K-12 biofilm or suspension cells; the *E. coli* Genechip antisense genome array contains probe sets for all 4290 open reading frames, rRNA, tRNA and 1350 intergenic regions, and the *E. coli* GeneChip Genome 2.0 array contains 10,208 probe sets for open reading frames, rRNA, tRNA and intergenic regions for four *E. coli* strains: MG1655, CFT073, O157:H7-Sakai and O157:H7-EDL933. The same type of microarray was used for each binary comparison. cDNA synthesis, fragmentation and hybridizations were as described previously (González Barrios *et al.*, 2006). Hybridization was performed for 16 h, and the total cell intensity was scaled to an average value of 500. The probe array images were inspected for any image artifact. Background values, noise values and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA control were used to monitor the labeling process. Corroborating the deletion mutations, the microarray signals of the *tnaA*, *sdiA* and *luxS* genes were very low in their respective microarray

Table 2 Experimental design for the seven sets of DNA microarray experiments. LB medium was used for all the experiments

Experiment	*(i)*	*(ii)*	*(iii)*	*(iv)*	*(v)*	*(vi)*	*(vii)*
Strain	BW25113 WT	BW25113 *tnaA*	BW25113 *tnaA*	BW25113 WT vs BW25113 *sdiA*	BW25113 *sdiA*	BW25113 *luxS*	BW25113 *luxS*
Exogenous signal	NA	1 mM Indole vs no indole	1 mM Indole vs no indole	NA	1 mM Indole vs no indole	100 μM AI-2 vs no AI-2	100 μM AI-2 vs no AI-2
Temperature	30 vs 37 °C	30 °C	37 °C	30 °C	30 °C	30 °C	37 °C
Cell type and culture time	7 h-biofilm cells, initial absorbance of 0.05	7 h-biofilm cells, initial absorbance of 0.05	7 h-biofilm cells, initial absorbance of 0.05	Suspension cells at an absorbance of 4.0, initial absorbance of 0.05	7 h-biofilm cells, initial absorbance of 0.05	3 h-suspension cells, initial absorbance of 0.5	3 h-suspension cells, initial absorbance of 0.5
Goal	Temperature and *E. coli* biofilm formation	Temperature and indole signaling	Temperature and indole signaling	Role of SdiA	Indole signaling without SdiA	Temperature and AI-2 signaling	Temperature and AI-2 signaling

Abbreviations: NA, not applicable; WT, wild type.

experiments. For each binary microarray comparison of differential gene expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 13 probe pairs (*P*-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the *P*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher (2- to 4.5-fold) than the standard deviation for the whole microarray (4.5-fold for condition (i) above, 1.8-fold for (ii), 1.3-fold for (iii), 2.5-fold for (iv), 1.6-fold for (v), 1.2-fold for (vi) and 1.3-fold for (vii) (Ren *et al.*, 2004b). Gene functions were obtained from the Affymetrix–NetAffx Analysis Center (https://www.affymetrix.com/analysis/netaffx/index.affx). The expression data were deposited in the NCBI Gene Expression Omnibus and are accessible through accession number GSE9923 (Edgar *et al.*, 2002).

Detection of indole, swimming motility assays and promoter transcriptional assays

Extracellular concentrations of indole were measured with reverse-phase high-pressure liquid chromatography as described previously (Lee *et al.*, 2007b). Swimming motility was measured as described previously (Lee *et al.*, 2007b). The motility halos were measured at 8 and 20 h. To measure the activity of promoters related to motility in the presence of indole, *E. coli* BW25113 *tnaA* with the *qseB*::*lacZ*, *flhD*::*lacZ* and *fliA*::*lacZ* fusion plasmids (pVS159, pVS182 and pVS183, Table 1) (Sperandio *et al.*, 2002) were used. Overnight cultures were diluted 1:100 in LB ampicillin ($100\,\mu g\,ml^{-1}$) and then grown to stationary phase to an absorbance of 4.0 at 600 nm at 30 or 37 °C. Indole (1 mM) or dimethylformamide alone was added at the beginning of culturing. The β-galactosidase activity was evaluated as described previously (Wood and Peretti, 1991) and was based on a protein concentration of 0.24 mg protein per ml per OD_{600} (Tao *et al.*, 2004). Each experiment was performed with two replicates from two independent cultures.

Antibiotic resistance and cell division assays

Overnight cultures of *E. coli* BW25113 and its isogenic *tnaA* (ΔKm) mutant (Table 1) were diluted 1:100 in LB and then grown to an absorbance of 0.35 (exponential-phase cells) and 4.0 (stationary-phase cells) at 600 nm at 30 or 37 °C. Indole (1 mM) or dimethylformamide alone was added at the beginning of the culture. Kanamycin at a final concentration of $0.1\,mg\,ml^{-1}$ for the exponential-phase cells and $2\,mg\,ml^{-1}$ for the stationary-phase cells was mixed with the cells and incubated at 30 or 37 °C for 15, 30 and 60 min without shaking, then cells were enumerated with LB agar plates. At least two independent cultures were used for each strain. To determine the impact of indole on cell division, BW25113 *tnaA* was grown in LB medium to an absorbance of 0.6 at 600 nm at 250 r.p.m., indole was added, and images were taken after 5 h incubation without shaking at 30 or 37 °C. Images were taken with a Zeiss Axiovert 200 microscope (Thornwood, NY, USA) with a 63× phase contrast objective. Also, the number of cells at a fixed absorbance of 4.0 at 600 nm at 30 or 37 °C was determined with LB agar plates. Each experiment was performed with three independent cultures.

Real-time PCR

To corroborate the DNA microarray data, the transcription level of three important genes, *uraA* (forward primer 5′-GACAATCCCGCTTAGTTTGC-3′; reverse primer 5′-CGCGACTTCATACCCTAACG-3′), *yceK* (forward primer 5′-TGCGTTTAATTGTGGT GAG C-3′; reverse primer 5′-GAATGGCAGATCAAG GATCG-3′) and *lsrA* (forward primer 5′-ACCGAA CGCTTGTTTAGTCG-3′; reverse primer 5′-TGTC GTCGGTAGACAGTTCG-3′), was quantified using qRT-PCR (Bansal *et al.*, 2007). The expression level of the housekeeping gene *rpoA* (forward primer 5′-CGCGGTCGTGGTTATGTG-3′; reverse primer 5′-GCGCTCATCTTCTTCCGAAT-3′) was used to normalize the expression data of interesting genes. Independent RNA samples from the DNA microarrays were used for these studies (96 qRT-PCR reactions based on three qRT-PCR reactions for each of four genes including the *rpoA* housekeeping gene, with and without signal and four different array conditions). qRT-PCR was performed in triplicate using a Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA). The relative fold changes were calculated from threshold cycle numbers measured using MyiQ software (Bio-Rad).

To determine the level of transcription for the virulence genes, the *E. coli* O157:H7 *luxS* mutant (Table 1) was grown overnight at 37 °C in LB medium supplemented with $10\,\mu g\,ml^{-1}$ tetracycline (Fisher Scientific, Fair Lawn, NJ, USA). The overnight culture was diluted to an absorbance of 0.05 at 600 nm in DMEM with glucose ($4.5\,g\,l^{-1}$) (Hyclone, Logan, UT, USA), and grown with and without 100 μM AI-2 (DPD) to an absorbance of ~1 at 30 or 37 °C. Cells were centrifuged at 10,000 *g* for 2 min and cell pellets were stored at −80 °C. RNA was isolated from the cell pellets using the RNeasy Mini kit (QIAGEN Sciences, Germantown, MD, USA). Using qRT-PCR, expression was determined for virulence gene *espA* (*LEE4* operon, forward primer 5′-GCCAAAATTGCTGATGTTCAGA-3′; reverse primer 5′-CGTCTTGAGGAAGTTTGGCTTT-3′) and virulence gene *eae* (*LEE5* operon, forward primer 5′-CGGATAACGCCGATACCATT-3′; reverse primer 5′-GCCTGAGCTACCCCATTCTTT-3′). Also, the housekeeping gene *rpoA* was used to normalize the LEE gene expression data. qRT-PCR was performed in

triplicate with two independent cultures (72 qRT-PCR reactions based on three qRT-PCR reactions for each of three genes including the *rpoA* housekeeping gene, two biological replicates, two temperatures and with and without AI-2).

Results

The aims of this research were to determine how the environment (temperature) affects indole and AI-2 signaling in *E. coli* and to investigate the mechanism by which indole and AI-2 control cell signaling by DNA microarrays. Also, the effect of indole on four important cell phenotypes, biofilm formation, motility gene transcription, antibiotic resistance and cell division as well as the effect of AI-2 on transcription of virulence genes were investigated as a function of temperature. In addition, we investigated the genes that are controlled by SdiA at 30 °C.

