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Effects of the Global Regulator CsrA on the BarA/UvrY Two-Component Signaling System

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The hybrid sensor kinase BarA and its cognate response regulator UvrY, members of the two-component signal transduction family, activate transcription of CsrB and CsrC noncoding RNAs. These two small RNAs act by sequestering the RNA binding protein CsrA, which posttranscriptionally regulates translation and/or stability of its target mRNAs. Here, we provide evidence that CsrA positively affects, although indirectly, *uvrY* expression, at both the transcriptional and translational levels. We also demonstrate that CsrA is required for properly switching BarA from its phosphatase to its kinase activity. Thus, the existence of a feedback loop mechanism that involves the Csr and BarA/UvrY global regulatory systems is exposed.

The BarA/UvrY two-component signal transduction system (TCS) of *Escherichia coli* consists of the membrane-bound sensor kinase BarA and its cognate response regulator UvrY (1). BarA, which belongs to the subfamily of tripartite sensor kinases (2, 3), senses and responds to the presence of formate and acetate but also to that of other short-chain fatty acids (4). Phosphorylated BarA catalyzes the transphosphorylation of UvrY (1), a typical response regulator of the FixJ family (1, 5), which in turn activates expression of the noncoding RNAs of the carbon storage regulation (Csr) system, CsrB and CsrC. These small regulatory RNAs possess repeated sequence elements that allow them to interact with multiple copies of the RNA binding protein CsrA and thereby prevent its regulatory interaction with its mRNA targets (6).

CsrA is a small, dimeric RNA binding protein that coordinates gene expression by positively or negatively regulating the translation, stability, and/or elongation of its target transcripts (7, 8). CsrA directly interacts with the 5′ untranslated leaders of target mRNAs at sites characterized by a GGA sequence that is often located within the loop of a short stem-loop structure (9–11). In this way, CsrA activates exponential-phase processes while it represses several stationary-phase functions (12). CsrA is widely distributed among eubacteria (13) and regulates expression of genes for virulence factors (14, 15), quorum sensing (16, 17), motility (18, 19), carbon metabolism (20, 21), biofilm formation (22, 23), cyclic di-GMP synthesis (24), and peptide uptake (10).

Curiously, activation of *csrB* transcription, which depends directly on UvrY-P, does not take place in a *csrA* mutant strain (25). Therefore, it has been suggested that CsrA has a positive effect on the BarA/UvrY TCS. In this study, we confirmed and extended these results by examining the effects of CsrA on either the expression or the activity of BarA and UvrY. Our results demonstrate that CsrA, apparently indirectly, is required for proper *uvrY* expression and also for activation of the BarA kinase activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this work are listed in Table 1. Strains IFC5010 (*csrA*::Kan^r *csrB-lacZ*) and IFC5016 (*uvrY*::Cam^r *csrA*::Kan^r *csrB-lacZ*) were constructed by P1*vir* transduction of the *csrA*::Kan^r allele from strain TR1-5 (25) into strains KSB837 (*csrB-lacZ*) (26) and UYKSB837 (*uvrY*::Cam^r *csrB-lacZ*) (25), respectively. Strain IFC5015 (*ackA*::*tetR*::*pta csrA*::Kan^r *csrB-lacZ*) was constructed by P1*vir* transduction of the *ackA*::*tetR*::*pta* allele from strain ECL5336 (27) into strain IFC5010. For strain IFC5017 (*barA*::Cam^r *csrA*::Kan^r *csrB-lacZ*) construction, the *barA* gene was deleted by homologous recombination using the lambda Red recombinase system (28). Briefly, a fragment amplified by PCR, using primers barAdel-Fw (5′-ATTTAACAGTGTGACCTTAATTGTCCCATAACGGAACTCCGTGTAGGCTGGAGCTGCTTC-3′) and barAdel-Rv (5′-CATAAACACAGGCACTTTGTCACCAATCTGAAACCAGCGTATGAATATCCTCCTTAGTTCC-3′) and plasmid pKD3 (28) as the template, was used to replace the *barA* allele with a chloramphenicol cassette in strain IFC5010.

To construct Amp^r-linked *lacZ* operon fusions, plasmid pAH125-bla was first generated by replacing the kanamycin resistance cassette of pAH125 (29) with an ampicillin resistance cassette. To this end, a *bla* PCR product was generated, using primers Amp-Prom-Fw (5′-GCGGCGCCTTCAAATATGTATCCGCTCATG-3′) and Amp-Rv (5′-GCGCGGCCGCGGTCTGACAGTTACCAATGC-3′) and plasmid pUC18 as the template, and cloned into the NarI-NotI sites of pAH125. Helper plasmid pINT-cat was constructed by replacing the ampicillin resistance cassette of plasmid pINT-ts (29) with the chloramphenicol resistance cassette. A 1.1-kb DNA fragment containing the *cat* gene, obtained from plasmid pKD3 (28) by HindIII digestion, was blunt ended and cloned into blunt-ended XmnI-BsaI sites of pINT-ts. Plasmid pAH-uvrY, containing an *uvrY-lacZ* operon fusion, was constructed by cloning a PCR-amplified fragment containing the upstream noncoding region through the first 4 codons (nucleotides [nt] −409 to +12 relative to the start of translation) (using primers uvrYP-fw-Pst [5′-AACTGCAGGGCGGCGGAGTATACCATAAG-3′] and uvrYP-Rv-BamHI [5′-CGGGATCCAGAACGTTGATCAAAGGAATATC-3′]) into the PstI-BamHI sites of pAH125-bla. Plasmid pUV-uvrY22, containing a translational *uvrY-lacZ* fusion under the control of the *lacUV5* promoter, was constructed by cloning the region

