The role of the QseC quorum-sensing sensor kinase in colonization and norepinephrine-enhanced motility of *Salmonella enterica* serovar Typhimurium

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Received 6 September 2007; received in revised form 30 September 2007; accepted 4 October 2007
Available online 12 October 2007

Abstract

Transcriptional analysis of *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) in the presence of the mammalian hormone norepinephrine revealed up-regulation of genes in the flagellar and chemotaxis regulon. Motility assays confirmed enhanced motility of wild-type *S*. Typhimurium in the presence of norepinephrine that could be blocked by the \(\alpha\)-adrenergic antagonist, phentolamine. Furthermore, a mutation in the *qseC* gene, encoding the sensor kinase of the two-component QseBC quorum-sensing system, also diminished motility of *S*. Typhimurium. To investigate the role of *S*. Typhimurium *qseC* \textit{in vivo}, 13-week old pigs were intranasally inoculated with equal concentrations \((1 \times 10^{9} \text{CFU})\) of wild-type *S*. Typhimurium and a *qseC* mutant. Over a 1-week competitive index experiment, the *qseC* mutant displayed decreased colonization of the gastrointestinal tract compared to the wild-type parent strain. Thus, this study has identified a role for the QseBC quorum-sensing signal transduction system in motility and swine colonization of *S*. Typhimurium. Cross-talk between cell–cell communication systems in *Salmonella* (quorum sensing) and host hormones may explain opportunistic behaviors of the pathogen, such as immune evasion and stress-induced recrudescence of *Salmonella*, during fluctuations of host hormone levels.

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\textit{Keywords:} Salmonella; Quorum sensing; Colonization; Swine; Motility

1. Introduction

The endocrine system in higher eukaryotes uses hormones as messengers for cellular communication within the body [1]. Therefore, distant cells can work in concert to respond to chemical signals and alter physiology. In an analogous manner, bacteria are able to communicate via cell-to-cell communication systems termed quorum sensing [2,3]. Quorum sensing utilizes small hormone-like molecules called autoinducers (AIs) to monitor the environment. As bacteria produce and release AIs, the signal molecules accumulate in the environment. Thus, the greater the cell density of a quorum-sensing bacterial species, the greater is the accumulation of the quorum-sensing signal in the environment. Accumulation of the quorum-sensing signal permits the bacterial cells to respond to the AI as a population instead of individual cells. Typically, the response to an AI involves the modulation of gene expression.

Multiple quorum-sensing systems, including the \textit{luxS}/AI-2 and AI-3 systems, are produced by \textit{Escherichia coli} (both commensal and pathogenic), \textit{Shigella} sp., \textit{Salmonella} sp., \textit{Klebsiella pneumoniae} and \textit{Enterobacter cloacae}, as well as microbial intestinal flora cultured from stools of healthy human volunteers [4,5]. It has been proposed that intestinal bacteria may utilize AI-2 and AI-3 for interspecies communication and may aid pathogenic bacteria by signaling the appropriate timing for expression of virulence determinants within the gastrointestinal tract [6].
Similar to AI-2 and AI-3 being produced by bacterial cells, epinephrine and norepinephrine are produced by the mammalian host for cellular communication. Epinephrine and norepinephrine are neurotransmitters released by the sympathetic nervous system. The gastrointestinal tract is highly innervated with the sympathetic nervous system controlling motility, secretion and vasoregulation [7–9]. Kaper and Sperandio [10] suggest that communication between bacteria and the host occurs through the quorum-sensing (bacteria) and neuroendocrine (host) systems. Thus, the presence of epinephrine/norepinephrine in the gastrointestinal tract, in addition to AI-3 (produced by the microbial flora), may serve as quorum-sensing signals to E. coli and Salmonella, alerting the pathogens of their preferred sites of colonization and invasion.

In E. coli, the QseBC two-component system modulates the quorum-sensing response to AI-3, epinephrine and norepinephrine [11]. QseC is a histidine sensor kinase and QseB is a response regulator that modulates motility in the presence of these quorum-sensing signals. In this study, the transcriptional induction of a large number of flagellar and chemotaxis genes in response to norepinephrine exposure prompted an investigation of the role of the QseC sensor kinase in motility and swine colonization of Salmonella enterica serovar Typhimurium (S. Typhimurium).

