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The Cyclic AMP-Dependent Catabolite Repression System of *Serratia marcescens* Mediates Biofilm Formation through Regulation of Type 1 Fimbriae[▽]

Eric J. Kalivoda,[1] Nicholas A. Stella,[1] Dawn M. O'Dee,[2] Gerard J. Nau,[2] and Robert M. Q. Shanks[1,2]*

Charles T. Campbell Laboratory of Ophthalmic Microbiology, Department of Ophthalmology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15213,[1] and Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15261[2]

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The mechanisms by which environmental carbon sources regulate biofilm formation are poorly understood. This study investigates the roles of glucose and the catabolite repression system in *Serratia marcescens* biofilm formation. The abilities of this opportunistic pathogen to proliferate in a wide range of environments, to cause disease, and to resist antimicrobials are linked to its ability to form biofilms. We observed that growth of *S. marcescens* in glucose-rich medium strongly stimulated biofilm formation, which contrasts with previous studies showing that biofilm formation is inhibited by glucose in *Escherichia coli* and other enteric bacteria. Glucose uptake is known to inversely mediate intracellular cyclic AMP (cAMP) synthesis through regulation of adenylate cyclase (*cyaA*) activity, which in turn controls fundamental processes such as motility, carbon utilization and storage, pathogenesis, and cell division in many bacteria. Here, we demonstrate that mutation of catabolite repression genes that regulate cAMP levels (*crr* and *cyaA*) or the ability to respond to cAMP (*crp*) confers a large increase in biofilm formation. Suppressor analysis revealed that phenotypes of a cAMP receptor protein (*crp*) mutant require the *fimABCD* operon, which is responsible for type 1 fimbria production. Consistently, *fimA* transcription and fimbria production were determined to be upregulated in a *cyaA* mutant background by using quantitative real-time reverse transcription-PCR and transmission electron microscopy analysis. The regulatory pathway by which environmental carbon sources influence cAMP concentrations to alter production of type 1 fimbrial adhesins establishes a novel mechanism by which bacteria control biofilm development.

Serratia marcescens is a ubiquitous gram-negative bacterium capable of causing disease in diverse organisms, including humans, coral, insects, and plants (2, 11, 43, 44, 52). The abilities of *S. marcescens* to cause nosocomial infections and survive in the environment are attributed to its ability to form biofilms, its broad metabolic capacity, and its high natural resistance to antimicrobials and cleaning agents (18, 21, 28, 47). Biofilms are surface-attached microbial communities that afford resistance to biocides and antibiotics and are commonly involved in medical-device-associated infections (14, 20, 37).

S. marcescens readily adheres to diverse substrates, such as contact lenses and epithelial cells (23). Such interactions can be mediated through type 1 fimbriae; these large surface pili are important virulence factors in many bacteria (29). Fimbriae are versatile adhesins capable of mediating attachment to eukaryotic cell surfaces, interactions with biotic and abiotic substrates, and bacterium-bacterium interactions (4, 11, 27, 29, 41, 46, 58, 65). Previous studies of *S. marcescens* have indicated that biofilms are regulated by quorum sensing, require type 1 fimbriae, and differ in morphology with respect to carbon and nitrogen sources (31, 58). However, little is known about the regulation of *S. marcescens* biofilm formation. Thus far, type 1 fimbriae are the only surface adhesins known to mediate *S. marcescens* biofilm formation, where they are required for the primary step in attachment to biotic surfaces (31) and abiotic surfaces (58). Regulation of type 1 fimbriae in *S. marcescens* has been shown to be largely independent of quorum sensing and dependent upon the *oxyR* transcription factor and two genes required for normal biofilm structure in strain MG1: *bsmA* and *bsmB* (31, 58). The *fimABCD* operon codes for these mannose-sensitive type 1 fimbriae in *S. marcescens* (31, 41, 58).

