Regulatory Circuitry of the CsrA/CsrB and BarA/UvrY Systems of *Escherichia coli*

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Received 27 March 2002/Accepted 14 June 2002

The global regulator CsrA (carbon storage regulator) is an RNA binding protein that coordinates central carbon metabolism, activates flagellum biosynthesis and motility, and represses biofilm formation in *Escherichia coli*. CsrA activity is antagonized by the untranslated RNA CsrB, to which it binds and forms a globular ribonucleoprotein complex. CsrA indirectly activates *csrB* transcription, in an apparent autoregulatory mechanism. In the present study, we elucidate the intermediate regulatory circuitry of this system. Mutations affecting the BarA/UvrY two-component signal transduction system decreased *csrB* transcription but did not affect *csrA*'-*lacZ* expression. The *uvrY* defect was severalfold more severe than that of *barA*. Both *csrA* and *uvrY* were required for optimal *barA* expression. The latter observation suggests an autoregulatory loop for UvrY. Ectopic expression of *uvrY* suppressed the *csrB*-*lacZ* expression defects caused by *uvrY*, *csrA*, or *barA* mutations; *csrA* suppressed *csrA* or *barA* defects; and *barA* complemented only the *barA* mutation. Purified UvrY protein stimulated *csrB*-*lacZ* expression approximately sixfold in S-30 transcription-translation reactions, revealing a direct effect of UvrY on *csrB* transcription. Disruption of *sdhA*, which encodes a LuxR homologue, decreased the expression of *uvrY*'-*lacZ* and *csrB*-*lacZ* fusions but did not affect *csrA*'-*lacZ*. The BarA/UvrY system activated biofilm formation. Ectopic expression of *uvrY* stimulated biofilm formation by a *csrB*-null mutant, indicative of a CsrB-independent role for UvrY in biofilm development. Collectively, these results demonstrate that *uvrY* resides downstream from *csrA* in a signaling pathway for *csrB* and that CsrA stimulates UvrY-dependent activation of *csrB* expression by BarA-dependent and -independent mechanisms.

To persist in nature, bacteria must be able to compete and survive under various growth conditions. To accomplish this task, they possess regulatory systems that permit them to recognize and adapt to a changing environment. In *Escherichia coli* and related species, the transition from exponential growth to stationary-phase growth is accompanied by striking physiological changes, which produce cells that are more stress resistant, slower metabolizing, and better at scavenging nutrients (23, 25). These adaptations are brought about largely through changes in gene expression that are coordinated through global regulatory networks (19, 39). The present study investigates interactions among three different types of global regulatory systems that affect stationary-phase gene expression.

The global regulatory system Csr (carbon storage regulator) represses a variety of stationary-phase genes (reviewed in reference 45). The central component of this system, CsrA, is a 61-amino-acid RNA binding protein. This protein inhibits glycogen biosynthesis and catabolism, gluconeogenesis, and biofilm formation, whereas it activates glycolysis, acetate metabolism, motility, and flagellum biosynthesis in *E. coli* (27, 47, 49, 58, 59, 63). The dramatic effect of CsrA on biofilm formation

is mediated primarily through its regulatory role in directing glycogen biosynthesis and catabolism (27). Homologues of *csrA* exhibit a broad phylogenetic distribution in the eubacteria (45), repress stationary-phase genes of *Pseudomonas fluorescens* (8) and genes involved in plant pathogenesis in *Erwinia carotovora* (11), and regulate genes involved in mucosal invasion by *Salmonella enterica* (4, 5).

CsrA is capable of posttranscriptional repression or activation, depending upon the particular RNA target. The mechanism by which CsrA represses glycogen synthesis in *E. coli* has been examined in considerable detail. CsrA binds to the untranslated leader of the *glgCAP* transcript, which encodes enzymes required for glycogen synthesis, at a site that overlaps the *glgC* Shine-Dalgarno sequence and a second site within a hairpin that is located upstream of the Shine-Dalgarno sequence (6). Thus, CsrA blocks ribosome binding and inhibits the initiation of *glgC* translation. Inhibition of translation probably contributes to the observed destabilization of *glgCAP* mRNA by CsrA (29, 30). CsrA positively regulates motility in *E. coli* by binding to and stabilizing the *flhDC* transcript, which encodes the subunits of a tetrameric DNA binding protein (FlhD₂C₂) that activates the expression of genes involved in flagellum biosynthesis, motility, and chemotaxis (59).

A second component of Csr is the 366-nucleotide untranslated CsrB RNA, which binds to ~18 CsrA subunits, forming a large globular ribonucleoprotein complex (31). In vitro transcription-translation studies of *glgCAP* expression and in vivo

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csrB disruption and overexpression studies have revealed that CsrB RNA functions as an antagonist of CsrA, apparently by sequestering this protein (20, 30, 31). A highly repeated sequence element that is located in the loops of predicted CsrB hairpins and is related to the sequences involved in *glgCAP* recognition sites (6) probably mediates the binding of CsrA to CsrB. The function of CsrB RNA as an antagonist of an mRNA binding global regulatory protein offers a novel paradigm for posttranscriptional control by prokaryotic regulatory RNA molecules (reviewed in references 14 and 57). We recently demonstrated that CsrA indirectly activates *csrB* transcription, indicative of an autoregulatory mechanism that determines the intracellular activity of CsrA without affecting its level (20).

Bacterial adaptation to environmental changes depends heavily upon two-component signal transduction systems. These typically consist of a membrane-bound sensor kinase which detects environmental changes and its cognate response regulator, which is phosphorylated by the kinase and thereby activated for specific DNA binding (24). Data from the *E. coli* K-12 genome sequence indicate that it possesses approximately 30 typical two-component systems (35). Recent studies have begun to reveal a relationship between homologues of the BarA/UvrY two-component system and the CsrA/CsrB system. HCN biosynthesis (*hcnABC*) and extracellular protease (*aprA*) in *P. fluorescens* CHA0 are regulated indirectly by GacA, a homologue of UvrY, via a posttranscriptional mechanism involving RsmA, a homologue of CsrA, and a potential CsrB homologue, RsmZ (8, 21). PrrB RNA, a 132-nucleotide transcript in *P. fluorescens* F113 that is similar to CsrB RNA, is itself regulated by GacS/GacA (1). The *barA* gene of *S. enterica* positively affects the expression of *hilA*, which encodes a regulator of genes of *Salmonella* pathogenicity island 1 (SPI1) (4). Mutations in *csrA* or *csrB* also affect *hilA* expression (4, 5). Furthermore, *gacS* and *gacA* (alternatively called *expS* and *expA*) of *E. carotovora* affect levels of RsmB, a homologue of CsrB (12, 26). BarA/UvrY-homologous systems in gram-negative pathogens control a variety of virulence functions (21, 42).

