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Minireview

Insights on *Escherichia coli* biofilm formation and inhibition from whole-transcriptome profiling

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Summary

Biofilms transform independent cells into specialized cell communities. Here are presented some insights into biofilm formation ascertained with the best-characterized strain, *Escherichia coli*. Investigations of biofilm formation and inhibition with this strain using whole-transcriptome profiling coupled to phenotypic assays, *in vivo* DNA binding studies and isogenic mutants have led to discoveries related to the role of stress, to the role of intra- and interspecies cell signalling, to the impact of the environment on cell signalling, to biofilm inhibition by manipulating cell signalling, to the role of toxin/antitoxin genes in biofilm formation, and to the role of small RNAs on biofilm formation and dispersal. Hence, *E. coli* is an excellent resource for determining paradigms in biofilm formation and biofilm inhibition.

Biofilm formation

Biofilms are a community of microorganisms attached to a surface by polysaccharides, proteins and nucleic acids (Sauer *et al*., 2007). *Escherichia coli* biofilm development is a complex process that leads to beautiful structures (Fig. 1) that are important for disease and for engineering applications [note the first engineered biofilm was created to secrete peptide antimicrobials to reduce corrosion (Jayaraman *et al*., 1999)]. These matrices are formed through at least five developmental stages that include (i) initial reversible attachment of planktonic cells to a solid surface, (ii) transition from reversible to irreversible attachment, (iii) early development of biofilm architecture, (iv) development of microcolonies into a mature biofilm and (v) dispersion of cells from the biofilm to return to the planktonic state (van Houdt and Michiels, 2005). Early steps in biofilm formation require the synthesis of different bacterial surface appendages including flagella that allow reversible attachment (Prüß *et al*., 2006) and cell motility which is a determinant of biofilm architecture (Wood *et al*., 2006). For irreversible attachment, flagella synthesis is repressed and adhesive organelles like curli fimbriae, encoded by the *csg* operon, and type I fimbriae, encoded by *fim* genes, are important for biofilm formation (Prüß *et al*., 2006). The mannose-sensitive, type I fimbriae also mediate adherence (Connell *et al*., 1996) and antibiotic-resistant pod formation (Anderson *et al*., 2003) that is important for invasion of host cells in some urinary tract infections, and bundle-forming pili and the EspA filament are important for biofilm formation by enteropathogenic *E. coli* (Moreira *et al*., 2006). Note that conjugation plasmids increase biofilm formation (Ghigo, 2001) in a manner independent of flagella, type I fimbriae, outer membrane autotransporter Ag43 (promotes autoaggregation) and curli (Reisner *et al*., 2003) due to an envelope stress response (Yang *et al*., 2008). This review focuses on *E. coli* biofilm formation and inhibition based on recent developments in the field (primarily whole-transcriptome profiling) with both pathogenic and non-pathogenic strains. More comprehensive reviews of *E. coli* biofilm formation are available such as that of Ghigo and colleagues (Beloin *et al*., 2008).

Whole-transcriptome profiling and stress response

Although DNA microarray technology may miss some aspects of biofilm development related to global averaging of heterogeneous cells (An and Parsek, 2007; Barken *et al*., 2008), whole-transcriptome profiling has provided robust insights into the biofilm mode of life (a schematic of newly characterized proteins related to biofilm formation is shown in Fig. 2). For *E. coli*, five single-time-point DNA microarrays have been used to explore the genetic basis of its biofilm formation (Schembri *et al*., 2003; Beloin *et al*., 2004; Ren *et al*., 2004a; Junker *et al*., 2006; Hancock and Klemm, 2007) and one temporal study has

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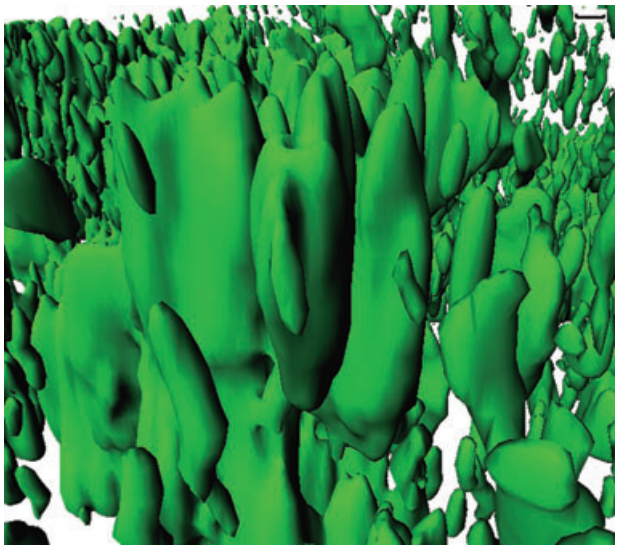

Fig. 1. *Escherichia coli* BW25113 biofilm as viewed using the green-fluorescent-protein-expressing plasmid pCM18, confocal microscopy and IMARIS software (conditions: Luria Bertani broth after 48 h at 37°C, flow rate of 10 ml h^{-1}). Scale bar (upper right) indicates 10 μm.

been completed (Domka *et al*., 2007). In the temporal study, six *E. coli* proteins related to the bacterial signalling molecule cyclic diguanylic acid (c-di-GMP, Fig. 3) were altered in a temporal manner (*yaiC*, *yliF*, *yciR*, *yddV*, *yeaJ* and *yjiU*); c-di-GMP has been linked to biofilm formation in several strains and its overproduction increases *E. coli* biofilm formation (Mendez-Ortiz *et al*., 2006). Of these, YciR has been linked to H-NS and curli formation via c-di-GMP control of the stationary-phase, stress-response, master controller RpoS, and YaiC has been linked to curli and cellulose via c-di-GMP (Weber *et al*., 2006).

One common trend in these biofilm transcriptome studies is that stress genes are induced. For example, 42 stress-related genes were identified in the temporal study (Domka *et al*., 2007), and five induced stress-response genes (*hslST*, *hha*, *soxS* and *ycfR*) were indentified in 7 h *E. coli* biofilm cells (Ren *et al*., 2004a). Follow-up studies on the putative outer membrane protein YcfR (renamed BhsA for influencing biofilm formation through hydrophobicity and stress response) indicate this protein mediates the stress response in *E. coli* by a mechanism that includes inducing indole synthesis (cells that lack BhsA are more sensitive to acid, heat, hydrogen peroxide and cadmium and have reduced indole concentrations) and that stress itself increases *E. coli* biofilm formation (Zhang *et al*., 2007).

