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The Crp-Activated Small Noncoding Regulatory RNA CyaR (RyeE) Links Nutritional Status to Group Behavior▽†

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Small noncoding regulatory RNAs (sRNAs) play a key role in regulating the expression of many genes in *Escherichia coli* and other bacteria. Many of the sRNAs identified in *E. coli* bind to mRNAs in an Hfq-dependent manner and stimulate or inhibit translation of the mRNAs. Several sRNAs are regulated by well-studied global regulators. Here, we report characterization of the CyaR (RyeE) sRNA, which was previously identified in a global search for sRNAs in *E. coli*. We demonstrated that CyaR is positively regulated by the global regulator Crp under conditions in which cyclic AMP levels are high. We showed by using microarray analysis and Northern blotting that several genes are negatively regulated by CyaR, including *ompX*, encoding a major outer membrane protein; *luxS*, encoding the autoinducer-2 synthase; *nadE*, encoding an essential NAD synthetase; and *yqaE*, encoding a predicted membrane protein with an unknown function. Using translational *lacZ* fusions to *yqaE*, *ompX*, *nadE*, and *luxS*, we demonstrated that the negative regulation of these genes by CyaR occurs at the posttranscriptional level and is direct. Different portions of a highly conserved 3′ region of CyaR are predicted to pair with sequences near the ribosome binding site of each of these targets; mutations in this sequence affected regulation, and compensatory mutations in the target mRNA restored regulation, confirming that there is direct regulation by the sRNA. These results provide insight into the mechanisms by which Crp negatively regulates genes such as *luxS* and *ompX* and provide a link between catabolite repression, quorum sensing, and nitrogen assimilation in *E. coli*.

Noncoding regulatory RNAs posttranscriptionally regulate gene expression in bacteria, archaea, and eukaryotes. The posttranscriptional regulation of gene expression by noncoding regulatory RNAs called small noncoding regulatory RNAs (sRNAs) in *Escherichia coli* is a model system for understanding this mechanism of regulating gene expression in bacteria. In *E. coli*, ~100 sRNAs have been identified (10, 66, 71). Many of the genes encoding sRNAs were identified because they were located in a conserved intergenic region and flanked by a recognizable promoter and/or Rho-independent terminator (2, 66). These sRNAs are typically ~100 bp long, and many of them have been shown to bind to the RNA chaperone Hfq (71).

Hfq is a member of the Sm-like family of RNA binding proteins and was first identified by Kajitani and Ishihama as the factor required for replication of the Q_β phage (29). Later, Tsui et al. found that an insertion mutation in *hfq* caused diverse pleiotropic phenotypes (61). Hfq has subsequently been shown to catalyze the binding of sRNAs to a complementary site in the targeted mRNAs (for a review, see reference 63).

Binding of an sRNA to a targeted mRNA frequently results in inhibition of translation and coupled degradation of the mRNA and the sRNA. One of the best-characterized examples of this mechanism of gene regulation involves the regulation of the *sodB* mRNA by the RyhB sRNA; Massé et al. have shown that expression of RyhB sRNA leads to coupled degradation of RyhB and the mRNA encoding *sodB* by RNase E (37). In other cases, the binding of an sRNA to a targeted mRNA leads to stabilization of the mRNA and stimulation of translation. The best-characterized example of this outcome is the regulation of the *rpoS* mRNA by the sRNA DsrA; DsrA pairing disrupts secondary structure in the *rpoS* mRNA that would otherwise block ribosome binding (for a review, see reference 35).

Many of the sRNAs that have been identified have been shown to be regulated by a specific global regulator. RyhB is regulated by the Fur repressor (38), RprA is regulated by the RcsC/RcsD/RcsB phosphorelay (34), OmrA and OmrB are regulated by the EnvZ/OmpR two-component system (21), and MicA and RybB are regulated by σ^E (16, 27, 50, 59). Thus, many different classes of transcriptional regulators regulate sRNAs, and it may be that most global regulators include at least one sRNA as part of the regulon. Here, we report that expression of the CyaR sRNA (previously designated RyeE) is regulated by the global regulator Crp and that activation of *cyaR* expression by Crp occurs under conditions in which cyclic AMP (cAMP) levels are known to be high. Two other groups of workers have recently reported regulation of CyaR by Crp (26, 49) in agreement with our findings. Moreover, we show here that the CyaR sRNA affects the expression of a number of genes; some of these genes, including *ompX* and *luxS*, were previously shown to be negatively regulated by Crp. We also demonstrate that the negative regulation of *luxS* by CyaR leads to a decrease in autoinducer-2 production by *E. coli*. This sRNA has been renamed CyaR to reflect its regulation.

MATERIALS AND METHODS

Bacterial strains and plasmids. All *E. coli* K-12 strains used in this study are derivatives of strain MG1655. All strains and plasmids used in this study are listed in Table 1. The primers and 5′-biotinylated probes used in this study

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant features	Reference or source
Strains		
BB170	*V. harveyi luxN*::Tn*5*	6
CV600	MG1655 Δ*lacX74* Δ*crp*::*cat*	C. Vanderpool
CV8000	MG1655 Δ*lacX74 imm*^{21} P_{cyaR}-*lacZ* (positions −76 to 64)	C. Vanderpool
DJ480	MG1655 Δ*lacX74*	D. Jin, NCI
DJ624	MG1655 Δ*lacX74 mal*::*lacI*^{q}	D. Jin, NCI
MG1655	Wild type	Lab strain collection
MG1225	MG1655 Δ*lacX74 mal*::*lacI*^{q} λRS P_{BAD}::*ompX*::*lacZ*	M. Guillier
NRD345	MG1655 Δ*cyaR*::*cat*	This study
NRD348	MG1655 Δ*lacX74 imm*^{21} P_{cyaR}-*lacZ* (positions −41 to 64)	This study
NRD351	MG1655 Δ*lacX74 imm*^{21} P_{cyaR}-*lacZ ilvD*::Tn*10* Δ*cyaA*	CV8000 + P1(SG2201)
NRD352	MG1655 Δ*lacX74 imm*^{21} P_{cyaR}-*lacZ* (positions −76 to 64) Δ*crp*::*cat*	CV8000 + P1(CV600)
NRD359	MG1655 Δ*lacX74 mal*::*lacI*^{q} Δ*cyaR*::*cat*	DJ624 + P1(NRD345)
NRD356	MG1655 Δ*crp*::*cat*	MG1655 + P1(CV600)
NRD377	MG1655 Δ*lacX74 mal*::*lacI*^{q} λRS P_{BAD}::*ompX*::*lacZ* Δ*cyaR*::*cat*	MG1225 + P1(NRD345)
NRD381	MG1655 Δ*luxS*::*cat*	This study
NRD389	MG1655 Δ*lsrACDBFG*::*kan*	This study
NRD390	MG1655 Δ*lacX74 mal*::*lacI*^{q} Δ*lsrACDBFG*::*kan*	DJ624 + P1(NRD389)
NRD392	MG1655 Δ*lacX74 mal*::*lacI*^{q} Δ*lsrACDBFG*::*kan* Δ*cyaR*::*cat*	NRD390 + P1(NRD345)
NRD399	*mal*::*lacI*^{q} Δ*araBAD lacI′*::P_{BAD}::*nadE′-lacZ*	This study
NRD400	*mal*::*lacI*^{q} Δ*araBAD lacI′*::P_{BAD}::*yqaE′-lacZ*	This study
NRD402	MG1655 Δ*lacX74 mal*::*lacI*^{q} Δ*lsrACDBFG*::*kan* Δ*luxS*::*cat*	NRD390 + P1(NRD381)
NRD406	*mal*::*lacI*^{q} Δ*araBAD lacI′*::P_{BAD}::*luxS*(T-7A)*′-lacZ*	This study
NRD407	*mal*::*lacI*^{q} Δ*araBAD lacI′*::P_{BAD}::*nadE*(T-3A C-2T A-1G)*′-lacZ*	This study
NRD408	*mal*::*lacI*^{q} Δ*araBAD lacI′*::P_{BAD}::*yqaE*(T7A C9G)*′-lacZ*	This study
NRD410	*mal*::*lacI*^{q} Δ*araBAD lacI′*::P_{BAD}::*nadE*(T-3A C-2T A-1G)*′-lacZ* Δ*ryeE*::*cat*	NRD407 + P1(NRD345)
NRD411	*mal*::*lacI*^{q} Δ*araBAD lacI′*::P_{BAD}::*yqaE*(T7A C9G)*′-lacZ* Δ*ryeE*::*cat*	NRD408 + P1(NRD345)
NRD413	*mal*::*lacI*^{q} Δ*araBAD lacI′*::P_{BAD}::*luxS′-lacZ*	This study
NRD415	*mal*::*lacI*^{q} Δ*araBAD lacI′*::P_{BAD}::*luxS*(T-7A)*′-lacZ* Δ*ryeE*::*cat*	NRD406 + P1(NRD345)
PM1205	*mal*::*lacI*^{q} Δ*araBAD lacI′*-P_{BAD}::*cat sacB*::*lacZ*	Pierre Mandin, NIH
SG2201	*ilvD*::Tn*10* Δ*cya*	Lab strain collection
Top10	ϕ80*lacZ*ΔM15 Δ*lacX74 recA1*	Invitrogen
Plasmids		
pBR-plac	Amp^r; *araBAD* promoter-based expression vector having a pBR322 origin	21
pNRD405	AatII-EcoRI *cyaR*-containing fragment cloned into the same sites in pBR-plac	This study
pNRD407	A44T site-directed mutation in *cyaR* in pNRD405	This study
pNRD408	T47A site-directed mutation in *cyaR* in pNRD405	This study
pNRD409	T50A site-directed mutation in *cyaR* in pNRD405	This study
pNRD410	G38C, G39A, and A40T site-directed mutations in *cyaR* in pNRD405	This study
pKD46	Amp^r; RepA101(Ts); λ *exo*, γ, and β expressed from an *araBAD* promoter	12

(Table 2) were supplied by Integrated DNA Technologies, Inc. Transduction was performed using phage P1*vir* as described by Miller (42). The Δ*cyaR* strain NRD345 and the Δ*luxS* strain NRD381 were generated by lambda Red recombinase-mediated gene replacement using the PCR product generated from template plasmid pKD3 and using the ryeEKO For and Rev primers and the luxSKO For and Rev primers, respectively. The *lsrACDBFG* deletion harbored by strain NRD389 was created by lambda Red recombinase-mediated gene replacement using the PCR product generated with the template plasmid pKD4 and the lsrACDBFGKO For and Rev primers.

lacZ translational fusions to *yqaE*, *nadE*, and *luxS* that were under control of the *araBAD* promoter were generated using a procedure developed by P. Mandin, NIH (P. Mandin and S. Gottesman, unpublished data). First, the transcription start sites of *yqaE*, *nadE*, and *luxS* were determined by 5′ rapid amplification of cDNA ends (5′ RACE) as described below. Primers pBAD-yqaE-lacZ For and pBAD-yqaE-lacZ Rev, primers pBAD-nadE-lacZ For and pBAD-nadE-lacZ rev, and primers pBAD-luxS4-lacZ and pBAD-DNA-RACE were then used to PCR amplify from cDNA or genomic DNA the sequence from the transcription start site to the first few codons of *yqaE*, *nadE*, and *luxS*, respectively. The ends of the PCR products had sequences that were homologous to the *araBAD* promoter at one end and homologous to *lacZ* at the other end. Each PCR product was inserted into the chromosome of strain PM1205 (Table 1) by lambda Red recombinase-mediated gene replacement, resulting in translational *lacZ* fusions under transcriptional control of the *araBAD* promoter.

lacZ translational fusions to *nadE* and *yqaE* that harbored point mutations in the translational initiation region were constructed by amplifying the *nadE′*- and *yqaE′-lacZ* translational fusions from NRD399 and NRD400, respectively, using primers carrying the mutations [primers nadE(T-3A C-2T A-1G)-lacZ For and deeplac or primers pBAD-X-lacZ For and yqaE(T7A C9G)-lacZ rev]. The PCR products were then recombined into the chromosome of PM1205 as described above to generate strains NRD407 [*nadE*(T-3A C-2T A-1G)*′-lacZ* fusion] and NRD408 [*yqaE*(T7A C9G)*′-lacZ* fusion]. The *luxS′-lacZ* translational fusion that had a point mutation in the translational initiation region was constructed by first amplifying two overlapping pieces of this region using primers luxS(T-7A) For and deeplac and primers pBAD-X-lacZ For and luxS(T-7A) Rev. The two PCR fragments were then joined by overlap extension PCR, and the resulting PCR product was recombined into the chromosome of strain PM1205 to generate strain NRD406.

