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Cell-to-cell signaling in *E. coli* and *Salmonella*

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Bacterial cell-to-cell signaling

In order to regulate energy expenditure, microbes rely on a variety of mechanisms to control gene expression in response to changing environmental conditions. One mechanism used by microorganisms was originally described as quorum sensing (QS). During QS, a bacterial cell produces and secretes a signaling molecule, called an autoinducer. As the density of the bacterial population increases, so does the concentration of secreted autoinducer molecules. When the concentration of the autoinducer reaches a critical threshold, it diffuses back into the cell and activates or represses certain target genes. This type of signaling enables bacteria to regulate genes in a manner that reflects population density. Bacteria are also able to detect signal molecules produced by other species of bacteria as well as hormones produced by their mammalian hosts. Therefore, cell-to-cell signaling involves more than just taking a bacterial census, but is also involved in communicating about the local environment and growth potential of a population of cells (6, 70).

As currently understood, *E. coli* and *Salmonella* utilize three main types of cell-to-cell signaling processes. In the LuxR process, *E. coli* and *Salmonella* detect an autoinducer synthesized by other types of bacteria. During the LuxS/AI-2 signaling system, *E. coli* and *Salmonella* participate in intra- and interspecies signaling. Finally, during the AI-3/epinephrine/norepinephrine system, *E. coli* and *Salmonella* recognize self-produced autoinducer, signal produced by other microbes, or the human stress hormones epinephrine or norepinephrine.

Overview: LuxR-I

Quorum sensing using the LuxR-I system was initially described as regulating the bioluminescence in *Vibrio fischeri* (47). Two proteins regulate the luciferase operon in *V. fischeri*, LuxI and LuxR. LuxI is responsible for the synthesis of the autoinducer molecule *N*-acyl-homoserine-lactone (AHL), called autoinducer 1 (AI-1). After synthesis, AI-1 freely diffuses across the bacterial membrane into the surrounding environment (33). As the number of bacteria in a population increases, so does the concentration of AI-1 molecules. At a particular threshold concentration, AI-1 diffuses back into the bacterial cell and binds to its specific receptor protein LuxR. LuxR complexed with AI-1 activates transcription of itself as well as transcription of the luciferase operon (16, 17, 33) (Fig. 1). In all of the

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LuxR-LuxI systems, bacteria produce the AHL molecule, autoinducer-1 (AI-1), which then binds to the LuxR protein to regulate transcription of different genes involved in a variety of phenotypes (12, 49).

AI-1

LuxI and its homologues synthesize autoinducer molecules by transferring a fatty acid chain from an acylated acyl carrier protein to *S*-adenosylmethionine, releasing the AHL and methylthioadenosine (58). AHLs consist of a homoserine lactone ring joined to a fatty acid side chain (Fig. 2A). Although each species of bacteria produces a distinct autoinducer molecule, variations in AHLs are found among different species of bacteria. For example, the length of acyl chain may contain four to 18 carbons, and acyl chains differ in the degree of saturation. Additionally, the AHL may be modified at the third carbon of the acyl chain and contain a hydrogen, hyrdroxyl, carbonyl, or oxo group (20, 42, 81).

Similar to the AHL, the receptor molecule (LuxR and its homologues) is species specific; however, some LuxR homologs can detect other related AHLs produced by other species of bacteria, but with lower specificity (68). The AHL receptor molecules detect nanomolar concentrations of its corresponding AHL molecule and are stabilized by this interaction. In the absence of its specific AHL, AHL receptor proteins are targeted to degradation (89, 90).

Overview of AHL/SdiA in *E. coli* & *Salmonella*

E. coli and *Salmonella* are unique in this cell-signaling process in that these bacteria rely on AHL detection for interspecies communication as opposed to intra-species communication that was the paradigm of this mechanism for many years (44). *E. coli* and *Salmonella* lack LuxI, and thus do not synthesize AHLs; however, both encode the protein SdiA that apparently recognizes and binds to AHLs produced by other species of bacteria. SdiA requires these AHL compounds to fold properly (86, 87).

SdiA detects a much broader range of AHLs than other LuxR homologs (68). SdiA is most strongly activated by 3O-AHLs with chains between six and eight carbons long (Fig. 2B), sensing concentrations as low as 1 nm to 5 nm of these AHLs. However, SdiA can also recognize oxoC10, 6, and 8 AHLs at approximately 50 nm (1, 30, 44) (Fig 2C). When a sulfur atom replaces the 3′-oxygen molecule in a laboratory-synthesized derivative, SdiA is also strongly activated (30); however, it is not know whether this molecule naturally exists in nature.

AI-1 signaling in *E. coli*

Initial experiments suggested that SdiA (suppressor of cell division inhibition) played a role in the regulation of cell division genes *ftsQAZ* in *E. coli* (79). These results were based on *sdiA* cloned into a multi-copy plasmid, yet the *sdiA* mutant has no apparent cell division defects (79). Additional experiments demonstrated that SdiA repressed the LEE and motility genes in enterohemorrhagic *E. coli* (EHEC) (31); however, these effects were observed only by overexpression of SdiA, and no *sdiA* mutant was examined (31).

The precise role of SdiA was elusive for many years until the discovery that SdiA did not sense self-produced AHLs, but AHLs produced by other bacterial species. Many LuxR-type proteins rely on the AHL autoinducer as a co-factor for proper folding, and that in the absence of AHLs, the protein is targeted for degradation (89, 90). Indeed, the NMR structure of the SdiA protein indicates that AHL-binding allows proper protein folding (86), and the phenotypes associated with SdiA expression are only observed in the presence of AHLs (44).