First studied with *Escherichia coli* but well conserved in prokaryotes, uptake of preferred carbon sources and repression of genes required for utilization of less preferred carbon sources are regulated by a pathway called the catabolite repression system (CRS) (9). Catabolite regulation is generally, though not always, regulated by the second messenger cyclic AMP (cAMP) (56). The cAMP-dependent regulatory pathway has global effects on fundamental processes, including flagellum- and pilus-based motility, cell division, carbon storage, and pathogenesis (9, 10, 12, 36). It has been shown that cells grown in glucose are inhibited for cAMP production, while cells grown in less favorable carbon sources produced elevated levels of cAMP (45). Glucose uptake into bacteria by the phosphoenolpyruvate sugar phosphotransferase system (PTS) is a key regulator of cAMP production. One of the components of the PTS, coded for by the *crr* gene, is enzyme IIA^{Glc}. The major regulatory signal of the PTS is mediated through the phosphorylation state of enzyme IIA^{Glc}. When glucose is limiting, phosphorylated enzyme IIA activates adenylate cyclase

* Corresponding author. Mailing address: University of Pittsburgh, Dept. of Ophthalmology, EEI 1015, 203 Lothrop St., Pittsburgh, PA 15213. Phone: (412) 647-3537. Fax: (412) 647-5880. E-mail: shanksrm@upmc.edu.

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TABLE 1. Strains and plasmids used in this study[a]

Designation	Description	Source or reference
Strain name or genotype		
Serratia marcescens		
RSS2	WT pigmented strain	Presque Isle Cultures
cyaA1	WT with *cyaA*::Tn mariner mutation	This study
cyaA2	WT with *cyaA*::Tn mariner mutation	This study
cyaA3	WT with *cyaA*::Tn mariner mutation	This study
cyaA4	WT with *cyaA*::pMQ118 mutation	This study
fimC4	*fimC*::pRMQS167	This study
cyaA2 fimC4	*cyaA2* with *fimC*::pRMQS167 mutation	This study
crr-1	WT with *crr*::pEJK1 mutation	This study
crr-1 fimC2	*fimC2* with *crr*::pEJK1	This study
crp-1	WT with *crp*::pMQ118 mutation	This study
crp-1 fimC2	*crp-1* with *fimC*::Tn mariner mutation	This study
fimC2	WT with *fimC*::Tn mariner mutation 50E2	58
WT with P_{lac}-*fimABCD*	WT with chromosomal P_{lac}-*fimABCD*	58
cyaA2 with P_{lac}-*fimABCD*	*cyaA2* with chromosomal P_{lac}-*fimABCD*	This study
E. coli		
S17-1 l-*pir*	*thi pro hsdR hsdM*$^+$ Δ*recA* RP4-2 Tc::Mu-Km::Tn*7 pir*	40
PirPlus	DH10B *pir-116*	Gentaur
BW26386	Δ*cyaA1404*::*FRT*	13
Plasmids		
pRMQS106	*CEN6 ARSH4 URA3 aacC1 gfp-3HA*; ColE1	59
pMQ118	Yeast *oriR6K rpsL* suicide vector; Kan^r	*
pMQ131	Yeast pBBR1 shuttle vector; *aphA3*	*
pMQ132	Yeast pBBR1 shuttle vector; *accC1*	*
pRMQS157	pMQ131 with *cyaA*	This study
pRMQS169	pMQ118 with P_{lac}-*fimA′*	58
pRMQS167	pMQ118 with internal *fimC* fragment	This study
pEJK1	pMQ118 with internal *crr* fragment	This study
pRMQS173	pMQ118 with internal *crp* fragment	This study
pRMQS174	pMQ118 with internal *cyaA* fragment	This study

[a] WT, wild type; *, R. M. Shanks and G. A. O'Toole, unpublished.

(CyaA) activity, which generates cAMP. In *Escherichia coli* and *Salmonella* spp., mutations in adenylate cyclase or PTS components impart severe reductions in the amount of intracellular cAMP (17, 32, 50). Generated cAMP mediates changes through binding with cAMP receptor protein (CRP), a global regulator of transcription (9, 10). CRP can act as both a positive and a negative regulator of transcription and is required for virulence by several organisms (3, 36, 60, 66).

The CRS of *S. marcescens* is largely uncharacterized. An *S. marcescens* strain deficient in adenylate cyclase activities has previously been identified, though the mutation was not mapped, nor was the gene cloned (67). This mutant strain produced only 40% of wild-type levels of intracellular cAMP and required exogenous cAMP for use of various carbon sources (67). Data from ectopic expression of *S. marcescens* genes in *E. coli* suggest that the production of a secreted phospholipase is controlled by the CRS (19). A mutation in the PTS component gene *ptsI* of *Serratia marcescens* has previously been identified in a screen for genes required for extracellular chitinase activity (64). The defect in chitinase activity was complemented with *ptsH* and *ptsI* on a plasmid, but a role for *crr* in this process was not determined (64).