Strain CV8000, which harbors a *cyaR-lacZ* transcriptional fusion that contains the full-length promoter of *cyaR* (long fusion), was obtained from Carin K. Vanderpool. The *cyaR-lacZ* fusion harbored by strain CV8000 was generated by amplifying a portion of the *cyaR* gene and the promoter upstream of *cyaR* from *E. coli* K-12 genomic DNA using the c14Eco and c14Bam primers and cloned into pRS1553 (54) digested with EcoRI and BamHI. Strain MG1655 was transformed with this plasmid, and the transformants were then infected with λRS468 phage (54) to generate recombinant phages harboring a *cyaR-lacZ* transcriptional fusion. Strain DJ480 was infected with these recombinant λ phages, and single lysogens were isolated (54).

Strain MG1225 carrying the *ompX′-lacZ* translational fusion was acquired from Maude Guillier and was constructed as follows. The *araBAD* promoter was amplified by PCR from pBAD24 using the EcopBAD2 and pBADompX prim-

TABLE 2. Primers and probes used in this study

Primer or probe	Sequence (5′–3′)
Primers	
c14Bam	CCGGGGATCCGATTACACAGGCTAAGGAGG
c14Eco	CACGGAATTCCGATTATTTTTCCCGGATGG
deeplac	CGGGCCTCTTCGCTA
EcopBAD2	CGACGAATTCTCCCGCCATTCAGAGAAGAAACC
luxS(T-7A) For	CGGAGGAGGCTAAATGCCGTTGTTAGATAG
luxS(T-7A) Rev	CTATCTAACAACGGCATTTAGCC
luxSKO For	GAAAGAGTTCAGAAAATTTTTAAAAAAATTACCGGAGGTGGCTAAGTGTAGGCTGG AGCTGCTTC
luxSKO Rev	TGAACTGGCTTTTTTCAATTAATTGTGAAGATAGTTTACTGACTACATATGAATATCC TCCTTAG
lsrACDBFGKO For	AATACATTTGTTCAAAACTCACCTGCAAAACTGAACGGGGGAAATGTGTAGGCTGGA GCTGCTTC
lsrACDBFGKO Rev	CGTCAGCGCGTTACCCCAATTGTAGATAAAATTGATAAATTCGCCCATATGAATATCC TCCTTAG
M13For(−20)	GTAAAACGACGGCCAG
nadE(T-3A C-2T A-1G)-lacZ For	CTTTTTATCGCAACTCTCTACTGTTTCTCCATCTTTTCTGTCTGGAGGGGTATGATGAC ATTGCAACAAGTCG
ompXSma	CGACTCCCGGGCAGTGCTGAAAGACATGCAATTTTTTTCATAACCACCTC
pBAD-DNA-RACE	ACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCAT
pBAD-luxS4-lacZ	AACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGTATGATCGACTGTGAA GCTATC
pBAD-nadE-lacZ For	ACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATCTTTTCTGTCTGGAGGG GTTC
pBAD-nadE-lacZ Rev	AACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACTTGTTGCAATGTCATTGA ACCC
pBADompX	CCAGATTATTCCTAATCACCAGACTAATGATTCCATCAATCCTGGATGGAGAAACAGT AGAGAGTTGCG
pBAD-yqaE-lacZ For	ACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATGCTTAAGAGATAAAATCTC TTTTTAAACAA
pBAD-yqaE-lacZ Rev	AACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGATGACGATTCTCCAGA AACC
pBR-placRyeE For	GCGTGACGTCGCTGAAAAACATAACCCATAAAATGCTAG
pBR-placRyeE Rev	GAGAATTCCCTTTTATTTCATTGTATTACGCGTAAAAAATAAGC
pBAD-X-lacZ For	GCGGATCCTACCTGACGC
RACE_nadE1	GTTGTTGCAATGTCATTGAAC
RACE_yqaE2	CCAAGCAGTGTCAACAGAA
RACE _ryeE1	GGAGGTGGTTCCTGGTACAG
RACE_universal	GATATGCGCGAATTCCTGTAGAACGAACACTAGAAGAAA
ryeEKO For	GGAAAATTCTTAGAAACCGATCACATACAGCTGCATTTATTAAGGGTGTAGGCTGGA GCTGCTTC
ryeEKO Rev	GAAATAAATCCTTTTATTTCATTGTATTACGCGTAAAAAATAAGCCATATGAATATCC TCCTTAG
ryeEA44T For	GCTAGCTGTACCAGGAACCTCCTCCTTAGCCTGTGTAATCTC
ryeEA44T Rev	GATATTACACAGGCTAAGGAGGAGGTTCCTGGTACAGCTAGC
ryeET47A For	CTAGCTGTACCAGGAACCACCACCTTAGCCTGTGTAATCTCC
ryeET47A Rev	GGAGATTACACAGGCTAAGGTGGTGGTTCCTGGTACAGCTAG
ryeET50A For	CTGTACCAGGAACCACCTCCATAGCCTGTGTAATCTCCC
ryeET50A Rev	GGGAGATTACACAGGCTATGGAGGTGGTTCCTGGTACAG
ryeEG38C-G39A-A40T For	CCCATAAAATGCTAGCTGTACCACATACCACCTCCTTAGCCTGTG
ryeEG38C-G39A-A40T Rev	CACAGGCTAAGGAGGTGGTATGTGGTACAGCTAGCATTTTATGGG
yqaE(T7A C9G)-lacZ rev	GTTTTCCCAGTCACGACGTTGTAAAACGACGATGACGATTCTCCACATACCCATATGT ACTCCCTATAAG
Probes	
cfaprobe1	CCACCAGCCATCCATATAACTTTCGCCTAACCCCA
yqaEprde1	CCCAACGGAACCCTTTACCGAGCAGCACGCCGAGC
flu-probe	CCCTGCAGGAGTGCATCGCCTTCACGGATGGTCAGGGTATCG
luxSprobe1	TCCCTCTTTCTGGCATCACTTCTTTGTTCGGCACG
mscLprobe1	CCCAGAGGAGGCATGATGATATCGGCAACCAGTGA
nadEprobe1	GGTTAATACGCGATCCGGTTGAATAAAGGCAATGG
ompXprobe1	TACGGAAGTACCTGCGGTGAAAGCCAGAACTGCGG
ryeEprobe1	TGGTTCCTGGTACAGCTAGCATTTTATGGGTTATG
uspGprobe1	CGGACATGTTGTTTAATGCGGGAAGGATCGATGGT

ers. The resulting PCR product and the ompXSma primer were used to amplify a sequence of *E. coli* K-12 strain MG1655 genomic DNA containing the first 10 codons of *ompX* and the 241 nucleotides directly upstream of the *ompX* start codon. The resulting PCR product was cloned into pRS414 (54) digested with EcoRI and SmaI. Recombinant lambda phages containing the *lacZ* translational fusion to *ompX* were generated, and the *lacZ* fusion was inserted into the chromosome of strain DJ480 as described above.

Plasmid pNRD405 was constructed by PCR amplification of *cyaR* from strain

MG1655 using the pBR-placRyeE For and pBR-placRyeE Rev primers and subsequently cloning the PCR product into the pBR-plac vector digested with AatII and EcoRI. *cyaR* site-directed mutants with A44T, T47A, T50A, and G38C G39A A40T mutations were constructed using a QuikChange II site-directed mutagenesis kit (Stratagene), template plasmid pNRD405, and primers ryeEA44T For and ryeEA44T Rev, primers ryeET47A For and ryeET47A Rev, primers ryeET50A For and ryeET50A Rev, and primers ryeEG38C-G39A-A40T For and ryeEG38C-G39A-A40T Rev, respectively. All of the *cyaR* site-directed mutants were confirmed by sequencing.

Culture media and growth conditions. All MG1655 derivatives were grown in liquid medium or agar plates containing Lennox broth or in M63 minimal medium supplemented with vitamin B_1 at a final concentration of 0.001% and glucose or glycerol at a final concentration of 0.2%. In some instances, LB medium was supplemented with glucose or arabinose at a final concentration of either 0.2 or 0.01%. Antibiotics were added to LB liquid medium or agar plates at the following concentrations: tetracycline, 25 mg liter^{-1}; chlorampenicol, 25 mg liter^{-1}; and ampicillin, 100 mg liter^{-1}. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to cultures at a final concentration of 100 μM.

β-Galactosidase assays. All strains were grown overnight at 37°C in LB liquid medium. Each overnight culture was then diluted 200-fold in fresh LB liquid medium or LB liquid medium supplemented with glucose and incubated at 37°C. β-Galactosidase assays were performed as described by Miller (42) using samples removed from the cultures during log phase (optical density at 600 nm [OD_{600}], 0.3 to 0.4), late log phase (OD_{600}, 1.0 to 1.2), and stationary phase (OD_{600}, 3.2 to 5.2). All β-galactosidase assays were performed at least twice.

Northern blotting. For determination of the effects of *cyaR* expression on the abundance of potential target mRNAs, overnight cultures of NRD359/pBR-plac or NRD359/pNRD405 (plac-*cyaR*$^+$) grown in LB liquid medium containing ampicillin were diluted 200-fold in fresh medium and incubated at 37°C. A 700-μl sample was removed from each culture when the OD_{600} was 0.5. IPTG was then added to each culture, and additional samples were removed 2, 5, 10, and 15 min after the addition of IPTG. RNA was extracted from the samples using the hot phenol method described by Massé et al. (37).

For examination of the role of Crp in the expression of *cyaR*, an overnight culture of strain MG1655 or NRD356 grown in LB liquid medium was diluted 200-fold in fresh LB medium and incubated at 37°C. When each culture reached log phase or late log phase, a 700-μl sample was removed from the culture and the RNA was extracted as described above.

To determine the effect of expression of CyaR from its native promoter on the abundance of the *yqaE*, *ompX*, *nadE*, and *luxS* mRNAs, overnight cultures of strain DJ624 or NRD359 grown in LB liquid medium were diluted 200-fold in fresh LB medium and incubated at 37°C. Samples (700 μl) were removed from log-, late-log-, and stationary-phase cultures of each strain, and the RNA was extracted from each sample as described above.

