SdiA, an \textit{N}-Acylhomoserine Lactone Receptor, Becomes Active during the Transit of \textit{Salmonella enterica} through the Gastrointestinal Tract of Turtles

Jenee N. Smith\textsuperscript{1*}, Jessica L. Dyszel\textsuperscript{1*}, Jitesh A. Soares\textsuperscript{1}, Craig D. Ellermeier\textsuperscript{2a}, Craig Altier\textsuperscript{3b}, Sara D. Lawhon\textsuperscript{4}, L. Garry Adams\textsuperscript{4}, Vjolca Konjufca\textsuperscript{5d}, Roy Curtiss III\textsuperscript{5c}, James M. Slauch\textsuperscript{2}, Brian M. M. Ahmer\textsuperscript{1*}

\textsuperscript{1} Department of Microbiology, The Ohio State University, Columbus, Ohio, United States of America, \textsuperscript{2} Department of Microbiology and College of Medicine, University of Illinois, Urbana, Illinois, United States of America, \textsuperscript{3} Department of Population Health and Pathobiology, North Carolina State University, Raleigh, North Carolina, United States of America, \textsuperscript{4} Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, Texas, United States of America, \textsuperscript{5} Department of Biology, Washington University, St. Louis, Missouri, United States of America

Abstract

\textbf{Background:} LuxR-type transcription factors are typically used by bacteria to determine the population density of their own species by detecting N-acylhomoserine lactones (AHLs). However, while \textit{Escherichia} and \textit{Salmonella} encode a LuxR-type AHL receptor, SdiA, they cannot synthesize AHLs. In vitro, it is known that SdiA can detect AHLs produced by other bacterial species.

\textbf{Methodology/Principal Findings:} In this report, we tested the hypothesis that SdiA detects the AHL-production of other bacterial species within the animal host. SdiA did not detect AHLs during the transit of \textit{Salmonella} through the gastrointestinal tract of a guinea pig, a rabbit, a cow, 5 mice, 6 pigs, or 12 chickens. However, SdiA was activated during the transit of \textit{Salmonella} through turtles. All turtles examined were colonized by the AHL-producing species \textit{Aeromonas hydrophila}.

\textbf{Conclusions/Significance:} We conclude that the normal gastrointestinal microbiota of most animal species do not produce AHLs of the correct type, in an appropriate location, or in sufficient quantities to activate SdiA. However, the results obtained with turtles represent the first demonstration of SdiA activity in animals.

Introduction

The functions of more than half of the genes present in \textit{Escherichia coli} and \textit{Salmonella enterica} serovar Typhimurium (hereafter referred to as S. Typhimurium) have never been determined, despite decades of genetic screens and selections. This failure is hypothesized to result from a lack of gene expression or gene function in the laboratory environment. A major component of the natural environment missing from most laboratory experiments is the presence of other microbial species. \textit{E. coli} and S. Typhimurium colonize and propagate in the intestines of animals in the presence of large numbers of other microbial species. We have previously identified genes of S. Typhimurium that are expressed specifically in the presence of signaling molecules produced by other microbes \cite{1-4}. These seven genes (two loci) are activated by SdiA, a member of the LuxR family of transcription factors, in response to \textit{N}-acylhomoserine lactones (AHLs) produced by other species.

The use of LuxR homologs to detect the AHL production of other species is unusual. The typical function of LuxR homologs is “quorum sensing”, a phenomenon by which bacteria sense their own population density \cite{5-7}. Many Gram-negative bacteria use this information to regulate the production of host colonization factors. For instance, \textit{Vibrio fischeri} uses this information to regulate genes that play a role in colonization of the squid, \textit{Euprymna scolopes} \cite{8-10}. Numerous plant and animal pathogens use quorum sensing to regulate host interaction genes \cite{11,12}. Presumably, this prevents the bacteria from alerting host immune responses before a population sufficient to overcome host defenses has been assembled.
AHLs are synthesized using enzymes of the LuxI or LuxM family [13–18]. AHLs vary widely in the length of their acyl chain (from four to 10 carbons) and can be modified at the 3-carbon position to have a carboxyl or hydroxyl group. Each LuxI homolog produces predominantly a single AHL variant, along with smaller quantities of closely related variants [19]. The LuxR family member of a species can detect nanomolar concentrations of the AHL produced by that species, providing some specificity to the system. However, LuxR homologs can often detect other related AHLs with lower sensitivity [20].

Salmonella encodes a LuxR homolog, SdiA, but does not encode an AHL synthase [3,4]. Instead, SdiA responds to AHLs produced by other microbial species [1,2]. SdiA detects a wider range of AHLs than other LuxR homologs, although with varying sensitivities. The most potent AHL is 3-oxo-3-octanoyl-homoserine lactone (oxoC8) and SdiA responds to this molecule at concentrations of 1 nM or higher [3,21]. The closely related AHL, 3-oxo-hexanoyl-homoserine lactone (oxoC6) can be detected at concentrations above 5 nM, and at 50 nM SdiA responds to oxoC4, oxoC10, oxoC12, and the unmodified AHLs C6 and C8. At concentrations above 1 µM SdiA responds to C4, C10, and C12 [3,21]. The three-dimensional structure of the N-terminus of SdiA bound to C8 has been determined using NMR, confirming that detection of AHLs by SdiA is direct [22].