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TABLE 1 *E. coli* strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
CF7789	MG1655 Δ*lacIZ* (MluI)	Michael Cashel
TR1-5CF7789	CF7789 *csrA*::Kan^r	25
KSB837	CF7789 ϕ(*csrB-lacZ*)	26
IFC5010	KSB837 *csrA*::Kan^r	This work
BAKSB837	KSB837 *barA*::Kan^r	25
UYKSB837	KSB837 *uvrY*::Cam^r	25
ECL5336	MC4100 *ackA*::Tet^r::*pta*	27
IFC5011	CF7789 ϕ(*uvrY-lacZ*) operon fusion	This work
IFC5012	TR1-5CF7789 ϕ(*uvrY-lacZ*) operon fusion	This work
IFC5013	CF7789 ϕ(P*lacUV5-uvrY22′-′lacZ*) leader fusion	This work
IFC5014	TR1-5CF7789 ϕ(P*lacUV5-uvrY22′-′lacZ*) leader fusion	This work
IFC5015	KSB837 *ackA*::*tetR*::*pta csrA*::Kan^r	This work
IFC5016	KSB837 *uvrY*::Cam^r *csrA*::Kan^r	This work
IFC5017	KSB837 *barA*::Cam^r *csrA*::Kan^r	This work
Plasmids		
pACT3	Low-copy-no. vector, Cam^r	31
pEXT21	Low-copy-no. vector, Sp^r	31
pBA29	*barA* in blunt-ended VspI site of pBR322, Tet^r	25
pAH125	CRIM vector for transcriptional lacZ fusions, Kan^r	29
pINT-ts	CRIM integration vector, Amp^r	29
pUV5	CRIM vector for translational fusions, Amp^r	30
pAH125-bla	CRIM vector for transcriptional *lacZ* fusions, Amp^r	This work
pINT-cat	CRIM integration vector, Cam^r	This work
pMX539	*uvrY* in SmaI site of pACT3, Cam^r	This work
pMX540	*uvrY* D54Q in SmaI site of pACT3, Cam^r	This work
pMX541	*uvrY* in blunt-ended NdeI site of pBA29, Tet^r	This work
pMX542	*barA* promoter in pEXT21, Sp^r	This work
pMX543	*uvrY* under the control of *barA* promoter in pEXT21, Sp^r	This work
pMX544	*csrA* in HindIII and PstI sites of pEXT21, Sp^r	This work
pMX545	*csrB* in pGEMT-Easy, Amp^r	This work
pAH-uvrY	*uvrY-lacZ* operon fusion, Amp^r	This work
pUV-uvrY22	*uvrY-lacZ* leader (plus 22 codons) fusion under the control of the *lacUV5* promoter Amp^r	This work

from nt −47 to +66 relative to the start of *uvrY* translation, amplified by PCR using primers uvrY-lead-Fw (5′-GGAATTCAATGACTAACTATC AGTAGC-3′) and uvrY-lead22-Rv (5′-CGGGATCCTCTTCCAGAATG CGTCG-3′), into the EcoRI-BamHI sites of plasmid pUV5 (30). All fusions were integrated into the CF7789 and TR1-5CF7789 chromosomes, as previously described (29), to generate strains IFC5011 to IFC5014.

To construct plasmids pMX539 and pMX541, the *uvrY* open reading frame and its promoter region were PCR amplified using primers uvrY-Prom549-Fw (5′-GGAATTCGCAGCATCAGCGTCAGC-3′) and UvrY-Rv-HindIII (5′-CCCAAGCTTCCGTACCACCAGCATCG-3′) and chromosomal DNA from strain MG1655 as the template and cloned into the SmaI site of plasmid pACT3 (31) and into the blunt-ended NdeI site of pBA29 (25), respectively. Plasmid pMX540, carrying the *uvrY*^{D54Q} mutant allele, was generated by site-directed mutagenesis of plasmid pMX539, using the QuikChange kit (Stratagene, CA) and the mutagenic primers uvrY-D54Q-Fw (5′-GTTGACGTGGTGCTAATGCAGATGAG TATGCCGGGC-3′) and uvrY-D54Q-Rv (5′-GCCCGGCATACTCATC TGCATTAGCACCACGTCAAC-3′). The correct amino acid replacement was confirmed by DNA sequencing. To construct plasmid pMX544, a fragment containing the *csrA* open reading frame and its promoter region was PCR amplified, using primers csrA1-Fw (5′-CCCAAGCTTGCC AGTGTGAAAGGCTGG-3′) and csrA1-Rv (5′-AACTGCAGGAATGAA CGGGAGTAAAGCG-3′) and chromosomal DNA from strain MG1655 as the template, and cloned into the HindIII and PstI sites of plasmid pEXT21 (31). To construct plasmid pMX543, which carries the *uvrY* gene under the control of the *barA* promoter and 5′ untranslated regions (5′-UTRs), a 1-kb fragment containing the *barA* promoter and 5′-UTRs was first PCR amplified, using the primers BarA-Forwd (5′-GGAATTCCCG ACCACACTGGCAGC-3′) and 5′-TGCTCTAGAAGATCTGATCATA TGGAGTTCCGTTATGGG-3′ and chromosomal DNA from strain MG1655 as the template, and cloned into EcoRI and XbaI sites of plasmid pEXT21 (31) to generate plasmid pMX542. Then, a 762-bp PCR-amplified fragment (using primers uvrY [5′-CCCGGATCCCATATGATCAAC GTTCTACTTGTTGATGACCACG-3′] and UvrY-Rv-HindIII [5′-CCC AAGCTTCCGTACCACCAGCATCG-3′] and chromosomal DNA as the template) containing the *uvrY* open reading frame was cloned into the NdeI-HindIII sites of pMX542 to generate pMX543.

To construct plasmid pMX545, a 376-bp DNA fragment containing the *csrB* gene was PCR amplified from chromosomal DNA of strain MG1655 and introduced by T/A cloning into pGEMt-Easy vector (Promega).

Bacteria were routinely cultured at 37°C in lysogeny broth (LB) medium. Media were supplemented with antibiotics at the following concentrations: chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; streptomycin, 100 μg/ml; and spectinomycin, 50 μg/ml. P1*vir* transduction was performed as previously described (32).

RNA extraction and Northern blotting. Total RNA was purified from samples taken at the indicated times by the hot-phenol extraction method, as described previously (33). Northern blot analysis was performed by fractionation of the purified RNA samples (5 μg) on 1.2% agarose-formaldehyde gels and transfer onto nitrocellulose membranes

(Amersham XL) by capillary transfer by using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Membranes were cross-linked using a cross-linking device (Stratalinker; Stratagene) and prehybridized for 3 h at 42°C in a buffer containing 5× Denhardt solution (34), 5× SSC, 0.2% SDS, 50% formamide, and 250 mg of sheared salmon sperm DNA per ml. Subsequently, a radiolabeled *csrB*-specific DNA probe, denatured at 90°C for 5 min, was added to the prehybridization buffer, and the membranes were incubated at 42°C overnight. The *csrB*-specific probe was obtained by digestion of plasmid pMX545 with EcoRI, separation of the fragments on agarose gels, and purification of the *csrB*-specific band by using the Qiagen agarose purification kit. Probe labeling was performed by using [α-^{32}P]dCTP and the Radprime kit (Invitrogen), according to the manufacturer's instructions. Membranes were washed twice with 50 ml of 2× SSC and 0.1% SDS at 37°C and twice with 0.2× SSC and 0.1% SDS at 42°C. Images were obtained using phosphorimager screens and analyzed using the Typhoon image scanner (Amersham Biosciences).

