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The Acyl-Homoserine Lactone Synthase YenI from *Yersinia enterocolitica* Modulates Virulence Gene Expression in Enterohemorrhagic *Escherichia coli* O157:H7

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The human pathogen enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 colonizes the rectoanal junction (RAJ) in cattle, its natural reservoir. Colonization at the RAJ poses a serious risk for fecal shedding and contamination of the environment. We previously demonstrated that EHEC senses acyl-homoserine lactones (AHLs) produced by the microbiota in the rumen to activate the *gad* acid resistance genes necessary for survival through the acidic stomachs in cattle and to repress the locus of enterocyte effacement (LEE) genes important for colonization of the RAJ, but unnecessary in the rumen. Devoid of AHLs, the RAJ is the prominent site of colonization of EHEC in cattle. To determine if the presence of AHLs in the RAJ could repress colonization at this site, we engineered EHEC to express the *Yersinia enterocolitica* AHL synthase gene *yenI*, which constitutively produces AHLs, to mimic a constant exposure of AHLs in the environment. The *yenI*$^+$ EHEC produces oxo-C6-homoserine lactone (oxo-C6-HSL) and had a significant reduction in LEE expression, effector protein secretion, and attaching and effacing (A/E) lesion formation *in vitro* compared to the wild type (WT). The *yenI*$^+$ EHEC also activated expression of the *gad* genes. To assess whether AHL production, which decreases LEE expression, would decrease RAJ colonization by EHEC, cattle were challenged at the RAJ with WT or *yenI*$^+$ EHEC. Although the *yenI*$^+$ EHEC colonized the RAJ with efficiency equal to that of the WT, there was a trend for the cattle to shed the WT strain longer than the *yenI*$^+$ EHEC.

Enterohemorrhagic *Escherichia coli* serotype O157:H7 (EHEC) is a human pathogen that causes complications that range from abdominal cramps and bloody diarrhea to the life-threatening sequelae known as hemolytic uremic syndrome (1–3). Although EHEC colonizes the large intestine and causes disease in humans, EHEC is a member of the transient normal bovine microbial flora and naturally colonizes the rectoanal junction (RAJ) mucosa of cattle and is then subsequently shed into the environment with the animal's feces (4). To colonize the host, EHEC forms attaching and effacing (A/E) lesions on epithelial cells (5). These lesions are characterized by the effacement of the epithelium's microvilli, the intimate attachment of bacteria to the epithelial cells, and the rearrangement of the host actin cytoskeleton to form a pedestal-like structure cupping the individual bacterium (6, 7). The majority of the genes required to form A/E lesions are encoded within a chromosomal pathogenicity island known as the locus of enterocyte effacement (LEE) (8, 9). The LEE is comprised of 41 genes, most of them organized in five major operons (9). These genes encode transcriptional regulators (10, 11), a type III secretion system (12), the adhesin intimin (13), and its receptor Tir (13), as well as several effector proteins (14–18).

A complex network of proteins and genes has been shown to regulate the LEE, including H-NS (19), GadX (20), Per (11), EtrA and EivF (21), QseA (22), SdiA (23), CpxR (24), LexA (25), Pch (26), Hha (27), and Ler (11, 28, 29). Ler, encoded by the first gene in the *LEE1* operon, is the transcriptional master regulator of the other LEE genes (11, 28, 29). The nucleoid-associated protein H-NS silences the LEE; however, Ler antagonizes H-NS to overcome silencing and to activate the LEE (19). Recently, a member of the LuxR protein family, the transcription factor SdiA, was shown to modulate transcription of the LEE by directly repressing the expression of *ler* (30, 31).

The first LuxR-I quorum sensing (QS) system was described in *Vibrio fischeri* (32). LuxI is a synthase, while LuxR is a cognate transcription factor. The LuxI synthase produces small chemical signaling molecules known as acyl-homoserine lactones (AHLs) that diffuse freely out of the bacteria into the environment. Once an external threshold concentration is reached, AHLs diffuse back into the cells and bind to their cognate cytoplasmic LuxR transcription factor. Ligand binding initiates an increase in LuxR protein stability and also promotes LuxR protein oligomerization (33). The AHL-LuxR complex then binds to target promoters and regulates their expression (33). For example, the LuxR-I system of *V. fischeri* activates the production of light by inducing the expression of genes important for bioluminescence (32). Since this initial discovery, homologs of the LuxR-I system have been found in over 50 bacterial species, including the human pathogens *Yersinia enterocolitica* and *Pseudomonas aeruginosa* and the plant pathogens *Erwinia carotovora* and *Agrobacterium tumefaciens* (33). A majority of these species encode both a synthase and a transcriptional regulator, but interestingly, a subset of species encode only the LuxR homologs but not their cognate LuxI synthases. The

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LuxR homolog SdiA present in *E. coli* and *Salmonella* spp. is an example of such "orphan" LuxR proteins.

