Transcriptional regulation of *sdiA* by cAMP-receptor protein, LeuO, and environmental signals in *Salmonella enterica* serovar Typhimurium

Amy L. Turnbull, Wook Kim, and Michael G. Surette

Abstract: The *sdiA* gene encodes for a LuxR-type transcription factor, which is active when bound to *N*-acyl homoserine lactones (AHLs). Because *Salmonella enterica* serovar Typhimurium does not produce AHLs, SdiA senses signals produced by other organisms. SdiA is not expressed constitutively, and response is limited to conditions in which elevated expression occurs, but little is known about the regulation of *sdiA* expression. Here we map the *sdiA* promoter and define several regulators that directly or indirectly act on the promoter. The major activator of *sdiA* expression is cAMP-receptor protein (CRP), and we define the CRP operator in the *sdiA* promoter using promoter and *crp* mutants. LeuO activates *sdiA* expression to a lesser extent than does CRP. We demonstrate that LeuO directly binds the *sdiA* promoter and the Rcs phosphorelay represses *sdiA* expression. In this study, NhaR, IlvY, and Fur affected *sdiA* expression indirectly and weakly. Expression in late-stationary phase depended on RpoS. AHL-dependent expression of the SdiA-regulated gene *rck* correlated to the observed *sdiA* transcriptional changes in regulator mutants. The data demonstrate that regulation of *sdiA* involves integration of multiple environmental and metabolic signals.

Key words: Salmonella enterica, SdiA, acyl homoserine lactone, quorum sensing.

Résumé : Le gène *sdiA* code un facteur de transcription de type LuxR qui est actif lorsque lié aux acyl-homosérine-lactones (AHL). Puisque *Salmonella enterica* du sérovar Typhimurium ne produit pas de AHL, SdiA détecte les signaux produits par d'autres organismes. SdiA n'est pas exprimé de façon constitutive et la réponse qui en dépend est limitée aux conditions où son expression est élevée, mais on connaît peu de choses de la régulation de l'expression de *sdiA*. Nous avons cartographie le promoteur de *sdiA* et nous avons défini plusieurs régulateurs qui agissent directement ou indirectement sur ce promoteur. Nous avons déterminé que CRP est le principal activateur de l'expression de *sdiA* et nous avons caractérisé le site opérateur de CRP sur le promoteur de *sdiA* à l'aide de mutants du promoteur et de *crp*. LeuO active l'expression de *sdiA* à un degré moindre que CRP. Nous avons démontré que LeuO se lie directement au promoteur de *sdiA*. Le système de phosphorelais Rcs réprime l'expression de *sdiA*. NhaR, IlvY et Fur affectent indirectement et faiblement l'expression de *sdiA*. Son expression lors de la phase stationnaire tardive dépend de RpoS. L'expression du gène *rck*, régulée par SdiA et dépendante des AHL, est corrélée avec les changements transcriptionnels de *sdiA* observés chez les mutants du régulateur. Les données démontrent que la régulation de *sdiA* implique l'intégration de multiples signaux environnementaux et métaboliques.

Mots-clés : Salmonella enterica, SdiA, acyl-homosérine lactone, détection du quorum.

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Introduction

Cell–cell signalling (quorum sensing) enables bacteria to communicate and receive information about other bacteria present in their environment (Ng and Bassler 2009; Njoroge and Sperandio 2009; Decho et al. 2010; Jakubovics 2010). Cell–cell signalling results from the production of signalling molecules and their accumulation in the surrounding environment. At some threshold concentration, the signalling molecules bind to receptors on or in the bacterial cell, leading to changes in gene expression in the responding cell (Keller and Surette 2006). Bacteria can use cell–cell signalling mechanisms to coordinate their behaviour through changes in gene expression to better suit a particular environment. One quorum-sensing system found in gram-negative bacteria uses *N*-acyl homoserine lactones (AHLs) as a signal (Ahmer et al. 2007; Gorshkov et al. 2010; Mei et al. 2010; Weeks et al.

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2010). AHLs are synthesized from *S*-adenosylmethionine and acyl-ACP, usually by LuxI family synthases (Laue et al. 2000; Miller and Bassler 2001; Ng and Bassler 2009). AHLs are typically sensed by LuxR family receptors, which, upon AHL binding, then bind DNA at specific sites and alter transcription of a set of genes (Gray and Garey 2001; Gorshkov et al. 2010; Mei et al. 2010; Weeks et al. 2010).

SdiA is a LuxR homologue found in *Salmonella*, *Escherichia*, and *Klebsiella* that lacks a cognate AHL synthase (Ahmer 2004). In *Salmonella*, SdiA is hypothesized to respond to signals produced by other organisms (Michael et al. 2001; Smith and Ahmer 2003). SdiA has relaxed binding specificity to AHLs, and synthetic homothiolactones are the strongest activators of SdiA found to date (Janssens et al. 2007). SdiA activates two operons in *Salmonella* in response to AHL binding, *rck* on the pSLT virulence-associated plasmid and *srgE* on the chromosome (Ahmer et al. 1998; Smith and Ahmer 2003). *rck* is a six-gene operon including *pefI* (transcription factor for upstream *pef* operon), *srgD* (putative transcription factor), *srgA* (DsbA homologue involved in Pef assembly in periplasm), *srgB* (putative lipoprotein), *rck* (adherence to laminin and fibronectin, also resistance to complement killing), and *srgC* (putative transcription factor).

Recent studies have demonstrated that SdiA can be active in the gut and that it responds to AHLs produced by other species. SdiA was activated during transit through turtles by AHLs produced by *Aeromonas hydrophila* (Smith et al. 2008) and through mice that were coinfected with *Yersinia enterocolitica* (Dyszel et al. 2010). However, activation of SdiA did not appear to confer a fitness advantage when compared with a *sdiA*$^-$ strain.

The SdiA regulon in *Escherichia coli* was identified initially using SdiA overexpressed from plasmids, but no regulation was detected when the *sdiA* gene was in its native context (Wei et al. 2001). Previously, our laboratory has shown that *sdiA* expression was increased in swarm cells (Kim and Surette 2006). In addition, it was observed that *sdiA* expression was increased in Luria–Bertani (LB) broth containing 2% NaCl when compared with LB with 0.5% NaCl. These results indicate that there is a differential expression of *sdiA* that is growth condition dependent. The regulators of *sdiA*, however, are not known. In this study, the regulation of *sdiA* transcription and *sdiA* promoter organization was investigated.

Materials and methods

Bacterial strains, plasmids, and growth conditions

All strains and plasmids and primers used in plasmid construction are listed in Table 1. For routine growth, strains were cultured in Luria–Bertani broth (LB-Miller (Invitrogen), 10 g peptone, 5 g yeast extract, and 10 g NaC, per litre) at 37 °C and 200 r·mim^{-1}. For experiments on *sdiA* gene expression, cells were grown in LB-marine (LM), which contains 2% NaCl. LBns refers to LB made with no NaCl. When required, kanamycin, chloramphenicol, and ampicillin were used at 50 μg·mL^{-1}, 12.5 μg·mL^{-1}, and 100 μg·mL^{-1}, respectively.

Construction of plasmids, mutants, and DNA fragments for electrophoretic mobility shift assays

All DNA manipulations were conducted using standard techniques (Sambrook and Russell 2001). *sdiA1*, *sdiA2*, and *sdiA3* reporters were constructed using the same 3′ primer as for *sdiA0*, sdiA5′B, as published previously (Kim and Surette 2006). All regions used in the construction of transcriptional reporters were PCR amplified, then digested with *Xho*I and *Bam*HI. *nhaR* and *ilvY* were cloned into pBAD18 using *Eco*RI and *Hin*dIII, *leuO* was cloned into pBAD18 using *Hin*dIII and *Sac*I, and *leuO* was cloned into pQE8 using *Bam*HI and *Hin*dIII. All restriction enzymes were from Invitrogen Canada. Specific gene deletions were constructed using the λ red recombinase system (Datsenko and Wanner 2000). All mutations, including those in strains obtained from the Salmonella Genetic Stock Centre, were moved into the *Salmonella* serovar Typhimurium background using the P22 phage and methods described previously (Maloy 1990).

Luciferase assays

For time-course measurements of gene expression and optical density, cultures were measured every 30 min in a Victor^2 1420 (PerkinElmer Life Sciences) as described by Bjarnason et al. (2003). Luciferase activity was expressed in counts per second. For all other measurements of gene expression, 100 μL of culture in biological triplicate was measured with a 1 s read time in a Trilux Scintillation Counter (PerkinElmer Life Sciences). Counts-per-second values were normalized to the optical density of the culture- or colony-forming units, yielding relative light units.

Primer extension

Cells were grown in LM at 37 °C at 200 r·min^{-1} and were harvested in late exponential phase. Because the *sdiA* gene is weakly expressed, a point mutant that has greater activity than the wild promoter was used (sdiA1-c1e4, see below). RNA was extracted from cells (QIAGEN RNeasy Mini-kit) and an on-column DNA digestion was performed. RNA (20 μg) was used in the following reactions. RNA was reverse transcribed into cDNA using Superscript II Reverse Transcriptase (Invitrogen) with the use of a Vic-labeled PZE06 primer (Applied Biosystems), which anneals to a site in *luxC*, yielding labelled cDNA (Bjarnason et al. 2003). RNA was digested using RNase I_f (NEB). cDNA was cleaned and was concentrated to 6 μL on a PCR purification column (Zymogen). A regular sequencing reaction of c1-e4 with the PZE06 primer and the primer extension reaction were compared by capillary electrophoresis (DNA Sequencing Facility, University of Calgary, Calgary, Alberta, Canada). On the electrophoretogram, the sequencing reaction was aligned with the sequencing reaction containing the labelled cDNA, allowing determination of the transcription initiation site as in the cDNA reaction.

Promoter point mutant library construction

Error-prone PCR of the wild-type *sdiA* promoter with primers used in the construction of *sdiA1* was conducted by adjusting $MnCl_2$ to 62.5 μmol·L^{-1} (Pritchard et al. 2005). The products were cloned into pCS26-PacI as described previously (Turnbull and Surette 2008). The ligation reaction was transformed into wild-type 14028 and plated onto LM agar. Colonies were imaged using an Alpha Imager FluorChem 8900 CCD camera (Alpha Innotech Corp.). Colonies were picked up using sterile toothpicks and were inoculated into 1 mL cultures in 96-well deep-well plates. These plates were

Table 1. Strains, plasmids, and primers used in this study.

