Chapter 9
Interkingdom Chemical Signaling in Enterohemorrhagic *Escherichia coli* O157:H7

Melissa M. Kendall

Abstract *Escherichia coli* is one of the most-studied species of bacteria due to its frequent incidence in diverse environments and hosts, as well as its use as a tool in molecular biology. Most *E. coli* strains are commensal, in that they colonize the host without causing disease; however, some strains of *E. coli* are pathogens and are able to cause diverse illnesses, including urinary tract infections, sepsis/meningitis, as well as intestinal disease that result in diarrhea (Kaper et al. 2004). Six categories of diarrheagenic *E. coli* are recognized, and these are classified in part based on how they interact with epithelial cells (Kaper et al. 2004). Of these, enterohemorrhagic *E. coli* O157:H7 (EHEC) is one of the most important pathogenic *E. coli* strains. EHEC causes major outbreaks of bloody diarrhea that can result in the development of fatal hemorrhagic colitis and hemolytic uremic syndrome (Karmali et al. 1983). EHEC colonizes the colon, where it forms attaching and effacing (AE) lesions on the intestinal epithelial cell. AE lesions are characterized by intimate attachment of EHEC to epithelial cells, effacement of the microvilli and rearrangement of the underlying cytoskeleton, which results in formation of a pedestal-like structure beneath the bacterium (Jerse et al. 1990; Jarvis et al. 1995; Kenny et al. 1997). Most of the genes involved in the formation of AE lesions are encoded within a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) (McDaniel et al. 1995). The LEE contains 41 genes that are organized in five major operons (*LEE1, LEE2, LEE3, LEE5*, and *LEE4*) (Elliott et al. 1998, 1999; Mellies et al. 1999). The LEE encodes a type three secretion system (T3SS) (Jarvis et al. 1995), an adhesin (intimin) (Jerse et al. 1990) and its receptor (Tir) (Kenny et al. 1997), as well as effector proteins (Kenny et al. 1996; Abe et al. 1997; McNamara and Donnenberg 1998; Elliott et al. 2001; Tu et al. 2003; Kanack et al. 2005). EHEC also encodes an arsenal of effector proteins located outside of the LEE that are important in EHEC virulence (Campellone et al. 2004; Deng et al. 2004; Garmendia et al. 2004, 2005; Gruenheid et al. 2004; Tobe et al. 2006).

M.M. Kendall (✉)
Department of Microbiology, Immunology, and Cancer Biology,
University of Virginia School of Medicine, Charlottesville, VA 22908, USA
e-mail: melissakendall@virginia.edu

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9.1 *Escherichia coli* O157:H7

Escherichia coli is one of the most-studied species of bacteria due to its frequent incidence in diverse environments and hosts, as well as its use as a tool in molecular biology. Most *E. coli* strains are commensal, in that they colonize the host without causing disease; however, some strains of *E. coli* are pathogens and are able to cause diverse illnesses, including urinary tract infections, sepsis/meningitis, as well as intestinal disease that result in diarrhea (Kaper et al. 2004). Six categories of diarrheagenic *E. coli* are recognized, and these are classified in part based on how they interact with epithelial cells (Kaper et al. 2004). Of these, enterohemorrhagic *E. coli* O157:H7 (EHEC) is one of the most important pathogenic *E. coli* strains. EHEC causes major outbreaks of bloody diarrhea that can result in the development of fatal hemorrhagic colitis and hemolytic uremic syndrome (Karmali et al. 1983). EHEC colonizes the colon, where it forms attaching and effacing (AE) lesions on the intestinal epithelial cell. AE lesions are characterized by intimate attachment of EHEC to epithelial cells, effacement of the microvilli and rearrangement of the underlying cytoskeleton, which results in formation of a pedestal-like structure beneath the bacterium (Jerse et al. 1990; Jarvis et al. 1995; Kenny et al. 1997). Most of the genes involved in the formation of AE lesions are encoded within a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) (McDaniel et al. 1995). The LEE contains 41 genes that are organized in five major operons (*LEE1, LEE2, LEE3, LEE5*, and *LEE4*) (Elliott et al. 1998, 1999; Mellies et al. 1999). The LEE encodes a type three secretion system (T3SS) (Jarvis et al. 1995), an adhesin (intimin) (Jerse et al. 1990) and its receptor (Tir) (Kenny et al. 1997), as well as effector proteins (Kenny et al. 1996; Abe et al. 1997; McNamara and Donnenberg 1998; Elliott et al. 2001; Tu et al. 2003; Kanack et al. 2005). EHEC also encodes an arsenal of effector proteins located outside of the LEE that are important in EHEC virulence (Campellone et al. 2004; Deng et al. 2004; Garmendia et al. 2004, 2005; Gruenheid et al. 2004; Tobe et al. 2006).