In addition, the envelope stress-response genes, such as *pspABCDE*, *cpxAR*, *rpoE* and *rseA*, were induced in *E. coli* 8-day-old biofilms cells compared with exponentially growing planktonic cells regardless of the presence

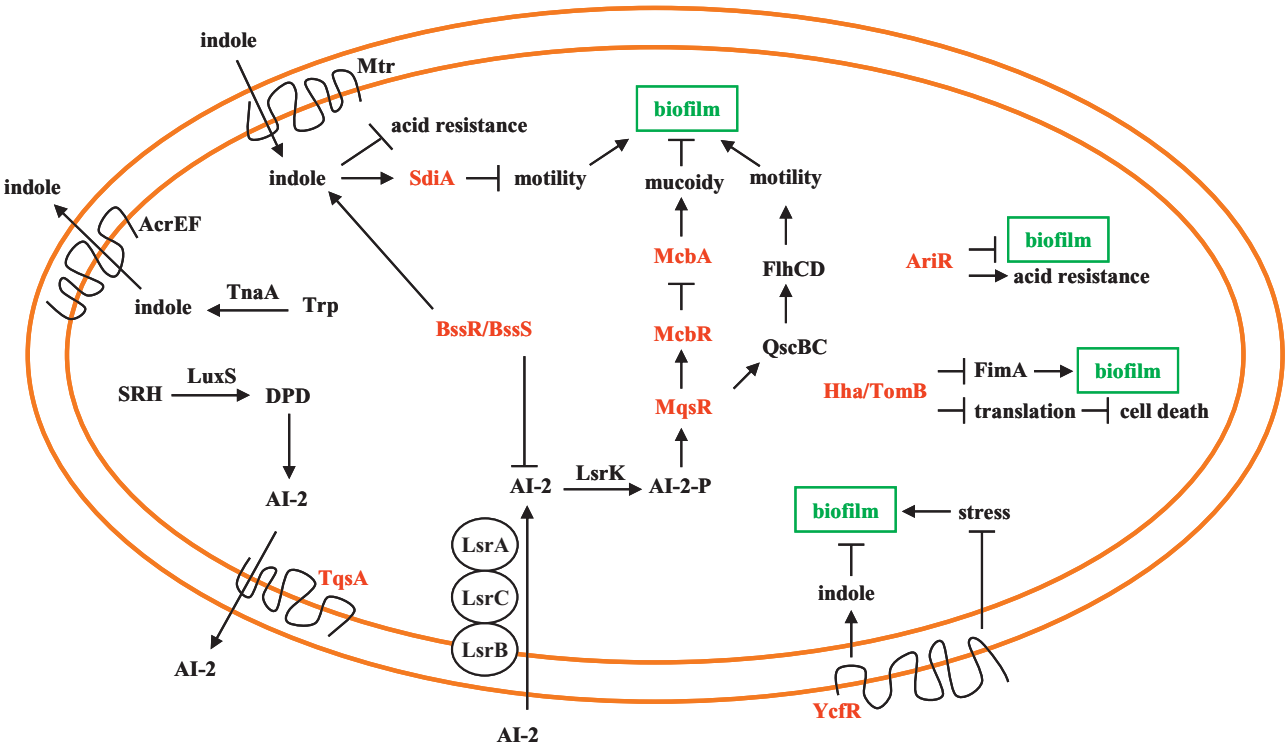

Fig. 2. Schematic of *E. coli* proteins related to biofilm formation. Proteins that were identified through whole-transcriptome studies and later characterized as described in this review are shown in red.

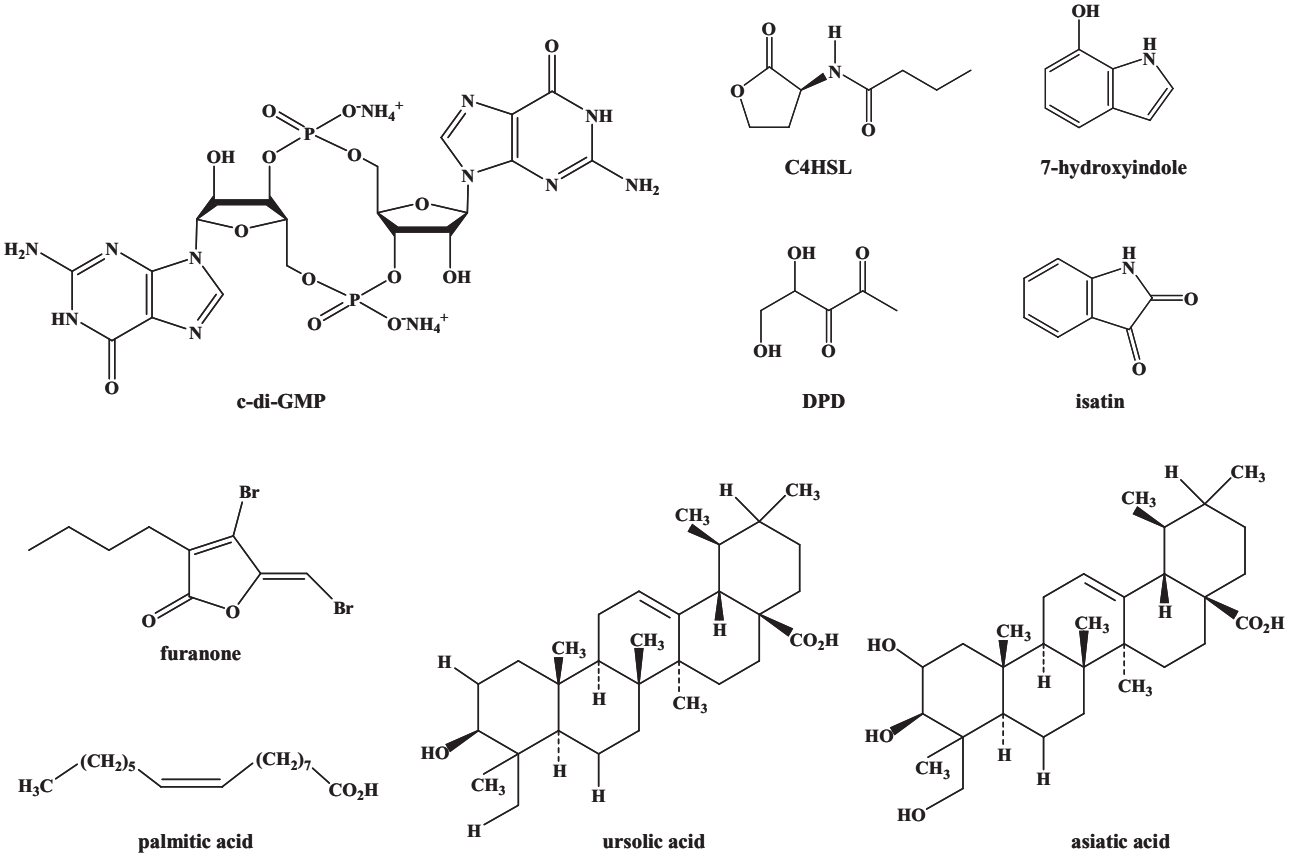

Fig. 3. Structure of biofilm-related compounds: cyclic diguanylic acid (c-di-GMP), *N*-butyryl-*L*-homoserine lactone (C4HSL), 4,5-dihydroxy-2,3-pentanedione (DPD), 7-hydroxyindole, isatin, (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (furanone), palmitic acid, ursolic acid and asiatic acid.

of a conjugative plasmid (Beloin *et al*., 2004). Furthermore, *rpoS* plays a key role during biofilm formation because it encodes the sigma S factor which regulates a number of stress-related genes, and *yeaGH* were also identified as putative stress-response genes (Schembri *et al*., 2003) as they are regulated by RpoS in *Salmonella enterica*. In addition, cold-shock protein regulators *cspABFGI* and the heat-shock protein regulator *htgA* were induced in a temporal fashion during biofilm formation (Domka *et al*., 2007).