SdiA has been shown to be involved in interspecies communication, as evidenced by the fact that SdiA is able to detect and respond to AHLs produced by other bacteria (30, 34–36). Dyszel and colleagues demonstrated that SdiA from *Salmonella enterica* serovar Typhimurium detects AHLs produced by the pathogen *Yersinia enterocolitica* in mice. To mimic a constant interaction with *Y. enterocolitica*, Dyszel et al. constructed an *S.* Typhimurium strain to constitutively express the *Y. enterocolitica* gene *yenI*, which encodes an AHL synthase. SdiA-dependent genes activated by AHLs conferred a fitness advantage in *S.* Typhimurium organisms carrying *yenI* and *sdiA* compared to those carrying *yenI* but not *sdiA*, implicating the importance of SdiA and AHLs in competition within a niche.

Additionally, recent evidence from studies in cattle indicates that EHEC may sense AHLs in an SdiA-dependent manner in order to discern the appropriate niche for bacterial colonization. An investigation of the bovine digestive tract determined that AHLs are present only in the rumen (30, 37). EHEC activates the *gad* acid resistance genes in response to AHLs, likely as a mechanism to safely passage through the cattle's acidic environment (30). Conversely, rumen-derived AHLs repress the LEE (23, 30). The rationale for this inhibition is that rumen colonization is unfavorable, and the absence of AHLs in the RAJ, the prominent site of colonization, alleviates SdiA-AHL mediated-repression of the LEE, thus promoting colonization at the RAJ. Ruminants known as "supershedders" shed high numbers of EHEC in their feces over a prolonged period, and EHEC strains isolated from supershedders more intimately colonize the RAJ (38). Epidemiologic studies have shown that supershedders account for approximately 95% of all EHEC shed into the environment (39–41). Since most humans become infected with EHEC either by ingestion of food products contaminated by infected animals (42–45) or through direct contact with infected animals (46–49), understanding how EHEC promotes intimate colonization at the RAJ in its natural host is crucial for development of preventive strategies to decrease EHEC shedding into the environment and consequent transmission to humans.

In this study, we explore the role of AHLs in colonization of the RAJ by EHEC by constructing an AHL-producing EHEC strain to mimic constant exposure to AHLs in the environment. Our data provide evidence that continuous exposure of EHEC to AHLs decreases A/E lesion formation on epithelial cells *in vitro* but does not prohibit colonization of EHEC at the RAJ in cattle. These data suggest that EHEC colonization at the RAJ is complex, with multiple factors contributing to efficient colonization.

MATERIALS AND METHODS

Strains and plasmids. All strains and plasmids used in this study are listed in Table 1. Unless otherwise stated, *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth at 37°C and 250 rpm. Where indicated, strains were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen). Medium was supplemented where necessary with the selective antibiotics streptomycin, kanamycin, ampicillin, and chloramphenicol, which were added to a final concentration of 100, 50, 100, or 30 μg/ml, respectively.

