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SdiA of *Salmonella enterica* Is a LuxR Homolog That Detects Mixed Microbial Communities

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Proteins of the LuxR family detect the presence of *N*-acylhomoserine lactones (AHLs) and regulate transcription accordingly. When AHLs are synthesized by the same species that detects them, the system allows a bacterium to measure the population density of its own species, a phenomenon known as quorum sensing. The *sdiA* genes of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium are predicted to encode LuxR homologs. However, these species do not appear to synthesize AHLs or any other molecule detected by SdiA. It has previously been demonstrated that overexpression of *sdiA* results in the activation of the *ftsQAZ* locus in *E. coli* and four other loci in *Salmonella* serovar Typhimurium. Here we report that transcriptional fusions to these five loci fall into two classes. The first class requires overexpression of *sdiA* for activation. The second class responds to *sdiA* expressed from its natural position in the chromosome if the appropriate AHLs are added to the culture. The only member of the second class is a series of P*rck-luxCDABE* fusions in *Salmonella* serovar Typhimurium. SdiA responds with highest sensitivity to AHLs that have a keto modification at the third carbon and an acyl chain length of 6 or 8 (half-maximal response between 1 and 5 nM). Growth of *Salmonella* in proximity to species known to synthesize these AHLs results in *sdiA*-dependent activation of the P*rck-luxCDABE* fusions. SdiA appears to be the first AHL receptor discovered that detects signals emanating exclusively from other species.

Numerous species of gram-negative bacteria use *N*-acylhomoserine lactone (AHL) signals to monitor their own population density (for reviews see references 14, 39, and 48). The prototypical example is the *Vibrio fischeri* LuxR/LuxI system (10, 12, 35). Proteins of the LuxR type have a domain for binding AHL and a second domain for binding DNA (44), while proteins of the LuxI type catalyze the final step in AHL synthesis (21, 26, 34, 36, 41, 51). A variety of AHL signaling molecules have been discovered. These differ primarily in acyl chain length and the nature of the substituents at the C-3 position. Each LuxI homolog makes a specific AHL, although many LuxI enzymes also make lesser amounts of related AHL molecules. In addition to the LuxI family of AHL synthases, a second type (LuxLM) has been described for *Vibrio harveyi* and *V. fischeri* (2, 17). These enzymes use biosynthetic substrates similar to those used by the LuxI family (22). More recently, a third type of AHL synthase has been proposed, although enzymatic studies are required to confirm this (32).

In a widely accepted model for quorum sensing, each bacterial cell in a population produces AHL. As the population density increases, the concentration of AHL also increases. Above a threshold concentration, representing a "quorum" of bacterial cells, the LuxR homolog binds AHL and activates transcription of target genes. One of the target genes is often the *luxI* homolog, which results in a positive feedback loop of increased AHL synthesis. Many bacterial behaviors have been shown to be regulated in a population density-dependent manner by AHLs, including plasmid conjugal transfer, protein secretion, exoenzyme and cytotoxin synthesis, antibiotic synthesis, capsular exopolysaccharide synthesis, biofilm formation, twitching motility, and swarming motility (9).

Escherichia coli and *Salmonella enterica* serovar Typhimurium encode a single *luxR* homolog named *sdiA* (1, 53). The genomic organizations of the *sdiA* region are identical in the two species (Fig. 1A). Upstream of *sdiA* is an uncharacterized open reading frame (ORF) named *yecC* which is similar to the ATP binding component of ABC transporters (1, 3). Downstream of *sdiA* is a gene named *uvrY* in *E. coli* and *sirA* in *Salmonella* serovar Typhimurium (27, 29). Further downstream is the *uvrC* gene, which encodes a DNA repair enzyme. Despite the name, *uvrY* plays no role in DNA repair but instead encodes a transcription factor of the FixJ family that controls virulence functions in all γ-proteobacterial pathogens examined to date (see references in reference 18). Our recent searches of genome databases suggest that orthologs of *uvrY* and *uvrC* are present in all members of the γ-*Proteobacteria*, while *sdiA* is located directly upstream only in *Escherichia*, *Salmonella*, and *Klebsiella* spp. Furthermore, it is striking that although these three genera possess a copy of *sdiA*, they are not known to synthesize the AHLs that are typically detected by LuxR homologs (reference 48 and this report). In fact, there are no AHL synthase genes (*luxI* or *luxLM* homologs) in any of the available genome sequences for these organisms. There-

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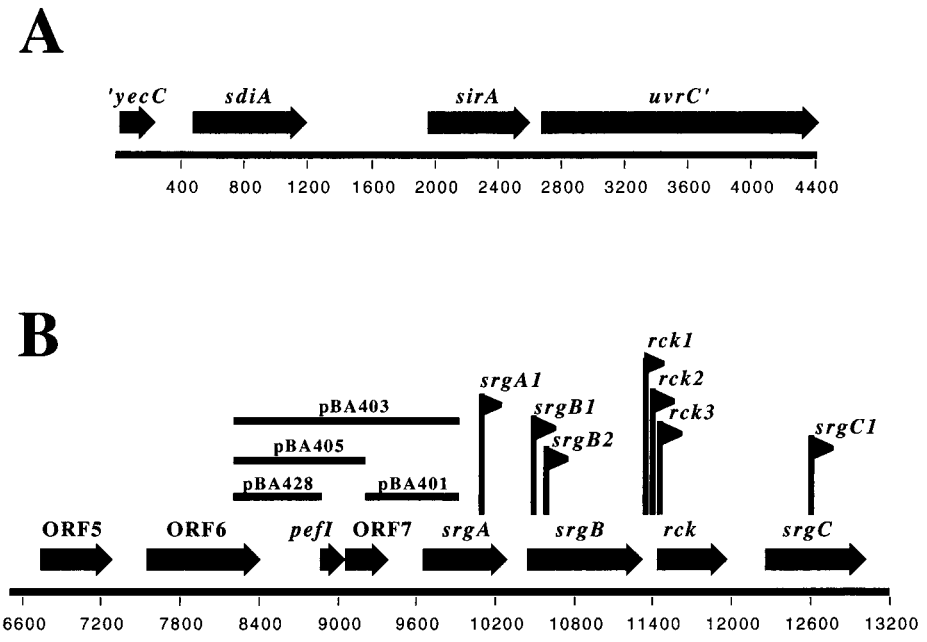

FIG. 1. ORF maps of the *sdiA* (A) and *rck* (B) regions of the *Salmonella* serovar Typhimurium genome derived from GenBank accession numbers U88651 (A) and L08613 (B). Numbers along the bottom of each map represent nucleotide positions.

fore, *Escherichia*, *Salmonella*, and *Klebsiella* appear to be unusual with regard to quorum sensing in that they encode a putative AHL receptor, SdiA, but not an AHL synthase.