In human urine, stress genes were also induced in asymptomatic bacteriuria *E. coli* during biofilm formation including both cold shock and heat shock (e.g. *cspAGH*, *ibpAB*, *pphA*, *soxS* and *yfiD*) (Hancock and Klemm, 2007); asymptomatic bacteriuria is a bacterial infection of urine that does not elicit the usual symptoms of an urinary tract infection. Note that stress tolerance is central to the ability of many bacterial pathogens to successfully colonize hostile host environments; for example, Hfq, a protein involved in the stabilization of small, non-coding RNAs (sRNAs), is critical to the ability of uropathogenic *E. coli* to form biofilms, to colonize effectively, and to persist in the urinary tract (Kulesus *et al*., 2008). Therefore, the newly identified relationship between stress tolerance and biofilm formation via whole-transcriptome profiling is important.

Escherichia coli genomic tools

To validate the whole-transcriptome studies, the isogenic *E. coli* K-12 library containing all non-lethal deletion mutations (3985 genes) created by the Genome Analysis Project in Japan (Keio collection) (Baba *et al*., 2006) is invaluable. Each Keio deletion mutant is designed with the ability to eliminate the kanamycin-resistance selection marker by expressing the FLP recombinase protein from pCP20 (Cherepanov and Wackernagel, 1995) as each kanamycin resistance gene is flanked by a FLP recognition target that is excised by FLP recombinase; hence, multiple mutations may be quickly constructed, too, using P1 transduction (Maeda *et al*., 2008) in a process termed Rapid Gene Knockout. Also available are pCA24N overexpression plasmids which contain His-tagged proteins which facilitate complementation studies as well as easy column chromatography-based methods for protein purification (Kitagawa *et al*., 2005). Tools for high-throughput

genetic screening of two simultaneous gene knockouts are also available for these libraries (Typas *et al*., 2008). Screening the Keio collection to discern proteins related to biofilm formation identified the importance of 110 genes primarily associated with cell surface structures and cell membrane including genes from flagella, fimbriae, motility, curli, and lipopolysaccharide operons (Niba *et al*., 2007).

Structure of biofilm regulator AriR

Another development in *E. coli* biofilms related to stress tolerance is the elucidation of the structure of one of the first biofilm proteins, YmgB, and the discovery that it is part of a fourth acid-resistance system in *E. coli* (YmgB was renamed AriR for regulator of acid resistance influenced by indole) (Lee *et al*., 2007a) (Fig. 4). Acid resistance is important for *E. coli* to pass through the low-pH environment of the stomach (pH 2 and below) to colonize the intestinal tract. The *E. coli* gene cluster *ymgABC* was identified through whole-transcriptome profiling studies related to biofilm development and cell signalling because its expression in many DNA microarrays matched that of well-known acid-resistance genes such as *gadABC* and *hdeABD* which were differentially regulated like the *ymg* operon (Lee *et al*., 2007a). Specifically, the furanosyl borate diester or derivative known as the quorum signal autoinducer 2 (AI-2) repressed *ymgAB* threefold (Ren *et al*., 2004b); in contrast, the biofilm inhibitor furanone from the alga *Delisea pulchra*, which masks AI-2 signalling, induced *ymgA* twofold (Ren *et al*., 2004b). Furthermore, deleting the AI-2 transporter gene *tqsA* repressed *ymgBC* fourfold (Herzberg *et al*., 2006), *ymgABC* were induced 14-fold at 15 h relative to 7 h biofilms (Domka *et al*., 2007), and the stationary-phase biofilm signal indole repressed *ymgABC* two- to fivefold (Lee *et al*., 2007b). In addition, deleting *ymgB* represses the acid-resistance loci *gadABCE* and *hdeB* (Lee *et al*., 2007a). Therefore, these results suggest strongly that the *ymgABC* gene cluster, and thus likely the AriR protein itself, plays an important role in *E. coli* biofilm formation and acid resistance as a result of AI-2 or indole signalling. Corroborating this hypothesis, phenotypic studies showed AriR represses biofilm formation in rich medium containing glucose, decreases cellular motility, and protects the cell from acid confirming that AriR plays a major role in acid resistance in *E. coli* (Lee *et al*., 2007a). The data show that these phenotypes are potentially mediated through interactions with the important cell signal indole, and gel shift assays suggest that AriR is a non-specific DNA-binding protein. *In vivo* DNA microarrays also show that AriR binds, either directly or indirectly via a second protein, genes important for biofilm formation. Surprisingly, the structure of the protein (1.8 Å resolution) shows that AriR is a biological dimer that is homologous to the *E. coli* global regulatory protein Hha (also a non-specific DNA-binding protein), despite its low protein sequence identity of only 9%. Note that Hha does not control acid resistance, and whole-transcriptome studies show Hha and AriR control different genes. Hence, AriR influences both acid resistance and biofilm formation and may have other functions, too.

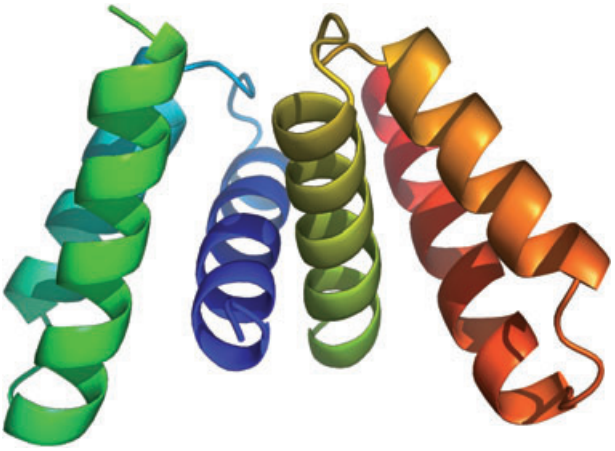

Fig. 4. Structure of AriR (YmgB) dimer (Lee *et al*., 2007a; figure courtesy of Wolfgang Peti). AriR forms a dimer in solution, as determined using size exclusion chromatography and was crystallized as a head-to-head dimer. The final model includes two protein molecules (each containing residues 25–86) and 31 water molecules; the N-terminal 24 amino acids of AriR are spontaneously cleaved. Each subunit of the AriR dimer consists of three α-helices, spanning residues 27–44 (α1), 50–62 (α2) and 67–84 (α3). Helices α2 and α3 are oriented in a near-perfect antiparallel fashion with respect to one another with helix α1 crossing in front of them at nearly a 90° angle. The tertiary structure of the monomer is maintained by an extensive network of hydrophobic interactions consisting almost exclusively of leucine, isoleucine and valine residues. The peripheral residues of the protein are primarily polar and charged. The dimerization contact is mediated predominantly by residues in helix α1, including Ser31, Leu34, Gly35, Val38, Thr39, Val48 and Met42, and results in the burial of 1326 Å^2 of solvent accessible surface. Like AriR, Hha is an all α-helical protein although it has four helices while AriR has three.