Indole and growth

To confirm that indole is nontoxic to the *E. coli* BW25113 *tnaA* isogenic mutant at low temperatures as was found previously with the wild-type strain at 37 °C (Lee *et al.*, 2007b), the specific growth rate was measured using the indole-deficient mutant with and without indole in LB; 1 mM indole was used, as wild-type *E. coli* BW25113 produces indole up to 0.6 mM during the stationary phase in LB medium at 37 °C (Lee *et al.*, 2007b) and at 30 °C. The specific growth rate was $1.05 \pm 0.02\,h^{-1}$ in the absence of indole whereas the growth rate was $1.03 \pm 0.01\,h^{-1}$ with indole at 30 °C, and at 37 °C, the specific growth rate was $1.46 \pm 0.05\,h^{-1}$ in the absence of indole whereas the growth rate was $1.38 \pm 0.06\,h^{-1}$ with indole. Hence, indole (1 mM) does not significantly change the specific growth rate of *E. coli* at 30 or 37 °C.

Temperature and inhibition of biofilm formation by indole

To investigate the effect of indole as a function of temperature, biofilm formation of the *E. coli* BW25113, *tnaA* and *sdiA* mutants was measured in LB medium at 25, 30 or 37 °C in the presence of indole (0, 0.25, 0.5 and 1.0 mM). We utilized the isogenic *tnaA* mutant, as this strain does not produce indole (Lee *et al.*, 2007b), and so the effect of exogenous indole should be more effective than with the wild-type strain. We also utilized the *sdiA* mutant, as we found that indole probably works through this regulator at 37 °C (Lee *et al.*, 2007b).

Biofilm formation of the BW25113 *tnaA* mutant was reduced in a dose-dependent manner reaching 10-fold by 1 mM indole at both 25 °C and at 30 °C (Figure 1b). Similarly, biofilm formation of the wild-type strain was reduced in a dose-dependent manner reaching 16-fold by 1 mM indole at 25 °C and sevenfold by 1 mM indole at 30 °C (Figure 1a). However, indole (1 mM) was far less effective in decreasing biofilm formation of both the wild-type strain and the *tnaA* mutant at 37 °C (Figure 1). Moreover, indole (up to 1 mM) does not affect the biofilm formation of the *sdiA* mutant at 30 and 37 °C (Figure 1c). Hence, these results show that indole requires SdiA to inhibit *E. coli* biofilm formation as well as show that indole exerts its effect on biofilm formation primarily at temperatures lower than 37 °C. These results also confirm our earlier indirect evidence that indole signaling is mediated by SdiA, which was based primarily on DNA microarrays (Lee *et al.*, 2007b). As reported previously (Lee *et al.*, 2007b), we found that the isogenic *sdiA* mutant formed more biofilm than its wild-type BW25113 strain at 25 or 30 °C (Figures 1c vs a), whereas others reported that the deletion of *sdiA* decreased biofilm formation for *E. coli* MG1655 vs nonisogenic UT481 (*sdiA*) (Suzuki *et al.*, 2002).

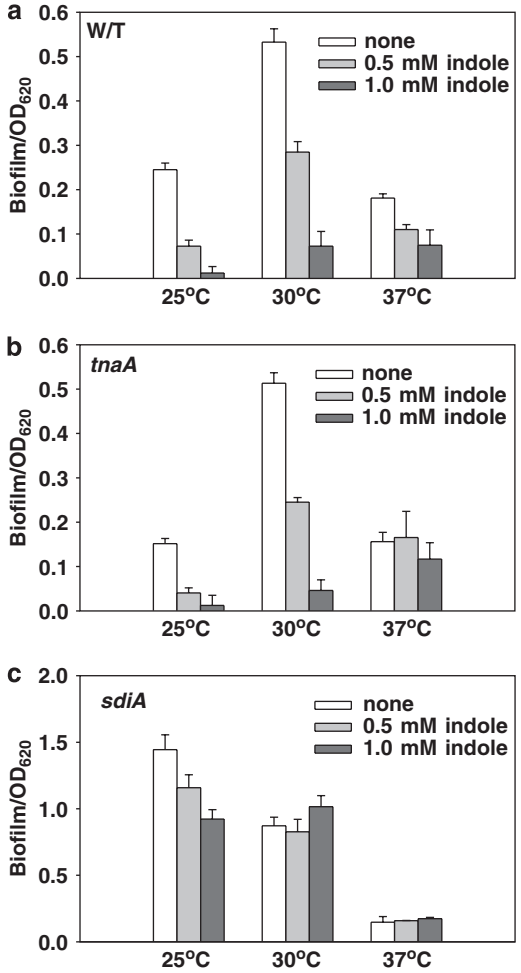

Figure 1 Effect of indole (0, 0.5 and 1.0 mM) on biofilm formation at 25, 30 and 37 °C in LB after 16 h for the *E. coli* BW25113 wild-type strain (WT) (**a**), BW25113 *tnaA* (**b**) and BW25113 *sdiA* (**c**). Each data point is the average of at least 12 replicate wells from two independent cultures, and one standard deviation is shown.

Temperature and differential gene expression in biofilm cells

To investigate the effect of temperature on *E. coli* biofilm formation without adding cell signals, DNA microarrays were used to determine differential gene expression for wild-type biofilm cells grown in LB at 30 °C vs biofilm cells grown at 37 °C. It was found that 252 genes were regulated significantly (more than 4.5-fold) at 30 vs 37 °C; 139 genes were induced and 113 genes were repressed (Table 3 and Supplementary Table 1). Most notable was that two indole-related genes (*tnaAB*) and the *tna* leader region (*tnaC*) were highly induced (4- to 14-fold) at 30 °C. Hence, indole genes are preferentially induced at 30 than at 37 °C, and biofilms are formed in a different manner at the two temperatures.

Uridine monophosphate (UMP) synthesis genes (*carA*, *pyrBI* and *upp*) were significantly induced (five- to ninefold) at 30 °C in biofilms (Table 3). Cell motility genes (*flgB, motAB* and *fliD*) were significantly repressed (5- to 12-fold) at 30 °C compared to 37 °C. Not surprisingly, many genes involved in translation, transport and metabolism were also induced and repressed due to the different culture temperatures (Supplementary Table 1).

Temperature and indole signaling

To study the effect of temperature on indole signaling in *E. coli* biofilms, DNA microarrays were used to determine differential gene expression of *tnaA* biofilm cells upon adding 1 mM indole vs no indole at 30 or 37 °C. As *E. coli* both synthesizes and degrades indole by its reversible tryptophanase (encoded by *tnaA*) (Newton and Snell, 1965), the indole-deficient *tnaA* knockout strain was used. To confirm the elimination of indole production, extracellular indole was measured in the microarray samples after 7 h culture in LB. The *tnaA* strain did not produce indole in LB, and the exogenous addition of indole (1 mM) was stably maintained and not degraded for 7 h at 30 or 37 °C. Hence, indole was not produced and metabolized in the absence of the *tnaA* gene and the changes in differential gene expression are due to the presence of indole.

We found that the addition of indole (1 mM) significantly regulates 186 genes (more than 2-fold) in biofilm cells at 30 °C (Supplementary Table 2); 96 genes were induced and 90 genes were repressed (differential transcription of the most important genes are shown in Table 3). However, at 37 °C, indole regulates far fewer genes in biofilm cells (59 genes more than twofold, 28 genes induced and 31 genes repressed) (Table 3 and Supplementary Table 3).

At 30 °C, genes involved in UMP biosynthesis (*carAB, pyrLBI, pyrC, pyrD, pyrF* and *upp*) and uracil transport (*uraA*) were the most significantly repressed genes (3- to 60-fold) upon adding indole (Table 3); recall that these genes were induced at 30 °C compared to 37 °C in biofilms of the wild-type strain. Therefore, at 30 °C, indole accumulates extracellularly in the stationary phase (Hirakawa *et al.*, 2005) and serves to repress UMP synthesis. Also, *ariA* (*ymgB*) along with the rest of the operon (*ycgZ* and *ymgABC*) involved in acid resistance through indole (Lee *et al.*, 2007c) were induced threefold upon adding indole (Table 3), which confirms that indole regulates the expression of the acid-resistance gene *ariA*. In addition, the biofilm stress regulator *bhsA* (Zhang *et al.*, 2007), nitrate reductase genes *narG, narUZYW* and cold-shock protein genes *cspB* and *cspI* were induced (two- to fourfold) (Table 3). Among the most repressed genes were those encoding the outer membrane porin proteins *ompT* (2.0-fold), *ompF* (3.7-fold), *yciD* (3.5-fold), *flgB* (6.5-fold), which is involved in flagellar biosynthesis, and *fimA*, which encodes the major subunit of type 1 fimbriae (4.3-fold) (Table 3). Also, *bssR* (*yliH*) that encodes a regulator of biofilm formation through indole (Domka *et al.*, 2006), was repressed 2.6-fold upon adding indole at 30 °C.