The sensor kinase BarA of *E. coli* was identified as a multicopy suppressor of an *envZ* defect in the expression of outer membrane proteins (38) and was recently shown to activate the transcription of *rpoS*, which encodes the stationary-phase sigma factor RpoS or σ^s (37). BarA is a member of the subclass of tripartite sensor kinases. These proteins consist of an N-terminal cytosolic domain, a canonical pair of transmembrane regions linked by a periplasmic bridge, a transmitter domain containing a conserved histidine residue, a central receiver domain with a conserved aspartate residue, and a C-terminal phosphotransfer domain with a conserved histidine residue (38, 42). Tripartite kinases catalyze the phosphorylation of their cognate response regulators via an ATP-His-Asp-His-Asp phosphorelay (18, 28). While the sensory mechanism of BarA remains unresolved, the *barA* gene has been implicated in the virulence of uropathogenic *E. coli* in the urinary tract (64; A.-K. Pernestig et al., submitted for publication). BarA was recently demonstrated to be a cognate kinase of UvrY, a response regulator of the FixJ family (42). The *uvrY* gene is cotranscribed with *uvrC*, which encodes a DNA repair enzyme, although UvrY plays no apparent role in DNA repair (36).

Comprehensive transcript profiling recently revealed that increased *sdiA* gene dosage causes *uvrY* transcript levels to increase by about 10-fold (60). The *sdiA* gene encodes a protein of the LuxR family, the members of which contain one domain for binding N-acylated homoserine lactones (AHL) and a second domain for binding DNA (54, 56). These proteins permit the bacterium to sense and respond to the resident microbial population by binding to AHL beyond a threshold concentration and activating or repressing the transcription of target genes, i.e., they mediate quorum sensing (17). *E. coli* and *Salmonella* are not known to synthesize AHL and there are no apparent AHL synthase genes in their genomes (33). However, SdiA of *S. enterica* has been proposed to be an AHL receptor that detects signals from other species (3, 33).

Here, we examine regulatory interactions of BarA/UvrY, CsrA/CsrB, and SdiA of *E. coli*. The UvrY response regulator of *E. coli* directly activates transcription of *csrB* and mediates the indirect effects of CsrA on *csrB*. The BarA sensor kinase and the DNA binding protein SdiA also regulate *csrB* transcription, apparently through effects on UvrY phosphorylation and *uvrY* expression, respectively. Autoregulatory loops characterize these systems. CsrA activates transcription of its RNA antagonist, CsrB (20), and we show that UvrY stimulates expression of *barA*, which encodes its cognate sensor kinase. A simple model for this regulatory circuitry is presented and discussed.

MATERIALS AND METHODS

Strains, plasmids, and phage. The bacterial strains, plasmids, and bacteriophage used in this study are listed in Table 1.

Media and growth conditions. Luria-Bertani medium (34) was used for routine cultures. Kornberg medium (1.1% K_2HPO_4 , 0.85% KH_2PO_4 , 0.6% yeast extract containing 0.5% glucose for liquid medium) was used to grow cultures for the gene expression assays and Northern blot analysis. M63 medium supplemented with glucose (0.4%); thiamine (5 μ g/ml); adenine and thymine (50 μ g/ml); calcium pantothenate (1 μ g/ml); and serine, glycine, and methionine (100 μ g/ml each) was used for the selection of *rel*⁺ *barA* mutants (55). Tryptone broth (pH 7.4) contained 1% tryptone and 0.5% NaCl. Colonization factor antigen medium (pH 7.4) contained 1% Casamino Acids, 0.15% yeast extract, 0.005% $MgSO_4$, and 0.0005% $MnCl_2$ (16). The following antibiotics were added, as required, at the indicated concentrations: chloramphenicol, 20 μ g/ml; kanamycin, 50 μ g/ml; ampicillin, 100 μ g/ml; and tetracycline, 10 μ g/ml, except that ampicillin and kanamycin were used at 50 and 40 μ g/ml, respectively, during the construction of the *uvrY*⁺-*lacZ* fusion, and kanamycin was used at 100 μ g/ml for the selection of *csrA*::*kanR* strains. All cultures that were used for gene expression assays were grown at 37°C with rapid rotary shaking (48).

Molecular and genetic techniques. Plasmid transduction or cotransduction of resistance markers, subcloning, PCR amplification, and molecular genetic techniques were performed by standard procedures (34, 50).

The plasmid pBarA was constructed by PCR amplification of the *barA* gene from -270 to the end of the *barA* coding region from *E. coli* MC4100 chromosomal DNA with *Pfu* polymerase and the primers 5'-GAGAATGCATACGCC AAAATGAGGACAG and 5'-GCGGATCCACTCGACAAGACATCCATTA. The resultant product was cloned directly into the pGEM-T vector (Promega) by using the T-overhang, with the *barA* gene position in a clockwise direction. A 0.5-kb *Bam*HI-*Eco*RI fragment containing *csrA* from pCSR10 (48), a 1.3-kb *Eco*RI-*Hind*III fragment containing *uvrY* from pCA9505 (36), and a 3.0-kb *Nco*I-*Not*I fragment containing *barA* from pBarA were treated with the Klenow fragment of DNA polymerase I and were individually subcloned into the blunt-ended *Vsp*I site of pBR322 to generate pCRA16, pUY14, and pBA29, respectively. The open reading frames of the above genes are oriented in the same direction as *bla* in the vector.