The effects of environmental carbon sources and carbon utilization regulatory proteins on biofilm formation have been documented for diverse bacterial species, including *S. marcescens* (1, 7, 25, 26, 34, 42, 51, 55). Exogenous cAMP stimulates biofilm formation in *E. coli* and related enteric bacteria; consistently, mutation of *cyaA* or *crp* leads to a decrease in biofilm production (25).

In this study, we demonstrate that biofilm formation by *S. marcescens* is regulated by components of the CRS. Conditions and mutations that reduce cAMP production or inhibit the ability of the bacterium to respond to cAMP greatly stimulate biofilm formation. The mechanism for increased biofilm formation was determined to be altered levels of type 1 fimbriae, which were upregulated when cAMP was reduced. We also show that catabolite repression genes are conserved between *S. marcescens* and other enterics but regulate biofilm formation in the opposite manner relative to what has been previously reported for *E. coli*.

MATERIALS AND METHODS

Bacterial strains, media, growth conditions, and experimental design. The microorganisms used in this study are listed in Table 1. All bacteria were grown in LB (0.5% yeast extract, 1% tryptone, 0.5% NaCl). M63 medium supplemented with casein amino acids (0.06%, wt/vol) and either glucose, citrate, sucrose, or glycerol (0.2%, vol/vol) was used for nutritional studies. LB and M63 broth were supplemented with adenosine 3′,5′-cyclic monophosphate (A9501; Sigma-Aldrich, Inc.) where noted. The antibiotics used were ampicillin (100 μg/ml), gentamicin (10 μg/ml), kanamycin (100 μg/ml), and tetracycline (10 μg/ml). Experiments were performed at 30°C unless otherwise noted. All experiments were performed using triplicate independent cultures, and all experiments were repeated at least twice on different days.

Mutagenesis, plasmid construction, and quantitative reverse transcription-PCR (qRT-PCR). Mariner transposon mutations were generated as previously described using pBT20 (58). The *fimC4* mutagenic construct was introduced into *S. marcescens* by using allelic replacement vector pMQ118 as previously described (58). Briefly, pMQ118 with an internal fragment of *fimC* was designed to recombine with the chromosomal *fimC* gene, yielding a disruption of the *fimC* gene; the same strategy was used to mutate *crp*, *crr*, and *cyaA* (Table 1). Insertion mutations were verified using PCR. Revertant strains were acquired as previously described (58). Strains with pMQ118 integrations were grown to saturation three to five times without selection to maintain pMQ118 in the chromosome. Single colonies from the resulting cultures were patched onto media with and without kanamycin. Colonies sensitive to kanamycin were tested phenotypically and by PCR to verify the loss of pMQ118 and the restoration of the wild-type gene and were referred to as "revertants."

The *cyaA* open reading frame of *S. marcescens* was cloned into pMQ131 by using *Saccharomyces cerevisiae* in vivo recombination and placed under the transcriptional control of P_{lac}, generating pRMQS157 (59). pMQ131 has pBBR1 replication elements for stable replication in *S. marcescens* (R. Shanks and G. A. O'Toole, unpublished). Chromosomal DNA was isolated with a commercial kit (Gentra D5500A). DNA was amplified using a high-fidelity polymerase (Phusion, New England Biolabs) and cloned using yeast in vivo cloning (59).

RNA preparation and qRT-PCR were performed as described previously, with cells grown to an A_{600} of 1.0, and *rplU* transcript levels were used as an internal normalization control as previously reported (58). Three independent cultures were grown on different days for each genotype. The primers used for transcriptional analysis were F-fimA-RT (ACTACACCCTGCGTTTCGAC) and R-fimA-RT (GCGTTAGAGTTTGCCTGACC) for *fimA*.