Strain, plasmid, or primer	Relevant characteristic(s)	Source or reference
Strains		
Wild type	*Salmonella* Typhimurium 14028	ATCC 14028
crp^{-}	Donor: *Salmonella* Typhimurium LT2 *crp*::Tn*10 trpB223*	Salmonella Genetic Stock Centre, strain PP1037
	Recipient: *Salmonella* Typhimurium 14028	
leuO	*leuO*::*cat*	This study
Δ*rpoS*	Deletion of *rpoS*	White and Surette 2006
rcsD	*rcsD*::*cat*	This study
E12	*rcsD*::Tn*10d*Cm	This study
ilvY	*ilvY*::*cat*	This study
nhaR	*nhaR*::*cat*	This study
R18	Donor: *Salmonella* Typhimurium *galE854 lky-1 fur-1 rpsL1266*	Salmonella Genetic Stock Centre, strain R18
	Recipient: *Salmonella* Typhimurium 14028	
Plasmids		
pCS26	Low-copy *luxCDABE* vector	Bjarnason et al. 2003
pBAD18	High-copy vector containing inducible promoter	Guzman et al. 1995
pBR322	High-copy vector	NEB
pQE8	Protein expressing vector	QIAGEN
pUC19	High-copy vector	NEB
sdiA0	pCS26 containing 14028 *sdiA* promoter, 709 nt	Kim and Surette 2006
sdiA1	pCS26 containing 14028 *sdiA* promoter, 377 nt	This study
sdiA2	pCS26 containing 14028 *sdiA* promoter, 237 nt	This study
sdiA3	pCS26 containing 14028 *sdiA* promoter, 137 nt	This study
pps:pBR	pBR322 containing entire *pps* gene	This study
leuO:pQE8	pQE8 containing *leuO* coding region	This study
leuO:pBAD	pBAD18 containing *leuO* coding region	This study
flhDC:*lux*	pCS26 containing *flhDC* promoter	—
lacZ:*lux*	pCS26 containing *E. coli* MG1655 *lacZ* promoter	—
ilvY:pBAD	pBAD18 containing *ilvY* coding region	This study
nhaR:pBAD	pBAD18 containing *nhaR* coding region	This study
sitA:*lux*	pCS26 containing *sitA* promoter	Bjarnason et al. 2003
rck:*lux*	pCS26 containing *rck* promoter	Kim and Surette 2006
Primers		
LeuOdel1	AGCCAGAAAAAGGGAGTTAAGCGTGACAGTG GAGTTAAATGTGTAGGCTGGAGCTGCTTC	This study
LeuOdel2	GGAATAAACCAGAATTTGTTTCTGATTTATTCT GCCCGGTTCCTCCTTAGTTCCTATTCCG	This study
RcsDdel1	CCTTCACCTTCAGCGTTGCTTTTACAGGTCG TAAACATAAGTGTAGGCTGGAGCTGCTTC	This study
RcsDdel2	TGTTCATGTATTGGGCTACCTTGCTACAGC AAGCTTTTGACCTCCTTAGTTCCTATTCCG	This study
IlvYdel1	CTAATTCGGTAAAATCTTCCAGAACGCATCA ATTAGCGGCGT GTA GGC TGG AGC TGC TTC	This study
IlvYdel2	GTGGATTTACGCGATCTAAAAACCTTCTTGC ATCTGGCGGCCTCCTTAGTTCCTATTCCG	This study
NhaRdel1	ATGAGCATGTCTCATATTAACTACAACCATC TTTATTATTGTGTAGGCTGGAGCTGCTTC	This study
NhaRdel2	CTACTGTAGTTTAAACAATGCCGAGTAGTC CGCATTGCAAACCTCCTTAGTTCC TATTCCG	This study
sdiA1-1	AGTCCTCGAGTACCCGCCAGTTCCTTGAGA	This study
sdiA2-1	AGTCCTCGAGCAAAAAGTGTAATGCCGCTG	This study
sdiA3-1	AGTCCTCGAGATCAATATCAAAGGCGTGAC	This study
LeuOqE8-1	AGTCGGATCCCCAGAGGTCAAAACCGAAAAG	This study
LeuOqE8-2	AGTCAAGCTTTATTCTGCCCGGTTTTATCG	This study

Table 1 (*concluded*).

Strain, plasmid, or primer	Relevant characteristic(s)	Source or reference
LeuObad1	AGTCAGCTCCGTGACAGTGGAGTTAAATATG	This study
LeuObad2	AGTCAAGCTT TATTCTGCCCGGTTTTATCG	This study
IlvYpad1	AGTCAAGCTTAAGCCTGTTCGGGTAATGGTAG	This study
IlvYbad2	AGTCGAATTCAAACCGGAGGTCTGTCTGTG	This study
NhaRbad1	AGTCGAATTCCATTGAACAGGGAGAGAAATG	This study
NhaRbad2	AGTCAAGCTTATACCGCAGCCGGAAGTATTAC	This study
ilvC1	AGTCCTCGAGGCTGGCCGAGATCTTCTTC	This study
ilvC2	AGTCGGATCCTAAAGCGGCATTTACCCAAC	This study
nhaA1	AGTCCTCGAGTGCACGTTTGATGAAAATGG	This study
nhaA2	AGTCGGATGCAAATGGGTGCAGATGTTTCAC	This study
leuL1	AGTCGGATCCGAACGATATGTGACATTAATTC	This study
leuL2	AGTCGGATCC TATTTAACTCCACTGTCACGC	This study
sdiA0-A1	AGTCGGATCCCGCGGATGCTGAGGATC	This study
sdiA1-A1	AGTCGGATCCGCTTCCGCCTGAGAG	This study
sdiA2-A3	AGTCGGATCCAATCATTATTATGAA	This study

grown overnight and glycerol was added, and they were frozen at –80 °C for future use. Plasmids were sequenced from strains expressing *sdiA* at different levels from the wild-type strain.

Screen to identify regulators of *sdiA* using random overexpression of genomic fragments and transposon mutagenesis

A random genomic overexpressing library of *Salmonella* serovar Typhimurium consisting of fragments (~12 kb) of the *Salmonella* serovar Typhimurium genome cloned into the high-copy vector pBR322 was a gift from Dr. Aaron White. This library was transformed into wild-type *Salmonella* serovar Typhimurium containing the *sdiA0*:*lux* construct. Transformants were plated onto LM agar and grown overnight, and luminescence pictures were taken. Colonies selected were those that expressed *sdiA* at levels significantly different from the wild-type strain, and a total of 44 plasmid inserts were sequenced.

A Tn*10d*Cm (Elliott and Roth 1988) random transposon library of *Salmonella* serovar Typhimurium 14028 carried in phage P22 was used to mutagenize *Salmonella* serovar Typhimurium containing the *sdiA0*:*lux* construct. Transductants were plated onto LM agar and grown overnight, and luminescence pictures were taken. Colonies selected were those that expressed *sdiA* at levels significantly different from the wild-type strain. Transposon insertion sites were determined using arbitrary primed PCR (Welsh and McClelland 1990).

LeuO purification

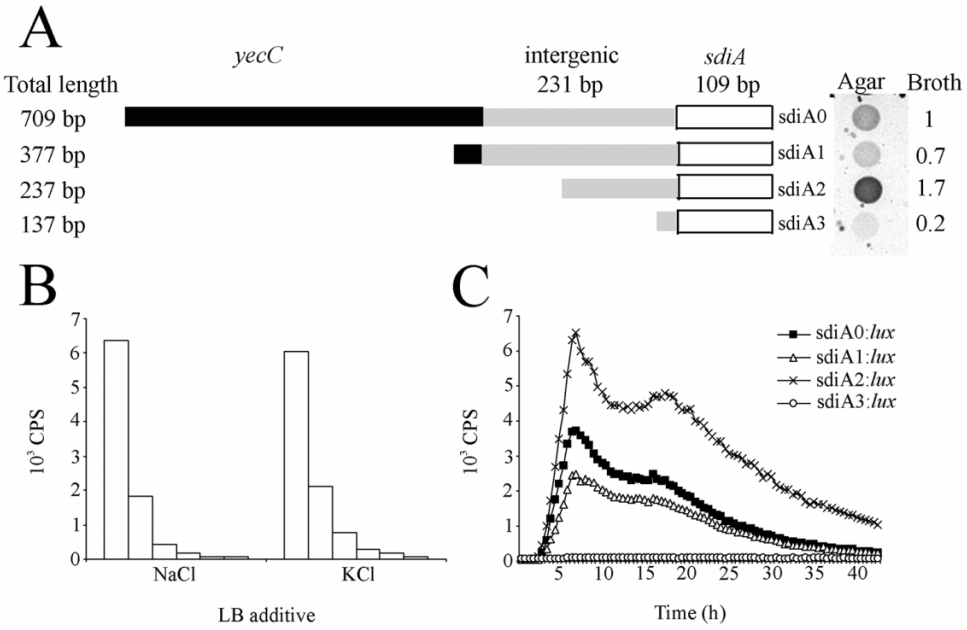
Histidine-tagged LeuO was purified using the pQE/pREP system (QIAGEN). pQE8-LeuO was constructed by cloning the *leuO* genes as a translational fusion into pQE8 amplified using LeuOqe8-1 and LeuOqe8-2 primers (Table 1). Both pREP4 and pQE8-LeuO were transformed into M15. This strain was grown overnight at 30 °C, 200 r·min^{-1} in LB with 0.5% glucose with kanamycin and chloramphenicol. LeuO expression was induced with 200 μmol·L^{-1} isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Cells were harvested by centrifugation for 10 min at 16 000*g*. The cells from a 100 mL culture were resuspended in 1 mL lysis buffer (50 mmol·L^{-1} NaH_2PO_4, 500 mmol·L^{-1} NaCl, 10 mmol·L^{-1} imidazole, pH 8). The protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to 1 mmol·L^{-1} final concentration, and lysozyme was added to 1 mg·mL^{-1}. Samples were incubated on ice for 30 min. Cells were flash frozen and thawed three times, followed by sonication, until clearing was observed. Insoluble cell debris was removed by centrifugation at 4 °C, 10 000*g*, for 25 min.

