IDENTIFICATION OF THE *SDIA* REGULON OF *ESCHERICHIA COLI* K-12 AND THE INFLUENCE OF *SDIA* ON *N*-ACYL HOMOSERINE LACTONE BIOSENSORS

A Thesis

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ABSTRACT

Quorum sensing is a process employed by bacteria in which they coordinate behavior by regulating gene expression by sensing their surrounding population density. To do this, many gram-negative bacteria encode a *luxI* homolog to synthesize a form of *N*-acylhomoserine lactone (AHL), and a *luxR* homolog to sense and respond to AHL accumulation in the environment. Species of the *Escherichia*, *Salmonella*, and *Klebsiella* genera encode the *luxR* homolog, *sdiA*, without the cognate *luxI* homolog or any other AHL signal synthase and instead use *sdiA* to detect AHLs synthesized by other species of bacteria. A number of biosensor reporter systems that incorporate a *luxR* homolog (such as *lasR*, *rhlR* or *ahyR*), and its target promoter have been constructed to detect particular AHLs. These are often based in *Escherichia coli* K-12 despite the fact that *E. coli* encodes a *luxR* homolog, in part because the organism does not produce AHLs. Here we report that *sdiA* of *E. coli* and *Salmonella enterica* serovar Typhimurium do not have an effect on the LuxR or LasR-based biosensor plasmids tested, but it activates the RhlR-based biosensor even in the absence of AHL and inhibits activation of the AhyR-based biosensor. Because of this, we have constructed a group of biosensor plasmids and isogenic controls based in an *E. coli sdiA* mutant to eliminate interference by *sdiA*. However, it is unclear which native genes in *E. coli* K-12 are regulated by *sdiA*. Previous assays to identify the *sdiA* regulon were based on overexpression of *sdiA*, which by-

passes the AHL requirement for *sdiA* regulation, and appears to permit binding of non-target promoters. We therefore performed a genetic screen to identify members of the *sdiA* regulon of *E. coli* K-12 that used chromosomal expression of *sdiA* and reporter fusions. A library of 10,000 random *luxCDABE* transcriptional fusions was screened for regulation by oxoC6 and three fusions were isolated. One fusion demonstrated decreased luminescence in the presence of AHL. The other two fusions showed an increase in luminescence and one was identified as *gadW*, a regulator of glutamate-dependent acid resistance (GDAR), a system exclusive to *E. coli* that is involved in the acid stress response. The other AHL-regulated regions encode hypothetical proteins of unknown function, though previous studies suggest they may be involved in stress responses also.

Dedicated to my loving family

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in memory of my Maxie

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LIST OF ABBREVIATIONS

AHL(s)	*N*-acyl homoserine lactone(s)
amp	ampicillin
AR	acid resistance
ATCC	American Type Culture Collection
LB	Luria-Bertani
C4	*N*-butanoyl-L-homoserine lactone
C6	*N*-hexanoyl-L-homoserine lactone
cam	chloramphenicol
EA	acidified Ethyl Acetate
GDAR	glutamate-dependant acid resistance
kan	kanamycin
OD	optical density
oxoC6	*N*-(3-oxo-hexanoyl)-L-homoserine lactone
oxoC8	*N*-(3-oxo-octanoyl)-L-homoserine lactone
oxoC12	*N*-(3-oxo-dodecanoyl)-L-homoserine lactone
RLU	relative light units
tet	tetracycline
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

Many species of bacteria have mechanisms by which they sense their own population density (4). This type of regulation is called quorum sensing and allows individuals of a population to synchronize their activities with others in their population, resulting in coordinated behavior that often resembles that of a multicellular organism (4). For this type of coordination, the bacteria synthesize and detect a small signal molecule, also known as a pheromone or autoinducer (AI), the nature of which varies depending on the specific species involved (16). The cells within a population produce a certain signal that either diffuses or is actively transported into the extracellular environment. As the population density increases, the concentration of AI increases and a threshold concentration is attained, which represents a quorum of cells. The signal is then bound by a receptor protein, which then regulates gene expression in response (2, 4).

LuxR

Gram-negative bacteria most often use various forms of *N*-acyl homoserine lactone, or AHL, as their quorum sensing signal (62). These molecules can differ in the length, oxidation, and saturation of the acyl side chain (Figure 1.1) (2). The exact

structure of the AHL depends on the bacterial species, as each synthesize and sense a specific AHL or set of AHLs (23).

The prototype for gram-negative quorum sensing is the LuxR system of *Vibrio fischeri* that colonizes the light organ of the Bobtail Squid (*Euprymna scolopes*) and uses the system to regulate production of bioluminescence (15, 23, 67). These bacteria use the signal synthase LuxI to synthesize *N*-(3-oxo-hexanoyl)-L-homoserine lactone (oxoC6) as their signal, which leaves the cell by diffusing freely across the bacterial membrane (17, 18, 54). A high population density of *V. fischeri* in the light organ of the Bobtail Squid leads to the accumulation of oxoC6, which is bound by the receptor protein, LuxR (29, 38, 54). With oxoC6 bound, LuxR multimerizes and is able to bind DNA and regulate expression of target genes (60). LuxR represses transcription of its own gene and activates transcription of the adjacent *luxICDABE* operon, which not only encodes more LuxI, but also the luciferase enzyme responsible for light production, and the proteins that synthesize the substrates for the luciferase reaction (15, 18, 19). Given that light production by an individual bacterium is likely to be ineffective, this presumably prevents individuals from wasting energy unless a suitably large population is present.

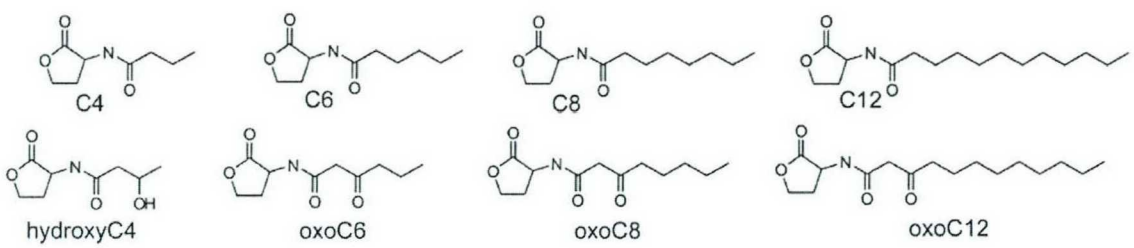

Figure 1.1 Structures of common AHLs. C4: *N*-butanoyl-L-homoserine lactone; hydroxyC4: *N*-(3-oxo-butanoyl)-L-homoserine lactone; C6: *N*-hexanoyl-L-homoserine lactone; oxoC6: *N*-(3-oxo-hexanoyl)-L-homoserine lactone; C8: *N*-octanoyl-L-homoserine lactone; oxoC8: *N*-(3-oxo-octanoyl)-L-homoserine lactone; C12: *N*-dodecanoyl-L-homoserine lactone; oxoC12: *N*-(3-oxo-dodecanoyl)-L-homoserine lactone.

LuxR Homologs

As the genomes of more organisms are sequenced and analyzed, the list of bacteria that encode a LuxR-type system continues to grow. Among these are animal and plant pathogens that use quorum sensing to regulate virulence, such as *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Erwinia carotovora*, and *Yersinia enterocolitica* (47, 52, 61-63). Similar to *V. fischeri*, it is assumed that these pathogens use quorum sensing to avoid virulence genes expression until the number of bacteria increases to a point where infection is likely to be more successful.

Many organisms that utilize this system have the *luxR* and *luxI* homologs encoded adjacent to one another (25). In the AHL bound form, the LuxR homolog increases expression from the promoter of its cognate signal synthase, generating a positive feedback loop (58). The promoter consensus sequence for the LuxR protein is known as the "*lux* box" (14). As more LuxR homologs have been discovered, similar "boxes" have been identified (24, 27). The boxes are usually very similar to each other; the elements are often 20 bp palindromes, centered around the -40 region (27). This demonstrates that though they regulate different types of genes, there is a high degree of similarity between the systems. In fact, it has been shown that in the presence of its cognate AHL, LasR, a LuxR homolog of *P. aeruginosa*, can bind and activate the *lux* box of *V. fischeri*, whereas LuxR with oxoC6 only weakly activates the *lasB* promoter of *P. aeruginosa* (27).

Although the basic characteristics of the system are conserved, parts of the LuxR system can often be duplicated or deleted. For example, *P. aeruginosa* encodes two

complete LuxR-type systems, both of which are involved with virulence gene expression: LasRI and RhlRI, which synthesize and detect *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (oxoC12) and *N*-butanoyl-L-homoserine lactone (C4), respectively (40, 49-51, 71). The organism also encodes at least one additional LuxR homolog named QscR, although there is no cognate signal synthase for QscR. This protein can interact with LasR and RhlR, affecting AHL synthesis and virulence (10, 41). In contrast to *P. aeruginosa* that encodes three LuxR homologs and two LuxI homologs, *E. coli*, *Salmonella*, and *Klebsiella* encode a single LuxR homolog, SdiA. These organisms encode no LuxI homolog and consistent with this absence, do not produce AHLs (46).

The *luxCDABE* Operon

The prevalence of LuxR homologs has sparked the desire to easily screen for and identify organisms that possess these quorum sensing systems by screening for the ability to produce AHLs. A variety of methods have been used for this purpose, such as thin layer chromatography (TLC), which can be used to screen for the production and also determine the identity of AHLs (55). Some organisms can be used to indirectly detect the presence of AHLs. These "biosensor strains" are often based in a species of bacteria possessing an easily-observed phenotype regulated by LuxR-type quorum sensing system. For example, CviR is the LuxR homolog of *Chromobacterium violaceum* that induces production of a purple pigment in response to C4, synthesized by CviI (61). In the case of biosensor strains, the bacteria are isolates or engineered strains that lack the LuxI homolog, so that the strain no longer produces AHL. Therefore, in these organisms a change in the phenotype indicates activation by its LuxR homolog in response to an

exogenous source of AHL. The biosensor strains can also reveal which AHL is produced because LuxR homologs usually only respond to the specific AHL synthesized by its cognate LuxI homolog. A biosensor strain can also be a host organism, such as *E. coli,* engineered to carry a LuxR-type system of another organism, usually on a plasmid. In response to the appropriate AHL, the LuxR homolog activates its target promoter (usually that of the *luxI* homolog), which then drives expression of a reporter system. The *lacZY* genes and the *luxCDABE* operon are often utilized in plasmid-based reporter systems.

Since its discovery, the *lux* bioluminescence operon has been studied extensively and utilized in a variety of laboratory applications, such as reporter fusions (73). These reporter plasmids are most often harbored in *E. coli* and are used to screen cultures or supernatants for the presence of AHLs, although some plasmids are broad host range and can be transformed directly into the organism of interest (72).

Winson *et al.* engineered a promoterless *lux* cassette from the *luxCDABE* operon of *Photorhabdus luminescens*, which they generated by removing common endonuclease restriction sites from within the operon and adding them to regions flanking the operon, allowing for the cassette to be removed from its source and cloned into another vector with ease (73). It was found that the *luxCDABE* operon of *P. luminescens* is more appropriate to use in such applications because it is functional in temperatures up to 45°C, whereas the *lux* operon of *V. fischeri* is not functional above 30°C (73).

This cassette was then used by the Winson lab to create a set of biosensor plasmids that incorporates a *luxR* homolog with the promoter of its cognate autoinducer

synthase gene fused to the *lux* cassette (Figure 1.2) (72). These plasmids are frequently used to demonstrate the presence of AHL, identify which AHLs are produced, and determine the conditions in which they are produced, as well as quantify AHL concentration.