In contrast, the most noticeable change at 37 °C upon indole addition to biofilm cells was that a hypothetical gene *yceK* was induced (10-fold), whereas *yceK* was repressed (4-fold) at 30 °C. *yceK* encodes a 75 aa protein of unknown function, which was verified to be a lipoprotein (Gonnet *et al.*, 2004). YceK has an N-terminal signal peptide, which is predicted to be cleaved by signal peptidase II between Gly15 and Cys16 (Juncker *et al.*, 2003). YceK has a paralog, YidQ, in *E. coli* K-12 and shares high identity with its homologs in *Salmonella*, *Yersinia*, *Enterobacter* and *Shigella*. *yceK* was also repressed eightfold in *E. coli* JM109 biofilms at 37 °C (Ren *et al.*, 2004a), but it was upregulated eightfold in EHEC biofilms at 30 °C (Lee *et al.*, 2007a). Also, deletion of the temperature-sensitive biofilm stress-regulator gene *bhsA* induced *yceK* 4.6-fold at 37 °C (Zhang *et al.*, 2007). Therefore, YceK may play a role in *E. coli* biofilm formation as a temperature sensor associated with indole. To investigate this role, we measured biofilm formation of the *yceK* knockout strain and found the deletion of *yceK* decreases biofilm formation at 37 °C by 2.6-fold, while there was no change in biofilm formation due to the deletion of *yceK* at 30 °C. Hence, YceK increases K-12 biofilm at 37 °C, but not at 30 °C. Similar to *yceK*, *yjjY* encoding a hypothetical protein showed the same pattern of gene expression in response to indole (Table 3).

Also, several ribosomal protein genes and *bhsA* were induced by indole at 37 °C (Table 3). Among the repressed genes, curli and fimbrial genes, *csgDEF* and *fimAC*, were repressed twofold by indole at 37 °C. Also, 12 membrane and transport genes were repressed about twofold by indole at 37 °C. However, indole did not directly regulate the gene expression of *sdiA* or *luxS* at both 30 and 37 °C.

Table 3 Partial list of the common differentially expressed genes for (i) biofilm cells of the BW25113 wild-type strain (WT) grown for 7 h at 30 vs 37 °C, (ii) biofilm cells of the *tnaA* mutant grown for 7 h with 1 mM indole vs no indole at 30 °C, (iii) biofilm cells of the *tnaA* mutant grown for 7 h with 1 mM indole vs no indole at 37 °C, (iv) suspension cells of WT vs *sdiA* mutant at an absorbance of 4.0 at 600 nm at 30 °C, (v) biofilm cells of the *sdiA* mutant grown for 7 h with 1 mM indole vs no indole at 30 °C, (vi) suspension cells of the *luxS* mutant with 100 μM AI-2 vs no AI-2 at 30 °C for 3 h and (vii) suspension cells of the *luxS* mutant with 100 μM AI-2 vs no AI-2 at 37 °C for 3 h

Gene	*b #*	*Fold changes*[a]							*Description*
		WT 30 vs 37 °C	tnaA *and indole vs no indole 30 °C*	tnaA *and indole vs no indole 37 °C*	*WT vs sdiA 30 °C*	sdiA *and indole vs no indole 30 °C*	luxS *and AI-2 vs no AI-2 30 °C*	luxS *and AI-2 vs no AI-2 37 °C*	
UMP synthesis pathway and uracil transport									
carA	b0032	**6.1**	**−19.7**	−1.6	**−8.6**	−1.9	−1.1	**9.8**	Carbamoyl-phosphate synthetase, glutamine (small) subunit
carB	b0033	3.7	**−9.2**	−1.2	**−9.8**	−1.9	−1.1	**7.5**	Carbamoyl-phosphate synthase large subunit
pyrC	b1062	3.2	**−5.7**	−1.1	−2.1	−2.1	−1.1	**3.0**	Dihydro-orotase
pyrD	b0945	1.0	**−3.0**	−1.1	−2.8	**−3.0**	−1.2	**5.7**	Dihydro-orotate dehydrogenase
pyrF	b1281	2.1	**−2.5**	1.1	**−3.0**	−1.4	−1.2	**2.0**	Orotidine-5-phosphate decarboxylase
pyrI	b4244	**6.1**	**−32.0**	−1.1	**−9.2**	−2.0	−1.1	**4.0**	Aspartate carbamoyltransferase, regulatory subunit
pyrB	b4245	**8.6**	**−32.0**	−1.2	**−7.5**	−1.1	−1.1	**2.5**	Aspartate carbamoyltransferase
pyrL	b4246	2.5	**−12.1**	1.7	**−4.6**	−1.2	−1.1	**2.3**	*pyrBI* operon leader peptide
uraA	b2497	1.6	**−59.7**[b]	−1.1[b]	**−3.7**	−2.1	−1.1[b]	**4.0**[b]	Uracil transport, uptake of uracil
upp	b2498	**5.3**	**−6.1**	1.5	−2.3	−1.6	−1.1	**3.2**	Uracil phosphoribosyltransferase
AHL- and indole-related									
sdiA	b1916	−1.2	1.2	−1.1	**119.4**	NA	−1.3	1.1	Transcriptional regulator of *ftsQAZ* gene cluster, indole regulator
tnaC	b3707	**11.3**	−1.1	−1.1	−1.7	1.5	1.0	1.9	Tryptophanase leader peptide
tnaA	b3708	**13.9**	NA	NA	−1.2	**3.2**	1.1	1.1	Tryptophanase
tnaB	b3709	**4.6**	−2.0	−1.1	−1.7	1.5	1.0	**2.3**	Low affinity tryptophan permease
ycgZ	b1164	1.7	**3.7**	−1.4	−1.1	−1.5	−1.1	1.1	Hypothetical protein
ymgA	b1165	1.7	**3.0**	−1.6	−1.1	1.0	−1.1	1.1	Hypothetical protein
ariA	b1166	1.4	**3.2**	−1.3	1.1	−1.2	−1.1	1.1	Indole inducing acid resistance gene, formerly YmgB
ymgC	b1167	−1.5	**4.0**	1.0	−1.0	1.0	−1.1	1.1	Hypothetical protein
bhsA	b1112	−1.7	**3.7**	**3.5**	−1.1	2.3	−1.1	−1.3	Biofilm stress regulator, formerly YcfR
bssR	b0836	−2.8	**−2.6**	−1.1	1.9	−1.4	−1.2	−1.3	Regulator of biofilm through indole signaling, formerly YliH
yceK	b1050	1.6	**−4.3**[b]	**9.8**[b]	−1.3	**29.9**	1.0[b]	1.4[b]	Putative lipoprotein with unknown function
yceJ	b1057	**−13.0**	**4.6**	−1.1	2.3	−1.3	1.0	−1.4	Cytochrome b561 homolog 2
yjjY	b4402	−2.0	**−2.1**	**5.3**	**1.5**	**11.3**	−1.1	1.7	Hypothetical protein
narG	b1224	1.1	**2.3**	1.4	1.9	1.0	1.0	1.0	Nitrate reductase 1-α subunit
narV	b1465	−1.1	**2.5**	1.1	1.6	1.5	−1.1	−1.5	Cryptic nitrate reductase 2-γ subunit
narW	b1466	1.6	**2.6**	1.4	1.7	1.4	−1.1	−1.1	Cryptic nitrate reductase 2-δ subunit
narY	b1467	1.2	**3.5**	−1.1	2.1	1.1	−1.1	−1.7	Cryptic nitrate reductase 2β subunit
narZ	b1468	1.7	**4.0**	−1.2	2.1	1.1	−1.1	−1.4	Cryptic nitrate reductase 2α subunit
napA	b2206	1.4	**−2.3**	−1.3	2.5	−1.2	1.1	−1.3	Periplasmic nitrate reductase, large subunit, in complex with NapB
napF	b2208	−1.6	**−4.3**	−1.1	2.3	−1.3	−1.1	1.1	Fe-S ferredoxin-type protein, electron transfer
cspI	b1552	−2.8	**2.5**	−1.1	2.1	1.7	−1.2	−1.7	Qin prophage; cold-shock-like protein
cspB	b1557	−2.1	**2.6**	1.0	1.2	1.5	−1.3	−1.2	Qin prophage; cold-shock protein
macA	b0878	−1.7	**4.6**	−1.1	1.7	−1.5	1.0	−1.1	Accessory protein to ABC-type macrolide transport protein MacB
entA	b0596	−1.7	**−4.0**	1.2	1.1	−1.6	−1.5	1.7	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
ansB	b2957	−2.0	**−4.3**	1.1	1.9	−2.0	1.1	1.0	Periplasmic L-asparaginase II
ompT	b0565	−1.3	**−2.0**	**−2.0**	−1.2	−1.4	1.0	1.3	DLP12 prophage; protease VII, outer membrane protein 3b (a)