Northern blot analysis of CyaR expression was performed by fractionating 3 μg of RNA on a Bio-Rad Criterion 10% Tris-borate-EDTA (TBE)-urea polyacrylamide gel that was prerun for 30 min at 55 V and subsequently run at 55 V for 3 h in 1× TBE. The fractionated RNA was then transferred to a 0.2-μm nylon membrane (Whatman Nytran N) by electroblotting at 200 mA for 2 h in 0.5× TBE. The RNA was cross-linked to the nylon membrane by using UV irradiation. The membrane was then hybridized with 5′-biotinylated ryeEprobe1 in ULTRAhyb solution (Ambion) at 42°C, and the CyaR sRNA was detected using a Brightstar Biotect kit (Ambion).

Northern blot analysis of *ompX*, *nadE*, *yqaE*, *luxS*, *mscL*, *uspG*, *cfa*, and *flu* expression was performed by fractionating 10 μg of total mRNA on a 1.2% agarose gel that was prerun at 12 V/cm for at least 5 min and subsequently run at 5 V/cm for 2 h in 1× morpholinepropanesulfonic acid (MOPS). After capillary transfer of the RNA to a 0.45-μm nylon membrane (Whatman Nytran SuPerCharge), the membrane was hybridized with ompXprobe1, nadEprobe1, yqaEprobe1, luxSprobe1, mscLprobe1, uspGprobe1, cfaprobe1, or flu-probe, and the mRNA was detected as described above.

5′ RACE. 5′ RACE was performed by using the method described by Argaman et al. (2), with the following modifications. One microgram of RNA ligated to the 5′ universal RNA adapter (5′-GAU AUG CGC GAA UUC CUG UAG AAC GAA CAC UAG AAG AAA-3′) or the 5′ pBAD-DNA-RACE RNA adapter (ACC TG CGC TTT TTA TCG CAA CTC TCT ACT GTT TCT CCA T) was reverse transcribed by using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions and the pBAD-luxS4-lacZ, RACE-nadE1, RACE-yqaE, or RACE-ryeE1 primer and then treated with RNase H. The cDNA was then amplified by PCR using the pBAD-luxS4-lacZ, RACE-nadE1, RACE-yqaE, or RACEryeE1 primer and the RACEUniversal or pBAD-DNA-RACE primer. The PCR products were then TOPO cloned into the pCR4-TOPO vector (Invitrogen), and the resulting plasmids were transformed into Top10 cells. At least five transformants harboring plasmids containing the amplified cDNA from the tobacco acid pyrophosphatase-treated RNA were sequenced using the M13For(−20) primer supplied with the TOPO cloning kit (Invitrogen).

Microarray analysis. The *cyaR* deletion strain NRD359 harboring the vector pBR-plac or plasmid pNRD405, which contains *cyaR* under control of a *lac* promoter, was grown overnight at 37°C in LB liquid medium containing ampicillin. Each strain was then diluted 200-fold in fresh medium and incubated at 37°C until the OD_{600} was 0.5. IPTG was then added to each culture to a final concentration of 100 μM. After 15 min of induction with IPTG, a sample was taken from each culture, and the RNA was extracted by the hot phenol method as described above. The RNA was treated with Turbo DNase (Ambion), and the RNA was then recovered as described above for 5′ RACE. The labeled cDNA was then prepared and hybridized to an *E. coli* Genome 2.0 array (Affymetrix) according to the manufacturer's instructions.

Analyses of the microarray data were performed as described by Massé et al., except that genes that were not present ($P > 0.05$) in three or more of the cDNA samples or had an average difference of less than twofold under the two conditions used for the two microarray experiments were eliminated from further consideration. The genes whose expression patterns were not reproducible in the two microarray experiments (i.e., genes that did not show a change in expression in the same direction under the two conditions in both microarray experiments) were also eliminated. Finally, the data for genes that gave a low signal (<300) in all of the microarrays were also removed from the data set. Of the remaining genes (Table 3), those with the greatest change and the most reproducible signal intensities (*yqaE*, *ompX*, *nadE*, and *luxS*) were analyzed further by performing Northern blot analysis as described above. Other genes with smaller changes in abundance that were of interest to us were also examined by Northern blotting (*uspG*, *mscL*, and *cfa*), but the changes were not significant and these genes were not studied further.

The expression of three genes (*yeeR*, *yeeS*, and *flu*) appeared to increase substantially upon *cyaR* overexpression in the microarray experiments (Table 3). These three genes are adjacent to each other in the *E. coli* genome and are transcribed in the same direction. Reexamination of the stocks of strains NRD359/pBR-plac and NRD359/pNRD405 used in the microarray experiments described above demonstrated that these two strains were dominated by different phase variants of *flu*, providing an explanation for the substantial changes in expression. Northern blotting of overnight cultures of NRD359/pBR-plac and NRD359/pNRD405 that were in the same phase with respect to *flu* expression (i.e., there was not rapid settling of cells in the cultures) demonstrated that there was no difference in *flu* expression in the presence and in the absence of CyaR expression. The negative regulation of *yqaE*, *ompX*, *nadE*, and *luxS* by CyaR was observed regardless of whether the two strains were in the same phase with respect to *flu* expression.

Autoinducer-2 assays. Autoinducer-2 assays were performed as described by Surette and Bassler (55) and Wang et al. (65). Strains NRD359 and NRD392 harboring pBR-plac or pNRD405 were grown overnight at 37°C in LB liquid medium containing ampicillin. Cells from 1 ml of each overnight culture were then pelleted, resuspended in 1 ml of fresh LB medium, and diluted and grown at 30°C in fresh LB medium containing IPTG at a final concentration of 100 μM. Samples were removed every hour, and a cell-free culture filtrate was prepared. Cell-free culture filtrates were stored at −20°C overnight.

In duplicate, 10 μl of a cell-free culture filtrate or fresh LB medium was added to a Corning Costar 3912 flat-bottom assay plate. Ninety microliters of AB medium containing *Vibrio harveyi* diluted 5,000-fold from an overnight culture was added to each well. The 96-well microplate was then incubated at 30°C, and bioluminescence was measured 2 h after inoculation and then every 0.5 h for an additional 3 h.

RESULTS

Northern blotting previously showed that CyaR sRNA was expressed well in LB liquid medium but was expressed poorly in liquid M63 minimal medium supplemented with glucose and vitamin B_1 (66). We confirmed this expression pattern using strain CV8000, a *lac* derivative of *E. coli* K-12 strain MG1655 harboring a *lacZ* transcriptional fusion to the promoter region of *cyaR* (positions −74 to 64) (Fig. 1). Consistent with the Northern blotting performed by Wassarman et al. (66), expression of *cyaR* was fivefold higher in a late-log-phase culture

TABLE 3. Genes regulated by CyaR

Gene	Locus	Expt 1			Expt 2			Avg change (fold)	Gene product
		Level when CyaR was not expressed[a]	Level when CyaR was expressed[a]	Change (fold)	Level when CyaR was not expressed[a]	Level when CyaR was expressed[a]	Change (fold)		
uspG	b0607	951.8	647.5	−1.47	1,232.6	448.8	−2.75	−2.1	Universal stress protein
modC	b0765	297.3	263.8	−1.13	451.2	111.8	−4.04	−2.6	Component of a molybdenum ABC-type transporter
ompX	b0814	31,805.2	4,250.6	−7.48	19,716	2,519.9	−7.82	−7.7	Outer membrane protein with unknown function
gsiB	b0830	1,507.9	447	−3.37	565.1	428.5	−1.32	−2.35	Component of a predicted glutathione ABC-type transporter
nadE	b1740	2,776.2	450.9	−6.16	3,628.7	767.8	−4.73	−5.4	NAD^+ synthase
yobD	b1820	388.8	176.5	−2.20	458.8	229.7	−2.00	−2.1	Conserved inner membrane protein with unknown function
yeeD	b2012	3,063.7	772.8	−3.96	182.6	170.5	−1.07	−2.5	Conserved predicted protein
gabD	b2661	531.3	222.6	−2.39	649	340.6	−1.91	−2.1	Succinic semialdehyde dehydrogenase
ygaU	b2665	1,307	864.6	−1.51	1,377.9	455.3	−3.03	−2.3	Protein with unknown function
yqaE	b2666	3,254.6	286.7	−11.3	2,304.7	390.2	−5.91	−8.6	Conserved predicted membrane protein
luxS	b2687	6,105.7	1,961.7	−3.11	5,417.7	1,589.3	−3.41	−3.3	Autoinducer-2 synthase
cysN	b2751	2,528.7	211.1	−12.0	127.3	24	−5.30	−8.6	Subunit of sulfate adenylyltransferase
cysH	b2762	2,125.2	133.2	−16.0	131.4	27	−4.87	−10.4	Phosphoadenylyl sulfate reductase
mscL	b3291	2,135.8	1,015.1	−2.10	2,005.3	1,029.4	−1.95	−2.0	Mechanosensitive channel that relieves pressure on the membrane
ibpB	b3686	3,952.1	438.7	−9.00	3,387.6	2,764.3	−1.23	−5.1	Heat shock protein
ibpA	b3687	5,866.2	1,651.1	−3.55	7,108.9	5,687.2	−1.25	−2.4	Heat shock protein
sbp	b3917	1,454.4	192.9	−7.54	78	14.1	−5.53	−6.5	Sulfate binding protein involved in sulfate import
ryeA	b4432	3,807.7	2,131	−1.79	5,880.6	2,309.7	−2.55	−2.2	sRNA
sfuC	b0066	97.8	147.8	1.51	120.3	339.3	2.82	2.2	Thiamine transporter
ykgC	b0304	119.7	160.3	1.34	103.3	345.3	3.34	2.3	Predicted oxidoreductase
fepA	b0584	467.8	1,247.3	2.67	1,360.1	1,997	1.47	2.1	Outer membrane receptor and transport protein for enterobactin
fepC	b0588	146.1	491.2	3.36	543	647.4	1.19	2.3	Fe^{3+}-enterobactin ABC-type transporter
flu	b2000	279	11,245	40.3	277.4	9,614	34.7	37.0	Outer membrane protein involved in autoaggregation
yeeR	b2001	151	1,897.1	12.6	130.6	1,461.4	11.2	12.0	Predicted membrane protein
yeeS	b2002	9	598.7	66.5	45.3	377.7	8.34	37.4	Predicted DNA repair protein

[a] Units are Affymetrix-reported values for gene signal.

(OD_{600}, 1.0 to 1.2) of strain CV8000 grown in LB liquid medium (254 Miller units) than in a late-log-phase culture grown in liquid M63 minimal medium (54 Miller units).

One possible source of the difference between expression of *cyaR* in LB liquid medium and expression of *cyaR* in M63 minimal medium containing glucose might be the difference in glucose levels. This possibility was tested by comparing the β-galactosidase activities of samples of cells taken from log-phase, late-log-phase, and stationary-phase cultures of CV8000 grown in LB medium or LB medium supplemented with glucose at a final concentration of 0.2%. The levels of expression of *cyaR* were similar for the cultures of CV8000 grown in M63 minimal medium containing vitamin B_1 and glucose and in LB liquid medium containing glucose in both the log phase (61 and 67 Miller units) and the late log phase (54 and 53 Miller units). However, the level of expression of *cyaR* was significantly higher in cultures of CV8000 grown in LB medium than in cultures grown in LB medium containing glucose in the late log phase (5-fold higher; 263 and 53 Miller units) and the stationary phase (22-fold higher; 691 and 32 Miller units) (Fig. 2A).