SdiA seems to integrate external stimuli such as temperature and pH (28, 73), which may allow enterohemorrhagic *E. coli* (EHEC) O157:H7 to colonize the gastrointestinal (GI) tract of cattle (15, 28, 40), the main reservoir for this bacterium (32). During passage through the cattle GI tract, EHEC encounters broad ranges in pH, and thus, must regulate gene expression to ensure survival and colonization (Fig. 3). Upon entering the rumen, EHEC is subjected to a neutral pH and AHLs (18). Here the AHLs activate SdiA which in turn increases expression of the *gad*-encoded acid resistance genes (28, 51). After passage through the rumen, EHEC traverses through the low pH (2.0 to 2.5) environment of the abomasum en route to the colon. Potentially, the up-regulation of the *gad* acid resistance genes in the rumen primes EHEC for entry into the acidic environment of the abomasum (pH 2.0 to 2.5) (51), the next stop for EHEC en route to the colon.

Additionally, SdiA directly regulates expression of the LEE genes. The LEE genes (and corresponding AE lesion formation) are necessary for EHEC colonization of the renal-anal-junction (RAJ) site in the colon (40). Five major operons (*LEE1-5*) comprise the LEE. The LEE encoded regulator (Ler) that is encoded in *LEE1* activates the other LEE operons (22, 57, 63). In the presence of AHLs, SdiA directly binds to *ler*, acting as a repressor of this gene, and consequently, the other LEE genes expression of the LEE (28). AHLs have not been detected in the RAJ, thus in the absence of AHLs, SdiA will be degraded, relieving repression of the LEE and allowing EHEC to successfully colonize the cattle. Accordingly, competition experiments demonstrated that an *sdiA* mutant does not colonize cattle as efficiently as WT EHEC (28).

In nonpathogenic *E. coli*, SdiA, in conjunction with indole, may regulate biofilm formation, motility formation, and indole production (36–38). However, more research is needed to elucidate this signaling pathway as differential regulation by SdiA could be observed in the presence or absence of AHLs (37), the compounds required by SdiA for proper folding and function (86, 87).

AI-1 signaling in *Salmonella*

Upon detecting AHLs produced by other species of bacteria, SdiA in *Salmonella* activates expression of two *srg* (SdiA-regulated gene) loci, the *rck* operon that encodes seven genes and the *srgE* gene (1, 61). The *rck* operon is located on the *Salmonella* virulence plasmid pSLT and contains six genes: *pefI*, *srgD*, *srgA*, *srgB*, *rck*, and *srgC* (1, 19, 44, 61) (Fig. 4). The *pefI* and *srgA* genes regulate expression and folding of the upstream *pef* operon (5, 41, 48). The *pef* operon encodes frimbriae that function in adhesion to the small intestine of mice (3, 19). SrgD and SrgC are putative transcription factors whose target genes have not been identified (19, 44). The lipoprotein SrgB has no known function (19). Rck, an 8-

stranded β-barrel protein localized to the outer membrane, (8) functions in resistance to complement killing (23, 24) by preventing the polymerization of complement component C9 on the bacterial cell envelope (25) as well as acting as an adhesin to host tissues (11). The second SdiA-regulated locus is encoded in the chromosome and consists of the gene *srgE* that is predicted to encode a protein containing a coiled-coil domain (2, 61). The function of SrgE has not yet been elucidated (61). Neither the *rck* operon nor the *srgE* gene are found in *E. coli* (1).

Similar to *E. coli*, SdiA in *Salmonella* appears to play a role in adapting and recognizing environmental conditions. In *Salmonella*, SdiA is induced at low pH (pH 4) under aerobic conditions (56), and the *rck* operon is expressed at 37°C, but not at 30°C or at 22°C (1). Contrary to *E. coli*, SdiA in *Salmonella* does not appear to play a role in cow colonization, as it is not activated during passage through the GI tract of cattle (62). SdiA is activated, however, during passage through the GI tract of the turtle, a reptile that is commonly associated with *Salmonella* (62). Potentially, the age or diet of cattle influences AHL production by its commensal microbiota (62), and therefore, colonization by *Salmonella*.

LuxS/AI2

Overview

Since the discovery of cell-to-cell signaling using AHLs, another cell-to-cell signaling system has been discovered. The *luxS* QS system is present in roughly half of all sequenced bacterial genomes (80) and was first characterized as the regulator of bioluminescence in *Vibrio harveyi* (70).

LuxS synthesizes AI-2

LuxS is a small metalloenzyme that catalyzes the terminal step in the essential activated methyl cycle in bacterial cells (Fig. 5). In this pathway, *S*-adenosylmethionine (SAM) is recycled ultimately to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (59, 60, 82–84). SAM is a major cellular methyl donor in bacterial cells, and transfer of its methyl group to various substrates produces the toxic by-product *S*-adenosylhomocysteine (SAH) (60). The nucleoside Pfs subsequently detoxifies SAH, yielding adenine and *S*-ribosylhomocysteine (SRH). In the final step of the pathway, LuxS coverts SRH to homocysteine and DPD. DPD is an extremely unstable compound that reacts with water and cyclizes to form several different furanones, one of which is thought to be the precursor of AI-2 (39, 60, 66, 82).

Crystal structures of LuxS have revealed that this protein is a homodimer with two identical active sites that are formed at the dimmer interface by residues from both subunits (27, 39, 52). Each active site contains a divalent metal ion, Fe^{2+}, that catalyzes an internal redox reaction (50, 88).