Table 3 Continued

Gene	*b #*	*Fold changes*[a]							*Description*
		WT 30 vs 37°C	tnaA *and indole vs no indole 30°C*	tnaA *and indole vs no indole 37°C*	*WT vs sdiA 30°C*	sdiA *and indole vs no indole 30°C*	luxS *and AI-2 vs no AI-2 30°C*	luxS *and AI-2 vs no AI-2 37°C*	
ompF	b0929	**6.1**	**−3.7**	−1.4	−1.2	−1.4	1.1	**2.3**	Outer membrane pore protein 1a (Ia;b;F)
yciD	b1256	2.5	**−3.5**	1.1	1.4	1.2	1.1	1.1	Outer membrane protein W; colicin S4 receptor
csgG	b1037	1.4	1.1	−1.6	**8.0**	−2.6	−1.2	−1.2	Curli production assembly/transport component
csgF	b1038	2.1	1.3	**−2.5**	**6.7**	−2.0	−1.2	−1.1	Curli production assembly/transport component *csgF* precursor
csgE	b1039	2.1	1.3	**−2.1**	**3.7**	−1.3	−1.4	−1.1	Curli production assembly/transport component *csgE* precursor
csgD	b1040	3.7	1.2	**−2.0**	**3.2**	−1.2	−1.2	−1.1	Probable *csgAB* operon transcriptional regulatory protein
csgB	b1041	−1.4	1.4	−1.4	**17.1**	−2.6	−1.1	−1.5	Minor curlin subunit precursor, similar to CsgA
csgA	b1042	−1.2	1.2	1.0	**8.0**	−1.5	1.0	−1.2	Curlin major subunit, coiled surface structures; cryptic
csgC	b1043	−3.7	1.6	−1.1	**6.5**	−1.9	1.0	−1.4	Putative curli production protein
ycgR	b1194	−3.0	1.1	1.1	**−6.5**	−1.7	−1.1	1.1	Protein with PIL domain (c-di-GMP-binding domain)
yhjH	b3525	−2.0	1.2	−1.2	**−5.3**	−2.0	1.0	1.0	Probable cyclic-di-GMP phosphodiesterase
putA	b1014	3.0	1.5	1.1	**−8.6**	2.3	1.1	**5.3**	proline dehydrogenase
putP	b1015	3.0	1.1	−1.2	**−3.2**	1.0	1.1	**5.3**	Major sodiumproline symporter
soxS	b4062	−3.5	−1.7	1.2	**−3.2**	1.1	1.0	1.1	Regulation of superoxide response regulon
sokC	b4413	ND	ND	−1.7	**−3.7**	−1.2	−1.1	−1.5	Antisense RNA blocking *mokC* (orf69) and *hokC* (*gef*) translation
rtT	b4425	ND	ND	1.3	**−3.5**	1.1	−1.1	1.6	*rtT* RNA; may modulate the stringent response
purK	b0522	−2.0	1.0	−1.1	**4.9**	−1.9	−1.1	1.0	Phosphoribosylaminoimidazole carboxylase
purE	b0523	−2.6	−1.1	1.0	**5.3**	−1.9	−1.1	1.0	Phosphoribosylaminoimidazole carboxylase
purR	b1658	−2.0	1.1	1.2	**3.5**	−1.2	−1.1	−1.1	Transcriptional repressor for *pur* regulon
hdeD	b3511	2.5	1.2	−1.1	**−3.2**	1.9	1.0	1.0	Acid-resistance membrane protein
mdtE	b3513	1.5	1.1	−1.1	**−5.3**	−1.3	1.1	1.1	Multidrug-resistance efflux transporter
yicE	b3654	−2.3	−1.3	1.1	**−6.5**	−1.7	−1.1	−1.1	Putative nucleobase transporter
purT	b1849	−3.7	−1.3	1.0	**6.1**	−1.9	−1.2	1.6	Phosphoribosylglycinamide formyltransferase 2
purC	b2476	1.0	−1.5	−1.1	**3.2**	−1.5	−1.1	1.3	Phosphoribosylaminoimidazole-succinocarboxamide synthetase
purM	b2499	−1.2	−1.5	−1.1	**3.0**	−2.1	1.0	1.5	Phosphoribosylaminoimidazole synthetase
purL	b2557	−1.9	−1.1	1.0	**4.3**	−1.7	−1.1	1.4	Phosphoribosylformyl-glycineamide synthetase
purD	b4005	−1.3	−1.1	−1.1	**4.9**	−2.3	−1.1	1.7	Phosphoribosylglycinamide synthetase
AI-2-related									
luxS	b2687	2.8	1.1	1.3	−1.2	2.1	NA	NA	*S*-ribosylhomocysteine lyase (AI-2 synthesis protein)
lsrK	b1511	−1.1	1.2	1.4	−1.3	2.1	**4.6**	−1.7	Autoinducer-2 (AI-2) kinase; upregulated in biofilms
lsrR	b1512	1.3	1.4	1.3	−1.1	1.9	**4.9**	**−2.3**	*lsrACDBFGE* operon regulator for autoinducer-2 (AI-2) uptake
lsrA	b1513	−1.5	1.0[b]	1.5[b]	1.1	2.0	**9.2**[b]	**−2.3**[b]	Autoinducer-2 (AI-2) uptake; essential for aerobic growth
lsrC	b1514	−1.1	1.1	1.4	1.1	1.9	**9.8**	−1.9	Autoinducer-2 (AI-2) uptake
lsrD	b1515	−1.1	1.2	1.5	1.5	1.7	**7.0**	−1.4	Autoinducer-2 (AI-2) uptake
lsrB	b1516	3.0	1.2	−1.2	−1.4	1.7	**5.3**	**−2.5**	Autoinducer-2 (AI-2) uptake
lsrF	b1517	2.8	1.3	1.1	−1.4	2.5	**4.6**	−1.7	Function unknown, involved in AI-2 catabolism
lsrG	b1518	2.1	1.1	1.0	−1.7	2.5	**6.1**	−1.7	Function unknown, involved in AI-2 catabolism
Motility, chemotaxis and fimbriae									
flgN	b1070	−1.6	1.1	1.1	**−3.7**	−1.4	1.0	1.1	Protein of flagellar biosynthesis
flgM	b1071	−1.6	1.1	−1.1	**−3.7**	−1.6	1.1	1.1	Anti-FliA (antisigma) factor; also known as RflB protein
flgB	b1073	**−12.1**	**−6.5**	−1.2	**−2.3**	−2.3	1.1	**2.0**	Flagellar biosynthesis, cell-proximal portion of basal-body rod
flgF	b1077	−1.6	−1.6	−1.1	**−2.8**	−2.5	1.1	**2.1**	Flagellar biosynthesis, cell-proximal portion of basal-body rod
flgK	b1082	−2.3	1.2	−1.1	**−4.6**	−2.1	1.1	−1.1	Flagellar biosynthesis, hook-filament junction protein 1

