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Events at the Host-Microbial Interface of the Gastrointestinal Tract III. Cell-to-cell signaling among microbial flora, host, and pathogens: there is a whole lot of talking going on

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Clarke, Marcie B. and Vanessa Sperandio. Events at the Host-Microbial Interface of the Gastrointestinal Tract. III. Cell-to-cell signaling among microbial flora, host, and pathogens: there is a whole lot of talking going on. *Am J Physiol Gastrointest Liver Physiol* 288: G1105–G1109, 2005; doi:10.1152/ajpgi.00572.2004.—Humans have an important association with their intestinal microbial flora. The microbial flora helps to shape the mammalian innate immune system, absorbs nutrients, and plays an intricate role on intestinal development. Microbes and mammals communicate with each other through an array of hormone and hormonelike chemical compounds. These “signals,” however, are hijacked by bacterial pathogens, such as enterohemorrhagic *Escherichia coli* (EHEC), to activate its virulence genes, colonize the host, and start the disease process. This review explores the cell-to-cell signaling events in the gastrointestinal tract that lead EHEC to regulate its virulence genes in a coordinate manner.

prokaryote; enterohemorrhagic *Escherichia coli*; quorum sensing; hormones; epinephrine; autoinducer;

PROKARYOTES AND EUKARYOTES have coexisted intimately for millions of years. It is estimated that in humans, the total microbial population within the gastrointestinal (GI) tract ($\sim 10^{14}$) exceeds the total number of mammalian cells ($\sim 10^{13}$) by at least an order of magnitude (1). The GI tract is the site of the largest and most complex environment in the mammalian host. The density of bacteria along the GI tract can vary greatly, with the majority of the flora residing in the colon (10^{11} to 10^{12} bacterial cells/ml). Given the enormous number and diversity of bacteria comprising the GI environment, it should not be surprising that the members of this community somehow communicate among themselves and with the host itself to coordinate various processes. The observation that the human bacterial flora is extremely important to development, as well as in shaping the innate immune system, further reinforces this suggestion (8).

Eukaryotes have a mixed existence with prokaryotes, having amicable and detrimental interactions. The human bacterial flora exemplifies an important beneficial interaction (8), whereas detrimental interactions with pathogens result in disease. Given these polar relationships, it may be asked, “at what levels do prokaryotes and eukaryotes communicate?” Eukaryotic cell communication occurs through hormones. In bacteria, signaling processes used to coordinate gene expression in response to changes in bacterial cell density have historically been referred to as quorum sensing (25).

QUORUM SENSING

Quorum sensing (QS) is a cell-to-cell signaling mechanism that refers to the ability of bacteria to respond to chemical

hormonelike molecules called autoinducers. When an autoinducer reaches a critical threshold, the bacteria detect and respond to this signal by altering their gene expression. Three major QS circuits have been described: one used primarily by Gram-negative bacteria, one used primarily by Gram-positive bacteria, and one that has been proposed to be universal. This universal system, called the LuxS system, has been detected in more than 55 species by sequence analysis or functional assays (25).

LuxS is an enzyme involved in the metabolism of *S*-adenosyl-methionine; it converts ribose-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). DPD is a very unstable compound that reacts with water and cyclizes into several furanones, one of which is thought to be the precursor of autoinducer-2 (AI-2) (16). The AI-2 structure has been solved by cocrystallizing this ligand with its receptor LuxP in *Vibrio harveyi* and is reported to be a furanosyl-borate-diester (3). However, LuxP homologs, as well as homologs from the *V. harveyi* signaling cascade, have only been found in *Vibrio* sp. suggesting that AI-2 recognition may vary among other bacterial species. The AI-2 receptor in *Escherichia coli* and *Salmonella* is the periplasmic protein LsrB. Cocrystallization of LsrB with AI-2 demonstrated that its ligand was not a furanosyl-borate-diester but a furanone [2R, 4S-2-methyl-2,3,3,4-tetrahydrofuran (R-THMF)] (12). This result is consistent with previous observations indicating that AI-2 fractions of *Salmonella* and *E. coli* only yielded several furanosyl compounds that did not contain boron (12, 21, 24).

