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Effect of *sdiA* on Biosensors of *N*-Acylhomoserine Lactones

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Many gram-negative bacteria synthesize *N*-acylhomoserine lactones (AHLs) and then use transcription factors of the LuxR family to sense and respond to AHL accumulation in the environment; this phenomenon is termed quorum sensing. Bacteria produce a variety of AHLs, and numerous bacterial reporter strains, or biosensors, that can detect subsets of these molecules have been constructed. Many of these are based on *Escherichia coli* because this species does not produce AHLs. However, both *Escherichia* and *Salmonella* spp. contain a LuxR homolog named SdiA that can detect exogenous AHL synthesized by other microbial species. In this study we have determined that *sdiA* of *E. coli* and *Salmonella* spp. can activate an RhlR-based biosensor plasmid in response to AHLs other than what the biosensor was designed to detect. SdiA does not activate LuxR-, LasR-, or AhyR-based biosensor plasmids, although the presence of *sdiA* in *E. coli* does interfere with the function of the AhyR-based biosensor. Because *sdiA* interferes with the function of two of the four reporters, we have constructed a set of *E. coli* biosensor strains that lack *sdiA*. The set includes control reporters that allow the *luxR* dependence of responses to be determined.

Bacteria have mechanisms by which they sense their own population density and adjust their behavior accordingly, termed quorum sensing (3). In this way individual bacteria can regulate their activities with others in their population, resulting in coordinated behavior that often resembles that of a multicellular organism. The prototype for gram-negative quorum sensing is the colonization of the light organ of the bobtail squid, *Euprymna scolopes*, by *Vibrio fischeri* (5, 6). The bacteria in this relationship use the LuxI enzyme to synthesize primarily *N*-(3-oxo-hexanoyl)-L-homoserine lactone (oxoC6) (7, 18). This molecule can diffuse freely across bacterial membranes and is bound by the LuxR transcription factor (10). A high population density of *V. fischeri* in the light organ leads to the accumulation of oxoC6, with the result being light production (22). The LuxR protein activates transcription of the *luxICDA BEGH* operon in response to oxoC6, producing the luciferase enzyme subunits, substrate synthesis enzymes, and more LuxI. Given that light production by an individual bacterium is likely to be ineffective, this presumably prevents individuals from wasting energy until a suitably large population has been assembled. Quorum sensing by the use of LuxR and LuxI homologs has now been documented in numerous gram-negative bacterial species, including many pathogens, which use quorum sensing to regulate genes involved with the colonization of eukaryotic hosts (21).

The LuxR/LuxI homologs from various bacterial species utilize *N*-acylhomoserine lactones (AHLs) that vary in length, oxidation, and saturation of the acyl chain. For example, *Pseudomonas aeruginosa* contains two pairs of LuxR/LuxI homologs, neither of which optimally detects oxoC6. LasI synthesizes *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (oxoC12), which is detected by LasR (15). RhlI synthesizes *N*-butanoyl-L-homoserine lactone (C4), which is detected by RhlR (16, 17).

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Bacterial species of the genera *Escherichia*, *Salmonella*, and *Klebsiella* are unique in that they have a LuxR homolog, SdiA, but they do not contain a LuxI homolog or any other enzyme family that can synthesize AHLs (1). The function of SdiA is most understood in *Salmonella enterica* serovar Typhimurium. In this organism, SdiA detects AHLs produced by other bacterial genera (14, 19). SdiA then activates two *Salmonella*-specific loci, the *rck* (resistance to complement killing) operon, which is carried on the *Salmonella* virulence plasmid, and *srgE* (*sdiA*-regulated gene), which is carried in the chromosome but is of unknown function (2, 19). The *rck* operon includes six genes, three of unknown function and three that play a role in adhesion to extracellular matrix and/or host cells and resistance to complement killing (1).