Table 3 Continued

Gene	*b #*	*Fold changes*[a]							*Description*
		WT 30 vs 37°C	tnaA *and indole vs no indole 30°C*	tnaA *and indole vs no indole 37°C*	*WT vs sdiA 30°C*	sdiA *and indole vs no indole 30°C*	luxS *and AI-2 vs no AI-2 30°C*	luxS *and AI-2 vs no AI-2 37°C*	
flgL	b1083	−2.3	1.0	−1.1	**−3.2**	−1.5	1.1	−1.1	Flagellar biosynthesis; hook-filament junction protein
cheZ	b1881	−2.8	−1.1	1.1	**−4.6**	−1.3	1.0	1.1	Chemotactic response; CheY protein phophatase; antagonist of CheY as switch regulator
cheY	b1882	1.0	1.3	1.0	**−4.3**	−1.5	1.1	1.2	Chemotaxis regulator transmits chemoreceptor signals to flagellar motor components
cheB	b1883	−1.9	1.4	−1.2	**−4.0**	−2.0	1.0	−1.2	Response regulator for chemotaxis (*cheA* sensor); protein methylesterase
cheR	b1884	−2.6	1.2	−1.2	**−3.7**	−1.7	1.0	−1.1	Response regulator for chemotaxis; protein glutamate methyltransferase
tap	b1885	−1.6	1.5	−1.2	**−4.9**	−1.7	1.1	1.0	Methyl-accepting chemotaxis protein IV, peptide sensor receptor
tar	b1886	−1.7	1.1	−1.1	**−5.3**	−1.9	1.1	1.1	Methyl-accepting chemotaxis protein II, aspartate sensor receptor
cheW	b1887	−2.8	−1.4	1.0	**−6.1**	−1.7	1.1	1.1	Positive regulator of CheA protein activity
cheA	b1888	−1.6	−1.4	−1.1	**−5.3**	−1.7	1.1	1.1	Sensory transducer kinase between chemo- signal receptors and CheB and CheY
motB	b1889	**−11.3**	−1.4	−1.1	**−4.3**	−1.9	1.0	1.1	Enables flagellar motor rotation, linking torque machinery to cell wall
motA	b1890	**−5.3**	1.1	1.0	**−4.3**	−2.1	1.0	1.1	Proton conductor component of motor; no effect on switching
tsr	b4355	−1.1	1.1	−1.1	**−3.0**	−1.6	1.1	−1.1	Methyl-accepting chemotaxis protein I, serine sensor receptor
fliZ	b1921	−3.2	1.1	−1.3	**−3.0**	−2.0	1.1	1.1	Hypothetical protein
fliA	b1922	−3.5	−1.4	−1.1	**−6.1**	−2.0	1.1	1.1	Flagellar biosynthesis; alternative sigma factor 28; regulation of flagellar operons
fliC	b1923	−1.5	−1.1	1.2	**−6.1**	−1.3	1.1	1.1	Flagellar biosynthesis; flagellin, filament structural protein
fliD	b1924	**−5.3**	1.1	−1.1	**−7.0**	−2.6	1.1	−1.2	Flagellar biosynthesis; filament capping protein
fliS	b1925	−2.8	1.6	−1.2	**−4.3**	−2.1	1.1	1.1	Flagellar biosynthesis; repressor of class 3a and 3b operons
fliT	b1926	−1.7	1.4	−1.1	**−3.2**	−1.7	1.0	−1.2	Flagellar biosynthesis; repressor of class 3a and 3b operons
fimA	b4314	2.0	**−4.3**	**−2.5**	**−3.7**	1.4	1.1	1.0	Major type 1 subunit fimbrin (pilin)
fimC	b4316	−2.8	−1.3	**−2.6**	**−3.0**	−1.7	1.0	1.5	Periplasmic chaperone, required for type 1 fimbriae

LB medium was used for all experiments. Complete list of significantly changed genes are shown in the supplementary tables. Raw data for the 14 DNA microarrays are available using GEO series accession number GSE 9923.
[a]Most significant changes are shown in boldface (NA: not applicable; ND: not detected, since genes not present).
[b]Verified gene expression by quantitative real time-PCR.

Verification of microarray results by qRT-PCR

Quantitative RT-PCR was used to verify gene expression for *uraA*, *yceK* and *lsrA* in four sets of the DNA microarray experiments (response due to indole of the *tnaA* mutant at 30 °C and at 37 °C and the response due to AI-2 of the *luxS* mutant at 30 and 37 °C). Using four sets of independent cultures, the qRT-PCR showed comparable changes in expression compared to the DNA microarrays. For the indole-*tnaA* array at 30 °C (ii), in the qRT-PCR experiment *uraA*, *yceK* and *lsrA* were repressed by 19.3-, 1.4- and 1.3-fold, respectively, whereas in the microarray experiments these genes were repressed by 60-, 4.3- and 1.5-fold, respectively; hence, the qRT-PCR corroborated the large inhibition of *uraA* expression at 30 °C while showing little differential expression for *yceK* and *lsrA* at 30 °C. Similarly, for the indole-*tnaA* array at 37 °C (iii), in the qRT-PCR experiment, *uraA*, *yceK* and *lsrA* are changed by -1.7-, 3.5- and 1.2-fold, respectively, whereas in the microarrays, gene expression was altered by -1.1-, 9.8- and 1.0-fold, respectively. For the AI-2-*luxS* array at 30 °C (vi), in the qRT-PCR experiment, *uraA*, *yceK* and *lsrA* are changed by -1.8-, -1.8- and 12.8-fold, respectively, whereas in the microarray these genes were changed by -1.1-, 1.0- and 9.2-fold, respectively. For the AI-2-*luxS* array at 37 °C (vii), in the qRT-PCR experiment, *uraA*, *yceK* and *lsrA* are changed by 1.2-, 2.4- and -2.7-fold, respectively, whereas in the microarray these genes were changed by 4.0-, 1.4- and -2.3-fold, respectively. Therefore, the DNA microarray data were verified by qRT-PCR.

Temperature and inhibition of flagellar gene transcription by indole

Previously, we showed indole decreases the swimming motility of nonpathogenic *E. coli* at 37 °C (Lee *et al.*, 2007b) as well as pathogenic EHEC at 37 °C (Bansal *et al.*, 2007). In the current microarray analysis, indole represses the flagellum gene *flgB* (6.5-fold) at 30 °C (Table 3). To study the genetic and temperature basis of this decrease in motility and flagella gene expression by indole, we used transcription reporters to probe directly the ability of indole to alter the transcription of genes that control motility, *qseB*, *flhD* and *fliA*, in the indole-deficient *tnaA* strain at 30 °C or 37 °C. In the absence of indole, promoter activity of the three genes at 37 °C was 4- to 13-fold lower than that at 30 °C (Figure 2), which partially agrees with a previous report that higher temperatures (37 °C) causes a rapid reduction in motility in several *E. coli* strains (Morrison and McCapra, 1961). However, swimming motility of the *E. coli* BW25113 used in this study was not changed upon increasing temperature from 30 to 37 °C (1.6 ± 0.1 vs 1.7 ± 0.1 cm at 20 h).

Upon the addition of 1 mM indole, transcription of the quorum-sensing flagella regulon *qseB* (Sperandio *et al.*, 2002) was repressed 3.1 ± 0.3-fold at 30 °C but was unchanged at 37 °C (Figure 2). Also, the addition of indole led to a 4.6 ± 0.4-fold decrease in transcription of *flhD* (master controller of the flagella regulon) at 30 °C but indole did not change its transcription at 37 °C (Figure 2). Similarly, there was a 5.7 ± 0.7-fold decrease of transcription of *fliA* (sigma factor σ^{28}) at 30 °C but only a 2.1 ± 0.2-fold decrease at 37 °C (Figure 2). These results clearly show a temperature dependence of indole on flagellar gene expression and partially explain the more significant effect of indole on biofilm inhibition at 30 °C than that at 37 °C. However, indole (0.5 mM) decreased the swimming motility of BW25113 *tnaA* at 30 °C (0.4 ± 0.1 vs 0.21 ± 0.02 cm at 8 h) and 37 °C (0.45 ± 0.05 vs 0.22 ± 0.03 cm at 8 h) by about twofold. Hence, indole may decrease motility by controlling transcription as well as translation of motility genes. This discrepancy, the fact that the transcription of the motility genes was altered by temperature but motility was not changed, may be related to the recent finding by us and others that many of the mutants in Keio collection are hypermotile, which is probably caused by IS insertion into *flhDC* (Hobman *et al.*, 2007).

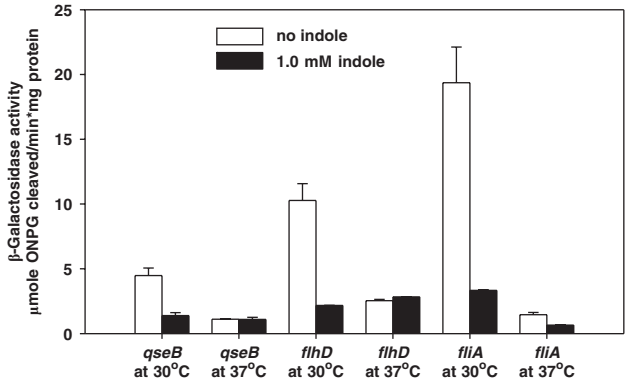

Figure 2 Effect of indole (1 mM) on the transcription of *qseB*::*lacZ*, *flhD*::*lacZ* and *fliA*::*lacZ* for *E. coli* BW25113 *tnaA* in LB at 30 and 37 °C as determined by β-galactosidase activity. Each data point was averaged from two independent cultures, and one standard deviation is shown.

Temperature and enhancement of antibiotic resistance by indole

As indole increases drug resistance by inducing multidrug exporter genes (Hirakawa *et al.*, 2005) and as SdiA controls multidrug resistance (Rahmati *et al.*, 2002), we investigated if temperature affects the ability of indole to alter antibiotic resistance by measuring the kanamycin resistance of the wild-type strain and the *tnaA* (ΔKm) mutant with and without indole (1 mM) at both 30 and 37°C. Using exponential-phase cells and contacting with 0.1 mg ml^{-1} kanamycin for 60 min, the addition of 1 mM indole to the *tnaA* mutant increased cell survival 30-fold at 30 °C (1.2 ± 0.5% with indole vs 0.04 ± 0.06% without indole), whereas at 37 °C, the addition of indole to the *tnaA* mutation increased

cell survival only by twofold ($0.004 \pm 0.002\%$ with indole vs $0.002 \pm 0.001\%$ without indole). In a similar experiment in which stationary-phase *tnaA* cells were compared to wild-type cells and both were contacted with $2\,mg\,ml^{-1}$ kanamycin for 30 min, the *tnaA* mutant deficient in indole production had fivefold lower survival ($6 \pm 8\%$) compared to the wild-type strain ($32 \pm 4\%$) at 30 °C, whereas at 37 °C, the *tnaA* mutation did not affect survival significantly ($15 \pm 2\%$) compared to the wild-type strain ($21 \pm 4\%$). Also, addition of 1 mM indole to the *tnaA* mutant partially restored kanamycin resistance at 30 °C ($15 \pm 10\%$) and fully restored kanamycin resistance at 37 °C ($25 \pm 9\%$). Therefore, indole increases antibiotic resistance and is more efficient at lower temperature.