Special precautions were required for two of the Plasmid transductions of this study, as follows. The *barA*::*kanR* mutation was transduced from AKP014 (42) into MG1655 with selection for kanamycin resistance. Because AKP014 is a *relA* mutant, and *relA* is separated by only 1.4 kb of DNA from *barA*, a *relA* wild-type

TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Description	Source or reference
Strains		
AKP014	MC4100 <i>barA::kanR</i>	42
AKP023	MC4100 <i>uvrY::cam</i>	42
BA MG1655 ^a	MG1655 <i>barA::kanR</i> (from AKP014)	This study
CAG18642	<i>zfh-3131::Tn10</i> ; 57.5 min (near <i>csrA</i>)	52
CF7789	MG1655 Δ <i>lacIZ</i> (<i>Mlu</i> I)	Michael Cashel
DHB6521	SM551 λ InCh1 (<i>Kan</i> ^r)	9
HS703	MC4100 <i>barA::λplacMu53</i> [ϕ (<i>barA-lacZ</i>)1010]	37
KSA712	CF7789 Δ (<i>att-lom</i>):: <i>bla</i> ϕ (<i>csrA</i> '-' <i>lacZ</i>)I(Hyb) Amp ^r Kan ^s	27
KSB837	CF7789 Δ (<i>att-lom</i>):: <i>bla</i> ϕ (<i>csrB-lacZ</i>)I(Hyb) Amp ^r Kan ^s	20
KSY009	CF7789 Δ (<i>att-lom</i>):: <i>bla</i> ϕ (<i>uvrY</i> '-' <i>lacZ</i>)I(Hyb) Amp ^r Kan ^s	This study
KSGA18	CF7789 ϕ (<i>glgA::lacZ</i>) (λ placMu15)	20
MG1655	Prototrophic	Michael Cashel
RG1-B MG1655 ^a	<i>csrB::cam</i>	20
SM551(= DHB6501)	F ⁻ λ ^s Δ <i>lac</i> (MS265) <i>mel</i> NalA ^r <i>supF58</i> (= <i>suIII</i> ⁺)	9
TR1-5 MG1655 ^a	<i>csrA::kanR</i>	47
UY CF7789 ^a	CF7789 <i>uvrY::cam</i> (from AKP023)	This study
WX2	Δ <i>lac-pro met pro zzz::Tn10 thy supD</i> r _K ⁻ m _K ⁻ <i>sdiA::kanR</i>	56 (L. Rothfield)
Plasmids		
pCBZ1	pGE593 ϕ (<i>csrB-lacZ</i>)	20
pCZ3-3	pMLB1034 ϕ (<i>glgC</i> '-' <i>lacZ</i>)	46
pUZ9	pMLB1034 ϕ (<i>uvrY</i> '-' <i>lacZ</i>)	This study
pCSR10	Minimal <i>csrA</i> in pUC19	47
pCRA16	<i>csrA</i> in blunt-ended <i>VspI</i> site of pBR322, Tet ^r	This study
pCA9505	Carries <i>uvrY</i> gene, Amp ^r	36
pUY14	<i>uvrY</i> in blunt-ended <i>VspI</i> site of pBR322, Tet ^r	This study
pBarA	<i>barA</i> in pGEM-T, Amp ^r	This study
pBA29	<i>barA</i> in blunt-ended <i>VspI</i> site of pBR322, Tet ^r	This study
pSdiA	<i>sdiA</i> in <i>EcoRI</i> site of pBR322, Amp ^r Tet ^r	Robert A. LaRossa
pSPT18-CsrB	Plasmid for the <i>csrB</i> riboprobe synthesis	20
pGE593	Vector for <i>lacZ</i> transcriptional fusions; Amp ^r	15
pMLB1034	Vector for <i>lacZ</i> translational fusions; Amp ^r	51
pGEM-T	T-cloning vector, Amp ^r	Promega
pBR322	Cloning vector; Amp ^r Tet ^r	50
Bacteriophages		
P1vir	Strictly lytic P1	Carol Gross
λ InCh1	For genomic insertions; <i>Kan</i> ^r	9

^a Strain designations containing the prefix TR or TR1-5, RG or RG1-B, UY, BA, or SA indicate that the mutant allele *csrA::kanR*, *csrB::cam*, *uvrY::cam*, *barA::kanR*, or *sdiA::kanR*, respectively, was introduced by P1vir transduction.

transductant was selected by virtue of its ability to grow on M63 supplemented medium and was designated BA MG1655. The *uvrY::cam* mutation was transduced from AKP023 (42) into CF7789. Because AKP023 is a *flhD* mutant and *flhD* is located 21 kb away from *uvrY*, a motile transductant was identified by the plate assay for motility and designated UY CF7789. The other *barA* or *uvrY* mutants were generated by P1vir transduction from BA MG1655 or UY CF7789, respectively.

Construction of a chromosomal *uvrY*'-'*lacZ* translational fusion. A 572-bp fragment containing the upstream regulatory region and 12 codons of *uvrY* was amplified from MG1655 DNA by PCR with the primers 5'-CAGCATCGCTTT CAGGCAGGAGACTTC and 5'-CAGTTCGTGGTCATCAACAAGTAGAA CG, treated with T4 DNA polymerase and polynucleotide kinase, and subcloned into the *Sma*I site of pMLB1034 (51). The resulting plasmid, pUZ9, contained 26 codons of *yecF*, which is upstream from and divergently oriented with respect to *uvrY* (7), the complete upstream flanking region of *uvrY*, 12 codons of the *uvrY* coding region, and an in-frame *uvrY*'-'*lacZ* translational fusion. DNA sequencing was performed to confirm the presence of the correct fusion and the absence of PCR-generated mutations. The *uvrY*'-'*lacZ* fusion in pUZ9 was moved into the *E. coli* CF7789 chromosome by using the λ InCh1 system as described elsewhere (9). The resulting strain, KSY009, which was chosen for subsequent studies, was Amp^r Kan^s and was no longer temperature sensitive. The presence of the *uvrY*'-'*lacZ* translational fusion was confirmed by PCR analysis, as recommended elsewhere (9). Oligonucleotide primers used in this study were synthesized by Integrated DNA Technologies Inc., Coralville, Iowa.

Motility assay. The plate assay was initiated by stabbing a colony from an overnight culture into semisolid agar (tryptone broth or colonization factor antigen medium solidified with 0.35% agar). The plates were kept in a humidified incubator at 30°C and examined at ~16 h of growth (59).

β -Galactosidase and total protein assays. β -Galactosidase activity was assayed in 10-min reactions, as described previously (46). Total protein was measured by the bicinchoninic acid method with bovine serum albumin as the protein standard (53).

Purification of His₆-tagged UvrY. His₆-tagged UvrY was purified as described previously, except that purified protein was dialyzed against 10 mM Tris-acetate (pH 8.0) containing 25% glycerol and concentrated in Centricon 10 units (Amicon) (42).