E. coli and *Klebsiella* spp. also carry *sdiA*, but the function of *sdiA* in these organisms is unclear (1). No experiments have been performed on the *sdiA* genes of *Klebsiella* spp., and the results from experiments performed with *E. coli* are difficult to interpret. To date, all information on *sdiA* in *E. coli* has been obtained using *sdiA* overexpression. SdiA overexpression in *E. coli* results in a large pleiotropic response that is probably not an accurate indication of the natural function (the pleiotropic effect of *sdiA* overexpression is not observed with *Salmonella*). The genes affected by *sdiA* overexpression in *E. coli* have never been demonstrated to respond significantly to *sdiA* expressed from its natural position in the chromosome. However, a plasmid-encoded transcriptional fusion to the *Salmonella* gene *srgE* is responsive to *sdiA* and AHL in *E. coli*. Therefore, although the target genes are not known, *sdiA* is functional in *E. coli* (1).

Despite the presence of *sdiA* in *E. coli*, this organism has been used as the host organism for several AHL biosensor strains. This is for good reason as *E. coli* has the advantages of not producing AHLs, being safe, and being easy to manipulate. *E. coli* carrying a plasmid that encodes both a LuxR homolog and a transcriptional fusion regulated by that LuxR homolog can be used as a biosensor that responds to those AHLs that are specifically bound by that particular LuxR homolog. A

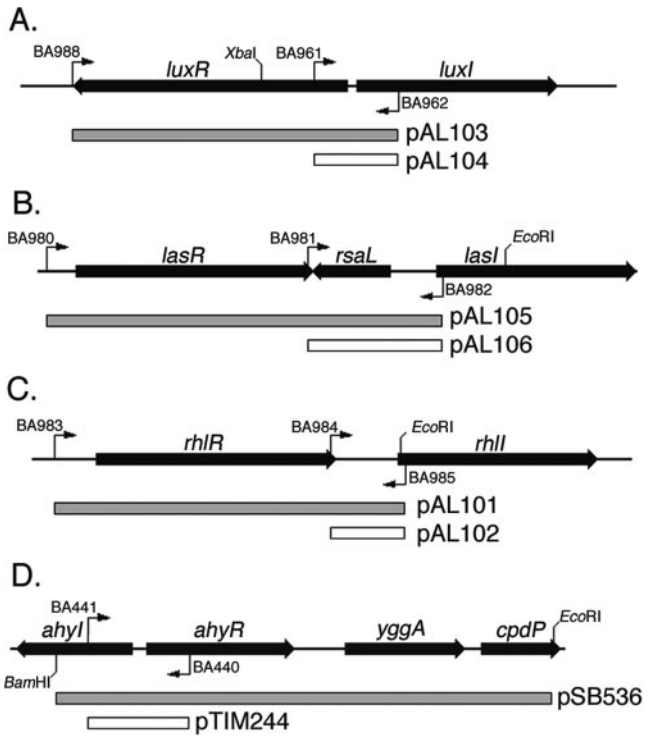

FIG. 1. Map of the DNA fragments used to make the biosensor plasmids. The PCR primers used and important restriction sites are shown. The DNA sequence of each primer is listed in Table 1. Templates: the *luxRI* region of *V. fischeri* (pSB401) (A), the *lasRI* region of *P. aeruginosa* (PA01) (B), the *rhlRI* region of *P. aeruginosa* (PA01) (C), and the *ahyRI* region of *A. hydrophila* (pSB536) (D).

description of a set of these biosensor strains was published previously (23, 24). The set consists of a plasmid carrying *luxR* and a *luxR*-regulated fusion that optimally detects oxoC6 (pSB401), a plasmid carrying *lasR* and a *lasR*-regulated fusion that optimally detects oxoC12 (pSB1075), and a third plasmid carrying *rhlR* and an *rhlR*-regulated fusion that optimally detects C4 (pSB406). A description of another plasmid that carries the *Aeromonas hydrophila* gene *ahyR* and an *ahyR*-regulated fusion (pSB536) was published separately (20). Like RhlR of *P. aeruginosa*, AhyR optimally detects C4. In each case, the fusion is to the *luxI* homolog fortuitously located adjacent to the *luxR* homolog (Fig. 1).