SdiA activates the expression of two srg (SdiA regulated gene) loci: the rck operon located on the S. Typhimurium virulence plasmid, pSLT, and the srgE gene located in the chromosome at 33.6 centimorgans [1,2]. The rck operon is directly downstream of the pef operon that encodes plasmid-encoded fimbriae [23]. These fimbriae play a role in adhesion to the small intestine of mice [24]. The rck operon contains six genes: pefI, srgD, srgA, srgB, rck, and srgC [3,23]. PefI is a regulator of the upstream pef operon [25]. SrgD is a putative transcription factor with a helix-turn-helix motif of the LuxR family, but its target genes have not been identified [3,23]. SrgA is a DsbA homolog that catalyzes disulfide bond formation of the Pef fimbrial subunit and other periplasmic proteins [26,27]. SrgB is a lipoprotein of unknown function [23]. Rck is an β-stranded β-barrel protein localized to the outer membrane [29,28]. This protein has dual functions. The first function provided its name: resistance to complement killing [29,30]. More specifically, Rck prevents the polymerization of complement component C9 on the bacterial surface [31]. Second, Rck serves as an adhesin to fibronectin and laminin [32]. The last gene in the rck operon, srgC, is a regulator of the AraC-family of transcription factors whose target genes are unknown [23]. The second srg locus consists of a single gene, srgE, which is predicted to encode a protein containing a coiled-coil domain [24,25]. As with the rck operon, srgE is not present in E. coli and was probably acquired horizontally by Salmonella. Despite the regulation of putative virulence genes by SdiA, we have previously reported that an sdiA mutant of S. Typhimurium is not attenuated in mouse, chicken, or cow models of infection [1].

The mammalian intestinal community is comprised of approximately 800 microbial species [33]. We hypothesized that members of this community utilize AHL-type quorum sensing and that the presence of these AHLs would provide an effective way for S. Typhimurium to detect the intestinal environment [1,4]. In this report we have tested this hypothesis. S. Typhimurium did not detect AHLs during transit through the intestinal tract of several species of animals, indicating that the normal microbiota of these hosts did not produce AHLs of the correct type or at a sufficient concentration to activate SdiA. However, SdiA was active during transit through turtles colonized by the AHL-producing organism, Aeromonas hydrophila.

**Results**

**Construction and testing of RIVET strains**

To test the hypothesis that Salmonella uses SdiA to detect the AHL production of other microorganisms within the gastrointestinal tract, we used the RIVET method (Recombination-based In Vivo Expression Technology) to record SdiA activity in vivo [34–37]. We constructed a srgE-tnpR-lacZ fusion in a strain that carries a res1-tnpR::res1 cassette elsewhere in the chromosome (strains are listed in Table 1). The tnpR-lacZ fusion was placed immediately after the srgE stop codon so that srgE remains functional. The tnpR gene encodes a resolvase that catalyzes a site-specific recombination event at res1 target sequences. Thus any condition or event that induces expression of srgE-tnpR-lacZ is permanently recorded in the genome by the deletion of the tnpR::res1 cassette. The deletion event is detected by screening the bacteria for a loss of tetracycline resistance.

To obtain the optimal fusion sensitivity for the srgE promoter three versions of this strain were constructed, each with a different tnpR ribosome binding site [38]. Once these strains were constructed, an sdiA::mTn3 mutation was transduced into each of them using phage P22HTint to create isogenic sdiA mutant controls. We have previously reported that even in the presence of AHLS, SdiA is not active when Salmonella is grown on LB agar plates [2]. However, SdiA is slightly active when grown in liquid broth, and is highly active when grown in motility agar [2]. Therefore, the functionality of the RIVET strains was tested under these same conditions. Either synthetic AHL (1 µM oxoC6) or the solvent control, acidified ethyl acetate (EA), was added to each medium. After overnight growth in liquid medium at 37 °C, serial dilutions were plated on agar plates to obtain individual colonies. Bacteria were recovered from motility agar by stabs with a sterile toothpick and streaking to isolation. The resulting colonies were then screened for loss of tetracycline resistance. The strains containing mutated tnpR ribosome binding sites showed no resolution under any condition and were not used further. The strains containing the wild-type tnpR ribosome binding site showed resolution and are the strains used for the remainder of the experiments in this report (JNS3206 is sdiA and JNS3226 is sdiA::mTn3, hereafter referred to as the RIVET strains). In media containing AHL, resolution was observed in 23% of wild-type bacteria recovered from motility agar, 2.5% of wild-type bacteria recovered from broth, and from 0.6% of bacteria grown on solid agar. No resolution was observed under any growth condition in the absence of AHL or from sdiA mutant colonies. The reason that only 23% of colonies resolve after overnight growth in vitro is not known, but indicates that the srgE gene is not highly expressed, or that truly optimal conditions have not yet been discovered. Regardless, resolution in the sdiA+ bacteria but not the sdiA mutant bacteria indicates SdiA activity. The observation that the highest resolution was observed after growth in motility agar is similar to what was observed previously using a lacZ fusion to srgE [2]. Therefore, expression of the srgE-tnpR-lacZ fusion can be recorded as a percentage of tetracycline susceptible colonies and this expression is dependent upon sdiA and AHL.