In vitro transcription-translation. Effects of UvrY protein on *csrB-lacZ* expression were examined by using S-30 extracts prepared from a *uvrY* mutant strain (UY CF7789), as previously described (31, 48), except that reaction volumes were scaled down to 28 μ l. Radiolabeled proteins were detected by fluorography with sodium salicylate (10), and methionine incorporation into the LacZ polypeptide was quantified by liquid scintillation counting of H₂O₂-solubilized gel sections (48).

Quantitative biofilm assay. The quantitative analysis of biofilm formation in microtiter plates by crystal violet staining was previously described in detail (27).

Northern hybridization. RNA isolation, riboprobe synthesis, and Northern blotting were conducted essentially as described elsewhere (20). RNA (5 μ g) was separated on formaldehyde agarose (1.2%) gels, blotted by capillary transfer

FIG. 1. Northern analysis of CsrB levels in *E. coli* K-12 MG1655 and isogenic *barA*- and *uvrY*-knockout mutants. RNA from cultures harvested at 2 h post-exponential phase of growth was probed for the CsrB transcript. (A) Blot representative of the observed results. An overexposed film is shown to reveal CsrB RNA from the *uvrY* mutant. (B) Quantification of the signal by phosphorimage analysis. Signals were normalized with respect to MG1655, which was assigned a value of 100%. Each bar represents the mean value obtained from two independent experiments. Where error bars depicting the standard deviations are not apparent, the standard deviations were below the resolution of the graph.

onto positively charged nylon membranes (Boehringer Mannheim), and immobilized by being baked at 120°C for 30 min. A digoxigenin-labeled *csrB* riboprobe was synthesized from pSPT18-*CsrB* by using the DIG Luminescent Detection kit for nucleic acids, as described by the manufacturer (Boehringer Mannheim). The blot was prehybridized and hybridized at a probe concentration of 50 ng/ml with Perfecthyb Plus hybridization buffer (Sigma Chemical). Signal detection used the commercial protocol (Boehringer Mannheim), except that incubation in blocking solution was extended for an additional 10 h. Chemiluminescent signals were detected with Kodak X-Omat AR film. In addition, signals were quantified by phosphorimaging with a GS-525 PhosphorImager (Bio-Rad). Phosphorimaging data were analyzed with Molecular Analyst (version 2.1.2) software (Bio-Rad). Prior to blotting, the gels were stained with ethidium bromide and rRNA bands were photographed. The resulting signals were quantified by densitometry, and values were used to correct for minor variations in sample loading.

RESULTS

Effects of *uvrY* and *barA* on the in vivo expression of *csrB*. Northern hybridization was used to determine if UvrY or BarA affects CsrB RNA levels (Fig. 1). RNA was isolated from MG1655 and its isogenic *uvrY* and *barA* mutants at ~2 h post-exponential phase, which is optimal for CsrB accumulation (20). CsrB RNA levels were decreased ~60% in the *barA* mutant and ~98% in the *uvrY* mutant relative to the parent strain. These results suggested that UvrY is an important regulator of *csrB* expression and that BarA influences *csrB* expression to a lesser extent.

To further determine if *uvrY* and *barA* regulate expression of

csrA and *csrB*, expression from chromosomal *csrA*'-'*lacZ* translational or *csrB-lacZ* transcriptional fusions was examined in wild-type or *uvrY* or *barA* mutant strains. The specific β -galactosidase activity from the *csrA*'-'*lacZ* fusion was not altered by either mutation (Fig. 2A), while the activity from the *csrB-lacZ* fusion was dependent upon both *uvrY* and *barA* (Fig. 2B). The *uvrY* mutation reduced *csrB-lacZ* expression ~95% or 20-fold, whereas the *barA* mutant exhibited a decrease of ~70%. BarA is a member of the subclass of tripartite sensor kinases, and UvrY has been shown previously to be the cognate response regulator for BarA (42). These results suggest that BarA-phosphorylated UvrY activates transcription of the *csrB* gene and that either unphosphorylated UvrY can activate *csrB* expression to a lesser extent or UvrY is activated by an alternative phosphoryl donor.

Effects of *csrA* and *uvrY* on *barA* expression. To further examine the regulatory interactions of the CsrA/CsrB and BarA/UvrY systems, the effects of CsrA and UvrY on *barA* expression were examined by monitoring expression of a chromosomal *barA-lacZ* transcriptional fusion in wild-type or *csrA* or *uvrY* mutant strains. The wild-type strains exhibited approximately twofold-greater β -galactosidase activity than did their isogenic *csrA* (Fig. 3A) or *uvrY* (Fig. 3B) mutants. We note that because HS703 was kanamycin resistant, this *csrA* isogenic strain pair was constructed by cotransduction of the *csrA::kanR* mutation along with a closely linked *tetR* marker from TR1-5 CAG18642 into HS703 (*barA-lacZ*). Tet^r transductants were screened for the *csrA* glycogen phenotype to distinguish *csrA* wild-type and mutant colonies, and isogenic *csrA* mutant and wild-type strains, which each contained the *tetR* marker that was used for cotransduction, were compared in the assays (Fig. 3A). We note that the *tetR* mutation itself increased *barA-lacZ* expression approximately twofold (compare the isogenic parent strains, CAG HS703 and HS703, in Fig. 3A and B). The gene disrupted by the *tetR* marker is *srlD* (*gutD*) and encodes the glucitol-6-phosphate dehydrogenase of the glucitol operon (40, 61). The basis of its effect on *barA* expression is unknown. In conclusion, UvrY and CsrA each exhibit modest stimulation of *barA* expression.

Effects of *csrA*, *barA*, and *uvrY* on *uvrY* expression. To determine if the expression of *uvrY* is influenced by *csrA*, we examined expression from a *uvrY*'-'*lacZ* translational fusion in *csrA* wild-type and mutant strains. Expression from this gene fusion was not affected by a *csrA* mutation (data not shown). Overexpression of *csrA* from a plasmid resulted in only slight activation of this fusion, which is not likely to be biologically relevant. Similarly, the disruption of *barA* or *uvrY* had no effect on the expression of this *uvrY*'-'*lacZ* fusion (data not shown).