A limitation of these biosensor plasmids is that an increase in light production does not necessarily indicate the presence of AHLs. This is because the luciferase enzyme and the enzymes that produce the substrate for the luciferase reaction require energy, oxygen, and reducing equivalents (8). Therefore, light production can increase and decrease independently of the *luxR* homolog and AHL. In this study, we have constructed a set of control plasmids that lack the *luxR* homolog of interest. This control plasmid set allows the *luxR* dependence of light production to be determined. Additionally, we have found that *sdiA* of *E. coli* interferes with two of the biosensors. A set of strains lacking *sdiA* and carrying the various biosensor plasmids or control plasmids has been assembled for general use.

TABLE 1. Oligonucleotides used

Name	DNA sequence
BA440	GGGATCACAGGCCAGCATATGGTTG
BA441	GAAGATGCTGGGCAGCATGTAATCC
BA961	GCTCTAGACAATAGGAACTTCCCTATGTGTCGTCGGCATTTATGT
BA962	GAGAGATCTCATTATAGTCATACCAACCTCCCTT
BA980	GCGTCTAGAGAATAGGAACTTCCCTCTCGGACTGCCGTACAACGT
BA981	GCTCTAGAGAATAGGAACTTCCCATTTGGGTCTTATTACTCTCTG
BA982	GAGAGATCTGCGCCGACCAATTTGTACGATCAT
BA983	GCTCTAGAGAATAGGAACTTCCCAACGGTGCTGGCATAACAGATA
BA984	GCTCTAGAGAATAGGAACTTCCCCGCGGCGCTGGGTCTCATCTGA
BA985	GAGAGATCTCAGCGATTCAGAGAGGAATTCGATC
BA988	GCTCTAGAGAATAGGAACTTCCCTTAATTTTTAAAGTATGGGCAA

Construction of AHL biosensor and control plasmids. Isogenic sets of plasmids that differ only in the presence or absence of the *luxR* homolog of interest were constructed. The plasmids carrying the *luxR* homolog contain fragments essentially identical to those used in the biosensor plasmids described previously (23, 24), whereas the control reporters carry an abbreviated, but otherwise identical, version of the full fragments, so that the *luxR* homolog is absent and only the *luxI* promoter fusion remains. Unlike the previous reporters, these sensors are all in the same plasmid backbone. The exception is the *ahyR* biosensor, for which we used the original reporter plasmid pSB536 and constructed only a control plasmid lacking the *ahyR* gene (20). All plasmids were constructed by using PCR to amplify the appropriate *luxR* homolog (*lasR*, *luxR*, or *rhlR*) along with the *luxR*-regulated promoter located adjacent to each homolog (the *lasI*, *luxI*, or *rhlI* promoter). Primers used are shown in Fig. 1, and the DNA sequence is listed in Table 1. The control plasmids were made by amplifying only the promoter region without the *luxR* homolog. The resulting fragments are shown in Fig. 1. These were cloned into the pCR 2.1-TOPO vector (Invitrogen) and then removed as EcoRI fragments. The EcoRI fragments were then cloned into pSB401 that had been digested with EcoRI, replacing the *luxRI′* fragment of pSB401. This places each fragment upstream of the promoterless *Photorhabdus luminescens luxCD ABE* operon in pSB401. The *ahyI* promoter fragment was cloned into the pSB377 EcoRI site to match the origin of replication and antibiotic resistance marker of the *ahyR*-containing plasmid pSB536 (20, 23, 24) (Fig. 1D). Each construct was confirmed using PCR screening, restriction digestion, and DNA sequencing. The plasmids were then transformed into wild-type and *sdiA* mutant strains of *E. coli* and *S. enterica* (WM54, JLD271, 14028, and BA612) (Table 2). Used in conjunction with the plasmids containing the functional *luxR* homolog (referred to as $luxR^+$, $lasR^+$, $rhlR^+$, or $ahyR^+$), the control plasmids of each set (lacking *luxR*, *lasR*, *rhlR*, or *ahyR*) allow the *luxR* dependence of any response to be determined. Comparison of the reporters in the presence and absence of *sdiA* allows the influence of *sdiA* to be determined.