β-Galactosidase activity. β-Galactosidase activity was assayed and expressed in Miller units as described previously (32). Cells were grown in LB broth, or LB with the pH adjusted and buffered to pH 5.0 with 0.1 M homopiperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (HOMOPIPES). When indicated, acetate or formate was used at a concentration of 7 mM.

Immunoblotting and generation of polyclonal anti-UvrY and anti-BarA. Cultures for Western blot analyses were grown aerobically at 37°C and harvested by centrifugation during mid-exponential growth. The cell pellet was resuspended in 100 μl lysis buffer (50 mM Tris-HCl, 4% SDS, pH 6.8) and boiled for 5 min. Aliquots of 10 μl were separated by SDS-PAGE (15% polyacrylamide gels for UvrY and 8% polyacrylamide gels for BarA), and the proteins were transferred to a Hybond-ECL filter (Amersham Biosciences). The filter was equilibrated in TTBS buffer (25 mM Tris, 150 mM NaCl, and 0.05% Tween 20) for 10 min and incubated in blocking buffer (1% milk in TTBS) for 1 h at room temperature. Polyclonal antibodies against UvrY and BarA, raised by subcutaneous immunization of rabbits with His_6-UvrY and His_6-BarA, were added at dilutions of 1:2,000 and 1:10,000, respectively, to the filter and incubated for 1 h at room temperature. The bound antibody was detected by using anti-rabbit IgG antibody conjugated to horseradish peroxidase and the ECL detection system (Amersham Biosciences).

RESULTS AND DISCUSSION

CsrA is required for activation of *csrB* transcription. It has been previously reported that acetate and formate, as well as short-chain fatty acids, act as stimuli for the sensor kinase BarA (4), leading to its autophosphorylation and transphosphorylation of the cognate response regulator UvrY (1). Phosphorylated UvrY (UvrY-P), in turn, activates transcription of the CsrB and CsrC small untranslated RNAs, which act by sequestering the CsrA global regulatory protein and antagonizing its regulatory activity (25, 35). Curiously, UvrY-dependent activation of *csrB* transcription, which takes place at the transition from exponential to stationary growth phase (25), was found not to occur in a *csrA* mutant as judged by Northern blotting and by *csrB-lacZ* (located at *att*λ) reporter expression (Fig. 1A and B), in agreement with a previous report (25). Moreover, it was found that *csrB* expression in the *csrA* mutant was fully restored by ectopic expression of CsrA using plasmid pMX544 (Fig. 1A and B). Because CsrA does not affect *csrB* stability (26) and because *csrB* transcription is activated directly by UvrY-P, it can be inferred that CsrA affects the activity and/or the expression of the components of the BarA/UvrY signaling system.

Acetate and formate are unable to activate BarA in a *csrA* mutant. Considering that a role of CsrA, an RNA binding protein, in the control of the BarA/UvrY phosphorelay cascade as not very probable, we hypothesized that CsrA might be required either for

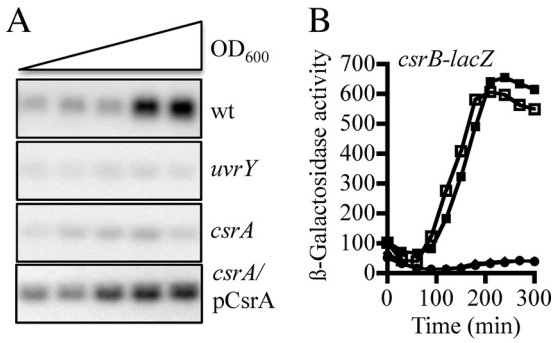

FIG 1 Effects of *csrA* and *uvrY* on *csrB* transcription. (A) Northern blot analysis of CsrB levels in the isogenic strains KSB837 (wild type [wt]), UYKSB837 (*uvrY*), IFC5010 (*csrA*), and IFC5010 carrying the *csrA*-expressing plasmid pMX544 (indicated as pCsrA). Cultures of these strains were grown in LB medium, and total RNA isolated from samples that were harvested throughout the growth curve (optical density at 600 nm [OD_{600}] of 0.3 to 2.0) was probed for the CsrB transcript. Experiments were repeated three times in their entirety with essentially identical results. (B) Overnight cultures of the wild-type strain (squares) and its isogenic *uvrY* (circles) and *csrA* (diamonds) mutant strains and the *csrA* mutant strain carrying the *csrA* expressing plasmid pMX544 (closed squares), all carrying the *csrB-lacZ* transcriptional fusion, were diluted to an OD_{600} of ~0.05 in LB medium, and the β-galactosidase activity was followed for 300 min. Note that the circles and diamonds extensively overlap. The average from four independent experiments is presented (standard deviations were less than 5% from the mean).

the production of the BarA-specific stimulus and thereby activation of the BarA/UvrY signaling cascade or for the expression of the *barA* and/or *uvrY* genes. To test the first possibility, we took advantage of the fact that although the BarA/UvrY TCS remains inactive when cells are grown at pH 5.0 (36), addition of acetate or formate to the growth medium results in the immediate activation of BarA/UvrY and thereby activation of *csrB* transcription (4). Therefore, strains KSB837 (wild type), IFC5010 (the isogenic *csrA* mutant), and IFC5010 carrying the *csrA*-expressing plasmid pMX544 were grown in LB buffered at pH 5.0 in the absence or presence of acetate and formate, and the expression of *csrB* was monitored by Northern blotting and by using a *csrB-lacZ* transcriptional fusion as a reporter. As expected, no activation of *csrB* transcription was observed in either of the tested strains at pH 5.0 (Fig. 2A and B). However, addition of acetate or formate to the growth medium resulted in the immediate activation of *csrB* transcription in the wild-type strain but not in the *csrA* mutant strain (Fig. 2A and B). Finally, ectopic expression of CsrA, using plasmid pMX544, in the *csrA* mutant strain restored *csrB* transcription almost to wild-type levels (Fig. 2A and B). It can therefore be concluded that a possible insufficiency of the BarA specific stimulus is not the cause of the above-described phenotype. This was further supported by the finding that the same increase in the concentration of extracellular acetate was attained by both the wild-type and the *csrA* mutant strains when grown at pH 7.0 and that no increase of extracellular acetate was observed in any of the two strains at pH 5.0 (Fig. 2C), in agreement with previously reported results (4, 30). Therefore, we concluded that the requirement for CsrA in the BarA/UvrY signaling cascade does not involve the synthesis of acetate, which acts as a physiological stimulus for BarA.