Complementation studies: effects of ectopic expression of *csrA*, *uvrY*, or *barA* on *csrB-lacZ* expression in *csrA*, *uvrY*, and *barA* mutants. We previously showed that CsrA is a strong activator of *csrB* transcription and provided evidence that this effect was indirect (20). Since *uvrY*, and to a lesser extent *barA*, activates *csrB* expression (Fig. 1 and 2) and *csrA* modestly stimulates *barA* expression (Fig. 3), complementation studies were conducted to further delineate the regulatory circuitry of this system. We found that multicopy plasmids containing either *csrA* or *uvrY* could restore *csrB-lacZ* expression in a *csrA* mutant background (Fig. 4). We also found that only *uvrY* could restore *csrB-lacZ* expression in a *uvrY* mutant (Fig. 4).

FIG. 2. Effects of the *uvrY* or *barA* null mutations on expression from *csrA'-lacZ* translational or *csrB-lacZ* transcriptional fusions. β-Galactosidase specific activities expressed from *csrA'-lacZ* in strains KSA712 (wild type), UY KSA712 (*uvrY::cam*), and BA KSA712 (*barA::kanR*) (A) and from *csrB-lacZ* in strains KSB837 (wild type), UY KSB837 (*uvrY::cam*), and BA KSB837 (*barA::kanR*) (B) are shown. β-Galactosidase activities in the wild-type, *uvrY::cam*, or *barA::kanR* strain are shown as closed circles, squares, or triangles, respectively. Growth (A_{600}) of the respective strains is depicted by open circles, squares, and triangles, respectively. This experiment was repeated in its entirety with essentially identical results.

Finally, *barA*, *uvrY*, or *csrA* was able to enhance *csrB-lacZ* expression in a *barA* strain background (Fig. 4). These results are consistent with a late or perhaps terminal role for UvrY in a signaling pathway to *csrB*. Because *csrA* stimulates *barA* expression (Fig. 3) and *csrB* expression in a *barA* mutant (Fig. 4), these results indicate that *csrA* influences *csrB* expression through BarA-dependent and -independent mechanisms. In addition, *csrA* has no effect on *csrB* expression in the *uvrY* mutant background, indicating that its role in *csrB* expression is completely dependent upon UvrY. The simplest model that accounts for all of these findings is that CsrA is involved in both BarA-dependent and -independent pathways for UvrY activation. We acknowledge that more complex explanations

have not been formally eliminated. Taken in context, the failure of *barA* to complement a *csrA* defect also allows for the possibility that CsrA may affect BarA function, e.g., CsrA may indirectly influence BarA activation.

Effects of *uvrY* and *barA* on CsrB-activated genes. To assess the effects of the *uvrY* and *barA* mutations on genes that are repressed by *csrA* and activated by *csrB*, we monitored expression of a chromosomal *glgCA'-lacZ* translational fusion and a plasmid-encoded *glgC'-lacZ* translational fusion. The effect of *uvrY* disruption was similar to that of *csrB* (20) and was a modest decrease in *glgCA'-lacZ* expression (Fig. 5A). Likewise, overexpression of *uvrY* yielded the opposite effect from *uvrY* and *csrB* disruption (Fig. 5B). Ectopic expression of *uvrY*

FIG. 3. Effects of *csrA* and *uvrY* mutations on expression from a *barA-lacZ* transcriptional fusion. (A) β-Galactosidase specific activities expressed from a *barA-lacZ* fusion in strains CAG HS703 (wild type) and TR1-5 CAG HS703 (*csrA::kanR*) are shown as closed and open circles, respectively. (B) Activities in strains HS703 and UY HS703 (*uvrY::cam*) are shown as closed and open circles, respectively. In each panel, growth (A_{600}) of the respective strains is depicted by closed or open squares. This experiment was repeated in its entirety with essentially identical results.

FIG. 4. Complementation studies: effects of ectopic expression of *csrA* (pCRA16), *uvrY* (pUY14), or *barA* (pBA29) on expression of a *csrB-lacZ* transcriptional fusion in isogenic *csrA*, *uvrY*, or *barA* mutants of KSB837. The vector control was pBR322 in each case. Specific β -galactosidase activities and growth (A₆₀₀) at 24 h are shown as bars and closed circles, respectively. This experiment was repeated with essentially identical results.

from the multicopy plasmid pUY14 in a *csrB*-null mutant strain resulted in modest to negligible effects on *glgCA'-lacZ* (Fig. 5C), suggesting that UvrY affects expression of *glgCAP* primarily through its role as an activator of *csrB*. The *uvrY* and *barA* mutations also decreased *glgC'-lacZ* expression from pCZ3-3 about twofold (data not shown).

UvrY activates *csrB* expression in vitro. To determine whether UvrY directly regulates the transcription of the *csrB* gene, in vitro transcription-translation of pCBZ1-encoded *csrB-lacZ* transcriptional fusion was examined in S-30 extracts prepared from the *uvrY* mutant, UY CF7789, in the presence of various concentrations of purified UvrY protein. The expression of the pCBZ1-encoded *csrB-lacZ* fusion was activated ~20-fold by *uvrY* in vivo, as determined in *uvrY* wild-type versus mutant strains (data not shown). As shown in Fig. 6, in vitro synthesis of the LacZ protein was stimulated approximately sixfold in the presence of 2.3 μ M UvrY protein subunits, a concentration that saturated the reaction. Synthesis of

the LacZ polypeptide was also detected in reactions with the control vector, pGE593. This was likely due to read-through transcription, as previously shown for similar derivatives of the vector pMLB1034 (29). However, in contrast to the reactions with pCBZ1, LacZ expression from the control vector was not stimulated by UvrY. Since pCBZ1 contained the upstream region of *csrB* (−242 to +4), this experiment indicates that UvrY activates *csrB* transcription, presumably by binding to *csrB* DNA. In this experiment, recombinant UvrY protein was used as isolated from the cell. Since BarA is required for maximal expression of *csrB* in vivo (Fig. 1 and 2), it is likely that phosphorylated UvrY activates *csrB* transcription. It is possible that UvrY protein was phosphorylated prior to or during the S-30 reaction or that unphosphorylated UvrY may bind to DNA and activate transcription, albeit at reduced affinity relative to the phosphorylated form.

Effects of *sdiA* on expression of *csrA*, *csrB*, and *uvrY*. SdiA is a LuxR homologue that possesses a putative AHL binding

FIG. 5. Effects of the *csrB* or *uvrY* null mutations and *uvrY* overexpression on a chromosomal *glgCA'-lacZ* translational fusion. β -Galactosidase activities expressed from *glgCA'-lacZ* in strains KSGA18 (closed circles) and UY KSGA18 (*uvrY::cam*) (open circles) (A), KSGA18[pBR322] (closed circles) and KSGA18[pUY14] (open circles) (B), and RG KSGA18[pBR322] (*csrB::cam*) (closed circles) and RG KSGA18[pUY14] (*csrB::cam*) (open circles) (C) are shown. In each panel, growth (A₆₀₀) of the respective strains is depicted by closed or open squares. This experiment was repeated in its entirety, with essentially identical results.