Recombinant DNA methods. Methods used for PCR amplification, plasmid purification, and transformations followed standard protocols as previously described (50). Oligonucleotide primers (Table 2) were designed by using Primer Express v1.5 (Applied Biosystems). Δstx_{2a}, *yenI*$^+$ Δstx_{2a}, and *vector*$^+$ Δstx_{2a} strains were constructed using λ Red mutagenesis (51). Briefly, to generate the Δstx_{2a} mutant in a wild-type EHEC strain (strain 86-24), PCR product was amplified using Phusion polymerase (Invitrogen), primers stx2AλRed F and stx2AλRed R, and pKD3 plasmid as the template. PCR product was digested with DpnI to remove the template DNA and then gel purified (Qiagen). Wild-type EHEC strains transformed with the helper plasmid pKD46 were prepared for electroporation and transformed with the resulting stx_{2a} PCR product. Colonies were screened for ampicillin sensitivity and chloramphenicol resistance. The chloramphenicol cassette was removed from Δstx_{2a} deletion candidates with the resolvase plasmid pCP20. Final verification was performed by PCR amplification and sequencing to yield the resolved Δstx_{2a} EHEC strain (YNN01). *yenI* and empty vector were chromosomally integrated into the YNN01 background to yield *yenI*$^+$ Δstx_{2a} and *vector*$^+$ Δstx_{2a} EHEC strains using the same λ Red mutagenesis method. Primers yenIλRed F and yenIλRed R were used to amplify the λP_R-*yenI*-FRT-kan-FRT or λP_R-FRT-kan-FRT cassettes from pJLD1600 and pJD500 (36), respectively, and the sequences homologous to the *lacI* integration site in the chromosome of Δstx_{2a} EHEC.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
86-24	Wild-type Stx2$^+$ EHEC strain (serotype O157:H7)	67
YNN01	86-24 Δstx_{2a}	This study
YNN02	YNN01 λP_R	This study
YNN03	YNN01λP_R-*yenI*	This study
Plasmids		
pKD3	λ-Red template plasmid	51
pKD46	λ-Red helper plasmid (recombinase)	51
pCP20	λRed resolvase plasmid	51
pJLD1600	λP_R-*yenI*-FRT-*kan*-FRT template plasmid	36
pJLD500	λP_R-FRT-kan-FRT vector template plasmid	36

Western blotting. Wild-type and *yenI*$^+$ strains were grown in high-glucose DMEM at 37°C in the presence of either 10 μM oxo-C6-HSL (synthesized in the Flack's laboratory) or an equivalent amount of dimethyl sulfoxide (DMSO) to an optical density at 600 nm (OD_{600}) of 1.0, and secreted proteins were prepared as previously described (12). Protein samples were electrophoresed in sodium dodecyl sulfate–12% polyacrylamide gels. Samples were subjected to Western blotting as described previously (50). Blots were probed with rabbit polyclonal antisera to EspA and EspB (Cocalico Biologicals) and visualized with enhanced chemiluminescence (Bio-Rad).

FAS assay. To assess A/E lesion formation, fluorescent actin staining (FAS) assays were performed as previously described (52). Briefly, HeLa cells were grown on coverslips in low-glucose DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37°C in 5% CO_2 and were infected with a 1:100 dilution of overnight static bacterial cultures for 6 h. The coverslips were washed, fixed, and permeabilized with 0.2% Triton X. Fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma-Aldrich) was used to visualize actin accumulation, and propidium iodide was added to stain bacteria and HeLa nuclei.

RNA extraction. Overnight cultures grown aerobically at 37°C in LB were diluted at 1:100 into high-glucose DMEM in the presence or absence of 10 μM oxo-C6-HSL and grown in triplicate to late exponential growth phase (OD_{600} = 1.0). Oxo-C6-HSL was dissolved in DMSO at 10 mM concentration and added directly to DMEM at 1:1,000 dilutions. For samples assessed without exogenous signals, the respective concentration of DMSO was used to ensure that the solvent did not alter gene expression. RNA from these biological replicates was extracted using

TABLE 2 Oligonucleotide primers used in this study

Primer	Sequence
stx2AλRed F	CTTTTTTATATCTGCGCCGGGTCTGGTGCTGATTACTTCAGCCAAAAGGAACACCTGTATGTGTAGGCTGGAGCTGCTTCG
stx2AλRed R	CATTAACAGAAGCTAATGCAAATAAAACCGCCATAAACATCTTCTTCATGCTTAACTCCTCATATGAATATCCTCCTTAG
yenIλRed F	AGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATCTGTAATCATAGTCATGATACGACTCACTATAGGGCG
yenIλRed R	AAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGTTGACCATGATTACGCCAAGC
stx2AUP	CTGCATTATGCGTTGTTAGCTCAG
stx2ADown	ATCCGCCGCCATTGCATTAAC
lacZF	TGCAAGGCGATTAAGTTGGGTAACG
IRlacIR	TGTGACCTGGCGTCAGCATTTTAAATCT

TRIzol (Invitrogen) and the RiboPure Bacteria RNA isolation kit (Ambion) according to the manufacturer's instructions.