Ni-NTA resin (QIAGEN) was prepared according to the manufacturer's instructions, and 1 mL was loaded into a column. The soluble fraction of 1 mL cell lysate (from 100 mL culture) was applied to the resin. LeuO was eluted using an elution buffer gradient (buffer 1: 50 mmol·L^{-1} NaH_2PO_4, 500 mmol·L^{-1} NaCl, 50 mmol·L^{-1} imidazole; buffer 2: 50 mmol·L^{-1} NaH_2PO_4, 500 mmol·L^{-1} NaCl, 500 mmol·L^{-1} imidazole). The gradient of imidazole was 50 mmol·L^{-1} to 500 mmol·L^{-1}. Fractions (500 μL) were collected until no elution buffer remained. Samples were kept on ice as an SDS–PAGE was run to determine which eluates contained LeuO. Fractions containing LeuO were pooled and dialyzed overnight (Pierce Slide-a-lyser, 10 000 MWCO) against 2 mmol·L^{-1} Tris–HCl (pH 8) and 0.2 mmol·L^{-1} EDTA. Samples were concentrated 20-fold by evaporation, and an equal volume of 100% glycerol was added (100 μL LeuO solution evaporated to 5 μL, with 5 μL glycerol added). LeuO was stored at –80 °C until further use.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were conducted with either purified protein (LeuO) or the soluble fraction of a cell lysate overexpressing a protein, and corresponding knockout mutant of the gene as a negative control (Hellman and Fried 2007). LeuO was used at 5.4 pmol per reaction. In all EMSAs, the DNA being tested for binding was at 10 ng per reaction; DNA consisted of PCR-amplified promoters in H_2O. Reaction volume was 10 μL with 1 μL 10× binding buffer, 1 μL 10× BSA (NEB), and 1 μg nonspecific DNA (herring sperm DNA, Invitrogen). Binding reactions were incubated on ice for 30 min. Samples were applied to a 6% DNA native PAGE with 0.5× Tris–borate–EDTA (TBE) buffer (pH 7.5). DNA was visualized with Sybr

Fig. 1. (A) *sdiA* promoter constructs showing upstream gene *yecC* (black), intergenic region (grey), and *sdiA* coding region (white). Total length indicates length of transcriptional fusions fused to promoterless *luxCDABE*. Right panel: Reporter expression on Luria–Bertani (LB), LB-marine (LM) agar plates, and expression in broth normalized to *sdiA0*. (B) Expression of *sdiA0* in decreasing NaCl or KCl. Bars from left to right show maximal expression values (occurring between 7.5 and 9.5 h) in LB with no NaCl with 0.5 mol·L^{-1}, 0.25 mol·L^{-1}, 0.125 mol·L^{-1}, 0.0625 mol·L^{-1}, 0.03125 mol·L^{-1}, and no salt. (C) Expression of *sdiA* reporters in LM broth. CPS, counts per second.



Green (Molecular Probes) in TBE (pH 7.5). For EMSAs using cell lysates, cultures were concentrated 100 times at the time of harvest, and in the EMSA reaction, 1% cell-free extracts (final concentration) were used. For these reactions, the nonspecific DNA was omitted because genomic DNA served as a nonspecific competitor. For all EMSAs, a positive control was used, consisting of a promoter for which published binding of the protein exists (P_{leuL}, P_{nhaA}, and P_{ilvC} for LeuO, NhaR, and IlvY, respectively), as well as a negative control consisting of a promoter with no known or predicted binding sites for these proteins. The DNA used in EMSAs was generated by PCR and was column purified (Zymogen) (Table 1). *sdiA0-A1*, *sdiA1-A2*, and *sdiA2-A3* were constructed using the corresponding primer pairs (Table 1). Binding buffers (10×) for the proteins tested were as follows: LeuO (0.2 mol·L^{-1} HEPES KOH pH 8, 0.7 mol·L^{-1} potassium glutamate, 1 mmol·L^{-1} DTT, 5 mmol·L^{-1} $MgCl_2$) (modified from Chen and Wu 2005); NhaR (0.2 mol·L^{-1} Tris–HCl pH 7.5, 10 mmol·L^{-1} EDTA, 1.5 mol·L^{-1} NaCl, 10 mmol·L^{-1} DTT, 10% glycerol) (modified from Goller et al. 2006); and IlvY (0.1 mol·L^{-1} Tris–HCl pH 7.5, 0.5 mol·L^{-1} KCl, 0.1 mol·L^{-1} $MgCl_2$, 1 mmol·L^{-1} EDTA, 10 mmol·L^{-1} DTT) (Rhee et al. 1998).

DNase I footprinting

DNase I footprinting of the *sdiA* promoter using LeuO was conducted according to Sambrook and Russell, with several modifications (Sambrook and Russell 2001; Wilson et al. 2001). Briefly, the 5′ primer used to construct *sdiA2* included a 3′ Vic label (Applied Biosystems) in PCR to produce Vic-labeled *sdiA2*. The binding reaction consisted of 100 ng sdiA2-Vic DNA, 83 pmol LeuO, and 1 µg nonspecific DNA. After the binding reaction incubation, 50 µL 10 mmol·L^{-1} $MgCl_2$ and 5 mmol·L^{-1} $CaCl_2$ were added, mixed gently, and incubated 1 min at room temperature. DNase I (Invitrogen), 2×10^{-4} U, was added and incubated for 1 min at room temperature. The reaction was stopped by adding 15 µL of 500 mmol·L^{-1} EDTA. DNA was recovered, concentrated on a column (Zymogen), and analyzed by capillary sequencing (DNA Sequencing Services, University of Calgary).

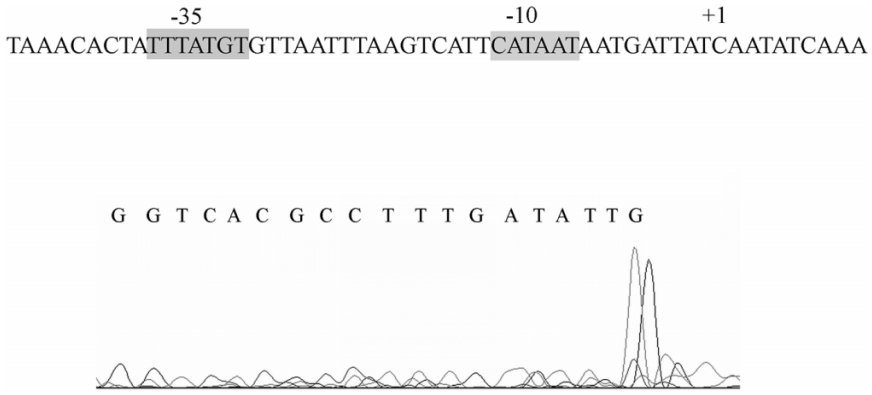
Results

Promoter reporter fusions of varying length suggest transcriptional regulation of *sdiA*

To identify regulatory regions within the *sdiA* promoter, four transcriptional reporters were constructed using the *sdiA* promoter and the promoterless luciferase operon. Each reporter starts at a different 5′ site within the *sdiA* promoter, whereas the 3′ end of the reporter remains constant (Fig. 1A). The four *sdiA* reporters were assayed in LM broth and on LM agar plates. In broth, *sdiA0*, *sdiA1*, and *sdiA2* had the same temporal expression patterns, with only the amplitude of expression being altered (Fig. 1C). Similar differences in expression between reporters on LM agar plates were observed (Fig. 1A). One interpretation of the expression differences is that the *sdiA* promoter contains several operators, and the four reporters have altered expression values due to the presence and absence of these operators.

To determine the dominant regulatory input on *sdiA* expression, the *sdiA0* reporter sequence was cloned into pUC19 and transformed into a strain carrying the *sdiA0* reporter. Expression of the reporter plasmid decreased (not shown). This suggests that *sdiA* expression depends on transcriptional activators. If regulation had been predominantly by repression, expression of the *sdiA0* reporter would have increased in this titration experiment.

Fig. 2. Mapping transcription start site with –10 and –35 regions onto the *sdiA* promoter based on results of primer extension using dye-labelled primer and capillary electrophoresis (see Materials and methods) (top panel). Electrophoretogram showing primer extension product (large peak) and a regular DNA sequencing reaction (smaller peaks) (bottom panel).



Preliminary studies indicated that expression of the *sdiA0* reporter increased in LB containing 2% NaCl. To more carefully examine possible osmoregulation, a titration of NaCl and KCl was performed in LBns broth (LB with no NaCl). *sdiA0* reporter expression was not specific to NaCl because similar expression values were obtained using KCl (Fig. 1B). At salt concentrations of less than 62.5 mmol·L^{-1} in LB, expression was below detection. This effect was not restricted to monovalent cations because divalent cations $MgCl_2$ and $CaCl_2$ induced the same level of expression as did NaCl and KCl at 125 mmol·L^{-1} and 62.5 mmol·L^{-1} (not shown).

Identification of the *sdiA* transcription start site

To determine the transcription start site, primer extension analysis was performed by reverse transcription of mRNA using a fluorescent dye-labelled primer that anneals to a site within *luxC* and is detected by capillary electrophoresis. Primer extension was repeated in four independent experiments on three different *sdiA* promoter–reporter constructs. A single transcription initiation site at a cytosine, 26 bp upstream of the ATG start codon, was observed as a dye peak at this position (Fig. 2); the second dye peak was pulled up by the first and was an artefact. The putative –10 region, 5′-CATAAT-3′, has five conserved bases with the σ^{70} consensus (5′-TATAAT-3′), including the two most conserved bases (McClure 1985). The putative –35 region, 5′-TTTATGT-3′, has four of seven matches to the σ^{70} consensus (5′-TTGACAT-3′); one mismatched base is to a conserved base and the remaining two mismatches from the consensus are not at highly conserved sites. The spacing between the putative –10 and –35 regions is 16 bp, in agreement with the consensus spacing (Fig. 2) (McClure 1985).