CsrA is required for proper *uvrY* expression. Next, we tested the possibility that CsrA affects the expression of *barA* and/or

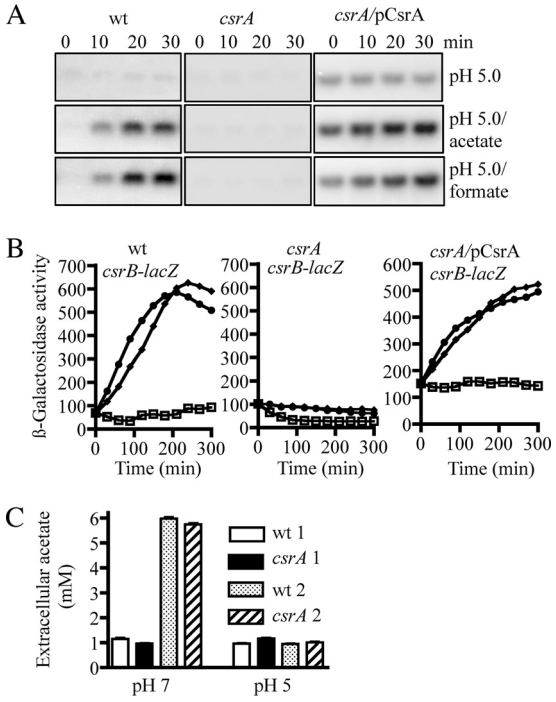

FIG 2 The synthesis of the BarA stimulus does not appear to be affected in the *csrA* mutant strain. (A) The isogenic strains KSB837 (wild type), IFC5010 (*csrA*), and IFC5010 carrying the *csrA*-expressing plasmid pMX544 (indicated as pCsrA) were grown in LB medium, the pH of which had been adjusted and buffered to 5.0 using 0.1 M homopiperazine-*N*,*N*′-bis-2-(ethanesulfonic acid) (HOMOPIPES). At an OD_{600} of 0.2, a sample was withdrawn, 7 mM acetate or formate was added to the medium, and samples were withdrawn every 10 min. Total RNA isolated from these samples was analyzed by Northern blotting using a CsrB-specific probe. The experiment was repeated three times in its entirety with essentially identical results. (B) Cultures of the isogenic strains KSB837 (wild type), IFC5010 (*csrA*), and IFC5010 harboring plasmid pMX544 (indicated as pCsrA), all carrying the *csrB-lacZ* transcriptional fusion, were diluted to an OD_{600} of ~0.05 in LB medium at pH 5.0 as described for Fig. 1B alone (squares) or in the presence of acetate (circles) or formate (diamonds), and the β-galactosidase activity was followed. The average from four independent experiments is presented (standard deviations were less than 5% from the mean). (C) Concentration of extracellular acetate. The KSB837 wild-type strain and IFC5010, its isogenic *csrA* mutant strain, were grown in LB at pH 7.0 or 5.0. Samples were withdrawn either at early exponential growth phase (designated 1) or at late exponential phase (designated 2), and the concentration of acetate was determined with the R-Biopharm acetic acid determination kit (Boehringer Mannheim). The averages from three independent experiments are presented and the standard deviations are indicated.

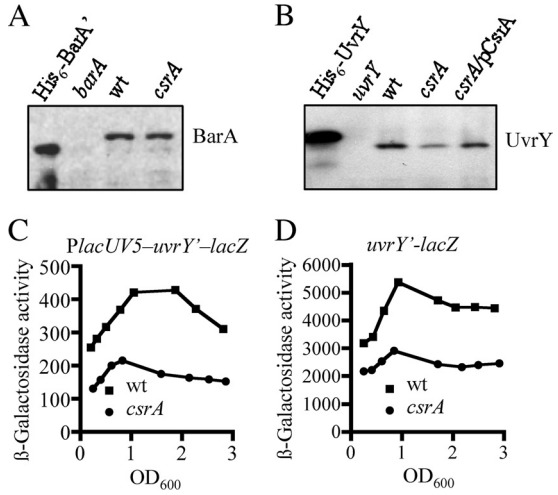

FIG 3 CsrA is required for proper *uvrY* expression. (A) Levels of BarA protein (102.550 Da) in the KSB837 (wild-type) and IFC5010 (*csrA*) strains as determined by Western blot analyses using BarA polyclonal antibodies. (B) Levels of UvrY protein (23.890 Da) in the KSB837 (wild-type) and IFC5010 (*csrA*) strains and in IFC5010 complemented with the *csrA*-expressing plasmid pMX544 (indicated as pCsrA), as determined by Western blot analyses using UvrY polyclonal antibodies. Purified His_6-tagged Bar′ (81.550 Da), a BarA version lacking the 198 first amino acid residues, and His_6-tagged UvrY (25.380 Da) (25), and extracts from *barA* (BAKSB837) and *uvrY* (UYKSB837) mutant cells were used in the first lanes of each Western blot. The molecular mass differences between purified and wild-type BarA and UvrY proteins are due to the His_6 tag and the absence of the first 198 amino acids in BarA′. (C and D) Effects of *csrA* on *uvrY* transcription and translation. Wild-type (wt) and *csrA* mutant cells carrying either a chromosomal P*lacUV5-uvrY′-′lacZ* translational fusion (strains IFC5013 and IFC5014, respectively) (C) or a chromosomal *uvrY′-′lacZ* operon fusion (strains IFC5011 and IFC5012, respectively) (D) were harvested at various times throughout growth and assayed for β-galactosidase activity. The β-galactosidase activity is presented as a function of growth density (OD_{600}). The averages from four independent experiments are presented (standard deviations were less than 5% from the mean).

uvrY. To this end, the amounts of BarA and UvrY proteins in the wild-type and *csrA* mutant strains were compared by Western blotting, using specific BarA and UvrY polyclonal antibodies. The two strains expressed similar amounts of BarA (Fig. 3A). On the other hand, the amount of UvrY protein was significantly lower in the *csrA* mutant than in the wild-type strain (Fig. 3B). Moreover, wild-type levels of UvrY were expressed in the *csrA* mutant complemented with the *csrA*-expressing plasmid pMX544 (Fig. 3B), indicating that CsrA affects, directly or indirectly, *uvrY* expression.