RT-qPCR. The primers used for the real-time PCR assays were designed by using Primer Express v1.5 (Applied Biosystems) (Table 2). The amplification efficiency and template specificity of each of the primers were validated, and reaction mixtures were prepared as previously described (53). Quantitative real-time reverse transcriptase PCR (RT-qPCR) was performed in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems). Using the ABI sequence Detection 1.2 software (Applied Biosystems), data were collected and normalized to endogenous levels of *rpoZ*. Data were analyzed by using the comparative critical threshold cycle (C_T) method and presented as fold changes compared to WT levels. Error bars represent the standard deviations of the $\Delta\Delta C_T$ values. Statistical significance was determined by Student's *t* test, and a *P* value of ≤ 0.05 was considered significant.

AHL detection. AHL extraction and detection were performed as previously described (54). Briefly, wild-type, *yenI*$^+$, and vector control strains were grown in high-glucose DMEM to late exponential phase. AHLs were extracted three times with ethyl acetate and concentrated to 50 μl. For analytical thin-layer chromatography (TLC), 1 μl of concentrated extracts from wild-type and vector strains or 10 μl of concentrated extract diluted at 1:100 from *yenI*$^+$ strains was applied to C_{18} reverse-phase TLC plates (200-μm layer; Whatman). The chromatograms were developed with 70% methanol, dried, and overlaid with a culture of the *Agrobacterium tumefaciens traI lacZ* (55) indicator strain and 60 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 16 h at 30°C. For positive control, 1 μl of 10 μM 3-oxo-C_6-HSL was also included.

Cattle experiments. Two groups of five mature Holstein steers were housed in quarantined facilities. All personnel followed strict biosafety procedures, and all procedures were approved by the Institutional Animal Care and Use and Biosafety Committees. O157 cultures were adjusted to the desired bacterial concentration by dilution in phosphate-buffered saline and confirmed by viable plate count. Cattle received a single rectal application of 10^9 CFU of wild-type or *yenI*$^+$ O157, as previously described (56). O157 was cultured from rectoanal junction mucosa swabs as previously described (39) on the days indicated in Results.

RESULTS

The canonical LuxR/LuxI-type quorum-sensing system encodes a transcription factor (LuxR) and an associated AHL synthase (LuxI). Several examples exist, however, whereby a LuxR regulator lacks an associated AHL synthase. In *E. coli*, for example, the LuxR regulator SdiA functions to regulate several important virulence and colonization genes yet lacks a cognate synthase. The EHEC homolog of SdiA senses AHLs synthesized by other bacteria rather than self-produced AHLs to modulate virulence gene expression (30). Previously, we demonstrated that an EHEC lacking *sdiA* colonizes the RAJ of cattle with reduced efficiency compared to EHEC carrying *sdiA* (30), suggesting that SdiA plays an important role to promote colonization at the RAJ. Additionally, we demonstrated that AHLs repress the LEE pathogenicity island *in vitro*. An absence of AHLs promotes alleviation of SdiA-AHL-mediated repression of the LEE and likely contributes to successful colonization of EHEC at the RAJ. To explore the effect of AHLs on EHEC virulence and colonization, we engineered an EHEC strain to constitutively express the *Yersinia enterocolitica* AHL synthase YenI to mimic a constant exposure of AHLs. The U.S. Department of Agriculture (USDA) prohibits the use of Shiga toxin-producing *E. coli* in cattle experiments; therefore, the stx_{2a} gene was deleted from both wild-type and *yenI*$^+$ EHEC strains. Initial studies confirmed that the chromosomal integration of *yenI* in EHEC did not affect growth (Fig. 1A). Additionally, we confirmed that the *yenI*$^+$ EHEC strain also produced endogenous AHLs as detected by the thin-layer chromatography comparisons with the wild type and the vector control (Fig. 1B).