The *E. coli sdiA* gene was initially isolated as a regulator of the $ftsQp_2$ promoter upstream of the *ftsQAZ* operon (53). However, this 5- to 13-fold up-regulation required that *sdiA* be expressed from a low-copy-number plasmid (pSC101 origin). SdiA expressed from its natural position in the chromosome had only a marginal effect on $ftsQp_2$ expression (40% higher expression in the wild type than in the *sdiA* mutant) (53). It was later demonstrated that *E. coli* SdiA activates *ftsQ* in response to AHL (43). This was a twofold stimulation using a strain in which *sdiA* was expressed from a plasmid. No experiments comparing a wild-type strain to an *sdiA* mutant with respect to detection of AHL and activation of *ftsQ* were reported (43). It was also reported that SdiA responds to an unidentified compound present in spent *E. coli* culture supernatants (43), although this effect was later explained by growth rate differences in fresh medium compared to spent medium (16).

A study with *E. coli* O157:H7 (enterohemorrhagic *E. coli* [EHEC]) recently determined that expression of *sdiA* from a plasmid causes repression of both motility and virulence factor expression (30). A second study, performed with *E. coli* K-12, also found that *sdiA* expressed from a plasmid causes repression of motility gene expression (54). Neither study reported the phenotype of a wild-type strain compared to that of an isogenic *sdiA* mutant. It was also reported that an SdiA-dependent ligand could be removed from the EHEC culture supernatant using immobilized SdiA as an affinity matrix (30).

In *Salmonella* serovar Typhimurium, a genetic screen was performed to identify genes regulated by SdiA (1). The *sdiA* gene was expressed under the control of the *araBAD* promoter on a multicopy plasmid (pJVR2) and placed in a *Salmonella* serovar Typhimurium *sdiA* mutant strain so that expression of *sdiA* was dependent on the presence of arabinose. Random *lacZY* transcriptional fusions (MudJ transposon insertions) were created in this strain and screened for a difference in expression on plates containing glucose versus arabinose. Ten MudJ fusions that respond to plasmid-borne *sdiA* but not to a vector control were identified. Although these fusions were responsive to *sdiA* overexpression, they were not active when *sdiA* was expressed from its natural position in the chromosome (1). This suggested that any putative ligand detected by SdiA was not present in pure cultures of *Salmonella* serovar Typhimurium and that overexpression of *sdiA* somehow bypasses the requirement for a ligand (1).

Seven of the 10 *sdiA*-responsive MudJ insertions are located within four genes on the 90-kb virulence plasmid of *Salmonella* serovar Typhimurium (*srgA*, *srgB*, *rck*, and *srgC* [Fig. 1B]). Sequence analysis suggests that *srgA* (*sdiA*-regulated gene) encodes a *dsbA* homolog, *srgB* encodes a putative lipoprotein, *rck* (resistance to complement killing) encodes a small outer membrane protein, and *srgC* encodes a putative *araC* type transcriptional regulator (13). The functions of *srgA*, *srgB*, and *srgC* are not known. However, *rck* has been studied in detail and appears to form an 8-stranded β-barrel in the outer membrane (7). The *rck* gene is not expressed well from the 90-kb plasmid of *Salmonella* serovar Typhimurium, but when expressed from a heterologous promoter in a rough background of either *Salmonella* serovar Typhimurium or *E. coli*, *rck* confers resistance to human complement (4, 20, 23, 24). The rough background is required because lipopoysaccharide is a redundant complement resistance factor. Rck also confers adhesiveness to epithelial cells and/or extracellular matrix when expressed from a plasmid in *E. coli* K-12 (7, 8, 24). The requirement for a heterologous promoter in these experiments is consistent with our observation that the *rck* promoter is *sdiA* dependent and is not active in pure culture. Given the lack of an obvious AHL

TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype	Source or reference
Strains		
14028	Wild-type *Salmonella* serovar Typhimurium	American Type Culture Collection
BA612	14028 *sdiA1*::mTn*3* (Amp^r)	1
UT481	*E. coli* K-12 Δ*lac*	53
WX2	UT481 *sdiA*::kan	53
BA1101	14028 *srg-5*::MudJ	1
BA1102	14028 *srgB2*::MudJ	1
BA1103	14028 *srgA1*::MudJ	1
BA1104	14028 *rck2*::MudJ	1
BA1105	14028 *rck3*::MudJ	1
BA1107	14028 *srgB1*::MudJ	1
BA1109	14028 *srgC1*::MudJ	1
BA1110	14028 *srg-6*::MudJ	1
BA1111	14028 *rck1*::MudJ	1
BA1112	14028 *srg-7*::MudJ	1
BA1303	14028 *srgA1*::MudJ *sdiA*::mTn*3*	1
Plasmids		
pWSK29	pSC101 cloning vector (Amp^r)	52
pWSK129	pSC101 cloning vector (Kan^r)	52
pBA306	pWSK29 *Salmonella* serovar Typhimurium *sdiA*$^+$ (Amp^r)	1
pBM1	pWSK129 *Salmonella* serovar Typhimurium *sdiA*$^+$ (Kan^r)	This study
pCX39	Mini-F′ carrying *ftsQAZ* promoter 2 (*sdiA* sensitive) fusion to *galK′-lacZYA*; Amp^r	53
pJVR2	*sdiA* under control of *araBAD* promoter; pACYC origin; Cm^r	1
pBA321	*sdiA* under control of *araBAD* promoter; ColE1 origin; Kan^r	This study
pSB401	*luxR*$^+$ *luxI*::*luxCDABE*; pACYC origin; Tet^r	57
pBA401	pSB401Δ*Eco*RI carrying ORF7-*srgA* intergenic region	This study
pBA403	pSB401Δ*Eco*RI carrying ORF6-*pefI* intergenic region, *pefI*, and ORF7	This study
pBA405	pSB401Δ*Eco*RI carrying ORF6-*pefI* intergenic region and *pefI*	This study
pBA428	pSB401Δ*Eco*RI carrying ORF6-*pefI* intergenic region	This study

synthase gene in the *E. coli* or *Salmonella* serovar Typhimurium genome, we hypothesized that SdiA is used to detect only the signals of other bacterial species (1). In this report we test and confirm this hypothesis.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. Bacteria were grown in Luria-Bertani (LB) medium or on LB plates containing 1.5% agar (EM Science) unless otherwise indicated. AB mannitol plates were used for growth of *Agrobacterium tumefaciens* as described previously (6). For filter disk assays the reporter strains were grown in 3 ml of LB soft agar (0.75% agar) overlaid on a standard LB plate. Tetracycline, chloramphenicol, ampicillin, and kanamycin were used at 20, 30, 100, and 60 μg/ml when appropriate. Glucose and arabinose were used at a final concentration of 0.2% unless otherwise indicated. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a final concentration of 80 μg/ml. AHLs with even-numbered side chains of 4 to 12 C atoms and with or without a 3-oxo group were synthesized according to the work of Chhabra et al. (5). The unsubstituted AHLs are abbreviated as C4, C6, C8, C10, or C12 based on acyl chain length. Those AHLs with a 3-oxo modification are abbreviated as oxoC4, oxoC6, oxoC8, oxoC10, or oxoC12. AHLs were added to media as dilutions from a 10 mM stock solution in acetonitrile. The final concentration of acetonitrile was always less than 1% and had no effect on the growth or AHL response of the reporter. All incubations were carried out at 37°C unless otherwise stated.