Subsequently, we asked whether CsrA affects *uvrY* expression at the translational level and/or at the transcriptional level, e.g., by modulating the expression of a transcriptional regulator. To this end, the β-galactosidase activity from a chromosomal *lacUV5-uvrY′–lacZ* translational reporter fusion, in which the constitutive *lacUV5* promoter replaced the native promoter, was monitored in the wild-type and *csrA* mutant strains. In this case, β-galactosidase activity in the *csrA* mutant was found to be approximately 50% of that in the wild-type strain (Fig. 3C). As mentioned above, CsrA regulates translation of its target mRNAs by interacting with their 5′ untranslated regions (9–11). However, analyses *in silico* did not reveal any apparent CsrA binding sites in the 5′-UTR of the *uvrY* transcript, which is suggestive of an indirect effect of CsrA on *uvrY* expression. The effect of CsrA on *uvrY* transcription was tested by using a chromosomal *uvrY′-lacZ* transcriptional fusion. It was observed that the β-galactosidase activity in the wild-type strain was almost 2-fold higher than the in the *csrA* mutant strain (Fig. 3D), suggesting that CsrA indirectly affects the transcription of *uvrY*. It should be noted that the *csrA* mutation, an insertion of a kanamycin cassette 10 amino acid residues before the stop codon of CsrA (21, 37), decreases but does not entirely eliminate CsrA activity (38). Therefore, the actual effect of CsrA on *uvrY* transcription and translation may be significantly greater than observed. Nonetheless, the above results suggest that CsrA is required for proper *uvrY* expression, affecting both transcription and translation of *uvrY*.

Overexpression of UvrY alone but not concurrently with BarA restores *csrB* transcription in a *csrA* mutant. We then asked whether ectopic expression of *uvrY* and/or *barA* from plasmids

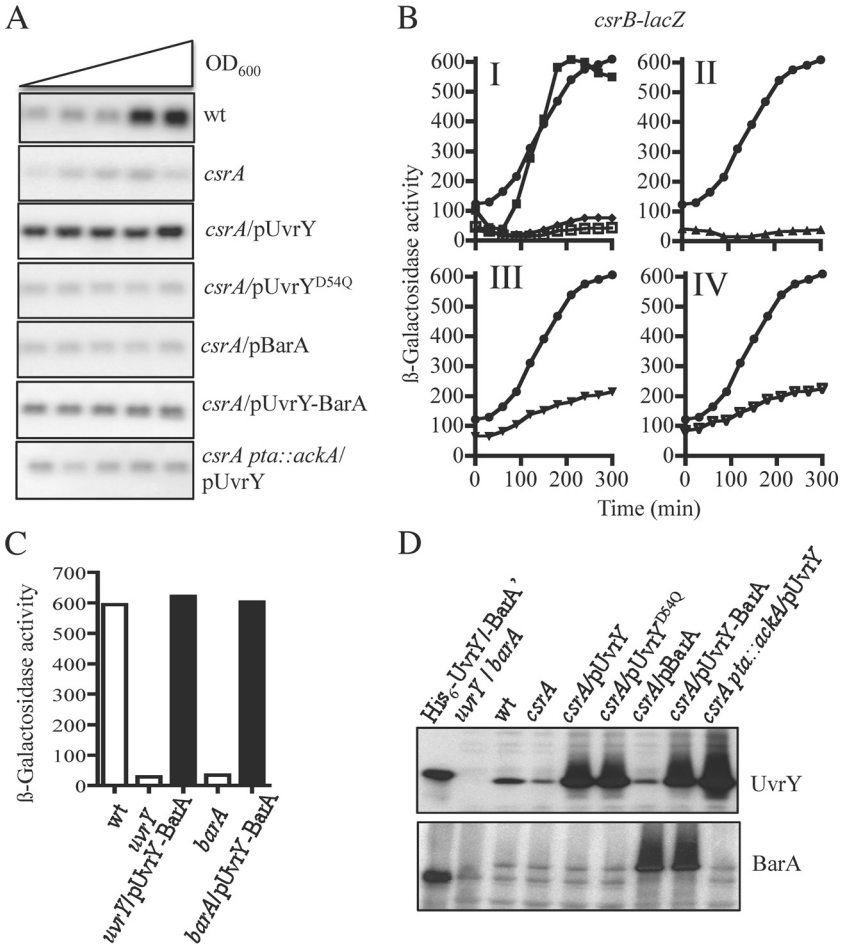

FIG 4 Acetyl-P, but not BarA, is responsible for UvrY phosphorylation in a *csrA* mutant. Effects of ectopic expression of *uvrY*, *uvrY*^{D54Q}, *barA*, and *uvrY barA* on *csrB* expression in isogenic *csrA* and *csrA pta*::*ackA* mutant strains are shown. (A) Cultures of the KSB837 wild-type strain and IFC5010, its isogenic *csrA* mutant strain, harboring or not plasmid pMX539 (expressing *uvrY* and indicated as pUvrY), pMX540 (expressing *uvrY*^{D54Q}, having Q substituted for the conserved phosphorylatable D, and indicated as pUvrY^{D54Q}), pBA29 (expressing *barA* and indicated as pBarA), or pMX541 (expressing both *uvrY* and *barA* and indicated as pUvrY-BarA), and IFC5015, the isogenic *csrA pta*::*ackA* triple mutant strain, harboring pMX539 (indicated as pUvrY) were grown in LB medium. Total RNA isolated from samples that were harvested throughout the growth curve (OD_{600} of 0.3 to 2.0) was probed for the CsrB transcript. (B) Overnight cultures of the *csrB-lacZ* transcriptional fusion-carrying wild type (panel I, closed squares), the *csrA* mutant strain (panel I, open squares), and the same mutant strain harboring plasmid pMX539 (expressing *uvrY*) (panels I to IV, circles), pBA29 (expressing *barA*) (panel I, diamonds), pMX540 (expressing *uvrY*^{D54Q}) (panel II, triangles), pMX541 (expressing both *uvrY* and *barA*) (panel III, triangles), and the isogenic *csrA pta*::*ackA* triple mutant strain harboring pMX539 (expressing *uvrY*) (panel IV, triangles) were diluted to an OD_{600} of ~0.05 in LB medium, and the β-galactosidase activity was followed for 300 min. The average from four independent experiments is presented (standard deviations were less than 5% from the mean). (C) Ectopic expression of *uvrY* and *barA* by pMX541 (indicated as pUvrY-BarA) restores *csrB* expression in *uvrY* and *barA* mutant strains. Cultures of the *csrB-lacZ* transcriptional fusion-carrying wild type (KSB837) and the isogenic *uvrY* (UYKSB837) and *barA* (BAKSB837) mutant strains both carrying or not the *uvrY*- and *barA*-expressing plasmid pMX541 were grown to an OD_{600} of ~2.0 in LB medium, and the β-galactosidase activity was assayed. The average from two independent experiments is presented. (D) Levels of UvrY (upper panel) and BarA (lower panel) proteins in KSB837 (wild type), IFC5010 (*csrA*), and IFC5010 harboring either of the following plasmids: pMX539 (indicated as pUvrY), pMX540 (indicated as pUvrY^{D54Q}), pBA29 (indicated as pBarA), pMX541 (indicated as pUvrY-BarA), and *csrA pta*::*ackA* (IFC5015) harboring pMX539 (indicated as pUvrY) as determined by Western blot analyses. Purified His_6-tagged BarA′ and UvrY proteins and extracts from *barA* (BAKSB837) and *uvrY* (UYKSB837) mutant cells were used in the first lanes of each Western blot.