Since *cyaR* expression was subject to a "glucose effect" in the experiments described above, we hypothesized that *cyaR* expression might be activated by cAMP receptor protein (Crp) in the presence of cAMP. In *E. coli*, glucose import prevents activation of cAMP synthesis by adenylate cyclase, which is encoded by the *cya* gene. In the absence of transport of glucose into *E. coli*, adenylate cyclase is activated, resulting in the synthesis of cAMP, which then binds to Crp (for a review, see reference 51). With cAMP bound, Crp is able to bind upstream of specific promoters, leading to activation of gene expression.

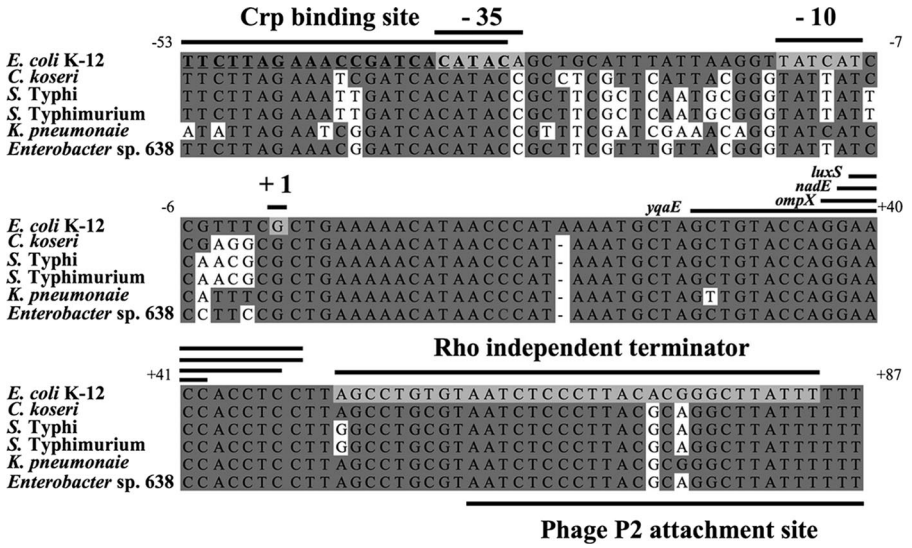

FIG. 1. Alignment of *cyaR* and the *cyaR* promoter region of several gammaproteobacteria. DNA sequence identity is indicated by dark gray shading. The predicted −10 region and −35 region of the *cyaR* promoter, the Rho-independent terminator, and the transcription start site mapped by 5′ RACE are indicated by light gray shading. The regions of CyaR predicted to pair with the *yqaE*, *ompX*, *nadE*, and *luxS* mRNAs are indicated by bars above the regions. The sequence of the predicted CRP binding site is indicated by bold type and is underlined. *C. koseri*, *Citrobacter koseri*; *K. pneumoniae*, *Klebsiella pneumoniae*.

To test whether *cyaR* expression is dependent on Crp and cAMP, isogenic derivatives of CV8000 carrying either a *cya* or *crp* deletion were constructed and tested to determine expression of the reporter fusion during growth in LB broth. Expression of *cyaR* was drastically reduced (<1% of wild-type expression) in late-log-phase cultures of strains having either Δ*cya* or Δ*crp* mutations (227 Miller units for a wild-type strain versus 0.7 and 1.8 Miller units for its Δ*cya* and Δ*crp* derivatives, respectively) (Fig. 2B).

We also examined the abundance of the CyaR sRNA in log-phase and late-log-phase cultures of MG1655 and its Δ*crp* derivative by Northern blotting. While the CyaR sRNA was easily detected by Northern blotting in cells from a late-log-phase culture of the parent strain, strain MG1655, it was not detected in cells of the *crp* mutant strain NRD356 (Fig. 2C, compare lanes 4 and 6), even though five times more RNA was loaded into the lanes containing Δ*crp* strain NRD356 (lanes 5 and 6) than into the lanes containing MG1655 (lanes 3 and 4).

Analysis of the *cyaR* promoter. To further characterize *cyaR* and regulation of its transcription, we mapped the transcription start site of *cyaR* by using 5′ RACE. Six of the seven independently cloned cDNAs of *cyaR* had the same transcription start site (Fig. 1). Based on the identified transcription start site and the predicted Rho-independent terminator, we predicted that the *cyaR* sRNA is 84 bp long. A comparison of the region encompassing *cyaR* and its predicted promoter in several gammaproteobacteria revealed a conserved −10 sequence and a highly conserved sequence that overlaps the predicted −35 region of the promoter (Fig. 1). This highly conserved sequence is centered at position −42.5 and has a high level of homology to the consensus sequence for Crp binding sites (5′-AAATGTGATCTAGATCACATTT-3′; the bases conserved in the Crp binding site upstream of *cyaR* are underlined) (15, 17, 47). To confirm that this conserved putative Crp binding site is required for *cyaR* expression, we constructed a *lacZ* transcriptional fusion to the *cyaR* promoter identical to the fusion constructed in CV8000, except that it lacked the region upstream of the Crp binding site of *cyaR* (positions −76 to −54) and the Crp binding site of *cyaR* was changed from 5′-TTCTTAGAAACCGATCACATAC-3′ to 5′-GGATCGGAATTCGATCACATAC-3′ (the bases that were changed are underlined). Cells carrying this reporter fusion expressed β-galactosidase at levels that were 18- and 27-fold lower than the levels expressed by cells with the wild-type fusion during log phase (3.3 versus 60 Miller units) and late log phase (7.8 versus 206 Miller units), respectively. The low level of residual activity of this mutant promoter, compared to the very low levels of expression in a strain with *crp* deleted, may have been due to the presence of the unaltered second half of the Crp binding site and the presence of three conserved residues in the 5′ end of the mutant Crp binding site (5′-GGATCGGAATTCGATCACATAC-3′; conserved residues are underlined). Our results are consistent with direct Crp/cAMP regulation of *ryeE*, which was renamed *cyaR* to reflect this regulation. Similar results have recently been reported by Johansen et al. for *E. coli* (27) and by Papenfort et al. for *Salmonella enterica* serovar Typhimurium (49).

Identification of the targets of the CyaR sRNA. As mentioned above, Hfq-binding sRNAs recognize specific mRNAs by base pairing. The binding of an sRNA to an mRNA results in either inhibition of translation and coupled degradation of the sRNA and targeted mRNA or an increase in translation and an increase in message stability. To identify genes that are either negatively or positively regulated by the CyaR sRNA, we used a microarray-based approach similar to that described by Massé et al. (39) and Guillier and Gottesman (20) to examine the effects of CyaR expression. Total genome expression was compared in Δ*cyaR* strain NRD359 carrying a vector control (pBr-plac) and Δ*cyaR* strain NRD359 carrying *cyaR* controlled

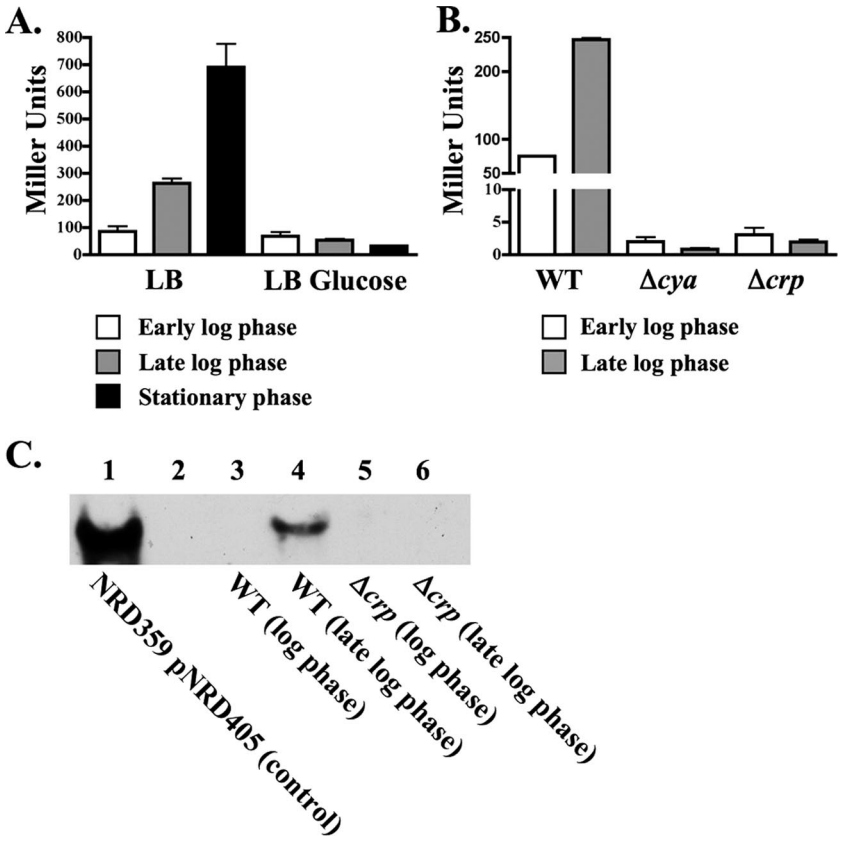

FIG. 2. Comparison of CyaR expression under different conditions. (A) β-Galactosidase assays of strain CV8000, which contains a *lacZ* transcriptional fusion to the *cyaR* promoter, grown at 37°C in LB liquid medium with or without glucose. β-Galactosidase assays were performed using samples taken from the cultures during log phase, late log phase, or stationary phase. (B) β-Galactosidase assays of strain CV8000 (WT) or derivatives of strain CV8000 harboring a *cya* deletion (NRD351) or a *crp* deletion (NRD352) grown at 37°C in LB liquid medium. β-Galactosidase assays were performed using samples taken from cultures of each strain during log phase or late log phase. The data in panels A and B are averages of three independent experiments. (C) Northern blot analysis of total RNA from a culture of strain NRD359/pNRD405 grown in LB liquid medium containing ampicillin and IPTG (control) (lane 1), from log-phase and late-log-phase cultures of wild-type strain MG1655 (WT) (lanes 3 and 4, respectively), or from log-phase and late-log-phase cultures of the Δ*crp* strain NRD345 (lanes 5 and 6, respectively) grown in LB liquid medium at 37°C. Five times more RNA was loaded in lanes 5 and 6 (Δ*crp*) than in lanes 3 and 4 (wild type). The ryeEprobe1 was used to detect RNA encoding *cyaR*.

by plac; samples were collected from log-phase cultures after 15 min of IPTG induction.

In the microarray experiments, the expression of 18 genes decreased on average at least twofold, and the expression of 7 genes increased at least twofold, not counting *cyaR* itself. Four of the genes whose mRNA levels decreased upon CyaR expression (*cysH*, *cysN*, *spb*, and *yeeD*) were expressed at very low levels in one of the two experiments and were not considered further. The reason for the difference in basal expression levels between experiments is not known, but we noted that many *cys* genes were expressed much better in experiment 1 than in experiment 2. Of the remaining 15 genes, *yqaE*, *ompX*, *nadE*, and *luxS* are four of the five genes showing the largest decreases in expression upon *cyaR* expression. The fifth gene, *ibpB*, and the upstream gene *ibpA*, both encoding heat shock proteins, were not examined further; the decreases in expression of these genes may suggest that there is a gradual decrease in expression of the heat shock regulon in response to CyaR overexpression, since other genes involved in the heat shock response were also downregulated, although to a lesser extent (see Table S1 in the supplemental material).