The Winson plasmids utilize the well-characterized LuxR quorum sensing system of *V. fischeri* and the LasR and RhlR systems of *P. aeruginosa.* The most widely used of the plasmids is pSB401, which encodes the *luxR* gene and the promoter of *luxI* (*luxRI'*) of *V. fischeri* fused to the promoterless *luxCDABE* cassette (*luxRI'*::*luxCDABE*) and responds to oxoC6 (Figure 1.2 A) (72). Similarly, pSB1075 encodes *lasRI'* also of *P. aeruginosa* and detects oxoC12, and pSB406 encodes *rhlRI'* of *P. aeruginosa* and detects C4 (Figure 1.2 B and 1.2 C) (72). However, pSB406 does not produce a large change in light production upon activation by AHL and so pSB536, which also detects C4, is often used instead. This plasmid encodes *ahyRI'* from *A. hydrophila* fused to the *lux* cassette (61).

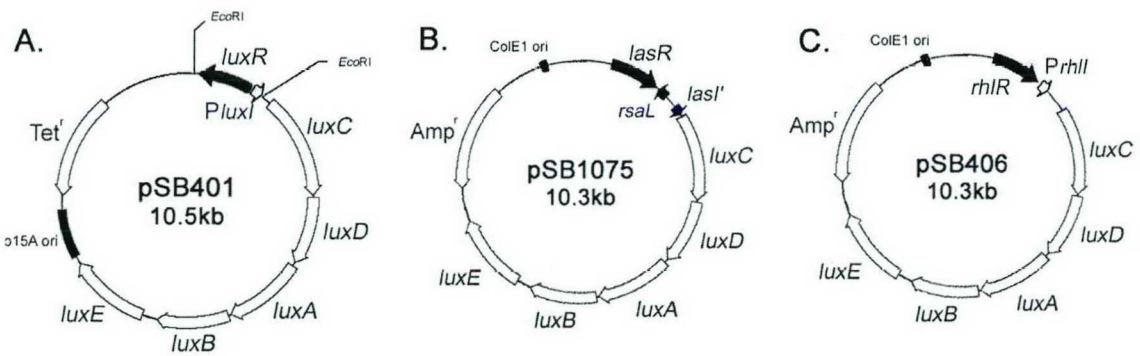

Figure 1.2 The *lux*-based biosensor plasmids constructed by Winson *et al.* (A) pSB401 contains the *luxRI'* region of *V. fischeri* fused to the *luxCDABE* reporter cassette and optimally detects oxoC6; (B) pSB1075 contains the *lasRI'* region of *P. aeruginosa* fused to the *luxCDABE* reporter cassette and optimally detects oxoC12; (C) pSB406 contains the *rhlRI'* region of *P. aeruginosa* fused to the *luxCDABE*

cassette and optimally detects C4 (72, 73). Adapted from Winson *et al.* 1998. FEMS Microbiol Lett **163**: 193-202.

Although *lux*-based reporter systems are extremely useful because luminescence is easy to monitor and quantify, there are problems associated with such *lux* plasmids. As with many transcriptional fusions, the response produced by the *luxCDABE* cassette is not always a direct result of the activation of the promoter being tested (20). Changes in light production are not strictly dependent on AHL or the receptor protein that activates the promoter, as metabolic and environmental conditions such as oxygen availability, temperature, and growth phase are able to effect the luminescence of any given *lux* fusion (30). These factors can influence promoter activity and are able to affect the luciferase reaction itself, independent of the promoter activity.

SdiA of *S.* Typhimurium

As previously mentioned, organisms such as *E. coli*, and those of the *Salmonella* and *Klebsiella* spp. encode the *luxR* homolog *sdiA*, but no *luxI* homolog and do not synthesize AHLs (1, 46). The *sdiA* gene of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) was identified with the knowledge that *E. coli* encoded *sdiA*, which had been identified in a previous study (68).

S. Typhimurium *sdiA* was found by probing a cosmid library with the *E. coli sdiA* gene (1). In order to isolate genes of the *sdiA* regulon, a MudJ library was created in an *sdiA* mutant strain of *S.* Typhimurium carrying plasmid-encoded *sdiA* under control of an arabinose promoter (1). 10,000 random mutants were screened for differences in β-galactosidase activity when grown on glucose versus arabinose (1). Ultimately, the

screen identified multiple genes located on the virulence plasmid and one gene in the chromosome (1, 59). The genes on the virulence plasmid are arranged in an operon of six genes, the *rck* operon (22, 46). Only two of the genes in the operon, *pefI* and *rck*, had been previously named and characterized. Rck is an outer membrane protein that prevents formation of the membrane attack complex of the classical complement pathway, thus conferring resistance to complement killing (2, 31-33). The *pefI* gene is a transcriptional regulator of the *pefBACD* operon, encoded immediately upstream, that is involved in the synthesis of the plasmid-encoded fimbriae, which mediates attachment to the intestinal epithelium (5-7, 22). The four uncharacterized genes in the *rck* operon have been named *srgA*, *B*, *C*, and *D* for *sdiA* regulated gene (Figure 1.4) (1, 22, 31-33). The *srgA* gene is a *dsbA* homolog that specifically acts on the PefA fimbrial subunit to generate a disulfide bond (3, 8, 22, 53). The remaining *srg* genes in the *rck* operon encode hypothetical genes of unknown function: *srgB* encodes a putative lipoprotein, *srgC* encodes a hypothetical protein of the AraC family of regulators, and *srgD* encodes a putative regulator of the LuxR family (though it does not contain a pheromone binding domain) (22).

The chromosomal gene that is regulated by *sdiA* is named *srgE* and is located in a *Salmonella*-specific region of the chromosome, which, because of the varied G + C content, is likely a mosaic of horizontal acquisitions (1). The *srgE* gene encodes a hypothetical protein that is predicted to have a coiled-coil domain, however the sequence does not align with any annotated proteins in the databases (2, 59). The genes that are known to be regulated by *sdiA* in *S.* Typhimurium have no orthologs in *E. coli.*

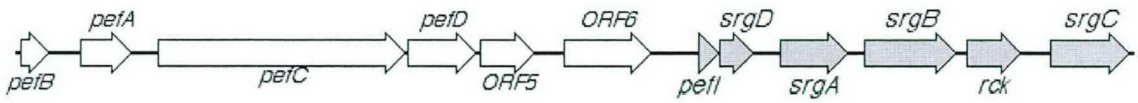

Figure 1.3 Map of the *sdiA*-regulated genes on the virulence plasmid of *S.* Typhimurium. A map of a portion of the virulence plasmid representing the region encoding the *pef* and *rck* operons. Shaded genes designate confirmed *sdiA*-regulated genes.

Though *S.* Typhimurium and *E. coli* do not encode a *luxI* homolog, it has been shown that *S.* Typhimurium *sdiA* is able to sense AHLs (46). Unlike most LuxR homologs, *sdiA* is promiscuous in that it detects a wide range of AHLs, but is most responsive to AHLs with a 3-oxo modification, and best senses oxoC6 and *N*-(3-oxo-octanoyl)-L-homoserine lactone (oxoC8) (46). This led to the hypothesis that *sdiA* detects mixed microbial communities. This is supported by the recent determination of the crystal structure of SdiA of *E. coli* (74). SdiA binds AHLs much less tightly than its homologs, leaving more room in the binding site when the protein is in active conformation, suggesting that the structure of the protein is "designed" to accept a variety of AHLs.

The role of *sdiA* in virulence is not completely understood and a phenotype for *sdiA* has not yet been characterized (2). However, preliminary results obtained in mice preinoculated with the AHL-producing *Yersinia enterocolitica* suggest that *sdiA* may provide a fitness advantage. More $sdiA^+$ *S.* Typhimurium than *sdiA* mutants were recovered from these mice, but not from mice infected with an isogenic strain of *Y. enterocolitica* lacking the *luxI* homolog (unpublished data). This finding demonstrates that *sdiA* detects AHLs produced by other species of bacteria during a coinfection.

The conditions under which SdiA functions are complex: the dependence on AHL varies with temperature and expression level of *sdiA*. When expressed from a plasmid, *sdiA* does not require AHL to activate its target genes, but when encoded in single copy as expressed from the chromosome, *sdiA* does require AHL. Additionally, the *rck* operon is activated only at 37°C and not at 30°C or 22°C (59). Similarly, *srgE* is expressed at 37°C and 30°C, but not at 22°C. Interestingly, at 30°C *sdiA* will activate expression of *srgE* even without AHL present (59). Thus, it seems that overexpression, and in the case of *srgE*, growth at 30°C bypasses the AHL requirement.

There are additional requirements for SdiA to activate target genes. In order for chromosomally encoded *sdiA* to respond to AHL and activate chromosomal transcriptional fusions to target genes, the cells must be grown in motility agar containing less than 0.5 % agar, though liquid medium generates a weak response in late log phase (59). However, when the target promoter is encoded on a plasmid, all of these restrictions seem to be eliminated and chromosomally encoded *sdiA* will activate the plasmid-based transcriptional fusions in an AHL-dependent manner (59).

SdiA of *E. coli*

The *sdiA* gene was identified first in *E. coli* in an experiment designed to better understand cell division regulation. MinC and D are proteins that are part of the *min* system in *E. coli* that ensures cell division occurs midcell by inhibiting septum formation at other locations, whereas MinE imparts topological specificity to the inhibition (13). This is done by repressing the formation of the Z-ring composed of FtsZ subunits, which

is key to the initiation of septum formation. When *minE* is deleted, MinC and D globally inhibit cell division, though the inhibition can be overcome by overexpression of *ftsZ*.

Wang *et al.* screened a plasmid library for genes that, like *ftsZ*, were able to relieve the inhibitory effects of MinC and D in the absence of *minE* (68). They isolated *sdiA* and determined that it increased expression of the P2 promoter of the *ftsQAZ* operon, thus increasing the cellular concentration of FtsZ, and named it suppressor of division inhibition A (68). However, the tests were performed with *sdiA* encoded on a plasmid and there is little effect on the *ftsQAZ* promoter when *sdiA* is expressed from the chromosome (68). In fact, cells still divide normally when *sdiA* is deleted from the chromosome (68). It was later shown that AHL enhanced the activation of the P2 promoter of *ftsQAZ* by plasmid-encoded *sdiA,* but the effect of AHL on chromosomal *sdiA* was not reported (57).

Although *sdiA* was first discovered in *E. coli*, the data obtained about its target genes and overall function in this organism are much less clear than in *S.* Typhimurium. The data available for gene regulation by *sdiA* in *E. coli* has primarily been generated from experiments that used plasmid-based expression of *sdiA* to identify the genes that it regulates (70). The overexpression of *sdiA* associated with the plasmid-based expression likely leads to pleiotropic effects in *E. coli* that are not seen in *S.* Typhimurium (2).

Wei *et al.* used a microarray to identify genes regulated by plasmid-based *sdiA* in *E. coli* K-12, but the genes were never confirmed to be regulated by chromosomally encoded *sdiA* (70). The overexpression that results from plasmid-based expression of *sdiA* likely not only bypasses the AHL requirement, but also may result in the protein

binding to promoters not normally bound. To date, no gene has been confirmed to be a member of the *E. coli sdiA* regulon. However, it was shown that *E. coli sdiA* is able to activate a *lux* fusion of the *S.* Typhimurium *srgE* gene and thus *sdiA* is indeed functional in *E. coli* (2, 59).

Direction of Current Research

E. coli K-12 has long been the preferred gram-negative background for many experiments because the strains used are easy to grow, easy to genetically manipulate, and nonpathogenic. The tradition of using *E. coli* extends as far as the history of bacterial genetics itself, which can lead to a false sense of comfort, as it is one of the most understood bacteria. Therefore, this organism is the preferred background for many plasmids, including those used in the investigation of quorum sensing that utilize a LuxR-type biosensor. The organism encodes a LuxR homolog, but does not produce AHLs, so it was assumed that *E. coli* was a "neutral" background for such studies. However, it has been shown that LuxR homologs are sometimes able to bind and even activate the promoters of other LuxR homolog regulated promoters (27). This raised the question as to whether *sdiA* of *E. coli* was able to activate the *luxI*-type promoters of the AHL sensing plasmids often harbored in *E. coli*. This combined with the inherent problems associated with the biosensor plasmids seemed to necessitate better controls for the existing biosensor plasmid systems.

