E. coli K-12 and EHEC Genes Regulated by SdiA

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Abstract

Background: Escherichia and Salmonella encode SdiA, a transcription factor of the LuxR family that regulates genes in response to N-acyl homoserine lactones (AHLs) produced by other species of bacteria. E. coli genes that change expression in the presence of plasmid-encoded sdiA have been identified by several labs. However, many of these genes were identified by overexpressing sdiA on a plasmid and have not been tested for a response to sdiA produced from its natural position in the chromosome or for a response to AHL.

Methodology/Principal Findings: We determined that two important loci reported to respond to plasmid-based sdiA, ftsQAZ and acrAB, do not respond to sdiA expressed from its natural position in the chromosome or to AHLs. To identify genes that are regulated by chromosomal sdiA and/or AHLS, we screened 10,000 random transposon-based luciferase fusions in E. coli K-12 and a further 10,000 in E. coli O157:H7 for a response to AHL and then tested these genes for sdiA dependence. We found that genes encoding the glutamate-dependent acid resistance system are up-regulated, by sdiA. Gene regulation by sdiA of E. coli is only partially dependent upon AHL.

Conclusions/Significance: The genes of E. coli that respond to plasmid-based expression of sdiA are largely different than those that respond to chromosomal sdiA and/or AHL. This has significant implications for determining the true function of AHL detection by E. coli.

Introduction

Prokaryotes have the ability to coordinate their gene regulation and behavior in response to population density, effectively acting as multicellular organisms. The detection of population density is referred to as quorum sensing [1,2]. A common mechanism of quorum sensing among the gram-negative bacteria is the synthesis and detection of a diffusible molecule of the LuxR family that regulates genes in response to N-acyl homoserine lactones (AHLs) [reviewed in [3,4,5]]. The prototypical example is the regulation of bioluminescence by Vibrio fischeri [reviewed in [6,7,8,9,10]]. This organism becomes luminescent when a high population density, represented by a quorum, is reached within the light organ of the squid Euprymna scolopes. V. fischeri measures its population density by producing N-(3-oxo-hexanoyl)-L-homoserine lactone (oxoC6) using the LuxI enzyme [11]. Because the oxoC6 can freely diffuse across the bacterial cell wall, the accumulation of AHL indicates a high population density [12,13]. The transcription factor LuxR binds oxoC6, dimerizes, and activates transcription of the luxICDABE operon resulting in luminescence [14,15,16]. LuxR/I systems have been found in numerous Gram-negative pathogens that colonize plants and animals and often regulate the pathogens’ host interaction genes [17]. Presumably it is advantageous for the bacteria to delay the expression of genes that are likely to stimulate the host immune response until after a significant population density has been reached. The LuxI enzyme from a particular species often produces AHLS that differ from oxoC6 in the length of the acyl chain, the degree of saturation, or the modification at the 3-carbon position. The cognate LuxR homolog detects the specific AHL variant made by its partner LuxI enzyme.

The genera Escherichia and Salmonella encode a single LuxR homolog named SdiA but do not encode an AHL synthase [18,19,20,21]. With some good fortune, the genes regulated by SdiA in Salmonella enterica serovar Typhimurium [hereafter referred to as S. Typhimurium] were identified without knowledge of the signal. Random MudJ insertions (which create lacZ transcriptional fusions) were isolated in a strain in which sdiA was conditionally expressed from a multicopy plasmid. Fusions that respond to plasmid-encoded sdiA were isolated in two loci, ogE and the rck operon [18,19,20]. Overexpression of sdiA had bypassed the requirement for AHL [19]. The fusions obtained were used to identify the signals required for activity of SdiA expressed from its natural position in the chromosome. However, this step was troublesome because the chromosomal transcriptional fusions respond to SdiA only under specific growth conditions [20]. For unknown reasons, plasmid-based fusions lack these environmental restraints. Therefore, plasmid-based fusions were used to identify the AHLS detected by sdiA under standard laboratory growth

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conditions [19]. SdiA was found to detect a wide range of AHLs at concentrations that are physiologically relevant [19,20,22,23]. SdiA can detect N-(3-oxo-octanoyl)-L-homoserine lactone (oxoC8) at 1 nM and oxoC6 at 5 nM. At 50 nM, SdiA can detect oxoC10 as well as the unmodified variants N-hexanoyl-L-homoserine lactone (C6), and N-acytely-L-homoserine lactone (C3) [19,21,24]. Once the appropriate AHLs were identified, growth conditions were screened that would allow chromosomal fusions to respond to chromosomal sdiA and AHL [20]. Our work with a gene encoded adjacent to sdiA, named sdiC (uvrY in E. coli), led to the discovery that growth in motility agar at 37°C allows optimal activity of both SdiA and SirA [20,25,26]. Like plasmid-based expression of sdiA, lowering the temperature to 30°C can cause sdiA-dependent activation in the absence of AHL [20]. However, this only occurs with the srgE locus because the sdi operon is not expressed at temperatures below 37°C. The lowered temperature may allow SdiA to oligomerize in the absence of signal. The mechanism by which motility agar enhances expression of chromosomal fusions (but not plasmid-based fusions) is not known.