Interspecies cell signalling: acylhomoserine lactones

Cell signalling plays a role in the formation of some biofilms (Davies *et al*., 1998; Stanley and Lazazzera, 2004). In *E. coli*, acylhomoserine lactones (AHLs) from other bacteria are sensed through SdiA so *E. coli* can detect signals that it does not synthesize (Michael *et al*., 2001; van Houdt *et al*., 2006). For example, SdiA of the close *E. coli* relative *S. enterica* is activated by AHLs present in the gastrointestinal tract of turtles (Smith *et al*., 2008). These exogenous AHLs such as *N*-butyryl-*L*-homoserine lactone (Fig. 3) from *Pseudomonas aeruginosa* reduce *E. coli* biofilm formation (Lee *et al*., 2007b). In addition,

N-hexanoyl-*L*-homoserine lactone from strains such as *Pseudomonas syringae* increase acid resistance of *E. coli* by 44% by inducing *gadA* by 33%; this increase in survival in a harsh (acidic) environment upon detecting other bacteria may give *E. coli* a competitive advantage (van Houdt *et al*., 2006).

Intraspecies cell signalling: AI-2

In contrast to AHLs, addition of purified AI-2 increases *E. coli* biofilm formation (González Barrios *et al*., 2006). This use of synthesized AI-2 with *E. coli* was the first direct proof that AI-2 controls biofilm formation as prior studies relied on conditioned medium (which contains substances other than AI-2) and *luxS* mutations (which affect both signalling and metabolism) to link AI-2 to biofilm formation (Hardie and Heurlier, 2008).

AI-2 is a bacterial species non-specific signal used by both Gram-negative and Gram-positive bacteria and synthesized by *S*-ribosylhomocysteine lyase (LuxS) (Schauder *et al*., 2001). LuxS converts *S*-ribosylhomocysteine into homocysteine and (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD, Fig. 3), which forms spontaneously into a family of AI-2 molecules (Hardie and Heurlier, 2008). As a bacterial communication signal, AI-2 appears to be exported by the transporter of quorum-sensing signal TqsA (Herzberg *et al*., 2006) [formerly YdgG; this protein was identified initially by biofilm transcriptome profiling (Ren *et al*., 2004a)]. AI-2 is internalized by a *lsr* operon-encoded system (Taga *et al*., 2003), and then controls a variety of genes (DeLisa *et al*., 2001; Sperandio *et al*., 2001; Ren *et al*., 2004b). The *lsr* operon of seven genes *lsrACDBFGE* is induced by phospho-AI-2 and regulated by LsrR, LsrK and GlpDK (Taga *et al*., 2001; 2003; Xavier and Bassler, 2005a). The regulator LsrR represses the AI-2 uptake operon *lsr*, which is derepressed by the binding of phospho-AI-2 to LsrR (Taga *et al*., 2003). Another regulator, LsrK, a cytoplasmic kinase, phosphorylates internal AI-2 into an activated molecule (Xavier and Bassler, 2005a). Note that both LsrK and LsrR also regulate other genes including sRNAs (Li *et al*., 2007). The glycerol uptake and metabolism system encoded by *glpDFK* genes also influences AI-2 signalling by regulating *lsr* transcription through LsrR (Xavier and Bassler, 2005a).

Some insights have been gained as to how AI-2 controls biofilm formation. In *E. coli*, AI-2 stimulates biofilm formation and changes its architecture by stimulating flagellar motility via the motility quorum-sensing regulator MqsR [formerly B3022 that was identified through biofilm transcriptome profiling (Ren *et al*., 2004a)] which acts through the two-component motility regulatory system QseBC (González Barrios *et al*., 2006) to transcriptionally regulate FlhDC, the master regulator of flagella and motility genes *fliLMNOPQR*, *fliAZ*, *flhBA* and *flgABCDMN* (Liu and Matsumura, 1994; Claret and Hughes, 2002; Clarke and Sperandio, 2005a). MqsR also induces expression of the transcription factor YncC (González Barrios *et al*., 2006); YncC inhibits the expression of periplasmic YbiM, which prevents overproduction of colanic acid (causing mucoidy) and prevents YbiM from inhibiting biofilm formation (Zhang *et al*., 2008). Colanic acid synthesis is induced in mature biofilms (Domka *et al*., 2007) and is important for the three-dimensional architecture of a biofilm but not for biofilm formation (Danese *et al*., 2000; Prigent-Combaret *et al*., 2000). YncC was renamed McbR for MqsR-controlled colanic acid and biofilm regulator, and YbiM was renamed McbA as it is the first gene regulated by McbR (Zhang *et al*., 2008).

These results are consistent with the recent finding that in the oral bacterium *Aggregatibacter actinomycetemcomitans*, AI-2 regulates its biofilm formation most likely through its QseBC system (Shao *et al*., 2007). Also, in the human gastric pathogen *Helicobacter pylori*, AI-2 controls motility by controlling genes upstream of the motility and flagellar regulator FlhA (Rader *et al*., 2007). Further proof that AI-2 controls motility in different genera is that AI-2 regulates transcription of the flagellin gene *flaA* in the human pathogen *Campylobacter jejuni* (Jeon *et al*., 2003). In addition, BssR (formerly YliH)/BssS (formerly YceP) [both identified through whole-transcriptome profiling (Ren *et al*., 2004a)] regulate *E. coli* biofilms by influencing AI-2 and indole concentrations in a divergent manner (Domka *et al*., 2006).