SdiA is not active during the transit of Salmonella through mice, chickens, pigs, a rabbit, a guinea pig, or a cow

To determine if SdiA is active during the transit of Salmonella through the gastrointestinal tract, the wild-type and sdiA mutant RIVET strains were mixed together in a 1:1 ratio and orally administered to six CBA/J mice. These mice are resistant to Salmonella infection and provide a long term model of intestinal
Salmonella and their black color on the XLD indicator plates and their sensitivity to sdiA colonization and persistence [39]. Inoculating a mixture of the RIVET strains. These colonies were confirmed to be indicator plates containing kanamycin to recover the Fecal samples were collected, homogenized and plated on XLD activity of these two strains within the gut of the same animal. The recovered colonies were then screened for sdiA1 are distinguished by the ampicillin sensitivity of the colony (the sdiA mutant allows a comparison of the resolvase activity of wild-type and sdiA mutants during transit through the mice (Table 2). Consistent with the lack of SdiA activity, both the wild-type and sdiA mutant were recovered in ratios that were not significantly different from the inoculated ratio indicating that the sdiA mutation did not confer a fitness phenotype in these mice (Table 2 and Figure 1). The lack of SdiA activity in the mouse gut was surprising, but colonization and persistence [39]. Inoculating a mixture of the wild-type and sdiA mutant allows a comparison of the resolvase activity of these two strains within the gut of the same animal. Fecal samples were collected, homogenized and plated on XLD indicator plates containing kanamycin to recover the Salmonella RIVET strains. These colonies were confirmed to be Salmonella by their black color on the XLD indicator plates and their sensitivity to ampicillin and tetracycline resistance. Wild-type and sdiA mutant are distinguished by the ampicillin sensitivity of the colony (the sdiA mutation encodes ampicillin resistance) and resolvase activity is detected by the loss of tetracycline resistance. Sufficient numbers of bacteria for screening were recovered from five of the six mice. Only one of 717 colonies recovered from the five mice was tetracycline susceptible, indicating that SdiA was not active during transit through the mice (Table 2). Consistent with the lack of SdiA activity, both the wild-type and sdiA mutant were recovered in ratios that were not significantly different from the ratio inoculated indicating that the sdiA mutation did not confer a fitness phenotype in these mice (Table 2 and Figure 1). The lack of SdiA activity in the mouse gut was surprising, but since compounds that activate AHL biosensor strains have been chemically extracted from the bovine rumen [40], we hypothesized that the presence of AHLs might differ among animal species. Therefore, the RIVET strains were orally administered to cows, pigs, rabbits, guinea pigs, and chickens, as described in the Materials and Methods. For some individual animals, we failed to recover the RIVET strains from their feces. However, the RIVET strains were recovered from the feces of one rabbit (of two), one guinea pig (of four), one cow (of three), six pigs (of six), and 12 chickens (of 12). No SdiA activity was observed during the transit of Salmonella through any of these animals as indicated by the fact that all recovered RIVET strains were tetracycline resistant (Table 2). Consistent with the lack of SdiA activity, the sdiA mutant did not appear to have a significant fitness defect during the transit through any of the animals (Table 2 and Figure 1). It should be noted that the sdiA mutant actually fared slightly better than the wild-type during transit through chicks. The sdiA+/sdiA− ratio was 57:43 at inoculation while it was 48:52 at recovery. This is a competitive index of 1.4. While this result was statistically significant, competitive indices of even 3 to 5 are considered minimal.

SdiA is active during the transit of Salmonella through turtles

Having determined that Salmonella did not detect AHLs in the gastrointestinal tracts of the birds and mammals tested above, we decided to test a reptile that is commonly associated with Salmonella, the turtle. Preliminary experiments indicated that SdiA was indeed activated during transit through turtles (not shown). To determine if AHL-producing bacteria could be recovered from turtles, cloacal swab samples were obtained from a group of eight turtles and the recovered material was plated on several media in order to culture a wide variety of bacterial species (LB, blood agar, XLD, lactose MacConkey). The recovered colonies were then tested in a cross-streak assay against the bioluminescent SdiA activity reporter strains 14028/pJNS25 (sdiA− srgE-luxCDABE) and BA612/pJNS25 (sdiA− srgE-luxCDABE) [2]. Luminescence of the wild-type reporter but not the sdiA mutant at the junction indicates SdiA activity [2]. Bacteria that can activate SdiA in the cross-streak assay were found in each of the eight turtles (Figure 2). Eighteen isolates were submitted to the Ohio State University medical center for automated identification by a Dade Microscan WalkAway 96s. All 18 isolates were found to be Aeromonas hydrophila.

After determining that all eight of the turtles contained Aeromonas hydrophila, the Salmonella RIVET strains were orally administered to these turtles and cloacal swab samples were collected over time. When the recovered colonies from all turtles at all time points were screened, it was observed that the ratio of wild-type to sdiA mutant recovered was changed during transit

Table 1. Strains and plasmids used.