Biofilm assays. Biofilms on glass were generated using 20- by 150-mm borosilicate glass test tubes (14-961-33; Fisher Scientific) with 5 ml of LB incubated overnight at 30°C on a TC-7 tissue culture roller at full speed, 56 rpm (New Brunswick Instruments). These biofilms were stained while rotating, using 6 ml of 0.1% crystal violet, and solubilized using 6 ml of 30% glacial acetic acid, and absorbance for 150-μl aliquots was determined at 590 nm with a Synergy 2 plate reader (Biotek). Triplicate independent cultures were grown for each strain or condition, and each experiment was repeated at least two times on different days per condition. Flow cell experiments were performed using the Kadouri system 2 method as previously described (39), using LB diluted 10-fold in water and a flow rate of 16.5 ml per hour. Flow cells were inoculated with 10^8 bacteria and incubated at room temperature for 24 h. Confocal images were obtained as previously described (58). Flow cell experiments were performed twice with consistent results.

Detection of cAMP. Intracellular cAMP levels were determined for stationary-phase bacteria as previously reported (53). Cells were adjusted to an optical density of 2.0 in a volume of 1 ml, washed twice in sterile distilled H_2O, resuspended in 1 ml of phosphate-buffered saline (PBS), and lysed using 50 mg of CelLytic Express (Sigma, St. Louis, MO). The lysate was spun briefly in a microcentrifuge, and the supernatant was tested for cAMP levels by using a competitive enzyme immunoassay (CA-201; Sigma, St. Louis, MO). These experiments were performed three times in triplicate independent cultures on different days.

Yeast agglutination assay. The kinetic yeast aggregation assay was performed with 1.5 ml PBS mixed with 500 μl of yeast solution (Sigma YSC2; 2%, wt/vol, in PBS) and 400 μl of bacteria in PBS (A_{600} = 1.0). Cultures were agitated vigorously and transferred to a cuvette (1 cm^2; Sarstedt), and the A_{600} was measured spectrophotometrically to assess the agglutination-dependent precipitation of cultures over time (Beckman DU-70).

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences determined in this study are EU153350 and EU183232.

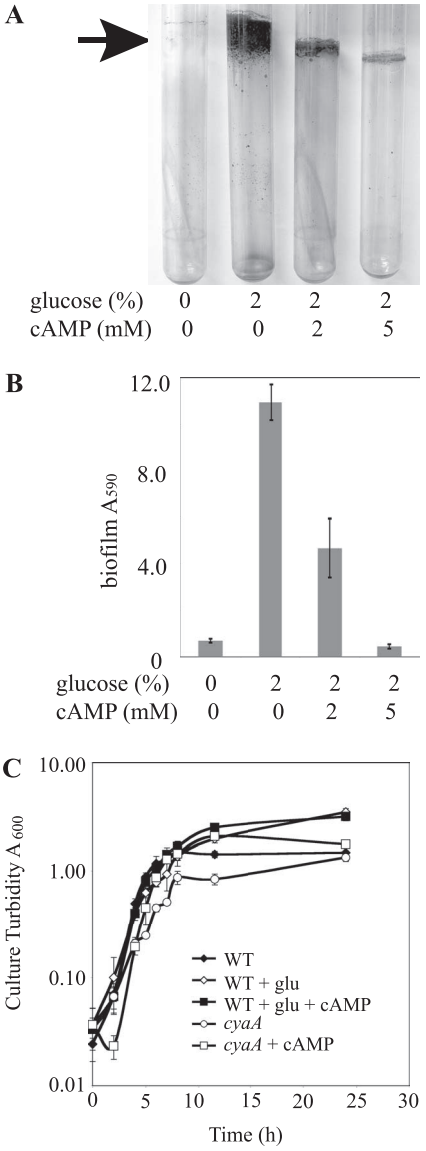

FIG. 1. Glucose stimulates *S. marcescens* biofilm formation. (A) Photographs of crystal violet-stained biofilms (black arrow) formed on glass test tubes. Biofilms were formed in at 30°C for 15 h under high-sheer conditions in LB supplemented with glucose or cAMP as indicated. (B) Biofilm levels from crystal violet-stained test tubes (results from three independent cultures per strain are shown, and error bars indicate 1 standard deviation). Growth in glucose-rich medium significantly increased crystal violet staining ($P > 0.01$). (C) Growth curve analysis shows culture turbidity as a function of time. Bacteria were grown in LB medium at 30°C and supplemented with 2% glucose (glu) or 10 mM cAMP as indicated. WT, wild type. This experiment was done with triplicate independent cultures. Error bars depict 1 standard deviation.