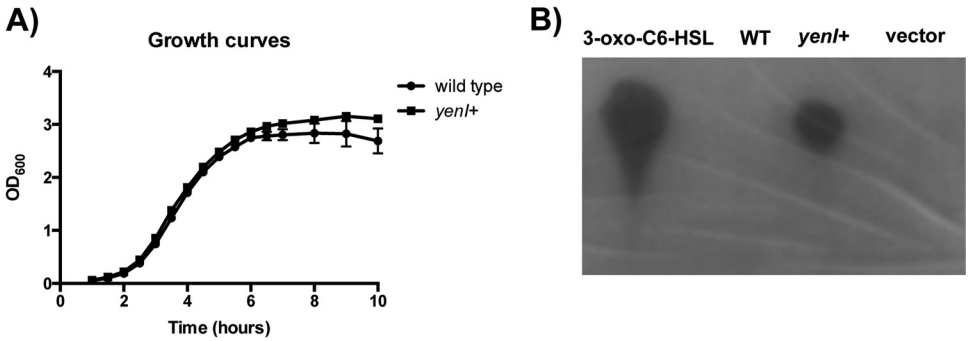

FIG 1 *yenI*$^+$ EHEC produces AHLs. (A) Growth curves of wild-type and *yenI*$^+$ strains grown in triplicate in high-glucose DMEM at 37°C. Optical density at 600 nm (OD_{600}) was measured at the indicated times. (B) *yenI*$^+$ EHEC produces endogenous AHLs, as shown by TLC of AHLs extracted from wild-type, *yenI*$^+$, and *vector*$^+$ strains; 3-oxo-C6-HSL was used as a positive control.

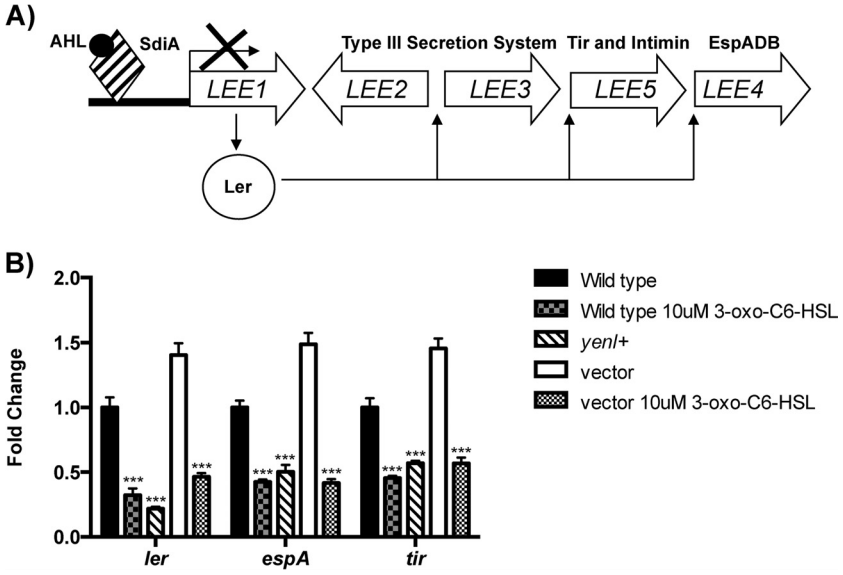

FIG 2 LEE regulation by AHLs. (A) Schematic of SdiA-AHL complex regulation of the LEE genes. SdiA complexed with AHLs binds to the *LEE1* promoter to repress its expression. *LEE1* encodes the Ler transcription activator of the LEE genes; hence, SdiA inhibition of *LEE1* inhibits transcription of all LEE genes in a cascade fashion. (B) RT-qPCR analyses of *ler* (*LEE1*), *espA* (*LLE4*), and *tir* (*LEE5*) transcription in the wild type, wild type with exogenous AHLs, *yenI*$^+$ strain (produces AHLs endogenously), wild type with vector (vector used to insert *yenI* into the EHEC chromosome, no AHLs, negative control), and wild type with vector plus endogenous AHLs. The mRNA levels are graphed as fold changes compared to WT levels. Statistical significance was determined by Student's *t* test; ***, $P \leq 0.001$.

Endogenous AHLs repress the transcription of the LEE. In response to exogenous AHLs, SdiA represses transcription of the LEE genes by directly binding to the promoter and repressing the master transcriptional regulator *ler* (30) (Fig. 2A). Consistent with our previous results, addition of exogenous AHLs reduced the expression of the LEE. The endogenous production of AHLs in the *yenI*$^+$ EHEC strain decreased expression of *ler*, *espA*, and *tir* (all of which are LEE genes) to a level similar to the one obtained with the addition of exogenous signal. Transcriptional repression of the LEE in the *yenI*$^+$ strain was dependent on endogenous AHLs, since chromosomal integration of the empty vector control had no effect on transcription of the LEE in the absence of exogenous AHL signal.