| Strain or plasmid | Genotype | Source or reference |
|-------------------|----------|---------------------|
| Strains           |          |                     |
| 14028             | Wild type Salmonella enterica serovar Typhimurium | American Type Culture Collection |
| BA612             | 14028 sdiA::mTn3 (amp′) | [4] |
| JNS3206           | J246 srgE10-tnpR-lacZY (kan′) | This study |
| JNS3226           | J246 srgE10-tnpR-lacZY sdiA1::mTn3 (kan′ amp′) | This study |
| J5198             | LT2 metE55 metA22 tnpR-lacZY sdiA1::mTn3 (kan′ amp′) | [56] |
| J5246             | 14028 srgE10-tnpR-lacZY (kan′ res1-tetRA-res1) | [37] |
| S17-1::pir        | 14028 srgE10-tnpR-lacZY sdiA1::mTn3 (kan′ amp′) | [60] |
| W3110             | E. coli K-12 F′ IN(mrD-rmtE)1 | [61] |
| Plasmids          |          |                     |
| pCE71             | FRT-tnpR-lacZY tnpR oriR6K (kan′), Contains wild type tnpR Shine Dalgarno. FRT orientation B. | [37] |
| pCE73             | FRT-tnpR-lacZY tnpR oriR6K (kan′), Contains mutated tnpR Shine Dalgarno mut168. FRT orientation B. | [37] |
| pCE75             | FRT-tnpR-lacZY tnpR oriR6K (kan′), Contains mutated tnpR Shine Dalgarno mut135. FRT orientation B. | [37] |
| pCP20             | cI857 λPy flp pSC101 oriTS (amp′ cam′) | [58] |
| pJNS25            | pJNS25 luxCDABE p15A ori (tet′) | [2] |
| pKD4              | FRT-kan-FRT oriR6K (amp′) | [57] |
| pKD46             | pBAD gam bet exo pSC101 oriTS (amp′) | [57] |

doi:10.1371/journal.pone.0002826.t001
through the turtles (a 49:51 ratio inoculated, a 56:44 ratio recovered, Table 2 and Figure 1). However, while statistically significant, this is only a 1.3-fold difference in competitive index that is not likely to be biologically relevant, especially when looking at the time course plotted in Figure 1B. Therefore, we do not feel that the sdiA mutant of Salmonella serovar Typhimurium has a significant fitness phenotype in turtles. However, the striking result is that the resolution observed in wild-type Salmonella was greater than in the sdiA mutant, indicating that SdiA was active during the transit of Salmonella through these turtles (Table 2 and Figure 3). This is the first observation of SdiA activity in vivo.

Table 2. SdiA activity and fitness during transit through different animal species.

| Number and type of animal | Percent wild-type inoculated | Total colonies screened | Percent wild-type recovered | Competitive Index | Mean of the log competitive index +/- Std Dev | Total colonies resolved | Percent of wild-types resolved | Percent of sdiA mutants resolved |
|---------------------------|-----------------------------|------------------------|-----------------------------|------------------|---------------------------------------------|------------------------|-----------------------------|-------------------------------|
| 5 mice                    | 52%                         | 717                    | 72%                         | 0.41             | −0.541+/−0.645                              | 1                     | 0.14%                       | 0%                            |
| 1 guinea pig              | 45%                         | 621                    | 54%                         | 0.70             | −0.155                                      | 0                     | 0%                          | 0%                            |
| 1 rabbit                  | 45%                         | 709                    | 50%                         | 0.82             | −0.086                                      | 0                     | 0%                          | 0%                            |
| 6 pigs                    | 45%                         | 1349                   | 53%                         | 0.73             | −0.105+/−0.176                              | 0                     | 0%                          | 0%                            |
| 1 calf                     | 89%                         | 1000                   | 96%                         | 0.34             | −0.469                                      | 0                     | 0%                          | 0%                            |
| 12 chicks                  | 57%                         | 2454                   | 48%                         | 1.44             | 0.176+/−0.150                               | 0                     | 0%                          | 0%                            |
| 8 turtles                  | 49%                         | 6793                   | 56%                         | 0.75             | −0.078+/−0.068                               | 715                   | 25.9%                       | 3.0%                          |

1Animals were inoculated with a 1:1 mixture of the wild-type and sdiA mutant Salmonella RIVET strains (a total of 9.7×10⁸ cfu for the mice, 6.0×10⁸ cfu for the guinea pigs and rabbits, 1.8×10⁹ cfu for the pigs, 1.3×10¹⁰ cfu for the calves, 7.0×10⁸ cfu for the chicks, and 3.1×10⁸ cfu for turtles).

2The inoculum was plated for single colonies and screened for ampicillin resistance to determine the actual percent wild-type inoculated.

3The “Total colonies screened” represents a compilation of all colonies recovered (sum of all time points) from all animals of each species. The colonies were recovered by plating fecal samples, unless noted otherwise, on XLD-kan and then screened for ampicillin and tetracycline resistance. Wild-type and sdiA mutant Salmonella are differentiated by ampicillin resistance and resolution of the resI-terRċ-res1 cassette is indicated by tetracycline sensitivity.

4The competitive index equals the output ratio (cfu of mutant/cfu of wild type) divided by the input ratio (cfu of mutant/cfu of wild type). A competitive index of 1.0 indicates that the wild-type and sdiA mutant had equal fitness during transit through the animal. The log of the competitive index represents a normal distribution so the mean of this value was calculated. A value of zero indicates that the wild-type and sdiA mutant had equal fitness during transit through the animal. For those animal species in which colonies were recovered from more than one animal, the standard deviation is shown. The wild-type was statistically more fit than the sdiA mutant in turtles, while the sdiA mutant had more fit than wild-type in chicks (student’s t test, p < 0.05). However, the differences are so small (competitive index of less than 3) that they are not likely to be biologically significant. For those animal species where only one individual shed colonies, the standard deviation could not be calculated, but a competitive index that is less than 3-fold different than 1.0 is unlikely to be biologically significant (0.33 to 3.0).

5All colonies from the mice were obtained from fecal samples. No colonies were from fecal samples.