2. Materials and methods

2.1. Bacterial growth media

The S. Typhimurium strains used in this study were grown in the following media: Luria-Bertani (LB) broth, Dulbecco’s modified Eagle medium (DMEM) (31053, Invitrogen Life Technologies, Carlsbad, CA) or SAPI minimal medium containing 2.77 mM dextrose, 6.25 mM ammonium nitrate, 1.84 mM monobasic potassium phosphate, 3.35 mM potassium chloride, and 1.01 mM magnesium sulfate, 30% porcine serum and 10 mM Hepes buffer [12,13]. Norepinephrine was used at concentrations of 50 μM and 2 mM. Norepinephrine is photosensitive, readily oxidized in the presence of oxygen and its half-life is very short. Initial experiments (microarray and real-time RT-PCR) utilized a higher concentration of norepinephrine due to overlap with other research projects in the laboratory. However, the motility assays were performed with a lower concentration of norepinephrine for comparison of quorum-sensing phenotypes to other microorganisms that have been previously published by other investigators. Phenolamine was used at a concentration of 200 μM. Antibiotics were used at the concentrations of 100 μg/ml for ampicillin, 30 μg/ml for nalidixic acid, and 30 μg/ml for chloramphenicol. Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO).

Cultures were grown and assays performed at 37 °C. Pre-conditioned DMEM (i.e. spent medium) was produced by growing a culture of BSX 8 for approximately 24 h. The culture was centrifuged at 10,000 × g for 20 min at 4 °C and the pre-conditioned DMEM supernatant was filter-sterilized through a 0.2 μm filter.

2.2. Construction of the qseC mutant by recombineering

Recombineering was performed to construct a strain containing the qseC knockout by transformation of a linear PCR product containing the cat gene encoding chloramphenicol acetyltransferase. The oBBI 88 (5’ CGATAGCT-GAATGAGTGACGTAGCTACTATTTCCATGCAGTATC-CTTACG) and oBBI 89 primer (5’ GCATAGAGCAGTGACGTAGCTACTATTTCCATGCAGTATC-CTTACG) pair containing FRT sites (underlined) were used to amplify the cat gene from pACYC184 (GenBank accession no. X06403). The 5’ end of both oBBI 88 and 89 have stop codons in all three reading frames to truncate protein translation. A purified oBBI 88/89 cat PCR product was used as a PCR template to amplify the qseC knockout fragment using primers oBBI 96 (5’ CGTGCAAGGCTCTGCTACCCGGTGATGAATGAGTCGGAGC) and oBBI 97 (5’ CTCGTTACCAATTACTAGCAGCAGTCGGAGC). Primers oBBI 96 (upstream) and 97 (downstream) contain 45 and 44 bp of qseC (STM3178) nucleotide sequence, respectively (bold). Following PCR purification of oBBI 96/97 cat, the fragment was transformed into arabinoose-induced BSX 7 (TT22971 (LT2) containing pKD46) [14]. Transformants were plated to LB chloramphenicol to select for a qseC knockout strain. The qseC::cat construct was moved to BSX 8 (p4232) [15] by P22 phage transduction, and the resulting qseC mutant was named BBS 10. The gene knockout was confirmed by PCR amplification of the qseC region.

2.3. Motility assay

Strains were grown overnight in DMEM containing 10 μM FeCl3 at 37 °C, 180 rpm. Strains were diluted 1:50 in DMEM and incubated at 37 °C, 180 rpm. At ~7 h, bacteria were pelleted and resuspended in an equal volume of DMEM. A 1 μl aliquot of bacteria was spotted onto DMEM 0.3% motility medium containing 10 μM FeCl3 and incubated at 37 °C for ~16–18 h. Where indicated, the concentrations of norepinephrine and phenolamine used in the motility medium were 50 and 200 μM, respectively.