The identification of the SdiA regulon of E. coli has been even more problematic than that of S. Typhimurium. Three genetic screens identified sdiA as a gene that, when expressed from a plasmid, could give rise to a particular phenotype. These included suppression of a cellular division block [27], resistance to mitomycin C [28], and resistance to quinolones [29]. Plasmid-based expression of sdiA caused these phenotypes by upregulating the fitQAB or acrAB loci. Additionally, when sdiA is expressed from a plasmid in E. coli, the strain becomes up to seven-fold more resistant to fluoroquinolones (norfloxacin, ofloxacin, and ciprofloxacin) and chloramphenicol, and two-fold more resistant to kanamycin and tetracycline [29]. An sdiA null mutant strain was up to three-fold more sensitive to the fluoroquinolones, but not chloramphenicol, nalidixic acid or tetracycline [29]. This increase in resistance was hypothesized to be due, at least in part, to sdiA increasing the expression of the acrAB genes that encode a multidrug efflux pump. When cellular protein levels were measured, plasmid-based expression of sdiA led to a 4.3-fold increase in AcrA levels and 4.5-fold increase in AcrB levels [29]. A chromosomal sdiA mutation led to a 50% decrease in AcrB but no difference in AcrA protein levels compared to wild-type. It is interesting to note that the overexpression of sdiA in an acrAB mutant did not abolish the increase in fluoroquinolone resistance, suggesting that sdiA might affect another efflux pump or pathway to drug resistance [29]. AHL was not used in any of the assays.

A microarray study was performed to identify the sdiA regulon of E. coli. This study used plasmid-based expression of sdiA in the absence of AHL [30]. These experiments identified 75 genes that were up-regulated and 62 genes that were down-regulated in response to plasmid-based expression of sdiA. The fitQAB and acrAB loci were among the genes identified, again confirming that these loci respond to plasmid-based expression of sdiA. The microarray study and a second independent study also showed that the uvrY gene (sdiA in Salmonella) is regulated by plasmid-encoded sdiA [30,31]. In Enterohaemorrhagica E. coli O157:H7 (EHEC), it was determined that the expression of EspD, intimin, and flagellar proteins were reduced by plasmid-based expression of sdiA [32]. The microarray study with E. coli K-12 also observed repression of flagellar genes by plasmid-based expression of sdiA [30]. However, for all of the genes discussed above, the effect of chromosomal sdiA was either not reported or was found to be less than two-fold.

We hypothesized that overexpression of sdiA causes a pleiotropic effect in E. coli that does not occur in S. Typhimurium [21]. Furthermore, we know that sdiA of E. coli is expressed from the chromosome and functional because it activates a plasmid-based srgE-lasCDAEB fusion from S. Typhimurium in response to AHLs [21]. Recently, Van Houdt et al., performed a genetic screen with E. coli in which 13,100 plasmid-based fusions were screened for a response to AHL during growth in LB broth at 30°C [33]. Six up-regulated and nine down-regulated promoters were identified and confirmed to be dependent upon sdiA expressed from its natural position in the chromosome. Interestingly, uvrY was the only gene that overlapped between this set of genes and the set derived from sdiA overexpression studies. The fold-induction or repression observed in this chromosomal sdiA based study was never more than 1.5-fold for any of the genes [33]. Additionally, a second microarray study was performed recently that compared wild-type E. coli to a sdiA mutant E. coli in late stationary phase at 30°C, although AHL was not included in the growth medium [34]. Forty genes were repressed by sdiA and 42 were activated. Except for the repression of flagellar genes, the vast majority of the genes identified in this study are different than those found in previous studies.

In this report, we examined the regulation of two loci, fitQAB and acrAB, that were previously determined to respond to plasmid-based expression of sdiA. We tested the hypothesis that these genes are regulated by sdiA expressed from its natural location in the chromosome if AHL is present. However, we observed no regulation of these genes in response to chromosomal sdiA and AHL. Therefore, we decided to perform a new genetic screen in E. coli using the information gained from our studies of the sdiA regulon of S. Typhimurium. We used a transposon to create chromosomal fusions in a wild-type background (in which sdiA is expressed from its natural position in the chromosome) and then screened the fusions for a response to AHL during growth in motility agar at 37°C. We performed this screen with both E. coli K-12 and EHEC. The AHL-responsive fusions were then tested for sdiA-dependence.

Results
Quinolone Resistance Is Not Increased by Chromosomal sdiA and/or AHL

It has been reported that plasmid-based expression of sdiA causes an increase in quinolone resistance in E. coli [29]. In this report, we tested the hypothesis that sdiA expressed from its natural position in the chromosome can increase resistance to quinolones in response to AHL. We used two assays to measure antibiotic resistance. The first was the E-Test strip assay, which utilizes a plastic strip that is coated with a gradient of antibiotic. Bacteria are spread on the surface of an agar plate and then the strip is placed on the plate. The minimum inhibitory concentration (MIC) is read from where the zone of growth inhibition intersects the strip. Using the E-Test strips, neither sdiA nor AHL had any effect on the MIC of S. Typhimurium, E. coli K-12, or EHEC for chloramphenicol, tetracycline, nalidixic acid, norfloxacin, ofloxacin, or ciprofloxacin (Figure 1). The strains used are described in Table 1.