6All colonies from the pigs were obtained from cloacal swabs. These data were also used to generate Figures 1 and 3.

7All colonies from the chicks were obtained from the cecum and distal large intestine. No colonies were from fecal samples.

8All colonies from the turtles were obtained from cloacal swabs. These data were also used to generate Figures 1 and 3.

9Statistically significant fitness difference between wild-type and sdiA mutant (p < 0.05).

To test the hypothesis that *Aeromonas hydrophila* is indeed the organism responsible for activation of SdiA during the transit of *Salmonella* through turtles, we hoped to obtain a group of turtles that lacked AHL-producing bacteria. Half of these turtles would then be inoculated with *Aeromonas hydrophila* and half would be inoculated with LB prior to performing the RIVET experiment. Unfortunately, the next two groups of turtles that we obtained (one group of 12 and another group of 17) all had *Aeromonas* in their feces. Next, we reasoned that newly hatched turtles would be less likely to have picked up *Aeromonas* from the environment and we would have a better chance of obtaining *Aeromonas*-free animals if

Figure 1. Fitness of the sdiA mutant in different animal species. (A) The log of the competitive index for each individual animal is plotted. The mean is indicated by the large horizontal line while the 95% confidence intervals are indicated by the smaller horizontal lines. A value of zero indicates that the wild-type and the sdiA mutant were of equal fitness during transit through the animal. (*) The wild-type was statistically more fit than the sdiA mutant during transit through turtles, while the sdiA mutant was statistically more fit than the wild-type during transit through chicks (p < 0.05). (B) Fitness of the sdiA mutant in turtles over time. The data from (A) is separated into time points. (*) The wild-type was statistically more fit than the sdiA mutant on day 7 (p < 0.05).
we ordered hatchlings. A group of 20 hatchlings was obtained and, unfortunately, all of these animals were colonized with *Aeromonas*. In a final attempt at obtaining *Aeromonas*-free turtles, we ordered 30 turtle eggs and hatched them under sterile conditions. More specifically, we placed the eggs on sterile vermiculite until hatching, and then placed them in sterile cages containing sterile water. The hatchlings were fed standard non-sterile turtle food to promote the development of a normal microbiota (the food was tested and shown to be free of *Aeromonas*). Remarkably, all of the hatchlings were found to have *Aeromonas* in their feces. We are not sure if the *Aeromonas* is transmitted from the mother to the embryo during the development of the egg, or if there was *Aeromonas* on the egg shell, or some other source of environmental contamination, but we were not able to obtain turtles that lacked *Aeromonas*.

Next, we attempted to clear the *Aeromonas* from adult turtles using Amikacin as described in the Materials and Methods. This was not successful. *Aeromonas* numbers would drop temporarily but the organism was never completely eradicated. Therefore, we can only conclude that SdiA was indeed activated during the transit of *S.* Typhimurium through turtles, and that the probable source of the AHLs was *Aeromonas hydrophila*.

**Discussion**

We have determined that SdiA of *S.* Typhimurium is activated in turtles, but not in mice, pigs, chickens, a cow, a guinea pig, and a rabbit. It appears that the normal microbiota of these particular animals did not produce AHLs, at least not in a location or at a concentration sufficient to activate SdiA. The lack of SdiA activity during the transit of *Salmonella* through the cow was particularly surprising because there is a report of chemical extraction of AHLs from the rumens of six of eight bovines [40]. It is certainly possible that AHLs are a normal component of the bovine rumen and certain other gastrointestinal regions of particular animals. We may not have observed SdiA activity in our bovine experiment (or other experiments) due to the age or the diet of the animals. Our calves were young, only three to four months old. While the animals have a functioning rumen at this point, AHL-producing species may not be part of the ecosystem at this age. The ages of the animals in the Erickson study were not reported. Diet may also play a role. Our calves were fed free choice grass and hay and a pelleted feed that was 12% protein and 3% fat that is primarily comprised of corn and cottonseed meal. The Erickson study used four different combinations of alfalfa hay and barley, but did not observe a statistically significant correlation between diet and AHLs. Further study will be required to determine the circumstances in which AHL production occurs in the bovine rumen.
It is possible that AHLs were indeed synthesized by the normal microbiota of some of the animals in this study, but that the AHLs were rapidly degraded by either host enzymes or enzymes produced by other microbes. While AHL-degrading enzymes have not been reported in the intestine, they have been found in soil and in the termite hindgut, and it would not be surprising to find them in the intestine [41–43]. While enzymatic degradation may occur, the Salmonella SdiA activity observed in turtles infected with Aeromonas argues that AHLs can survive the turtle intestinal environment in a concentration sufficient to activate SdiA. It will be interesting to determine the half-life of AHLs in the intestines of various animal species and to determine the distances over which signaling can occur.

The SdiA activity observed during the transit of Salmonella through turtles was likely due to the AHL production of Aeromonas hydrophila. This organism is a major fish pathogen that causes septicaemia and Ulcerative Disease Syndrome and is among the most troublesome of bacterial pathogens in aquaculture [44–46]. It also causes gastroenteritis, peritonitis, and meningitis in humans [47]. Many of the Aeromonas virulence factors are regulated by the AhyR/AhyI quorum sensing system in which AhyI is an AHL molecule to activate AhyR. In this way, the population of organisms can simultaneously express virulence factors via AhyR and attack the host [48–50].