Autoinducer 2 has also been shown to influence enterohaemorrhagic *E. coli* (EHEC) (Bansal *et al*., 2008) which is not surprising as the gastrointestinal tract is colonized by hundreds of bacterial species (Collier-Hyams and Neish, 2005) that produce a diverse range of signals including AI-2 (Clarke and Sperandio, 2005b). Understanding EHEC infections is important given that there are over 73 000 EHEC infections annually in the USA which lead to 2000 hospitalizations and 60 deaths, the economic cost of which is $405 million (Frenzen *et al*., 2005). EHEC forms a biofilm on various surfaces (Ryu and Beuchat, 2005; Uhlich *et al*., 2006), and sloughing of the biofilm may cause contamination (Ryu and Beuchat, 2005); however, an effective means of preventing its biofilm formation has not been elucidated, and there is no effective treatment for EHEC infections as antibiotic treatment increases the risk of haemolytic uraemic syndrome and renal failure (Wong *et al*., 2000; Tarr *et al*., 2005). It was found that AI-2 attracts EHEC in agarose plug chemotaxis assays, increases swimming motility and increases EHEC attachment to HeLa cells (Bansal *et al*., 2008). Whole-transcriptome profiling shows exposure to AI-2 alters the expression of 23 locus of enterocyte effacement (LEE) genes directly involved in the production of virulence

determinants, as well as other genes associated with virulence (e.g. 46 flagellar/fimbrial genes, 24 iron-related genes), in a temporally defined manner (Bansal *et al*., 2008). Another recent study (Kendall *et al*., 2007) using higher glucose concentrations that may have masked the effects of AI-2 (Wang *et al*., 2005) observed *espA* (LEE4) and *eae* (LEE5) are altered upon exposing EHEC to AI-2, and a proteome analysis showed AI-2 increases EHEC virulence using both epithelial cells and nematodes (Kim *et al*., 2007). These results suggest that AI-2 is an important signal in EHEC infections of the human gastrointestinal tract.

Interspecies cell signalling: AI-2

Note that AI-2 signalling also occurs between bacterial species. For example, *E. coli* senses AI-2 that is produced by *Vibrio harveyi* to assess changes in its cell population (Xavier and Bassler, 2005b). In addition, *P. aeruginosa* responds to AI-2 and modulates its gene expression pattern including pathogenicity, although it does not itself produce AI-2 (Duan *et al*., 2003). AI-2 also regulates at extraordinarily low concentrations the dual-species biofilm formation of two Gram-positive human oral commensal bacterial strains, *Actinomyces naeslundii* T14V and *Streptococcus oralis* 34 (Rickard *et al*., 2006).

Intraspecies cell signalling: indole

Escherichia coli produces indole by tryptophanase (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 *E. coli* biofilms (Lee *et al*., 2007b) and works in a quorum-sensing fashion (Lee *et al*., 2007c) as it satisfies the four criteria for cell signals (Winzer *et al*., 2002): (i) the putative signal must be produced during a specific stage [indole is produced primarily in the stationary phase (Wang *et al*., 2001)], (ii) the putative signal must accumulate extracellularly and be recognized by a specific receptor [indole is a known extracellular signal (Wang *et al*., 2001; Hirakawa *et al*., 2005) that is exported by AcrEF (Kawamura-Sato *et al*., 1999) and is imported by Mtr (Yanofsky *et al*., 1991)], (iii) the putative signal must accumulate and generate a concerted response [indole has been shown to delay cell division (Chant and Summers, 2007)], and (iv) the putative signal must elicit a response that extends beyond the physiological changes required to metabolize or detoxify the signal [indole has been shown to control biofilms (Lee *et al*., 2007b) and cell division (Chant and Summers, 2007) which are not related to indole metabolism].

It was first reported that indole stimulates biofilm formation for *E. coli* S17-1 (Di Martino *et al*., 2003). However, indole was subsequently shown to decrease significantly the biofilm formation of nine non-pathogenic *E. coli* strains (Domka *et al*., 2006; Lee *et al*., 2007b; Zhang *et al*., 2007) as well as decrease the biofilm formation of pathogenic *E. coli* O157:H7 (Lee *et al*., 2007c). The AHL-binding protein SdiA is necessary for this biofilm response with indole (Lee *et al*., 2007b; 2008a). Indole decreases *E. coli* biofilms by reducing motility (Domka *et al*., 2006; Bansal *et al*., 2007; Lee *et al*., 2007b), repressing acid-resistance genes (Lee *et al*., 2007b), reducing chemotaxis (Bansal *et al*., 2007) and reducing attachment to epithelial cells (Bansal *et al*., 2007) (note this is opposite that of AI-2). Indole also controls plasmid stability (Chant and Summers, 2007). Whereas AHL increases acid resistance 1.4-fold (van Houdt *et al*., 2006), indole reduces acid resistance 500-fold (Lee *et al*., 2007b). 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).

Promiscuous cell signalling: indole

Indole is also a promiscuous signal as it alters the phenotypes of non-*E. coli* strains. For example, indole increases biofilm formation of *P. aeruginosa* and *P. fluorescens* even though these pseudomonads do not produce indole (Lee *et al*., 2007b). Furthermore, in *P. aeruginosa*, indole alters extensively gene expression in a manner opposite that of AHLs by repressing genes that encode for the *mexGHI-opmD* multidrug efflux pump and by repressing genes involved in the synthesis of quorum sensing-regulated virulence factors including pyocyanin (*phz* operon), 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) signal (*pqs* operon), pyochelin (*pch* operon) and pyoverdine (*pvd* operon) (Lee *et al*., 2008b). Corroborating these whole-transcriptome results, indole decreases production of pyocyanin, rhamnolipid, PQS and pyoverdine and enhances antibiotic resistance (Lee *et al*., 2008b). Further evidence that indole is a signal that affects bacteria that do not synthesize it is shown in co-cultures of *E. coli* with *P. fluorescens* cells engineered to remove indole by oxidizing it; removal of indole results in a 12-fold increase in the number of *E. coli* cells (Lee *et al*., 2007b); this engineered dual species biofilm represents the first synthetic gene circuit successfully used to control biofilm formation. Therefore, it appears that the mechanism by which procaryotes manipulate the biofilm signal indole is through the relaxed substrate range of many dioxygenases and monooxygenases found in bacteria that bring about indole hydroxylation (Rui *et al*., 2005); that is, some of the oxygenases bacteria use for catabolism (Fishman *et al*., 2005) have also evolved to regulate concentrations of the cell 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. Furthermore, *E. coli* may use indole to reduce the virulence of strains such as *P. aeruginosa.*

Temperature-specific signals

Recently it was found temperature affects indole and AI-2 signalling in *E. coli* which suggests *E. coli* may use primarily indole signalling outside the human host and AI-2 signalling inside the host (Lee *et al*., 2008a). It was found that indole addition results in more extensive differential gene expression at 30°C than at 37°C and that indole reduces biofilm formation (without affecting growth) more significantly at 25°C and 30°C than at 37°C. In contrast to indole, the addition of the AI-2 precursor DPD leads to more extensive differential gene expression at 37°C than at 30°C (Lee *et al*., 2008a). Furthermore, compared with 37°C, at 30°C indole more significantly decreased flagella-related promoter activity, enhanced antibiotic resistance and inhibited cell division. Also, the addition of AI-2 induces the transcription of virulence genes in EHEC at 37°C but not at 30°C (Lee *et al*., 2008a).