To confirm the negative regulation of *yqaE*, *ompX*, *nadE*, and *luxS* by CyaR, we examined the expression of these genes under conditions similar to those used for the microarrays, except that RNA was isolated from the cultures at additional time points (0, 2, 5, 10, and 15 min after IPTG was added to cultures of NRD359/pBR-plac and NRD359/pBR-*cyaR* [pNRD405]). The levels of mRNAs for *ompX*, *yqaE*, and *nadE* clearly decreased or the mRNAs disappeared within 2 min upon induction of CyaR expression, and the levels of mRNA for *luxS* showed a marked decrease within 5 min upon induction of CyaR expression (Fig. 3A), consistent with the hypothesis that there is direct regulation of the stability of these mRNAs by CyaR. As expected, *cyaR* expression was not induced until after IPTG was added, and the abundance of the CyaR sRNA continued to increase after IPTG induction (Fig. 3B).

Northern blotting was also performed under these conditions for three other genes of interest, *mscL*, *uspG*, and *cfa*,

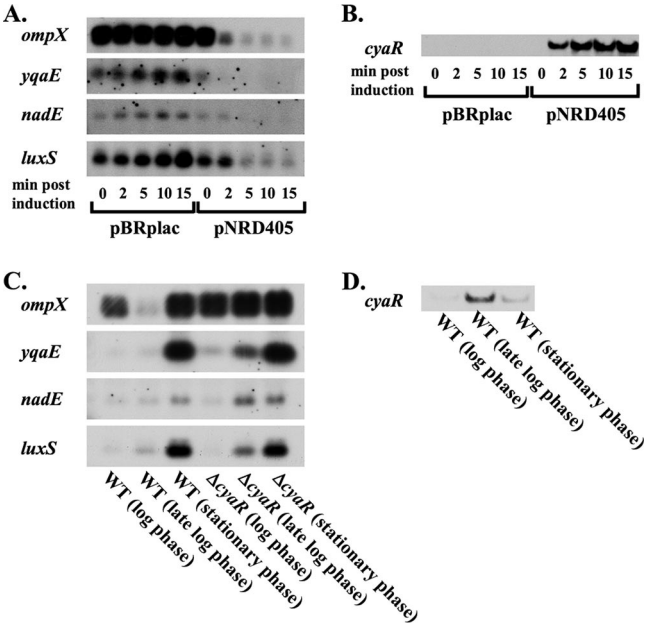

FIG. 3. Northern blot analysis of *ompX*, *nadE*, *yqaE*, and *luxS* expression in the presence or absence of *cyaR* expression. (A and B) The *cyaR* deletion strain NRD359 harboring an empty vector (pBR-plac) or a plasmid containing *cyaR* under a *lac*-based promoter (pNRD405) was grown at 37°C in LB liquid medium containing ampicillin to an OD_{600} of 0.5, and a sample of each culture was removed. IPTG was then added to each culture, and additional samples were removed from each culture 2, 5, 10, and 15 min after the addition of IPTG. RNA was extracted by the hot phenol method, fractionated on a 1.2% agarose gel (A) or a 10% TBE-urea gel (B), and transferred to a nylon membrane. 5′-Biotinylated probes were used to detect mRNA-encoding genes showing negative regulation by *cyaR* in the microarray experiments whose results are shown in Table 3, including *ompX*, *yqaE*, *nadE*, and *luxS* (A) or the CyaR sRNA (B). (C and D) Strain DJ624 (WT) and Δ*cyaR* strain NRD359 (Δ*cyaR*) were grown at 37°C in LB liquid medium, and samples were removed from each culture at log phase (at an OD_{600} between 0.3 and 0.4), late log phase (at an OD_{600} between 0.9 and 1.2), and stationary phase (at an OD_{600} more than 5.0; 6 h postinoculation). RNA was extracted, fractionated, and transferred to a nylon membrane as described above. 5′-Biotinylated probes were used to detect *ompX*, *yqaE*, *nadE*, *luxS* and *ompA* (loading control [data not shown]) (C) or the CyaR sRNA (D). Based on the RNA ladder run alongside the mRNA samples for the Northern blots shown in panel A, the mRNAs encoding *ompX*, *nadE*, *yqaE*, and *luxS* are estimated to be approximately 900 and 600 bp (*ompX*), 1,000 bp (*nadE*), 300 bp (*yqaE*), and 700 bp (*luxS*) long, respectively, indicating that all of the transcripts detected encoded only a single gene. Two *ompX* mRNAs that were different sizes were detectected because *ompX* is transcribed from two different promoters located 170 bp apart (40).

whose expression decreased 2.0-, 2.1-, and 1.9-fold upon *cyaR* expression in the microarray experiments described above. However, we did not detect decreases in *mscL*, *uspG*, or *cfa* expression upon overexpression of *cyaR* (data not shown).

The four mRNAs confirmed by Northern blotting, the *ompX*, *yqaE*, *nadE*, and *luxS* mRNAs, were selected for further study. The functions of these mRNAs are discussed briefly here, but no obvious common theme was suggested by the results for this group of genes. OmpX and YqaE are both membrane proteins, a class of proteins that many other sRNAs have been found to regulate (8, 11, 21, 27, 43, 50, 59, 62, 64). *ompX*, whose expression was reduced almost eightfold upon CyaR expression, encodes an outer membrane protein with an unknown function that is not essential (40), and *yqaE*, whose expression was reduced almost ninefold upon CyaR expression, is predicted to encode a conserved inner membrane protein. Another gene that was expressed at much lower levels after CyaR expression was *nadE*. *nadE* is an essential gene and encodes an ammonia-dependent NAD synthetase, which synthesizes NAD from nicotinic adenine dinucleotide (24, 53, 69). Finally, *luxS*, which encodes the autoinducer-2 synthase involved in quorum sensing, was also significantly downregulated upon CyaR expression. Autoinducer-2-mediated quorum sensing has a significant influence on the expression of many genes in *E. coli*, including genes involved in biofilm formation and motility (14, 18, 33).

Consistent with regulation of CyaR by cAMP and Crp, several of the downregulated transcripts (including *ompX* and *luxS*) have previously been shown to be negatively regulated by Crp (19, 65, 72). LuxS was previously shown by Wang et al. (65) to be negatively regulated by *crp*, but it lacked a sequence upstream of the coding sequence for *luxS* that was able to bind CRP in vitro. Thus, the regulation by CyaR explains this discrepancy.

For the mRNAs whose levels increased after CyaR expression, the most dramatic increases (*flu*, *yeeR*, and *yeeS*) were found to be due to phase variation and not CyaR (see Materials and Methods). For other mRNAs there were modest changes or relatively low levels of expression were observed, and these mRNAs were not analyzed further.

CyaR expression results in decreases in the abundance of *yqaE*, *ompX*, *nadE*, and *ompX* mRNAs during late log phase in wild-type *E. coli* cells. In the microarray and Northern blot experiments described above, target mRNAs were identified and confirmed to be negatively regulated by CyaR under CyaR overexpression conditions (compare Fig. 2, lane 1 [1 μg of RNA isolated from NRD359/pNRD405 expressing *cyaR* for 15 min], to lane 4 [3 μg of RNA isolated from a late-log-phase culture of MG1655]). To assess the effect of CyaR expression from its native promoter on the abundance of these mRNAs, we compared the levels of *yqaE*, *ompX*, *nadE*, and *luxS* mRNAs in cells taken from log-phase, late-log-phase, and stationary-phase cultures of a $cyaR^+$ strain, DJ624, and its Δ*cyaR* derivative NRD359 by Northern blotting. Consistent with our observation that CyaR expression is highest during late log phase, the levels of all four of these mRNAs were much lower in strain DJ624 than in its Δ*cyaR* derivative during late log phase, while no significant differences were observed during the log and stationary phases (Fig. 3C). No significant differences in the levels of the *ompA* mRNA, which was used as a control in these experiments, were observed between the strains in any growth phase (data not shown).

The negative regulation of *yqaE*, *ompX*, *luxS*, and *nadE* by CyaR occurs at the posttranscriptional level and is direct. To determine whether *yqaE*, *luxS*, and *nadE* were negatively regulated by CyaR at the posttranscriptional level, we constructed translational *lacZ* fusions to these genes and placed these fusions under the control of a different promoter, the *araBAD* promoter. Replacing the normal promoter of *yqaE*, *luxS*, and *nadE* with the *araBAD* promoter allowed us to decouple posttranscriptional regulation of these genes from transcriptional regulation and to control the expression of the fusions by altering the concentration of arabinose in the medium. A strain harboring a lambda prophage carrying a *lacZ* translational

fusion to *ompX* was obtained from Maude Guillier and was used to examine whether *ompX* was negatively regulated by CyaR. The *ompX-'lacZ* translational fusion in this strain was also under the control of the *araBAD* promoter, but it still had its native proximal promoter; the two promoters of *ompX* were previously determined to be at positions −220 and −50 relative to the start codon (40).

Since we did not want to affect the posttranscriptional regulation of *yqaE*, *luxS*, and *nadE* by altering the 5′ end of the mRNAs that normally encode these genes, we mapped the 5′ end of each of the mRNAs by using 5′ RACE and then constructed the fusions in such a way as to allow transcription from the *araBAD* promoter to begin at the mapped position 1 of the genes. The transcription start sites of *yqaE*, *luxS*, and *nadE* were mapped to positions −57, −83, and −22 relative to the initiation of translation, respectively. Surprisingly, the transcription start site for *luxS* is within the divergent gene for MicA, an sRNA that negatively regulates expression of the major outer membrane protein OmpA (62). As a result, the first 15 bp of the *luxS* mRNA is complementary to the first 15 bp of the MicA sRNA, an unusual arrangement.

lacZ translational fusions to *yqaE*, *luxS*, and *nadE* were constructed and placed in the chromosome under control of the *araBAD* promoter such that transcription began at the mapped transcriptional start site for these genes. As mentioned above, the *ompX* translational *lacZ* fusion is under the control of its native proximal promoter. The fusion genes were expressed in a Δ*araBAD* Δ*cyaR* strain harboring either an empty vector (pBR-plac) or a plasmid expressing CyaR from a *lac* promoter (pNRD405), and the amounts of translated fusion proteins were determined by β-galactosidase activity assays. Expression of CyaR from the plasmid reduced the translation of *yqaE*, *nadE*, *luxS*, and *ompX* 4.7-, 1.7-, 5.2-, and 14.3-fold, respectively (Fig. 4).

The sequence of CyaR and the 5′ end of mRNAs encoding *yqaE*, *nadE*, *luxS*, and *ompX* were examined, and possible regions of base pairing were determined (see Fig. S1 in the supplemental material). CyaR is predicted to base pair with *ompX*, *luxS*, and *nadE* mRNAs in a region that includes the ribosome binding site of these genes, whereas CyaR is predicted to base pair with the *yqaE*-encoding mRNA immediately downstream of the start codon for *yqaE*. Based on these predictions of base pairing, several site-directed *cyaR* mutants were constructed by using QuikChange site-directed mutagenesis and the template plasmid pNRD405. The resulting mutants with the A44U, U47A, and U50A single mutations and the G38C G39A A40U triple mutation in CyaR were then tested to determine their abilities to negatively regulate the translational fusions as described above. The U50A change was not predicted to disrupt base pairing with any of the four targets (see Fig. S1 in the supplemental material), and, consistent with this prediction, a plasmid expressing this mutant CyaR was able to downregulate all four fusions (see Fig. S2 in the supplemental material). The A44U substitution in CyaR reduced the negative regulation of *ompX* and eliminated the negative regulation of *luxS* (Fig. 4B and 4D). Papenfort et al. recently reported that an A44U substitution in CyaR also reduces the ability of this molecule to negatively regulate *ompX* in *S. enterica* serovar Typhimurium (49). The U47A substitution in CyaR also eliminated the ability of CyaR to negatively regulate *luxS*, but it had less effect on *ompX* (Fig. 4B and 4D). Both the A44U and U47A CyaR mutants were still able to negatively regulate the *yqaE* and *nadE* mRNAs (Fig. 4A and 4C). In contrast, the G38C G39A A40U triple substitution (Fig. 4) reduced the ability of CyaR to negatively regulate *yqaE* and *nadE* expression and had no effect on the ability of CyaR to negatively regulate *luxS* and *ompX* expression. Since each of the substitutions in CyaR (A44U, U47A, or G38C G39A A40U) reduced the expression of only a subset of the four mRNAs regulated by CyaR, these mutations in CyaR most likely acted by disrupting base pairing between CyaR and the affected mRNAs rather than by reducing active CyaR levels. However, it is also worth noting that these mutations did not totally eliminate regulation, except regulation of *luxS*.