2.4. RNA extraction and analysis by DNA microarray and real-time RT-PCR assays

Total RNA was isolated using the RNeasy Midi kit and RNase-free DNase set (Qiagen, Valencia, CA) from S. Typhimurium BSX 8 and BBS 10 grown to mid-log phase (OD600 = 0.4) in SAPI serum medium containing 0 or 2 mM norepinephrine and frozen in RNAProtect Bacteria.
Reagent (Qiagen). Following RNA extraction from triplicate experiments, a second DNase I digestion was performed using the Turbo DNase-free kit (Ambion, Austin, TX). RNA integrity, quality and quantity were assessed using the Agilent Bioanalyzer 2100 and RNA Nano 6000 Labchip kit (Agilent Technologies, Palo Alto, CA).

For DNA microarray analysis, RNA was labeled with aminooethyl dUTP via first strand cDNA synthesis followed by aminoethyl coupling to either Alexa 555 or 647 fluorescent molecules (Invitrogen) according to Protocol M007 from the Pathogen Functional Genomics Resource Center at The Institute for Genomic Research (TIGR; http://pfgrc.tigr.org/protocols.shtml). Labeled cDNA was hybridized to Salmonella typhimurium/typhi DNA microarrays (version 2) developed and manufactured by TIGR (http://pfgrc.tigr.org/desc.shtml) using Protocol M008 (http://pfgrc.tigr.org/protocols.shtml). The ScanArray Express (Perkin-Elmer Life Sciences, Boston, MA) was used to scan the microarray slides and signal intensities were quantified using ScanArray Express 3.0 software. Background-corrected data were normalized using the Lowess function on a minimum of three biological replicates.

Real-time RT-PCR was performed using the Quantitect SYBR Green RT-PCR kit (Qiagen) and the Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primer sequences are given in Table 1. RT-PCR transcripts were amplified in duplicate from triplicate experiments with the following thermal cycling parameters: 3 min at 95°C, 40 cycles of 15 s at 95°C and a hold for 5 s at each temperature increment. Relative quantification of gene amplification by real-time RT-PCR was evaluated using the comparative CT method as described by Livak and Schmittgen [16]. The gyrase subunit B gene, gyrB, was used as the endogenous normalization control. The ΔCT values were calculated by determining the difference in threshold values for target and reference in each sample. Calculation of ΔΔCT involved the subtraction of no norepinephrine ΔCT value from ΔCT value of the 2 mM norepinephrine. Fold differences in gene expression of the target gene are presented as 2−ΔΔCT. Statistical analyses of the ΔCT values were analyzed by SAS Analyst (Cary, NC) using the two-sample t-test for the means. Results were considered significant when P<0.05.

2.5. IPEC-J2 invasion assays

IPEC J2 cells (passages 52–57) were derived from porcine jejunal epithelial cells and were a kind gift from Dr. Bruce Schultz (Department of Anatomy and Physiology, Kansas State University, Manhattan, KS). The cells were grown and maintained in 50% DMEM and 50% Nutrient Mixture F12 (1:1 DMEM/F12; Invitrogen Life Technologies), 5% fetal bovine serum (FBS), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenous acid, 5 ng/ml epidermal growth factor, 100 μg/ml streptomycin and 100 units/ml penicillin. The cells were seeded into 24-well cell culture plates (BD Falcon, BD Biosciences, San Jose, CA) and maintained in an atmosphere of 5% CO2 at 37°C for 2 days. The day of the experiment, cells were washed thrice with PBS and a day well of cells was used to determine cell density (~3 × 104 cells/well) using Trypan Blue staining and a hemocytometer.