Diverse roles in signaling have been attributed to AI-2 in other organisms by comparing *luxS* mutants with wild-type strains and complementing these mutants either genetically or with spent supernatants (reviewed in Ref. 25). However, one also has to take into consideration that a knockout of *luxS* seems to affect the synthesis of at least two autoinducers, AI-2 and AI-3 (21). AI-3 is chemically different than AI-2; AI-2 is a very polar furanone that does not bind to C-18 columns. Nonetheless, AI-3 binds to C-18 columns and can only be eluted with methanol (21). Electrospray mass spectrometry analysis of the AI-3 fraction showed a major peak with a mass of 213.1 Da and minor peaks at 109.1, 164.9, 176.1, 196.1, 211.1, 214.1, and 222.9 Da (21). All of these are different from AI-2 (3), suggesting that AI-3 is a novel compound (21).

The activity of both signals can be differentiated by using biological tests specific to each signal. For example, AI-3 shows no activity for the AI-2 bioassay (21), which is predicated on the production of bioluminescence in *V. harveyi* (22). On the other hand, AI-3 activates the transcription of the

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enterohemorrhagic *E. coli* (EHEC) virulence genes, whereas AI-2 has no effect in this assay (21). The only two phenotypes shown to be AI-2 dependent, using either purified or in vitro synthesized AI-2, are bioluminescence in *V. harveyi* (16) and expression of the *lsr* operon (encoding the ABC transporter responsible for AI-2 uptake) in *S. typhimurium* (23).

The *luxS* gene is present in an array of bacterial species, including several species of bacteria that colonize the GI tract either temporarily or long term. These include commensal and pathogenic *E. coli*, *Salmonella enterica* serotypes Typhimurium and Typhi, *Shigella flexneri*, *Helicobacter pylori*, *Campylobacter jejuni*, *V. cholerae*, *Enterococcus faecalis*, *S. aureus*, *Clostridium difficile*, *C. perfringens*, *Bacillus* species, and *Streptococcus* species (25). It has recently been shown, using anaerobically cultured stools from healthy human volunteers, that the microbial intestinal flora produce both AI-2 (using the *V. harveyi* bioluminescence assay) and AI-3 (using the EHEC virulence gene transcription AI-3-dependent bioassay) (21). To obtain further information regarding which intestinal commensals and pathogens are able to produce AI-2 and AI-3, freshly isolated strains from patients were tested (M. Sircili and V. Sperandio, unpublished observations). With the use of the bioassays described above, AI-2 and AI-3 activity was observed in spent supernatants from enteropathogenic *E. coli* strains from serogroups O26:H11 and O111ac:H9, *Shigella* sp, and *Salmonella* sp. Activity from both autoinducers was also detected in normal flora bacteria such as a commensal *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* (Sircili and Sperandio, unpublished observations). These results suggest that AI-3 production is not limited to EHEC and that both AI-2 and AI-3 may be involved in interspecies signaling among intestinal bacteria and could play a role in the pathogenesis of disease caused by these other bacteria.

QS REGULATION OF THE EHEC VIRULENCE GENES

EHEC O157:H7 is responsible for major outbreaks of bloody diarrhea and hemolytic uremic syndrome (HUS) throughout the world. EHEC has a very low infectious dose (as few as 50 colony-forming units), which is one of the major contributing factors to EHEC outbreaks. Treatment and intervention strategies for EHEC infections are still very controversial, with conventional antibiotics usually having little clinical effect and possibly even being harmful [by increasing the chances of patients developing hemolytic uremic syndrome (HUS)] (10).