To confirm that the A44U substitution in CyaR disrupts the base pairing between CyaR and the *luxS* mRNA, a mutant with a compensatory mutation in the *luxS-lacZ* translational fusion was prepared and assessed as described above. As predicted, the compensatory mutation in the *luxS-lacZ* fusion restored the ability of the A44U CyaR mutant and eliminated the ability of wild-type CyaR to negatively regulate *luxS* expression (Fig. 5A), consistent with our model for pairing. A mutation in *ompX* that would have compensated for the A44U substitution in CyaR was not constructed, since Papenfort et al. recently reported that a compensatory mutation in the predicted pairing region in *ompX* restored the ability of the A44U CyaR mutant to negatively regulate *ompX* in *S. enterica* serovar Typhimurium (49).

Compensatory mutations for the G38C G39A A44U mutant CyaR were also constructed in the predicted pairing region of the *nadE* and *yqaE* mRNAs. As predicted, these compensatory mutations increased the ability of the G38C G39A A44U mutant CyaR and decreased the ability of the wild-type CyaR to negatively regulate the *nadE-lacZ* and *yqaE-lacZ* fusions (Fig. 5B and C). In fact, the triple mutation and compensating mutations led to regulation of *yqaE* that was tighter than the regulation observed for the wild-type molecule (Fig. 5 C).

Expression of CyaR results in a decrease in autoinducer production. Since expression of CyaR results in a dramatic decrease in the translation of *luxS* encoding autoinducer-2 synthase (Fig. 4D), we examined the effect of CyaR expression on the production of autoinducer-2 by *E. coli*. Autoinducer-2 levels are measured by collecting culture filtrates from the producer strain and measuring their abilities to activate expression of the *lux* genes in *V. harveyi* (55).

Strain NRD359, a derivative of strain DJ624 that harbors a *cyaR* deletion, was transformed with pBR-plac or pNRD405, which transcribes *cyaR* from a *lac* promoter. The transformed strains were grown at 30°C, and culture filtrates were prepared from each culture every hour after inoculation as described in Materials and Methods. The relative amounts of autoinducer-2 in culture filtrates were then determined by measuring the amount of light produced by *V. harveyi* strain BB170, a response to the concentration of autoinducer-2 present in the culture filtrate (56).

As NRD359/pBR-plac and NRD359/pNRD405 approached late log phase (4 h postinoculation), autoinducer-2 could be detected in the cell-free culture filtrates of these strains, and the autoinducer-2 levels peaked at 5 h postinoculation (Fig. 6A and 6B). When the peak autoinducer-2 levels were present, the levels

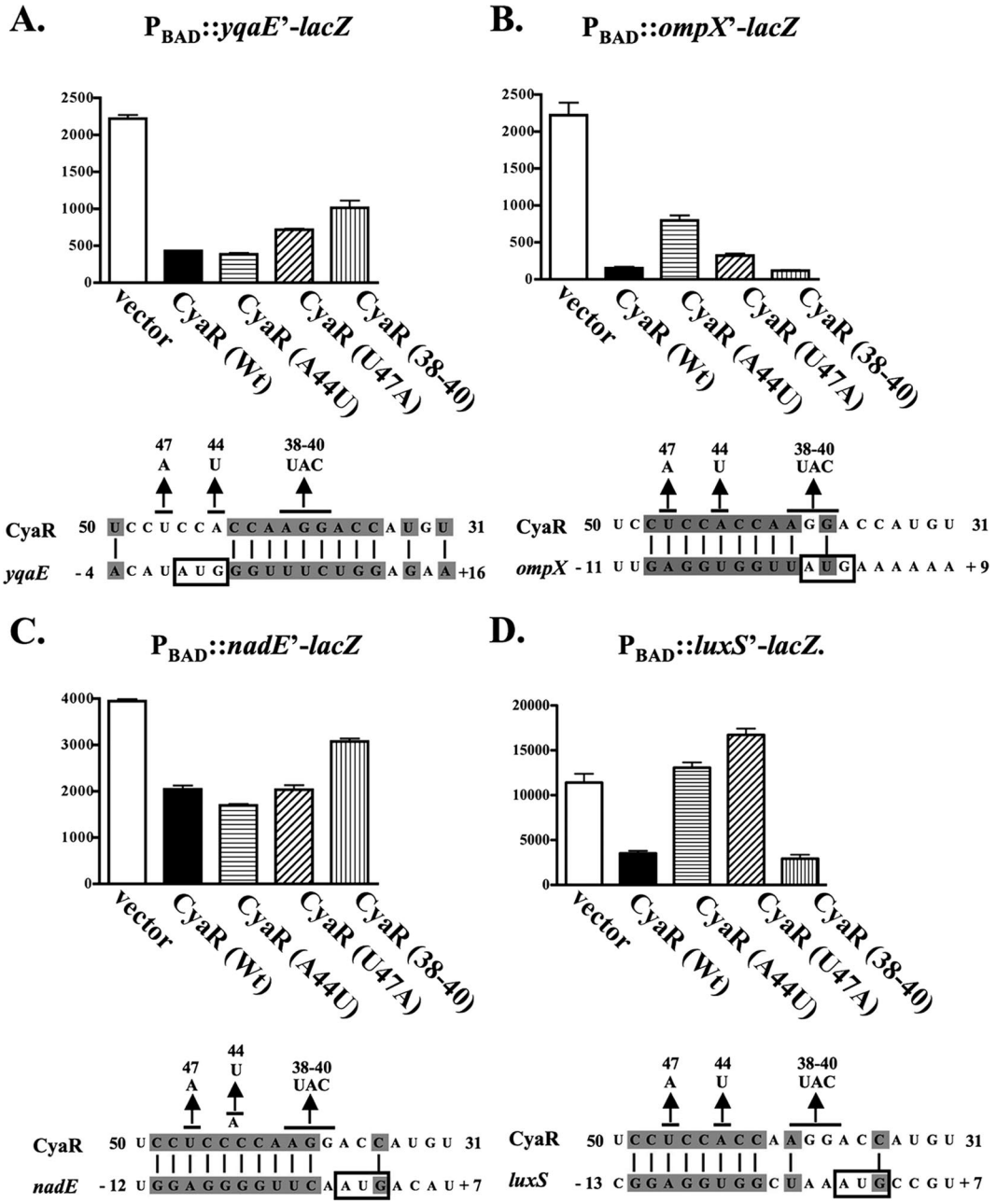

FIG. 4. β-Galactosidase assays of *yqaE-lacZ*, *ompX-lacZ*, *nadE-lacZ*, and *luxS-lacZ* translational fusions in the presence or absence of wild-type or mutant CyaR expression. Overnight cultures of (A) NRD400 (*yqaE-lacZ* fusion), (B) NRD377 (*ompX-lacZ* fusion), (C) NRD399 (*nadE-lacZ* fusion), or (D) NRD413 (*luxS-lacZ* fusion) harboring pBR-plac (vector), pNRD405 (wild-type *cyaR* [Wt]), pNRD407 (*cyaR* A44T), pNRD408 (*cyaR* T47A), or pNRD410 (*cyaR* G38C G39A A40T [CyaR (38-40)]) were diluted 200-fold in fresh LB medium containing IPTG (100 μM), ampicillin (100 mg liter^{-1}), and arabinose (0.01%) or (in the case of NRD377) no arabinose. These strains were then incubated at 37°C for 6 h. The OD_{600} of each culture was determined, and β-galactosidase assays were performed as described in Materials and Methods. The data are the averages of three independent experiments. Below the graphs showing the results of the β-galactosidase activity assays are diagrams showing the predicted pairing between CyaR and *yqaE* (A), *ompX* (B), *nadE* (C), or *luxS* (D) mRNA. The start codon of *yqaE*, *ompX*, *nadE*, and *luxS* is indicated by a box, and the base substitutions in CyaR are indicated above the CyaR sequence.

were 2.8-fold lower in the strain expressing *cyaR* (NRD359/pNRD405) than in the strain lacking *cyaR* (NRD359/pBR-plac) (Fig. 6B), consistent with regulation of *luxS* by CyaR. These results were obtained in two independent experiments.

To confirm this significant difference in autoinducer-2 production in the presence and absence of CyaR expression, we constructed strain NRD392, a derivative of strain DJ624 that harbors an *lsrACDBFG* deletion and a *cyaR* deletion. *lsrACDBFG* encodes a transporter that mediates the uptake of autoinducer-2 from the medium; the transcription of *lsrACDBFG* is induced by autoinducer-2 (57). Deletion of *lsrACDBFG* reduces the uptake of autoinducer-2 by *E. coli* (57) and thus allows higher levels of