BSX 8 and BBS 10 were grown stationary in DMEM/F12 medium devoid of antibiotics at 37°C for 18 h. Bacterial populations were estimated by spectrophotometry at 600 nm optical density and enumerated following serial dilution plating of each strain. Bacteria were

## Table 1

| Gene | Norepinephrine | Fold change | P-value | Primer sequences (5’–3’)
|------|----------------|-------------|---------|-----------------------------
|      | 0 mM           | 2 mM        |         |                             |
|      | Average CT     | S.D.        | Average CT | S.D.                        |
| flmA | 18.03          | 0.13        | 15.96    | 0.31                        | 3.6 <0.0001 CCGAGTATCGTCAGATGGTGTG TGTTGTTTCTTCACTACCAAG CGGTCGTTCACGCCCAAGTAG TTCGATGTATCCGGTTCTGATGT CACCAAGAAGGCACCAATAAG GAATGTTGGTTGCTTGAATTTG GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGCTATCCTGAAGG CCGTATAACCACTACTTGCAC GCATCTCGGGAAGATTTACG CGAACATGGC...
incubated with PBS-washed IPEC J2 cells (MOI = 200) in a final volume of 200 μl DMEM/F12 medium devoid of antibiotics for 2 h at 37 °C, 5% CO2. To remove extracellular bacteria, cells were washed thrice with PBS and incubated in medium containing 50 μg/ml of gentamicin for 2 h. To enumerate intracellular Salmonella, the cells were washed twice with PBS, and 100 μl of 1% Triton X-100 was added to each well for 15 min of incubation at 37 °C. Serial dilution plating was performed on each sample and plated on LB agar plates for overnight growth at 37 °C. The percent of bacteria that invaded the IPEC J2 cells was determined by dividing the number of intracellular bacteria by the number of bacteria in the inoculum and multiplying by 100. Statistical analysis was performed by SAS Analyst on four experiments each with three replicates using the two-sample t-test for the means. Results were considered significant when P < 0.05.

2.6. Competitive index (CI) animal experiment

Six conventionally raised male and female piglets from Salmonella spp. fecal-negative sows were weaned at 12 days of age, shipped to the National Animal Disease Center, Ames, IA, and raised in two groups of three in an isolation facility. Pigs were randomly assigned groups such that each group of three pigs was represented by a single offspring from three different sows. Bacteriologic culture of rectal swabs was performed multiple times to confirm that all pigs were negative for Salmonella spp. At 13 weeks of age (day 0), all six pigs received an intranasal inoculation of 1 ml PBS containing two S. Typhimurium strains, BSX 8 (1.2 × 10^9 CFU (colony-forming units)) and BBS 10 (1.3 × 10^9 CFU). Rectal temperatures and clinical symptoms (lethargy, loss of appetite and diarrhea) were recorded for each animal daily. Pig fecal samples were obtained on days 1–7 and 14 for quantitative and qualitative Salmonella culture analysis. The qseC mutant strain was differentiated from the wild-type (WT) strain based upon chloramphenicol resistance. On days 7 and 14, pigs (n = 3) were necropsied and six tissue samples (cecum, ileal Peyer’s Patch, ileocecal lymph nodes, tonsil, liver, and spleen) were placed on ice for quantitative and qualitative Salmonella culture. For calculation of the CI, the output ratio (qseC CFU/WT CFU) was divided by the input ratio (1.0833) of the mutant to the wild-type strain. To incorporate samples that only had qualitative data (samples that had too few Salmonella for quantitative detection) into the calculations for CI and P-value, the highest possible value was given for the level of detection. For example, the lowest possible value for quantitative detection was 50 CFU/g of feces and 20 CFU/g of tissue. Therefore, samples negative in the quantitative analysis but positive in the qualitative testing are represented by a “+” and were given the highest possible value for a quantitative negative/qualitative positive sample (50 CFU for fecal or 20 CFU for tissues) to determine CI and P-values. The level of detection for the qualitative assay was 1 CFU/g; therefore, quantitative negative/qualitative negative samples are represented by a “−” and were given the highest possible value of 1 CFU/g to determine CI and P-values. For statistical analysis, the CI data (output/input ratio) was analyzed by SAS Analyst using the one-sample t-test for a mean. Most of the samples at 14 day post-inoculation were only qualitatively positive; thus, only the data for Salmonella colonization of the tonsils at day 14 is presented, since CI and P-values could not be calculated for the other tissues. All liver and spleen tissue samples were negative for Salmonella.