FIG. 6. In vitro transcription-translation of the *csrB-lacZ* transcriptional fusion carried on pCBZ1. Reaction mixtures contained pCBZ1 (*csrB-lacZ*) or vector only (1.6 μ g), as indicated. Reactions were conducted in the absence or presence of UvrY protein, in an S-30 extract from UY CF7789 (*uvrY::cam*). (A) Labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The position of an unlabeled standard of β -galactosidase (LacZ) is shown. (B) Incorporation of [³⁵S]methionine into the LacZ polypeptide was determined by liquid scintillation counting. Approximately 5.5×10^3 cpm is equivalent to 1 pmol of LacZ polypeptide per reaction per h.

domain and a second domain for binding DNA (54, 56). Genomic array studies indicated that an increase in the copy number of *sdhA* significantly increases the levels of *uvrY* mRNA (60). To determine if *csrA*, *csrB*, or *uvrY* expression is altered by *sdhA*, we examined expression from chromosomal *csrA'-lacZ*, *csrB-lacZ*, and *uvrY'-lacZ* fusions in isogenic *sdhA* mutant and wild-type strains. We also compared expression from these fusions in strains containing a plasmid clone of *sdhA*, pSdhA, or the cloning vector, pBR322. No significant effect of SdhA was observed for *csrA'-lacZ* expression (Fig. 7A and B). However, expressions from *csrB-lacZ* and *uvrY'-lacZ* fusions were partially dependent upon *sdhA*, as they were decreased by the *sdhA* mutation (Fig. 7C and E) and increased 1.5- and ~6-fold, respectively (Fig. 7D and F), by *sdhA* overexpression. In order to determine whether *sdhA* regulates *csrB* expression via its effect on *uvrY*, a complementation test was conducted. Expression from the *csrB-lacZ* fusion in the *uvrY* mutant was no longer affected by *sdhA* overexpression (data not shown), suggesting that *sdhA* stimulates *csrB* expression through its effects on *uvrY*. Regulation via the highly conserved SdhA homologue of *S. enterica* was recently found to respond to AHL,

FIG. 7. Effects of *sdhA* disruption and overexpression on expression of chromosomal *uvrY'-lacZ* and *csrA'-lacZ* translational fusions and a *csrB-lacZ* transcriptional fusion. (A and B) Expression from *csrA'-lacZ* in KSA712 and SA KSA712 (*sdhA::kanR*) (A) and KSA712[pBR322] and KSA712[pSdhA] (B). (C and D) Expression from *csrB-lacZ* in strains KSB837 and SA KSB837 (*sdhA::kanR*) (C) and KSB837[pBR322] and KSB837[pSdhA] (D). (E and F) Expression of *uvrY'-lacZ* in strains KSY009 and SA KSY009 (*sdhA::kanR*) (E) and KSY009[pBR322] and KSY009[pSdhA] (F). In each panel, β -galactosidase activities in the wild-type strain are shown as closed circles, whereas activities in *sdhA::kanR* or *sdhA*-overexpressing strains are shown as open circles. Growth (A_{600}) of the respective strains is depicted by closed or open squares. This experiment was repeated in its entirety, with essentially identical results.

the most active of which was *N*-(β -ketocaproyl)-L-homoserine lactone (33). Nevertheless, we did not observe any effect of this compound, over a broad concentration range, on *lacZ* fusions for *csrA*, *csrB*, or *uvrY*, in *sdhA* wild-type or mutant strains (data not shown).

Effects of *uvrY*, *barA*, or *sdhA* on biofilm formation. We recently showed that CsrA represses biofilm formation, while CsrB activates this process (27). The effects of *uvrY*, *barA*, and *sdhA* on biofilm formation were monitored in static cultures by the microtiter plate assay, which measures the binding of crystal violet to adherent cells of the biofilm (27). The most striking observation was that the ectopic expression of *uvrY* activated biofilm formation severalfold (Fig. 8B). This effect was almost as great as that of a *csrA*-knockout mutation. In addition, *uvrY* overexpression activated biofilm formation in a *csrB* mutant strain background, indicating that UvrY has effects on biofilm formation that are mediated independently of *csrB*. More modest effects were observed for knockouts of *csrB*, *uvrY*, *barA*, and *sdhA*, which were still statistically significant (Fig. 8A). The parent strain (MG1655) formed approximately three-

FIG. 8. Effects of *csrB*, *uvrY*, *barA*, and *sdiA* null mutations and ectopic expression of *uvrY*, *barA*, and *sdiA* on 24-h biofilms grown in microtiter wells. (A) Biofilm formation by the parent strain MG1655 and isogenic mutants, as indicated. (B) Effects of increased gene dosage of *uvrY* (pUY14), *barA* (pBA29), and *sdiA* (pSdiA) on biofilm formation by MG1655. (C) Same as panel B, except in a *csrB*-null mutant (RG1-B MG1655). Bars show the averages and standard errors of three experiments, with three samples per experiment. Double asterisks indicate statistically significant differences between strains of a given set ($P < 0.01$).

fold-more biofilm than each of these mutants. Overexpression of *barA* caused an unexplained modest decrease in biofilm formation. The increased gene dosage of *sdiA* caused a modest increase in biofilm formation, which was not determined to be statistically significant. In a repetition of this entire experiment, all of the above trends, including the relatively modest ones, were found to be reproducible.

DISCUSSION

The RNA binding protein CsrA and the untranslated RNA CsrB constitute a posttranscriptional regulatory system that has profound effects on central carbon metabolism, motility, and multicellular behavior of *E. coli* (reviewed in reference 45). This system also has provided a model for the elucidation of several homologous systems in other gram-negative bacteria, referred to as CsrA/CsrB, RsmA/RsmB, or RsmA/RsmZ, which exhibit additional interesting and important regulatory roles (1, 4, 5, 8, 12, 32, 43). We recently reported that CsrA is autoregulatory and indirectly activates transcription of the gene for its RNA antagonist, CsrB (20). The simplest explanation for this role of CsrA was that CsrA either activates a transcriptional activator or inhibits a transcriptional repressor of *csrB*. The present study supports the former hypothesis and delineates the intervening signaling circuitry by which CsrA activates *csrB* transcription, namely, the BarA/UvrY two-component signal transduction system.