Temperature and inhibition of cell division by indole

SdiA regulates *ftsQAZ* and cell division (Wang *et al.*, 1991), and high concentrations of indole (4 mM) terminate cell division by an unknown mechanism (Chant and Summers, 2007). Using microscopy to investigate the impact of temperature on the inhibition of cell division by indole, we found 4 mM indole increases the length of BW25113 *tnaA* approximately threefold (0.7 ± 0.1 vs $1.9 \pm 0.5\,\mu m$) at 37 °C and fourfold (0.6 ± 0.2 vs $2.1 \pm 0.3\,\mu m$) at 30 °C. This slightly greater affect at 30 °C was corroborated by determining the number of cells at a fixed absorbance with and without indole (1 mM was used as this is a more physiologically relevant concentration than 4 mM indole, and 4 mM indole inhibits growth). As the absorbance is fixed, the samples with indole should require lower cell numbers to reach the same light scattering due to their larger size. As expected, 1 mM indole decreased the number of cells by 40% for the indole-deficient *tnaA* mutant compared to no indole ($0.63 \pm 0.02 \times 10^9$ vs $1.04 \pm 0.05 \times 10^9$ cells per ml per OD) at 30 °C, whereas at 37 °C, indole decreased the number of cells by only 22% compared to no indole (0.63 ± 0.07 vs $0.81 \pm 0.07 \times 10^9$ cells per ml per OD). Similarly, at fixed absorbance, the wild-type strain had less cells than the *tnaA* mutant because it synthesizes indole ($0.8 \pm 0.1 \times 10^9$ vs $1.04 \pm 0.05 \times 10^9$ cells per ml per OD) at 30 °C, whereas there are nearly the same number at 37 °C ($0.70 \pm 0.03 \times 10^9$ vs $0.81 \pm 0.07 \times 10^9$ cells per ml per OD). Therefore, our results confirm that indole increases the size of *E. coli* and that indole is more active at lower temperatures.

SdiA and indole signaling at 30 °C

As *E. coli* strongly responded to AHLs through SdiA at 30 °C but responded only very weakly at 37 °C (Van Houdt *et al.*, 2006), as indole did not affect biofilm formation significantly at 37 °C (Figure 1) and as indole accumulates in the stationary phase (Hirakawa *et al.*, 2005), DNA microarrays were performed with stationary BW25113 *sdiA* suspension cells (absorbance of 4.0 at 600 nm) at 30 °C and compared with the wild-type strain grown at the same conditions to determine the genes that SdiA controls. It was found that deletion of *sdiA* induced 40 genes and repressed 42 genes more than threefold (Table 3 and Supplementary Table 4) at 30 °C; hence, SdiA is a global regulator.

Bacterial flagellar biosynthesis genes *flgMN, flgKL, fliDST*, *fliAZ* and *fliC* were repressed by SdiA from three- to sevenfold at 30 °C (Table 3); these genes are induced by AI-2 at 37 °C (Ren *et al.*, 2004b). Also, the chemotaxis genes *cheRBYZ, cheAW, motAB, tar, tap* and *tsr* were repressed from three- to sixfold at 30 °C (Table 3) and are also induced by AI-2 at 37 °C (Ren *et al.*, 2004b). These results imply the inter-relatedness of SdiA-mediated and AI-2 signaling. Also, two fimbriae-related genes, *fimAC*, were repressed from three- to fourfold by SdiA (Table 3). Repression of flagellar biosynthesis genes, chemotaxis genes and fimbriae-related genes by SdiA at 30 °C is consistent with the increased biofilm formation phenotype of the *sdiA* mutant compared to the wild-type strain (Lee *et al.*, 2007b). The same UMP biosynthesis genes (*carAB, pyrLBI* and *pyrF*) and the uracil importer gene *uraA* were repressed by SdiA from 3- to 10-fold at 30 °C, whereas these genes were most repressed upon adding indole to the *tnaA* mutant at 30 °C that expressed SdiA (Table 3). In addition to the biofilm results (Figure 1), these whole-transcriptome results again support that the signal indole requires SdiA (Lee *et al.*, 2007b).

The most induced genes by SdiA at 30 °C include a group of purine biosynthesis genes, *purEK, purHD, purC, purM, purL, purR* and *purT* (three- to sixfold) (Table 3). Among them, the purine biosynthesis genes *purEK, purHD, purM* and *purT* were repressed by AI-2 at 37 °C (Ren *et al.*, 2004b). Note that at 30 °C, deleting the AI-2 synthesis gene *luxS* did not affect purine biosynthesis gene expression (Wang *et al.*, 2005). These results show that SdiA represses pyrimidine biosynthesis genes (that is, UMP) and at the same time induces purine biosynthesis genes at 30 °C. Another induced locus by SdiA is the curli formation gene, *csgABCDEFG* (3- to 17-fold) (Table 3).

To confirm the role of SdiA in indole signaling, DNA microarrays were used to determine the differential gene expression profile of biofilm cells of the *sdiA* mutant upon adding 1 mM indole vs no indole at 30 °C (Table 3 and Supplementary Table 5); if SdiA is important for mediating indole signaling, then the *sdiA* mutant should respond differently than the *tnaA* mutant with indole. It was found that the addition of indole 1 mM to the *sdiA* mutant significantly induced only 30 genes and repressed 11 genes at 30 °C compared to the *tnaA* mutant which had five times more genes differentially expressed upon the addition of indole. Furthermore, unlike the indole and AI-2 DNA microarrays with

both the *tnaA* and *luxS* mutants (Table 3), the addition of indole to the *sdiA* mutant did not affect genes related to UMP and the pattern of gene expression was markedly different (Table 3), which corroborates that SdiA is necessary for indole signaling. The addition of indole at 30 °C to the *sdiA* mutant induced strongly *yceK* (30-fold) and *yjjY* (11-fold). Also, 11 adaptation-related genes including *tnaA* and five small regulatory RNA genes were induced upon the addition of indole to this strain (Supplementary Table 5).

Temperature and AI-2 signaling

To determine whether temperature plays a major role in signaling by other molecules, we investigated the effect of AI-2 signal addition on gene expression at 30 and 37 °C. To ensure exogenous AI-2 was taken up by the cells, we first investigated the time at which externally added AI-2 is imported by the BW25113 *luxS* mutant. AI-2 (100 μM) was used in a recent study (Kendall *et al*., 2007) and shown to be physiologically relevant (De Keersmaecker *et al*., 2005), so we added 100 μM AI-2 during the exponential phase (at an absorbance of 0.5 at 600 nm) and measured the extracellular AI-2 activity every hour. More than 90% of the exogenous AI-2 (100 μM) was absorbed by the *luxS* strain between 2 and 3 h after adding AI-2 during the middle of the exponential growth phase (Figure 3); the disappearance of AI-2 from the medium suggests that exogenous AI-2 (100 μM) was readily metabolized by *E. coli* cells and that 100 μM AI-2 was physiologically relevant for this AI-2 study. Hence, the transcriptome analysis was performed at 3 h after adding 100 μM AI-2 at 30 or 37 °C to ensure AI-2 uptake had occurred. Under these conditions, addition of AI-2 at 37 °C significantly induced 27 genes and repressed 36 genes more than twofold, while addition of AI-2 at 30 °C only induced nine genes and repressed three genes more than twofold (Table 3 and Supplementary Tables 6 and 7). The nine