Based on our previous observation that SdiA of S. Typhimurium appears to be most active in motility agar [20], we tested the hypothesis that sdiA would be involved in antibiotic resistance during growth in motility agar. A dilution series of each antibiotic was added to molten motility agar at 55°C. The motility agar was dispensed into the wells of 96-well plates and allowed to cool to room temperature. The various bacterial strains were then inoculated into the motility agar by stabbing the center of each well. The MICs were determined in the presence and absence of AHLs. We also included two variables that have made a difference in past publications, i.e., plasmid-based expression of sdiA versus
chromosomal expression of sdiA, and growth at 30°C versus 37°C. In E. coli K-12, EHEC, and S. Typhimurium we observed no AHL-dependent increase in antibiotic resistance at either temperature (Figures 2 and 3). However, using plasmid-encoded sdiA we did observe 2-fold changes in response to some antibiotics. Some of these effects appeared to be partially or completely due to the vector used to encode sdiA, while other effects were due to sdiA and not the vector (Figures 2 and 3). Thus, we have confirmed the previously published results that plasmid-encoded sdiA can lead to small changes in antibiotic resistance but we observe no effect of AHL or sdiA on antibiotic resistance when sdiA is expressed from its natural position in the chromosome.

The Expression of acrAB Is Not Increased by Chromosomal sdiA and/or AHL

When expressed from a plasmid, sdiA has been shown to increase the expression of the acrAB locus in E. coli K-12 [29,30]. To test the hypothesis that acrAB can respond to chromosomal sdiA and AHL, we constructed a chromosomal merodiploid acrA+/acrZ::lacZY fusion in E. coli K-12 and an isogenic sdiA mutant and grew them in the presence of AHL or EA. As seen in Figure 4 there was no significant difference in β-galactosidase activity between the wild-type and sdiA mutant strains at either 30°C or 37°C. However, when sdiA was expressed from a plasmid we observed an increase of up to two-fold in acrA expression compared to the vector control (Figure 4), confirming the previously published results [29,30]. AHL slightly increased the activity of plasmid-encoded sdiA (Figure 4).

The Expression of ftsQAZ Is Not Increased by Chromosomal sdiA and/or AHL

The ftsQAZ operon has an essential role in cell division. Therefore, we made a chromosomal lacZ::ftsQAZ transcriptional fusion immediately after the stop codon of ftsQAZ but before the

Figure 1. Antibiotic resistance of S. Typhimurium, E. coli K-12, and EHEC as measured by E-Test strips. The graphs show the MIC of the wild-type strains and their respective isogenic sdiA mutants (BA612, JNS21, and DL1, respectively). Each bar is the average of two separate experiments performed in triplicate and error bars represent standard deviation.

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| Strain or Plasmid | Genotype | Source or Reference |
|------------------|----------|---------------------|
| Strains          |          |                     |
| 14028            | Wild-type Salmonella enterica serovar Typhimurium | ATCC            |
| 700927           | Wild-type Escherichia coli O157:H7 (EHEC) | ATCC            |
| AL4001           | BA4000 gatW4001::mTn5luxkan2 | This study      |
| BA612            | 14028 sdiA1::mTn3 | [18]             |
| BA4000           | nal resistant mutant of BW25113 | This study      |
| BW20767          | E. coli leu-52::IS10 recA1 creC510 hisDR17 endA1 zbf-5::mTn15luxkan2 the RP4-2-tet:Mu-1kan::Tn7 | [44]             |
| BW25113          | Δ(araD-araB)567 ΔlacZ4787(r::lacIQ) rph-1 Δ(rhaD-rhaB)568 hisDR154 | [43]             |
| DL1              | 700927 sdiA25::EZ-Tn5::kan-2> | This study      |
| JLD271           | WMS4 sdiA271::cam | [42]             |
| JLD370           | WMS4 acrA::acrA::lacZYA integrant | This study      |
| JLD373           | JLD370 sdiA271::cam | This study      |
| JLD404           | nal resistant mutant of 700927 | This study      |
| JLD604           | JLD404 file604::mTn5luxkan2 | This study      |
| JLD605           | JLD404 gatE605::mTn5luxkan2 | This study      |
| JLD607           | JLD404 yhdD607::mTn5luxkan2 | This study      |
| JLD610           | JLD404 hdeA608::mTn5luxkan2 | This study      |
| JLD800           | AL4001 sdiA271::cam | This study      |
| JLD803           | JLD604 sdiA::pRE112 | This study      |
| JLD804           | JLD605 sdiA::pRE112 | This study      |
| JLD806           | JLD607 sdiA::pRE112 | This study      |
| JLD809           | JLD610 sdiA::pRE112 | This study      |
| JLD3000          | MG1655 ftsZ::FRT-cam-FRT | This study      |
| JLD3004          | WMS4 ftsZ::FRT-cam-FRT | This study      |
| JLD3011          | WMS4 ftsZ::lacZYA | This study      |
| JLD3013          | JLD3011 sdiA271::cam | This study      |
| JNS21            | MG1655 sdiA25::EZ-Tn5::kan-2> | This study      |
| MG1655           | Wild-type Escherichia coli K-12 | E. coli Genetic Stock Center |
| S17::pir         | E. coli recA pro hisDR ·<RP4-2-tet:Mu-1kan::Tn7> ·pir | [45]             |
| WMS4             | E. coli K-12 ΔlacX74 | Bill Metcalf |
| Plasmids         |          |                     |
| pBAD33           | Arabinose-conditional expression vector, p15A (Cam') | [46]             |
| pCE36            | FRT lacZYA+ tns or R6K (Kan') | [47]             |
| pCLF3            | FRT-cam-FRT on R6K (Amp') | [48]             |
| pCP20            | cI857 ΔPR /p pSCI101 oriTS (Amp' Cam') | [49]             |
| pCX16            | pGB2 carrying E. coli sdiA (Spec') | [27]             |
| pGB2             | pSC101 cloning vector (Spec') | [27]             |
| pKD46            | pBAD gam bet exo pSCI101 oriTS (Amp') | [43]             |
| pJLD1203         | pSB401 fliE-luxCDABE (Tet') | This study      |
| pJLD1505         | acrA-lacZYA fusion in pVJK112 (Kan') | This study      |
| pJLD2000         | Central portion of sdiA in pRE112 (Cam') | This study      |
| pJVR2            | sdiA under control of araBAD promoter; pACYC origin (Cam') | [18]             |
| pRE112           | Suicide vector, sdiA, ori R6K (Cam') | [50]             |
| pSB401           | luxR' luxC-luxCDABE; pACYC origin (Tet') | [51]             |
| pUT mTs5 lux kan2 | Suicide vector, ori R6K, mini-Tn5 Km2 luxCDABE transposon, mob' (RP4) (Amp’ Kan') | [41]             |
| pVJK112          | lacZYA transcriptional fusion vector, ori R6K (Kan') | [52]             |