pMX539 and pBA29 (25) restores *csrB* expression in the *csrA* mutant strain. It was found that expression of *uvrY* but not *barA* did restore *csrB* expression (Fig. 4A and B), in agreement with a previous report (25). Notably, the presence of the *uvrY*-expressing plasmid in the *csrA* mutant strain resulted in elevated amounts of CsrB RNA even at the early exponential phase of growth (Fig. 4A), an effect that could be attributed to the vast overexpression of UvrY (Fig. 4D). Moreover, overexpression of an UvrY^{D54Q} mutant protein, in which the phosphorylatable aspartate residue was replaced with a glutamine residue, using plasmid pMX540 was not able to restore *csrB* transcription, indicating that phosphorylation of UvrY is required for its activation as a transcriptional regulator (Fig. 4A and B). In this respect, it is relevant to mention that phosphorylation of UvrY has been shown to occur either by BarA-P or directly by acetyl-P. Unexpectedly, it was found that, in contrast to the UvrY-expressing plasmid pMX539, plasmid pMX541, which expresses both BarA and UvrY, failed to activate *csrB* transcription in the *csrA* mutant strain (Fig. 4A and B). To ensure that UvrY and BarA were readily expressed by plasmid pMX541, we examined whether this plasmid was able to complement an *uvrY* mutant and a *barA*

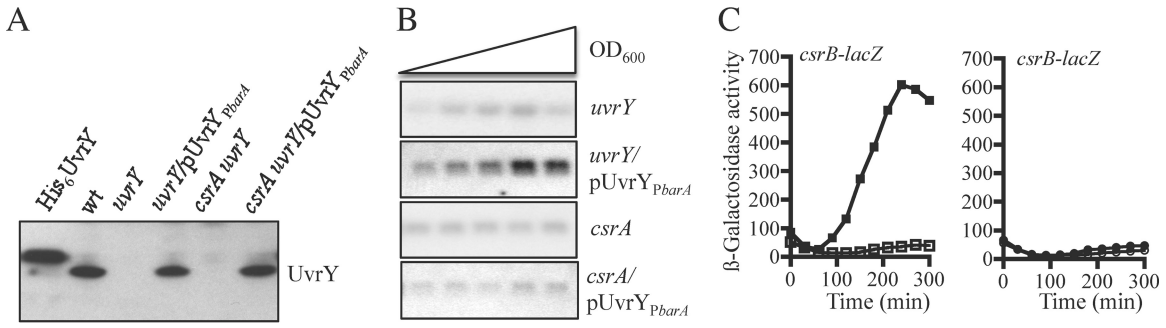

FIG 5 Release of *uvrY* expression from *csrA* control is not sufficient for activation of *csrB* transcription in a *csrA* mutant. (A) Levels of UvrY protein in KSB837 (wild type), UYKSB837 (*uvrY*), UYKSB837 carrying plasmid pMX543 (having *uvrY* under control of the *barA* promoter and 5′-UTR, and indicated as $pUvrY_{PbarA}$), IFC5016 (*uvrY csrA*), and IFC5016 carrying plasmid pMX543 (indicated as $pUvrY_{PbarA}$) as determined by Western blot analyses. Purified His_6-tagged UvrY protein was used in the first lane of the Western blot. (B) Northern blot analysis of CsrB levels in UYKSB837 (*uvrY*), UYKSB837 harboring pMX543 (indicated as $pUvrY_{PbarA}$), IFC5010 (*csrA*), and IFC5016 harboring pMX543 (indicated as $pUvrY_{PbarA}$). Cultures of these above strains were grown in LB medium, and RNA isolated from samples that were harvested throughout the growth curve (OD_{600} of 0.3 to 2.0) was probed for the CsrB transcript. (C) Overnight cultures of UYKSB837 (*uvrY*) (open squares), UYKSB837 harboring pMX543 (closed squares), IFC5010 (*csrA*) (open circles), and IFC5010 harboring pMX543 (closed circles), all carrying the *csrB-lacZ* transcriptional fusion, were diluted to an OD_{600} of ~0.05 in LB medium, and the β-galactosidase activity was followed for 300 min. The average from four independent experiments is presented (standard deviations were less than 5% from the mean).

mutant for *csrB* expression. It was found that *csrB* expression was restored in both mutant strains (Fig. 4C), indicating that pMX541 expresses functional BarA and UvrY proteins. Moreover, Western blot analyses, using specific UvrY and BarA polyclonal antibodies, revealed that similar amounts of UvrY protein were expressed by pMX539, pMX540, and pMX541 and that similar amounts of BarA were expressed by pBA29 and pMX541 (Fig. 4D). Thus, expression and functionality of the pMX541 expressed BarA and UvrY does not provide an explanation for the above result. The above results, in combination with the fact that BarA, like other tripartite two-component sensors, has been shown to be capable of having both a kinase and a phosphatase activity on it is cognate regulator (39–41), prompted us to speculate that BarA may remain locked in its phosphatase state and fail to be activated as a kinase in the *csrA* mutant. In such a scenario, phosphorylation of the pMX539-expressed UvrY in the *csrA* mutant strain should rely on acetyl-P. Moreover, the vast overexpression of UvrY (Fig. 4D) should overwhelm the phosphatase activity of the chromosomally expressed BarA, permitting the accumulation of significant amounts of UvrY-P and culminating in activation of *csrB* transcription. On the other hand, when comparable amounts of BarA and UvrY proteins are expressed, i.e., when pMX541 was used, the phosphatase activity of BarA should dephosphorylate the acetyl-P-dependent UvrY-P and thereby cancel its transcriptional regulation.