Plasmid constructions. To identify the promoter region of the *rck* operon, episomal transcriptional fusions were constructed (Fig. 1B). The putative promoter regions were amplified using *Pfu* Turbo DNA polymerase (Stratagene) with *Salmonella* serovar Typhimurium strain ATCC 14028 genomic DNA as the template. Each primer had an *Eco*RI site in the 5′ end. The resulting DNA fragments were gel purified using Qiagen gel extraction columns and cloned into the *Srf*I site of pCR-script Amp (Stratagene). One PCR product that gave rise to pBA428 was instead cloned into pCR-Blunt-II-Topo, which has *Eco*RI sites in the vector flanking the cloning site. The *Eco*RI fragment of each clone was removed, gel purified, and ligated into pSB401 that had been digested with *Eco*RI and exposed to phosphatase. pSB401 is a reporter vector containing a p15A origin of replication, a tetracycline resistance marker, and a promoterless *luxCDABE* operon from *Photorhabdus luminescens* (57). Upstream of the luciferase operon is an *Eco*RI fragment containing *luxR* and the *luxI* promoter from *V. fischeri*. This *Eco*RI fragment was removed and replaced with regions of DNA hypothesized to encode the *rck* promoter (see Fig. 1B). According to the numbering system of GenBank accession number L08613, which is used in Fig. 1B, pBA401 contains nucleotides 9276 to 9976, pBA403 contains nucleotides 8178 to 9976, pBA405 contains nucleotides 8178 to 9300, and pBA428 contains nucleotides 8178 to 8910. pBM1 was constructed by cloning the *sdiA*-containing *Pst*I fragment of pBA306 (1) into the *Pst*I site of pWSK129 (52). Orientation of inserts was determined by restriction mapping. One clone containing the *sdiA* gene oriented opposite the *lac* promoter of pWSK129 was saved and named pBM1. pBA321 was constructed by digesting pJVR2 (1) with *Xba*I and *Sac*I, gel purifying the *sdiA*-containing fragment, and ligating it to pBAD18-Kn (19) that had been digested with *Xba*I and *Sac*I. Plasmids were introduced into the appropriate strains using electroporation with a Bio-Rad Gene Pulser II.

Assay of luciferase activity. Luciferase activity was measured in liquid culture using a Turner Designs TD-20/20 luminometer. The optical density of the culture at 550 nm was measured using either a Spectronic 20D+ or a Beckman DU-64 spectrophotometer. All luminometer samples consisted of 10 μl of liquid culture placed into a 12- by 75-mm polystyrene tube. The samples were oxygenated by "ratcheting" the sample tube across a tube rack prior to insertion into the luminometer.

Expression of luciferase activity in soft agar plates was imaged and quantified using a C2400–32 intensified charge-coupled device camera with an Argus 20 image processor (Hamamatsu Photonics) or a Luminograph LB980 photon video camera (E. G. & G. Berthold). Images were captured with a Macintosh G4 computer and Adobe Photoshop 5.0 software.

For microplate format dose response assays, a logarithmic dilution series of each AHL was first made in LB medium containing the appropriate antibiotics (final volume, 100 μl) from a 10 mM stock solution in acetonitrile. The sensor strain was then diluted 1:100 from an overnight culture into LB broth with 0.6% agar, and 100 μl was added to the test microplate wells to give a final concentration of 0.3% agar. Luciferase expression was quantified after 6 h using a Victor^2 1420 multilabel counter (Wallac).

RESULTS

Identification of the *rck* promoter region. Previously, seven MudJ fusions clustering in or around the *rck* gene on the *Salmonella* serovar Typhimurium virulence plasmid were isolated based on their transcriptional responsiveness to *sdiA*

TABLE 2. Strains tested for activation of the reporter strain 14028/pBA428

Strain	Greatest fold induction	Source[a]	AHL(s) produced	Reference(s)[d]
Agrobacterium tumefaciens ATCC 23308	None	DCC	C6, oxoC8	15, 59
Citrobacter freundii strain 849	None	DCC	None detected	
Chromobacterium violaceum ATCC 31532	10[b]	Simon Swift	C6	33
Escherichia coli O157:H7 ATCC 35150	None	DCC	None detected	
Escherichia coli O157:H3 strain 317	None	DCC	None detected	
Escherichia coli O157:H7 ATCC 43895	None	DCC	None detected	
Escherichia coli O157:H12 Strain 316	None	DCC	None detected	
Escherichia coli O157:H7 ATCC 4388	None	DCC	None detected	
Escherichia coli O157:H7 ATCC 700927	None	ATCC	None detected	
Escherichia coli MG1655	None	EGSC	None detected	
Hafnia alvei 1058	8	IIISC	Unknown	
Klebsiella pneumoniae subsp. *ozaenae* ATCC 11296	None	Valley Stewart	None detected	
Klebsiella oxytoca ATCC 13182	None	Valley Stewart	None detected	
Klebsiella pneumoniae subsp. *pneumoniae* ATCC 13883	None	Valley Stewart	None detected	
Klebsiella pneumoniae subsp. *rhinoscleromatis* ATCC 13884	None	Valley Stewart	None detected	
Klebsiella terrigena ATCC 33257	None	Valley Stewart	None detected	
Klebsiella planticola ATCC 33531	None	Valley Stewart	None detected	
Klebsiella ornithinolytica JCM6096	None	Valley Stewart	None detected	
Klebsiella pneumoniae CDC 2665-69	None	DCC	None detected	
Providencia alkafaciens CDC 671-66	None	DCC	None detected	
Proteus vulgaris ATCC 12454	None	DCC	None detected	
Pseudomonas aeruginosa PAO1	3.0[b]	Dieter Haas	C4, C6, oxoC6, oxoC8, oxoC10, oxoC12	28, 37, 38, 42, 56
Pseudomonas aeruginosa PAK	2.5[b]	Shouguang Jin	C4, C6, oxoC6, oxoC8, oxoC10, oxoC12	28, 37, 38, 42, 56
Salmonella serovar Gallinarum ATCC 9184	None	ATCC	None detected	
Salmonella serovar Pullorum ATCC 9120	None	ATCC	None detected	
Salmonella serovar Typhi ATCC 19430	None	ATCC	None detected	
Salmonella serovar Typhi ATCC 33458	None	ATCC	None detected	
Salmonella serovar Typhimurium 14028	None	Laboratory collection	None detected	
Salmonella serovar Typhimurium SL1344	None	Laboratory collection	None detected	
Salmonella serovar Typhimurium SR-11	None	Laboratory collection	None detected	
Shigella flexneri	None	DCC	None detected	
Vibrio fischeri ESRI	11[c]	Karen Visick	C6, oxoC6, C8	11
Yersinia enterocolitica 10460	13	Steve Atkinson	C6, oxoC6	50
Yersinia enterocolitica 10460 *yenI*::kan	None	Steve Atkinson	None detected	50