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transcription terminator (see Materials and Methods). Using this construct we were able to confirm the previously published observation that expression of \textit{sdiA} from a plasmid increases \textit{ftsQAZ} expression by up to four-fold (Figure 5). However, when \textit{sdiA} is expressed from its natural position in the chromosome it has no effect on \textit{ftsQAZ}, even in the presence of AHL at either 30°C or 37°C (Figure 5).

Identification of AHL-Responsive Transcriptional Fusions in \textit{E. coli} K-12 and EHEC

To identify genes that are regulated in response to AHLs in \textit{E. coli} K-12 and EHEC we constructed random mTn5 transposon mutants, as described in Materials and Methods. The transposon creates a transcriptional luciferase \textit{(luxCDABE)} fusion upon insertion. Each mutant was screened for AHL-responsive luciferase activity by patching into two adjacent wells of a 96-well plate filled with motility agar containing either AHL (oxoC6) or the solvent control, acidified ethyl acetate (EA). The plates were incubated at 37°C and luminescence readings of each well were recorded after 9 hours. We screened 10,000 \textit{E. coli} K-12 mutants and 10,000 EHEC mutants. After the initial screening, candidate mutants were tested again for AHL responsiveness in triplicate using a more precise procedure in which molten motility agar is seeded with a liquid overnight culture of the mutant in question, rather than stabbing the mutant into the wells (see Materials and Methods).

One mutant in \textit{E. coli} K-12 and four in EHEC showed a consistent change in luciferase expression in the presence of o xoC6.
compared to the solvent control, EA. One of the fusions was down-regulated 7-fold by oxoC6 while four were up-regulated by up to 7-fold (Figure 6). The DNA sequence of each transposon insertion site was determined using the mutant chromosomal DNA as the template and two different sequencing primers that bind within the transposon sequence (primers are listed in Table 2). The two sequencing reactions yielded the same result in each case with regard to the transposon insertion point (Figure 7). The single AHL-regulated fusion in \textit{E. coli} K-12 (AL4001) was inserted in the \textit{gadW} gene. In EHEC there were three transposon-based fusions that were up-regulated in response to AHL. JLD605 contained an insertion in ECs4392 (ortholog of \textit{E. coli} K-12 \textit{gadE/yhiE}); JLD607 contained an insertion in ECs4388 (ortholog of \textit{E. coli} K-12 \textit{yhiD}); and JLD610 contained an insertion in ECs4390 (ortholog of \textit{E. coli} K-12 \textit{hdeA}). The one fusion in EHEC that was down-regulated, JLD604, was inserted just upstream of ECs2675, encoding a hypothetical protein, but the transposon orientation was anti-sense suggesting that expression was being driven from the ECs2676 gene promoter (ortholog of \textit{E. coli} K-12 \textit{fliE}). Because of the unusual location of the fusion in JLD604, we cloned the \textit{fliE} promoter region into pSB401 to form a \textit{fliE-luxCDABE} transcriptional fusion. When this construct, pJLD1203, was placed into wild-type and \textit{sdiA} mutant \textit{E. coli}, we observed that the \textit{fliE} promoter is indeed repressed by \textit{sdiA} (data not shown).

**AHL Responses Are \textit{sdiA}-Dependent**

We hypothesized that the AHL-responsiveness of each fusion was dependent upon the \textit{sdiA} gene. To test this, an isogenic \textit{sdiA} mutation was placed into each of the fusion strains, as described in Materials and Methods. As expected, the response of each fusion...
to AHL in motility agar was eliminated by the sdiA mutation (Figure 6). Interestingly, there was a substantial amount of AHL-independent SdiA activity observed with each fusion. Therefore, in *E. coli* K-12 and EHEC, SdiA is partially active even in the absence of AHL. This has been observed previously with the *srgE* gene of *Salmonella*, but only at the lower temperature of 30°C [20]. Previous reports have observed that *sdiA*-regulated fusions in *E. coli* K-12 are more responsive at 30°C than at 37°C [33,34].