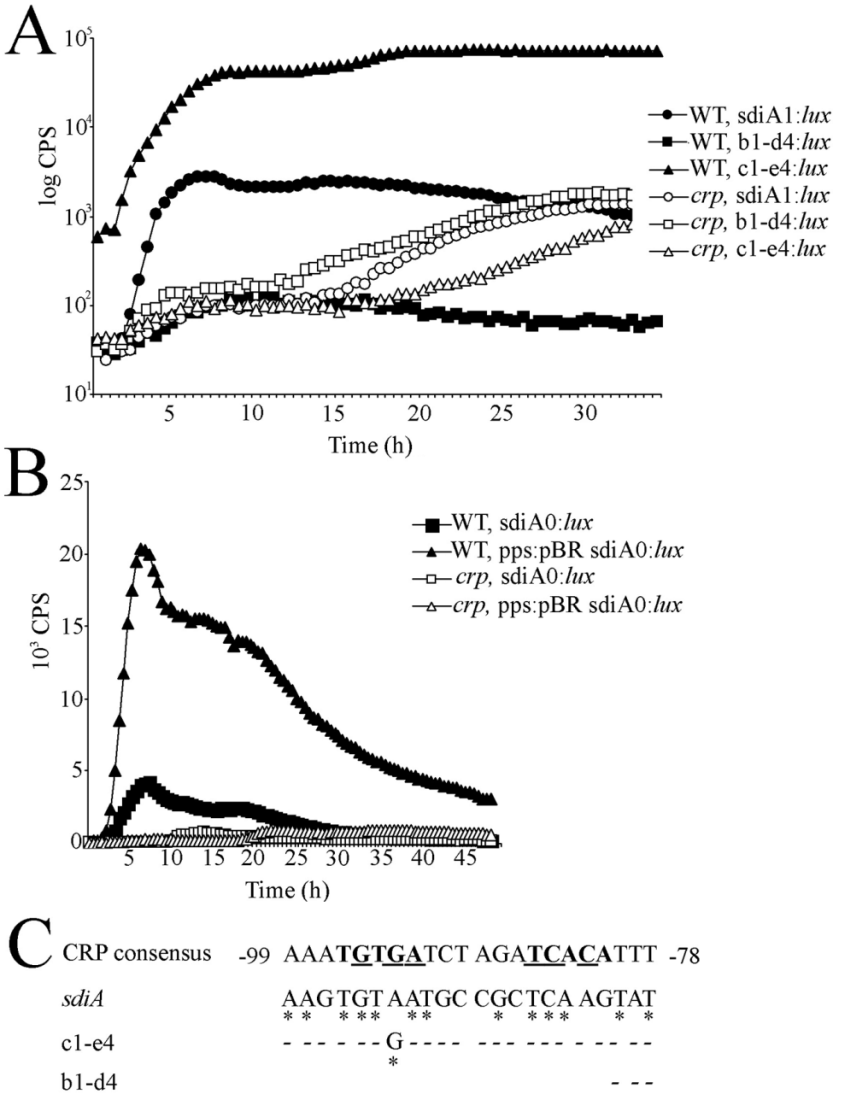
cAMP receptor protein is an activator of *sdiA*

A promoter point mutant library consisting of 700 individual *sdiA1* transcriptional fusions was generated using error-prone PCR (Pritchard et al. 2005). This library contained two mutants within a 13 bp region (sdiA1-b1d4 and sdiA1-c1e4). sdiA1-b1d4 had an A-to-G mutation at –83, generating an *Xho*I site (CTCGAG) during error-prone PCR. During cloning of this promoter using *Xho*I, it was truncated at –80. This promoter mutant had no expression above background (Fig. 3A). Interestingly, sdiA1-b1d4 was missing only 23 bp off the 5′ end of *sdiA2* (Fig. 3C), which had the greatest expression of the four native *sdiA* reporters. Thus, removal of these 23 bp turned off *sdiA* expression, indicating that this region likely contained an activator binding site. A computer search showed that this region contained a putative 22 bp cAMP receptor protein (CRP) consensus sequence, with four of five conserved bases in the conserved core of the first half site and three of five conserved bases in the second half site (Fig. 3C) (Gunasekera et al. 1992; Cameron and Redfield 2008). The sdiA1-b1d4 promoter mutant was missing all but the last three bases of the predicted 22 bp CRP binding site. The second mutant, sdiA1-c1e4, contained an A-to-G mutation at –93, which increased *sdiA* expression 27-fold in broth (Fig. 3A). This mutation was isolated twice in independent error-prone PCRs. The mutation in sdiA1-c1e4 converted a TGTAA nucleotide sequence to the CRP conserved core of the first 11 bp half site (TGTGA). Thus, the CRP binding site was converted to the CRP consensus sequence, resulting in increased *sdiA* expression.

To test whether CRP was indeed activating *sdiA*, the wild-type and mutant promoters were tested in a CRP mutant (*crp*$^-$). Expression of all *sdiA* reporters decreased in *crp*$^-$, except for sdiA1-b1d, which was already at basal levels; maximal expression values were decreased 13-fold for *sdiA0*, 3-fold for *sdiA1*, and 5-fold for *sdiA2* (not shown). All wild-type reporters had the greatest expression after 15 h in the *crp*$^-$ strain, indicating that CRP may not be responsible for expression in late-stationary phase, whereas exponential phase expression was off. sdiA1-c1e4, which had 27-fold increased expression in the wild-type background, was similar to the *sdiA1* in *crp*$^-$, indicating that the increased expression observed in sdiA1-c1e4 is due to CRP (Fig. 3A). Similarly, expression of *sdiA1* was reduced to sdiA1-b1d levels in the *crp*$^-$ background. CRP activates *sdiA* expression, and its activation is responsible for the majority of *sdiA* expression in exponential and early stationary phases of growth.

Phosphoenolpyruvate synthase (*pps*) was found previously in a screen to identify genes that altered *sdiA* expression when overexpressed (Kim 2004). When present on a multicopy plasmid, *pps* increased *sdiA0* expression 4-fold; however, this was not observed in a *crp* mutant background (Fig. 3B). The increased *sdiA0* expression resulting from *pps* overexpression is mediated through CRP.

Fig. 3. (A) Gene expression of *sdiA1* and promoter mutants sdiA1-b1d4 and sdiA1-c1e4 in wild type (WT) and *crp*⁻. (B) *pps*:pBR in WT and *crp*⁻ with the *sdiA0* reporter compared with *sdiA0* expression in these strains with empty pBR322. (C) Alignment of the cAMP receptor protein (CRP) consensus sequence with a region of the *sdiA* promoter, and promoter point mutants. Position of DNA fragment in relation to transcriptional start site (+1) is shown numerically. The asterisks denote identity between *sdiA* promoter and CRP consensus sequence. Nucleotides in bold indicate those that form the core of the CRP binding site, and underlined nucleotides are those that directly contact the CRP protein. CPS, counts per second.

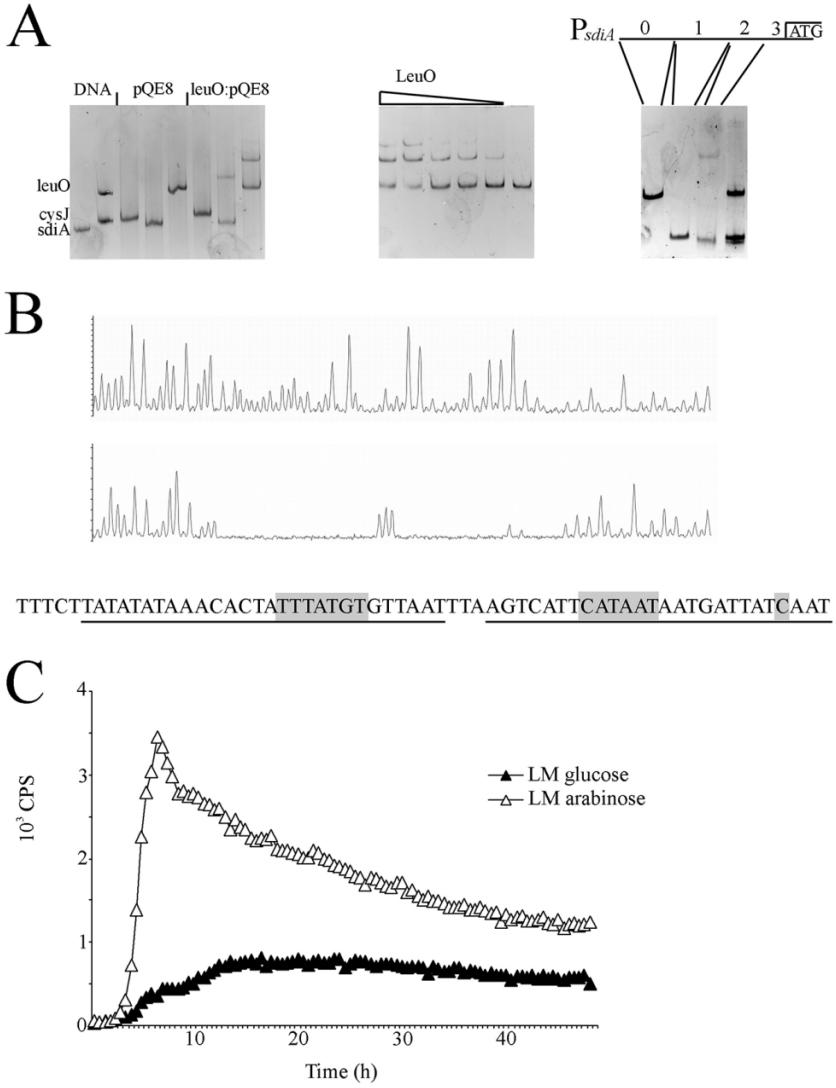


Because CRP is a regulator of carbon metabolism, the effects of different carbon sources were tested on *sdiA* expression. Adding acetate or citrate to the medium increased maximal expression values 4- and 2-fold, respectively (not shown). Glucose addition reduced maximal expression to 0.8 of that in LM, and to nearly 6-fold less than expression in acetate. The effects of carbon sources on *sdiA* expression in wild type provide further evidence that CRP is regulating this promoter.