The activation of the srgE-tnpR fusion during the transit of Salmonella through turtles was dependent on the sdiA gene. This is consistent with in vitro cross-streak assays in which Salmonella can detect Aeromonas in an sdiC-dependent fashion (Figure 2). However, the fitness phenotype in turtles was extremely small. When a 49:51 mixture of sdiA+ and sdiA− RIVET strains was given to turtles, a 56:44 mixture was recovered in the feces indicating that the sdiA mutant fared slightly worse than the wild-type in this environment. This is a competitive index of 0.75 which is very close to 1.0. While this result was statistically significant, it is not considered significant in the field of bacterial pathogenesis where even 3 to 5-fold differences in competitive indices are considered minimal. Given that S. Typhimurium is not a serovar that is typically associated with turtles, S. Typhimurium may not have sdiC-regulated genes that play a role in this animal. It would be interesting to determine if sdiA mutants of turtle-associated serovars such as Munchen, Arizonae, or Newport [51–53] have a phenotype in Aeromonas-infected turtles. While not typically associated with turtles, S. Typhimurium is an exceptionally broad host-range pathogen, causing gastroenteritis in humans, cattle, pigs, poultry, horses, and sheep. It is quite common in humans, accounting for 26% of all U.S. isolates [54]. It will be interesting to determine if serovar Typhimurium detects AHL-producing pathogens in any of these other hosts and if sdiA confers a fitness advantage in any of these coinfection situations.

Materials and Methods

Bacterial strains and media

All bacterial strains and plasmids used are listed in Table 1, E. coli, Salmonella, and Aeromonas were grown in Luria-Bertani (LB) broth at 37°C unless otherwise indicated (EMD chemicals, Gibbstown NJ). LB agar plates and LB motility agar were made by adding agar to 1.2% or 0.25%, respectively (EMD chemicals). For recovery of Salmonella and Aeromonas from animal tissues or feces, xylose-lysine-deoxycholate (XLD) agar plates were used (EMD chemicals). The antibiotics ampicillin (amp), kanamycin (kan), or tetracycline (tet) were added to concentrations of 150, 100, and 20 μg/ml respectively, when needed (Sigma-Aldrich, St. Louis MO). N-(3-oxo-hexanoyl)-L-homoserine lactone (oxoC6) was obtained from Sigma-Aldrich and dissolved in ethyl acetate that had been acidified by the addition of 0.1 ml glacial acetic acid per liter (EA) [55]. The stock concentration of oxoC6 was 1 mM and it was used at a final concentration of 1 μM. Solvent controls were performed by using EA alone at 0.1%. All plasmids were prepared from JS190, a r m2 Salmonella, before electroporation into restriction-proficient Salmonella strains [56].

Construction of srgE-tnpR-lacZY fusions

A FRT site was inserted between the stop codons and the transcription terminator of the srgE gene using a Red recombination with PCR primers BA723 (TTGTATGGGGCTATATAAAA-GAAATGAACTATGTAATCTCCTTAG) and BA908 (TGCAATGCGAGCTTGCGTACCCAGACGGTGTGTAGGCTGGAGCTGCTTC). The first 30 nucleotides of BA723 bind the end of the srgE gene (nt 13544–13515 of Genbank accession number AE008767), pointing downstream, and ending with the two stop codons which are underlined. (The srgE gene has two adjacent stop codons.) The next 20 nucleotides match the Priming Site 2 sequence of Datsenko and Wanner’s mutagenesis system [57]. The first 30 nucleotides of BA908 bind downstream of the srgE gene (nt 15275–15304 of Genbank accession number AE008767), pointing upstream. The next 20 nucleotides match the Priming Site 1 sequence of Datsenko and Wanner’s mutagenesis system [57]. The insertion of the antibiotic resistance marker was verified using PCR with two different primer sets, each containing a primer that binds within the antibiotic resistance gene and a second that binds either upstream or downstream of the intended insertion site. The antibiotic resistance cassette was then removed using the temperature sensitive plasmid pCP20 carrying the FLP recombinate [58]. Kanamycin susceptible strains containing pCP20 were electroporated with the pCE71, pCE73, and pCE73 suicide plasmids followed by selection on LB kan plates at 37°C. These suicide plasmids encode a FRT site followed by a promoterless tnpR-lacZY [37]. A Flp-mediated site-specific recombination recombines the FRT site on the suicide vector with the srgE10-FRT site in the chromosome resulting in integration of the vector into the chromosome [56]. Growth of the resulting colonies at 37°C was sufficient to cure pCP20 and this was confirmed by screening for ampicillin sensitivity. Integration of the tnpR-lacZY fusion plasmid into the srgE10-FRT site was confirmed using PCR. Once the srgE10-tnpR-lacZY fusion strains were confirmed, an sdiA1::minFtn3 mutation was introduced from BA612 by transduction using P22HTint [59].

Animals

Female CBA/J mice (8–10 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). Female Hartley albino guinea pigs (retired breeders) and female New Zealand albino Rabbits (250 g) were obtained from Charles River Laboratories (Wilmington, MA). All animals were caged separately. Red-eared slider turtles (Trachemys scripta elegans) of mixed sex were obtained from Concordia Turtle Farms (Wildsville, LA). Turtles were kept in individual cages containing water and a basking support. The cages, water, and basking supports were sterilized and changed daily. All experiments performed with mice, guinea pigs, rabbits, and turtles were performed at Ohio State University with IACUC approved protocol #2006A0037.