To determine if temperature affects the *E. coli* K-12 and EHEC *sdiA*-regulated fusions, each strain was assayed in liquid broth at 37°C and 30°C (Figures 8 and 9, respectively). Expression in the wild-type background was several orders of magnitude higher than in the *sdiA* mutant background at both temperatures. However, much of this was AHL-independent. All fusions except for *gadW* demonstrated higher overall levels of expression at 30°C than at 37°C. The maximal fold-induction between EA and AHL was also greater at 30°C than at 37°C for all fusions. The *gadW* fusion is induced 6-fold by AHL at 37°C and 25-fold at 30°C. The *fliE* fusion is repressed 5-fold by AHL at 37°C and 18-fold at 30°C. The *gadE* fusion is only induced 2.2-fold by AHL at 37°C but is induced 16-fold at 30°C. The *yhiD* and *hdeA* fusions are the least regulated being induced 2 to 5-fold by AHL under all conditions. Interestingly, at 37°C there is no difference in AHL-dependent maximum fold-induction between growth in motility agar (Figure 6) or liquid broth (Figure 8). This is different than what is observed in *Salmonella*, where motility agar enhances AHL-dependent regulation [20]. However, two overall trends remained the same at the two temperatures: i) there is substantial basal activity of SdiA even in the absence of AHL; and ii) the response to AHL is completely dependent upon *sdiA*.

We also tested the fusions on solid LB agar using cross-streak assays (Figure 10). The fusions were clearly regulated by oxoC6 but not the solvent control EA. Four of the fusions were up-regulated in the presence of chromosomal *sdiA* (*gadW, gadE, yhiD*, and *hdeA*), whereas the *fliE* promoter was down-regulated. This regulation was entirely dependent upon *sdiA*.

**Acid Resistance Phenotypes of *E. coli* K-12 and EHEC**

The four up-regulated genes (*gadW, gadE, yhiD*, and *hdeA*) identified in our screen in *E. coli* K-12 and EHEC are known to be involved in the glutamate dependent acid resistance system (AR-2), and are located within the acid fitness island (AFI) (Figure 7). Interestingly, this island is not present in *Salmonella*. AR-2 uses a pair of glutamate decarboxylases (gadA and gadB) and an antiporter (gadC) to increase the pH of the cell [35,36,37]. The activation of four genes in the AFI led us to hypothesize that *sdiA* might enhance the glutamate dependent acid resistance phenotype in *E. coli* K-12 and EHEC. To test this hypothesis we performed acid resistance assays as previously described [37]. Wild-type and isogenic *sdiA* mutants of *E. coli* K-12 and EHEC were grown in LB broth containing 1 μM oxoC6 or EA. The strains were subcultured 1:100 into LB broth containing either 1 μM oxoC6 or EA. The cultures were incubated with shaking at 30°C (A) and 37°C (B). Samples were removed from the cultures at time points for β-galactosidase assays. Each strain was assayed in triplicate and error bars represent standard deviation. * denotes p<0.05 compared to the adjacent solvent control. doi:10.1371/journal.pone.0008946.g004
broth with glucose to repress another acid resistance system (AR-1) and then sub-cultured into minimal E medium (MEM) with glucose and glutamate at pH 2.0 at either 30°C or 37°C. Cultures were sampled at zero, one, and two hours and plated for cfu. In E. coli K-12 at 30°C, the sdiA gene provided a 9-fold increase in survival (Figure 11b). A much smaller 2 to 3-fold survival phenotype was observed at 37°C and with EHEC (Figure 11). The addition of AHL to the growth and challenge media did not significantly increase the acid resistance phenotype, suggesting that the basal level of AHL-independent SdiA activity is sufficient for acid resistance.

Discussion

Numerous studies have utilized plasmid-based expression of sdiA in order to study the SdiA regulon of E. coli [27,28,29,30,31,32]. The rationale for using plasmid-encoded sdiA was that the AHL(s) that bind SdiA had not yet been discovered. In this report we addressed the question of whether the two most intensively studied loci identified in previous studies (ftsQAZ and acrAB) would respond to sdiA expressed from its natural position in the chromosome in the presence of AHL. Surprisingly, while we were able to replicate the observations that the genes respond to plasmid-encoded sdiA, the genes do not respond to chromosomal sdiA and/or AHL, at either 30°C or 37°C, in liquid broth or in motility agar. With regard to the antibiotic resistance phenotype, we were able to confirm small changes in antibiotic resistance when sdiA is expressed from a plasmid, but saw no differences in antibiotic resistance between a wild-type strain and an sdiA mutant of E. coli K-12, EHEC, or S. Typhimurium, at 30°C or 37°C, on solid agar or motility agar. Therefore we conclude that ftsQAZ and acrAB are not part of the E. coli SdiA regulon under the conditions tested.

 Rather than continuing to individually test previously discovered genes for a response to chromosomal sdiA and AHL, we decided to start from the beginning and screen random transposon-based luciferase fusions for those that respond to AHLS. With this approach, both sdiA and the fusion are chromosomal during the screen. We screened 10,000 fusions in E. coli K-12 and 10,000 fusions in EHEC for a response to AHL. We identified gadW in E. coli K-12 and gadE, yhdD and hdeA in EHEC as being activated in response to AHL, and we found fliE in EHEC as being repressed in response to AHL. The response of these fusions to AHL was sdiA-dependent. The genes activated by sdiA suggest a role for SdiA in regulation of the glutamate dependent acid resistance system, AR-2. Surprisingly, this phenotype was independent of EHEC even though the addition of...
AHL increases expression of the genes in the acid fitness island (AFI), suggesting that basal levels of SdiA activity are sufficient to increase acid resistance. This is the third study that identified genes of the AFI as being regulated by chromosomally expressed sdiA [33,34]. Oddly, the gad genes were not identified using plasmid-encoded sdiA and microarrays [30]. We determined that the gadE and hdeA promoters are responsive to plasmid-encoded sdiA (data not shown), so it is not clear why they were not identified in the microarray studies. However, both microarray studies identified numerous flagellar genes as repressed by plasmid-encoded sdiA [30,34]. Additionally, flagellar gene expression is down-regulated by plasmid-encoded sdiA in EHEC [32]. The repression of our mTn5luxkan2 fusion in fliE of EHEC confirms these observations.