RESULTS

Glucose stimulates formation of hyperbiofilms that are sensitive to exogenous cAMP. We observed that the addition of supplemental glucose to LB medium dramatically increased attachment of *S. marcescens* to glass test tubes under high-sheer conditions (Fig. 1A and B). No obvious biofilm was formed by the wild type grown in LB, but a robust biofilm was clearly visible with the addition of glucose (Fig. 1A). As this result differs from what has been previously reported for several enteric bacterial species (25), we decided to study the effect of glucose on *S. marcescens* biofilm formation.

The presence of glucose in growth medium has been shown to inhibit the production of cAMP by some bacteria (9, 45). We hypothesized that *S. marcescens* grown in glucose-rich medium formed enhanced biofilms because of physiological changes brought about either by a reduction in cAMP levels or through

an increase in growth. To help differentiate between these two models, we assessed bacterial growth (Fig. 1C). At 15 h, when biofilm formation was analyzed, there was a slight though significant increase ($P > 0.01$ at 11.5 and 24 h) in the amount of culture turbidity when the growth medium was supplemented with glucose compared to that for cultures grown in LB alone (Fig. 1C). To test the model in which robust biofilm formation was a result of reduced cAMP levels, increasing concentrations of exogenous cAMP were added to the growth medium. Figure 1A and B show that glucose-induced biofilm formation can be reversed by the addition of exogenous cAMP in a dose-dependent manner. To ensure that this effect is not due to inhibition of bacterial growth by cAMP, we observed that wild-type cultures grown in LB supplemented with both glucose and 10 mM cAMP grew just as well as the culture grown with LB with additional glucose (Fig. 1C). Together, these data suggest that glucose-stimulated biofilm formation is mediated by the cAMP-regulated CRS rather than caused by minor changes in growth. Mutation of predicted catabolite repression genes was used to further test this model.

Catabolite repression proteins regulate *S. marcescens* biofilm formation. We had previously isolated transposon mutations in a predicted class I adenylate cyclase homolog (*cyaA*) in a screen for genes that modulate biofilm formation in *S. marcescens*, though these mutants were not described (58). The predicted CyaA polypeptide from the sequenced Db11 strain shared high amino acid identity with CyaA proteins from several enteric pathogens, including *Yersinia pestis* (87%), *Shigella flexneri* (85%), and *Salmonella enterica* (85%). An internal region of the *cyaA* gene was cloned into the pMQ118 suicide/allelic expression vector and sequenced (GenBank accession number EU153350). This sequence was ~98% identical to the corresponding DNA of sequenced *S. marcescens* strain Db11. Three independent transposon mutations in the adenylate cyclase (*cyaA*) open reading frame were found, and allele numbers were assigned (*cyaA1*, *-2*, and *-3*). *cyaA2* had the most 5′ integration site and was used throughout this study.

When cultures of *cyaA* mutants were grown in glass test tubes rotated at high speed, bacteria adhered dramatically to the sides of the tube at the air-liquid interface in a manner akin to that of the wild-type strain grown with excess glucose (Fig. 2A and Table 2). Biofilms formed under these high-sheer conditions were stained with crystal violet and quantified spectrophotometrically; a >10-fold increase was observed in the *cyaA2* mutant (Fig. 2A and Table 2). In addition, the culture was full of bacterial aggregates that precipitated when the culture was stationary, suggesting increased bacterium-to-bacterium interactions. The increased interactions of *cyaA* mutant cells were confirmed using an assay where pelleted bacteria were vortexed and their resuspension kinetics were determined (data not shown). When the wild-type *cyaA* gene was added in *trans* to the *cyaA2* mutant strain on a multicopy plasmid, biofilms returned to wild-type levels (Fig. 2A and Table 2). Growth curve analysis indicated that the hyperbiofilm phenotype was not a result of an increased growth rate caused by the *cyaA* mutant (Fig. 1C).