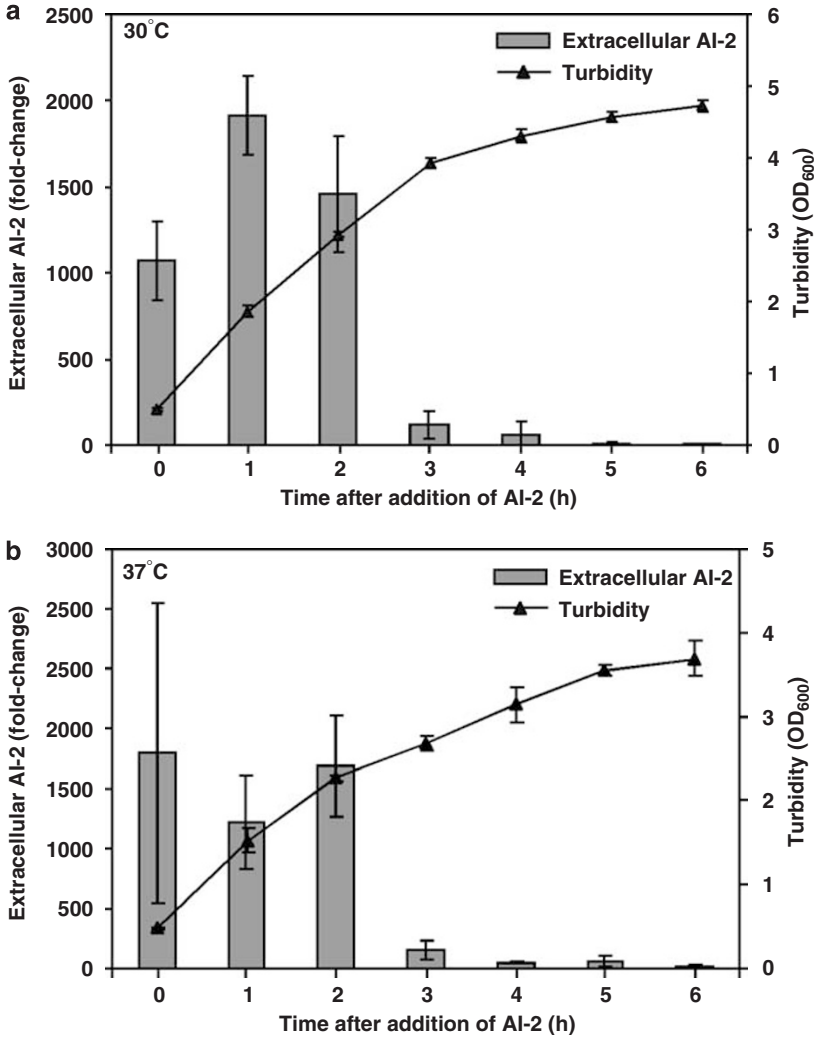

Figure 3 AI-2 uptake assay at 30 °C (**a**) and 37 °C (**b**). BW25113 *luxS* was grown in LB medium to an absorbance of 0.5 at 600 nm, and 100 μM AI-2 was added. Supernatants were collected, and the *V. harveyi* autoinducer assay was performed to determine the amount of extracellular AI-2 remaining. Two independent cultures were used to generate the AI-2 uptake profile.

induced genes by AI-2 at 30 °C include the *lsr* locus (*lsrKRACDBFG*) with eight genes (from 4.6- to 9.8-fold), which are involved in AI-2 uptake and internalization (Table 3), and *tynA* encoding copper amine oxidase (2.5-fold). This result suggests that at 30 °C the AI-2 signal still positively regulates AI-2 transport and exogenous AI-2 was imported into *E. coli* cells and metabolized by cells, but this signal does not generate any further impact on bacterial gene expression.

In contrast to 30 °C, addition of AI-2 at 37 °C induced much more gene expression including flagellar genes *flgB* and *flgF* (twofold), UMP/uracil synthesis genes (*carAB* 7.5- to 9.5-fold; *pyrLBI, pyrD* and *pyrF* two- to sixfold; and *upp* encoding uracil phosphoribosyltransferase threefold) and the uracil transport gene *uraA* (fourfold) (Table 3). Addition of AI-2 at 37 °C after 3 h-incubation caused *lsrR* and *lsrAB* to be repressed 2.3- to 2.5-fold. This is consistent in that the AI-2 uptake experiment indicated that the bacterial cells import AI-2 rapidly (after 2-h of incubation); after 3 h of incubation, most exogenous AI-2 is intracellular (Figure 3). So repression of *lsrR* and *lsrAB* reflects rapid uptake of exogenous AI-2.

Since the addition of AI-2 induces transcription of two virulence genes (*espA* and *eae*) in the EHEC *luxS* strain (Kendall *et al.*, 2007), the temperature dependence of AI-2 signaling was also assessed by monitoring the expression of these two genes in the same EHEC *luxS* strain at 30 and 37 °C. Our data agreed well with the previous results (Kendall *et al.*, 2007) in that AI-2 significantly induced the expression of both *espA* (11.8 ± 0.6-fold) and *eae* (6.57 ± 0.02-fold) relative to controls at 37 °C. However, no significant change in expression was observed at 30 °C. These results are in good agreement with our microarray data (Table 3) and strongly suggest that AI-2 signaling occurs primarily at 37 °C but not 30 °C.

Discussion

Here we demonstrate that the *E. coli* signal indole functions mainly at low temperatures. Five sets of evidence, (i) whole-transcriptome gene expression, (ii) biofilm formation, (iii) promoter activity, (iv) cell division and (v) antibiotic resistance, show indole functions as a signal more significantly at 25 and 30 °C than at 37 °C. In addition, two sets of evidence show AI-2 functions primarily at 37 °C: whole-transcriptome studies and virulence gene transcription. We also show that SdiA is necessary for *E. coli* to control its biofilm formation with indole in the same way as SdiA is necessary for *E. coli* to alter its biofilm formation upon addition of AHLs (Lee *et al.*, 2007b), that indole addition to the *sdiA* mutant does not alter gene expression in the same way as the *tnaA* mutant, and that five times more genes are controlled upon indole addition when *sdiA* is not deleted. Also, our whole-transcriptome studies reveal that indole-, SdiA- and AI-2-based signaling in *E. coli* are intertwined through UMP biosynthesis and uracil transport.

E. coli produces indole by tryptophanase (encoded by *tnaA*) that can reversibly convert tryptophan into indole, pyruvate and ammonia (Newton and Snell, 1965). Indole is a signal (Wang *et al.*, 2001; Di Martino *et al.*, 2003) that inhibits biofilms (Lee *et al.*, 2007b) and works in a quorum-sensing fashion (Lee *et al.*, 2007a). Indole decreases *E. coli* biofilms by influencing motility, acid resistance, chemotaxis and attachment to epithelial cells (Domka *et al.*, 2006; Bansal *et al.*, 2007; Lee *et al.*, 2007a, b) and controls plasmid stability (Chant and Summers, 2007) (summarized in Figure 4). Also, indole induces the expression of multidrug exporter genes and increases drug resistance (Hirakawa *et al.*, 2005), and tryptophanase activity has been linked to the killing of nematodes by pathogenic *E. coli* (Anyanful *et al.*, 2005). In our study, indole clearly enhances antibiotic resistance primarily at 30 °C. Furthermore, Chant and Summers (2007) observed that indole prevents cell division in *E. coli*. In this study, we have confirmed this phenomenon by microscopy and also found that the result is temperature dependent. In addition, indole increases the biofilm formation of *Pseudomonas aeruginosa* that does not synthesize indole (Lee *et al.*, 2007b). Recently we observed that indole diminishes production of *P. aeruginosa* virulence factors by repressing quorum-sensing-related genes (unpublished data). Therefore, *E. coli* probably utilizes indole as a tool to protect itself from antibiotics and to compete with other bacteria in mixtures. In this study, we have discovered another important aspect of indole signaling in *E. coli*: it is temperature dependent.

Recent DNA microarray studies show that temperature shifts from 37 to 23 °C alter extensively gene expression (total 423 genes) involved in the utilization of iron, carbohydrate and amino acids (White-Ziegler *et al.*, 2007) and alter the general stress response of *E. coli* (White-Ziegler *et al.*, 2008). Here we show significant changes in gene expression (252 genes) in biofilm cells due to a difference in temperature (30 and 37 °C, Supplementary Table 1), which confirms that temperature is important for gene expression in *E. coli*. Low temperatures (23 °C) also induced *pyrB* and *pyrL* (White-Ziegler *et al.*, 2008), which agrees with our microarray data for biofilm cells grown in LB at 30 °C vs biofilm cells grown at 37 °C (Table 3).