Figure 6. Regulation of AHL-regulated genes of E. coli K-12 and EHEC in motility agar containing either 100 nM oxo-C6 or the solvent control, EA at 37°C. Luminescence in relative light units (RLU) was measured using a Wallac Victor™ 1420 multimode plate reader at the time intervals noted. Each strain was assayed in triplicate and error bars represent standard deviation. A) AL4001/JLD800 (gadW), B) JLD604/JLD803 (fliE), C) JLD605/JLD804 (gadE), D) JLD607/JLD806 (yhiD), E) JLD610/JLD809 (hdeA). doi:10.1371/journal.pone.0008946.g006

Table 2. Oligonucleotides used.

| Oligo | Sequence | Description                                      |
|-------|----------|--------------------------------------------------|
| BA184 | GATGTCGTGCAAGGCCGATTAAGTTG | For sequencing lacZ fusion junctions             |
| BA247 | GAGTCATTCAATATTGGCAGGTAAACAC | For sequencing mTn5luxkan2 insertion sites (anneals to luxC) |
| BA408 | GCCATGGCCGGGCTGATTGAGGTA | 3' oligo for amplifying E. coli yecC-sdiA region |
| BA409 | GGTCACCGCCGCTGCCCAACGGCCTTA | 5' oligo for amplifying E. coli yecC-sdiA region |
| BA502 (C1) | TTATACGCAAGGCGACAAGG | binds within FRT-cam-FRT |
| BA1090 | GAATGTATGTCCTGCGTCTTGAGTA | For sequencing mTn5luxkan2 insertion sites (anneals to luxC) |
| BA1168 | ATCCCCAGATCTCTTGCTGATAAGGGAAGG | 5' primer for making the FRT site at the end of ftsZ |
| BA1505 | GCCGGGTTCACCGAGAGTGAATTT | For amplifying the fliE promoter region of EHEC |
| BA1506 | AAATGGCACCACATCTGATCGGA | For amplifying the fliE promoter region of EHEC |
| BA1533 | TCTAGAAGGTCGGTCTGAAATCTCTTGCG | For amplifying internal fragment of sdiA in EHEC with xbaI site |
| BA1534 | CCCGGGGGACTATACACCAATATTACCTGAG | For amplifying internal fragment of sdiA in EHEC with smal site |
| BA1817 | GAGCCTGGGAACCCAAATCTGCAATCTCATGGAATATCTCTCATTAG | 3' primer for making the FRT site at the end of ftsZ |
| BA1818 | ACCAGGCGTCGTGAGTGGCCAAAAGATT | binds within ftsZ pointing downstream |

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by determining that fliE is repressed by chromosomal sdiA. As the EHEC strain tested here is not motile, we did not see any phenotype of this repression. It is also not clear why our screen failed to identify more flagellar genes as being repressed. We chose the conditions of our screen based on experience with S. Typhimurium where motility agar at 37°C is a good condition for observing sdiA-dependent activation of chromosomal fusions. In hindsight, SdiA of E. coli does not appear to be more active in motility agar than in liquid medium (compare Figure 6 to Figures 8 and 9). SdiA of E. coli also appears to be more active at 30°C than at 37°C. Therefore, it might be worthwhile to take an iterative approach and repeat the screening process in liquid broth at 30°C.

It is quite possible that the SdiA regulon changes depending on the environmental or metabolic conditions. Thus, conditions may exist that allow chromosomal sdiA to activate the acrAB, fliQAZ, or other genes. These conditions might remove barriers to individual target gene expression, or may increase SdiA expression or activity. Consistent with this possibility, the sdiA gene of Salmonella is upregulated during swarming motility [38].

Figure 7. A) Acid fitness island of E. coli. The transposon insertion in E. coli K-12, AL4001, is within gadW at nucleotide 3662317 of Genbank accession number U00096. The transposon insertions in the EHEC strains are shown on the same map but the nucleotide positions are from Genbank accession number BA000007. JLD605 is within gadE at nucleotide 4401036; JLD607 is within yhiD at nucleotide 4397949; JLD610 is within hdeA at nucleotide 4398821. B) JLD604 is just upstream of EcS2675 in the anti-sense orientation at nucleotide 3662317.
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Figure 8. Regulation of AHL-regulated genes of E. coli K-12 and EHEC in liquid cultures at 37 C containing either 1 μM oxo-C6 or the solvent control, EA. Luminescence in relative light units (RLU) and OD590 were measured using a Wallac Victor2 1420 multimode plate reader at the time intervals noted. Each strain was assayed in triplicate and error bars represent standard deviation. A) AL4001/JLD800 (gadW), B) JLD604/JLD803 (fliE), C) JLD605/JLD804 (gadE), D) JLD607/JLD806 (yhiD), E) JLD610/JLD809 (hdeA).
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However, until conditions are identified that allow chromosomal sdiA to activate a particular gene, that gene should not be considered to be a confirmed member of the sdiA regulon. It was recently discovered that S. Typhimurium SdiA becomes active in the Peyer’s patch so from mice infected with Yersinia enterocolitica [23]. In vivo environments like this may provide the most promising conditions for testing the sdiA-dependence of a particular gene. It will be interesting to determine if E. coli SdiA becomes active in a similar situation and to determine if any of the potential SdiA regulon members like acrAB and ftsQAZ become responsive to chromosomal sdiA in this setting. Furthermore, it will be of interest to determine the in vivo situation in which the gad genes and fliE are regulated by SdiA and play a role in the bacterium’s fitness.