A plasmid that lacked the *luxR* homolog would allow the true *luxR*-dependent response of a biosensor to be determined. With the removal of the *luxR* homolog, any change in light production would demonstrate *luxR*-independent changes in expression.

We have constructed a set of plasmid-based AHL biosensor strains-using the same quorum sensing systems encoded in the standard biosensor plasmids described above, but we include control plasmids that lack the respective *luxR* homolog of each biosensor system. These control plasmids are used in conjunction with their respective biosensor plasmids and allow the true *luxR*-dependent response of a biosensor to be determined (43).

The biosensors and controls will also allow investigation of the effects that *sdiA* can have on the plasmid biosensors. With respective *luxR* homologs eliminated, activation of the fusions can be compared in wild-type versus *sdiA* mutant backgrounds to determine whether *sdiA* has any effect on the function of these biosensors.

Additionally, because virtually nothing is known about the role of s*diA* in *E. coli* or the genes it regulates, we set out to identify the *sdiA* regulon of this organism. Having gained a better understanding of *sdiA* and its functioning in *S.* Typhimurium, we now hope to apply this knowledge to better investigate the role of *sdiA* in *E. coli* (2). Previous attempts to identify *sdiA*-regulated genes in *E. coli* relied upon overexpression, which appears to produce pleiotropic effects. We generated a library of random chromosomal *lux* fusions, in a wild-type background where *sdiA* is expressed from its natural position in the chromosome. The library was screened to identify genes that gave differences in light production in the presence of AHL, demonstrating regulation in response to AHL, presumably by *sdiA*.

CHAPTER 2

THE EFFECT OF *SDIA* ON BIOSENSORS OF *N*-ACYL HOMOSERINE LACTONES

Introduction

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 (62). These quorum sensing systems utilize various *N*-acyl homoserine lactones (AHLs) synthesized by LuxI homologs as their signal (Figure 1.1).

However, species of the *Escherichia*, *Salmonella*, and *Klebsiella* genera are unique in that they encode the *luxR* homolog *sdiA*, but no *luxI* homolog or any other AHL synthase (46). Of these organisms, *sdiA* is most understood in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), where it detects AHLs produced by other bacterial species (46, 59). The function of *sdiA* in *E. coli* and *Klebsiella* remains unclear. Experiments have been performed on *sdiA* of *E. coli*, but are difficult to interpret because most of the information was obtained using *sdiA* overexpression. Overexpression of *sdiA* in *E. coli* results in pleiotropic effects that are not seen in *S.* Typhimurium (2).

Despite the presence of *sdiA* in *E. coli*, this organism has been used as the host organism for several AHL biosensor strains, as *E. coli* K-12 is advantageous to use as the background strain because its genetics are the most understood out of all gram-negatives, it is easy to manipulate, it is nonpathogenic, and it does not produce AHLs. *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 set of these biosensor strains was published previously that incorporates the promoterless *luxCDABE* cassette as a reporter system (Figure 1.2) (72, 73). The set contains the frequently-used pSB401, a plasmid carrying *luxR* and the *luxR*-regulated *luxI* promoter of *V. fischeri* fused to the *luxCDABE* cassette (*luxRI'*::*luxCDABE*), which optimally detects oxoC6 (Figure 1.2 A) (72, 73). Also in the set is pSB1075 encoding *lasRI'*::*luxCDABE* to optimally detect oxoC12, and pSB406 encoding *rhlRI'*::*luxCDABE* to optimally detect C4, both of which are luxR homologs of *P. aeruginosa* (Figure 1.2 B and 1.2 C) (72, 73). Another plasmid, pSB536, was published separately that carries the *Aeromonas hydrophila* gene *ahyR* and the *ahyI* promoter fused to the same promoterless *luxCDABE* cassette (*ahyRI'*::*luxCDABE*) (61). Like RhlR of *P. aeruginosa*, AhyR optimally detects C4, but this plasmid produces a larger change in luminescence upon activation than pSB406 and so is more often used as the C4 biosensor plasmid (61).

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 (30). Therefore, light production can increase and decrease independently of the *luxR* homolog and AHL. We have constructed a set of control plasmids that lack the *luxR* homolog of interest. These control plasmids 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.

Materials and Methods

Bacterial strains and media. The bacterial strains and plasmids used in this study are listed in Table 2.1. Bacteria were grown in Luria-Bertani (LB) broth (EMD Chemicals Inc., Gibbstown, NJ) or on LB agar plates (EMD Chemicals) at 37°C unless otherwise noted. Antibiotics (Sigma, St. Louis, MO) were added to the media as appropriate at the following concentrations: tetracycline (tet), 20 μg/ml; ampicillin (amp), 100 μg/ml; kanamycin (kan), 60 μg/ml; and chloramphenicol (cam), 30 μg/ml. AHLs C4, oxoC6 (Sigma), and oxoC12 (9) were dissolved in acidified Ethyl Acetate (EA) (49) to make stock solutions at concentrations of 1 μM and stored at -20°C. EA was used as the solvent control at a final concentration of 0.1 %.

Plasmid construction. Oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) and are listed in Table 2.2. DNA sequencing was performed by the Plant Microbe Genomics Facility at the Ohio State University. Primers contained a 1/2 FRT site on the 5' end and an *Xba*I site on the 3' end (for use in work in

progress). The biosensor inserts were PCR amplified using pSB401 as the template for the LuxR system, *P. aeruginosa* as the template for the LasR and RhlR systems, and pSB536 for the AhyR system (Figure 2.1) (61, 72). The PCR products were separated by gel electrophoresis and gel purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA). The PCR inserts were cloned into pCR 2.1-TOPO using the Invitrogen TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) to generate the pRL1000 series of plasmids. These were transformed into One Shot TOP10 chemically competent cells. All plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). The inserts were removed from the cloning vector by *Eco*RI (Invitrogen) digestion. For the Lux, Las, and Rhl reporter sets, the *Eco*RI fragments were ligated with T4 DNA ligase (Fermentas, Hanover, MD) into pSB401 (72) that had been digested with *Eco*RI (to remove the *luxRI'* cassette) and dephosphorylated with either calf intestinal alkaline phosphatase (CIAP) (Invitrogen) or shrimp alkaline phosphatase (SAP) (USB, Cleveland, OH). For the Ahy reporter set, pSB536 was used as the $ahyR^+$ biosensor plasmid (61). To match the origin of replication and antibiotic resistance marker, the $ahyR^-$ *Eco*RI fragment was cloned into pSB377 digested with *Eco*RI and dephosphorylated with SAP (72). Clones were screened for insert and orientation via PCR and constructs were confirmed via sequence analysis. Plasmids were chemically transformed into *E. coli* strains and electroporated into *S.* Typhimurium strains.

Table 2.1 Bacterial strains and plasmids used for the biosensor project

Strain or plasmid	Genotype or description	Source or reference
Strains		
WM54	*E. coli* K-12 Δ*lacX74*	Bill Metcalf
JLD271	WM54 *sdiA271*::cam	This study
PAO1	Wild-type *Pseudomonas aeruginosa*	(34)
14028	Wild-type *Salmonella enterica* serovar Typhimurium	ATCC
BA612	14028 *sdiA1*::mTn*3*	(1)
Plasmids		
pSB377	*luxCDABE* transcriptional fusion vector; amp^r; ColE1 origin	(73)
pSB401	*luxRI'::luxCDABE*; tet^r; p15A origin	(72)
pSB1075	*lasRI'::luxCDABE*; amp^r; ColE1 origin	(72)
pSB536	*ahyRI'::luxCDABE*; amp^r; ColE1 origin	(61)
pTIM244	PCR fragment amplified from pSB536 template using primers BA440 and BA441 and cloned into pSB377/*Eco*RI	This study
pJNS25	P*srgE::luxCDABE* cloned into pSB401/*Eco*RI	(59)
pRL1001	PCR fragment amplified from pSB401 template using primers BA988 and BA962 and cloned into pCR 2.1-TOPO; *luxR*$^+$ insert	This study
pRL1002	PCR fragment amplified from pSB401 template using primers BA961 and BA962 and cloned into pCR 2.1-TOPO; *luxR*$^-$ insert	This study
pRL1003	PCR fragment amplified from PA01 template using primers BA983 and BA985 and cloned into pCR 2.1-TOPO; *lasR*$^+$ insert	This study
pRL1004	PCR fragment amplified from PA01 template using primers BA984 and BA985 and cloned into pCR 2.1-TOPO; *lasR*$^+$ insert	This study
pRL1005	PCR fragment amplified from PA01 template using primers BA980 and BA982 and cloned into pCR 2.1-TOPO; *rhlR*$^+$ insert	This study
pRL1006	PCR fragment amplified from PA01 template using primers BA981 and BA982 and cloned into pCR 2.1-TOPO; *rhlR*$^-$ insert	This study
pAL101	*rhlRI'::luxCDABE*; *rhlR*$^+$ insert from pRL1005 cloned into pSB401/*Eco*RI	This study
pAL102	*rhlI'::luxCDABE*; *rhlR*$^-$ insert from pRL1006 cloned into pSB401/*Eco*RI	This study
pAL103	*luxRI'::luxCDABE*; *luxR*$^+$ insert from pRL1001 cloned into pSB401/*Eco*RI	This study
pAL104	*luxI'::luxCDABE*; *luxR*$^-$ insert from pRL1002 cloned into pSB401/*Eco*RI	This study
pAL105	*lasRI'::luxCDABE*; *lasR*$^+$ insert from pRL1003 cloned into pSB401/*Eco*RI	This study
pAL106	*lasI'::luxCDABE*; *lasR*$^-$ insert from pRL1004 cloned into pSB401/*Eco*RI	This study

Luminescence readings and analysis. 96-well microtiter plates (Corning #3654) were used in the assays that had black walls to minimize light contamination from adjacent wells and clear bottoms to allow measurement of optical density (OD) (Corning Inc., Corning, NJ). Overnight cultures were subcultured 1:100 into microtiter plates containing LB broth supplemented with the appropriate antibiotic. AHLs were added to the media at the noted concentrations. Plates were incubated at 37°C for 9 hrs in a moist chamber. The Wallac Victor^2 1420 multimode plate reader was used to measure and record the OD_{590nm} and light production of each well. Data were analyzed using Prism 3.0 graphing software.

Cross-streak assays were performed by dripping 20 µl of overnight culture down an LB agar plate that had a perpendicular cross streak of 20 µl of EA or AHL at a concentration of 10 µM. The plates were incubated at 37°C for 6 hrs. Light production by the streaks was imaged using a C2400-32 intensified charge-coupled camera (CCD) with an Argus 20 image processor (Hamamatsu Photonics) (11).

Table 2.2 Oligonucleotides used for the biosensor project

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

Results

Because of the potential problems associated with current biosensors hosted in *E. coli*, we sought to create a set of biosensors with controls that would allow the effect of the *luxR* homolog on the fusion to be determined. For these, we utilized four well characterized LuxR-type systems that optimally sense three different AHLs: the LuxR system of *V. fischeri*, which senses oxoC6; the LasR and RhlR systems of *P. aeruginosa,* which sense oxoC12 and C4, respectively; and the AhyR system of *A. hydrophila,* which senses C4. The plasmid set contains isogenic pairs of plasmids that differ from each other only by the presence or absence of the gene encoding the *luxR* homolog.

Construction of AHL biosensor and control plasmids. The LuxR homologs that were used in this study are those that are most commonly used in biosensors. In fact, the plasmids encoding the *luxR* homolog contain fragments essentially identical to those used in the biosensor plasmids published previously (72, 73). The control plasmids 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. The 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* promoters). 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 (61). The plasmids were then transformed into wild-type and *sdiA* mutant strains of *E. coli* and *S.* Typhimurium (WM54, JLD271, 14028, and BA612 respectively, Table 2.1).