LeuO binds the *sdiA* promoter and is an activator of *sdiA*

An initial transposon mutagenesis screen was carried out to identify putative regulators of *sdiA* expression (Kim 2004). Transposon insertions in *leuO* resulted in decreased *sdiA* promoter activity. To characterize this putative *sdiA* regulator, a deletion mutant was constructed. *sdiA* expression was reduced by 30% in a Δ*leuO* background for all reporter lengths, although the temporal expression pattern was not affected (not shown). LeuO is a LysR-type transcriptional regulator identified as an activator of the leucine biosynthetic operon *leuLABCD* (Henikoff et al. 1988; Hertzberg et al. 1980). To test if LeuO was acting directly at the *sdiA* promoter, EMSAs and DNase I footprinting were performed using purified LeuO. LeuO bound the *sdiA* promoter, whereas no binding was detected with a negative control, the *cysJ* promoter for which no predicted LeuO binding sites exist (Fig. 4A, left). LeuO was found to bind the region of *sdiA* between *sdiA2* and *sdiA3* (Fig. 4A, right). Furthermore, at increased LeuO concentrations, this protein bound *sdiA* at two sites because two species of higher molecular mass were detected, whereas at lower LeuO concentrations, only one species was detected (Fig. 4A, middle). Footprinting revealed

Fig. 4. LeuO binds the *sdiA* promoter. (A) Left panel: Crude electrophoretic mobility shift assay (EMSA) with 10 ng DNA standards and positive control *leuO* promoter, negative control *cysJ* promoter, and test *sdiA1* (lanes 1 and 2); 50 ng protein from the soluble fraction of lysates with empty vector (pQE8, lanes 3–5) and LeuO-expressing vector (*leuO*:pQE8, lanes 6–8) tested on *cysJ* (lanes 3 and 6), *sdiA1* (lanes 4 and 7), and *leuO* (lanes 5 and 8). Middle panel: LeuO binds *sdiA* in two binding sites. Titration of LeuO from left to right in pmol: 2.7, 1.4, 0.7, 0.34, 0.17, 0 per lane with 10 ng sdiA1 (32.5 fmol). Right panel: LeuO binds between *sdiA2* and *sdiA3*. Each reaction used 5.4 pmol LeuO with 10 ng DNA representing fragments of the *sdiA* promoter. From left to right: the region of DNA contained uniquely in *sdiA0* (lane 1), the 5′-most region of DNA in *sdiA1* but not *sdiA2* (lane 2), the 5′-most region of unique DNA in *sdiA2* but absent in *sdiA3* (lane 3), and DNA controls (lane 4). (B) Electrophoretograms from DNase I footprinting on 100 ng DNA without LeuO (top) and with 83 pmol LeuO (bottom). DNA sequence shows LeuO binding sites mapped onto the *sdiA* promoter region (underlined) with +1, –10, and –35 sequences shaded for reference. (C) Expression of b1-d4 (irresponsive to cAMP-receptor protein) in wild type overexpressing LeuO with *leuO*:pBAD18 (LB-marine (LM) arabinose) compared with uninduced *leuO*:pBAD18 (LM glucose). CPS, counts per second.



two binding sites, both between *sdiA2* and *sdiA3*, corroborating the EMSA results (Fig. 4B). The 5′-most binding site was 28 bp long and covered the –35 sequence, and the 3′ binding site was 26 bp long and covered the –10 sequence and the +1. There is a 3 bp space between binding sites (Fig. 4B).

Further evidence that LeuO was activating came from overexpressing LeuO in pBAD18. Because *sdiA* is regulated by CRP, and carbon source affects the activity of CRP, the promoter mutant b1-d4, which lacks a CRP binding site, was tested in a strain carrying *leuO*:pBAD. Overexpressing LeuO increased maximal b1-d4 expression 4-fold (Fig. 4C). As a control, expression of *leuL* was tested in *leuO*:pBAD and it was found that overexpressing LeuO increased *leuL* expression 10-fold (not shown).

The Rcs phosphorelay negatively regulates *sdiA*

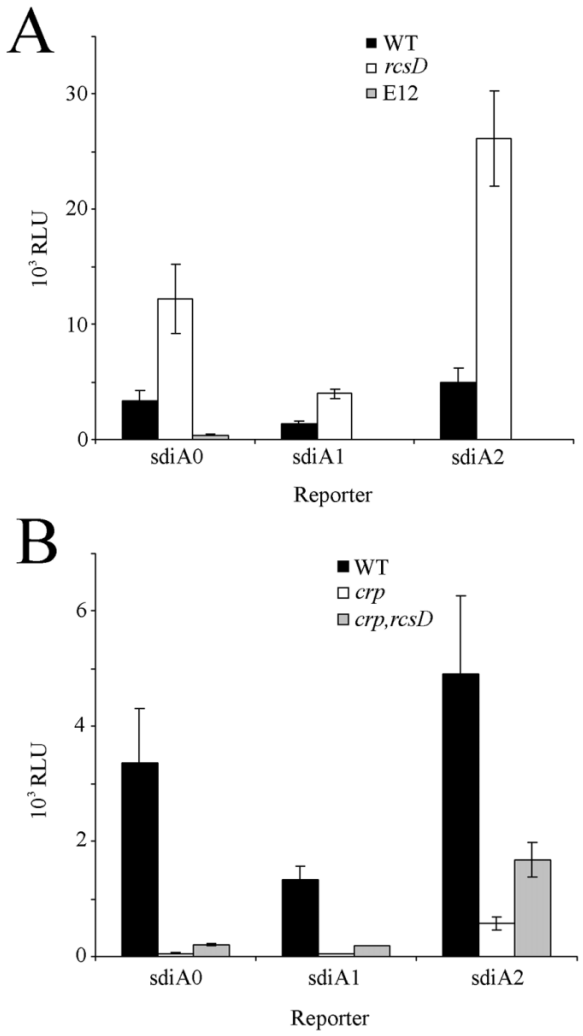
Another putative regulator identified in the transposon screen was RcsD. Strain E12 has decreased expression of *sdiA* reporters relative to the parent strain. This strain has a transposon insertion in the coding region for the linker domain of the RcsD hybrid sensor kinase. This mutation resulted in a truncated RcsD with transmembrane domains, a periplasmic sensory domain, and a histidine kinase-like do-

main, but it lacks the phosphotransfer domain. This results in an activated Rcs phosphorelay (Kim 2004). A deletion mutant was made, Δ*rcsD*, and its effects on *sdiA* expression were compared with those of the transposon mutant (E12) and wild type. Expression was measured in Rcs-inducing conditions by growth at 23 °C and high osmolarity on agar plates. For all *sdiA* reporters, expression was between 3- and 5-fold greater in Δ*rcsD* compared with wild type, whereas in E12, *sdiA0* expression was 9-fold lower (Fig. 5A). This suggests RcsD negatively regulates *sdiA* expression.

To test a known output of Rcs activity, expression of the *flhDC* promoter, which is repressed by RcsB (Francez-Charlot et al. 2003), was measured in Δ*rcsD* and E12. Compared with wild type, expression of *flhDC* was increased 1.7-fold at its maximum in Δ*rcsD* and decreased 2.3-fold at its maximum in E12, indicating a less-active Rcs phosphorelay in Δ*rcsD* and a more active system in E12 (not shown). Examination of colony morphology after growth in inducing conditions corroborated these results; E12 colonies appeared mucoid, whereas Δ*rcsD* colonies were slightly drier than were wild type. These results suggest that the Rcs phosphorelay represses *sdiA* expression in inducing conditions; however, we cannot determine from this if RcsB is acting directly on the promoter or through another regulator.

Because it was shown that CRP regulates *sdiA* and that an active Rcs-system-making capsule may be altering carbon metabolism in the cell, interactions between CRP and the Rcs system were examined. To measure CRP activity, expression of the *lacZ* promoter from *E. coli* MG1655 fused to *luxCDABE* was measured. CRP activity in wild type was the same regardless of the salt concentration, and activity in Δ*rcsD* in LBns was the same as that of wild type (not shown). However in LM, CRP activity increased 2.7-fold in Δ*rcsD* compared with wild type and Δ*rcsD* in LBns. Thus, a portion of increased *sdiA* expression observed in Δ*rcsD* could be due to increased CRP activity. To isolate the repression of *sdiA* by the Rcs system, *sdiA* expression was tested in *crp, rcsD* double mutants in Rcs-inducing conditions. In these conditions, *sdiA0* and *sdiA1* expression were at background levels in *crp*⁻ grown in LM, and *sdiA2* was the only reporter to have expression. However, *sdiA* expression in all reporter constructs was detectable in *crp*, *rcsD*, indicating that the Rcs system was repressing *sdiA* in *crp*⁻ (Fig. 5B). In this Rcs-inducing condition, growth in LM, expression of *sdiA2* was 8-fold lower in *crp*⁻ and 3-fold lower in *crp,rcsD* compared with wild type. Thus, the Rcs system negatively regulates *sdiA* expression, and an active Rcs system increases CRP activity.

Fig. 5. (A) Expression of *sdiA0*, *sdiA1*, and *sdiA2* in wild type (WT), Δ*rcsD*, and E12 (*rcsD*::Tn*10*) after 2 days of growth on LB-marine, at room temperature. Expression values are in relative light units (RLU) (counts per second normalized to 10^7 cfu). (B) *sdiA0*, *sdiA1*, and *sdiA2* expression in WT, *crp*⁻, and *crp*, *rcsD* in Rcs-inducing conditions. Expression values are in RLU (counts per second normalized to 10^7 cfu).



IlvY, NhaR, and Fur are indirect activators

To further characterize the regulatory effects of putative regulators identified in the random overexpressing library screen, deletion mutants and overexpression of *nhaR* and *ilvY* were tested for effects on *sdiA* expression. IlvY overexpression increases *sdiA* expression, with the largest effects in stationary phase (Fig. S1A)[1]. Conversely, NhaR overexpression decreases *sdiA* expression (Fig. S1A)[1]. However, the effects of both regulators are lessened in deletion mutants. Reproducibly, but not significantly, Δ*ilvY* has slightly decreased *sdiA* expression, whereas Δ*nhaR* has statistically significant increased expression (Student's *t* test, $p < 0.05$; Fig. S1)[1]. To test whether these proteins were acting directly at the promoter, crude EMSAs were performed. Although IlvY bound its positive control P_{ilvC} and NhaR bound its positive control P_{nhaA}, neither protein bound P_{sdiA} (Fig. S1)[1]. Thus, NhaR and IlvY are likely acting indirectly through other pathways to alter *sdiA* expression.