2.7. Quantitative and qualitative Salmonella culture analysis

For quantitative bacteriology, 1 g of pig feces was combined with 5 ml PBS, vortexed and 100 μl directly plated to brilliant green agar with sulfadiazone (BGS; Difco, Detroit, MI) containing nalidixic acid, ferric ammonium citrate (0.8 g/l), and sodium thiosulfate (6.8 g/l) with and without chloramphenicol. For tissue samples, 1 g of each tissue was combined with 2 ml of PBS in a whirlpak bag, pounded with a mallet, and homogenized in a stomacher (Seward, Westbury, NY) for 1 min. One hundred microliters of the resulting solution was aliquoted onto BGS containing nalidixic acid, ferric ammonium citrate, and sodium thiosulfate with and without chloramphenicol. One hundred microliters of a 10-fold dilution of each fecal and tissue sample were also plated, and additional dilutions were performed when colony-forming units reached >300/plate. Following 24 h of incubation at 37 °C, black colonies were enumerated and a single colony from each plate was confirmed to be Salmonella by serogroup antisera agglutination (Beckton, Dickinson and Co., Sparks, MD). The total number of CFU for each quantitative tissue or fecal sample was calculated per gram of sample by obtaining the number of Salmonella per plate and multiplying by the dilution factor.

Qualitative bacteriology of Salmonella was performed as follows: 1 g (fecal) or 100 μl (homogenized tissue) samples were inoculated in 10 ml of GN-Hajna (GN; Difco) broth and tetraethionate (TET; VWR, Rutherford, NJ) broth for 24 and 48 h of growth at 37 °C, respectively. Following incubation, 100 μl of each culture was transferred to 10 ml Rappaport–Vassiliadis medium (RV; Difco) and incubated at 37 °C for 18 h. The cultures were streaked on BGS containing nalidixic acid, ferric ammonium citrate, and sodium thiosulfate with and without chloramphenicol. Colonies suspicious for Salmonella were confirmed by serogroup antisera agglutination.

3. Results

3.1. S. Typhimurium motility genes are up-regulated during exposure to norepinephrine

To investigate the transcriptional response of S. Typhimurium to norepinephrine exposure, DNA microarray analysis was performed using total RNA isolated from
S. Typhimurium grown in SAPI serum medium with and without 2 mM norepinephrine. A number of Salmonella genes in the flagellar and chemotaxis regulon were up-regulated in the norepinephrine-exposed Salmonella culture (data not shown). Since flagellar assembly is dictated by a hierarchy of flagellar gene transcription [17], real-time RT-PCR was employed to measure the gene expression of early, middle, and late genes of the flagellar assembly cascade during exposure to norepinephrine (Table 1). Similar to the microarray data, real-time RT-PCR revealed up-regulation of the \textit{fliA} and \textit{fliY} (middle genes) as well as \textit{fljB} (late gene). The “early” \textit{flhC} gene of the master flagellar \textit{flhDC} operon was also transcriptionally activated.

3.2. Enhanced motility of Salmonella during norepinephrine exposure

To confirm the up-regulation of motility genes in response to norepinephrine, a phenotypic assay for motility was performed. S. Typhimurium was spotted on DMEM motility medium with and without 50 \(\mu\)M norepinephrine. As shown in Fig. 1, norepinephrine present in the medium enhanced the motility of S. Typhimurium. Phentolamine, an \(\alpha\)-adrenergic antagonist, eliminated the norepinephrine-enhanced motility of S. Typhimurium, indicating that Salmonella has an adrenergic receptor that responds to norepinephrine similar to the epinephrine receptor (QseC) described in \textit{E. coli} [18]. In addition to norepinephrine, the motility of S. Typhimurium was enhanced on DMEM motility medium containing 10\% pre-conditioned DMEM from the wild-type strain, indicating that an AI is present in the pre-conditioned medium (data not shown).