Six domestic female pigs (30–35 pounds) were obtained from North Carolina State University Farms. Pigs were housed three to a stall and food and water were provided ad libitum. This
The overnight cultures were centrifuged at 4,000 rpm and the pellets were washed three times with sterile saline and re-suspended in fresh LB lacking antibiotics. A 1:1 mixture of cultures was used to inoculate the animals intragastrically (200 μl for mice, 100 μl for concentrate for calves [1 ml resuspended in 100 μl], 4 ml for pigs [1 ml diluted to 4 ml], 10 ml for calves, 1 ml for turtles, guinea pigs, and rabbits). Dilution plating of the inoculum was used to determine the actual dose administered. Screening the isolated colonies for tetracycline resistance indicated that in each experiment no colonies resolved before the beginning of the experiment. The percentage of wild-type Salmonella in each inoculum was determined by screening the colonies for ampicillin resistance with the wild-type being amp sensitive and the sdiA::mTn3 mutant being amp resistant.

For most animals, fresh fecal samples were collected at time points and resuspended in phosphate-buffered saline (PBS) followed by serial dilution and plating on XLD-kan agar. Isolated colonies were then screened for ampicillin and tetracycline resistance. For the turtle experiments, cloacal swabs were collected, vortexed in 1 ml PBS, and plated on XLD-kan. For pigs, a Jorvet fecal sampling loop (Livestock Concepts) was inserted into the rectum, withdrawn, and the feces removed by agitating the loop in a 15 ml conical tube containing 1 ml PBS. Chicks were sampled in groups of three each day post-infection for a total of four days. They were euthanized and fecal samples were collected directly from the cecum and distal large intestine.

Clearance of Aeromonas from turtles

In an attempt to eradicate Aeromonas from turtles, the following four steps were performed every 48 hours for two weeks. First, a cloacal swab from each turtle was collected and tested for the presence of Aeromonas and Salmonella by plating on XLD, LB, and blood agar. Second, the turtles received an IM injection in the front leg of the antibiotic Amikacin at a dose of 5 mg/kg. The injections were performed in alternating legs. Third, immediately after the injection the turtle was bathed in 0.2% chlorhexidine gluconate (except around the eyes) and rinsed with sterile water to remove any external Aeromonas or Salmonella from the animal. Fourth, the animal was placed into a new sterile container containing sterile water. On the days when no injections were performed the turtles were moved to a new sterile container containing sterile water.

Acknowledgments

We gratefully acknowledge the technical assistance of Shinichiro Shoji, Amber Lindsay, and Darren Lucas at OSU, Mitsu Suyemoto at North Carolina State, and Alan Patranella at Texas A&M. We thank Roberta Pugh at Texas A&M for incredible hospitality. We thank Michael McClelland, Andreas Baumler, and Renee Tsolis for numerous helpful conversations.

Author Contributions

Conceived and designed the experiments: CA LGA RCI BMMA. Performed the experiments: JNS JLD JAS CA SL VK. Analyzed the data: JNS JLD BMMA. Contributed reagents/materials/analysis tools: CDE JS. Wrote the paper: JNS JLD BMMA.

References

1. Ahmer BM (2004) Cell-to-cell signalling in Escherichia coli and Salmonella enterica. Mol Microbiol 52: 953–945.
2. Smith JN, Ahmer BM (2003) Detection of other microbial species by Salmonella expression of the SdiA regulon. J Bacteriol 185: 1357–1366.
3. Michael B, Smith JN, Swift S, Heffron F, Ahmer BM (2001) SdiA of Salmonella enterica is a LuxR homolog that detects mixed microbial communities. J Bacteriol 183: 5733–5742.
4. Ahmer BM, Recousijk Jr, Timmers CD, Valentine PJ, Heffron F (1998) Salmonella typhimurium encodes an SdiA homolog, a putative quorum sensor of the LuxR family, that regulates genes on the virulence plasmid. Journal of Bacteriology 180: 1185–1193.
5. Fauza C, Winans SC, Greenberg EP (1996) Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum sensing transcriptional regulators. Annual Reviews of Microbiology 50: 727–751.
6. Greenberg EP (2003) Bacterial communication and group behavior. J Clin Invest 112: 1288–1290.
7. Waters CM, Bassler BL (2005) Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 21: 319–346.
8. Visick KL, Foster J, Dojojo J, McFall-Ngai M, Ruby EG (2000) Vibrio fischeri lux genes play an important role in colonization and development of the host light organ. J Bacteriol 182: 4578–4586.
9. Visick KL, McFall-Ngai M (2000) An exclusive contract: specificity in the Vibrio fischeri-Euprymna scolopes partnership. J Bacteriol 182: 1779–1787.
10. Lapp C, Urbansowski M, Greenberg EP, Ruby EG (2003) The Vibrio fischeri quorum-sensing system aims and lux sequentially induce luminescence gene expression and are important for persistence in the squid host. Mol Microbiol 50: 319–331.
11. Swift S, Williams P, Stewart GSAB (1999) N-Acylhomoserine lactones and quorum sensing in proteobacteria. In: Danny GM, Winans S, eds. Cell-