[a] DCC, Ohio State University Department of Microbiology Culture Collection; EGSC, *E. coli* Genetic Stock Center; ATCC, American Type Culture Collection; IIISC, Institute of Infections and Immunity Strain Collection, Nottingham, United Kingdom.
[b] Test strain incubated for 16 h at 28°C prior to cross-streaking with reporter strains and incubation at 37°C for 10 h.
[c] Test strain incubated for 16 h at 24°C prior to cross-streaking with reporter strains and incubation at 37°C for 10 h.
[d] References refer to identification of particular AHLs in a species, not a specific strain.

overexpression (1) (Fig. 1B). To test whether these MudJ insertions were disrupting a single operon, the polarity of each MudJ insertion on the downstream genes was assessed using Northern hybridization. A MudJ insertion in each of the four genes was placed into a strain that allows arabinose-dependent overexpression of *sdiA* (BA612/pJVR2). RNA was isolated from each strain after growth in either glucose or arabinose. Northern blot analysis using probes specific to each individual gene confirmed that each MudJ insertion is polar on the transcription of each of the downstream genes and that expression is dependent on arabinose (data not shown). This demonstrates that *srgA*, *srgB*, *rck*, and *srgC* are expressed as a polycistronic transcript with no detectable internal start sites.

To identify the promoter region of the *rck* operon, plasmid-based transcriptional fusions to individual fragments of DNA found upstream of *srgA* were constructed. The resulting constructs form transcriptional fusions between the DNA of interest and the *luxCDABE* operon of *P. luminescens*. Each reporter plasmid (pBA401, pBA403, pBA405, and pBA428) (Fig. 1B) was then placed into an arabinose-conditional *sdiA* strain (BA612/pBA321). The luminescence resulting from each construct was compared during growth in glucose to that during growth in arabinose. A fragment containing the DNA region between ORF7 and *srgA* was not responsive to *sdiA* overexpression (pBA401 [data not shown]). However, all fragments that contained the region between ORF6 and *pefI* were responsive to *sdiA* overexpression (data not shown for pBA403 and pBA405; data for the smallest plasmid, pBA428, are shown in Fig. 2). No fragment was responsive when cloned in the opposite orientation with respect to the *luxCDABE* genes (data not shown). In total, this demonstrates that the promoter for the *rck* operon is unidirectional and lies between ORF6 and *pefI* (Fig. 1B and 2). The results also indicate that *pefI* and ORF7 are previously unrecognized members of the *rck* operon. Both of these genes are homologous to transcription factors.

Testing of fusions for a response to chemically synthesized AHLs. All previous reports on *sdiA* have utilized plasmid-based expression of *sdiA* to obtain regulatory effects greater than 40% on any particular transcriptional fusion (1, 43, 53). We hypothesized that SdiA is used to detect AHLs synthesized by other species of bacteria and that overexpression of *sdiA* bypasses the requirement for AHL (1). To test this hypothesis, we screened all previously identified *sdiA*-regulated transcriptional fusions for a response to chemically synthesized AHLs in the absence of *sdiA* overexpression. The fusion collection con-

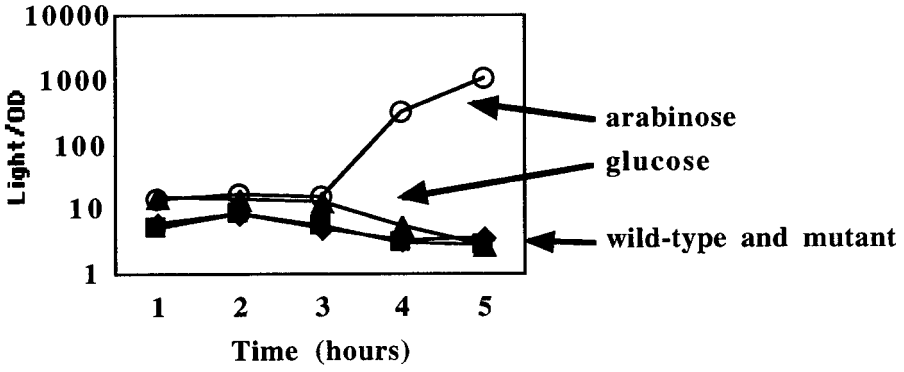

FIG. 2. Expression of the P*rck-luxCDABE* fusion, pBA428, in various strain backgrounds. Levels of expression are identical in the wild-type (14028/pBA428 [◆]) and *sdiA* mutant (BA612/pBA428 [■]) backgrounds. In a strain where *sdiA* is plasmid borne under the control of the arabinose-dependent *araBAD* promoter (BA612/pBA428/pBA321), P*rck-luxCDABE* expression is high in the presence of 0.2% arabinose (▲) and low in the presence of 0.2% glucose (53). Results shown are means of results from triplicate cultures. Error bars representing standard deviations were smaller than the symbols used and are therefore not shown.

sists of a plasmid-based *E. coli* P2*ftsQ-lacZYA* fusion (pCX39 [53]), 3 plasmid-based *Salmonella* serovar Typhimurium P*rck-luxCDABE* fusions (pBA403, pBA405, and pBA428 [this study]), and 10 *Salmonella* serovar Typhimurium MudJ insertion mutations, of which 3 are uncharacterized and 7 are located within the *rck* operon (1). Each fusion was tested using a filter disk assay. Individual reporter strains were placed in a soft agar layer on top of an LB plate. For the *lacZ* fusions X-Gal was included in the plates at 80 μg/ml. This relatively high concentration of X-Gal was chosen because it allowed perceptible detection of the background level of β-galactosidase activity coming from the MudJ fusions. Any further increase in β-galactosidase activity would then be readily detected visually. Filter disks impregnated with 100 pmol of synthetic AHLs were placed on top of each plate. At various times during incubation at 37°C the plates were examined for either blue halos surrounding the filter disks or, in the case of the *luxCDABE* fusions, luminescence surrounding the filter disks. This methodology was chosen because a concentration gradient of AHL is formed as the AHL diffuses away from the filter disk, so that all concentrations can be tested simultaneously.