AI-2

The structure of AI-2 has been solved by co-crystallization of the ligand with its receptor LuxP in *Vibrio harveyi* (7). In *V. harveyi*, AI-2 is a furanosyl-borate diester, (2*S*,4*S*)-2-

methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF borate) (7). LuxP homologues and homologues from the *V. harveyi* signaling cascade appear to be unique to this genera, suggesting that AI-2 recognition varies among bacterial species. Indeed, co-crystallization of AI-2 and its receptor, the periplasmic protein LsrB, in *Salmonella*, revealed that *Salmonella* recognizes a chemically distinct form of AI-2, (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxyte-tetrahydrofuran (*R*-THMF) (45) (Fig. 6).

AI-2 production is dependent on growth conditions, such as nutrient availability, pH, osmolarity, oxygen availability, growth rate and stress factors- both intracellular and from the environment (heat shock, amino acid limitation) (13, 70).

AI-2 signaling in *Salmonella* and *E. coli*

In *Salmonella enterica* serovar Typhimurium, AI-2 regulation involves genes that encode an ABC transporter named Lsr (LuxS-regulated) (Fig. 7). Seven genes comprise the *lsr* operon, *lsrACDBFGE*, and AI-2 activates its transcription (72). This transporter is also present in *E. coli*, and in both species, the Lsr transporter is homologous to sugar transporters. As extracellular AI-2 concentrations increase, AI-2 binds the periplasmic protein LsrB and is subsequently imported inside the cell by the Lsr ABC transport system. Once it is inside the cell, AI-2 is modified by phosphorylation. Phosphorylated AI-2 is thought to interact with LsrR, a SorC-like transcription factor. Upon binding AI-2, LsrR represses expression of the *lsr* operon (71, 72).

AI-2 and virulence

Many phenotypes have been attributed to cell signaling via AI-2; however, the precise role of AI-2 in bacterial cell-to-cell signaling is debated. Some studies have suggested that AI-2 is involved in biofilm formation, motility (14, 21, 26); however, another report examining a *luxS* mutant in W3110 strain did not see any effects on growth, motility, or biofilm formation (78). Additional experiments compared *luxS* mutants with wild-type strains and genetically complemented mutants or added preconditioned media to bacterial cultures and found that AI-2 signaling affected expression of the LEE-encoded type three secretion system and motility in EHEC (64, 65). However, using purified and *in vitro*- synthesized AI-2, it has been revealed that the signaling molecule affecting TTSS and motility in EHEC is not AI-2, but a distinct autoinducer, AI-3, that is not dependent upon *luxS* for synthesis (66, 76) (see below). Therefore, the role of AI-2 in pathogenesis needs to be examined further.

AI-2 and cell metabolism

In contrast, some researchers have suggested that the role of AI-2 in *Salmonella* and *E. coli* functions primarily in metabolism because of the similarities in function and sequence homology of the *lsr* operon to other sugar transporters (74, 78, 82, 83). Similar to other carbon sources, the synthesis and import of AI-2 is strictly controlled. In both *E. coli* and *Salmonella typhimurium*, AI-2 production is dependent on the amount of carbohydrates in the medium and is growth phase dependent. Cells in exponential phase produce and secrete AI-2, whereas those entering stationary phase no longer produce AI-2 (6, 69). Furthermore, AI-2 is not imported in the presence of glucose because the *lsr* operon is not transcribed due

to cAMP-CAP-mediated repression; however, when glucose becomes limiting, cells import AI-2 (77, 85).

Gene expression profiles also seem to suggest that the function of AI-2 is primarily metabolic. For instance, the *lsr* operon was not induced when *E. coli* was grown in medium containing glucose, whereas, in glucose-free medium, the operon was expression (78). Moreover, the *lsr* operon was induced only in the absence of glucose, and that the *luxS* mutation in *E. coli* primarily affected genes related to AI-2 production and transport (78). When a *luxS* mutant was compared to WT EHEC as well as the *luxS* mutant with DPD added, the majority of genes with an altered profile are associated with central metabolism and core biological processes (35). Finally, a study using phenotype microarrays observed that the *luxS* mutation resulted in numerous metabolic changes, especially in those processes that involve nitrogen and carbon metabolism (76).

AI-3/epinephrine (epi)/norepinephrine (NE) signaling system

Overview

The third major type of cell-to-cell signaling involves inter-kingdom signaling between prokaryotic and eukaryotic cells. In this system, the autoinducer-3 (AI-3) produced by the commensal GI microflora and/ or epinephrine (epi) and norepinephrine (NE) produced by the host (66) interact with a two-component regulatory system to activate transcription of genes involved in pathogenesis (64, 67). This signaling system was initially characterized in EHEC (66), but it is not unique to this strain (53, 76).

Signaling molecules AI-3, epi, NE

AI-3 quorum sensing system was discovered through its association with the *luxS* system. Although AI-3 does not directly depend upon *luxS* for synthesis, a *luxS* mutation has several effects on AI-3 production. A mutation in the *luxS* gene affects AI-3 synthesis by altering cellular metabolism (Fig. 8). More specifically, the *luxS* mutation requires that the cell utilize oxaloacetate, instead of SAM, for *de novo* synthesis of methionine. Exclusive use of this pathway may alter cellular metabolism and cellular concentrations of amino acids, potentially lead to reduced tyrosine levels and, consequently, diminished AI-3 concentrations (76).

Additional studies have demonstrated that AI-3 is a chemically distinct molecule from AI-2. AI-2 is a polar furanone that does not bind to C-18 columns, whereas AI-3 binds to C-18 columns and can only be eluted with methanol (66). Moreover, electrospray mass spectrometry also revealed differences between the structures of AI-3 and AI-2 (7, 66). AI-2 and AI-3 activity can be differentiated using two different assays. The AI-2 assay is based on the production of bioluminescence in *V. harveyi*, and AI-3 does not show any activity for this assay. Conversely, the AI-3 activates transcription of the EHEC virulence genes, and AI-2 has no effect on this assay.