To verify the effect of the *cyaA* mutation on surface attachment, biofilms were generated under continuous flow conditions at room temperature and observed using confocal laser scanning microscopy. Whereas both the wild type (Fig. 2B) and the *cyaA2* mutant (Fig. 2C) produce biofilms on glass coverslips, the *cyaA2* mutant exhibited exaggerated biofilm formation. An example of the difference 24 h after induction of biofilm formation is shown (Fig. 2B versus C). A site-directed *cyaA* mutant, *cyaA4*, exhibited phenotypes similar to those noted above, indicating that the transposon itself is not required for the hyperattachment phenotypes (data not shown). Together, these results suggest that *cyaA* mutants have elevated levels of both cell surface and bacterium-to-bacterium interactions. Although this shows that mutation of a predicted catabolite repression gene confers phenotypes similar to those

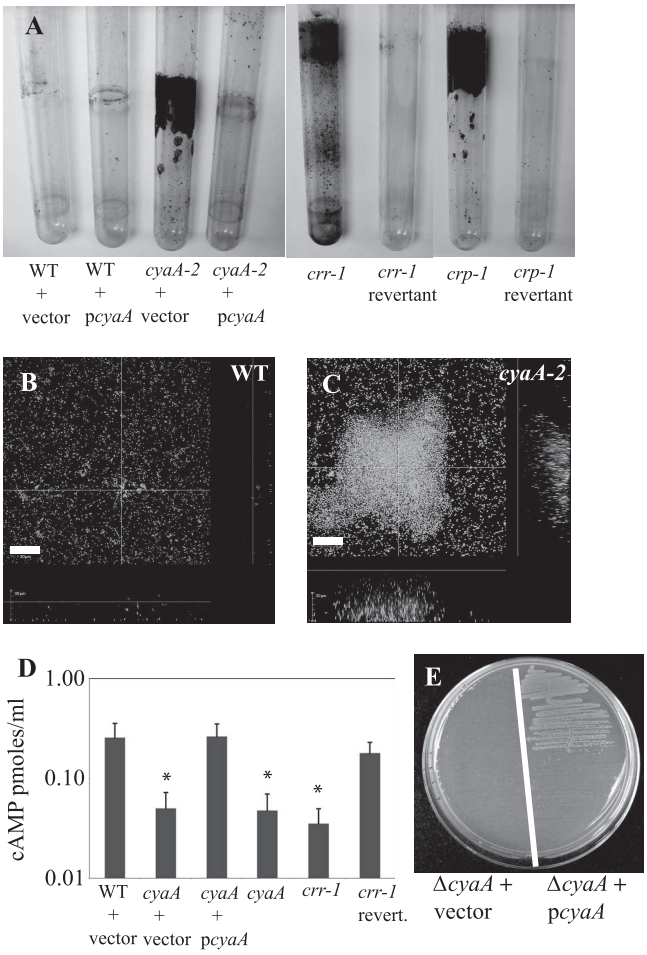

FIG. 2. *S. marcescens* catabolite repression genes regulate biofilm formation and cAMP production. (A) Biofilms formed on test tubes that were grown at 30°C on a rotor for 14 to 15 h. Results are shown for mutation and complementation of *cyaA*, *crr*, and *crp* mutant strains. These tubes are representative images of reproducible biofilm phenotypes. WT, wild type. (B, C) Confocal microscopy of Syto-9-stained biofilms formed on a glass coverslip under constant-flow conditions at room temperature for 24 h. Panel B is a micrograph of the wild type, and panel C shows the *cyaA2* mutant strain. Bar = 30 μm. This experiment was repeated with consistent results with two replicates per genotype per experiment. (D) Intracellular levels of cAMP, as determined by ELISA. Results are shown for a representative experiment done with triplicate independent samples per genotype. Error bars represent 1 standard deviation. *, $P < 0.05$. (E) Complementation of *E. coli* Δ*cyaA* mutant phenotypes for growth on M63 medium with glycerol as a sole carbon source by the *S. marcescens cyaA* gene on a multicopy plasmid.