The *E. coli* P2*ftsQ-lacZYA* fusion, pCX39, failed to respond to AHL when present in either *E. coli* or *Salmonella* serovar Typhimurium (data not shown). All of the *Salmonella* serovar Typhimurium MudJ fusions also failed to respond to AHL (data not shown). This includes the MudJ insertions that lie within the *rck* operon. Only the P*rck-luxCDABE* fusions pBA403, pBA405, and pBA428 responded to any of the AHLs (Fig. 3). The response did not occur in the isogenic *sdiA* mutant control and is therefore *sdiA* dependent. AHLs with a 3-oxo modifi-

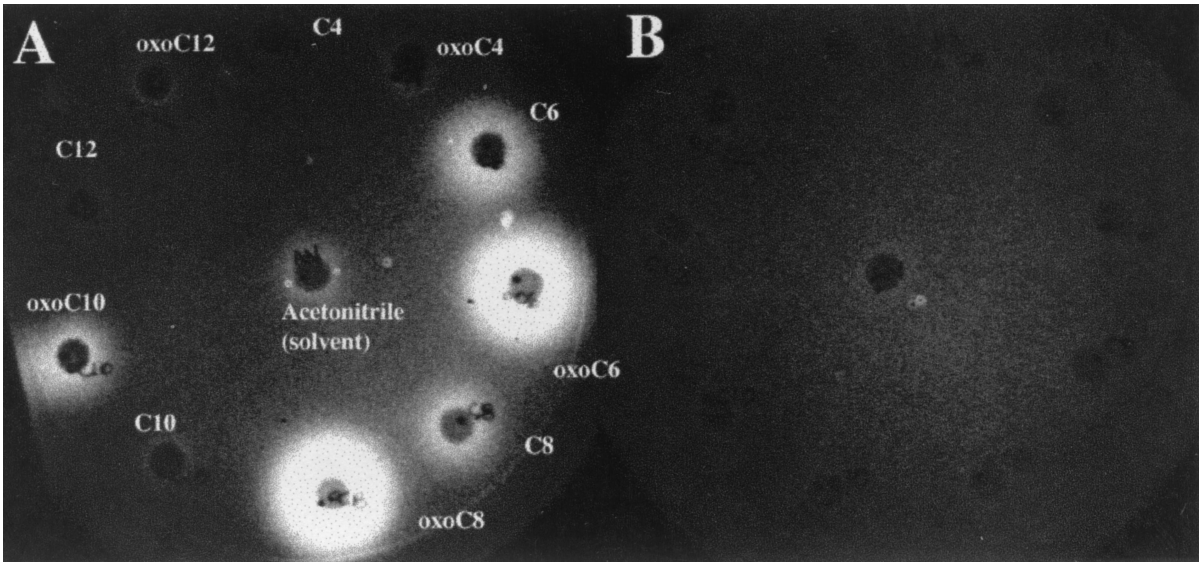

FIG. 3. Testing of individual chemically synthesized AHLs for activation of a P*rck-luxCDABE* fusion in either a wild-type background (14028/pBA405) (A) or the isogenic *sdiA* mutant background (BA612/pBA405) (B) using a filter disk assay. A soft agar overlay containing the appropriate reporter strain was poured onto an LB agar plate and allowed to harden at room temperature for 2 h. Filter disks impregnated with 100 pmol of the indicated AHL were then placed onto the overlay, and the plate was incubated at 37°C for 8 h. Similar results were obtained using reporter plasmids pBA403 and pBA428 (data not shown).

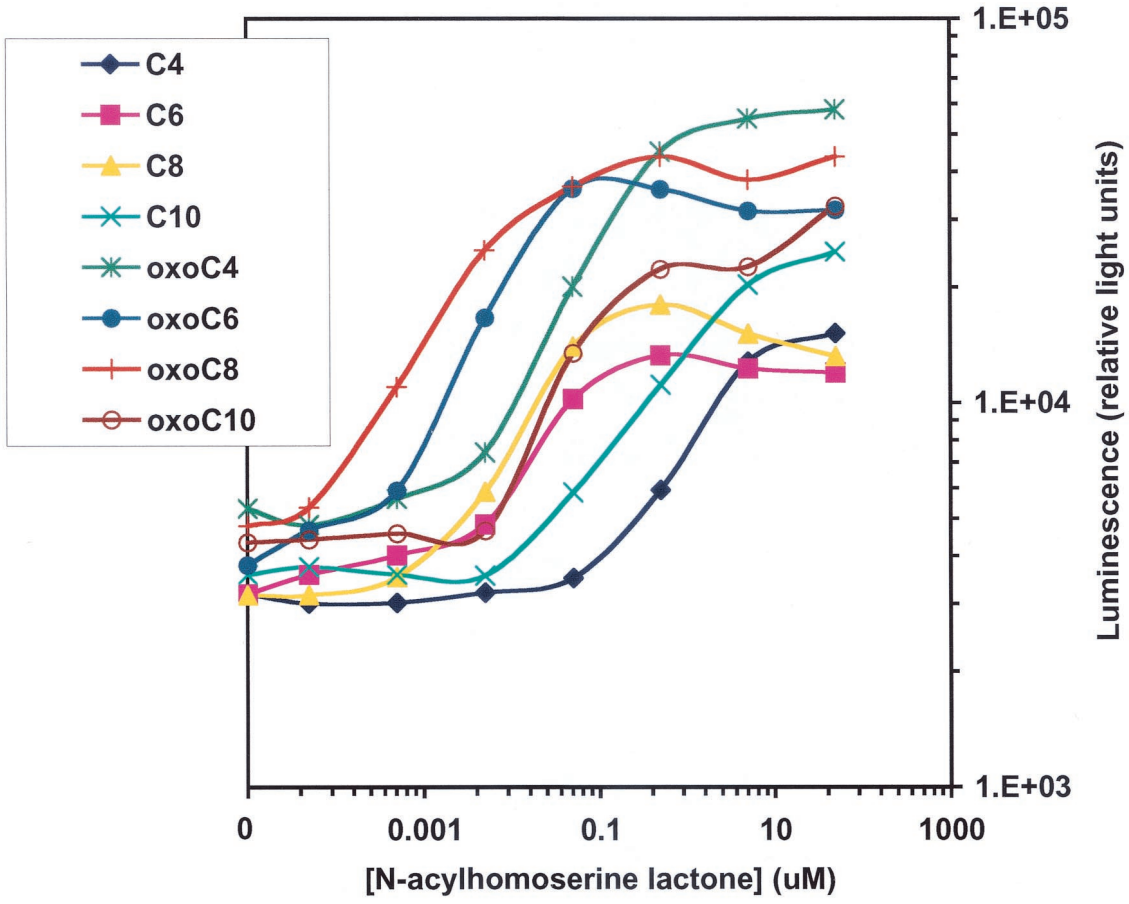

FIG. 4. Quantitation of *Salmonella* serovar Typhimurium responses to chemically synthesized AHLs. The reporter strain 14028/pBA428 was incubated in LB broth with 0.3% agar containing varying amounts of each AHL. The responses of triplicate cultures were measured after 6 h of incubation at 37°C. Error bars are not shown for clarity, but standard deviations did not exceed 32% of any value. This experiment is representative of experiments performed on four separate occasions.