AI-3 is an aromatic aminated signal, but its final structure has not yet been elucidated (29). Because Epi/NE can replace AI-3 in the regulation of EHEC virulence gene regulation and because the regulatory effects of epi/NE and AI-3 can be inhibited by adrenergic receptor

antagonists, it has been hypothesized that AI-3 may be structurally similar to epi/NE (66) (Fig. 9). In addition to EHEC, *Salmonella* and several species of pathogenic and nonpathogenic bacteria produce AI-3 (76).

AI-3/ epi/ NE receptor, QseC

The membrane-bound protein QseC (quorum sensing in *E. coli*) is a bacterial adrenergic receptor that directly interacts with AI-3 and epi/NE (10). QseC has two transmembrane domains, a histidine sensor kinase (HK) domain, and an ATPase domain. The HK domain allows autophosphorylation upon sensing AI-3 and, especially, epi/NE. QseC also contains an ATPase domain that enables it to phosphorylate QseB. The action of QseC can be blocked by phentolamine, an antagonist of α-adrenergic receptors (10). The QseC sensor is conserved among other enteric bacteria of the genera including *Shigella*, *Salmonella*, and *Yersinia* (10).

AI-3/epi/NE signaling in *E. coli*

QseBC plays an important role in EHEC pathogenesis and inter-kingdom signaling (29). Upon binding AI-3 and epi/NE, QseC augments its phosphorylation state and then activates a complex regulatory cascade that includes its response regulator QseB (9, 10, 65–67) (Fig. 10). Rabbit and bovine infection models have shown that recognition of these three signals is essential for *in vivo* virulence expression (10, 53, 75). QseBC activates transcription of the flagella regulon responsible for swimming motility in EHEC (9), production of Shiga toxin, expression of the LEE genes, as well as another two-component system, QseEF that is involved in AE lesion formation (29, 54, 55).

Recently, the AI-3/epi/NE quorum sensing has been implicated in biofilm formation in enteropathogenic *E. coli* (EPEC) (46). Microcolony formation in an initial step in biofilm development, and in EPEC, it is mediated by several adhesins including the bundle-forming pilus and the EspA (46). Expression of *espA* is controlled by the AI-3/epi/NE quorum sensing system, suggesting that biofilm formation is regulated, at least in part, by AI-3.

AI-3/ epi/NE signaling in *Salmonella*

The AI-3/epi/NE signaling system *S.* typhimurium appears very similar to that of EHEC. *S.* typhimurium encodes a functionally interchangeable homologue of the EHEC QseC (87% similarity) that similarly regulates virulence gene expression (43). A *S.* typhimurium *qseC* mutant is defective for colonization of the swine GI tract (4) and attenuated for systemic disease in mice (53). Moreover, microarray and real-time RT-PCR data indicated that in response to norepinephrine, motility genes as well as early-, mid-, and late-genes involved in flagellar synthesis were up-regulated in *S.* Typhimurium (4). As in EHEC, the QseBC two-component system in *S.* Typhimurium seems necessary for optimal induction of motility in response to norepinephrine (4). Norepinephrine also regulates genes encoding a lipid A modification system, iron transport, and type three secretion (34). The phenotypes observed in response can be blocked by propranolol (34).

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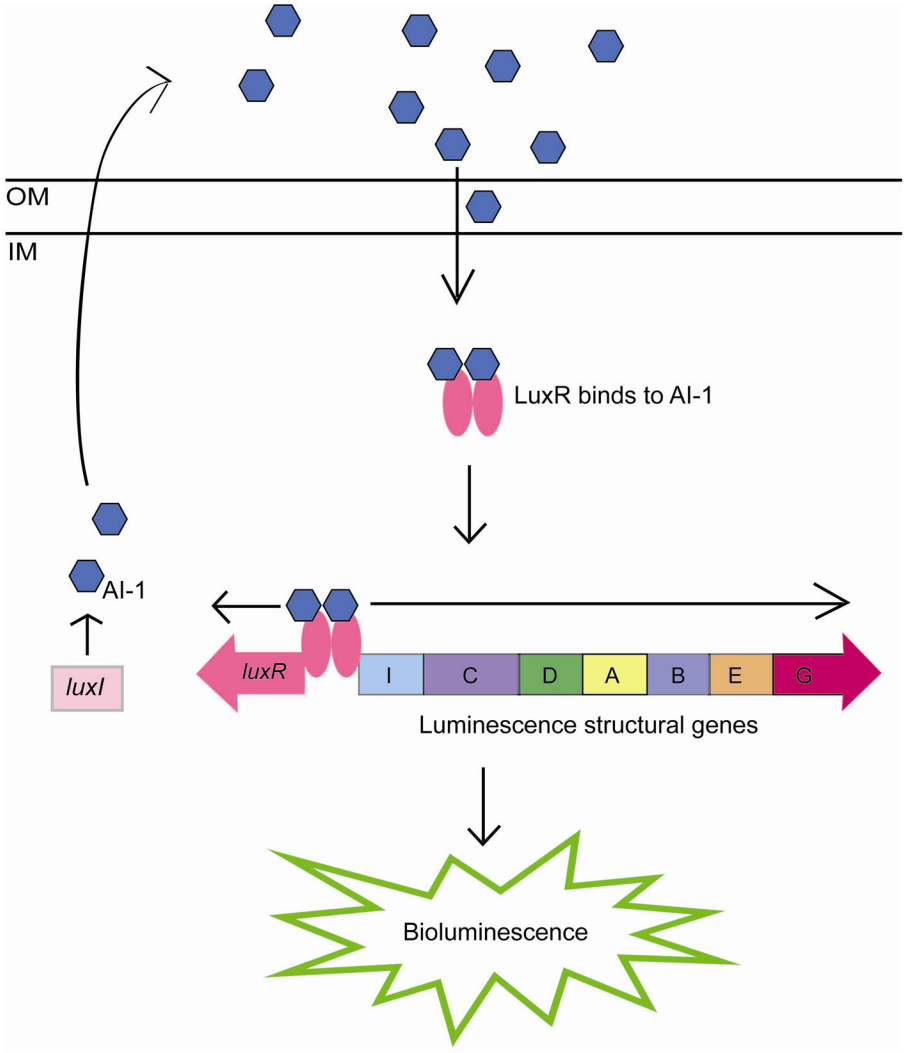