Materials and Methods

Bacterial Strains and Media

All bacterial strains and plasmids are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth (EM Science). Agar was added to 0.3% (motility agar), or 1.2% (agar plates) as indicated. M9 minimal glucose medium was made as described previously [39]. When necessary, media were supplemented with appropriate antibiotics at the following concentrations (micrograms per milliliter): ampicillin, 100; kanamycin, 50; tetracycline, 20; nalidixic acid 75; and chloramphenicol, 30. N-hexanoyl-DL-homoserine lactone (C6) and N-(3-Oxohexanoyl)-L-homoserine lactone (oxoC6) (Sigma) were dissolved in acidified ethyl acetate (EA) and used at the concentrations noted in the text. EA is 0.1 ml glacial acetic acid per liter of ethyl acetate [40].

Constructing and Screening Transposon Based Luciferase Fusions for AHL Responsiveness in EHEC and E. coli K-12

Transposon mutagenesis was performed by mating S17Δpir+ pUTmTn5luxkan2 [41] with JLD404, a spontaneous nalidixic acid resistant mutant of EHEC strain ATCC 700927. The two strains were mated on an LB plate overnight at 37°C. The cells were then scraped from the agar, resuspended in LB broth, dilution plated onto LB agar containing kanamycin and nalidixic acid (100 µg/ml and 75 µg/ml, respectively) and incubated at 37°C overnight. Ten thousand of the resulting mutants were then stabbed individually into the wells of black 96 well plates containing 200 µl of LB 0.3% motility agar supplemented with either 100 nM oxoC6 or 0.01% EA and incubated at 37°C for 9 hours. Luminescence for each well was measured using the Wallac Victor2 plate reader (Perkin Elmer). Mutants that had a two-fold difference between EA and oxoC6 were struck to isolation on LB kan nal. Ten thousand mutants of E. coli K-12 strain BA4000 were constructed and screened in the same way, except that the initial screen was performed using 1 mM oxoC6 and 0.1% EA.

Mutants that had a two-fold difference between EA and oxoC6 were struck to isolation on LB kan nal. Ten thousand mutants of E. coli K-12 strain BA4000 were constructed and screened in the same way, except that the initial screen was performed using 1 mM oxoC6 and 0.1% EA.

Cross Streak Assays

Mutants were grown overnight in LB kan nal at 37°C. The next day 20 µl of 10 mM oxoC6 and 20 µl of EA were dripped down
10 μl of each overnight culture was then dripped down the plate perpendicular to the EA and AHL cross streaks, in that order. The plates were then incubated at 37 °C for 7 hours. Expression of luciferase by bacteria on plates was imaged and quantitated using a C2400-32 intensified charge-coupled device camera with an Argus 20 image processor (Hamamatsu Photonics).

DNA Manipulation

Genomic DNA was isolated from overnight cultures of the mutants using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). The transposon insertion site in the genomic DNA was sequenced twice, each time using a different primer that binds within the transposon. All primers are listed in Table 2. DNA sequencing was performed by the Plant Microbe Genomics Facility at the Ohio State University.

Construction of sdiA Mutants

A EZ-Tn5<kan-2> (Epicentre Biotechnologies) mutation was isolated in the E. coli sdiA gene. To do this the yecC-sdiA region was amplified using PCR with Pfu DNA Polymerase (Stratagene) and primers BA408 and BA409 with MG1655 as template. The PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen). The yecC-sdiA fragment was removed from pCR-Blunt II-TOPO using XbaI and SfiI and cloned into pRE112 digested with the same enzymes resulting in pJS12. pJS12 was mutagenized in vitro with EZ-Tn5<kan-2> and transformed into S17pir selecting LB kan. Location of EZ-Tn5<kan-2> inserts were determined using PCR screening followed by DNA sequencing. One isolate, pJS18, was saved for future use and contains EZ-Tn5<kan-2> after nucleotide 1994484 of Genbank accession number U00096.2

Figure 10. Cross streak assays of the E. coli K-12 and EHEC lux fusions. The chromosomal lux fusions and their respective sdiA mutants were grown in broth overnight. The strains were dripped down the plate perpendicular to 20 μl of EA then 20 μl of 10 μM oXoC6 (diagrammed in Panel A for all panels). Plates were incubated at 37 °C for 7 hours then light emission was imaged using a C2400-32 intensified charge-coupled device camera with an Argus 20 image processor. A) AL4001/JLD800 (gadW), B) JLD604/JLD803 (fliE), C) JLD605/JLD804 (gadE), D) JLD607/JLD806 (yhiD), E) JLD610 / JLD809 (hdeA).