Cell Signalling in Bacteria. Washington D.C.: ASM Press, pp 291–313.
12. Smith D, Wang JH, Swanton JE, Davenport P, Price B, et al. (2006) Variations on a theme: diverse N-acetyl homoserine lactone-mediated quorum sensing mechanisms in gram-negative bacteria. Sci Prog 89: 167–211.
13. Basler BL, Wright M, Showalter RE, Silverman MR (1993) Intercellular signalling in Vibrio harveyi: sequence and function of genes regulating expression of luminescence. Molecular Microbiology 9: 773–786.
14. Gilson L, Kuo A, Dunlap PV (1995) AimC, a new family of autoinducer synthesis proteins. Journal of Bacteriology 177: 6946–6951.
15. Hanzelka BL, Greenberg EP (1996) Quorum sensing in Vibrio fischeri: evidence that N-acylhomoserine thiolactone is the amino acid substrate for autoinducer synthesis. Journal of Bacteriology 178: 2091–2102.
16. More MI, Finger LD, Stryker JL, Fucq C, Eberhard A, et al. (1996) Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science 272: 1655–1658.
17. Schaefer AL, Val DL, Hanzelka BL, Cronan JE, Greenberg EP (1996) Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified Vibrio fischeri LuxI protein. Proceedings of the National Academy of Sciences of the United States of America 93: 9505–9509.
18. Milton DL, Chalker VJ, Kirke D, Hardman A, Campara M, et al. (2001) The LuxM homologue VanM from Vibrio anguillarum directs the synthesis of N-(3-hydroxyhexanoyl)homoserine lactone and N-hexanoylhomoserine lactone. Journal of Bacteriology 183: 3537–3547.
19. Fucq C, Eberhard A (1999) Signal generation in autoinduction systems: synthesis of acylated homoserine lactones by Lux-type proteins. In: Dunny GM, Winans SC, eds. Cell-Cell Signaling in Bacteria. Washington D.C.: American Society for Microbiology, pp 211–230.
20. Steinböck L, Venturi V (2005) Detection of quorum-sensing N-acetyl homoserine lactone signal molecules by bacterial biosensors. FEMS Microbiol Lett 256: 1–9.
21. Jansens JC, Metzger K, Daniels R, Pusec D, Verhoeven T, et al. (2007) Synthesis of N-acetyl homo-serine lactone analogues reveals strong activators of SdiA, the Salmonella enterica serovar Typhimurium LuxR homologue. Appl Environ Microbiol 73: 535–544.
22. Yao Y, Martínez-Yamout MA, Dickerson TJ, Brosgan AP, Wright PE, et al. (2006) Structure of the Escherichia coli quorum sensing protein SdiA: activation of the folding switch by acyl homoserine lactones. J Biol Chem 335: 262–273.
23. Friedrich MJ, Kimsey NE, Vila J, Kashef RJ (1995) Nucleotide sequence of a 13.9 kb segment of the 90 kb virulence plasmid of Salmonella typhimurium. FEMS Microbiol Lett 176: 495–501.
24. Baumber AJ, Tsolis RM, Bowe FA, Kusters JG, Hoffmann S, et al. (1996) The pfam bimolecular orientation of Salmonella typhimurium mediates adhesion to murine small intestine and is necessary for fluid accumulation in the infant mouse. Infection & Immun 64: 61–68.
25. Nicholson B, Lov D (2000) DNA methyltransferase-dependent regulation of pf expression in Salmonella typhimurium. Molecular Microbiology 35: 728–742.
26. Bouman CW, Kohli M, Killoran A, Touchie GA, Kashef RJ, et al. (2003) Characterization of SrgA, a Salmonella enterica serovar Typhimurium virulence plasmid-encoded parologue of the dixsulfide oxidoreductase DsbA, essential for biogenesis of plasmid-encoded fimbriae. J Bacteriol 185: 991–1000.
27. Lin D, Rau CV, Slauch JM (2008) The Salmonella SPHI type three secretion system responds to periplasmic disulfide bond status via the flagellar apparatus and the RodCDE system. J Bacteriol 190: 87–97.
28. Girillo DM, Heffernan EJ, Wu L, Hardwood J, Firehr J, et al. (1996) Identification of a domain in Rck, a product of the Salmonella typhimurium virulence plasmid, required for both serum resistance and cell invasion. Infection & Immun 64: 2019–2023.
29. Hackett J, Wyk P, Reeves P, Mathan V (1987) Mediation of serum resistance in Salmonella typhimurium by an 11-kilodalton polypeptide encoded by the cryptic plasmid. Journal of Infectious Disease 155: 540–549.
30. Heffernan EJ, Harwood J, Firehr J, Guiney D (1992) The Salmonella typhimurium virulence plasmid complement resistance gene rck is homologous to a family of virulence-related outer membrane proteins, including pagC and ail. Journal of Bacteriology 174: 84–91.
31. Heffernan EJ, Reed S, Hackett J, Firehr J, Roudier C, et al. (1992) Mechanism of resistance to complement-mediated killing of bacteria encoded by the Salmonella typhimurium virulence plasmid gene rck. Journal of Clinical Investigation 90: 953–964.
32. Crago AM, Korakonis V (1999) Binding of extracellular matrix laminin to Escherichia coli expressing the Salmonella outer membrane proteins Rck and PagC. FEMS Microbiology Letters 176: 495–501.
33. Backedt F, Ley VA, Somenburg J, Peterson DA, Gordon JI (2005) Host-bacterial mutualism in the human intestine. Science 307: 1915–1920.
34. Camilli A, Beattie DT, Mekalanos JJ (1994) Use of genetic recombination to identify Vibrio cholerae genes induced during infection. Molecular Microbiology 18: 671–683.