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 (referred to as *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.

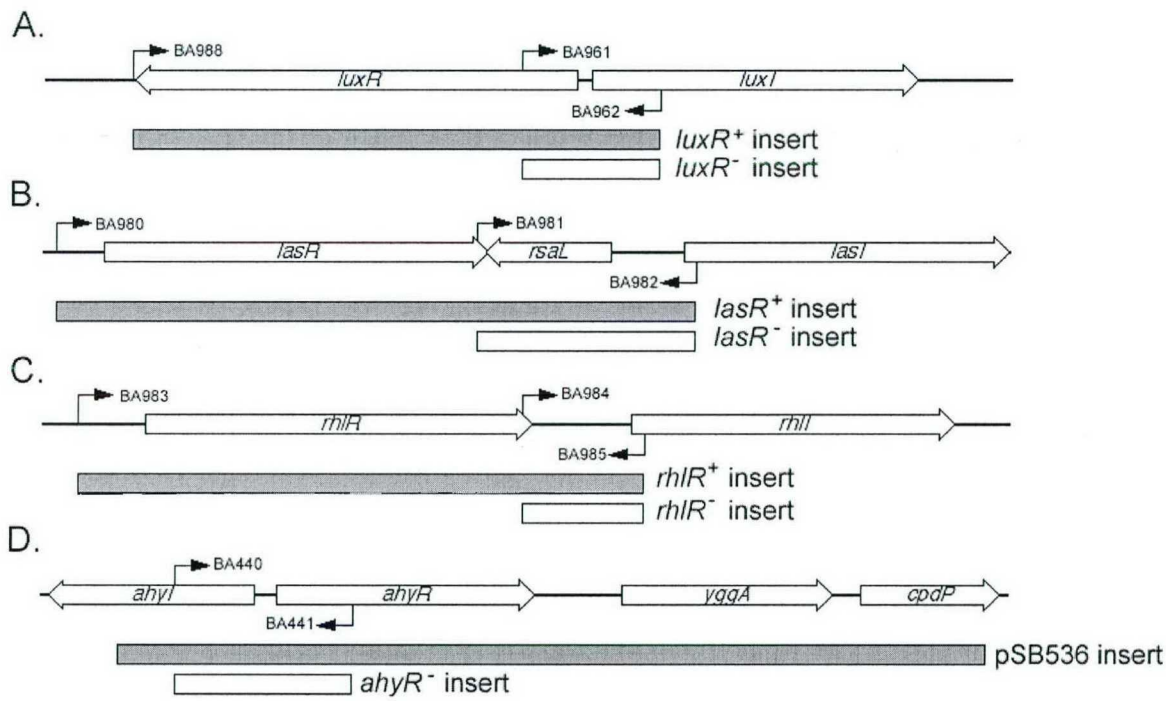

Figure 2.1 Map of the DNA fragments used to make the biosensor plasmids. The PCR primers used to amplify each insert are listed in Table 2.2. Templates used for each biosensor set are as follows: (A) the *luxRI* region of *V. fischeri* encoded by pSB401; (B) the *lasRI* region of *P. aeruginosa* (PA01); (C) the *rhlRI* region of *P. aeruginosa* (PA01); (D) the *ahyRI* region of *A. hydrophila* encoded by pSB536.

The Lux and Las reporter sets. The *luxR*$^+$ and *lasR*$^+$ biosensors respond to 10 nM oxoC6 or oxoC12 in *E. coli*, respectively, while the isogenic *luxR*$^-$ and *lasR*$^-$ plasmids do not detect AHL (Figure 2.2 A and 2.2 B). 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 *S.* Typhimurium as the host strain (the wild-type 14028 versus the *sdiA* mutant BA612, Figure 2.3 A and 2.3 B). For unknown reasons, the basal level of luminescence of the *luxR*$^-$ control plasmid is higher than the *luxR*$^+$ plasmid. However, this does not affect the utility of the control plasmid in determining the *luxR*-dependence of reporter activity. Therefore, the Lux and Las reporter sets respond appropriately to the presence and absence of exogenous AHLs and *sdiA* does not contribute to, or interfere with, AHL detection.

The Ahy reporter set. In the absence of *sdiA*, the Ahy reporter set 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 grown in the presence of the solvent control or oxoC6 (Figure 2.2 D). However, the maximal fold-difference achieved did not exceed four-fold at 100 μM C4, which does not make this a particularly useful biosensor, especially in light of the results obtained with the Rhl reporter set (see below). Therefore, this reporter was not tested further in *S.* Typhimurium. For unknown reasons, the basal level of luminescence from the *ahyR*$^-$ plasmid is more than an order of magnitude higher than the *ahyR*$^+$ plasmid. This is similar to what was observed with the Lux reporter set, though as with this set, the control plasmid shows no AHL-dependent activation of the *ahyI* promoter in the absence of *ahyR*.

Unlike with the Lux or Las reporter sets, 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* (Figure 2.2 D). 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.

The Rhl reporter set. In an *E. coli sdiA* mutant background, the *rhlR*$^+$ biosensor detects 1 μM C4 or 10 μM oxoC6, while the *rhlR*$^-$ plasmid does not detect AHLs (Figure 2.2 C). The values for the *rhlR*$^-$ plasmid in the *sdiA*$^-$ background are not visible in Figure 2.2 C because the values at every point were slightly above or below zero. As with the Lux and Ahy reporter sets, the *rhlR*$^+$ and *rhlR*$^-$ plasmids have different basal levels of luminescence, though in this case the *rhlR*$^+$ is higher. In contrast to the *ahyR*$^+$ plasmid, the fold-induction of the *rhlR*$^+$ plasmid nears three orders of magnitude (913-fold) in response to 100 μM C4, making it much more sensitive than the *ahyR*$^+$ plasmid described above. Similar results for the Rhl reporter set were seen using an *sdiA*$^-$ *S.* Typhimurium host strain (Figure 2.3 C).

The results for the Rhl reporter set are dramatically different when tested in an *sdiA*$^+$ background. The presence of *sdiA* in either the *E. coli* or *S.* Typhimurium host strain causes the *rhlR*$^+$ and the *rhlR*$^-$ plasmid to respond to AHL. As seen in Figure 2.2 C and 2.3 C, the *rhlI* promoter is activated in the *sdiA*$^+$ host, with and without the presence of *rhlR*, even in the solvent control (EA). The addition of AHL increases luminescence of either plasmid only by another two or three-fold (Figure 2.2 C and 2.3 C).

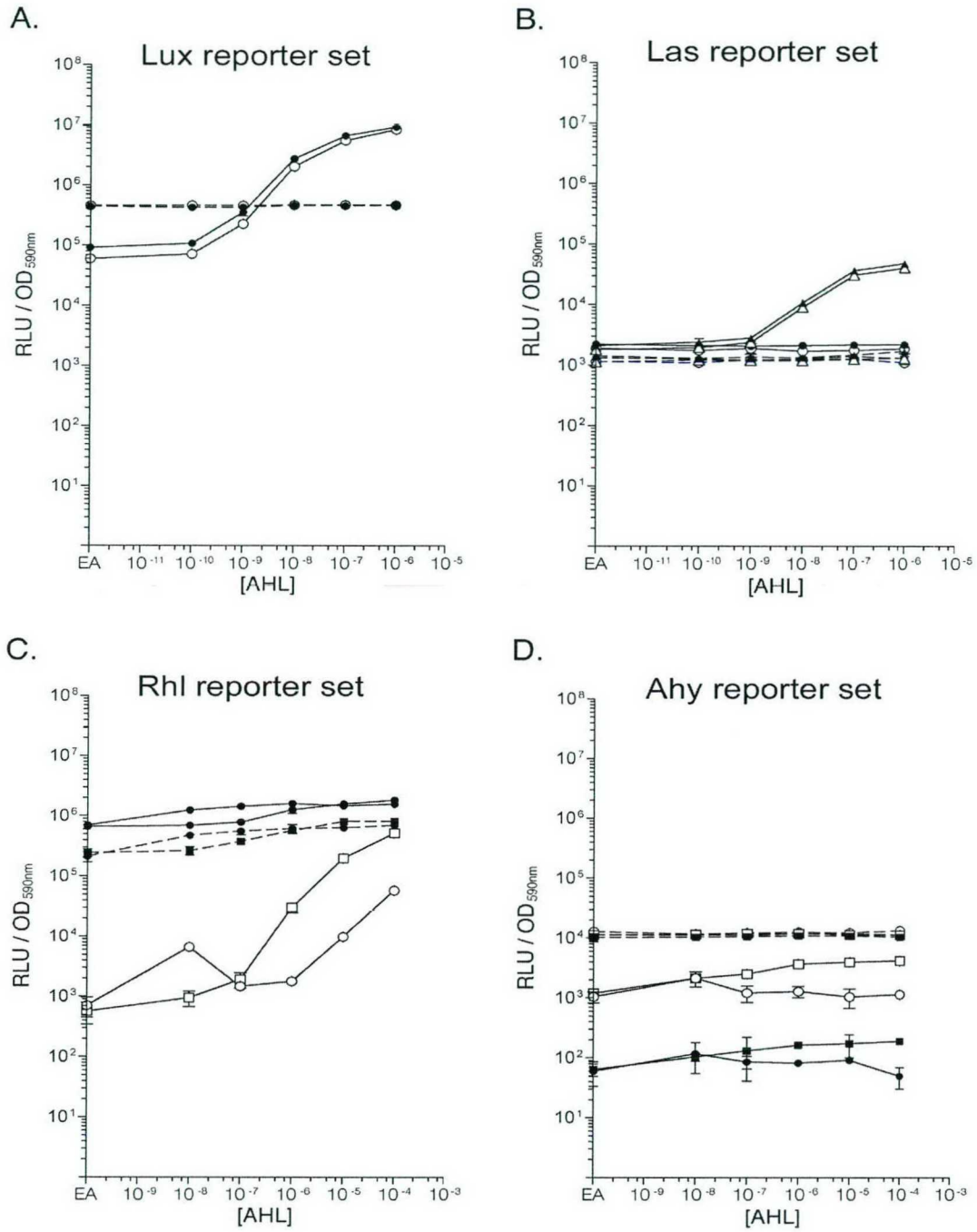

Figure 2.2 Response of the reporter plasmid sets to AHL and *sdiA* in *E. coli* K-12. Optical density at 590 nm (OD_{590nm}) and luminescence in relative light units (RLU) were measured using a Wallac Victor^2 1420 multimode plate reader. Data were analyzed using Prism 3.0 graphing software. Each point is the mean of triplicate cultures, with error bars representing standard deviation. "EA" on the X-axis represents an AHL concentration of zero in which there is only the solvent ethyl acetate. (A) Lux reporter set: pAL103 (*luxR*$^+$) and pAL104 (*luxR*$^-$). (B) Las reporter set: pAL105 (*lasR*$^+$) and pAL106 (*lasR*$^-$). (C) Rhl reporter set: pAL101 (*rhlR*$^+$) and pAL102 (*rhlR*$^-$). (D) Ahy reporter set: pSB536 (*ahyR*$^+$) and pTIM244 (*ahyR*$^-$). Closed symbols: *sdiA*$^+$; Open symbols: *sdiA*$^-$; Solid lines: biosensor plasmid (*luxR* homolog$^+$); Dashed lines: control plasmid (*luxR* homolog$^-$); Circles: oxoC6; Triangles: oxoC12; Squares: C4.

Effects of LuxR homologs on an *sdiA*-regulated promoter. In addition to investigating the effects of *sdiA* on the promoters of *luxI* homologs, we tested the ability of *luxR* homologs to activate the *S.* Typhimurium *sdiA*-regulated gene, *srgE*, which is regulated by *sdiA* in an AHL-dependent manner (46). A plasmid-based *lux* fusion created in a previous study was used to monitor activation of *srgE*. The plasmid pJNS25 was made by cloning the *srgE* promoter region into pSB401 upstream of the promoterless *luxCDABE* cassette (*srgE::luxCDABE*) (59).