3.3. The QseC sensor kinase regulates S. Typhimurium motility

The gene “cascade” for motility is regulated by the master regulators, FlhDC [17]. Since the \textit{flhDC} genes are regulated by the two-component system QseBC, a \textit{qseC} mutant of S. Typhimurium was constructed to investigate the role of the QseC sensor kinase in norepinephrine-enhanced motility of S. Typhimurium. A dramatic decrease in motility zone diameter was observed for the \textit{qseC} mutant compared to the wild-type parent strain in the presence of norepinephrine, suggesting that the \textit{qseBC} quorum-sensing system is required for optimal induction of motility in response to norepinephrine (Fig. 2). Monitoring of bacterial growth by optical density revealed similar growth rates for the \textit{qseC} mutant and wild-type strains (data not shown).

3.4. QseC is involved in S. Typhimurium colonization of swine

In addition to transcriptional regulation of flagellar assembly, the QseBC quorum-sensing system also regulates the expression of virulence genes and is important for \textit{E. coli} pathogenicity in a rabbit model [18]. Since enteric...
pathogens may recognize the in vivo conditions of the host gastrointestinal tract via quorum-sensing systems to thereby activate motility and virulence gene expression, an in vivo competition assay was performed to determine if the S. Typhimurium qseC gene is important for swine colonization. Fecal samples on days 1–7 post-inoculation revealed a significant decrease in shedding of the qseC mutant compared to the wild-type strain (Table 2).

Furthermore, fewer colony-forming units of the qseC mutant than the wild-type strain were recovered from the tonsils, ileal Peyer’s Patches, ileocecal lymph nodes, and cecum at 7 days post-inoculation (dpi) (Table 3). A significant decline in tonsil colonization by the qseC mutant was also observed at 14 dpi (CI = 0.154, P-value = 0.023). Since the majority of the colonization data for the ileal Peyer’s Patches, ileocecal lymph nodes, and cecum at 14 dpi was qualitative (+/−), the CI and P-value could not be determined for the Salmonella strains in these organs. Thus, the competitive disadvantage of the qseC mutant in vivo indicates an important role for the quorum-sensing sensor kinase, QseC, in swine colonization. To determine if the qseC gene is important for host cell invasion, an in vitro invasion assay using the porcine intestinal epithelial cell line IPEC J2 was conducted. A significant (P < 0.05) four-fold decrease in bacterial invasion of the IPEC J2 cells by the qseC mutant (0.19% invasion) compared to the parent strain (0.77% invasion) was observed.

4. Discussion

Mammalian cells communicate through hormones, and in a similar manner, bacteria produce hormone-like
molecules for cell-to-cell communication termed quorum sensing. Cross-talk between these cell communication molecules may occur when bacteria reside in a mammalian host [4], suggesting that host hormones such as norepi-
molecules may occur when bacteria reside in a mammalian sensing. Cross-talk between these cell communication molecules for cell-to-cell communication termed quorum

In our study, real-time RT-PCR revealed significant flagellar assembly via the synthesis of the external filament. and

and fliA

and late (fliB) genes. An alternative reason for the low level of differential expression within the motility genes is that the qseC mutant has decreased motility compared to the wild-type strain. Our interest in the norepinephrine-enhanced motility of S. Typhimurium was initiated by the identification by DNA microarray analysis of a large number of motility genes (>20) transcriptionally activated in the presence of norepinephrine.