Figure 1.

Model of LuxI/ LuxR quorum sensing in *Vibrio fischeri*. LuxI synthesizes AI-1 and diffuses outside of the cell. When cell density, and therefore, AI-1 concentration, is high, AI-1 diffuses back into the cell where it binds to LuxR. LuxR complexed with AI-1 subsequently activates transcription of itself as well as the luciferase operon.

IM, inner membrane; OM, outer membrane.

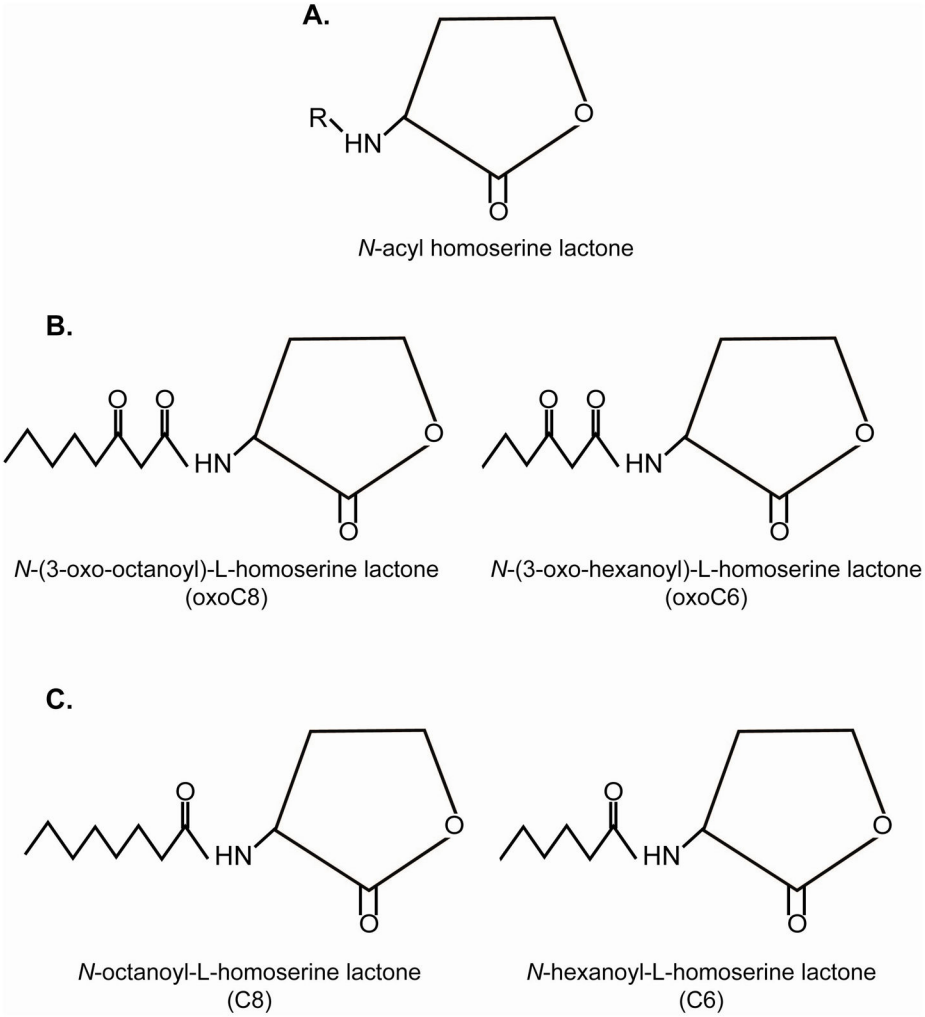

Figure 2.

Structures of AI-1. **A.** AI-1 is composed of a homoserine lactone ring bound to a fatty acid side chain; **B.** SdiA detects oxoC8 and oxoC6 with greatest sensitivities; **C.** SdiA can also detect other forms of AI-1 such as C8 and C6, although with less sensitivity.