EHEC colonizes the large intestine, where it causes attaching and effacing (AE) lesions. The AE lesion is characterized by the destruction of the microvilli and the rearrangement of the cytoskeleton to form a pedestallike structure, which cups the bacteria individually. The genes involved in the formation of the AE lesion are encoded within a chromosomal pathogenicity island named the locus of enterocyte effacement (LEE). The LEE region contains five major operons: *LEE1*, *LEE2*, *LEE3*, *tir* (*LEE5*), and *LEE4*; these encode a type III secretion system (TTSS), an adhesin (intimin), and this adhesin's receptor (Tir), which is translocated to the epithelial cell through the bacterial TTSS. The LEE genes are directly activated by the LEE-encoded regulator (Ler), which is the first gene in the *LEE1* operon (10). Transcription of the LEE genes is further

positively and negatively modulated by GrlA and GlrR, respectively, which are encoded in a small operon downstream of *LEE1* (4). EHEC also produces a potent Shiga toxin (Stx) that is responsible for the major symptoms of hemorrhagic colitis and HUS. There are two types of Stx, Stx1, and Stx2, which are most frequently associated with human disease. Both of the genes encoding Stx1 and Stx2 are located within the late genes of a λ -like bacteriophage and are transcribed when the phage enters its lytic cycle. Disturbances in the bacterial membrane, DNA replication, or protein synthesis (which are the targets of conventional antibiotics) may trigger an SOS response in the bacterial cells that signals the bacteriophage to enter the lytic cycle (10). The phage replicates, Stx is produced, and the phage lyses the bacteria, thereby releasing Stx in the host.

The LEE genes, the flagella regulon, as well as the genes encoding Stx in EHEC are activated through QS (19–21). This QS system is dependent on the presence of the *luxS* gene, and with the use of purified and “in vitro” synthesized AI-2, it has been demonstrated that AI-2 does not have any role in activating these genes and that the signaling molecule activating all of these virulence phenotypes in EHEC is in fact the AI-3 autoinducer (21). Sperandio et al. (20) reported that transcription of all of the LEE operons is activated by the presence of AI-3 in supernatants from wild-type EHEC, commensal *E. coli*, and MG1655 (K-12) strains but not from an isogenic EHEC *luxS* mutant or from K-12 strain DH5 α (has a frameshift mutation in the *luxS* gene) (20). Type III secretion (TTS) could not be detected in the EHEC *luxS* mutant (21), and this phenotype could be restored by genetic complementation with the *luxS* gene cloned on a plasmid or by providing AI-3 exogenously (21).

In addition to activation of TTS, Sperandio et al. (19) reported that ~10% of the common genome between EHEC and *E. coli* K-12 are differentially expressed between a wild-type EHEC and its isogenic *luxS* mutant [EHEC has 1.3 Mb of DNA absent in K-12, and K-12 has 0.53 Mb of DNA that is absent in EHEC (13, 19)]. Among the QS-regulated genes and phenotypes noted in these studies were the genes encoding flagella and motility (19). Specifically, it was shown that transcription of *flhDC* (the master regulator of the flagella regulon) and the *mot* operon (encoding motility genes) is decreased in a *luxS* mutant compared with wild-type and complemented strains. Transcription of these genes as well as motility could be restored by addition of the AI-3 signal exogenously, further confirming that regulation of flagella expression and motility is being controlled by a QS-signaling mechanism (19, 21). QS regulation of *flhDC* expression has far-reaching implications beyond flagella expression, given that FlhDC has been shown to also regulate bacterial cell division and several metabolic processes (14).

Given the widespread nature of the *luxS*/AI-3 system in bacteria, an interesting extrapolation is that the AI-3/*luxS* QS system might have evolved to mediate microflora-host interactions but ended up being exploited by EHEC to activate its virulence genes. In this manner, the AI-3/*luxS* system alerts EHEC as to when it has reached the large intestine, where large numbers of commensal bacteria, which contain the AI-3/*luxS* QS system, are resident.