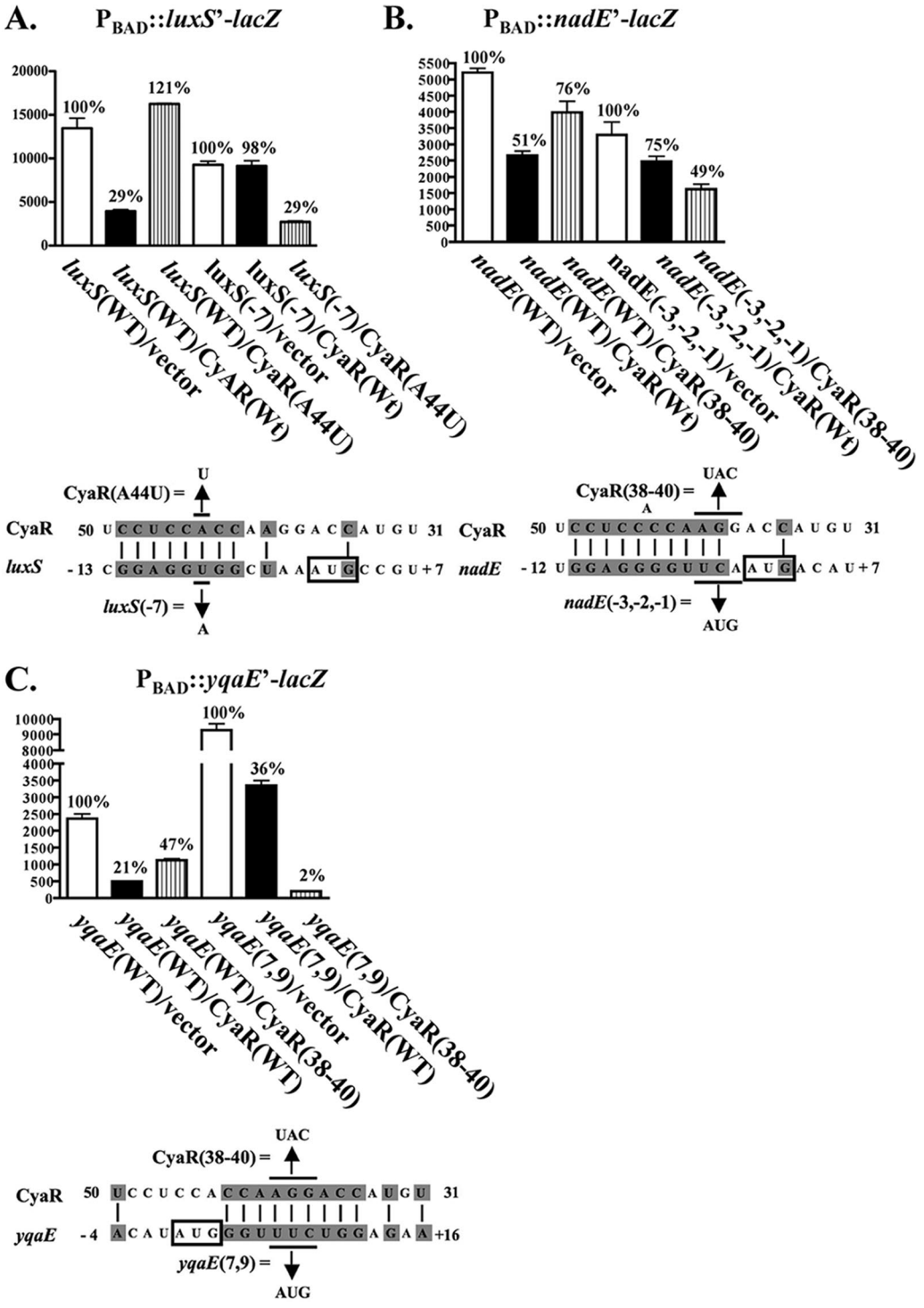

FIG. 5. β-Galactosidase assays measuring the effect of compensatory mutations in the translation initiation regions of *luxS*, *nadE*, and *yqaE* on restoration of negative regulation by CyaR. Overnight cultures of (A) NRD413 (*luxS-lacZ* fusion), NRD415 [*luxS* (T-7A)-*lacZ* fusion], (B) NRD399 (*nadE-lacZ* fusion), NRD410 [*nadE* (T-3A; C-2T; A-1G)-*lacZ* fusion], (C) NRD400 (*yqaE-lacZ* fusion), or NRD411 [*yqaE* (T7A;C9G)-*lacZ* fusion] harboring pBR-plac (vector), pNRD405 (wild-type *cyaR*), pNRD407 (*cyaR* A44T), or pNRD410 (*cyaR* G38C G39A A40T [CyaR(38–40)]) were diluted 200-fold in fresh LB liquid medium containing IPTG (100 μM), ampicillin (100 mg liter^{-1}), and arabinose (0.01%). After the strains were incubated at 37°C for 6 h, the OD_{600} of each culture was determined, and β-galactosidase assays were performed as described in Materials and Methods. The variant of the *lacZ* translational fusion examined and the variant of CyaR expressed, if any (bases changed in the *lacZ* translational fusion or in *cyaR* are indicated), are indicated on the *x* axis. The percentage of β-galactosidase activity relative to the activity of the strain harboring the empty vector is indicated above each bar. Below the graphs showing the results of the β-galactosidase assays are diagrams showing the predicted pairing between CyaR and the mRNA encoding *luxS* (A), *nadE* (B), or *yqaE* (C) and the base substitutions in *cyaR* and the compensatory base changes made in the mRNAs encoding the genes. The start codon of each gene is indicated by a box. The results are averages of two independent experiments. Wt and WT, wild type.

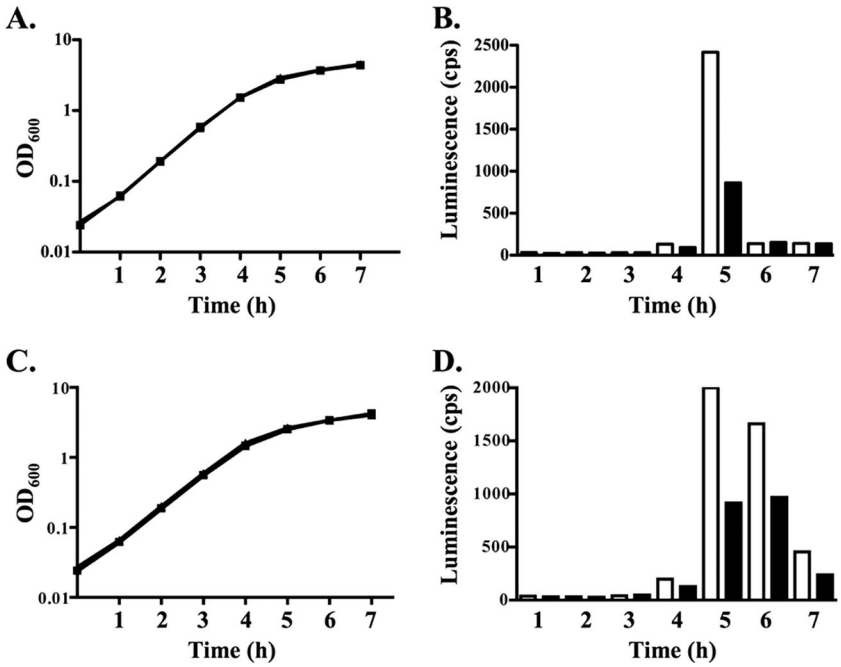

FIG. 6. Comparison of autoinducer-2 production in the presence and absence of CyaR expression. (A and C) Growth at 30°C of the Δ*cyaR* strain NRD359 (A) or the Δ*lsrACDBFG* Δ*cyaR* strain NRD392 (C) harboring the vector pBR-plac (squares) or the *cyaR*-expressing plasmid pNRD405 (triangles). (B and D) Autoinducer-2 production by NRD359/pBR-plac (open bars) and NRD359/pNRD405 (filled bars) (B) or by NRD392 pBR-plac (open bars) and NRD392/pNRD405 (filled bars) (D). Overnight cultures of strains NRD359/pBR-plac, NRD359/pNRD405, NRD392/pBR-plac, and NRD392/pNRD405 grown in LB medium containing ampicillin were pelleted, the supernatants were removed, and the cells were resuspended in LB medium. The washed cells were then diluted 200-fold in LB medium containing IPTG and incubated at 37°C. Every hour after inoculation the OD_{600} of each culture was determined and samples were removed. Cell-free culture filtrates were then prepared, and the relative amounts of autoinducer-2 were determined as described in Materials and Methods. Each experiment was performed twice, and the data are representative of the two experiments.

autoinducer-2 to remain in the culture medium for a longer period of time. We transformed NRD392 with pBR-plac or pNRD405 and grew the resulting strains at 30°C. We collected cell-free culture filtrates and measured the autoinducer-2 levels with the *V. harveyi* BB170 reporter strain as described above.

At late log phase, autoinducer-2 could be detected in the culture filtrates of strains NRD392/pBRplac and NRD392/pNRD405 (Fig. 6C and 6D), and at 5 h postinoculation the autoinducer-2 levels were 2.2-fold lower in the culture filtrates of the strain expressing *cyaR* (NRD392/pNRD405) than in the culture filtrates of the strain lacking *cyaR* (NRD392/pBR-plac). Moreover, a significant difference in autoinducer levels between the culture filtrates of the strain expressing *cyaR* and the culture filtrates of the strain lacking *cyaR* was still observed 7 h after inoculation. A similar difference in autoinducer levels between strains NRD359/pBR-plac and NRD359/pNRD405 was observed in an independent experiment (data not shown).

We also examined the production of autoinducer-2 in a wild-type strain of *E. coli* (DJ624) and a Δ*cyaR* derivative of this strain by growing these strains at 30°C in LB medium, preparing cell-free culture filtrates, and measuring the level of autoinducer-2 as described above. No significant difference in autoinducer-2 production between the wild-type strain and its Δ*cyaR* derivative was found (data not shown). This may not be surprising given the transient expression of CyaR in *E. coli* strains grown in LB medium (Fig. 3C).

DISCUSSION

We showed that the expression of the RyeE sRNA, renamed CyaR here, is activated by Crp under conditions in which cAMP levels are high and that CyaR directly negatively regulates the expression of several genes, including *yqaE*, *ompX*, *nadE*, and *luxS*, at the posttranscriptional level.

Crp is one of the best-studied regulatory proteins. It has been shown that when cAMP is present, Crp activates the expression of numerous genes which are primarily involved in the transport and catabolism of sugars and amino acids (19, 72, 73). In *E. coli*, the synthesis of cAMP by adenylate cyclase is inhibited by import of the preferred catabolite glucose, and the levels of cAMP and therefore the DNA binding activity of Crp are high when poor carbon sources are available and low when glucose is available (25). Consistent with this pattern, expression of a *cyaR-lac* fusion (Fig. 2A) is increased in LB medium compared to the expression in LB medium containing glucose, and expression is completely dependent upon both *cya* (for synthesis of cAMP) and *crp* (Fig. 2B and C). Additionally, upstream of *cyaR* and overlapping the predicted −35 region of the *cyaR* promoter in *E. coli* K-12 is a conserved sequence similar to the consensus sequence of a Crp binding site; in our experiments, alteration of this Crp binding site resulted in a drastic decrease in expression from the *cyaR* promoter. In parallel experiments with *Salmonella*, the *cyaR* promoter was

shown to directly bind Crp, further supporting the hypothesis that there is direct regulation by cAMP and Crp (49). Another sRNA, Spot 42, is negatively regulated by cAMP and Crp (44), and Spot 42 downregulates expression of *galK*. This has been interpreted as a mechanism for adjusting expression of Gal epimerase, which is needed for cell wall synthesis, relative to expression of Gal kinase, which is needed only for galactose metabolism, when glucose rather than galactose is used by cells as a carbon source.

CyaR is an Hfq-binding RNA, and other sRNAs have been shown to both positively and negatively regulate gene expression by pairing with specific mRNA targets. The results of microarray experiments, Northern blotting, and experiments with translational fusions all are consistent with the hypothesis that CyaR negatively regulates *ompX*, *yqaE*, *nadE*, and *luxS* at a posttranscriptional level by pairing with the mRNA. As mentioned above, several of these genes that have been shown to be negatively regulated by CyaR, including *luxS* and *ompX*, have also been shown to be negatively regulated by Crp (19, 65). Our results are consistent with these findings and provide a mechanism for the negative regulation of these genes via Crp activation of CyaR.

A large number of outer membrane proteins have been shown to be subject to regulation by Hfq-binding small RNAs (8, 11, 20, 27, 43, 50, 59, 62, 64). Some of this regulation serves as a feedback regulatory mechanism for the sigma E regulon (27, 50, 59), while in other cases (20) it is unclear what the functions of the regulatory effects are. OmpX, an outer membrane protein with an unknown function, had been suspected to be regulated by an sRNA, because the level of *ompX* mRNA increases significantly in an *hfq* mutant (22). Our work and recently described work of Papenfort et al. (49) and Johansen et al. (26) clearly confirmed this and demonstrated that CyaR regulates OmpX in both *E. coli* and *Salmonella*, thus leading to downregulation of OmpX in the presence of a poor carbon source or under low-glucose conditions.

ompX has a long untranslated leader, and the primary region of pairing is predicted to be positions −2 to −9 relative to the translation start site of *ompX* (see Fig. S1 in the supplemental material). A translational fusion containing 241 nucleotides upstream of the start site and 30 nucleotides of the *ompX* coding region was downregulated sixfold when CyaR was overexpressed from a *lac* promoter (Fig. 4B). Therefore, the 5′ end of *ompX* extending from the transcription start site to the 10th codon of the gene is sufficient for regulation; this region contains the predicted pairing region. The results of experiments carried out by Papenfort et al. to examine CyaR regulation of OmpX in *Salmonella* (49) are consistent with this predicted pairing. The A44U mutation in CyaR previously shown by Papenfort et al. to disrupt pairing between CyaR and OmpX in *Salmonella* had similar effects in *E. coli* (Fig. 4B).