9.2 Regulation of the LEE Expression

Regulation of the LEE is complex and tightly regulated. The LEE pathogenicity island encodes genes for three regulators, Ler, GrlA, and GrlR (Mellies et al. 1999; Deng et al. 2004). Ler is encoded in *LEE1* and is a master regulator of the LEE (Mellies et al. 1999; Sperandio et al. 2000; Sánchez-SanMartín et al. 2001; Haack et al. 2003; Russell et al. 2007). Expression of Ler is directly or indirectly regulated by multiple proteins (Friedberg et al. 1999; Sperandio et al. 2002a, b; Umanski et al.

2002; Iyoda and Watanabe 2004, 2005; Sharma and Zuerner 2004; Iyoda et al. 2006; Sharp and Sperandio 2007; Kendall et al. 2010), including GrlR that represses *ler* transcription and GrlA that activates *ler* transcription (Deng et al. 2004; Barba et al. 2005; Russell et al. 2007). Moreover, expression of the LEE and LEE-associated genes is subject to further regulation at the transcriptional and posttranscriptional levels in response to diverse environmental cues, including nutrients and stress responses (Sperandio et al. 2003; Mellies et al. 2007; Bhatt et al. 2009, 2011; Lodato and Kaper 2009; Shakhnovich et al. 2009; Kendall et al. 2011, 2012; Njoroge et al. 2012; Pacheco and Sperandio 2012) as well as host hormones present in the gastrointestinal (GI) tract (Sperandio et al. 2003).

9.3 Shiga Toxin

The mortality associated with EHEC infections stems from the production and release of a potent Shiga toxin. EHEC expresses Shiga toxin in the intestine, and this inhibitor of mammalian protein synthesis is absorbed systemically and binds to receptors found in the kidneys and central nervous system, causing HUS, seizures, cerebral edema, and/or coma (Karmali et al. 1983). The genes encoding Shiga toxin are located within a lambdoid bacteriophage and are transcribed when the phage enters its lytic cycle (Neely and Friedman 1998; Neely and Friedberg 2000; Wagner et al. 2001). Disturbances in bacterial envelope, DNA replication, or protein synthesis (which are targets of conventional antibiotics) initiate an SOS response in EHEC that triggers the bacteriophage to enter the lytic cycle and produce Shiga toxin. Consequently, treatment of EHEC infections with conventional antimicrobials is contraindicated (Davis et al. 2013).

9.4 Chemical Signaling in EHEC

Bacterial pathogens rely on environmental cues derived from the host, as well as from the resident microbiota, to properly coordinate expression of traits important for pathogenesis. Quorum sensing is a cell-to-cell signaling mechanism through which bacteria synthesize and/or respond to bacterial-produced chemical signals called autoinducers (AIs). As concentrations of AI molecules change, bacteria modulate gene expression. Quorum sensing was first characterized in *Vibrio fisheri* and is based on the LuxI and LuxR proteins (Nealson et al. 1970). LuxI is a cytoplasmic protein that synthesizes the AI molecules, which then diffuses freely out of the bacterial cell. Once a particular threshold concentration of AI molecules is reached in the extracellular environment, the AI molecules diffuse back into the bacterial cells, where they interact with the transcription factor LuxR. Interaction between LuxR and its cognate AI promotes LuxR stability and oligomerization, which enables LuxR to bind target promoters and control gene expression.