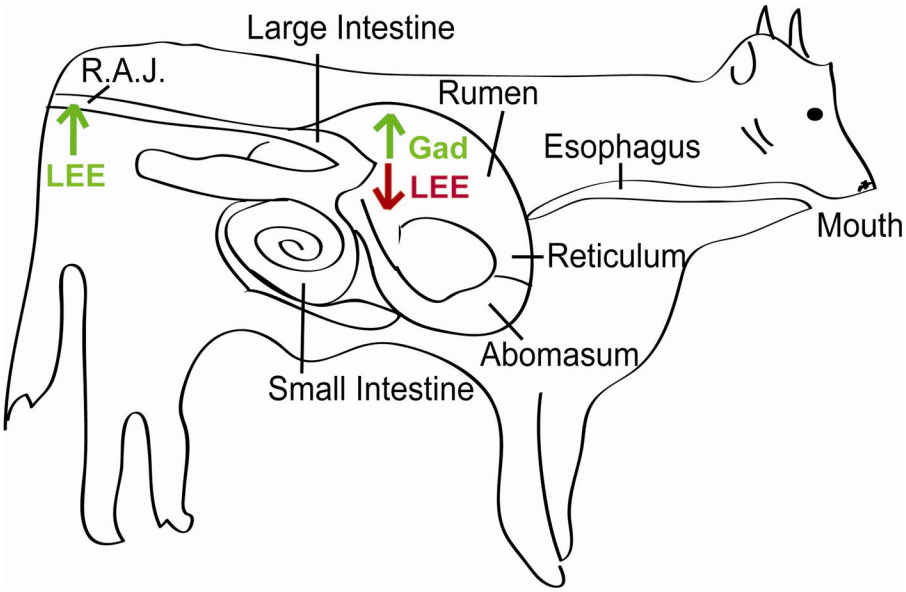

Figure 3.

Model of SdiA-AHL dependent EHEC gene expression in the GI of cattle. Once EHEC enters the rumen, it encounters AHLs. In the presence of AHLs, SdiA is functionally stable and acts to increase expression of acid-tolerance genes in the *gad* operon and represses expression of the LEE genes. Up-regulation of the *gad* genes allows EHEC to survive passage through the acidic abomasum. AHLs have not been detected in the colon, thus SdiA is unstable, and EHEC can activate the LEE and colonize the renal anal junction of the colon.

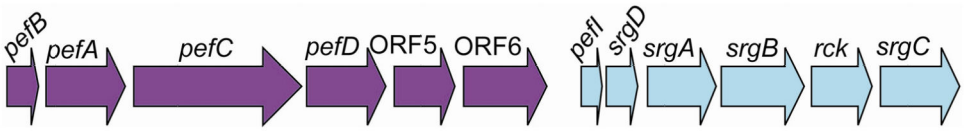

Figure 4.

SdiA in *Salmonella* activates expression of the *rck* operon. The *pefI* and the *srgA* genes encoded in this operon affect the transcription and folding, respectively, of plasmid-encoded fimbriae that are encoded by the upstream *pef* genes. SrgA plays a role in protein folding in the *pef*-encoded fimbriae by catalyzing disulfide bond formation (5, 41).

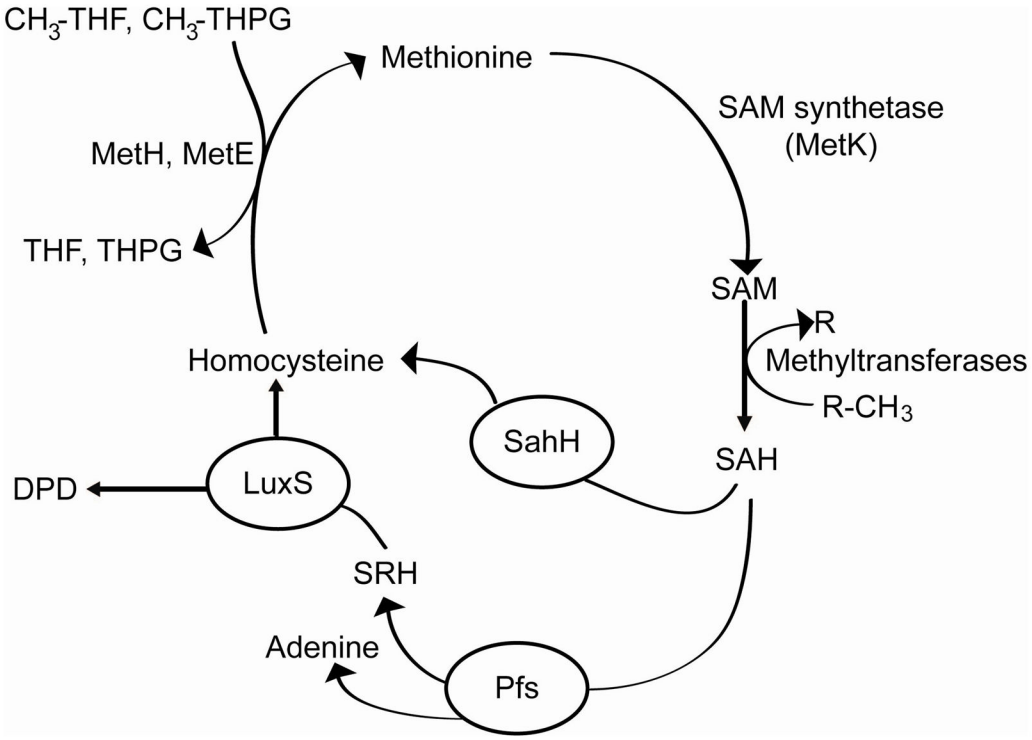

Figure 5.

Synthesis of AI-2. In the activated methyl cycle, SAM is converted ultimately to homocysteine and DPD. LuxS catalyzes the last step in the pathway, resulting in DPD. AI-2 is derived from the unstable compound DPD.