cation and acyl chain lengths of 6 or 8 induced the greatest luminescence (Fig. 3). Of the three P*rck-luxCDABE* reporter plasmids, pBA428 contains the smallest promoter region and was chosen for further study of *sdiA*-dependent responses to AHL.

Quantification of the *sdiA*-dependent response of *Salmonella* serovar Typhimurium to AHLs. Dose-response experiments were performed to quantify the response of SdiA to each synthetic AHL using the 14028/pBA428 reporter (Fig. 4). It was determined that *Salmonella* serovar Typhimurium does not respond well to AHLs in liquid LB medium but responds best in LB semisolid agar (0.3, 0.75, or 1.5% agar). In liquid cultures *sdiA*-dependent responses were 3- to 5-fold, while on semisolid agar the responses reached 14- to 17-fold (data not shown). Therefore, we chose to grow the reporter strain in 0.3% agar in 96-well plates for the dose-response experiments for which results are shown. The plates were incubated at 37°C for 6 h to reach optimal response levels. The P*rck-luxCDABE* reporter responded to all of the AHLs tested at concentrations greater than 1 μM (Fig. 4). The two most potent AHLs tested were oxoC6 and oxoC8, which exhibited half-maximal responses between 1 and 5 nM (Fig. 4). Detectable concentrations in this range are similar to values obtained for other LuxR homolog reporter systems, suggesting that these are physiologically relevant detection limits (15, 31, 37, 58, 59).

***Salmonella* serovar Typhimurium responds in an *sdiA*-dependent manner to the presence of AHL-synthesizing bacterial species.** Numerous gram-negative bacterial species are known to synthesize the AHLs that are most readily detected by SdiA (oxoC6 and oxoC8). Therefore, we tested the ability of the *Salmonella* serovar Typhimurium reporter strain (14028/pBA428) and an isogenic *sdiA* mutant (BA612/pBA428) to respond to the presence of other bacterial species on an LB agar plate using a cross-streak assay. Several species that elicit *sdiA*-dependent responses were identified (Table 2 and Fig. 5A and B). The best responses were obtained with *Yersinia enterocolitica* and *Hafnia alvei* (induction ratios, 8- to 13-fold). It is not known what AHLs are synthesized by *Hafnia*, but AHL type activity has been detected from this genus before (49). *Y. enterocolitica* synthesizes both C6 and oxoC6, which are consistent with the specificity preferences of SdiA (50). An isogenic *yenI* mutant of *Y. enterocolitica* that lacks AHL synthetic capabilities was unable to elicit a response from the *Salmonella* serovar Typhimurium P*rck-luxCDABE* reporter (Fig. 5C and D). It is noteworthy that at a distance from the test species, the wild-type and *sdiA* mutant reporters produce equal levels of