***yenI*$^+$ EHEC forms fewer A/E lesions.** Since endogenous AHLs reduced expression of the LEE, we next used fluorescent actin staining (FAS) to investigate whether *yenI*$^+$ EHEC has a reduced ability to form A/E lesions on epithelial cells compared to the wild type. HeLa epithelial cells were infected for 6 h with wild-type, *yenI*$^+$, or vector-only EHEC strains. Wild-type and vector control EHEC formed A/E lesions on 88% and 87% of the HeLa cells, respectively (Fig. 3). In contrast, *yenI*$^+$ produced significantly fewer A/E lesions, infecting only 3% of the HeLa cells. These results suggest that endogenous AHLs decrease the ability of EHEC to form A/E lesions. Congruently with the decrease in LEE expression (Fig. 2) and A/E lesion formation, the *yenI*$^+$ strain was unable to secrete the EspA and EspB LEE type three secreted proteins (Fig. 3C).

Endogenously produced AHLs activate *gad* gene expression. Although AHLs repress LEE gene expression, they activate expression of the *gad* acid resistance genes (30). Here, again, we show that transcription of the *gadA*, *gadC*, and *gadE* genes is significantly increased by both exogenous and endogenous (*yenI*$^+$) AHLs. These data combined suggest that the *yenI*$^+$ EHEC behaves as if it is constantly in the presence of this signal.

Wild-type and *yenI*$^+$ EHEC organisms colonize the RAJ mucosa of cattle similarly. Our *in vitro* studies suggest that endogenous AHL production decreases LEE expression and consequently reduces A/E lesion formation on HeLa cells. Next, we investigated if *yenI*$^+$ EHEC colonizes the host *in vivo* less efficiently than does the wild type. Five steers were challenged with a single rectal application of wild-type or *yenI*$^+$ EHEC. Wild-type or *yenI*$^+$ EHEC was cultured on day 0 prior to challenge and then on days 1, 3, 7, 10, 14, 17, 21, 24, 28, and 42 postchallenge. All animals were O157 culture negative prior to challenge, and all animals were culture positive for at least 14 days postchallenge. The wild-type and *yenI*$^+$ EHEC colonized the bovine RAJ similarly, although the wild-type tended to persist slightly longer than the *yenI*$^+$ EHEC (see Fig. 5).

DISCUSSION

EHEC can colonize multiple hosts. In order to survive host defenses and successfully colonize specific niches, EHEC must rely on environmental cues to modulate appropriate gene expression. Gram-negative bacteria use the chemical signal acyl-homoserine lactones to monitor their own population density and mount appropriate responses (33). EHEC can hijack this bacterial cell-to-cell communication by sensing AHLs produced by other bacteria in the environment to regulate their genes for survival and colonization in its natural reservoir, cattle (30). To establish colonization in cattle at the RAJ (4), EHEC must survive the acidic environment of the stomachs and activate A/E lesion formation at the RAJ. Construction of a type III secretion system for injection of bacterial effector proteins into the host cells to form A/E lesions requires a lot of energy resources; therefore, expression of the LEE genes that are required for A/E lesions has to be tightly regulated to prevent waste of energy in the wrong gastrointestinal compartment. Previous findings suggest that activation of acid resistance genes by AHLs synthesized by other bacteria present in the rumen of cattle prepares EHEC to survive the acidic environment of cattle