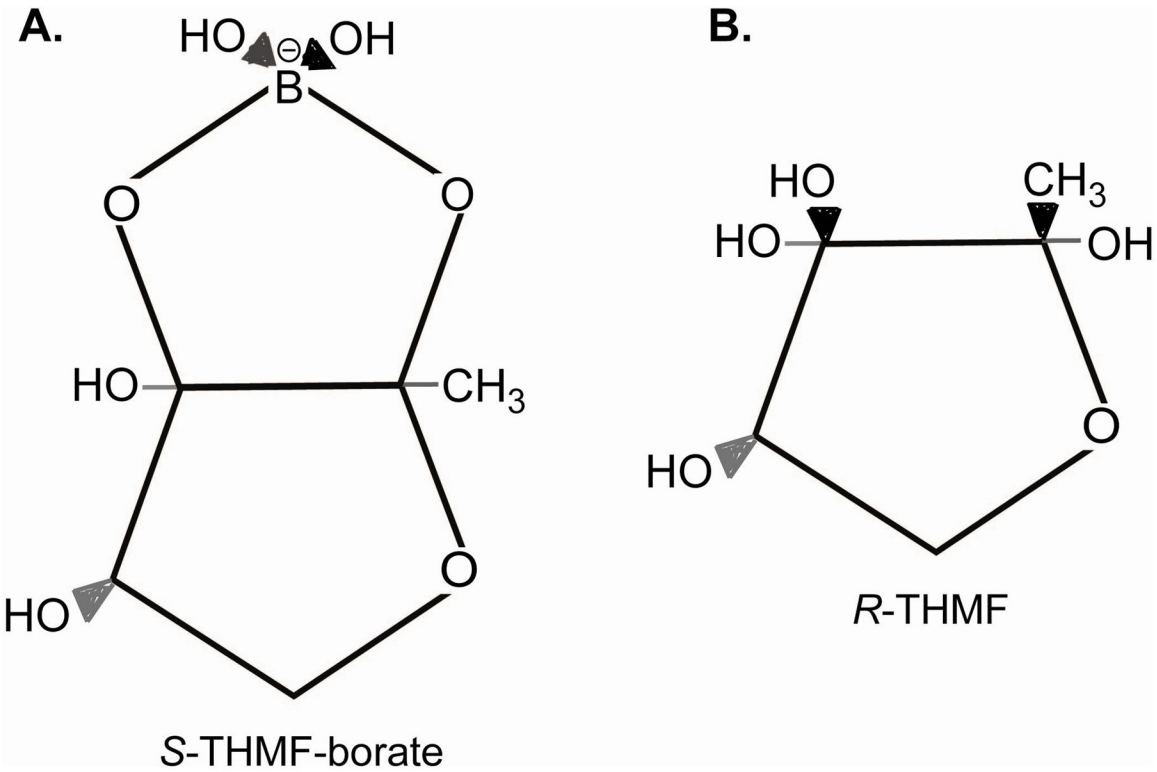

Figure 6.

Structure of AI-2 signaling molecules. **A.** AI-2 produced by *Vibrio harveyi* is (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF borate); **B.** AI-2 produced by *Salmonella typhimurium* is (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxyte-tetrahydrofuran (*R*-THMF).

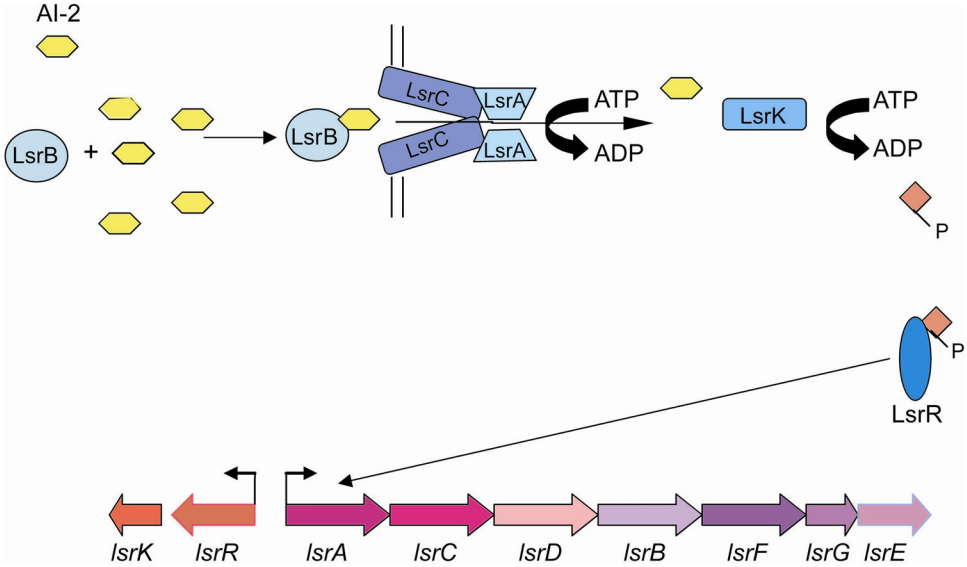

Figure 7.

The LuxS/AI-2 signaling pathway. The *lsrACDBFGE* genes are transcribed in an operon, whereas *lsrK* and *lsrR* are divergently transcribed. LsrB binds AI-2 which is then imported by the Lsr ABC transport system. Inside the cell, AI-2 is phosphorylated by LsrK and is thought to interact with LsrR, relieving the repression of the *lsr* operon.