A previous study by Volf et al. (2002) suggested that Fur represses *sdiA* by binding a putative Fur box. In that study, direct binding was not demonstrated, and expression in a *fur*⁻ strain was not tested. Furthermore, because the proposed Fur box overlaps with the predicted –10 sequence (this study), the effects of iron were assayed using our transcriptional fusions and growth conditions. In *fur*⁻, *sdiA* expression was increased 1.7-fold (Student's *t* test, $p < 0.005$); however,

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/w11-101.

because *sdiA* expression decreased in iron-limiting conditions and the same ratio of expression in high and low iron was observed in wild type and *fur*⁻ (1.5-fold increased expression in high iron for both backgrounds, although this was not significant for either background), these effects appear to be independent of Fur (Fig. S2)[1]. For comparison, expression of *sitA*, a Fur-regulated promoter, was greater in iron-limiting conditions (Fig. S2)[1]. Fur does not appear to directly regulate *sdiA*.

RpoS-dependent *sdiA* expression in stationary phase

Because *sdiA* has considerable expression throughout stationary phase, we tested whether RpoS is responsible for *sdiA* expression. The Δ*rpoS* mutation resulted in decreased expression for all *sdiA* reporters. Notably, *sdiA* promoter activity rapidly decreased to background levels between 25 and 30 h, indicating that *sdiA* expression was RpoS dependent during this time (Fig. 6). RpoS may control the expression of a stationary-phase activator of *sdiA*. Alternatively, RpoS could be directly responsible for *sdiA* expression, because some promoters can be both RpoS and RpoD dependent (Tanaka et al. 1993; Espinosa-Urgel et al. 1996).

Regulatory input at the *sdiA* promoter affects SdiA output

The regulation of *sdiA* expression characterized in the previous sections was determined using promoter–reporter fusions. To test whether observed differences in *sdiA* expression among mutants and media conditions resulted in actual differences in SdiA levels, SdiA-regulated *rck* expression was measured. Because SdiA requires AHL binding for activity, all media included supernatant from an AHL-producing strain. *sdiA* expression changes in *crp, leuO*, and *rcsD* mutants were reflected in *rck* expression (Table 2). Thus, the observed changes in *sdiA* expression in mutant strains resulted in actual changes in SdiA levels, reflected by altered *rck* expression.

Discussion

sdiA, encoding a LuxR-type transcriptional regulator of two operons in *Salmonella* serovar Typhimurium, is subject to complex transcriptional regulation. In this study, we show that CRP and LeuO are the major and minor activators of *sdiA*, respectively. Both act directly on the *sdiA* promoter. The Rcs phosphorelay represses *sdiA* expression; however, its effects may be at least partially indirect. The temporal pattern of *sdiA* expression is characterized by its two peaks: one in exponential phase and the other in early stationary phase. *sdiA* expression in stationary phase is RpoS dependent. Expression is also strongly induced by increasing osmolarity.

LeuO is a LysR-type transcriptional regulator with an unidentified coinducer (Maddocks and Oyston 2008). In *Salmonella* serovar Typhimurium, LeuO is required during the early stages of systemic murine infection and was identified as a virulence factor in a screen using *Caenorhabditis elegans* (Tenor et al. 2004; Lawley et al. 2006). LeuO has been shown to be a global regulator in *Salmonella* and *E. coli*, regulating diverse genes with a common role in stress response and virulence (Hernández-Lucas et al. 2008; Shimada et al. 2009). The length of the binding sites, DNA sequence preference, and location within the promoter agree with previously published binding results for LeuO and other LysR-type transcriptional regulators (Fernández-Mora et al. 2004; Chen et al. 2005; Maddocks and Oyston 2008). Additionally, the presence of a hypersensitive region between LeuO binding sites has been observed previously, and this is indicative of a bend in DNA upon LeuO binding, consistent with other LysR-type transcriptional regulators (De la Cruz et al. 2007; Maddocks and Oyston 2008).

CRP, a global regulator in bacteria, is the main activator of *sdiA* expression (Saier et al. 1996). In a *crp* mutant, expression can be observed only if a second mutation in *rcsD* is introduced. CRP–cAMP binds a 22 bp sequence with 2-fold symmetry and induces a 80° to 90° bend in the DNA (Schultz et al. 1991; Gunasekera et al. 1992). The co-inducer for CRP is cAMP, synthesized by Cya in response to intracellular glucose depletion (Fandl et al. 1990; Kao et al. 2005). CRP–cAMP activates hundreds of genes during glucose limitation (Perrenoud and Sauer 2005; Nanchen et al. 2008). Overexpression of *pps*, encoding the first enzyme in gluconeogenesis, leads to elevated cAMP, which would result in greater CRP–cAMP activation of *sdiA* (Kao et al. 2005). Interestingly, no predicted CRP binding site is present in the *sdiA* promoter of *E. coli* (Brown and Callan 2004).

Fig. 6. Time course of expression of *sdiA2* reporter in wild-type (WT) and Δ*rpoS* strains. Note the rapid decrease in *sdiA* expression between 20 and 30 h. CPS, counts per second.

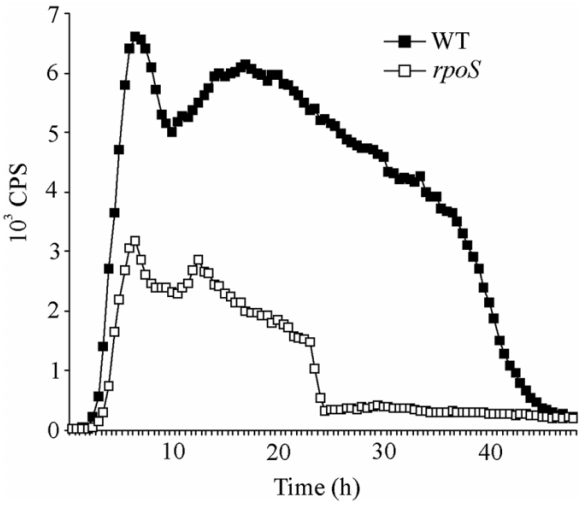


Table 2. Maximal expression of *sdiA0* reporter and *rck* reporter normalized to wild-type expression.

Strain	*sdiA0*	*rck*
Wild type	1	1
crp⁻	0.04	0.05
Δ*leuO*	0.7	0.2
Δ*rcsD*[a]	3.6	3.0

[a]Grown in Rcs phosphorelay-inducing conditions and compared with wild type grown in similar conditions.

As a regulator of central carbon metabolism, CRP is important during infection; *crp* mutants are avirulent and many CRP-regulated genes have been identified as important for virulence during infection (Curtiss and Kelly 1987; Kennedy et al. 1999). It has been shown that *sdiA* is expressed during infection when AHL-producing bacteria are present in the intestines (Smith et al. 2008; Dyszel et al. 2010). The role of CRP may be to activate *sdiA* in the small intestine, where *Salmonella* would have the greatest opportunity to sense AHLs.

The Rcs phosphorelay was found to repress *sdiA*. This system consists of inner membrane hybrid sensor kinase RcsC, phosphotransfer protein RcsD, response regulator RcsB, and response regulator chaperone RcsA (Takeda et al. 2001). The Rcs signalling system is present only in the *Enterobacteriaceae*, and within this family the RcsCDB genes are absent in endosymbionts (Erickson and Detweiler 2006; Huang et al. 2006). Although the precise environmental signal(s) are not known, this system is thought to respond to perturbations in outer membrane integrity (Clarke et al. 1997; Hagiwara et al. 2003). Generally, the genes activated by phospho-RcsB are colanic acid capsule biosynthetic genes (requiring RcsA), and the genes repressed by phospho-RcsB are flagellar biosynthetic genes (RcsA independent). The RcsB regulon in *Salmonella* serovar Typhimurium grown in high osmolarity consists of 89 genes (Mariscotti and Garcia-del Portillo 2009). The Rcs signalling system represses *sdiA*; however, direct binding was not demonstrated. All *sdiA0*, *sdiA1*, and *sdiA2* were all repressed, indicating that RcsB or another regulator is acting on the region of DNA contained in *sdiA2*.

A novel connection between the Rcs system and Crp was found: Crp activity was elevated in Δ*rcsD* strains. Blocking glycolysis has been shown to activate the Rcs phosphorelay, and the study by El-Kazzaz et al. (2004) also suggested that a metabolite of O-antigen synthesis activates the Rcs system. It is difficult to speculate as to how glycolytic intermediates would vary in Δ*rcsD* in leading to glucose depletion and increased CRP activity.

From the data presented above, it is fathomable that regulation of *sdiA* results in expression during passage through the gut. This would be the most practical environment for *Salmonella* to detect other microbial species, because the intestine hosts a complex microbial population consisting of more than 500 species (Eckburg et al. 2005). Alignment of 300 bp of the region upstream of the *sdiA* coding region with six other species in the *Enterobacteriaceae* with *sdiA* homologues showed high conservation of the 5′ LeuO binding site, with 16 of 28 bases conserved in all sequences; eight bases varied by one other base and only six bases were poorly conserved (data not shown). There was less conservation of the entire Crp binding site. Among *Salmonellae*, sequence identity of the intergenic region was >95%.

Recently, SdiA-mediated quorum sensing has been demonstrated to occur during murine infection when *Salmonellae* are in a mixed infection with AHL-producing *Y. enterocolitica* (Dyszel et al. 2010). Although *sdiA* has been demonstrated independently by two laboratories to have a slight negative effect on murine infection (Volf et al. 2002; Dyszel et al. 2010), an engineered *sdiA*$^+$ *Salmonella* strain that produced AHLs quickly outcompeted *sdiA*$^-$ during murine infection (Dyszel et al. 2010). The regulation of *sdiA* is complex, resulting from the input of multiple transcriptional regulators and environmental conditions. The integration of these signals may lead to unique expression of *sdiA* in the intestine, allowing *Salmonella* to sense and modulate its behaviour in response to other microbial species present.