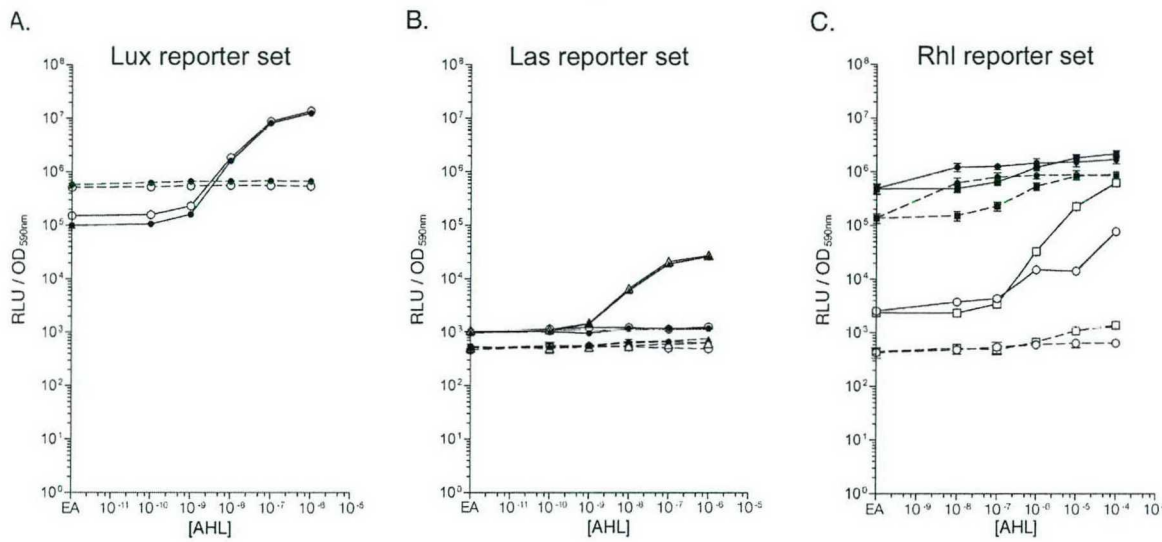

Figure 2.3 Response of the reporter plasmid sets to AHL and *sdiA* in *S.* Typhimurium. Data were obtained, analyzed, and plotted as described for *E. coli* in Figure 2.2. (A) Lux reporter set: pAL103 (*luxR*$^+$) and pAL104 (*luxR*$^-$). (B) Las reporter set: pAL105 (*lasR*$^+$) and pAL106 (*lasR*$^-$). (C) Rhl reporter set: pAL101 (*rhlR*$^+$) and pAL102 (*rhlR*$^-$). Closed symbols: *sdiA*$^+$; Open symbols: *sdiA*$^-$; Solid lines: biosensor plasmid (*luxR* homolog$^+$); Dashed lines: control plasmid (*luxR* homolog$^-$); Circles: oxoC6; Triangles: oxoC12; Squares: C4.

To test for activation of the *srgE* promoter, a *S.* Typhimurium *sdiA* mutant carrying pJNS25 was transformed with a plasmid encoding a *luxR* homolog or an isogenic plasmid lacking the luxR homolog. For this purpose, we utilized the TOPO plasmids generated in the original cloning of the biosensor regions. The plasmids containing the *luxR*$^+$, *luxR*$^-$, *lasR*$^+$, *lasR*$^-$, *rhlR*$^+$, and *rhlR*$^-$ amplified regions were transformed into BA612 + pJNS25, as was the cloning vector pCR 2.1-TOPO as a vector control. Cross-streak and microplate assays were then used to determine activation of the *srgE:: luxCDABE* fusion.

Both methods demonstrate that the plasmid encoding *lasR* activates *srgE::luxCDABE* in the presence of 10 μM oxoC12, but not in the presence of the solvent control EA (Figure 2.4). This difference in light production was not observed when the TOPO plasmid lacked *lasR*. Cross-streak assays showed that *lasR* activates the *srgE* promoter only in the presence of oxoC12 and not in the presence of oxoC6 or C4 (data not shown).

Similar results were obtained when testing the effects of *rhlR* on the *srgE* promoter. With 10 μM C4, the presence of *rhlR* on the TOPO plasmid resulted in significant activation of the *srgE::luxCDABE* fusion. With EA, the presence of *rhlR* had no effect on light production. When *rhlR* was not present, there was no change in luminescence even with AHL present. Cross-streak assays demonstrated that this *rhlR*-dependent activation of the *srgE* promoter only occurred in the presence of C4, and not oxoC6 or oxoC12 (data no shown).

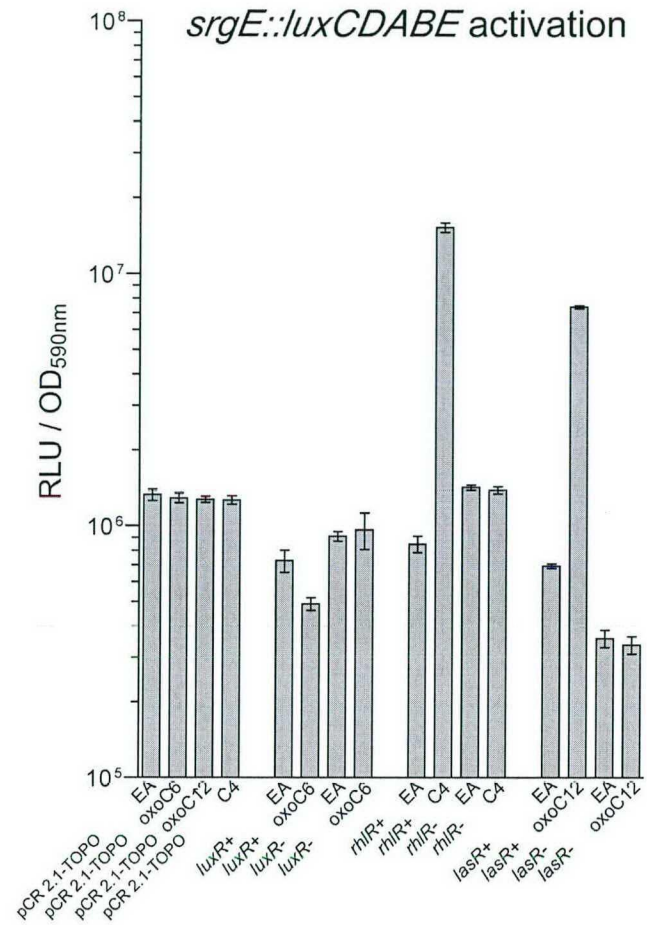

Figure 2.4 Effect of *luxR* homologs on an *sdiA*-regulated promoter of *S.* Typhimurium. Light production generated by *srgE::luxCDABE* was measured in the presence of AHL or the solvent control, EA. BA612 + pJNS25 (*S.* Typhimurium s*diA* mutant carrying plasmid-based *srgE::luxCDABE*) was transformed with the vector control, pCR 2.1-TOPO, or with plasmids containing the reporter inserts. Cultures were grown in microtiter plates at 37°C for 9 hrs and placed into a Wallac Victor^2 1420 multimode plate reader to measure luminescence in relative light units (RLU) and optical density at 590 nm (OD_{590nm}). Data were analyzed with Prism 3.0 graphing software. Each bar is the mean of triplicate cultures, with error bars representing standard deviation.

The data generated for the effects of *luxR* on the *sdiA*-regulated fusion are somewhat less clear because of inconsistent results obtained from cross-streak assays. In one cross-streak assay, the presence of *luxR* clearly increased the amount of light generated from the *srgE::luxCDABE* plasmid when crossed against 10 μM oxoC6. However, another cross-streak did not show this activation (data not shown). All cross-

streaks suggested that in the presence of EA, C4, and oxoC12, *luxR* does not have an effect on light produced from the fusion. However, the microplate assay shows that the presence of *luxR* slightly decreases the light produced by *srgE::luxCDABE*. This response required oxoC12 and did not occur in the presence of EA or in the absence of *luxR*. Because of the quantitative nature of the microplate assay and because it was performed in triplicate, it is assumed that these results more accurately portray the effects of *luxR* on the *srgE* promoter.

Discussion

The plasmids that were constructed in this study show that biosensor plasmids can indeed be affected by *sdiA* of the host strain. Whether or not *sdiA* affects the results and in what way depends on the biosensor being tested. The biosensor systems that utilize the LuxR system from *V. fischeri* or the LasR system of *P. aeruginosa* are not affected by the presence of *sdiA* in the host. This is not the case for the biosensors utilizing the AhyR system from *A. hydrophila* or the RhlR system from *P. aeruginosa*.

In this study, we demonstrate that *sdiA* of *E. coli* interferes with the $ahyR^+$ biosensor (pSB536) by decreasing the luminescence produced in response to AHL, though the presence of *sdiA* had no effect on the basal level of light produced from the $ahyR^-$ plasmid. Because interference by *sdiA* requires the presence of *ahyR*, this suggests that SdiA and AhyR may form inactive heterodimers in the cell forming. 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 in response to AHL. Therefore, pSB536 should only be used in an *sdiA* mutant background. However, the results obtained with the Rhl reporter set suggest that the *rhlR*$^+$ plasmid is a much better biosensor, once *sdiA* of the host is removed.

In the Rhl reporter set, the presence of *sdiA* in the host strain strongly activates the *rhlI* promoter, with the addition of AHL increasing expression only by another two or three-fold. Essentially, *sdiA* is constitutively activating the *rhlI* promoter to a level that is achieved by *rhlR* only in the presence of 100 μM C4 (Figure 2.2 C). Interestingly, Winson *et al.* used *sdiA*$^+$ *E. coli* host strains when describing the *rhlR* biosensor system, and they too observed only a two to three-fold induction in response to AHL (72). Based on these 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 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 (42). 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, the crystal structure of *E. coli* SdiA solved by Yao *et al.* demonstrated that when this protein is in the active conformation, the AHL in the binding site is bound less tightly than other LuxR homologs (74). This is understandable, as *sdiA* responds to 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 detects AHLs produced by other species (2, 46, 59).

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 may take place that allows activation of transcription. Since the *rhlI* promoter is not a native promoter for SdiA, for unknown reasons the 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 (39, 44). 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 (44). Future biochemical studies of SdiA and RhlR will be required to determine the mechanism by which *sdiA* causes activation of the *rhlI* promoter. However, regardless of the mechanism of interference, *sdiA* should not be present in RhlR system biosensor strains.

In this report, we have created 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.* (72, 73). 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 and may pose potential problems for other biosensor strains that have not yet been fully characterized. Thus, an *sdiA*$^{-}$ strain should always be used when utilizing *E. coli* as the background for any *luxR*-type biosensor systems.

CHAPTER 3

IDENTIFICATION OF THE *SDIA* REGULON OF *ESCHERICHIA COLI* K-12

Introduction

LuxR-type quorum sensing systems are found in a large number of gram negative bacteria, many of which are animal and plant pathogens that use quorum sensing to regulate virulence (62). As these systems are being discovered and thoroughly characterized, it is becoming more apparent that *luxR* homologs and this type of regulation are far more prevalent than originally estimated. In most cases, the autoinducer produced by a *luxI* homolog is key to synchronization of a population's behavior. However, organisms such as *E. coli*, *Salmonella* spp., and *Klebsiella* spp. encode the *luxR* homolog *sdiA*, but no *luxI* homolog and do not synthesize AHLs and so cannot be regulating gene expression in response to their own population density (46).

SdiA is most understood in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), where it has been shown that *sdiA* has the ability to sense AHLs produced by other bacteria, allowing it to respond to mixed microbial communities (59). In *S.* Typhimurium, *sdiA* has been found to regulate genes likely involved in virulence, although a phenotype, during infection or otherwise, has not yet been identified.