EHEC relies on quorum sensing to control expression of genes encoding motility and virulence (Sperandio et al. 1999, 2001, 2002a, b, 2003). Initial studies suggested that the AI molecule called AI-2 was the signal that mediated quorum sensing-dependent virulence gene expression in EHEC (Sperandio et al. 1999, 2001); however, additional studies revealed that a distinct molecule, AI-3, was actually the signal responsible for activating expression of the LEE-encoded T3SS and motility genes (Sperandio et al. 2003). The molecule AI-2 is synthesized by a small metalloenzyme LuxS. Specifically, LuxS converts *S*-ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). DPD is a very unstable compound that reacts with water and cyclizes to form several different furanones (Schauder et al. 2001; Winzer et al. 2002; Sperandio et al. 2003), one of which is thought to be the precursor of AI-2 (Schauder et al. 2001). AI-3 does not directly depend upon *luxS* for synthesis; however, a mutation in the *luxS* gene affects AI-3 production by altering cellular metabolism (Walters et al. 2006). Subsequent studies that incorporated biochemical assays have conclusively demonstrated that AI-2 and AI-3 are distinct molecules. For example, the polar furanone AI-2 does not bind to C_{18} columns, whereas AI-3 binds to C_{18} columns and can only be eluted with methanol (Sperandio et al. 2003). Moreover, electrospray mass spectrometry also revealed differences between the structures of AI-2 and AI-3 (Chen et al. 2002; Sperandio et al. 2003). AI-2 activity leads to 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, whereas AI-2 does not influence EHEC virulence. Significantly, the eukaryotic hormones epinephrine and norepinephrine (epi/NE) can substitute for AI-3 to activate EHEC virulence gene expression, including the LEE genes, and adrenergic receptor antagonists inhibit the regulatory effects of epi/NE and AI-3 (Clarke et al. 2006). Thus, although the final structure of AI-3 has not yet been elucidated, it has been hypothesized that AI-3 may be structurally similar to epi/NE (Sperandio et al. 2003) (Fig. 9.1a, b).

9.5 Infectious Disease and Hormones

Eukaryotic cell-to-cell signaling is based on a variety of hormones, which are essential for eukaryotic development and homeostasis. Significantly, the hormones epinephrine and norepinephrine also promote EHEC growth and are co-opted as signals that EHEC uses to modulate expression of virulence traits (Lyte and Ernst 1992; Lyte et al. 1996; Freestone et al. 2000; Sperandio et al. 2003). Epinephrine and norepinephrine belong to the class of hormones called catecholamines. These hormones are derived from the amino acid tyrosine and are composed of a catechol and a side-chain amine. Epinephrine and norepinephrine are the most abundant catecholamines in the human body and are involved in the fight or flight response. Epinephrine and norepinephrine are present at micromolar concentrations in the intestine (Eldrup and Richter 2000) and play important roles in physiology of the GI tract by modulating smooth muscle contraction, submucosal blood flow, and

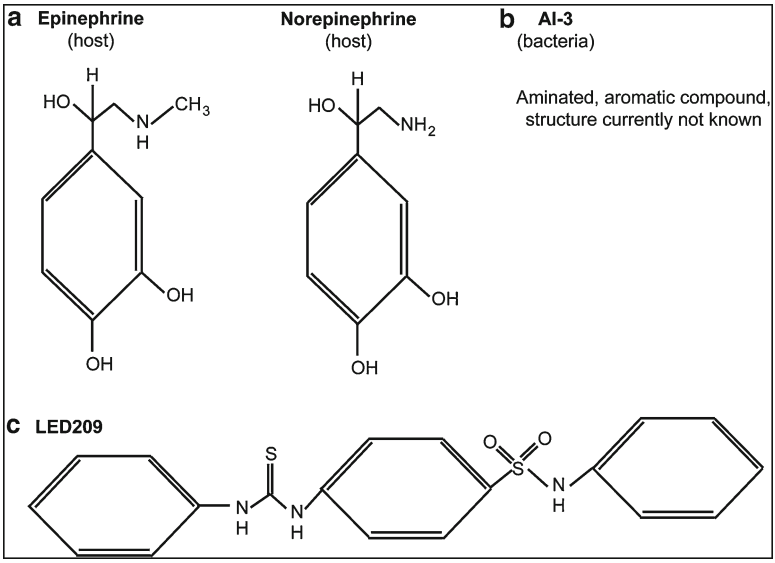

Fig. 9.1 Structures involved in adrenergic signaling. (**a**) Host hormones epinephrine and norepinephrine. (**b**) The structure of AI-3 has not been solved, but may resemble epinephrine and norepinephrine. (**c**) The structure of LED209 that inhibits QseC signaling