BACTERIAL-HOST CELL-TO-CELL COMMUNICATION THROUGH CROSS TALK BETWEEN THE BACTERIAL AI-3 AND THE HOST EPINEPHRINE/NOREPINEPHRINE SIGNALING SYSTEMS

The EHEC *luxS* mutant, unable to produce AI-3 and unable to express the LEE-encoded TTS system at normal levels, nonetheless still produced AE lesions on epithelial cells that were indistinguishable from those seen with wild type (21). The *luxS* mutant was still responding to eukaryotic cell signals to activate expression of the LEE genes. These signals were identified as the hormones epinephrine and norepinephrine. Finally, it has been demonstrated that the EHEC response to epinephrine and norepinephrine signaling is specific, given that it can be blocked by β - and α -adrenergic antagonists (such as propranolol and phentolamine, respectively). Epinephrine and norepinephrine can substitute for AI-3 to activate transcription of the LEE genes, type III secretion, and AE lesions on epithelial cells. Taken together, these results suggest that AI-3 and epinephrine/norepinephrine cross talk and that these compounds may use the same signaling pathway. As further evidence, regulation of the flagella regulon is also under AI-3 and epinephrine/norepinephrine control, and one can block EHEC response to both AI-3 and epinephrine/norepinephrine using propranolol. Specifically, propranolol can prevent formation of the AE lesions by the wild-type EHEC and the *luxS* mutant in epithelial cells (21).

Norepinephrine has been previously reported to induce bacterial growth (6), and there are reports in the literature that imply that norepinephrine might function as a siderophore (6). Recently, norepinephrine has been implicated as inducing expression of enterobactin and iron uptake in *E. coli*, suggesting that this is the mechanism involved in growth induction (2). However, the role of norepinephrine in bacterial pathogenesis seems to be more complex, because several reports suggested that this signal also activates virulence gene expression in *E. coli*, such as Stx (11), by an unknown mechanism of induction.

Both epinephrine and norepinephrine are present in the GI tract. Norepinephrine is synthesized within the adrenergic neurons present in the enteric nervous system (ENS) (7). Although epinephrine is not synthesized in the ENS, being synthesized in the central nervous system and in the adrenal medulla, it acts in a systemic manner after being released by the adrenal medulla into the bloodstream, thereby reaching the intestine (15). Both hormones modulate intestinal smooth muscle contraction, submucosal blood flow, and chloride and potassium secretion in the intestine (9). There are currently nine known human adrenergic receptors, partitioned into three subclasses: α_1 , α_2 , and β . Freddolino et al. (5) recently reported the three-dimensional structure of human β_2 -adrenergic receptor and predicted that the ligand-binding sites for epinephrine and norepinephrine are broadly similar. Taken together, there is extensive evidence in the literature that both epinephrine and norepinephrine are recognized by the same receptors and that both catecholamines have important biological roles in the human GI tract.

There seems to be a cross communication between the *luxS*/AI-3 bacterial QS system and the epinephrine/norepinephrine host signaling system. EHEC could respond to both a bacterial QS signaling system and a mammalian signaling

system to “fine tune” transcription of virulence genes at different stages of infection and/or different sites of the gastrointestinal tract. Given that eukaryotic cell-to-cell signaling occurs through hormones and bacterial cell-to-cell signaling occurs through QS, it is tempting to propose that QS might be a language by which bacterial and host cells communicate. Inasmuch as the host hormones epinephrine and norepinephrine signal to EHEC, it remains to be determined whether AI-3 exerts any functional effects on eukaryotic cell signaling.

EHEC QS SIGNALING CASCADE

Concerning the EHEC AI-3/epinephrine/norepinephrine signaling cascade, a transcriptional regulator from the LysR family, designated as QS *E. coli* regulator (Qse)A (18) has been recently identified. QseA is transcriptionally activated through QS and, in turn, binds to and directly activates transcription of the LEE-encoded regulator (F. Sharp and V. Sperandio, unpublished observations), (18). In addition, QseA also activates transcription of the *grlRA* operon (R. Russell and V. Sperandio, unpublished observations); also involved in the regulation of the LEE genes. These results suggest that QseA regulates transcription of the LEE in more than one level. Consequently, an EHEC *qseA* mutant has a striking reduction in type III secretion but has no defect in flagellation or motility, suggesting that QseA only regulates the LEE genes and plays no role in the flagella regulon (18).