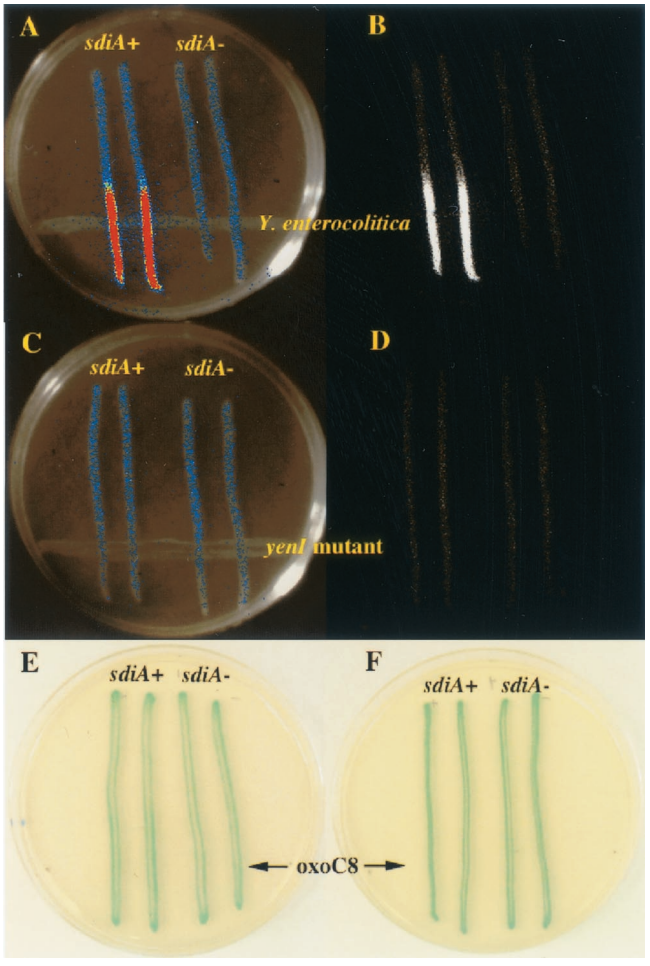

FIG. 5. Detection of *Y. enterocolitica* by *Salmonella* serovar Typhimurium. *Y. enterocolitica* strain NCTC 10460 is struck across the bottom of the plate (A and B). The wild-type *Salmonella* serovar Typhimurium reporter strain 14028/pBA428 is struck in duplicate perpendicular to the *Y. enterocolitica* on the left side of the plate (A through D). The isogenic *sdiA* mutant, BA612/pBA428, is struck in duplicate on the right side of the plate (A through D). Raw luminescence data are shown in panels B and D, while the pseudocolored version of the same data is shown in panels A and C. The luminescence of the wild-type reporter strain near the *Y. enterocolitica* strain is 13-fold greater than that of the *sdiA* mutant control (A and B). No response is obtained using an isogenic *yenI*::kan mutant of *Y. enterocolitica* (C and D). These data show that SdiA expressed from its natural position in the chromosome of *Salmonella* serovar Typhimurium can detect the physical proximity of other species that are capable of synthesizing AHLs. (E) MudJ insertions in the *rck* operon fail to respond to AHL. The wild-type *Salmonella* serovar Typhimurium reporter strain BA1103 (14028 *srgA1*::MudJ) is struck in duplicate on the left side of the LB kanamycin–X-Gal plate, while the isogenic *sdiA* mutant, BA1303, is struck in duplicate on the right side of the plate. Across the bottom is 20 μl of 10 μM oxoC8. All other *Salmonella* serovar Typhimurium MudJ insertions previously shown to respond to *sdiA* overexpression also failed to respond when present in the wild-type 14028 background in this assay (data not shown). (F) The *E. coli* P2*ftsQ-lacZYA* reporter fails to respond to AHL. The wild-type *E. coli* reporter strain UT481/pCX39 is struck in duplicate on the left side of the LB ampicillin–X-Gal plate, while the isogenic *sdiA* mutant, WX2/pCX39, is struck in duplicate on the right side of the plate. Across the bottom is 20 μl of 10 μM oxoC8. The *E. coli* P2*ftsQ-lacZYA* reporter and the *Salmonella* serovar Typhimurium MudJ insertions also failed to respond in cross-streak assays against *Y. enterocolitica*, in filter disk assays with synthetic AHL, and in liquid cultures supplemented with synthetic AHL (data not shown).

background luminescence. These results demonstrate that *Salmonella* serovar Typhimurium can detect the physical proximity of other species in an *sdiA*-dependent manner.

A. tumefaciens, *Chromobacterium violaceum*, and *V. fischeri* are known to produce the AHLs that *sdiA* most readily detects but failed to elicit a response from 14028/pBA428 using the standard cross-streak assay. However, by preincubation of the test strain on the plate for 16 h at lower temperatures, responses to both *C. violaceum* and *V. fischeri* were obtained (Table 2). *A. tumefaciens* was tested on both LB plates and AB mannitol plates at 22, 30, and 37°C. However, this species failed to stimulate the *Salmonella* serovar Typhimurium reporter strain under any condition (data not shown). This is probably due to the observation that the *A. tumefaciens* quorum-sensing system is activated by plant-derived octopine compounds which were not present in the assay (15).

Optimal assay conditions for SdiA and AI-2. To date, we have found that the maximal response from 14028/pBA428 is 14- to 18-fold using a cross-streak assay at 37°C in which chemically synthesized oxoC8 is spread across the plate, rather than any particular bacterial species (20 μl of 10 μM oxoC8 [data not shown]). Using this optimal assay the P2*ftsQ-lacZYA* reporter (pCX39) and the *Salmonella* serovar Typhimurium MudJ insertions all failed to respond to AHL (Fig. 5E and F).

AI-2 is a molecule produced by numerous bacterial species, including *E. coli* and *Salmonella* serovar Typhimurium, that induces *V. harveyi* luminescence genes (45). *E. coli* and *Salmonella* serovar Typhimurium were found to produce maximal levels of AI-2 during the exponential phase of growth in agitated LB broth containing 0.5% glucose (46). To determine if SdiA can respond to AI-2 production, the luminescence of the P*rck-luxCDABE* reporter (pBA428) was measured during growth under these conditions (in the absence of any exogenous AHL). Under these conditions, which are optimal for AI-2 production, the wild-type and *sdiA* mutant strains (14028/pBA428 and BA612/pBA428, respectively), produced identical levels of background luminescence throughout the growth curve (data not shown). This indicates that SdiA does not respond to AI-2.

Expression of *sdiA* from pSC101-based vectors results in SdiA activity in the absence of ligand. The first studies of *sdiA* that were performed in *E. coli* used low-copy-number pSC101-based vectors to express *sdiA* (43, 53). However, because *sdiA*-dependent activation of the *ftsQ* reporter (pCX39) does not occur in a wild-type background (even in the presence of AHL), we tested the possibility that the expression of *sdiA* from a low-copy-number vector may result in artificial activation (i.e., activation in the absence of ligand). Because only the P*rck-luxCDABE* fusions in *Salmonella* serovar Typhimurium respond to both conditions, the responses of the pBA428 reporter to chromosomal and plasmid-borne *sdiA* were compared. Expression of *sdiA* from a low-copy-number vector resulted in greater activation of the *rck* promoter than addition of AHL to a chromosomal copy of *sdiA* (Fig. 6). However, plasmid-borne *sdiA* still responded to AHL by further increasing the activity of the *rck* promoter. The concentration of oxoC8 resulting in half-maximal activation was between 1 and 5 nM regardless of whether *sdiA* was plasmid borne (Fig. 6).

Members of the *Escherichia*, *Salmonella*, and *Klebsiella* genera do not activate AHL biosensors. Members of the *Esche-*

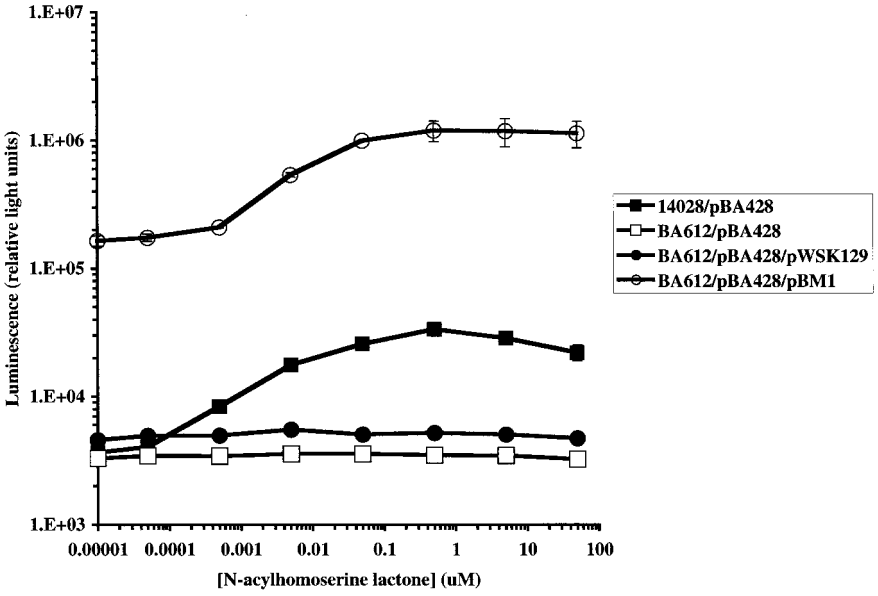

FIG. 6. Comparison of P*rck-luxCDABE* (pBA428) activation when *sdiA* is expressed from its natural position in the *Salmonella* serovar Typhimurium chromosome and when it is expressed from a pSC101-derived plasmid (pBM1). Strains were incubated for 6 h at 37°C in LB broth with 0.3% agar containing various concentrations of AHL before measurement of luminescence. Error bars represent standard deviations of triplicate samples.

richia, *Salmonella*, and *Klebsiella* genera are known to contain *sdiA* orthologs in their genomes. To test the hypothesis that SdiA does not detect signals emanating from the same species that contain *sdiA*, representatives of these three genera were tested for activation of the pBA428 reporter using the cross-streak assay (on an LB plate at 37°C). All seven type species of *Klebsiella*, six different strains of *E. coli* O157, the *E. coli* K-12 strain MG1655, a strain of *Shigella flexneri*, three strains of *Salmonella* serovar Typhimurium, two strains of *Salmonella enterica* serovar Typhi, and one strain each of *Salmonella enterica* serovar Gallinarum and *Salmonella enterica* serovar Pullorum were tested. All strains tested negative for the ability to elicit a response from the *Salmonella* serovar Typhimurium P*rck-luxCDABE* reporter under the conditions employed (Table 2).