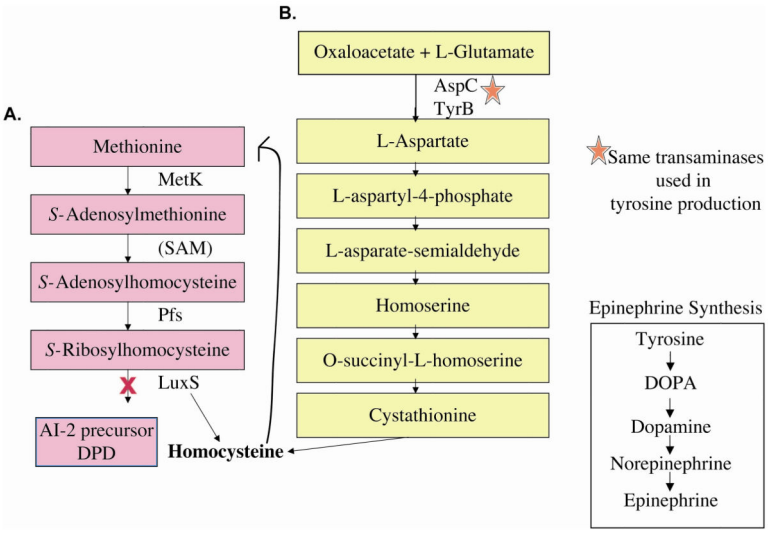

Figure 8.

Pathways for synthesis of homocysteine in *E. coli*. **A.** Methionine is important in the cell for production of the vital metabolic enzyme SAM (involved in the methylation of lipids, RNA, DNA, and protein) and *de novo* synthesis of methionine requires homocysteine. **B.** The *luxS* mutant cannot produce homocysteine through SRH hydrolysis; therefore, the oxaloacetate/L-glutamate pathway must be used. Oxaloacetate, L-glutamate, and the AspC and TyrB transaminases are used to produce aspartate, which than can proceed through a series of reactions, that result in the synthesis of homocysteine. Exclusive use of this pathway may lead to altered metabolism and amino acid content in the *luxS* mutant, leading to reduced AI-3 synthesis.

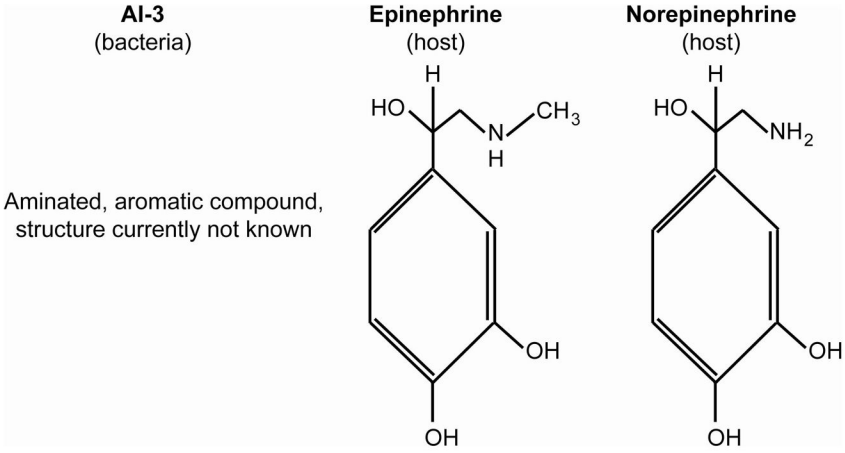

Figure 9.

The AI-3, epinephrine, and norepinephrine signaling molecules. The structure of AI-3 is not known, but may resemble the aromatic compounds epinephrine and norepinephrine.

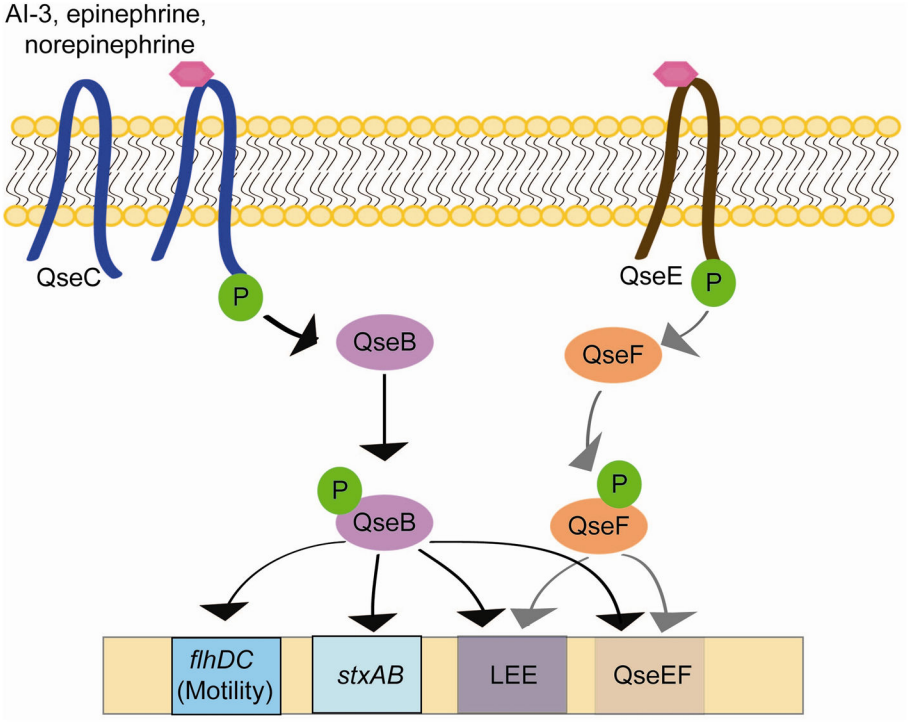

Figure 10.

The AI-3/epi/NE signaling pathway. AI-3, epinephrine, and norepinephrine bind the membrane receptor protein QseC, resulting in autophosphorylation. Subsequently, QseC phosphorylates its response regulator QseB, initiating a complex phosphorelay signaling cascade that results in expression of the flagellar biosynthesis and motility genes (*flhDC*), Shiga toxin (*stxAB*), the locus of enterocyte effacement (LEE), and a second two-component system, QseEF that also promotes expression of the LEE.