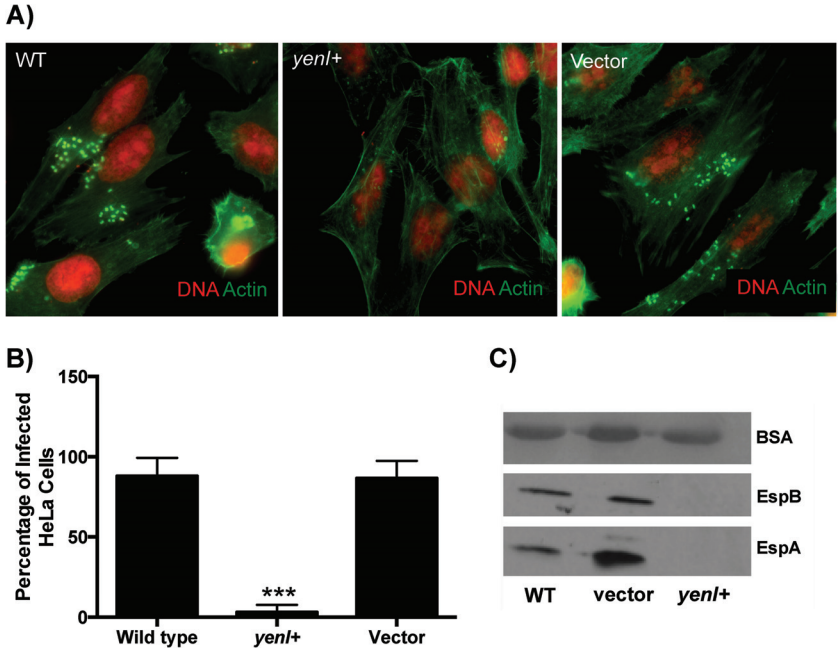

FIG 3 AHLs decrease A/E lesion formation by EHEC. (A) Fluorescent actin staining (FAS) assays. HeLa cells were infected with wild-type, *yenI*$^+$, and vector control strains for 6 h. Actin was stained in green with FITC-phalloidin, and HeLa cell nuclei and bacteria were stained in red with propidium iodide. A/E lesion formations were visualized as bright green cupping the red bacteria. (B) Quantification of infected cells or cells with A/E lesions. (C) Western blots of the secreted proteins of wild-type, vector, and *yenI*$^+$ strains probed with antiserum against EspB and EspA; bovine serum albumin (BSA) was used as a loading control. Statistical significance was determined by Student's *t* test; ***, $P \leq 0.001$.

stomachs while decreasing the LEE, since the rumen is not a preferable niche for colonization (30). Interestingly, the RAJ contains no AHLs (30, 37). The absence of AHLs in the RAJ alleviates AHL-mediated repression of the LEE to allow for A/E lesion formation on the mucosal epithelial cells at the RAJ, favoring colonization at the RAJ. These data suggest that AHLs produced by the bacterial population in cattle guide EHEC to survive and establish a niche in an appropriate environment once inside the cattle.

If AHLs provide cues to EHEC that the rumen is an unfavorable environment for colonization, then an interesting question to explore is whether AHLs present in the RAJ would decrease bacterial colonization of EHEC at the RAJ and decrease subsequent shedding of EHEC into the environment. We explored this idea by creating an EHEC strain that contains the LuxI-like synthase gene *yenI* from *Y. enterocolitica* to imitate the constant exposure to AHLs that occurs in the environment. *yenI*$^+$ EHEC produced endogenous AHLs that significantly decreased expression of the LEE genes while increasing expression of the *gad* acid resistance genes (Fig. 4). Lowered transcriptional expression of the LEE also resulted in a significantly decreased production of secreted proteins and, consequently, A/E lesion formation on HeLa cells (Fig. 3). Although the *in vitro* data are consistent with our previous data demonstrating the importance of AHLs in the downregulation of the LEE, *yenI*$^+$ EHEC colonizes the RAJ similarly to the wild type, albeit there is a trend that animals infected with *yenI*$^+$ EHEC shed EHEC for a shorter time than those infected with WT (Fig. 5). Diffusion of AHLs into the large environment of the RAJ rather than an enclosed system *in vitro* may account for the lack of AHL-mediated repression of EHEC colonization of this site.

The data also suggest that unidentified signals sensed by SdiA are contributing to EHEC colonization at the RAJ, as supported by our previous findings that the wild-type EHEC outcompeted the mutant lacking *sdiA* in colonization at the RAJ (30). Since AHLs are not naturally present in the RAJ, it is also possible that there are enzymes or factors expressed by the epithelial cells in the RAJ that can readily degrade AHLs. For example, a class of AHL-degrading enzymes found in mammals called paraoxonases (PONs) has been shown to degrade AHLs and inhibit the quorum-sensing regulation of bacteria (57). PON2 has the highest enzymatic capacity to degrade AHLs compared to other PONs (58, 59). Interestingly,

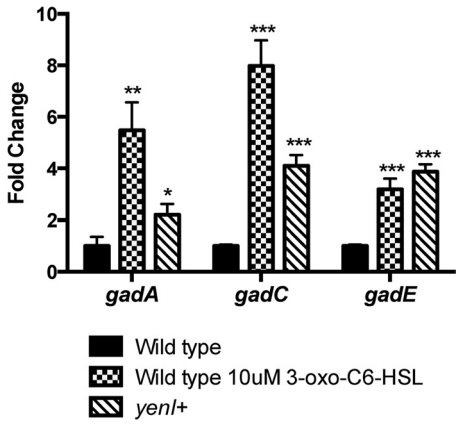

FIG 4 AHLs increase *gad* gene expression in EHEC. RT-qPCR analyses of *gadA*, *gadC*, and *gadE* transcription in the wild type, in the wild-type strain with exogenous AHLs, and in the *yenI*$^+$ strain (produces endogenous AHLs). The mRNA levels are graphed as fold changes compared to WT levels. Statistical significance was determined by Student's *t* test; **, $P \leq 0.01$; ***, $P \leq 0.001$.