TABLE 2. Strains and plasmids used

Strain or plasmid	Genotype or description	Source or reference[a]
Strains		
WM54	*E. coli* K-12 Δ*lacX74*	Bill Metcalf
JLD271	WM54 *sdiA271*::Cam	Wanner mutagenesis, deleting nt 8137 to 8856 of accession no. AE000284 and replacing with Cam^r of pKD3 (4)
PAO1	Wild-type *P. aeruginosa*	9
14028	Wild-type *S. enterica* serovar Typhimurium	American Type Culture Collection
BA612	14028 *sdiA1*::mTn*3*	2
Plasmids		
pSB377	*luxCDABE* transcriptional fusion vector, Amp^r ColE1 origin	24
pSB401	*luxR*$^+$ *luxI*::*luxCDABE*; Tet^r p15A origin	23
pSB1075	*lasR*$^+$ *lasI*::*luxCDABE*; Amp^r ColE1 origin	23
pSB536	*ahyR*$^+$ *ahyI*::*luxCDABE*; Amp^r ColE1 origin	20
pTIM244	*ahyI*::*luxCDABE*; Amp^r ColE1 origin	PCR with pSB536 template and oligos BA440 and BA441; cloned into pSB377/EcoRI
pAL101	*rhlR*$^+$ *rhlI*::*luxCDABE*; Tet^r p15A origin	PCR with PA01 template and oligos BA983 and BA985; cloned into pSB401/EcoRI
pAL102	*rhlI*::*luxCDABE*; Tet^r p15A origin	PCR with PA01 template and oligos BA984 and BA985; cloned into pSB401/EcoRI
pAL103	*luxR*$^+$ *luxI*::*luxCDABE*; Tet^r p15A origin	PCR with pSB401 template and oligos BA988 and BA962; cloned into pSB401/EcoRI
pAL104	*luxI*::*luxCDABE*; Tet^r p15A origin	PCR with pSB401 template and oligos BA961 and BA962; cloned into pSB401/EcoRI
pAL105	*lasR*$^+$ *lasI*::*luxCDABE*; Tet^r p15A origin	PCR with PA01 template and oligos BA980 and BA982; cloned into pSB401/EcoRI
pAL106	*lasI*::*luxCDABE*; Tet^r p15A origin	PCR with PA01 template and oligos BA981 and BA982; cloned into pSB401/EcoRI

[a] Abbreviations: nt, nucleotides; oligos, oligonucleotides.

LuxR and LasR. The *luxR*$^+$ and *lasR*$^+$ biosensors respond to 10 nM oxoC6 and 10 nM oxoC12 in *E. coli*, respectively, while the isogenic *luxR*- and *lasR*-negative plasmids do not detect AHL (Fig. 2A and 2B). Under the conditions tested, the presence or absence of *sdiA* did not have an effect on the response to AHL by either the biosensor or the control plasmids. Similar results were observed using *Salmonella* as the host strain (the wild-type strain 14028 versus the *sdiA* mutant strain BA612) (Fig. 3A and 3B). For unknown reasons, the basal level of luminescence of the *luxR*-negative control plasmid is higher than that of the *luxR*$^+$ plasmid. However, this does not affect the utility of the control plasmid in determining the *luxR* dependence of reporter activity. Therefore, the *luxR* and *lasR* biosensors are able to respond appropriately to the presence and absence of exogenous AHLs, and *sdiA* does not contribute to, or interfere with, AHL detection.