Preliminary results suggest that during a mixed infection with *Yersinia enterocolitica*, wild-type *S.* Typhimurium are more fit than the isogenic *sdiA* mutants (unpublished data).

The *sdiA* regulon of *S.* Typhimurium was identified with a genetic screen that utilized overexpression of *sdiA.* A library of random chromosomal MudJ fusions was screened for regulation by plasmid-encoded *sdiA* (1). Although unknown at the time, overexpression of *sdiA* permitted activation of regulated genes without the presence of AHL. Once these genes were identified, they were used to characterize the conditions in which *sdiA* is most active. It was determined that *sdiA* is active in the presence of AHL and responds best to oxoC8 and oxoC6 (59). The highest *sdiA* activity is seen in LB containing 0.2 - 0.5 % agar, as opposed to solid agar plates or liquid broth. In order for *sdiA*-dependent activation of the fusions to be AHL-dependent, the bacteria must be grown at 37°C (59).

Less is known about the function of *sdiA* in *E. coli*. DNA microarrays have been performed in an attempt to characterize the *sdiA* regulon of *E. coli* (70). However, the experiment was performed in the absence of AHL because at the time they were performed, the AHL to which *sdiA* responds was not yet known. Additionally, these screens used plasmid-based expression of *sdiA,* which appears to generate pleiotropic effects. Wei *et al.* identified 137 genes that were regulated by *sdiA* overexpression (70). When our lab tested some of these genes using chromosomal fusions and natural levels of *sdiA* expression, regulation was not observed (unpublished data).

With the understanding gained from investigations in *S.* Typhimurium, we are now attempting to characterize the *sdiA* regulon of *E. coli* via a new genetic screen that uses AHL, chromosomal *sdiA*, and chromosomal transcriptional fusions.

Materials and Methods

Bacterial strains and media. The bacterial strains and plasmids used in this study are listed in Table 3.1. Bacteria were grown in Luria-Bertani (LB) broth or on LB agar plates (agar concentration 1.5 %) (EMD Chemicals Inc., Gibbstown, NJ). Motility agar was made by adding 0.3 % Agar agar (EMD Chemicals) to LB broth. All incubations were carried out at 37°C unless otherwise noted. Antibiotics (Sigma, St. Louis, MO) were added to the media as appropriate at the following concentrations: ampicillin (amp), 100 μg/ml; chloramphenicol (cam), 30 μg/ml; kanamycin (kan), 60 μg/ml; and tetracycline (tet), 20 μg/ml. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (EMD Chemicals) was used at a final concentration of 40 μg/ml. oxoC6 (Sigma) was dissolved in acidified Ethyl Acetate (EA) (49) to make a stock solution of 1 mM, stored at -20°C, and was used at a final concentration of 1 μM, unless otherwise noted. EA was used as the solvent control at a final concentration of 0.1 %.

Construction of a mutant library. A mutant library was created by mating the donor strain BW20767 + pUT mTn5 *lux kan*2 with the recipient BA4000. Overnight cultures of both strains were grown in 5 ml LB broth with antibiotic, where appropriate. Cells were washed and 100 μl of each strain was mixed gently, plated on LB without antibiotics, and incubated overnight at 37°C. The resulting lawn was harvested with LB broth, washed,

and concentrated to 1 ml, from which dilutions were plated onto LB kan plates. The procedure was done until approximately 10,000 mutants had been obtained. The donor strain carries a suicide vector with a conditional origin of replication that requires the π (Pi) protein for maintenance of the plasmid, which is provided by the host cell, where it is chromosomally encoded by the *pir* gene, located on a λ prophage. On this plasmid is a transposon that encodes a kanamycin (kan) resistance gene and a promoterless *luxCDABE* operon. Also on the plasmid is the transposase gene, which is encoded adjacent to, and not within, the transposon. When this plasmid is conjugated into a *pir*$^-$ host cell, the transposon transposes from the plasmid and inserts randomly into the host chromosome, generating a *lux* transcriptional fusion. This system is advantageous because the transposase is lost with the plasmid and once the transposon is inserted into the chromosome, the fusion is stable.

Luminescence readings and analysis. Wells of 96-well microtiter plates with black walls and clear bottoms (Corning #3654) (Corning Inc., Corning, NJ) were filled with LB kan motility agar and either 1 μM oxoC6 or an equivalent volume of the solvent control EA. The plates were left at room temperature for 1-2 hrs to allow the medium to solidify. Mutant colonies from the library were picked with sterile toothpicks and stabbed into the center of a well containing EA, then the adjacent well containing 1 μM oxoC6. Microtiter plates were incubated in a humid chamber, and removed at the points indicated to measure luminescence. The light production of each well was measured using the Wallac Victor^2 1420 multimode plate reader and the resulting data were analyzed using Prism 3.0 graphing software. Mutants that produced a two-fold or greater difference in

light production between the EA and the oxoC6 well were located on the microtiter plate, picked from the EA well, and inoculated into 5 ml LB kan. These cultures were grown overnight and tested again by seeding motility agar 1:100 in triplicate.

DNA manipulation. Oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) and are listed in Table 3.2. DNA sequencing was performed by the Plant Microbe Genomics Facility at the Ohio State University. Genomic DNA was isolated from overnight cultures of the mutants using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). Insertion regions were sequenced two times using the mutant genomic DNA as the template and primers BA247 and BA1090 specific for the transposon. Both primers anneal to *luxC* and are directed into the gene(s) upstream of the *lux* operon of the transposon (Table 3.2). DNA sequences were analyzed using MacVector and "dna Tools Xplorer" software. The insertion locations were identified by comparing the sequences to the published genome sequence of *E. coli* K-12. Primers were generated for the MG1655 sequences that flanked the region containing the promoter of interest. The location and direction of the chromosomal fusions were confirmed by PCR using a *lux* primer and the appropriate primer for the region.

Table 3.1 Bacterial strains and plasmids used for the *sdiA* regulon project

Strain or plasmid	Genotype or description	Source or reference
Strains		
WM54	*E. coli* K-12 Δ*lacX74*	Bill Metcalf
JLD271	WM54 *sdiA271*::cam	(43)
BA4000	nal resistant mutant of BW25113	This study
BW20767	RP4-2-tet::Mu-1kan::Tn*7* integrant *leu-63*::IS*10 recA1 creC510 hsdR17 endA1 zbf-5 uidA*(ΔMluI)::*pir*$^+$ *thi*	(45)
BW25113	Δ(*araD-araB*)567 Δ*lacZ4787*(::*rrnB*-3) *lacIp*-4000(*lacIQ*) *rph*-1 Δ(*rhaD-rhaB*)568 *hsdR514*	(12)
MG1655	*E. coli* K-12	ATCC
AL4001	BA4000 mutant containing transcriptional fusion to the promoterless *luxCDABE* cassette in the location demonstrated in Figure 3.2 A	This study
AL4008	BA4000 mutant containing transcriptional fusion to the promoterless *luxCDABE* cassette in the location demonstrated in Figure 3.2 B	This study
AL4016	BA4000 mutant containing transcriptional fusion to the promoterless *luxCDABE* cassette in the location demonstrated in Figure 3.2 C	This study
Plasmids		
pUT mTn*5 lux kan*2	Suicide vector, *ori* R6K, mini-Tn*5* Km2 *luxCDABE* transposon, *mob*$^+$ (RP4); Amp^r Kan^r	(73)
pSB401	*luxRI'::luxCDABE*, Tet^r p15A origin	(72)
pVIK112	*lacZYA* transcriptional fusion vector; Kan^r oriR6K	(37)
pAL312	PCR fragment amplified from MG1655 with primers BA1494 and BA1495 to represent mutant AL4001 transposon insertion region; cloned into pSB401/*Eco*RI in forward orientation	This study
pAL313	AL4001 insertion region cloned in opposite orientation of pAL312	This study
pAL326	PCR fragment amplified from MG1655 with primers BA1501 and BA1502 to represent mutant AL4008 transposon insertion region; cloned into pSB401/*Eco*RI in forward orientation	This study
AL327	AL4008 insertion region cloned in opposite orientation of pAL326	This study
pAL338	PCR fragment amplified using MG1655 as the template with primers BA1503 and BA1504 to represent mutant AL4016 transposon insertion region; cloned into pSB401/*Eco*RI in forward orientation	This study
pAL339	AL4016 insertion region cloned in opposite orientation of pAL338	This study
pAL412	AL4001 insertion region cloned into pVIK112/*Eco*RI in forward orientation	This study
pAL413	AL4001 insertion region cloned in opposite orientation of pAL412	This study
pAL426	AL4008 insertion region cloned into pVIK112/*Eco*RI in forward orientation	This study
pAL427	AL4008 insertion region cloned in opposite orientation of pAL426	This study
pAL438	AL4016 insertion region cloned into pVIK112/*Eco*RI in forward orientation	This study
pAL439	AL4016 insertion region cloned in opposite orientation of pAL438	This study

Regions of interest were amplified from MG1655 with *Taq* polymerase (Gene Choice Inc., Frederick, MD). The PCR products were recovered after gel electrophoresis using the QIAquick Gel Extraction Kit (Qiagen) then cloned into pCR 2.1-TOPO using the Invitrogen TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). These cloning reactions were transformed into One Shot TOP10 chemically competent cells (Invitrogen) and plated on LB kan X-gal. White colonies were picked and screened for insert using PCR.

Plasmid construction. Inserts were removed from the TOPO vector with *Eco*RI (Invitrogen) and ligated into either pSB401 or pVIK112 digested with *Eco*RI and Shrimp Alkaline Phosphatase (USB, Cleveland, OH). The ligation reactions were transformed into either TOP10 cells or BW20767 (λpir+). All plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). Presence and orientation of insert in each plasmid was confirmed via PCR and DNA sequencing. The *lux*-based fusions were then transformed into chemically competent WM54 and JLD217.

Table 3.2 Oligonucleotides used for the *sdiA* regulon project

Oligonucleotide name	DNA sequence
BA184	GATGTGCTGCAAGGCGATTAAGTTG
BA247	GAGTCATTCAATATTGGCAGGTAAACAC
BA1090	GAATGTATGTCCTGCGTCTTGAGTA
BA1494	TGCTCAGTATCGGTGAATTTACCCC
BA1495	TGGCACGGGAAACTTTGTGCTCTCA
BA1501	ATTATAGTGCCGTGGATCCTGGCATATAGCCC
BA1502	ATGGAGAGCGAGAACTCTCCATTCTTGACACC
BA1503	TACATCCAGCCAGCAACATAAGCGGCTTTCTC
BA1504	CGGGTTCAGGGTTTTCAATAACTCGCGTGAAG

Results

The LuxR homolog SdiA was first discovered in *E. coli*, though in this organism its regulon is still uncharacterized. Genes once thought to be regulated by *sdiA*, such as *ftzQAZ*, have never been confirmed to be part of the *sdiA* regulon and appear to be pleiotropic results of overexpressing *sdiA*. Identification of *sdiA* in *E. coli* lead to the discovery of *sdiA* in *S.* Typhimurium, where it is most characterized. Previous experiments involving genes of the *S.* Typhimurium *sdiA* regulon have revealed conditions in which *sdiA* is most active. We sought to better characterize the *E. coli sdiA* regulon using the knowledge gained from *S.* Typhimurium while avoiding the potential problems associated with the previous *E. coli* screens. We chose to perform this genetic screen using chromosomal *sdiA* and the growth conditions that promote *sdiA* activity. Therefore, the screen was performed in motility agar, containing oxoC6 (or EA for control), and run at 37°C to optimize *sdiA* activity. The strains used in the experiment were constructed so that both *sdiA* and the random transcriptional fusions were expressed from the chromosome. With these conditions, we hoped to get the most accurate data to date on the *sdiA* regulon of *E. coli.*

Construction and screening of a mutant library. A library of 10,000 random mutants was generated in an *E. coli* K-12 derivative, using transposon mutagenesis as described in Materials and Methods, which creates random transcriptional fusions. This library was screened by patching individual colonies into two adjacent wells of a 96-well plate filled with motility agar containing either 1 μM oxoC6 or an equal volume of the solvent

control EA. The plates were incubated at 37°C in a humid chamber and luminescence readings of each well were recorded at 3, 6, 9 and 12 hrs.