OmpX belongs to a family of outer membrane proteins that have been implicated in promoting bacterial adhesion and entry into and survival in mammalian cells. While the data for *E. coli* are not compelling (deletion of *ompX* did not affect adherence to human HEp-2 cells and indirectly stimulated adherence [40, 48]), in *Enterobacter cloacae* overexpression of OmpX increased invasion of rabbit illeal tissue (13). OmpX of *E. cloacae* has 83% protein identity to *E. coli* OmpX, and the proposed binding site for *cyaR* upstream of *ompX* is conserved in *E. cloacae*. Therefore, it seems reasonable to predict that the regulation of OmpX by CyaR has a role in downregulating adherance under low-glucose conditions. This could be part of a starvation response, for instance, in which cells should move to new environments in search of nutrients.

Another target of CyaR that is also involved in the behavior of bacteria at cell surfaces and during infection is *luxS*. LuxS synthesizes autoinducer-2 (56), a signal molecule used in quorum sensing in *E. coli* and a wide range of bacteria (5, 52). In *E. coli*, autoinducer-2 influences the expression of many genes, including genes involved in transport and processing of the autoinducer, in motility, and in biofilm development (14, 18, 33, 65). Autoinducer-2 has been shown to increase the mass and thickness of biofilms (18, 33). LuxS activity also impacts methionine metabolism, since homocysteine, a precursor of methionine, is generated in the reaction catalyzed by LuxS that produces autoinducer-2 (52).

The *luxS* mRNA has a long 5′ untranslated region that begins 83 bp upstream of the AUG start codon. CyaR has a region of base pairing with the *luxS* 5′ untranslated region that overlaps the ribosome binding site and involves the same region of CyaR used for *ompX* regulation; this region is conserved in the *luxS* leader (see Fig. S1 in the supplemental material). Consistent with this, the A44U mutation that disrupts pairing between CyaR and the *ompX* mRNA also disrupts pairing between CyaR and the *luxS* mRNA (Fig. 4D), and a compensatory mutation in the pairing region of the *luxS* mRNA restores negative regulation by the mutant form of CyaR (Fig. 5A). The effect of downregulation of *luxS* mRNA by CyaR overexpression is a decrease in the amount of autoinducer-2 produced by *E. coli* (Fig. 6B and 6D). In *E. coli*, autoinducer-2 simulates biofilm formation. Thus, as observed for *ompX*, it seems possible that downregulation of *luxS* under glucose-limiting conditions may help cells decrease biofilm formation, increase planktonic behavior, and move in search of nutrients.

The sequence encoding the long 5′ end of the *luxS* mRNA extends into *micA*. As a result, the *luxS* mRNA and the MicA sRNA share 15 bp of complementary sequence. Thus, the sigma E-regulated promoter of *micA* lies within the sequence encoding the *luxS* mRNA, and at least some of the promoter of *luxS* must lie within *micA*. A possible consequence of this arrangement is that expression from the promoter of one of these genes may interfere with expression of the other gene. Another potential consequence of this arrangement is that the 15-bp complementarity between these genes may facilitate the posttranscriptional regulation of *luxS* by MicA. It is interesting that the same region of MicA was suggested by Johansen et al. to base pair with the *ompX* mRNA, which these authors identified as a target of negative regulation by MicA and which is negatively regulated by CyaR (26).

Another confirmed target, *nadE*, is an essential gene whose product uses ammonia to catalyze the last step in NAD synthesis (24, 53, 69). A sequence immediately upstream of *nadE* that has the potential to pair with CyaR was found; however, this sequence is not well conserved in other closely related enterobacteria, including *S. enterica* serovar Typhimurium and *S. enterica* serovar Typhi (Fig. 1), even though the region of CyaR that pairs with this sequence is absolutely conserved in these species (Fig. 1). Thus, *nadE* may not be a target of

negative regulation by CyaR in these other species of enterobacteria. Nonetheless, the results of our experiments with the translational *lacZ* fusion to *nadE* demonstrated that the regulation of *nadE* by CyaR is posttranscriptional and is direct (Fig. 5B). None of the other genes in the pathway for NAD biosynthesis or recycling (*nadC*, *nadD*, *nadA*, *nadB*, and *nadR*) showed any significant effect of CyaR overproduction in our arrays, suggesting that it is not NAD synthesis per se, but possibly limiting NadE ammonia use under limiting C or energy source conditions, that is the physiological basis for this regulation. This is not the first example of a protein involved in nitrogen metabolism that is regulated by catabolite repression. Crp was previously shown to decrease the expression of glutamine synthetase (36, 60), which catalyzes the synthesis of glutamine from glutamate and ammonia, and to activate the expression of GlnHPQ, a high-affinity glutamine transporter (36). Possibly because *nadE* is an essential gene, the downregulation of *nadE* by CyaR is less dramatic than the downregulation seen with other targets (Fig. 5). While it is unusual for targets of these small RNAs to be essential, one essential gene, *ftsZ*, has previously been shown to be regulated by the small RNA DicF (58).

The fourth gene for which there is strong and reproducible regulation by CyaR is the gene encoding YqaE, a highly conserved predicted membrane protein with an unknown function. In plants, this gene has been found to be a stress-induced gene that is expressed at higher levels at low temperatures and under salt stress and dehydration conditions (41). There is some evidence that in *E. coli yqaE* may be regulated by RpoS; the level of expression of *yqaE* is higher in a wild-type strain than in an *rpoS* mutant when the organisms are challenged with a hyperosmotic shock (nearly fivefold higher) or a decrease in the pH of the growth medium (threefold higher) (67). There is a substantial amount of pairing between CyaR and the region immediately downstream of the start codon of *yqaE* in *E. coli* that is highly conserved (see Fig. S1 in the supplemental material). This pairing between CyaR and YqaE has been confirmed (Fig. 5C).

As mentioned above, the sequences upstream of *ompX*, *nadE*, and *luxS* that are capable of pairing with CyaR overlap the ribosome binding sites of these genes, while the predicted pairing region of *yqaE* is immediately downstream of its start codon. The extent of base pairing between CyaR and the translation initiation regions of *ompX*, *luxS*, *nadE*, and *yqaE* is substantial, and the base pairing should efficiently block ribosome binding to these mRNAs when the level of CyaR expression is high.

The base pairing tests whose results are shown in Fig. 4 and 5 provided some hints about the complexity and redundancy of regulation by sRNAs of the sort examined here. The lengths of the predicted pairing "seed" (continuous pairing section) are 10 bp for *nadE*, 9 bp for *yqaE* and *ompX*, and 8 bp for *luxS*. Disrupting the seed by a single nucleotide change did not change regulation for *nadE* (Fig. 4C), changed regulation modestly for *ompX* (Fig. 4B), and changed regulation drastically for *luxS* (Fig. 4D and 5A). Even a change of 3 nucleotides led to only a partial release of regulation for *yqaE* (Fig. 4A and 5C) and *nadE* (Fig. 5B). While all of these experiments were done under CyaR overproduction conditions and it is possible that CyaR can pair elsewhere on the mRNA, it seems clear that there is not an absolute requirement for extensive seed pairing. Why *luxS* is particularly sensitive to single nucleotide changes remains to be explored. The results of further in vivo pairing tests should help in the development of a more complete understanding of sRNA function.

As mentioned above, Crp has been shown to affect the expression of many genes. Most of the genes that have been shown to be directly regulated by Crp are involved in the transport or catabolism of sugars or amino acids. Interestingly, all of the genes identified here as genes that are indirectly regulated by Crp via the CyaR sRNA are involved in processes other than sugar and amino acid transport and catabolism. The genes that are indirectly negatively regulated by Crp via the CyaR sRNA are involved in or associated with other cellular processes, such as adherance (*ompX*), NAD biosynthesis (*nadE*), and quorum sensing (*luxS*). The function of the predicted membrane protein encoded by *yqaE* remains unknown.

While *E. coli* utilizes Crp to directly regulate transport and catabolism of sugars and amino acids and utilizes CyaR to indirectly regulate other processes via Crp, other species of nonenteric gammaproteobacteria, such as *Pseudomonas aeruginosa* and *Pseudomonas putida*, divide the regulation of these different processes between two different regulatory proteins, Vfr and Crc. *P. aeruginosa* Vfr is 91% similar to the *E. coli* Crp protein, binds cAMP, and binds to a DNA sequence that is similar to, but significantly different from, the consensus Crp binding sites (30). Vfr directly regulates the expression of genes involved in the production of exotoxin A, protease production, quorum sensing, type IV pilus formation, and twitching motility (1, 7, 30, 68). On the other hand, Crc is responsible for repressing the expression of genes involved in the catabolism of nonideal catabolites, when ideal catabolites, including tricarboxylic acid intermediates, are present (23, 45, 46, 70). Crc has been shown to repress the expression of genes by a cAMP-independent mechanism.

Why does *E. coli* regulate some genes directly by Crp and other genes indirectly by Crp via the CyaR sRNA? Certainly most of the genes directly regulated by Crp are positively regulated, while the genes described here are negatively regulated; it may be easier to evolve negative regulation by this activator protein via an sRNA intermediate. Another possibility is that this division of labor allows *E. coli* to rapidly decrease expression of a subset of genes by actively destroying the mRNAs, which may be particularly important for genes with longer mRNA half-lives, although this possibility has not been specifically investigated for these targets. Another possibility is that the process of regulating genes through CyaR sRNA provides *E. coli* with another way of fine-tuning the expression of its target genes in response to different factors.

***cyaR* as a phage attachment site.** The *cyaR* gene of *E. coli* and its upstream binding site for Crp is highly conserved in other enterobacteria (49), and it is downstream of a conserved gene that is designated *yegQ* in *E. coli* and *Salmonella*. However, Papenfort et al. noted that the gene downstream of *cyaR* varies (49). The variation downstream is likely the result of a second function of the *cyaR* gene, as a phage attachment site. As originally noted by Wassarman et al. (66), the 3′ end of the small RNA overlaps an attachment site for phage P2, and *E. coli* K-12 has a fragment of a cryptic prophage at this site. While the *att* site is clearly conserved (Fig. 1), the prophage is

not present in some enterobacteria, while a large number of prophage genes are present in other enterobacteria (for instance, avian pathogeneic *E. coli* [28]). In at least one strain, a P2 prophage also has introduced a restriction/modification gene cluster at this location (32). Many tRNAs serve as phage attachment sites, as does tmRNA (*ssrA*) (9, 31), and recently the sRNA RyeB was reported to serve as an integration site for phages in *S. enterica* serovar Typhimurium (4). With the addition of CyaR, it is clear that sRNAs, like tRNAs, are bacteriophage attachment sites.

It has been suggested that a conserved sequence and/or structure associated with tRNAs may be an attractive conserved site for phage lysogenization (9, 31). Certainly, the conservation of *cyaR* in a broad range of enterobacterial species suggests that phages related to P2 may make use of this site in all of these species.

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