CsrA is required for activation of the BarA kinase activity. The above hypothesis, that UvrY is autophosphorylated at the expense of acetyl-P and is not transphosphorylated by BarA in the *csrA* mutant, was then tested. To this end, a *pta*::*ackA* mutation was inserted into the *csrA* mutant strain in order to block the synthesis of acetyl-P (42), and the effect of the UvrY-overexpressing plasmid pMX539 on *csrB* expression was probed. It was found that although similar amounts of UvrY were expressed in the *csrA pta*::*ackA* triple mutant strain and in the *csrA* mutant (Fig. 4D), no activation of *csrB* transcription occurred, as judged by Northern blotting and by the *csrB-lacZ* reporter fusion (Fig. 4A and B). This suggests that in the *csrA* mutant strain, UvrY is phosphorylated exclusively at the expense of acetyl-P rather than being transphosphorylated by BarA.

Subsequently, we explored the intriguing possibility that BarA remains inactive as a kinase in the *csrA* mutant. We argued that if the activity of BarA is not affected by CsrA, then reestablishing the levels of UvrY protein in the *csrA* mutant should restore *csrB* transcription. Therefore, we constructed a *uvrY* gene carrying low-copy-number plasmid (pMX543), where the promoter and 5′-UTR sections of *uvrY* were replaced with the ones of *barA* (Fig. 5A), the expression of which was not affected by CsrA (Fig. 3A). This plasmid was transformed into UVKSB837, a *uvrY* mutant, or IFC5016, a *csrA uvrY* double mutant strain, and the amount of UvrY was examined by Western blotting (Fig. 5A). It was found that similar amounts of UvrY protein were expressed from the plasmid in both of these strains. Subsequently, we tested whether pMX543 was able to complement *csrB* expression in these mutant strains. Interestingly, pMX543 restored *csrB* expression in the *uvrY* mutant but not in the *csrA uvrY* mutant (Fig. 5B and C). A possible explanation for this finding is that CsrA is also required for the kinase activity of BarA.

To provide further support to the above conclusions, we argued that ectopic expression of UvrY, but not that of the mutant $UvrY^{D54Q}$, which is unable to be phosphorylated, in a *csrA* mutant should restore *csrB* expression when the cells are grown at pH 5.0 in the presence of acetate, which results in the production of elevated amounts of acetyl-P (43). On the other hand, the addition of formate, which acts exclusively via BarA, should be without effect. Accordingly, overexpression of UvrY in the *csrA pta*::*ackA* triple mutant, which is not able to convert acetate to acetyl-P, should not restore *csrB* expression when cells are grown at pH 5.0 in the presence of either acetate or formate. Indeed, *csrB* transcription in the *csrA* mutant strain grown at pH 5.0 was restored by the ectopic expression of UvrY in the presence of only acetate but not formate, whereas overexpression of $UvrY^{D54Q}$ was without effect (Fig. 6A and B). Also, no activation of *csrB* transcription was observed in the *csrA pta*::*ackA* mutant strain transformed with pMX539 when the cells were grown at pH 5.0 in the presence of either acetate or formate (Fig. 6A and B). We therefore concluded that when UvrY is overexpressed in the *csrA* mutant, acetyl-P-dependent phosphorylation of UvrY is responsible for the activation of *csrB* transcription.

Finally, we reasoned that if CsrA was required for switching

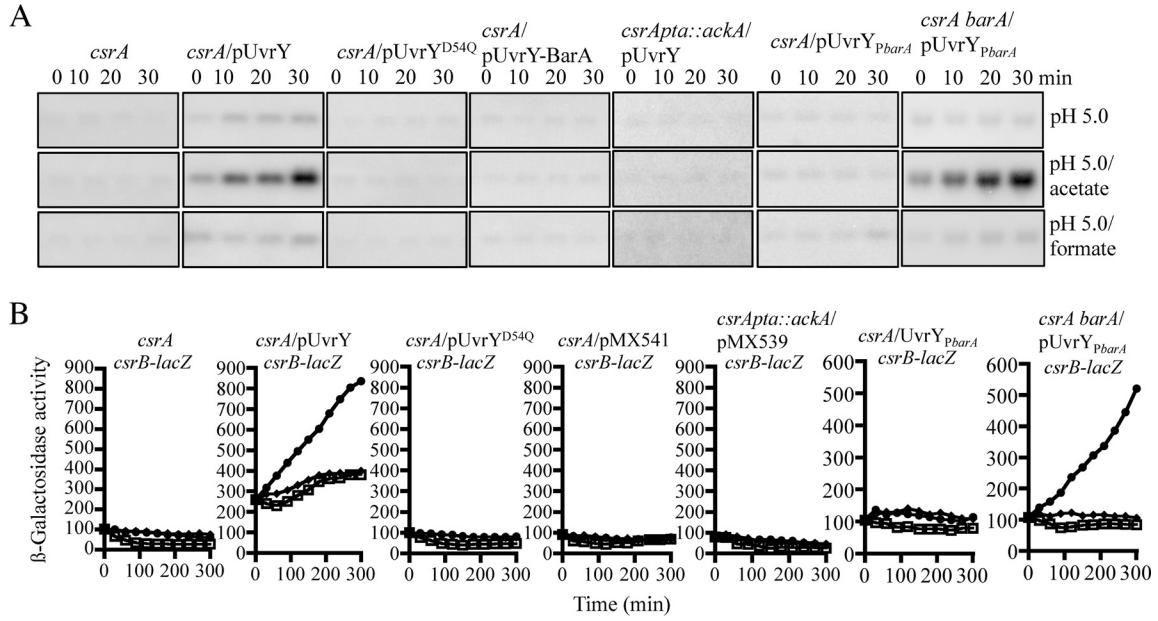

FIG 6 Effect of *csrA* on the activity of BarA. (A) Cultures of IFC5010 (*csrA*) harboring or not plasmid pMX539 (expressing *uvrY* and indicated as pUvrY), pMX540 (expressing $uvrY^{D54Q}$ and indicated as $pUvrY^{D54Q}$), pMX541 (expressing both *uvrY* and *barA* and indicated as pUvrY-BarA), or pMX543 (having *uvrY* under control of the *barA* promoter and 5′-UTR and indicated as $pUvrY_{PbarA}$), IFC5017 (*csrA barA*) harboring pMX543 (indicated as $pUvrY_{PbarA}$), and IFC5015 (*csrA pta*::*ackA*) harboring pMX539 (indicated as pUvrY) were grown in LB medium the pH of which had been adjusted and buffered to 5.0, using 0.1 M homopiperazine-*N*,*N*′-bis-2-(ethanesulfonic acid) (HOMOPIPES). At an OD_{600} of 0.2, a sample was withdrawn, 7 mM acetate or formate was added to the medium, and samples were withdrawn every 10 min. Total RNA isolated from these samples was analyzed by Northern blotting using a CsrB-specific probe. The experiment was repeated three times in its entirety with essentially identical results. (B) Overnight cultures of the *csrB-lacZ* transcriptional fusion carrying IFC5010 (*csrA*), IFC5010 harboring plasmid pMX539 (indicated as pUvrY), pMX540 (indicated as $pUvrY^{D54Q}$), pMX541 (indicated as pUvrY-BarA), or pMX543 (indicated as $pUvrY_{PbarA}$), IFC5017 (*csrA barA*) harboring pMX543 (indicated as $pUvrY_{PbarA}$), and IFC5015 (*csrA pta*::*ackA*) harboring pMX539 (indicated as pUvrY) were diluted to an OD_{600} of ~0.05 in LB medium at pH 5.0 as described for Fig. 1B alone (squares) or in the presence of acetate (circles) and formate (diamonds). The average from four independent experiments is presented (standard deviations were less than 5% from the mean).