Each mutant was screened for differences in the amount of light produced in the presence of EA and in the presence of 1 μM oxoC6. Mutants that produced a two-fold or greater difference in luminescence were isolated for further screening. Motility agar was used in the screen because it has been shown that *sdiA* of *S.* Typhimurium is most active in this type of medium, rather than solid agar or broth. However, the strain mutagenized is non-motile, so the luminescence was generated from growth of a single stab in the well. Light differences could result from slight variations in the way the mutant was stabbed into the two wells. Therefore, after the initial screening, isolated mutants were seeded rather than stabbed into wells containing motility agar with either EA or oxoC6, and screened again in triplicate.

Results of the library screen. Three mutants were identified that gave consistent and significant fold differences and were named AL4001, AL4008, and AL4016 (Figure 3.1). AL4001 and AL4016 showed increased promoter activation in the presence of oxoC6, with maximum differences of approximately eight and three-fold, respectively (Figure 3.1 A and C). In contrast, AL4008 showed a decrease in promoter activity in the presence of oxoC6, with approximately a two-fold maximum difference (Figure 3.1 B).

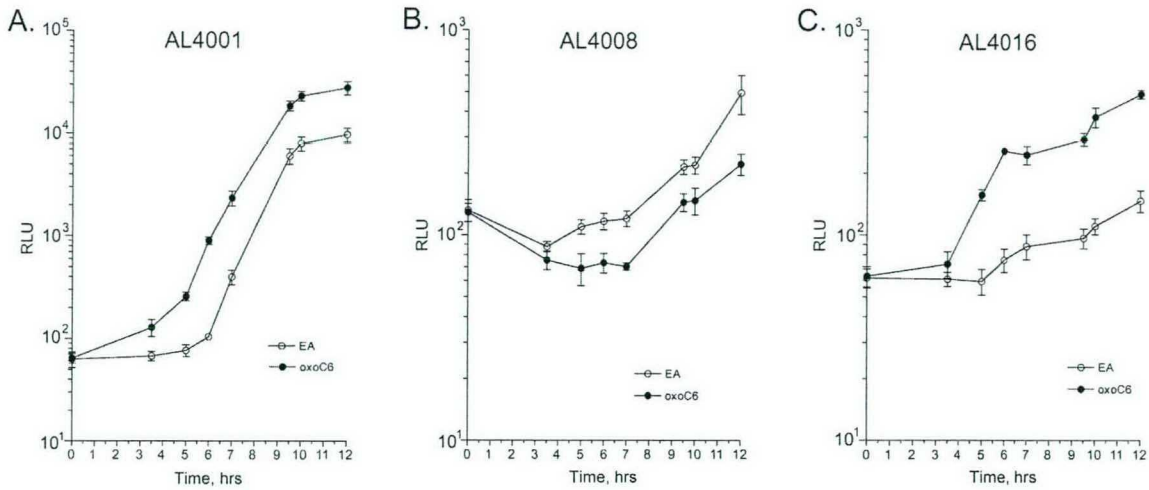

Figure 3.1 Growth curves of AHL-regulated mutants of *E. coli* K-12. Response of *E. coli* K-12 mutants containing random *luxCDABE* fusions to oxoC6 and the solvent control, EA. Optical density at 590 nm (OD_{590nm}) and luminescence in relative light units (RLU) were measured using a Wallac Victor^2 1420 multimode plate reader at the time intervals noted. Data were analyzed using Prism 3.0 graphing software. Each point is the mean of triplicate cultures, with error bars representing standard deviation.

Identification and characterization of isolated mutants. The transposon insertion sites were sequenced using the mutant chromosomal DNA as the template and two different primers, both specific for the transposon. BLAST analysis was used to identify the regions of the *E. coli* K-12 chromosome that contained the promoters of interest. Based on the point of insertion and orientation of the surrounding genes, the promoter or promoters likely responsible for the AHL-dependent change in light production were identified.

In the mutant AL4001, the transposon inserted within the *gadW* gene, which is part of the glutamate-dependant acid resistance system of *E. coli* (Figure 3.2). GadW is involved with the repression of this system, the regulation of which is very complex. The insertion points of the remaining two mutants, AL4008 and AL4016, are located in uncharacterized genes. The transposon of AL4008 inserted in a region containing numerous genes that encode hypothetical proteins of unknown function (Figure 3.2 B).

These genes are possibly arranged in an operon, and so might all be regulated by *sdiA*. If the AL4008 region is not an operon, it could have a number of promoters that must be tested for *sdiA* regulation individually. Sequencing of AL4016 revealed that the transposon is in the *yncG* region, which encodes a putative glutathione-S transferase (GST) like protein (Figure 3.2 C).

For each mutant, the insertion location was confirmed by PCR and the regions containing the putative promoter(s) were amplified from MG1655 (Figure 3.2). The PCR products were cloned into the pSB401 backbone to make *lux* promoter fusions in both orientations. The plasmids were transformed into a wild-type *E. coli* strain, WM54, and the isogenic *sdiA* mutant, JLD271. These strains will be used to investigate the *sdiA*-dependent regulation of the three promoter sets, as well as the AHL-dependence of the response. The PCR products were also cloned into the suicide vector pVIK112 to make *lacZYA* promoter fusions that can be integrated into the chromosome.

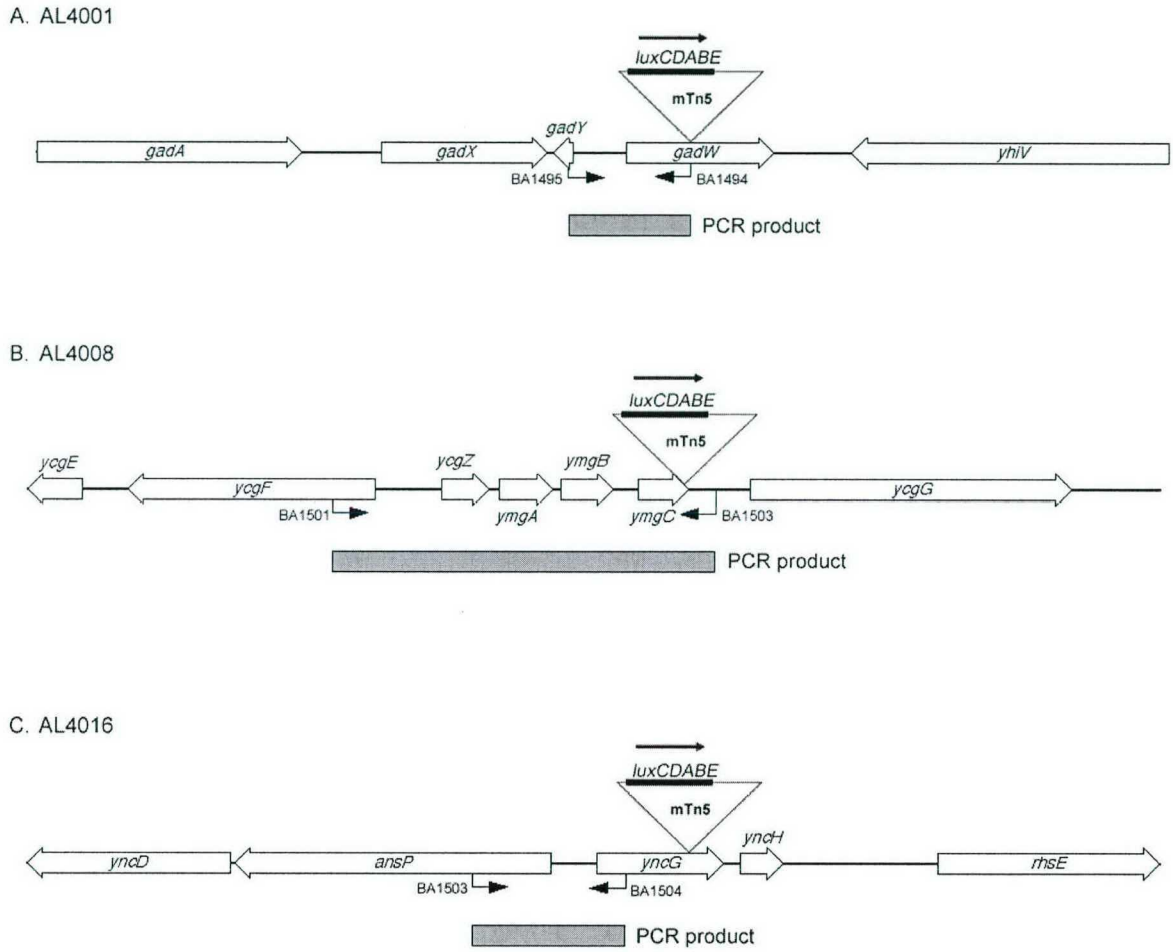

Figure 3.2 Maps of the transposon insertion regions of the AHL-regulated mutants of *E. coli* K-12. The genomic DNA isolated from each mutant was used as the template for sequencing reactions, using primers BA247 and BA1090 (see Materials and Methods). Sequencing results are represented by boxes pictured above the maps with the arrows below them indicating the direction of the promoter driving transcription of the *luxCDABE* cassette. The PCR primers used to amplify each region for cloning are listed in Table 3.2.

Discussion

Here we present a screen to identify genes in the *sdiA* regulon of *E. coli* K-12. Transposon mutagenesis was used to generate a library of random chromosomal *lux* transcriptional fusions that were then screened for regulation by exogenous AHL. Using this method, we isolated three mutants containing fusions to promoters that are

potentially regulated by *sdiA*. With the exception of *gadW*, none of the genes isolated have a known function.

Glutamate-dependent acid resistance (GDAR) in *E. coli* involves the structural proteins encoded by *gadA* and the *gadBC* operon, where *gadA* and *gadB* encode isomers of the glutamate decarboxylase and *gadC* encodes a glutamate / γ-aminobutyrate (GABA) antiporter (21). This system confers resistance to a pH as low as 2.5 and is induced in conditions such as exposure to low pH or entry into stationary phase. GadW, GadX, and GadE are the primary regulators of GDAR in *E. coli*, though the overall regulation is extremely complex. It is known to involve at least 11 regulators, including RpoS, H-NS, and CRP-cAMP, most of which act on the central regulator GadE through different (though sometimes overlapping) regulatory cascades (21). The importance of a particular regulator depends on the environmental and metabolic conditions (21).

The *gadW* gene is encoded directly downstream of *gadX* and *gadA*. However, *gadA, gadX*, and *gadW* are not arranged in an operon and are usually transcribed individually, though there is evidence that *gadA* and *gadX* can be cotranscribed, as can *gadX* and *gadW* (26, 64, 66).

The region containing *gadA, gadX* and *gadW* also encodes other genes involved in acid resistance, most of which are in fact induced by low pH (35, 65). Published genome sequences of several pathogenic strains of *E. coli* were analyzed and it was found that the genomic organization of the region is highly conserved though the cluster could not be found in any other species of bacteria (35). Analysis of this region in the various pathogenic strains as well as *E. coli* K-12 revealed that the G + C content of the region is

significantly lower than that of the whole genome (35). Because the 13 kb region encodes a distinct cluster of genes with related functions, but with a G + C content different than that of the surrounding DNA and of the genome as a whole, Hommais *et al.* suggest that this region constitutes an acid "fitness island" (28, 35). Such fitness islands enhance the adaptation for survival in a particular niche and they suggest that an acid fitness island could be extremely useful in maintaining pH homeostasis within the host gastrointestinal tract (28, 35).