Additionally, the QseBC two-component system is responsible for the transcriptional activation of the flagella regulon in response to QS (17). It is well known that many two-component systems act to positively regulate their own transcription. QseBC is no exception to this rule and has also been shown to autoactivate its own transcription (M. B. Clarke and V. Sperandio, unpublished observations). Transcriptional autoregulation could serve several purposes, including the amplification of signal or providing an additional threshold for gene activation. This additional level of control could allow the bacterial cell to activate the energetically expensive production of flagella through QseBC only under appropriate conditions.

The QseBC two-component system is activated by QS through AI-3 (21). Early studies (17, 21) indicated that an isogenic mutant in the *qseC* sensor kinase was unable to respond to AI-3 or epinephrine given exogenously. Interestingly, the motility of a *luxS* mutant can be restored either by the addition of AI-3 or epinephrine (17, 21), and the transcription of *flhDC* is also activated by both signals. Motility and *flhDC* transcription in a *qseC* mutant, however, are unable to respond to the presence of either AI-3 or epinephrine, indicating that QseC may be sensing the presence of these cross-signaling compounds (21).

Although QseBC regulates both its own transcription and that of *flhDC*, it plays no role in the regulation of other QS phenotypes, such as the LEE genes (17). Because flagella and motility are not the only phenotypes controlled by QS in EHEC, it is hypothesized that there are several other regulators involved in this signaling cascade.

Finally, three other genes in this signaling cascade have also been identified recently: *qseD* (encoding another regulator of the LysR family) and *qseE* and *qseF* (encoding a second two-component system), which are involved in regulating AE