The *E. coli* quorum-sensing regulator, SdiA (240 aa), belongs to the LuxR/UhpA family of transcriptional regulators and regulates several genes involved in cell division (Wang *et al.*, 1991). SdiA is stabilized upon binding with AHLs (Yao *et al.*, 2006), which are not synthesized by *E. coli* cells (Michael *et al.*, 2001); hence, SdiA may be responsible for detecting signals from other bacteria

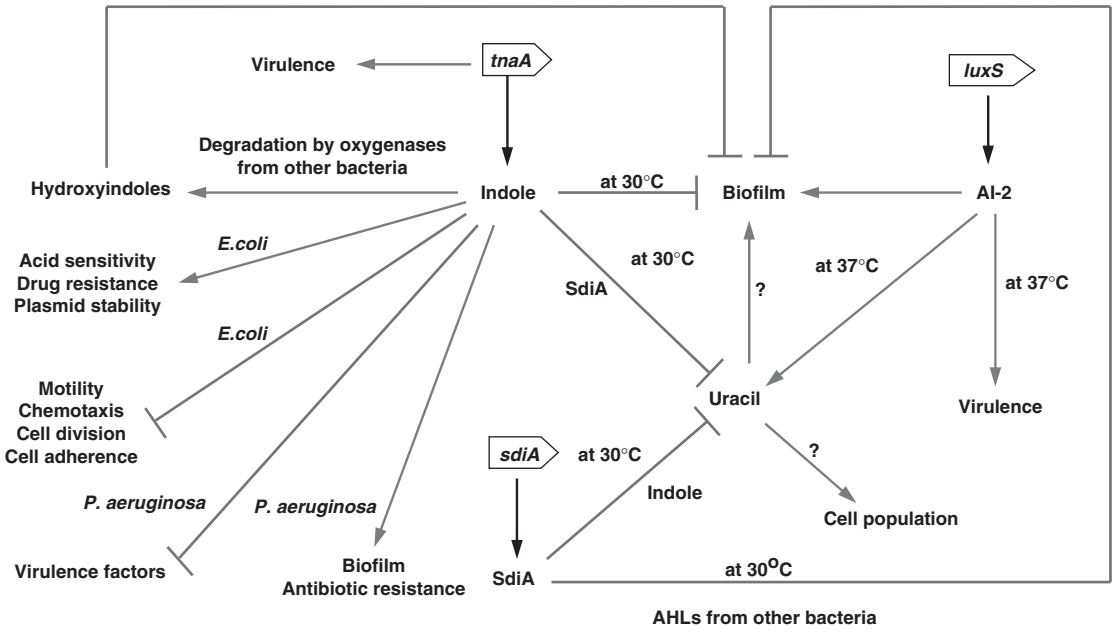

Figure 4 Conceptual model of the interaction of *E. coli* signals (AHLs, AI-2 and indole) on cellular phenotypes. → indicates induction of gene expression or stimulation of a phenotype, ⊥ indicates repression of gene expression or repression of a phenotype and black arrows indicate expression. Temperatures are indicated where there is a clear difference in phenotype.

(Ahmer, 2004; Van Houdt *et al*., 2006). In addition, *E. coli* strongly responded to AHLs at 30 °C, but very weakly at 37 °C (Van Houdt *et al*., 2006). Also, we reported that indole is an interspecies biofilm signal whose action appears to be mediated by SdiA (Lee *et al*., 2007b) and our results here confirm this (Figure 1). Therefore, SdiA detects AHLs from other bacteria (to at least control *E. coli* biofilm formation) (Lee *et al*., 2007b), and indole signaling requires the quorum-sensing protein SdiA; however, unlike AI-2 signaling, indole signaling is primarily detected at temperatures lower than 37 °C.

To understand the function of SdiA, Wei *et al*. (2001) studied global gene expression upon over-expressing SdiA using exponentially grown *E. coli* at 37 °C (absorbance was 0.45 at 600 nm, therefore these cells had a low concentration of extracellular indole); they found changes in efflux pump genes and genes involved in DNA repair and replication and that SdiA reduced *tnaA* transcription fourfold. Also, the deletion of *sdiA* results in a significant decrease in gene transcription of the *lsr* operon (AI-2-regulated genes) in cells at 37 °C in the late exponential phase (absorbance of 1.8 at 600 nm, personal communication with Professor William Bentley). In this study, stationary-phase cells (absorbance of 4.0 at 600 nm) at 30 °C in which the extracellular indole concentration was high (400 μM in the *sdiA* mutant and 446 μM in the wild-type strain) was used, and we found that SdiA regulated similarly UMP and uracil synthesis compared to the addition of indole at 30 °C (Table 3). Therefore, the function of SdiA is dependent on cell growth and is closely linked to indole signaling at 30 °C as well as AI-2 signaling at 37 °C. At 30 °C, although highly speculative, we hypothesize indole binds SdiA, changes its conformation, and this pair repress UMP and uracil synthesis transcription (Figure 4).

Additionally, SdiA at 30 °C repressed (five- to sixfold) two recently identified genes *ycgR* and *yhjH*, which are involved in the bacterial second messenger cyclic-di-GMP signal pathway (Table 3). *ycgR* encodes cyclic-di-GMP receptor protein with a PIL domain (c-di-GMP-binding domain), which affects exopolysaccharide synthesis and flagella- and pili-based motility (Ryjenkov *et al*., 2006). *yhjH* encodes a probable cyclic-di-GMP phosphodiesterase with an EAL domain which is involved in cyclic-di-GMP degradation (Rahman *et al*., 2007). Whether SdiA regulates bacterial biofilm formation through cyclic-di-GMP at 30 °C deserves to be investigated further. In addition, SdiA repressed the proline-symporter gene *putP* and its regulator encoded by *putA* 3.2- and 8.6-fold (Table 3), respectively, at 30 °C. SdiA also repressed *soxS* (3.2-fold); *soxS* encodes the regulator of the two-component system SoxRS, which responds to superoxide (Amábile-Cuevas and Demple, 1991), and *soxS* is one of the most-induced genes in *E. coli* biofilms (Ren *et al*., 2004a). Interestingly, SdiA repressed two regulatory RNA genes, *sokC* and *rtT*, by 3.7- and 3.5-fold, respectively, at 30 °C. *sokC* encodes an anti-sense RNA which blocks the translation of the small toxic membrane polypeptide HokC (Poulsen *et al*., 1991), and *rtT* RNA may be released from the primary *tyrT* transcript during tRNA processing, which possibly has a modulatory effect on the stringent response (Bosl and Kersten, 1991). Among

the 42 most repressed genes by SdiA at 30 °C, 20 of them encode transporters or membrane-associated proteins. For example, *hdeD* encoding an acid-resistance membrane protein, *mdtE* encoding a multidrug-resistance efflux transporter, and *yicE*, encoding a putative nucleotide transporter, were repressed from three- to sixfold by SdiA at 30 °C (Table 3). All three of these genes are repressed by AI-2 at 37 °C (Ren *et al.*, 2004b).

The AI-2 signal is involved in not only intraspecies but also cross-species communication (Surette and Bassler, 1998). Extracellular AI-2 accumulates during the exponential phase and decreases precipitously upon entry into the stationary phase (Xavier and Bassler, 2005). AI-2 signaling is involved in biofilm formation and motility (Ren *et al.*, 2004b; González Barrios *et al.*, 2006; Herzberg *et al.*, 2006), regulation of metabolic processes in *E. coli* (Wang *et al.*, 2005) and regulation of LEE4 in EHEC (*espA*, one of the LEE pathogenicity islands) (Kendall *et al.*, 2007). Our transcriptome data and qRT-PCR of two virulence genes (*espA* and *eae*) show that AI-2 signaling is involved in UMP and uracil synthesis at 37 °C and suggest that the control of pathogen genes by AI-2 is temperature dependent.

Our whole-transcriptome studies show that the UMP and uracil synthesis genes were divergently regulated in a temperature-dependent manner by indole, AI-2 and SdiA (note that four independent sets of microarray data show the importance of UMP, Table 3). Although highly speculative, these results suggest that a building block of RNA, uracil, may report the status of three bacterial signals, AHLs, AI-2 and indole, to *E. coli*. The effect of uracil on bacterial quorum sensing may be general, as we have found by screening 5850 *P. aeruginosa* transposon mutants for altered biofilm formation that uracil and UMP synthesis influences the formation of three quorum-sensing compounds related to LasR (elastase) and RhlR (pyocyanin, rhamnolipids), as well as influences swarming and PQS formation (unpublished results).

A network of transcriptional regulation is emerging based on AHLs, AI-2, uracil and indole in *E. coli* (Figure 4). Although AI-2 is heat labile and exists transiently during the exponential phase (Surette and Bassler, 1998), we show here that AI-2 regulates global gene expression primarily at 37 °C. In contrast, indole is stably maintained after the exponential phase and mainly regulates gene expression at 30 °C, and indole signaling is associated with the quorum-sensing sensor SdiA. It appears that AI-2 signaling dominates during rapid growth (exponential phase for planktonic cells) in the human host (37 °C), whereas indole functions during slow growth (stationary phase) outside the human host (less than 37 °C). Therefore, the environment (temperature) is an important cue for indole and AI-2 signaling in *E. coli*. Furthermore, as *V. harveyi* uses shared regulatory components to discriminate multiple autoinducers and LuxR controls all the quorum-sensing-regulated genes (Waters and Bassler, 2006), *E. coli* also has the capability to discern multiple signals upon changes in its environment, and the LuxR homolog SdiA is associated with multiple signals (for example, indole, AHLs and AI-2).

Acknowledgements

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