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References

Ahmer, B.M.M. 2004. Cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica.* Mol. Microbiol. **52**(4): 933–945. doi:10.1111/j.1365-2958.2004.04054.x. PMID:15130116.

Ahmer, B.M.M., Van Reeuwijk, J., Timmers, C.D., Valentine, P.J., and Heffron, F. 1998. *Salmonella typhimurium* encodes an SdiA homolog, a putative quorum sensor of the LuxR family, that regulates genes on the virulence plasmid. J. Bacteriol. **180**(5): 1185–1193. PMID:9495757.

Ahmer, B.M., Smith, J.N., Dyszel, J.L., and Lindsay, A. 2007. Methods in cell-to-cell signaling in *Salmonella*. Methods Mol. Biol. **394**: 307–322. doi:10.1007/978-1-59745-512-1_15. PMID: 18363242.

Bjarnason, J., Southward, C.M., and Surette, M.G. 2003. Genomic profiling of iron-responsive genes in *Salmonella enterica* serovar Typhimurium by high-throughput screening of a random promoter library. J. Bacteriol. **185**(16): 4973–4982. doi:10.1128/JB.185.16.4973-4982.2003. PMID:12897017.

Brown, C.T., and Callan, C.G. , Jr 2004. Evolutionary comparisons suggest many novel cAMP response protein binding sites in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **101**(8): 2404–2409. doi:10.1073/pnas.0308628100. PMID:14983022.

Cameron, A.D.S., and Redfield, R.J. 2008. CRP binding and transcription activation at CRP-S sites. J. Mol. Biol. **383**(2): 313–323. doi:10.1016/j.jmb.2008.08.027. PMID:18761017.

Chen, C.-C., and Wu, H.-Y. 2005. LeuO protein delimits the transcriptionally active and repressive domains on the bacterial chromosome. J. Biol. Chem. **280**(15): 15111–15121. doi:10.1074/jbc.M414544200. PMID:15711009.

Chen, C.-C., Chou, M.-Y., Huang, C.-H., Majumder, A., and Wu, H.-Y. 2005. A *cis*-spreading nucleoprotein filament is responsible for the gene silencing activity found in the promoter relay mechanism. J. Biol. Chem. **280**(6): 5101–5112. doi:10.1074/jbc.M411840200. PMID:15582999.

Clarke, D.J., Holland, I.B., and Jacq, A. 1997. Point mutations in the transmembrane domain of DjlA, a membrane-linked DnaJ-like protein, abolish its function in promoting colanic acid production via the Rcs signal transduction pathway. Mol. Microbiol. **25**(5): 933–944. doi:10.1111/j.1365-2958.1997.mmi528.x. PMID:9364918.

Curtiss, R., III, and Kelly, S.M. 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. **55**(12): 3035–3043. PMID:3316029.

Datsenko, K.A., and Wanner, B.L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U.S.A. **97**(12): 6640–6645. doi:10.1073/pnas.120163297. PMID:10829079.

De la Cruz, M.A., Fernández-Mora, M., Guadarrama, C., Flores-Valdez, M.A., Bustamante, V.H., Vazquez, A., and Calva, E. 2007.

LeuO antagonizes H-NS and StpA-dependent repression in *Salmonella enterica* ompS1. Mol. Microbiol. **66**(3): 727–743. doi:10.1111/j.1365-2958.2007.05958.x. PMID:17908208.

Decho, A.W., Norman, R.S., and Visscher, P.T. 2010. Quorum sensing in natural environments: emerging views from microbial mats. Trends Microbiol. **18**(2): 73–80. doi:10.1016/j.tim.2009.12.008. PMID:20060299.

Dyszel, J.L., Smith, J.N., Lucas, D.E., Soares, J.A., Swearingen, M.C., Vross, M.A., et al. 2010. *Salmonella enterica* serovar Typhimurium can detect the acyl homoserine lactone production of *Yersinia enterocolitica* in mice. J. Bacteriol. **192**(1): 29–37. doi:10.1128/JB.01139-09. PMID:19820103.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., et al. 2005. Diversity of the human intestinal microbial flora. Science, **308**(5728): 1635–1638. doi:10.1126/science.1110591. PMID:15831718.

El-Kazzaz, W., Morita, T., Tagami, H., Inada, T., and Aiba, H. 2004. Metabolic block at early stages of the glycolytic pathway activates the Rcs phosphorelay system via increased synthesis of dTDP-glucose in *Escherichia coli*. Mol. Microbiol. **51**(4): 1117–1128. doi:10.1046/j.1365-2958.2003.03888.x. PMID:14763984.

Elliott, T., and Roth, J.R. 1988. Characterization of Tn*10d*-Cam: a transposition-defective Tn*10* specifying chloramphenicol resistance. Mol. Gen. Genet. **213**(2–3): 332–338. doi:10.1007/BF00339599. PMID:2847006.

Erickson, K.D., and Detweiler, C.S. 2006. The Rcs phosphorelay system is specific to enteric pathogens/commensals and activates *ydeI*, a gene important for persistent *Salmonella* infection of mice. Mol. Microbiol. **62**(3): 883–894. doi:10.1111/j.1365-2958.2006.05420.x. PMID:17010160.

Espinosa-Urgel, M., Chamizo, C., and Tormo, A. 1996. A consensus structure for σ S-dependent promoters. Mol. Microbiol. **21**(3): 657–659. doi:10.1111/j.1365-2958.1996.tb02573.x. PMID:8866487.

Fandl, J.P., Thorner, L.K., and Artz, S.W. 1990. Mutations that affect transcription and cyclic AMP-CRP regulation of the adenylate cyclase gene (*cya*) of *Salmonella typhimurium*. Genetics, **125**(4): 719–727. PMID:2168849.

Fernández-Mora, M., Puente, J.L., and Calva, E. 2004. OmpR and LeuO positively regulate the *Salmonella enterica* serovar Typhi *ompS2* porin gene. J. Bacteriol. **186**(10): 2909–2920. doi:10.1128/JB.186.10.2909-2920.2004. PMID:15126450.

Francez-Charlot, A., Laugel, B., Van Gemert, A., Dubarry, N., Wirowski, F., Castanié-Cornet, M.-P., et al. 2003. RcsCDB His-Asp phosphorelay system negatively regulates the *flhDC* operon in *Escherichia coli*. Mol. Microbiol. **49**(3): 823–832. doi:10.1046/j.1365-2958.2003.03601.x. PMID:12864862.

Goller, C., Wang, X., Itoh, Y., and Romeo, T. 2006. The cation-responsive protein NhaR of *Escherichia coli* activates *pgaABCD* transcription, required for production of the biofilm adhesin poly-β-1,6-*N*-acetyl-D-glucosamine. J. Bacteriol. **188**(23): 8022–8032. doi:10.1128/JB.01106-06. PMID:16997959.

Gorshkov, V., Petrova, O., Gogoleva, N., and Gogolev, Y. 2010. Cell-to-cell communication in the populations of enterobacterium *Erwinia carotovora* ssp. *atroseptica* SCRI1043 during adaptation to stress conditions. FEMS Immunol. Med. Microbiol. **59**(3): 378–385. PMID:20528924.

Gray, K.M., and Garey, J.R. 2001. The evolution of bacterial LuxI and LuxR quorum sensing regulators. Microbiology, **147**(8): 2379–2387. PMID:11496014.

Gunasekera, A., Ebright, Y.W., and Ebright, R.H. 1992. DNA sequence determinants for binding of the *Escherichia coli* catabolite gene activator protein. J. Biol. Chem. **267**(21): 14713–14720. PMID:1321815.

Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J. Bacteriol. **177**(14): 4121–4130. PMID:7608087.

Hagiwara, D., Sugiura, M., Oshima, T., Mori, H., Aiba, H., Yamashino, T., and Mizuno, T. 2003. Genome-wide analyses revealing a signalling network of the RcsC-YojN-RcsB phosphorelay system in *Escherichia coli*. J. Bacteriol. **185**(19): 5735–5746. doi:10.1128/JB.185.19.5735-5746.2003. PMID:13129944.

Hellman, L.M., and Fried, M.G. 2007. Electrophoretic mobility shift assay (EMSA) for detecting protein–nucleic acid interactions. Nat. Protoc. **2**(8): 1849–1861. doi:10.1038/nprot.2007.249. PMID:17703195.

Henikoff, S., Haughn, G.W., Calvo, J.M., and Wallace, J.C. 1988. A large family of bacterial activator proteins. Proc. Natl. Acad. Sci. U.S.A. **85**(18): 6602–6606. doi:10.1073/pnas.85.18.6602. PMID:3413113.

Hernández-Lucas, I., Gallego-Hernández, A.L., Encarnación, S., Fernández-Mora, M., Martinez-Batallar, A.G., Salgado, H., et al. 2008. The LysR-type transcriptional regulator LeuO controls expression of several genes in *Salmonella enterica* serovar Typhi. J. Bacteriol. **190**(5): 1658–1670. doi:10.1128/JB.01649-07. PMID:18156266.

Hertzberg, K.M., Gemmill, R., Jones, J., and Calvo, J.M. 1980. Cloning of an *Eco*RI-generated fragment of the leucine operon of *Salmonella typhimurium.* Gene, **8**(2): 135–152. doi:10.1016/0378-1119(80)90033-5. PMID:6987127.

Huang, Y.-H., Ferrieres, L., and Clarke, D.J. 2006. The role of the Rcs phosphorelay in *Enterobacteriaceae*. Res. Microbiol. **157**(3): 206–212. doi:10.1016/j.resmic.2005.11.005. PMID:16427772.

Jakubovics, N.S. 2010. Talk of the town: interspecies communication in oral biofilms. Mol. Oral Microbiol. **25**(1): 4–14. doi:10.1111/j.2041-1014.2009.00563.x. PMID:20331790.