AhyR. In the absence of *sdiA*, the *ahyR* reporter system works as expected in *E. coli*. During growth in the presence of C4 at 100 nM or higher concentrations, the *ahyR*$^+$ plasmid produces more luminescence than when it is grown in the presence of the solvent control or oxoC6 (Fig. 2D). However, the maximal difference achieved did not exceed fourfold at 100 μM C4. This attribute of the system does not make it particularly useful as a biosensor, and we have found that the *rhlR* system is much better (see below). Therefore, this reporter was not tested further in *Salmonella*. For unknown reasons, the basal level of luminescence from the *ahyR*-negative plasmid is more than an order of magnitude higher than the *ahyR*$^+$ plasmid. This is similar to what was observed with the *luxR* reporter set. As with the *luxR* set, the control plasmid shows no AHL-dependent activation of the *ahyI* promoter in the absence of *ahyR*.

Unlike with *luxR* or *lasR*, the presence of *sdiA* interferes with the function of the *ahyR*$^+$ biosensor, pSB536. Reporter activity of the *ahyR*$^+$ plasmid is reduced between 10- and 100-fold by the presence of *sdiA* (Fig. 2D). Reporter activity of the control plasmid is similar in the presence and absence of *sdiA*, indicating that SdiA does not directly activate or repress the *ahyI* promoter itself. Because interference by *sdiA* requires the presence of *ahyR*, this suggests that SdiA and AhyR may oligomerize in the cell, forming inactive complexes. Alternatively, SdiA may compete with AhyR for binding to the *ahyI* promoter but fail to activate transcription. Regardless of the mechanism, these results show that *sdiA* of *E. coli* interferes with the *ahyR*-dependent activation of the *ahyI* promoter. Therefore, if used, the *ahyR* reporter system should be used only in an *sdiA* mutant background, though the *rhlR* reporter system is preferable.

RhlR. In an *E. coli sdiA* mutant background, the *rhlR*$^+$ biosensor detects 1 μM C4 or 10 μM oxoC6, while the *rhlR*-negative plasmid does not detect AHLs (Fig. 2C). The values for the *rhlR*-negative controls in the *sdiA* mutant background are not visible in Fig. 2C because the values at every point were slightly above or below zero. This indicates that, as with the *luxR* and *ahyR* plasmid pairs, the *rhlR*$^+$ and *rhlR*-negative plasmids have different basal levels of luminescence. In this case that of the *rhlR*$^+$ plasmid is higher. However, the fold induction of the *rhlR*$^+$ nears three orders of magnitude (913-fold) in response to 100 μM C4, making the *rhlR* biosensor much more useful than the *ahyR* biosensor described above. Similar results