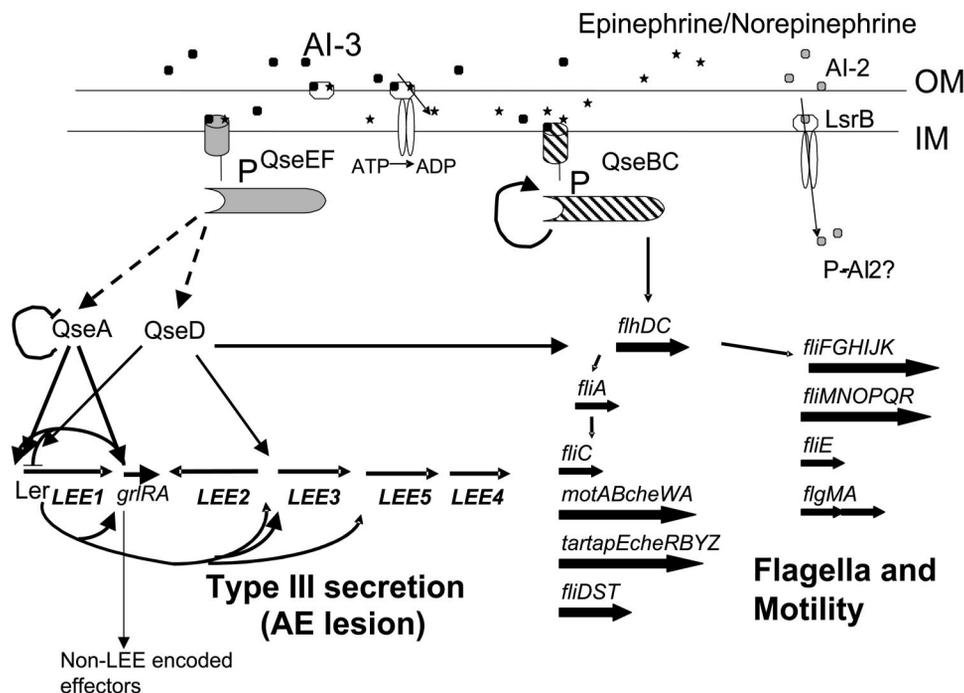


Fig. 1. Model of the AI-3/epinephrine/norepinephrine quorum sensing (QS) signaling cascade in enterohemorrhagic *Escherichia coli* (EHEC). Both AI-3 and epinephrine/norepinephrine seem to be recognized by the same receptor, which is probably in the outer membrane of the bacteria. These signals might be imported to the periplasmic space where they interact with two major sensor kinases. QS *E. coli* regulator (Qse)C might be the sensor kinase transducing these signals toward activation of the flagella regulon, whereas QseE might be sensor kinase transducing these signals to activate transcription of the LEE genes. QseB phosphorylates the QseC response regulator, which binds to the promoter of *flhDC* to activate expression of the flagella regulon. QseB also binds to its own promoter to positively autoregulate its own transcription. QseE is the sensor kinase, and its predicted response regulator is QseF. At what levels QseF regulates transcription of the LEE genes remain to be established. QseA is 1 of the transcriptional factors involved in the regulation of *ler* (*LEE1*) transcription in 2 levels, by binding and activating transcription of *LEE1* and by activating transcription of the *grlRA* operon, where *GrlA* and *GrlR* positively and negatively regulate expression of *ler*, respectively, and other effector proteins encoded outside of the LEE. Then, in a cascade fashion, *Ler* activates transcription of the other LEE genes. QseD is a second LysR-like regulator involved in modulating expression of the LEE and flagella genes. EHEC also possess an *lsr* operon involved in recognition and uptake of AI-2; however, the role of AI-2 signaling in EHEC remains to be addressed.

lesion formation (F. Sharp, N. Neading, and V. Sperandio unpublished observations). These data suggest that both AI-3 and epinephrine/norepinephrine are recognized by the same receptor, which is probably in the outer membrane of the bacterium (due to the nonpolar nature of these signals) (21). These signaling molecules might be imported into the periplasmic space where they then would interact with more than one sensor kinase. The interaction of AI-3 and epinephrine with more than one sensor kinase would also impart a “timing” mechanism to this system, which is a desirable feature given that it would be inefficient for EHEC to produce both the LEE TTSS and flagella simultaneously. Therefore, it is hypothesized that EHEC activates expression of the flagella regulon first through QseBC and then the LEE genes at a later time through QseEF. A model of the EHEC AI-3 QS signaling cascade is depicted in Fig. 1. The AI-3-dependent QS signaling cascade is present in all enterobacteriaceae (*E. coli*, *Salmonella* spp., *Shigella* spp., and *Yersinia* spp.). The most striking feature is that the genes encoding the transcriptional factors of this cascade are always in the exact same context in the chromosome of all these strains and share high levels of identity among these different species, suggesting that this signaling cascade is functionally conserved in enterobacteriaceae.

In conclusion, treatment of EHEC infections with conventional antimicrobials is highly controversial, because it is well

documented that antimicrobials activate the Stx phage to enter the lytic cycle, thereby producing and releasing Stx (10). There are now preliminary data (21) indicating that β -adrenergic antagonists, such as propranolol, can inhibit the entire signaling cascade in EHEC, rendering it unable to induce flagellation, motility, and AE lesion formation in response to either AI-3 and/or epinephrine/norepinephrine (21). These results thus suggest an exciting possible alternative for the treatment of EHEC infections by using β -adrenergic antagonists. Additionally, once the AI-3 structure is solved, it will allow the design of antagonists to AI-3. These studies may help generate a whole new class of antimicrobials that can block both AI-3 and epinephrine signaling to bacterial pathogens. Finally, these antimicrobials will not only be useful against EHEC but possibly also against other pathogens such as enteropathogenic *E. coli*, *Salmonella*, *Shigella*, and *Yersinia pestis*, all of which harbor this signaling cascade.

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