The organization of the signaling circuitry that connects the CsrA/CsrB and BarA/UvrY regulatory systems was defined by

several kinds of evidence. First, the steady-state levels of CsrB RNA were extremely deficient in *csrA* (20) and *uvrY* (Fig. 1) mutants. The in vivo expression of a *csrB-lacZ* transcriptional fusion containing the region from -242 to +4 bp of *csrB*, relative to the start of transcription, is also highly dependent upon *csrA* (20) and *uvrY* (Fig. 2). Since this fusion is capable of expressing only the first 4 nucleotides of the natural *csrB* transcript, the latter experiment establishes that UvrY activates *csrB* transcript initiation, as opposed to stabilizing CsrB RNA. A *barA* mutant is partially defective for CsrB accumulation and *csrB-lacZ* expression (Fig. 1 and 2), but this effect of *barA* is considerably less severe than those of *csrA* (20) or *uvrY* (Fig. 1 and 2) mutations. Second, *barA*, *uvrY*, and *csrA* itself (20) have no effects on the expression of *csrA* (Fig. 2). CsrA and UvrY each stimulate *barA-lacZ* expression ~1.5 to 2-fold (Fig. 3). Mutations in *csrA*, *barA*, or *uvrY* itself did not significantly affect *uvrY-lacZ* expression (data not shown). Third, complementation studies with multicopy plasmids showed that *uvrY* suppresses the defects in *csrB-lacZ* expression that are caused by *csrA*, *barA*, or *uvrY* mutations (Fig. 4). A *csrA* plasmid clone suppresses the defects of *csrA* or *barA* mutants but has no effect in a *uvrY* mutant (Fig. 4). Finally, a plasmid clone of *barA* suppresses the *barA* defect but does not affect *csrB-lacZ* expression in strains defective for *uvrY* or *csrA* (Fig. 4). Because ectopic expression of *csrA* has no effect on *csrB* if *uvrY* is defective, while ectopic expression of *uvrY* suppresses a *csrA* defect, these complementation experiments provide strong genetic evidence that the effects of CsrA on *csrB* transcription are mediated through UvrY. It should be reemphasized that CsrA

FIG. 9. Summary of the regulatory interactions of CsrA/B, BarA/UvrY, and SdiA. CsrA activates *csrB* transcription indirectly (20). This effect of CsrA requires functional UvrY, which directly activates *csrB* transcription. The effect of CsrA on *csrB* is mediated in part by activation of *barA* expression but apparently also involves a BarA-independent, UvrY-dependent mechanism, shown as [X]. UvrY also activates the expression of *barA*, in an autoregulatory loop. SdiA activates the expression of *uvrY* and, to a lesser extent, that of *csrB*. Finally, CsrB RNA binds to ~18 subunits of CsrA protein and antagonizes its regulatory effects in the cell (31, 45).

does not affect *csrB* by regulating the expression of the *uvrY* gene, although it is a modest activator of the expression of its cognate kinase, BarA (Fig. 3). Fourth, purified CsrA protein failed to regulate *csrB-lacZ* expression in S-30 transcription-translation assays, indicating that CsrA indirectly activates *csrB* expression (20), while recombinant UvrY protein activated the same *csrB-lacZ* fusion approximately sixfold (Fig. 6). To our knowledge, the latter result represents the first biochemical evidence that UvrY or any of its homologues directly activates gene expression and positions UvrY immediately upstream from *csrB* in a signaling pathway. Fifth, mutations in *uvrY* or *barA* result in a reduction of the expression of *glgC'-lacZ* and *glgCA'-lacZ* translational fusions (Fig. 5 and data not shown), which are repressed by CsrA and activated by CsrB (20, 47, 63). While these effects of *barA* and *uvrY* were modest, they were in agreement with the modest effects noted for CsrB (20). The relatively weaker effects of CsrB than of CsrA have been observed thus far for all Csr-regulated genes. They are consistent with the finding that CsrB levels in the cell are sufficient to bind only ~30% of the CsrA protein, assuming full occupation of the approximately 18 CsrA binding sites on CsrB (20, 31). The relatively lower level of CsrB in the cell than of CsrA may also account for the modest effects of BarA/UvrY, since UvrY effects on *glgCA'-lacZ* were largely dependent on the presence of a functional *csrB* gene (Fig. 5). Sixth, comparisons of *sdiA* wild-type, mutant, and overexpressing strains confirmed that SdiA activates expression of a *uvrY'-lacZ* translational fusion (Fig. 7). In addition, *sdiA* was found to activate *csrB* expression through its effect on *uvrY*.

The model shown in Fig. 9 incorporates the findings of this study in context with our previous findings on the regulatory circuitry of the Csr system. The RNA binding protein CsrA is the key regulator of the Csr system and indirectly activates the transcription of its RNA antagonist, CsrB, ~20-fold (20). Although CsrA binds to CsrB RNA, it does not alter its chemical

decay rate, which has a half-life of ~2 min (20). The effects of CsrA on *csrB* expression are completely dependent upon UvrY (Fig. 4), which is a direct activator of *csrB* expression (Fig. 6). While *barA* is involved in the circuitry, mutagenesis (Fig. 1 and 2) and complementation (Fig. 4) studies suggest that CsrA can activate *csrB* expression independently of BarA. UvrY is a member of the FixJ family of response regulators, and promoter binding by purified FixJ has been shown elsewhere to require phosphorylation (2, 13, 44). In addition, BarA has been shown elsewhere to phosphorylate UvrY (42). Thus, activation of UvrY by BarA likely occurs by phosphorylation. The relative effects of CsrA (20), UvrY, and BarA on *csrB* expression and complementation studies with these genes collectively suggest that a BarA-independent mechanism also activates UvrY. The model in Fig. 9 also depicts the finding that UvrY stimulates *barA* expression, indicative of a positive autoregulatory loop within this system. Finally, the LuxR homologue SdiA activates *uvrY* expression and in this way affects *csrB* expression. Although the sigma factor RpoS or σ^s is important in stationary-phase regulation and *rpoS* transcription was reported elsewhere to be activated by *barA* (37), we previously found that neither CsrA protein nor CsrB RNA levels were significantly affected by *rpoS* disruption (20).