Motility genes are expressed in a cascade with “early, middle, and late” genes expressed in sequential order [17]. The early genes, flhDC, encode the FlhDC master regulator complex which dictates the σ70-dependent transcription of the middle genes. Expression of the middle genes results in the assembly of the flagellar motor intermediate structure known as the hook-basal body. The fliA gene is a middle gene that encodes the alternate sigma factor, FliA (σ28), required for transcription of the late genes that complete flagellar assembly via the synthesis of the external filament. In our study, real-time RT-PCR revealed significant transcriptional activation of the early flhC gene and middle fliA and fliY genes in response to norepinephrine exposure. The most highly expressed motility gene in our microarray analysis was the late gene fljB, and real-time RT-PCR confirmed a 15-fold increase in gene expression during norepinephrine exposure. However, most of the differential expression of the motility genes observed in the microarray assays was not dramatic between the two conditions tested (+/− norepinephrine). Several explanations may account for the low level of differential expression within the motility genes. The regulatory cascade of motility genes results in signal amplification at each step; hence, when the late genes are expressed, their expression is greater compared to early and middle genes. Our quantitative RT-PCR data illustrated signal amplification with the lowest to highest fold transcriptional induction observed in the sequential order of early (flhC), middle (fliA and fliY), and late (fliB) genes. An alternative reason for the low level of differential expression of the motility genes is that during bacterial growth, AI-3 is presumably synthesized and secreted into the medium. The presence of AI-3 in the medium may induce expression of motility genes even in the absence of norepinephrine and, therefore, diminish the differential gene expression measured in the presence vs. absence of norepinephrine. The presence of 5% or 10% pre-conditioned medium enhances motility of S. Typhimurium on DMEM motility medium, indicating that even when diluted, the level of AI-3 is sufficient to effect gene expression. Further yet, motility genes may be maximally expressed on semi-solid medium (motility assays) compared to a broth culture (microarray assays), since the flagellum appears to be able to sense wetness on a surface and affect gene expression via the transcription inhibitor, FlgM [26]. However, in their investigation of gene expression during swarming, Wang et al. [27] were unable to measure a swarming-specific induction of flagellar gene expression on swarm medium. Lastly, our gene expression analysis did not measure post-transcriptional mechanisms that may regulate motility.

Several reports have correlated motility with virulence gene expression in Salmonella [28–31]. Our in vivo competition experiment comparing the colonization potential of the qseC mutant to the wild-type parent strain in swine indicates that the competitive fitness of the qseC mutant is not as robust as the wild-type strain. Furthermore, an in vitro invasion assay with the porcine intestinal epithelial cell line IPEC J2 suggests an invasion deficiency for the qseC mutant. Hence, we have demonstrated multiple phenotypes associated with the qseC gene: motility, cellular invasion, and in vivo competitive fitness. Whether the decrease in swine colonization by the qseC mutant is due to impaired motility, invasion or another yet unidentified colonization/virulence phenotype is currently unknown. Although this investigation identified a colonization deficiency for the qseC mutant, additional animal experiments need to be performed to determine whether a qseC mutant is attenuated for virulence in the swine model of infection. Nonetheless, the competition experiment suggests that sensing AI-3/epinephrine/norepinephrine in the swine gastrointestinal tract environment is important for optimal colonization.

The ability of Salmonella to sense and respond to norepinephrine produced by the host has potential food safety consequences. Salmonella has a ubiquitous distribution in animals, including pigs. The on-farm prevalence of a pig shedding Salmonella in feces is 6–8% [32,33]. Although pigs colonized with Salmonella can exhibit clinical symptoms, they are usually asymptomatic carriers of the pathogen. Salmonella-carrier pigs are a serious food safety issue because they have the potential to shed the pathogen in their feces, contaminating their neighboring penmates and the environment. A possible result of in vivo exposure of Salmonella to norepinephrine (produced by stressed pigs during transportation and marketing) is recrudescence from carrier pigs and shedding during lairage, resulting in pen contamination and exposure of concurrent and subsequent pigs to the pathogen immediately prior to entering the processing plant. Hence, a scenario could be envisioned whereby Salmonella are shed in the environment by stressed carrier pigs; the Salmonella, expressing their virulence genes (due to norepinephrine
exposure), are ingested by stressed, Salmonella-free pigs that have decreased peristaltic activity of the gastrointestinal tract (due to stress-induced norepinephrine release), resulting in the “primed” pathogens being retained in their preferred site of invasion. The α-adrenergic antagonist, phentolamine, eliminated the norepinephrine-enhanced motility of the wild-type strain. Thus, a potential strategy for Salmonella intervention is to disrupt colonization via quenching of the quorum signal, thereby inhibiting quorum sensing.

Acknowledgments

The authors are thankful to Ann Marie Jensen, Kellie Winter, Jolita Utche, Emily Morrison, Greg Filer and David Meek for their expert technical assistance and to the Pathogen Functional Genomic Resource Center at The Institute for Genomic Research for microarray slides and protocols. This research was supported by USDA, ARS CRIS funds. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendations or endorsement by the U.S. Department of Agriculture.

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