TABLE 2. Biofilm formation is regulated by cAMP and cAMP-associated proteins

Genotype or condition[a]	Biofilm level[b]	Growth level[c]
No bacteria	0.12 ± 0.02	ND
WT	0.79 ± 0.08	++/++
WT with pMQ131	0.70 ± 0.34	++/++
WT with pRMQS157	0.62 ± 0.32	ND
cyaA	10.12 ± 1.06	−/+
cyaA with pMQ131	13.88 ± 2.02	ND
cyaA with pRMQS157	0.82 ± 0.26	++/++
crr	4.96 ± 0.54	+/+
crr revertant	0.59 ± 0.15	++/++
crp	19.04 ± 0.95	−/−
crp revertant	0.46 ± 0.15	++/++
fimC	0.25 ± 0.05	++/++
fimC with 2% glucose	0.63 ± 0.09	ND
cyaA fimC	0.58 ± 0.17	ND
crr fimC	0.60 ± 0.06	ND
crp fimC	0.58 ± 0.17	ND
WT with P_{lac}-*fimABCD*	0.44 ± 0.11	ND
WT with P_{lac}-*fimABCD* plus glucose	0.35 ± 0.02	ND
cyaA with P_{lac}-*fimABCD*	0.46 ± 0.05	ND
cyaA with 2% mannose	2.67 ± 1.10	ND
cyaA with 2% methyl α-D-mannopyranoside	0.42 ± 0.06	ND

[a] WT, wild type. pMQ131 is an empty vector. pRMQS157 is pMQ131 with *cyaA* from *S. marcescens*.

[b] Values are mean A_{590} levels ± standard deviations for crystal violet-stained biofilms from triplicate independent cultures. Experiments were repeated at least three times, and results from a representative experiment are shown.

[c] The symbol(s) before the slash represents growth in M63-0.2% citrate-0.006% casein amino acids for 48 h at 30°C. The symbol(s) after the slash indicates growth in the same medium supplemented with 1 mM cAMP. ND, not determined; ++, ≥50% of wild-type growth (A_{600}); +, 5 to 49% of wild-type growth; −, no obvious growth (<5% of wild-type growth).

caused by growth of the wild-type strain with glucose, we wished to confirm that this *cyaA* homolog codes for a protein that regulates cAMP levels.

The *cyaA* gene codes for an adenylate cyclase. Our model for increased levels of biofilm formation by *cyaA* mutants predicts that increased biofilm formation is a result of reduced cAMP levels. Enzyme-linked immunosorbent assays (ELISAs) were used to determine whether the *cyaA* gene identified is required for regulation of cAMP production. Levels of cAMP were reproducibly fivefold lower in the *cyaA2* mutant strain than in the wild-type strain ($P < 0.05$) (Fig. 2D). This defect was restored by the addition of p*cyaA* (pRMQS157) (Fig. 2D).

To further assess whether the *S. marcescens cyaA* gene codes for a functional adenylate cyclase, we determined whether it could complement mutant defects of a *cyaA* mutation of *E. coli*. The *E. coli cyaA* gene codes for a bona fide adenylate cyclase (9). CyaA activity is known to be essential for *E. coli* growth on many carbon sources, including glycerol (9). We found that whereas a Δ*cyaA* mutant of *E. coli* with an empty vector (pMQ131) was unable to grow on M63 minimal medium with glycerol as a sole carbon source, the same strain with the *S. marcescens cyaA* gene on a plasmid (pRMQS157) was able to grow on this medium (Fig. 2E). Mutations in *S. marcescens cyaA* also confer carbon source utilization defects in *S. marcescens* that can be rescued by pRMQS157 or exogenous cAMP, as expected from an adenylate cyclase mutant (Table 2). These findings support the model in which the *cyaA* gene of *S. marcescens* codes for a functional adenylate cyclase.

Mutations of catabolite repression genes *crr* and *crp* increase *S. marcescens* biofilm formation. To confirm that the cAMP-dependent CRS is important for biofilm formation in *S. marcescens*, mutations were made in other genes involved in the catabolite repression signaling machinery. Predicted homologs of the PTS gene (*crr*) and the CRP gene (*crp*) were disrupted by targeted mutagenesis, generating the *crr-1* and *crp-1* mutant strains (Table 1). The *crp* gene from our strain of *S. marcescens* was cloned (GenBank accession number EU183232), and sequence analysis suggests that it codes for a protein with predicted 99% amino acid identity with CRP from *E. coli*, 98% with *Yersinia pestis*, and 95% with CRP from *Vibrio cholerae*. The *crr* homolog from *S. marcescens* has already been sequenced by another group (64). For a complementation control, "revertant" strains in which the suicide plasmids that had disrupted *crr* and *crp* were lost and the wild-type genes were restored were made.