There have been other studies published that attempted to characterize the *sdiA* regulon of *E. coli* K-12 (or genes regulated by AHL in general). Wei *et al.* used a DNA microarray to compare transcript levels produced by wild-type *E. coli* and those produced in a strain overexpressing *sdiA* (70). In the overexpression strain, *sdiA* was expressed from its natural position in the chromosome and from a high-copy number plasmid, which resulted in a 30-fold increase in detected *sdiA* transcripts (70) This microarray identified 75 genes that were activated and 62 genes that were repressed by overexpression of *sdiA* (70). The genes that our screen identified as AHL-regulated genes were not among those identified by the microarray.

The experimental conditions used here to identify the *sdiA* regulon differed greatly from those used by Wei *et al.* and likely contribute to discrepancies in the results obtained. When the microarray analysis was performed, the *sdiA* ligand was still unknown, so it was performed without the addition of AHL. This is problematic because it has been shown that, like other LuxR homologs, *sdiA* requires AHL for maximal activity (59). For the *sdiA* microarray, Wei *et al.* used transcripts generated by a strain

expressing *sdiA* at much higher levels than normal. Overexpression of *sdiA* in *E. coli* appears to bypass the AHL requirement for activation, which probably permitted results to be obtained without the addition of AHL. It is likely that not all of the genes identified in the screen are truly members of the *E. coli sdiA* regulon but are instead pleiotropic results that seem to occur when *sdiA* is overexpressed in *E. coli* (70). Wei *et al.* did not demonstrate that any of the genes identified in the screen were regulated by chromosomal levels of *sdiA*, nor was regulation observed when our lab tested a number of the promoters for regulation by chromosomal *sdiA* (unpublished data).

In the genetic screen performed for the present study, *sdiA* was expressed from its natural position in the chromosome, as were the promoter fusions of the mutant library that was screened. This was to increase the likelihood that the promoters identified were actually regulated by *sdiA*. In addition, motility agar containing 10 μM oxoC6 was used, ensuring high *sdiA* activity to identify as many regulated genes as possible.

Recently, a genetic screen was performed by van Houdt *et al.* for genes regulated by AHLs in *E. coli* K-12 (36). Unlike the microarray performed by Wei *et al.*, this experiment used chromosomal expression of *sdiA* to avoid possible false positives that can result from overexpression. A promoter trap library was constructed in which 1-3 kb fragments of MG1655 chromosomal DNA were inserted upstream of a promoterless *gfp* gene (36). From this library, 13,000 clones were screened for promoters that responded to *N*-hexanoyl-L-homoserine lactones (C6). They identified six genes that were upregulated and nine genes that were downregulated in response to C6, none of which were those identified in the *E. coli* microarray performed by Wei *et al.* (36, 70). The

response of each of the promoters was then confirmed to be regulated in an *sdiA*-dependent manner (36). However, van Houdt *et al.* performed the experiment at 30°C and the response of almost all of the promoters was eliminated at 37°C, which is interesting given that 37°C is required for AHL and *sdiA*-dependent activation of certain promoters in *S. typhimurium*. Our screen was performed at 37°C instead of 30°C, which may be a reason our assay identified fewer genes. Similar to our screen, the screen performed by van Houdt *et al.* identified an acid resistance gene of the GDAR system, *gadA,* which encodes a glutamate decarboxylase, a key enzyme of the GDAR system. This gene is located upstream of *gadW*, the GDAR gene identified in our screen, though they are not part of an operon. Interestingly, the *gadA* promoter was one of only two that were still regulated by AHL at 37°C instead of 30°C in the van Houdt screen. Additionally, the other two AHL-responsive regions identified by our screen were not isolated by the van Houdt screen.

There are reasons other than variations in growth conditions that can explain why we did not obtain the same results, as did the van Houdt *et al.* assay. Differences in sensitivities of the reporters used as well as that of the methods used to measure the activity of the fusions can lead to different results, especially when considering that different criteria were used to determine what level of activation was significant. It is likely that our screen does not represent the *sdiA* regulon in its entirety. As mentioned previously, the strain used in our screen was not motile and so did not swim to fill the wells of motility agar into which they were patched. The initial round of library screenings potentially could have missed several *sdiA*-regulated fusions simply because the stabs in the two wells were not in the exact same location, depth, angle, etc.

In addition, it was expected that patching 10,000 mutants would screen approximately 63.2 % of the approximately 5,000 nonessential genes encoded by *E. coli*. Therefore, slightly over one-third of the nonessential genes were not even screened and this number assumes no mutants were patched more than once. However, because the mutants were obtained via plate matings, it is possible that some mutants had "siblings" that were patched, meaning that we may have patched fewer than 10,000 unique mutants.

Though the genes identified in our study have not yet been confirmed to be regulated by *sdiA*, literature searches revealed no obvious relation to *sdiA* nor any reason for these to be regulated by *sdiA* in response to AHL. However, the search revealed that these genes have been identified in various screens related to stationary phase and stress responses. Often these assays identified more than one of the genes isolated in the screen presented here, suggesting that they are regulated by the same conditions. Only one of the insertions regions identified in this screen, in the *gadW* gene, has been characterized.

As mentioned previously, the literature search revealed that a number of genetic screens identified the genes encoded in our three regions potentially regulated by *sdiA*. Although *gadW* has been identified in a number of screens and characterized extensively, information on the other genes is scarce as they were identified in genetic screens, but were not characterized further. Many of these experiments primarily sought to identify genes that were regulated in response to entry into stationary phase or to other stresses.

Shimada *et al.* measured the expression from stationary-phase promoters in order to test their newly-constructed promoter cloning vector (56). In this study, *ycgZ* of the AL4008 region (Figure 3.2) was selected for testing based on unpublished identification

of stationary phase promoters. Here they determined that expression of *ycgZ* is dependent on *rpoS*, as are *gadA* and *gadB*, though unlike these GDAR genes, there is still some expression of *ycgZ* upon entrance into stationary phase in the absence of *rpoS*. They also found that *ycgZ* was highly expressed in stationary phase, with expression levels increasing over 10-fold (56).

A DNA microarray was performed to analyze the response of *E. coli* K-12 to hydrogen peroxide (75). The assay was performed to identify the regulon of OxyR, a regulatory protein that activates genes of the oxidative stress response, by analyzing the transcripts produced by a wild-type strain and an isogenic *oxyR* mutant in response to hydrogen peroxide (75). Three of the four genes present in the AL4008 region were among the genes most strongly induced by hydrogen peroxide (Figure 3.2). The *ymgB* and *ycgZ* genes were among the 30 most strongly induced in a wild-type background, with induction ratios of 20 and 11, respectively, whereas *ymgB* and *ymgA* were among the 30 most highly induced genes in the *oxyR* mutant background, with induction ratios of 17 and 14, respectively (75).

Patten *et al.* performed microarray analysis to identify *rpoS*-dependent genes by comparing stationary phase transcripts generated in a wild-type strain to those generated in an *rpoS* mutant (48). This screen also identified the genes of the AL4008 region, including *ymgC*, which was not identified in the Zheng *et al.* screen. This is probably due to differences in the threshold levels that each study considered significant. Patten *et al.* considered 4-fold increase in the mean expression ratio (MER) significant, whereas Zheng *et al.* only reported the 30 most strongly induced genes (48, 75). Patten *et al.*

report that *rpoS* induces *ycgZ, ymgA, ymgB,* and *ymgC* of the AL4008 region by approximately 8, 23, 5.5, and 12-fold, respectively (48). The AL4001 gene *gadW* is induced approximately four-fold in response to *rpoS* (48). The acid resistance genes *gadA, gadB,* and *gadX* were also identified in the screen which is expected as it is widely known that GDAR is induced upon entry into stationary phase, regardless of the pH (48).

Another genome-wide analysis of RpoS regulation was performed by Weber *et al.* to characterize the general stress response network in *E. coli* K-12 (69). As in the Patten *et al.* microarray, *rpoS*-dependent genes were identified by comparing transcripts generated in wild-type and *rpoS* mutant strains, though this study examined three different growth conditions (69). In addition to analyzing genes regulated in response to transition into stationary phase, transcript levels from conditions representing osmotic stress and acid stress were also assayed. Interestingly, by using these conditions, Weber *et al.* identified genes from all three regions identified by our screen; *gadW* of AL4001, *ycgZ* and *ymgA* of AL4008, and *yncG* of AL4016 were all found to be induced at least two-fold by *rpoS* in all three conditions tested (69).

Numerous DNA microarrays performed to isolate genes involved in stationary phase or other stress responses identified genes that we identified as part of the *sdiA* regulon. It is interesting that these genes of unknown function were repeatedly identified as genes involved in stress responses or stationary phase response of *E. coli* K-12, along with *gadW*, a regulator of the acid response that is known to be upregulated in stationary phase.

An underlying factor linking the genes identified in our screen seems to be the stationary phase sigma factor encoded by *rpoS*. Results from experiments performed by our lab indicate that *sdiA* is not regulated by RpoS, nor is the activity of *sdiA* in response to AHL (unpublished data). The regulon of RpoS is still not completely characterized and its overall role in stress response is not yet entirely understood. It is not hard to imagine that there are other conditions of stress yet to be identified that are encountered by the cell that elicit responses regulated by RpoS.

Once the genes we identified as responsive to AHL are confirmed to be regulated by *sdiA*, further experiments must be performed to elucidate the role that RpoS plays in their regulation. It is possible that RpoS is involved in their regulation in some conditions, while *sdiA* and AHL are responsible for it in others. Expression of the genes of the AL4008 region was decreased in the presence of AHL, though upregulated in the stress responses tested in the microarrays described. If these microarray data are indeed correct, it suggests that RpoS and *sdiA* may play antagonistic roles in the regulation of the promoter(s).

Regardless of the role that RpoS plays in the regulation of the promoters identified by our screen, the first step after dependence on *sdiA* and AHL is confirmed, is to determine if *sdiA* might be more active in the conditions used for the microarrays. The plasmid-based fusions can be used to determine the activity of the promoters of interest in response to various stress conditions. These will be tested in conditions such as transition into stationary phase, osmotic stress, acid stress, and exposure to hydrogen peroxide. It is important that the activities of the promoters be determined in a wild-type strain as well

as in an *sdiA* and an *rpoS* mutant. A double mutant, lacking *sdiA* and *rpoS,* must also be constructed to determine if the promoters of interest are completely dependent on either regulator for expression. The suicide plasmids that were constructed here to generate chromosomal *lacZYA* fusions to the promoters of interest will become important to determine promoter activity at the natural position in the chromosome. As past research with *sdiA* has demonstrated, experiments relying upon plasmid-based expression to investigate regulation often produce results not representative of actual regulation.

It is not clear why *sdiA*, as a homolog of the LuxR quorum sensing protein, would be responsible for the regulation of genes exclusively related to stress response and that are potentially also under the control of RpoS. Based on data obtained from *sdiA* of *S.* Typhimurium, it is assumed that *sdiA* senses the presence of other organisms. The host presents a range of stressful conditions. Detection of other bacteria via AHLs could potentially be another way for the cell to determine that multiple stress responses are required. Redundancy of regulation in pathways that are essential to survival is often seen in bacteria. The majority of the genes identified as potential members of the *sdiA* regulon are of unknown function and may well encode genes that are meant to increase survival within a host.

It has been said that bacteria entering a host's gastrointestinal tract are in stationary phase, suggesting that the potentially *sdiA*-regulated genes are already being expressed (21). It is possible that, though the genes are regulated by *rpoS*, regulation by *sdiA* can by-pass other levels of regulation in the event that an AHL-producing organism is detected. These genes may be of particular importance during a coinfection, resulting

in *sdiA* out-competing other regulators for control of the expression of its target genes. In order to elucidate the role played by *sdiA* in *E. coli*, during infection or otherwise, the regulation of the genes identified in this screen must be further characterized. Once more information is obtained, hypotheses can be made about the relationship between *sdiA*, *rpoS* and the general stress response in *E. coli*, as well as if and why the genes of the *sdiA* regulon have multiple levels of regulation. Until then, the information generated by this screen raises far more questions than answers.

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