Uridine monophosphate and indole and AI-2

By using a whole-transcriptome approach, it was also found that indole decreases uridine monophosphate (UMP) biosynthesis (*carAB*, *pyrLBI*, *pyrC*, *pyrD*, *pyrF* and *upp*) and uracil transport (*uraA*) at 30°C in *E. coli* (but not at 37°C) whereas AI-2 induces UMP biosynthesis at 37°C (but not at 30°C) (Lee *et al*., 2008a). Also, like indole, SdiA represses these same set of genes at 30°C. Additional experiments with *P. aeruginosa* have demonstrated that uracil addition increases quorum-sensing phenotypes and increases virulence in this strain via UMP biosynthesis (Ueda *et al*., 2008). These results suggest that in *E. coli*, a building block of RNA, uracil or a derivative of uracil may report the status of the bacterial signals, AHLs, AI-2 and indole.

Interkingdom signals

Cell signalling is also promiscuous across kingdoms. Examples were given above for interactions with various signals between different species of bacteria that affect biofilm formation; however, interactions also occur between pathogenic *E. coli* and its host that affect biofilm formation. As shown by a novel two fluorophore chemotactic assay, EHEC is attracted to the human hormones adrenaline and noradrenaline (as with AI-2) (Bansal *et al*., 2007). In addition, adrenaline and noradrenaline increase EHEC biofilm formation as well as increase motility and attachment to epithelial cells (Bansal *et al*., 2007). Noradrenaline also increases adhesion of EHEC to caecal mucosa (Chen *et al*., 2003), colonic mucosa (Green *et al*., 2004) and ileum (Vlisidou *et al*., 2004). Corroborating these results, whole-transcriptome profiling of EHEC in biofilms indicates that adrenaline and noradrenaline induce the expression of genes involved in surface colonization and virulence while indole decreases their expression (Bansal *et al*., 2007). Adrenaline and noradrenaline have also been shown to directly induce virulence genes (e.g. LEE genes) in EHEC (Sperandio *et al*., 2003) through receptor QseC. Taken together, these results suggest that adrenaline and noradrenaline increase EHEC infection while indole attenuates the process.

Biofilm inhibitors related to cell signalling

Advances in deciphering *E. coli* cell signalling have led to discoveries for inhibiting biofilms. For example, the recognition that indole inhibits biofilm formation (Lee *et al*., 2007b) as a quorum-sensing cell signal (Lee *et al*., 2007c) led to an investigation of the impact of hydroxy indoles on biofilm formation (Lee *et al*., 2007c). Given that indole controls biofilms (Lee *et al*., 2007b) and is present up to 700 μM (Domka *et al*., 2006), it was hypothesized (Lee *et al*., 2007c) that hydroxylated indoles may play a role in biofilm formation as many bacterial oxygenases such as dioxygenases from *Pseudomonas putida* PpG7 (Ensley *et al*., 1983), *Ralstonia pickettii* PKO1 (Fishman *et al*., 2005), *Pseudomonas mendocina* KR1 (Tao *et al*., 2004) and *Burkholderia cepacia* G4 (Rui *et al*., 2005) readily convert indole to oxidized compounds such as 2-hydroxyindole, 3-hydroxyindole, 4-hydroxyindole, isatin, indigo, isoindigo and indirubin (Rui *et al*., 2005). In Luria–Bertani medium (LB) on polystyrene with quiescent conditions, 7-hydroxyindole (Fig. 3) decreased EHEC biofilm formation 27-fold and decreased K-12 biofilm formation eightfold without affecting the growth of planktonic cells (Lee *et al*., 2007c). 5-Hydroxyindole also decreased biofilm formation 11-fold for EHEC and sixfold for K-12. In contrast, isatin (indole-2,3-dione, Fig. 3) increased biofilm formation fourfold for EHEC while it had no effect on K-12. Whole-transcriptome analysis revealed that isatin represses indole synthesis by repressing *tnaABC* 7- to 37-fold in EHEC, and extracellular indole levels were found to be 20-fold lower (Lee *et al*., 2007c). Furthermore, isatin repressed the AI-2 transporters *lsrABCDFGKR*, while significantly inducing the flagellar genes *flgABCDEFGHIJK* and *fliAEFGILMNOPQ* (which led to a 50% increase in motility). 7-Hydroxyindole induces the biofilm inhibitor/stress regulator *bshA* and represses *cysADIJPU*/*fliC* (which led to a 50% reduction in motility) and *purBCDEFHKLMNRT*. Isogenic mutants showed

7-hydroxyindole inhibits *E. coli* biofilm through cysteine metabolism. 7-Hydroxyindole (500 μM) also stimulates *P. aeruginosa* PAO1 biofilm formation twofold; therefore, hydroxyindoles are interspecies bacterial signals, and 7-hydroxyindole is a potent EHEC biofilm inhibitor.

Similarly, the realization that uracil or a derivative is intertwined with the *E. coli* AI-2 and indole cell signalling pathways (Lee *et al*., 2008a) as well as quorum sensing in *Pseudomonas* (Ueda *et al*., 2008), led to the discovery that the uracil analogue, 5-fluorouracil, inhibits *E. coli* biofilms; for example, 10 μM 5-fluorouracil inhibits biofilm formation fivefold while decreasing growth by 10% (author's unpublished data). 5-Fluorouracil also affects *P. aeruginosa* PA14 by decreasing dramatically its quorum-sensing phenotypes, reducing biofilm formation and reducing virulence (Ueda *et al*., 2008). Notably, 5-fluorouracil is already approved for treatment of human colon cancer (Wiebke *et al*., 2003); so it is relatively non-toxic to humans.

Given that AI-2 directly increases *E. coli* biofilm formation (González Barrios *et al*., 2006), compounds that mask AI-2 signalling should decrease biofilm formation. Indeed (*5Z*)-4-Bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (furanone, Fig. 3) of the alga *D. pulchra* inhibits *E. coli* biofilm formation by blocking AI-2 signalling (Ren *et al*., 2001) and the same genes induced by AI-2 are repressed by this furanone (Ren *et al*., 2004b). This organism makes more than 20 halogenated furanones (de Nys *et al*., 1993) to prevent biofilm formation so that it may conduct photosynthesis, and its effects with non-*E. coli* strains are well studied (Rasmussen and Givskov, 2006). The mechanism by which furanones inhibit biofilm formation is by displacing AHL from LuxR (Manefield *et al*., 1999) and by decreasing the DNA-binding activity of LuxR which blocks all three quorum-sensing systems of *V. harveyi* (HAI-1 acylated homoserine lactone, AI-2 and CAI-1) (Defoirdt *et al*., 2007). By interfering with quorum-sensing signalling and biofilm formation, these compounds have been shown to protect shrimp grown in aquaculture (Defoirdt *et al*., 2006) and to protect mice (Hentzer *et al*., 2003).