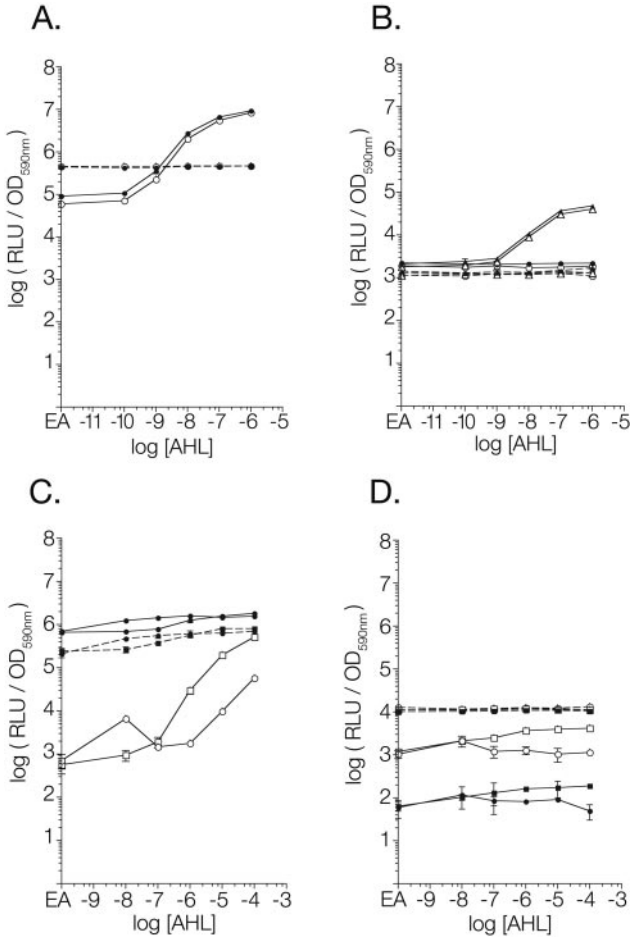

FIG. 2. Response of biosensors to AHL and *sdiA* in *E. coli*. Overnight cultures of each strain were subcultured 1:100 into 200 μl of LB broth containing AHL or the solvent control (0.1% ethyl acetate) in a 96-well plate with black side walls. The plate was incubated at 37°C for 9 hours before being placed into a Wallac Victor2 multimode plate reader for recording of the culture optical density at 590 nm (OD_{590nm}) and the luminescence of the culture in relative light units (RLU). Data were analyzed using Prism 3.0 graphing software. (A) LuxR biosensor set pAL103 (*luxR*$^+$) and pAL104 (*luxR* negative). (B) LasR biosensor set pAL105 (*lasR*$^+$) and pAL106 (*lasR* negative). (C) RhlR biosensor set pAL101 (*rhlR*$^+$) and pAL102 (*rhlR* negative). (D) AhyR biosensor set pSB536 (*ahyR*$^+$) and pTIM244 (*ahyR* negative). Closed symbols, *sdiA*$^+$; open symbols, *sdiA* negative; solid lines, *luxR* homolog present; dashed lines, *luxR* homolog absent; circles, oxoC6; triangles, oxoC12; squares, C4. EA on the *x* axis represents an AHL concentration of zero in which there is only the solvent ethyl acetate. Each point represents the mean of results from triplicate cultures, with error bars representing standard deviations.