Janssens, J.C.A., Metzger, K., Daniels, R., Ptacek, D., Verhoeven, T., Habel, L.W., et al. 2007. Synthesis of *N*-acyl homoserine lactone analogues reveals strong activators of SdiA, the *Salmonella enterica* serovar Typhimurium LuxR homologue. Appl. Environ. Microbiol. **73**(2): 535–544. doi:10.1128/AEM.01451-06. PMID:17085703.

Kao, K.C., Tran, L.M., and Liao, J.C. 2005. A global regulatory role of gluconeogenic genes in *Escherichia coli* revealed by transcriptome network analysis. J. Biol. Chem. **280**(43): 36079–36087. doi:10.1074/jbc.M508202200. PMID:16141204.

Keller, L., and Surette, M.G. 2006. Communication in bacteria: an ecological and evolutionary perspective. Nat. Rev. Microbiol. **4**(4): 249–258. doi:10.1038/nrmicro1383. PMID:16501584.

Kennedy, M.J., Yancey, R.J., Jr, Sanchez, M.S., Rzepkowski, R.A., Kelly, S.M., and Curtiss, R., III 1999. Attenuation and immunogenicity of Δ*cya* Δ*crp* derivatives of *Salmonella choleraesuis* in pigs. Infect. Immun. **67**(9): 4628–4636. PMID:10456909.

Kim, W. 2004. Physiological differentiation in swarming *Salmonella*. Ph.D. thesis, University of Calgary, Calgary, Alta.

Kim, W., and Surette, M.G. 2006. Coordinated regulation of two independent cell-cell signalling systems and swarmer differentiation in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **188**(2): 431–440. doi:10.1128/JB.188.2.431-440.2006. PMID:16385032.

Laue, B.E., Jiang, Y., Chhabra, S.R., Jacob, S., Steward, G.S., Hardman, A., et al. 2000. The biocontrol strain *Pseudomonas fluorescens* F113 produces the *Rhizobium* small bacteriocin, *N*-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone, via HdtS, a putative novel *N*-acylhomoserine lactone synthase. Microbiology, **146**(10): 2469–2480. PMID:11021923.

Lawley, T.D., Chan, K., Thompson, L.J., Kim, C.C., Govoni, G.R., and Monack, D.M. 2006. Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse. PLoS Pathog. **2**(2): e11. doi:10.1371/journal.ppat.0020011. PMID:16518469.

Maddocks, S.E., and Oyston, P.C.F. 2008. Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. Microbiology, **154**(12): 3609–3623. doi:10.1099/mic.0.2008/022772-0. PMID:19047729.

Maloy, S.R. 1990. Experimental techniques in bacterial genetics. Jones and Bartlett Publishers, Boston, Mass., USA.

Mariscotti, J.F., and Garcia-del Portillo, F. 2009. Genome expression analyses revealing the modulation of the *Salmonella* Rcs regulon by the attenuator IgaA. J. Bacteriol. **191**(6): 1855–1867. doi:10.1128/JB.01604-08. PMID:19124574.

McClure, W.R. 1985. Mechanism and control of transcription initiation in prokaryotes. Annu. Rev. Biochem. **54**(1): 171–204. doi:10.1146/annurev.bi.54.070185.001131. PMID:3896120.

Mei, G.Y., Yan, X.X., Turak, A., Luo, Z.Q., and Zhang, L.Q. 2010. AidH, an alpha/beta-hydrolase fold family member from an *Ochrobactrum* sp. strain, is a novel *N*-acylhomoserine lactonase. Appl. Environ. Microbiol. **76**(15): 4933–4942. doi:10.1128/AEM.00477-10. PMID:20525860.

Michael, B., Smith, J.N., Swift, S., Heffron, F., and Ahmer, B.M.M. 2001. SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities. J. Bacteriol. **183**(19): 5733–5742. doi:10.1128/JB.183.19.5733-5742.2001. PMID:11544237.

Miller, M.B., and Bassler, B.L. 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. **55**(1): 165–199. doi:10.1146/annurev.micro.55.1.165. PMID:11544353.

Nanchen, A., Schicker, A., Revelles, O., and Sauer, U. 2008. Cyclic AMP-dependent catabolite repression is the dominant control mechanism of metabolic fluxes under glucose limitation in *Escherichia coli*. J. Bacteriol. **190**(7): 2323–2330. doi:10.1128/JB.01353-07. PMID:18223071.

Ng, W.L., and Bassler, B.L. 2009. Bacterial quorum-sensing network architectures. Annu. Rev. Genet. **43**(1): 197–222. doi:10.1146/annurev-genet-102108-134304. PMID:19686078.

Njoroge, J., and Sperandio, V. 2009. Jamming bacterial communication: new approaches for the treatment of infectious diseases. EMBO Mol. Med. **1**(4): 201–210. doi:10.1002/emmm.200900032. PMID:20049722.

Perrenoud, A., and Sauer, U. 2005. Impact of global transcriptional regulation by ArcA, ArcB, Cra, Crp, Cya, Fnr, and Mlc on glucose catabolism in *Escherichia coli*. J. Bacteriol. **187**(9): 3171–3179. doi:10.1128/JB.187.9.3171-3179.2005. PMID:15838044.

Pritchard, L., Corne, D., Kell, D., Rowland, C., and Winson, M. 2005. A general model of error-prone PCR. J. Theor. Biol. **234**(4): 497–509. doi:10.1016/j.jtbi.2004.12.005. PMID:15808871.

Rhee, K.Y., Senear, D.F., and Hatfield, G.W. 1998. Activation of gene expression by a ligand-induced conformational change of a protein–DNA complex. J. Biol. Chem. **273**(18): 11257–11266. doi:10.1074/jbc.273.18.11257. PMID:9556617.

Saier, M.H., Jr., Ramseier, T.M., and Reizer, J. 1996. Regulation of carbon utilization. ASM Press, Washington, D.C., USA.

Sambrook, J. and Russell, D.W. 2001. Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA.

Schultz, S.C., Shields, G.C., and Steitz, T.A. 1991. Crystal structure of a CAP–DNA complex: the DNA is bent by 90 degrees. Science, **253**(5023): 1001–1007. doi:10.1126/science.1653449. PMID:1653449.

Shimada, T., Yamamoto, K., and Ishihama, A. 2009. Involvement of the leucine response transcription factor LeuO in regulation of the genes for sulfa drug efflux. J. Bacteriol. **191**(14): 4562–4571. doi:10.1128/JB.00108-09. PMID:19429622.

Smith, J.N., and Ahmer, B.M.M. 2003. Detection of other microbial species by *Salmonella*: expression of the SdiA regulon. J. Bacteriol. **185**(4): 1357–1366. doi:10.1128/JB.185.4.1357-1366.2003. PMID:12562806.

Smith, J.N., Dyszel, J.L., Soares, J.A., Ellermeier, C.D., Altier, C., Lawhon, S.D., et al. 2008. SdiA, an *N*-acylhomoserine lactone receptor, becomes active during the transit of *Salmonella enterica* through the gastrointestinal tract of turtles. PLoS ONE, **3**(7): e2826. doi:10.1371/journal.pone.0002826. PMID:18665275.

Takeda, S., Fujisawa, Y., Matsubara, M., Aiba, H., and Mizuno, T. 2001. A novel feature of the multistep phosphorelay in *Escherichia coli*: a revised model of the RcsC → YojN → RcsB signalling pathway implicated in capsular synthesis and swarming behaviour. Mol. Microbiol. **40**(2): 440–450. doi:10.1046/j.1365-2958.2001.02393.x. PMID:11309126.

Tanaka, K., Takayanagi, Y., Fujita, N., Ishihama, A., and Takahashi, H. 1993. Heterogeneity of the principal σ factor in *Escherichia coli*: the *rpoS* gene product, σ^{38}, is a second principal σ factor of RNA polymerase in stationary-phase *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **90**(8): 3511–3515. doi:10.1073/pnas.90.8.3511. PMID:8475100.

Tenor, J.L., McCormick, B.A., Ausubel, F.M., and Aballay, A. 2004. *Caenorhabditis elegans*-based screen identifies *Salmonella* virulence factors required for conserved host–pathogen interactions. Curr. Biol. **14**(11): 1018–1024. doi:10.1016/j.cub.2004.05.050. PMID:15182677.

Turnbull, A.L., and Surette, M.G. 2008. L-Cysteine is required for induced antibiotic resistance in actively swarming *Salmonella enterica* serovar Typhimurium. Microbiology, **154**(11): 3410–3419. doi:10.1099/mic.0.2008/020347-0. PMID:18957594.

Volf, J., Sevcik, M., Havlickova, H., Sisak, F., Damborsky, J., and Rychlik, I. 2002. Role of SdiA in *Salmonella enterica* serovar Typhimurium physiology and virulence. Arch. Microbiol. **178**(2): 94–101. doi:10.1007/s00203-002-0424-4. PMID:12115054.

Weeks, J.N., Galindo, C.L., Drake, K.L., Adams, G.L., Garner, H.R., and Ficht, T.A. 2010. *Brucella melitensis* VjbR and C12-HSL regulons: contributions of the *N*-dodecanoyl homoserine lactone signaling molecule and LuxR homologue VjbR to gene expression. BMC Microbiol. **10**(1): 167. doi:10.1186/1471-2180-10-167. PMID:20529360.

Wei, Y., Lee, J.M., Smulski, D.R., and Larossa, R.A. 2001. Global impact of *sdiA* amplification revealed by comprehensive gene expression profiling of *Escherichia coli*. J. Bacteriol. **183**(7): 2265–2272. doi:10.1128/JB.183.7.2265-2272.2001. PMID:11244066.

Welsh, J., and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. **18**(24): 7213–7218. doi:10.1093/nar/18.24.7213. PMID:2259619.

White, A.P., and Surette, M.G. 2006. Comparative genetics of the rdar morphotype in *Salmonella.* J. Bacteriol. **188**(24): 8395–8406. doi:10.1128/JB.00798-06. PMID:17012381.

Wilson, D.O., Johnson, P., and McCord, B.R. 2001. Nonradiochemical DNase I footprinting by capillary electrophoresis. Electrophoresis, **22**(10): 1979–1986. doi:10.1002/1522-2683(200106)22:10<1979::AID-ELPS1979>3.0.CO;2-A. PMID:11465496.