chloride and potassium secretion (Horger et al. 1998). In addition to the central nervous system and adrenal medulla, the adrenergic neurons that are present in the enteric nervous system are the major sources of epinephrine and norepinephrine (Furness 2000; Purves et al. 2001). Additionally, immune cells including T cells, macrophages, and neutrophils produce and secrete epinephrine and norepinephrine (Flierl et al. 2008). Therefore, bacterial infections may results in increased epinephrine and norepinephrine concentrations in the GI tract due to the stress of the infection in conjunction with the immune response. Finally, the commensal GI microbiota also contribute to the generation of biologically active norepinephrine (and to a lesser extent epinephrine) in the lumen of the GI tract (Asano et al. 2012).

9.6 Bacterial Adrenergic Receptors

The mammalian adrenergic receptors that bind epinephrine and norepinephrine and transmit signals are called G-coupled protein receptors. GPCRs are transmembrane receptors are coupled to heterotrimeric guanine-binding proteins (G proteins). EHEC does not encode G proteins; therefore, EHEC senses epinephrine and norepinephrine via a different mechanism. The main signaling transduction systems in bacteria are two component systems (TCSs) (Clarke et al. 2006). TCSs are critical for bacteria to sense and respond to changes in the environment. A typical TCS is composed of a histidine sensor kinase (HK) located in the cytoplasmic membrane that perceives a

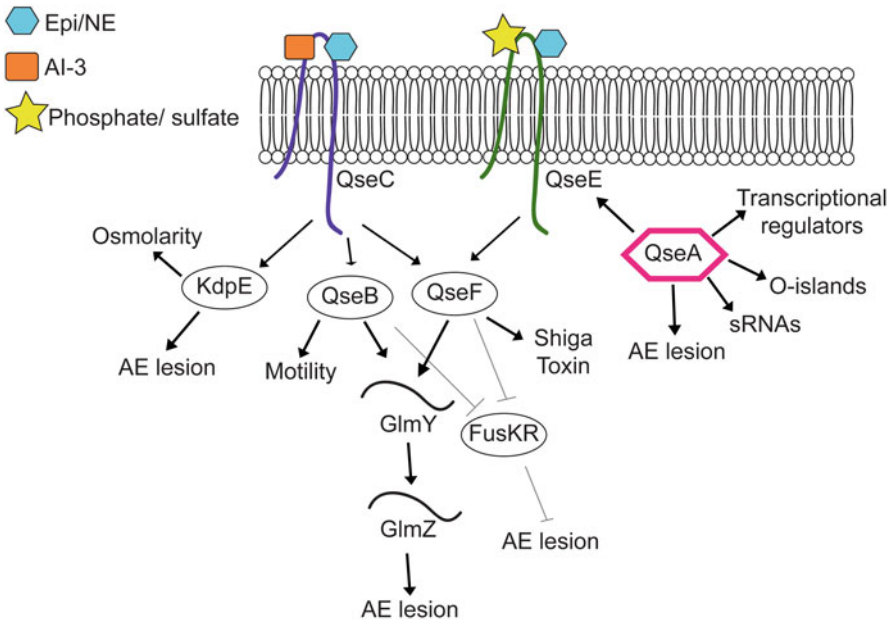

Fig. 9.2 Summary of the epinephrine/NE/AI-3 signaling cascade in EHEC. *Arrows* indicate positive regulation and *lines* with *bars* indicate negative regulation

stimulus and a cytoplasmic response regulator (RR) that controls the output (Jung et al. 2012). Upon sensing a specific environmental cue, the kinase autophosphorylates at a specific histidine residue and subsequently transfers this phosphate to an aspartate residue on its cognate RR. RRs are transcription factors that mediate the output of this signaling cascade by binding DNA to promote or repress gene expression (Jung et al. 2012). EHEC encodes two adrenergic receptors, QseC and QseE (Clarke et al. 2006; Reading et al. 2009), that upon sensing epinephrine and norepinephrine initiate a complex signaling cascade, which results in coordinated expression of virulence genes (Sperandio et al. 1999, 2002a, b, 2003; Clarke et al. 2006; Kendall et al. 2007; Reading et al. 2007, 2009; Hughes et al. 2009; Njoroge and Sperandio 2012; Pacheco et al. 2012; Gruber and Sperandio 2014) (summarized in Fig. 9.2).