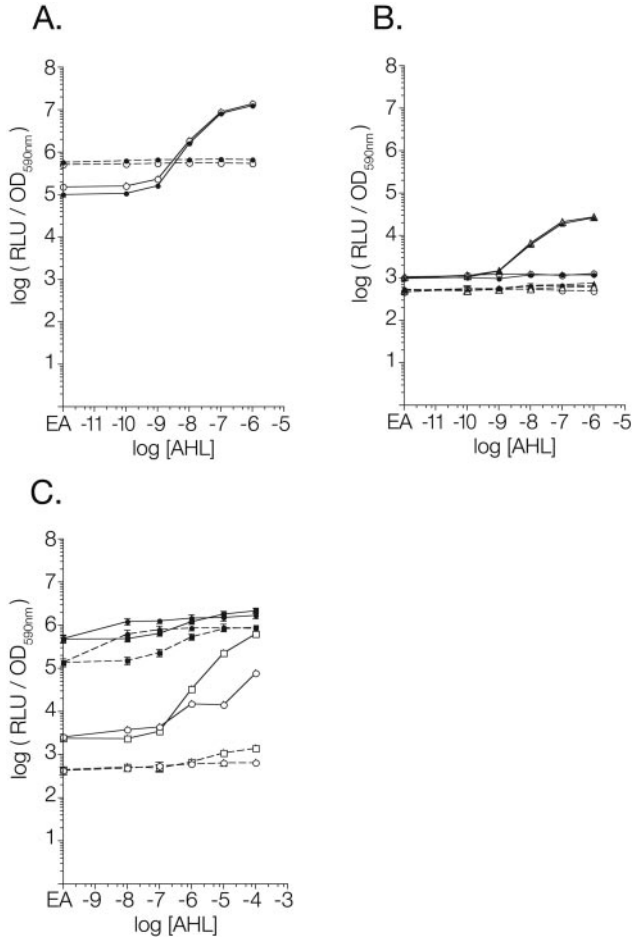

FIG. 3. Response of biosensor plasmids to AHL and *sdiA* in *Salmonella*. Data were obtained and analyzed as described for *E. coli* in the legend to Fig. 2. (A) LuxR biosensor set pAL103 (*luxR*$^+$) and pAL104 (*luxR* negative). (B) LasR biosensor set pAL105 (*lasR*$^+$) and pAL106 (*lasR* negative). (C) RhlR biosensor set pAL101 (*rhlR*$^+$) and pAL102 (*rhlR* negative). Closed symbols, *sdiA*$^+$; open symbols, *sdiA* negative; solid lines, *luxR* homolog present; dashed lines, *luxR* homolog absent; circles, oxoC6; triangles, oxoC12; squares, C4. EA on the *x* axis represents an AHL concentration of zero in which there is only the solvent ethyl acetate. Each point represents the mean of results from triplicate cultures, with error bars representing standard deviations. OD_{590nm}, optical density at 590 nm.

with the *rhlR* reporters were seen by using *Salmonella* host strains (Fig. 3C).

The most striking result is that the presence of *sdiA* in the host strain strongly activates the *rhlI* promoter, with the addition of AHL increasing expression only a further two- to threefold. Essentially, *sdiA* is constitutively activating the *rhlI* promoter to a level that is achieved by *rhlR* only in the presence of 100 μM C4 (Fig. 2C). Interestingly, Winson et al. used *sdiA*$^+$ *E. coli* host strains when describing the *rhlR* biosensor system, and they too observed only a two- to threefold induction in response to AHL (23). Based on our results, we believe that they were observing almost full induction of the reporter, even in the absence of AHL, due to the presence of *sdiA* in the host strain. With the removal of *sdiA*, the *rhlR* reporter system becomes much more useful, with a 53-fold response to 1 μM C4 and a 913-fold response to 100 μM C4.

Of the LuxR homologs discussed here, RhlR is the most closely related to SdiA (12). Given that *sdiA* is activating the *rhlI* promoter, it appears that RhlR and SdiA may have an overlapping DNA binding specificity, although they have diverged in the AHLs detected. While RhlR optimally detects C4, SdiA optimally detects oxoC6 and oxoC8, the same AHLs detected by LuxR and TraR. However, SdiA binds a fairly wide range of AHLs at what are likely to be physiologically relevant concentrations, possibly because SdiA has no corresponding

AHL synthase gene and instead is used to detect AHLs produced by other species (1, 14, 19). Most intriguing is that *sdiA* is activating the *rhlI* promoter in the absence of AHL. One possibility is that SdiA binds DNA regardless of AHL. Upon binding AHL, a conformational change that allows activation of transcription may take place. Since the *rhlI* promoter is not a native promoter for SdiA, for unknown reasons binding of SdiA alone may be sufficient for activation. This would be consistent with recent findings in which AhyR and RhlR were found to bind DNA regardless of the presence of AHL (11, 13). Additionally, an in vivo DNA methylation protection assay suggested that RhlR binds its target DNA sequence in a different conformation when AHL is present. The AHL-bound conformation was competent for transcription activation, while the other conformation was not (13). Future biochemical studies of SdiA and RhlR will be required to determine the mechanism by which *sdiA* causes activation of the *rhlI* promoter. Whatever the mechanism, *sdiA* should not be present in *rhlR* biosensor strains.

In this report we have provided to the scientific community a description of an isogenic set of biosensor strains that detect a range of AHLs. This set utilizes the transcription factors LasR, LuxR, and RhlR and was based on the original set published by Winson et al. (23, 24). We have added a control strain for each transcription factor that lacks the *luxR* homolog, thus allowing a definitive determination of *luxR* dependence for the observed responses. Additionally, we have determined that *sdiA* in the *E. coli* host strain is a serious impediment to the use of the RhlR biosensor. To avoid any potential complications arising from *sdiA*, the entire reporter set is available in an *sdiA* mutant background.

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