BarA from its phosphatase to its kinase activity, the simultaneous overexpression of UvrY and BarA in the *csrA* mutant, using plasmid pMX541, should not restore *csrB* expression when cells are grown at pH 5.0 in the presence of acetate or formate. The same result should be expected when wild-type levels of UvrY are reestablished in the *csrA* mutant by plasmid pMX543. This is because BarA should remain locked on as a phosphatase, dephosphorylating the acetyl-P-dependent UvrY-P and thereby cancelling its regulatory effect. In fact, no activation of *csrB* transcription was detected in the pMX541 or pMX543 carrying *csrA* mutant strain (Fig. 6A and B). On the other hand, reestablishing the wild-type levels of UvrY by plasmid pMX543 in a *csrA barA* double mutant, where no UvrY-P-dephosphorylating activity is present, should restore *csrB* expression in the presence of acetate but not formate. Indeed, *csrB* transcription in the pMX43-carrying *csrA barA* double mutant grown at pH 5.0 was restored to wild-type levels in the presence of acetate but not formate (Fig. 6A and B). Taken together, these results indicate that in the *csrA* mutant, BarA fails to be activated as a kinase but functions as a phosphatase even in the presence of its stimulus. It thus appears that one or more genes, whose expression is regulated by CsrA, may be needed for proper activation of BarA. Thus far, we have been unsuccessful in screening a plasmid-based genomic library for genes that would complement *csrB* expression in the *csrA* mutant.

Conclusions. In this study, we investigated the effect of the CsrA global regulator on the expression and activity of the components of the BarA/UvrY signaling system. This was motivated by the earlier observation that *csrB* expression, which relies on the BarA-to-UvrY phosphorelay, did not take place in a *csrA* mutant (25). Our results demonstrate that the CsrA protein is required for the proper expression of the UvrY response regulator and also for the adequate switch from the phosphatase to the kinase activity of the BarA sensor kinase (Fig. 7), providing an explanation for the above observation.

We provided evidence that CsrA positively affects *uvrY* expression at both the transcriptional and posttranscriptional levels. The effect of CsrA, an RNA binding protein, on *uvrY* transcription plausibly may be mediated via the regulation of expression of a transcriptional factor. Previously, SdiA and Crp have been reported to activate, respectively, *uvrY* transcription in *E. coli* and *csrB* transcription in *Yersinia pseudotuberculosis* (44, 45). However, CsrA modestly represses *sdiA* translation in *E. coli* (46), and Crp does not activate *csrB* expression (A. Pannuri and T. Romeo, unpublished data). Thus, these two regulators cannot account for the positive effects of CsrA on *uvrY* transcription. Another candidate is LexA, which coordinates the SOS response (47, 48), because a LexA binding site is predicted to be located between nt −120 and −139 upstream of the *uvrY* start site (49). CsrA-dependent modulation of *uvrY* translation might also be indirect, because no apparent CsrA binding sequences are present in the 5′-UTR of the *uvrY* transcript. In fact, the RNA DEAD box helicase DeaD was recently shown to be required for *uvrY* translation (50), although no link between CsrA and *deaD* expression is known at this time. Therefore, it is of great importance to clarify how these

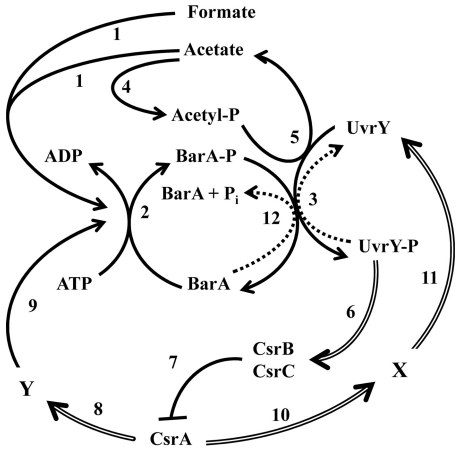

FIG 7 Model for the regulatory circuitry of the BarA/UvrY TCS and Csr system. Under stimulatory conditions, acetate and formate act as physiological signals that activate BarA (1), leading to its autophosphorylation at the expense of ATP (2) and transphosphorylation of UvrY (3). UvrY can also autophosphorylate at the expense of acetyl-P (5), which is produced from acetate (4). Phosphorylated UvrY (UvrY-P) activates expression of the noncoding CsrB and CsrC RNAs (6), which bind and sequester the CsrA protein (7) and thereby prevent its regulatory interaction with the mRNA targets. On the other hand, free CsrA regulates the expression of factor Y (8), which is required for properly switching BarA from its phosphatase to its kinase activity (9). At the same time, CsrA positively affects *uvrY* expression (11) by controlling the expression of the regulator(s) (X) (10). Finally, under nonstimulatory growth conditions or in a *csrA* mutant strain, BarA acts as a UvrY-P phosphatase (12), enabling the silencing of the system. Reactions under stimulatory and nonstimulatory conditions are indicated with solid and dotted lines, respectively. Double lines indicate effects on gene expression.

proteins are integrated into the Csr/UvrY circuitry, which allows for a global response through the CsrA protein.

Finally, we provide evidence that CsrA plays a significant role in the mechanism that enables BarA to switch from its phosphatase activity to its kinase activity. It is therefore tempting to speculate that, in addition to the BarA stimulus, a protein whose expression is regulated by CsrA is needed for proper activation of BarA. In this respect, it is relevant to mention that proper regulation of the kinase activity of GacS, the BarA homolog in *Pseudomonas aeruginosa*, requires the presence of the hybrid sensor kinases RetS and LadS (51–53). However, no homologs of these proteins exist in *E. coli*. Hence, identification of the protein(s) or other factors involved in the regulation of BarA signaling would greatly enhance our understanding of the Csr/BarA-UvrY regulatory network.

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