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which is near the center of sdiA. S17 admiration of JNS18 was mated with E.
coli K-12 strain MG1655 and EHEC strain 700927 selecting on M9 glucose kan and screening for cam sensitive. The resulting sdiA mutants of E. coli K-12 and EHEC were named JNS21 and DL1, respectively. A second sdiA mutation in E. coli K-12 was also available, the sdiA271 mutation moved into other K-12 strains using phage P1-mediated transduction.

Since EHEC does not have a transducing phage, we used single crossover disruptions of sdiA to quickly mutate the sdiA gene of the strains carrying AHL-regulated mTn5 fusion. A 378 bp internal fragment of the sdiA gene from EHEC, accession number BA000007, was amplified by PCR with an XbaI site at the 3' end and a Smal site at the 5' end using Tag DNA polymerase and primers BA1533 and BA1534. The DNA fragment was then cloned into a TOPO TA cloning kit (Invitrogen). The sdiA fragment was digested out of pCR4-TOPO with XbaI and Smal and ligated into pRE112 that had been cut with the same enzymes. The ligation was transformed into S17pir cells. The resulting clones were screened by PCR for the presence and orientation of insert. One of the positive clones was selected for further use and named pJLD2000. The EHEC mTn5 fusion mutants were mated with S17pir+pJLD2000 and plated on M9 glucose kan cam.

**Liquid Assays for lux Fusions**

LB kan cultures for the sdiA+ strains or LB kan cam cultures for the sdiA mutant strains were grown shaking at 37°C or 30°C overnight. They were then subcultured 1:100 in triplicate into LB kan with either 1 μM oxo-C6 or the appropriate volume of EA (0.1%) as a solvent control. They were grown with shaking at 37°C or 30°C and at time points 200 μl from each culture was placed in a black clear 96 well plate. Both the OD550 and the luminescence were measured using a Wallac Victor plate reader.

**E-Test Strip Assays**

The minimum inhibitory concentration of each antibiotic was measured according to manufacturer's instructions (AB bioMerieux). The strains were grown on an LB agar plate at 37°C with 1 μM C6 or without AHL (EA). Strains were then diluted in 0.85% NaCl solution, to an OD550 of 0.55 to 0.6. Using a sterile cotton tip applicator the strains were spread onto LB+EA and LB+AHL plates. After drying for 5 minutes, the E-Test strip was applied to the plate. The plates were then incubated at 37°C for 18 hours and read according to manufacturer's instructions. The antibiotics tested were chloramphenicol, ciprofloxacin, nalidixic acid, norfloxacin, ofloxacin and tetracycline.

**Motility Agar Antibiotic Resistance Assays**

Strains were grown overnight in either LB broth with oxo-C6 or LB broth with EA. 10 μl of each culture was mixed with 140 μl of LB motility agar (0.3% agar) containing either 1 μM oxo-C6 or 0.1% EA, placed into the well of a 96 well plate, and incubated overnight at 37°C. This is the overnight growth plate. In a separate 96 well plate, 10 μl of an antibiotic dilution series was mixed with 140 μl of LB motility agar containing either 1 μM oxo-C6 or 0.1% EA. This is the antibiotic plate. The antibiotic plate was inoculated with bacteria from the overnight growth plate by stabbing the wells of the overnight growth plate with a sterile toothpick and then stabbing the antibiotic plate. The antibiotic plates were incubated overnight at 30°C or 37°C. The MIC was read as the concentration where the strain showed no visible growth from the stab mark. Each strain was assayed in triplicate. The antibiotics tested were chloramphenicol, nalidixic acid, norfloxacin, ofloxacin, ciprofloxacin and tetracycline.

**Construction of acrA'/acrA-lacZ reporter Strains**

The intergenic region between acrA and acrR from MG1655 was amplified by PCR with primers BA537 and BA538. The PCR product spans nucleotides 484610 to 485235 of Genbank where...
accesion number AE000155. The promoter region was then cloned into pCR-Blunt II-TOPO (Invitrogen). The promoter region was removed from the TOPO cloning vector by digesting with EcoRI and cloned into pVK112 also digested with EcoRI. The resulting clones were then screened for insert and orientation by PCR. The correct construct, pJLD1505, was then transformed into BW2076. BW2076+pJLD1505 was mated with WM54 on an LB plate at 37°C overnight. The cells were resuspended and plated on M9+glucose kanamycin X-gal and incubated at 37°C for 48 hours. The resulting transconjugants were streaked to isolation on the selection medium. One isolate was named JLD370 and saved for further use. An isogenic sdiA mutant was made by transducing the sdiA271::cam from JLD271 into JLD370 with P1 phage. The resulting strain was named JLD375.

Construction of the ftsZ-lacZ Fusion
Primers were designed to match the end of ftsZ including the stop codon, but before the transcriptional terminator. These primers, BA1168 and BA1817, also contained the P1 and P2 priming sites for pCLF3, respectively. Using these primers a cassette containing a chloramphenicol resistance gene flanked by regions of sequence identity to ftsZ was amplified from pCLF3 using Tag DNA polymerase, agarose gel purified (Qiagen gel extraction kit) and electroporated into arabinose induced MG1655. The correct construct, pJLD1505, was then transformed into BW2076 by PCR. One isolate was named JLD370 and saved for further use. An isogenic sdiA mutant was made by transducing the sdiA271::cam from JLD271 into JLD370 with P1 phage. The resulting strain was named JLD375.

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