9.7 The QseBC TCS

The gene encoding QseC was first identified in an array that compared gene expression between wild type (WT) EHEC and a *luxS* mutant (Sperandio et al. 2002a, b), and subsequent studies revealed that QseC directly senses, and

autophosphorylates, in response to host-derived epinephrine and norepinephrine as well as the bacterial-derived AI-3 (Clarke et al. 2006). QseC is a global regulator in EHEC and influences expression of more than 400 genes in response to epinephrine and AI-3 (Hughes et al. 2009). QseC directs expression of genes involved in cell metabolism, virulence, motility and stress responses (Hughes et al. 2009). To mediate these responses, QseC phosphorylates three distinct RRs, QseB, QseF, and KdpE (Hughes et al. 2009). QseB activates genes encoding flagella and motility (Sperandio et al. 2002a, b); QseF coordinates expression of genes encoding AE lesions and stress responses (Reading et al. 2007); and KdpE regulates genes encoding potassium uptake, osmolarity, and AE lesion formation (Nakashima et al. 1992; Hughes et al. 2009; Njoroge et al. 2012). Adrenergic signaling is essential for EHEC virulence during infection, as a *qseC* mutant strain is attenuated for virulence in rabbit-infection models (Clarke et al. 2006; Rasko et al. 2008).

9.8 The QseEF TCS

A second TCS involved in adrenergic signaling was identified in a microarray study that compared differential gene expression in WT and the *luxS* mutant EHEC strains. This TCS was renamed QseEF, where QseE is the HK and QseF is the RR (Reading et al. 2007). The *qseE* and *qseF* genes are encoded within a polycistronic operon that also contains the *yfhG* gene, which encodes an uncharacterized protein, as well as *glnB*, which encodes the PII protein involved in nitrogen regulation (Reading et al. 2007). QseE senses epinephrine and norepinephrine, as well as the environmental signals phosphate and sulfate, but does not sense AI-3. Therefore, QseE functions to sense strictly host-derived signals, in contrast to QseC that senses host- and bacterial-derived molecules (Clarke et al. 2006; Reading et al. 2009). Finally, QseC activates transcription of *qseEF*, and therefore, in the epinephrine and norepinephrine signaling cascade, QseE is downstream of QseC (Reading et al. 2007).

QseEF regulates expression of genes involved in the SOS response and Shiga toxin production, as well as transcription of genes encoding for other TCSs, including RcsBC and PhoPQ (Reading et al. 2009, 2010; Njoroge and Sperandio 2012). Additionally, QseEF influences AE lesion formation through regulation of EspFu/TccP (Reading et al. 2007). EspFu/TccP is an effector encoded outside of the LEE that enhances AE lesion formation (Campellone et al. 2004; Garmendia et al. 2004). The LEE-encoded T3SS translocates EspFu into the host cell where it mimics the eukaryotic SH2/SH3 adapter protein and leads to actin polymerization during AE lesion formation (Campellone et al. 2004). Bioinformatic analyses revealed that QseF contains a σ^{54} activator domain, whereas the *espFu* gene contains a conserved σ^{70} promoter, suggesting that QseF regulation of EspFu was indirect. Moreover, purified QseF did not bind to the espFu promoter in electrophoretic mobility shift assays. Together, these data confirmed that QseEF regula-

tion of EspFu requires an intermediate factor. Subsequent studies revealed that QseF regulates the sRNA GlmY, which is located immediately upstream from the *qseGFglnB* operon (Reichenbach et al. 2009). More recently, Gruber and Sperandio reported that GlmY, acting in concert with a second sRNA GlmZ, is the link between QseF and EspFu (Gruber and Sperandio 2014). Interestingly, GlmY and GlmZ promote *espFu* translation through cleavage of the transcript and negatively regulate expression of the *LEE4* and *LEE5* operons through destabilization of the mRNA (Gruber and Sperandio 2014).