Like brominated furanones from algae, food ingredients have been found to inhibit the biofilm formation of *E. coli.* For example, the ground-beef fatty acids palmitic acid (Fig. 3), stearic acid, oleic acid and linoleic acid inhibit AI-2 activity and decrease *E. coli* biofilm formation (Soni *et al*., 2008). Similarly, furocoumarins such as bergamottin from grapefruit inhibit both AHL and AI-2 activities as well as decrease biofilm formation by EHEC (Girennavar *et al*., 2008).

Note the shortage of structural information about the family of compounds that comprise AI-2 has slowed the development of AI-2-based, quorum-sensing inhibitors (Lowery *et al*., 2008). However, propyl-DPD and butyl-DPD have been shown to inhibit DPD induction of *lsr* for *Salmonella typhimurium* while the same compounds stimulate bioluminescence in *V. harveyi* (Lowery *et al*., 2008). In addition, two sulfone-based compounds that inhibit bio-luminescence in *V. harveyi* were identified via virtual structure-based screening (Li *et al*., 2008).

Biofilm inhibitors not related to cell signalling

Screening 13 000 samples of compounds purified from plants resulted in the identification of another biofilm inhibitor, ursolic acid (Fig. 3), that is not toxic to *E. coli* and other bacteria as well as to hepatocytes (Ren *et al*., 2005). Whole-transcriptome analysis showed ursolic acid-induced genes related to chemotaxis and motility (*cheA*, *tap*, *tar* and *motAB*), heat-shock response (*hslSTV* and *mopAB*), and unknown functions (e.g. *b1566* and *yrfHI*) and that ursolic acid repressed genes related to cysteine synthesis (*cysK*) and sulfur metabolism (*cysD*); however, the antibiofilm effect of ursolic acid was not related to AI-2 (Ren *et al*., 2005). This manuscript and one by Rather and co-workers showed sulfur metabolism is related to biofilm formation as mutations in both *cysB* (Ren *et al*., 2005) and *cysE* (Sturgill *et al*., 2004) increase biofilm formation.

A related compound, asiatic acid (Fig. 3), was found to be even more effective than ursolic acid (Garo *et al*., 2007) and whole-transcriptome studies showed it also involves sulfur metabolism. Furthermore, aqueous fish muscle extract, composed primarily of fish muscle α-tropomyosin, was shown recently to significantly decrease attachment of a range of *E. coli* that cause urinary tract infections (Vejborg and Klemm, 2008).

Toxin–antitoxin pairs

Toxin–antitoxin (T–AT) pairs consist of a stable toxin and a labile antitoxin. Toxin–antitoxin pairs appear to be involved in antiphage defence (Pecota and Wood, 1996) and other possible functions include 'genomic junk', growth rate control, programmed cell death and persister formation (Magnuson, 2007). The value of T–AT pairs to the cell has been questioned since after deleting five T–AT pairs, it was shown the five best-studied *E. coli* T–AT pairs do not influence bacterial fitness of planktonic cells (Tsilibaris *et al*., 2007). However, MqsR is highly toxic as a deletion of the antitoxin B3021 is lethal, and it appears MqsR is part of a T–AT pair that consists of MqsR (toxin) and B3021 (Shah *et al*., 2006). As MqsR has been linked to biofilm formation via AI-2 (González Barrios *et al*., 2006) and via McbR (Zhang *et al*., 2008), T–AT pairs are clearly important for biofilm formation.

Further evidence of this link between T–AT pairs and biofilm formation is provided by Hha and YbaJ (renamed TomB for toxin overexpression modulator in biofilms)

(García-Contreras *et al*., 2008). Both Hha and TomB are highly induced in biofilms as found by whole-transcriptome profiling (Ren *et al*., 2004a), and Hha expression is toxic and TomB diminishes its toxicity (García-Contreras *et al*., 2008). Hha decreases biofilm formation by repressing type I fimbriae via *fimA* and *ihfA* and by inhibiting their translation via rare tRNAs (García-Contreras *et al*., 2008). Hha expression also induces ClpP and ClpX proteases that degrade many antitoxins, allowing free toxins to exert their inhibitory effects (García-Contreras *et al*., 2008). Note that decreases in translation efficiency activate toxins (Christensen *et al*., 2004). Hha also activates the prophage genes *rzpD*, *yfjZ*, *alpA* and *appY* which actively lyse cells (García-Contreras *et al*., 2008). Hence, Hha is toxic indirectly by activating other toxins by changing translation efficiency (García-Contreras *et al*., 2008). Therefore, one of the most important roles of the nebulous T–AT pairs is to help control biofilm formation.

Small RNA and biofilm dispersal

Biofilm dispersal is important for disseminating the strain; however, for the bacterium to leave the solid matrix in which it is both protected and entrapped, it may be necessary to sacrifice part of the biofilm and have some cells undergo autolysis (Webb *et al*., 2003). Hence, programmed cell death may make sense for the biofilm and the primitive tissue that this collection of cells represents but not for planktonic cells (Webb *et al*., 2003). Biofilm dispersal for *P. aeruginosa* involves prophage (Webb *et al*., 2003) and in *Pseudoalteromonas tunicata* involves the autolytic protein AlpA (Mai-Prochnow *et al*., 2006). In *E. coli*, along with cell toxicity and biofilm formation, Hha appears to control biofilm dispersal. Initial evidence is that Hha leads to decreased biofilm in flow cells and to the formation of plaques (García-Contreras *et al*., 2008); cell lysis via Hha may aid biofilm dispersal by forming holes in the biofilm matrix.

Some of the more than 60 sRNAs identified in *E. coli* (Kulesus *et al*., 2008) are related to biofilm dispersal. One of the first sRNA systems discovered in which sRNAs bind a regulator protein to control its activity is the carbon storage regulation system that consists of CsrA, the transcription regulator protein that binds specific mRNA to repress or activate transcription (Fig. 5), and the CsrA-binding sRNAs CsrB and CsrC that serve to titrate CsrA by binding nine CsrA dimers (Liu *et al*., 1997; Mercante *et al*., 2006). CsrA represses gluconeogenesis, glycogen metabolism, peptide transport and production of the adhesion poly β-1,6-*N*-acetyl-D-glucosamine while it activates glycolysis, acetate metabolism and flagellum biosynthesis (Baker *et al*., 2007). CsrA also represses biofilm formation and increases biofilm dispersal (Jackson *et al*., 2002). In addition, this remarkable protein represses the global regulator Hfq that acts as a RNA chaperone by promoting sRNA–mRNA base pairing in *E. coli* (Baker *et al*., 2007). The Csr system is widespread in eubacteria and is also known as the Rsm (repressor of secondary metabolites) system (Babitzke and Romeo, 2007).