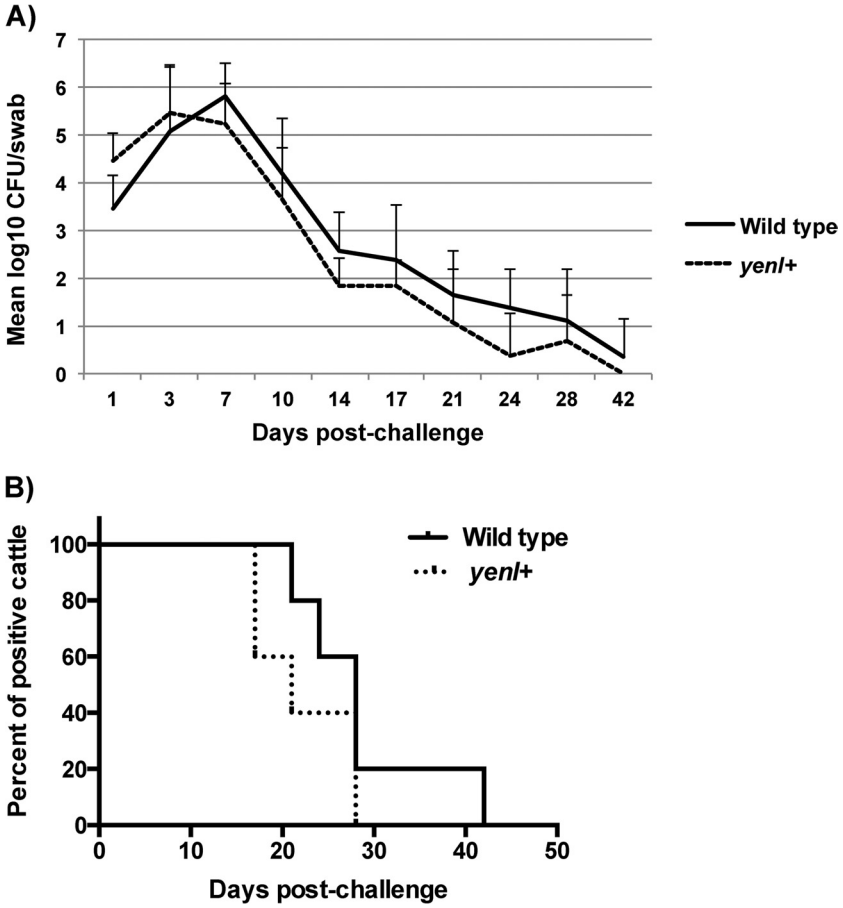

FIG 5 Cattle carriage of EHEC after challenge. The rectoanal junction (RAJ) mucosas of 10 Holstein steers were dosed with a single application of 10^9 CFU of either wild-type or *yenI*$^+$ EHEC. The challenge strains were cultured from RAJ mucosal swabs on the indicated postchallenge days. (A) Mean log CFU of wild-type or *yenI*$^+$ strain per swab from cattle throughout the experiment. (B) Percentage of animals (inoculated with either wild-type or *yenI*$^+$ strain) culture positive with EHEC postchallenge throughout the period of 56 days.

various tissues, including epithelial cells of the gastrointestinal tract, where EHEC colonizes humans and AHLs are absent (60), express PON2 (61). This suggests that EHEC utilizes other available signals such as epinephrine or norepinephrine (62–64) to modulate the LEE genes to promote colonization in the human gut. Paraoxonases are also found in cattle (65), and bovine serum has been shown to degrade AHLs (59). This infers that either paraoxonases or other similar AHL-degrading enzymes could degrade the AHLs produced by *yenI*$^+$ strains in the RAJ and, as a result, EHEC is utilizing other, more-abundant signals to promote colonization.

Other non-LEE factors may also be important for colonization of EHEC at the RAJ. For example, curli and other fimbriae have been implicated as important for colonization of EHEC in cattle (66). This demonstrates how EHEC can utilize an array of complex mechanisms and signals to regulate genes required for colonization in cattle. Further identification of new potential signals and elucidation of the mechanisms used by EHEC to colonize its natural host will help develop better preventive strategies to reduce EHEC colonization at the RAJ and consequent shedding of EHEC into the environment and transmission to humans.

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