The above strains were also tested for the ability to activate other AHL reporter systems. In cross-streak assays on LB plates at 37°C, all of the *Escherichia*, *Klebsiella*, and *Salmonella* strains failed to activate a LasR reporter (JM109/pSB1075 [57]), an AhyR reporter (DH5α/pSB536 [47]), and a LuxR reporter (JM109/pSB401 [57]) (data not shown). Therefore, these particular members of the *Escherichia*, *Salmonella*, and *Klebsiella* genera do not produce quantities of AHL that are detectable with these reporter systems and growth conditions.

DISCUSSION

Despite decades of research on *E. coli* and *Salmonella* serovar Typhimurium, microbiologists are still unable to assign, or even convincingly predict, functions for approximately 30% of the ORFs in the *E. coli* genome. A further 30% of the ORFs are predicted to encode proteins that belong to recognized families, but their specific functions remain undetermined (3). It seems likely that the functions of many of these genes are not observable using pure cultures. In nature, such bacteria do not normally exist as pure cultures and a percentage of their genetic capacity is almost certainly involved with "mixed-community" interactions.

In this report we have determined that *Salmonella* serovar Typhimurium can respond to the physical proximity of other bacterial species in an *sdiA*-dependent manner. This response requires that the other bacterial species be able to synthesize AHLs. *Salmonella* serovar Typhimurium detects chemically synthesized AHLs at nanomolar concentrations in an *sdiA*-dependent manner but fails to synthesize detectable quantities of these molecules under the conditions tested. SdiA also does not respond to AI-2 or any other molecule present in its own culture supernatant. Therefore, *sdiA* appears to encode a receptor that exclusively detects the signal molecules of other species.

This is the first study in which significant SdiA activity has been detected without the use of plasmid-based expression. In fact, expression of *sdiA* from even a low-copy-number plasmid vector leads to higher levels of *rck* promoter activation than does addition of AHL to a wild-type cell. Studies of other LuxR homologs indicate that binding of AHL promotes dimerization or some higher form of oligomerization, which is required for promoter activation (40, 44, 55, 60). Therefore, our working model for SdiA is that in pure culture (in the absence of AHL) SdiA is found primarily as a monomer in the cell. Because monomers and dimers are in equilibrium, a small percentage of the SdiA in a cell may be found as dimers even in the absence of AHL. When *sdiA* is expressed from a plasmid, the concentration of the SdiA monomer is elevated and there is a corresponding increase in the equilibrium concentration of the dimer. The number of dimers in this situation appears to exceed the number achieved with chromosomal expression of *sdiA* even in the presence of AHL (based on the

level of reporter activation shown in Fig. 6), although the equilbrium still greatly favors the monomeric form. When AHL is added to cells with plasmid-borne *sdiA*, a high number of monomers in the cell are converted to dimers, with a corresponding increase in reporter activity (Fig. 6). Figure 6 also demonstrates that the half-maximal response to oxoC8 occurs between 1 and 5 nM regardless of whether *sdiA* is expressed from the chromosome or from a plasmid.

It is unclear why the *ftsQ* locus and the three uncharacterized loci in *Salmonella* serovar Typhimurium respond to plasmid-borne *sdiA* but fail to respond to chromosomal *sdiA* and AHL. The question arises whether these loci ever respond to SdiA and AHL in nature. It is possible that these are only weakly regulated or indirectly regulated promoters that never respond significantly in nature. Alternatively, it is possible that environmental conditions exist that increase the expression of *sdiA* from the chromosome and/or remove competitive regulatory influences. The regulation of genes that respond to plasmid-borne *sdiA*, and the regulation of *sdiA* itself, must be studied in natural environments to address these questions.

It is also not clear why the plasmid-based P*rck-luxCDABE* fusions respond to chromosomal *sdiA* and AHL, but the MudJ insertions in the *rck* operon do not. The explanation cannot be a difference in sensitivity between the *luxCDABE* fusions and the *lacZ* fusions, because in Fig. 5E and F the background levels of *lacZ* activity are clearly observed (by including high levels of X-Gal in the plate), yet there is no increase in activity near the AHL cross-streak. (Even twofold differences in β-galactosidase activity are easily detected visually.) We have also eliminated the polarity of the MudJ insertions as a viable explanation (unpublished data). Given that there are three putative transcription factors encoded within the *rck* operon, there may not be a simple answer. Regardless, the *sdiA*-dependent activation of the P*rck-luxCDABE* fusions reported here is the first demonstration of a phenotype for *sdiA* expressed from its natural position in the chromosome.

It has been hypothesized that quorum sensing may be used by pathogenic species to prevent the expression of virulence factors until the population of bacteria has increased to a point at which victory is ensured. In the commonly studied quorum-sensing systems, the areas of colonization have only low populations of other bacterial species (e.g., the squid light organ for *V. fischeri*, plant wound sites for *Erwinia* and *Pseudomonas* species, human lung tissues for *P. aeruginosa*). In contrast, the intestinal environment has microbial population densities reaching 10^{11} cells/g of fecal contents (reviewed in reference 25). Given that *Salmonella* serovar Typhimurium is most often associated with the intestinal environment, we hypothesize that instead of using LuxR homologs to gauge the population density of its own species, *Salmonella* serovar Typhimurium (and possibly *E. coli* and *Klebsiella* species as well) appears to have dispensed with any AHL synthase genes it may have had in the past and instead uses SdiA to detect the AHLs produced by other species of bacteria. If this is true, then SdiA is technically not a "quorum sensor" but instead is strictly an AHL receptor. Interestingly, there are no reports of AHLs in the intestinal environment, and no members of the normal intestinal flora have been reported to synthesize AHLs. We are currently examining the intestinal environment for the presence of AHLs and screening individual members of the normal flora for AHL production.

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