The UvrY/BarA two-component signal transduction system was recently recognized in *E. coli*, and biochemical and genetic evidence was presented to demonstrate direct phosphotransfer from BarA to UvrY (42). BarA of *E. coli* was reported elsewhere to be involved in the bacterial adaptive responses against hydrogen peroxide-mediated stress by activating transcription of the *rpoS* gene, which encodes a sigma factor involved in the expression of stationary-phase and stress response genes (23, 37). The effect of UvrY on *rpoS* has not yet been examined. Two-component systems homologous to BarA/UvrY have been identified in other gram-negative species and are known as BarA/SirA in *S. enterica*, ExpS/ExpA or GacS/GacA in *E. carotovora*, and GacS/GacA in *Pseudomonas* (references 22 and 42 and references therein). Genetic evidence for relationships between the Csr-homologous (Rsm) systems and the BarA/UvrY-homologous (GacS/GacA) systems has been obtained from *Pseudomonas* and *Erwinia* species. Transcription of *rsmB* was under the positive control of *gacA* (*expA*) in *Erwinia carotovora* subsp. *carotovora* (12, 26). In this bacterium, mutants with mutations in *gacS* or *gacA* possessed very low levels of RsmB RNA compared with the wild-type strain but possessed similar levels of *rsmA* mRNA and RsmA protein (12). In contrast, Hyytiäinen et al. (26) noted that *rsmA* transcription is elevated in the *expA* (*gacA*) mutant of this species during exponential growth. A recent report by Heeb et al. (21) revealed that a small RNA, which might be a *csrB* homologue of *P. fluorescens* CHA0, is activated through *gacA/gacS* and suggested that this RNA may affect RsmA by a titration effect, similar to the CsrA/CsrB model. Both *E. carotovora* and *P. fluorescens* are plant-associated species, and the GacS/GacA and Rsm systems affect the stationary-phase expression of genes encoding a variety of secreted virulence proteins of *E. carotovora* and protease and hydrogen cyanide production in *P. fluorescens*. The *csrA*, *csrB*, and *barA* genes of the mammalian pathogen *S. enterica* serovar Typhimurium regulate expression of the regulatory gene *hilA* and several related genes that encode components of the SPI1 type III secretion

apparatus, which is required for mucosal invasion (4, 5). While the *barA* and *csrB* genes of *S. enterica* were originally reported to be unrelated (4), both genes affect expression of *hilA* and related genes. In the context of the results from *E. coli* and *E. carotovora*, it is plausible that regulatory interactions also occur between these genes in *S. enterica*. Thus, it appears that BarA/UvrY systems are intimately related to the CsrA/CsrB systems in a variety of γ -proteobacteria. Despite the important roles played by these systems, the environmental stimulus recognized by BarA remains to be established.

The present investigation confirms the result from a genomic array study, which had indicated that *sdiA* overexpression results in elevated levels of *uvrY* transcript in *E. coli* (60). SdiA is a member of the LuxR family of proteins, which regulate quorum sensing. SdiA facilitates the binding of RNA polymerase to the *ftsQP2* promoter and requires an intact α -subunit C-terminal domain for this effect (62). A binding site for SdiA was demonstrated within the -35 region of the *ftsQ* promoter of *E. coli*, and the sequence 5'-AAAAG NNNNNNNNGAAAA-3' was reported to comprise an SdiA box. A similar sequence overlaps the -35 region of the *uvrY* promoter (data not shown), and we predict that this is an SdiA box that is required for activation of *uvrY* transcription. Unlike other gram-negative species, in which LuxR homologues bind to AHL, *E. coli*, *Salmonella*, and *Klebsiella* spp. are not known to synthesize the AHL and have no apparent AHL synthase genes, which correspond to *luxI* or *luxLM* homologues (3). Nevertheless, Michael et al. (33) provided evidence that SdiA is an AHL receptor of *S. enterica*, which may detect signals emanating from other species. This suggests that SdiA may provide a means of recognizing other species, e.g., within the intestinal tract, and modulating *csrB* expression through effects on *uvrY*. This is an attractive hypothesis, in view of the proposed role of CsrB as an activator of *hilA* expression in *S. enterica* (4, 5). However, we tested the most active AHL derivative that was investigated by Michael et al. (33) and found that it had no effect on the expression of our *csrB-lacZ* fusion in *E. coli* (data not shown). Therefore, the environmental signal that permits SdiA of *E. coli* to regulate *uvrY* expression and, as a consequence, *csrB* transcription remains to be determined.

We recently reported that CsrA is a repressor of biofilm formation, while CsrB activates biofilm formation (27). In addition, the gratuitous induction of *csrA* in a preformed biofilm caused it to disperse by liberating viable planktonic cells (27). The effect of CsrA on biofilm formation was mediated largely through its role as a regulator of intracellular glycogen synthesis and turnover in the stationary phase of growth. The most striking finding noted upon examination of biofilm formation by strains in the present study was that ectopic expression of *uvrY* caused a severalfold increase in biofilm formation, which was almost as great as the increase caused by a *csrA* mutation (Fig. 8). UvrY was able to activate biofilm formation independently of CsrB. Recently, the *gacA* gene of *Pseudomonas aeruginosa*, which is homologous to *uvrY*, was reported to activate biofilm formation (41). In that study, a number of possible factors that are required for biofilm formation were examined, including twitching and swarming motility, alginate biosynthesis, and autoinducer production, but none accounted for the regulatory effect of GacA. While the effect of UvrY on

E. coli biofilm formation remains to be elucidated, UvrY effects on *glgA* expression and glycogen accumulation (data not shown) appear to be too modest to account for this role of UvrY. Thus, the mechanisms by which UvrY and GacA activate biofilm formation by *E. coli* and *P. aeruginosa*, respectively, have not yet been determined.

ACKNOWLEDGMENTS

We thank Herbert E. Schellhorn for providing the bacterial strain HS703, Robert A. LaRossa for the *sdiA* plasmid, Lawrence Rothfield for the *sdiA* mutant WX2, Mike Cashel for strain CF7789, and Dana Boyd for λ InCh materials and advice.

This project was supported in part by grants from the National Institutes of Health (GM-59969), the National Science Foundation (MCB-9726197), the Swedish Science Research Council, and CONACyT (37342-N).

This research was conducted in part at the Department of Molecular Biology and Immunology, University of North Texas Health Science Center at Fort Worth.

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