Concluding remarks

Whole-transcriptome profiling has elucidated much in regard to *E. coli* biofilm formation. Differential gene expression of biofilm cells versus planktonic cells initially identified important biofilm proteins (e.g. Hha as a toxin and regulator of translation efficiency, TomB as an antitoxin, BhsA as a membrane mediator of stress, MqsR as an AI-2 mediator, BssR/BssS as signal-controlled biofilm regulators and TqsA as the AI-2 exporter). Follow-up approaches such as transcriptome profiling using isogenic mutants and *in vivo* DNA-binding studies led to discoveries related to how these proteins affect biofilm formation (e.g. McbR/McbA as regulators of colanic acid, AriR as a new acid-resistance protein, the importance of rare tRNAs and the importance of T–AT pairs).

Escherichia coli as a reference system has also been important for discerning the role of small RNAs on biofilm formation (e.g. CsrB), and for discerning the role of toxins and antitoxins for biofilm formation (e.g. Hha and MqsR). It is interesting that toxin/antitoxin genes such as *hok/sok* that were postulated to protect *E. coli* cells from phage (Pecota and Wood, 1996) are now being related to biofilm dispersal and cell lysis (e.g. *hha/tomB*) (García-Contreras *et al*., 2008). It seems the cell is capable of taking the weapons of its enemy (phage) and using it to control its physiology (cell death) in a social manner (altruism).

Escherichia coli has also been instrumental in discerning the role of both procaryotic and eucaryotic signals on biofilm formation. It is with this strain that the role of AI-2 on biofilm formation was clearly shown (by direct addition of enzymatically synthesized compound), and EHEC has been a good model system for discerning the importance of interspecies and interkingdom signalling. To date, little research has been performed on the effect of plant signals on EHEC biofilm formation and this is important in regard to its pathogenicity.

Much research has been aimed at finding effective ways for the prevention, control or eradication of biofilms (Labbate *et al*., 2004), and advances have also been made in *E. coli* biofilm inhibition and in antivirulence measures. To date, there are few known antivirulence compounds (Cegelski *et al*., 2008); antivirulence compounds are an important way to fight infectious diseases because unlike antimicrobials, antivirulence compounds do not affect growth and so there is less chance of developing resistance (Hentzer *et al*., 2002). Here, we have

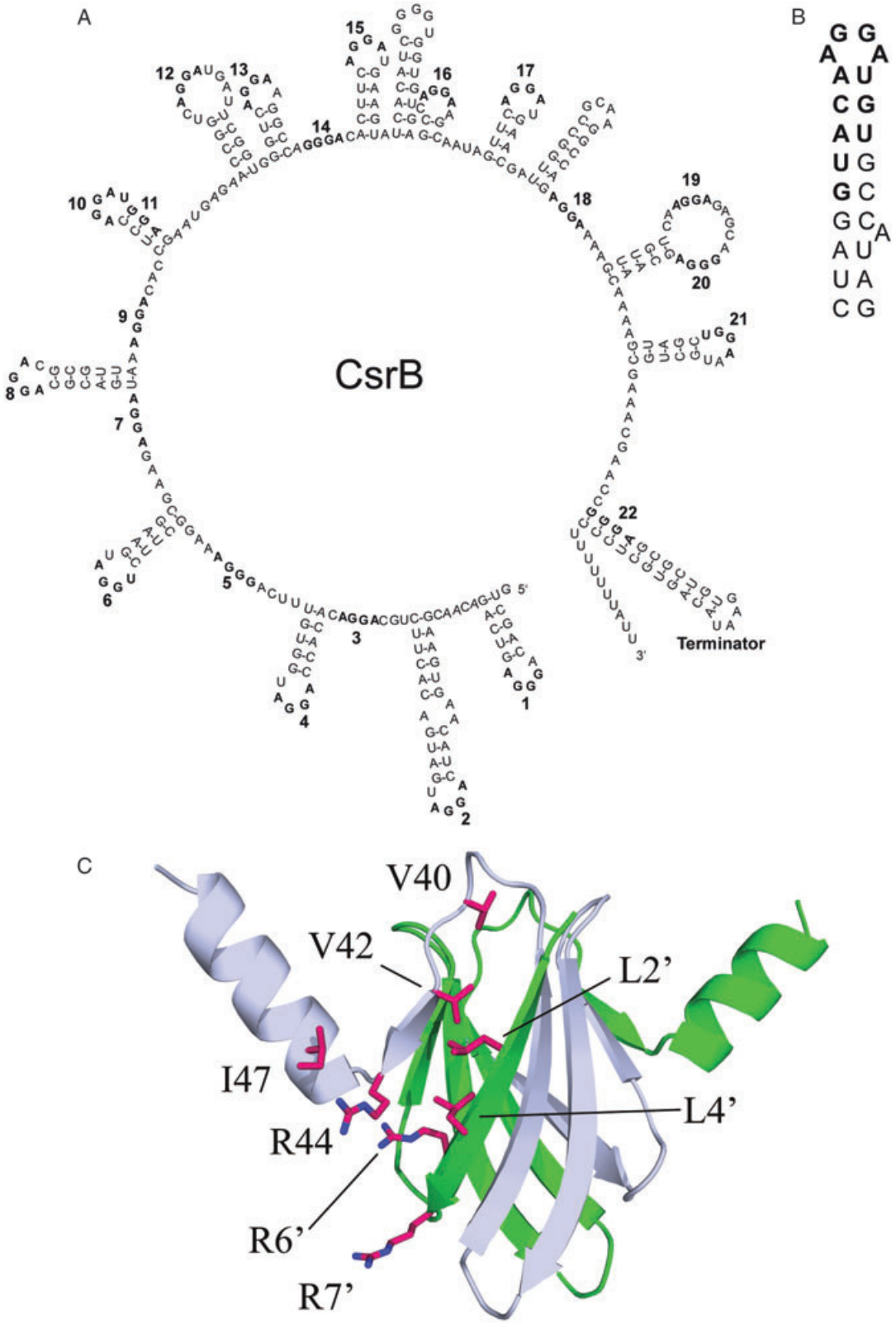

Fig. 5. CsrA regulatory protein and sRNA CsrB which binds CsrA (reprinted with permission from Elsevier from Babitzke and Romeo, 2007).
A. Secondary structure of sRNA CsrB showing 22 GGA regions for binding CsrA proteins.
B. CsrA consensus-binding sequence.
C. Structure of CsrA dimer with possible CsrB-binding residues shown.

shown that several non-toxic antibiofilm (antivirulence) compounds exist for *E. coli* including brominated furanones (Ren *et al*., 2001), ursolic acid (Ren *et al*., 2005), indole derivatives (Lee *et al*., 2007c) and 5-fluorouracil (Ueda *et al*., 2008). It is expected that there will be much activity in this area to find ever more potent compounds and that mixtures of these compounds will be required for efficacy in inhibiting biofilms.

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