9.9 Interplay Between QseBC and QseEF Sensing Systems

Single deletion strains of *qseC* or *qseE* are able to modulate gene expression in an epinephrine-dependent manner, whereas, *qseC/qseE* double mutant does not respond to epinephrine (Njoroge and Sperandio 2012). These findings suggest that QseC and QseE are the only adrenergic receptors in EHEC. QseC and QseE display convergent regulation of some target genes while differentially regulating others (Njoroge and Sperandio 2012). For example, QseB also promotes expression of GlmY, and thus regulates EspFu expression (Gruber and Sperandio 2014). Moreover, QseBC and QseEF negatively regulate expression of the TCS FusKR (Pacheco and Sperandio 2012). The HK FusK senses fucose in the GI tract, which EHEC uses to determine its location in the GI tract and correctly time expression of the LEE genes (Pacheco and Sperandio 2012). Further characterization of these regulatory cascades will provide a clearer understanding of how EHEC coordinates expression of these TCSs in order to precisely regulate virulence genes.

9.10 The Transcriptional Regulator QseA

QseA is a LysR-family transcriptional regulator that is activated by the AI-3/epi/NE signaling cascade (Sperandio et al. 1999, 2002a, b). QseA plays an important role in promoting ECHE virulence. QseA activates transcription of *ler*, and hence all the LEE genes (Sperandio et al. 2002a, b). The *LEE1* operon contains two promoters, a distal P1 promoter, and a proximal P2 promoter (Mellies et al. 1999; Sperandio et al. 2002a, b). QseA binds both promoters to regulate *ler* expression (Sperandio et al. 2002a, b; Kendall et al. 2010). Consistent with the transcriptional data, a *qseA* mutant strain formed significantly less AE lesions compared to WT EHEC (Sperandio et al. 2002a, b). Subsequent studies demonstrated that QseA regulates *grlRA* transcription in a Ler-dependent and Ler-independent mechanism and also showed that QseA regulon extends beyond the LEE and includes genes encoded in O-islands, which are regions of the chromosome unique to EHEC (Hayashi et al. 2001; Perna et al. 2001), other transcriptional regulators, sRNAs, as well as *qseE* (Reading et al. 2007; Kendall et al. 2010).

9.11 Disruption of AI-3/Epi/NE Signaling as an Antivirulence Strategy

Bacterial infections may lead to severe morbidity and mortality; however, the ability to treat these diseases with conventional antibiotics is becoming more and more limited. This is due primarily to the fact that antibiotics have lost their effectiveness as many bacteria are becoming resistant, often to multiple types of antibiotics. Conventional antibiotics disrupt essential functions, including DNA replication and protein synthesis, and thus place selective pressure on bacteria to develop resistance. An alternative approach may be to develop anti-virulence drugs that target bacterial virulence, but that does not inhibit bacterial growth or lead to death of the bacterial cell (Rasko and Sperandio 2010).

QseC homologues are present in over 25 plant and animal pathogens (Rasko et al. 2008). Thus, disrupting QseC signaling may be an effective strategy to inhibit virulence. Indeed, a high throughput screen identified a small, synthetic compound called LED209 (Fig. 9.1c) that blocked QseC signaling and prevented virulence expression not only in EHEC, but also in enteroaggregative *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Francisella tularensis* (Rasko et al. 2008; Curtis et al. 2014). LED209 functions as a prodrug that inhibits virulence by binding to and allosterically modifying QseC to disrupt activity (Curtis et al. 2014). LED209 specifically targets QseC and does not inhibit pathogen growth, suggesting that LED209 will not place selective pressure on pathogens to evolve resistance. An issue with an inhibitor of adrenergic signaling is that it may present adverse effects on the host. Significantly, LED209 did not present toxicity in cell culture or in rodents (Curtis et al. 2014), and future studies will need to be performed to confirm non-toxicity and efficacy in humans. Finally, some bacterial infections, including infections caused by *Clostridium difficile* and *Salmonella*, are associated with antibiotic use that disrupts the resident microbiota. Therefore, another important issue to be addressed concerns the effects of LED209 on the resident GI microbiota (Curtis and Sperandio 2011). Nevertheless, these recent findings underscore the potential of disrupting chemical signaling as a novel and effective antivirulence approach to treat diverse infectious diseases.

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