The *crr* gene codes for a PTS component, $EIIA^{Glc}$, that regulates adenylate cyclase activity in *E. coli* and *S. enterica* serovar Typhimurium. To determine whether the *crr-1* mutant had reduced cAMP levels, ELISAs were performed. Analysis of triplicate independent cultures revealed that the *crr-1* mutant had reduced levels of intracellular cAMP that were similar to *cyaA2* levels; both exhibited ~5-fold reductions compared to the wild type and the *crr* revertant (Fig. 2D). Moreover, mutation of *crr* confers a *cyaA* mutant-like biofilm phenotype (Fig. 2A and Table 2). Normal biofilm formation was observed in the *crr* revertant, demonstrating that the increased biofilm phenotype was dependent on the *crr-1* mutation rather than a secondary mutation elsewhere in the chromosome (Fig. 2A and Table 2).

CRP is a transcription factor required for the CRS, and its activity is regulated by cellular pools of cAMP through the formation of a cAMP-CRP complex. Mutation of *crp* leads to altered nutritional requirements for *S. marcescens* growth in minimal medium that could not be rescued by exogenous cAMP (Table 2). We observed that the *crp-1* mutant exhibited a significant increase in biofilm formation ($P < 0.001$) (Fig. 2A and Table 2). As with the *crr* revertant mentioned above, a restoration of normal biofilm formation in the *crp* revertant strain indicates that the increased biofilm phenotype was dependent on the *crp-1* mutation rather than on a second-site mutation (Fig. 2A and Table 2).

Type 1 fimbriae are necessary for the enhanced biofilm phenotype exhibited by *cyaA*, *crr*, and *crp* mutants. We hypothesized that the hyperbiofilm phenotype caused by growth in glucose or by mutation of catabolite repression genes was due to altered gene expression of a biofilm-promoting factor(s). The increased expression of this factor would enable *S. marcescens* to better adhere to abiotic substrates. To determine the mechanism by which the CRS regulates biofilm formation, we took a genetic approach. Mutations of *crp*, *cya*, or *crr* led to matte colonies that were very dense and slid across the surface of an agar plate when prodded, whereas wild-type colonies were smooth, shiny, and pliable. We predicted that the mechanisms for increased biofilm formation and altered colony morphology were dependent upon the same factor(s). This colony morphology phenotype was chosen for suppressor anal-

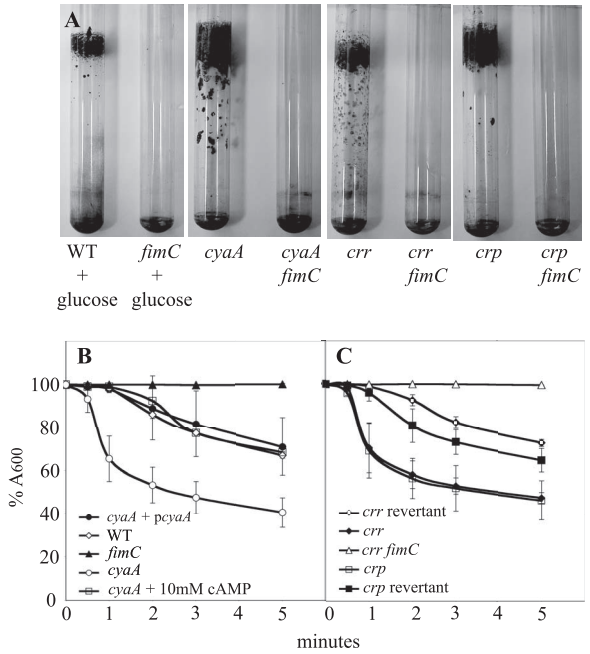

FIG. 3. Fimbriae are necessary for catabolite repression mutant biofilm phenotypes. (A) Mutation of *fimC* suppressed the hyperbiofilm phenotype caused by high levels of glucose or mutation of catabolite repression genes. For panels B and C, a kinetic assessment of yeast agglutination was performed. Positive agglutination results in lower absorbance readings. Here, absorbance is shown relative to time zero. WT, wild type. (B) The ability of *S. marcescens* to agglutinate yeast was dependent on fimbriae (*fimC*). Mutation of *cyaA* led to an increase in yeast agglutination that can be complemented by wild-type *cyaA* on a plasmid (pRMQS157) or by the addition of exogenous cAMP. (C) Kinetic agglutination of yeast by *crr-1*, mutants, and control strains. A *crr fimC* double mutant did not aggregate yeast (identical results were found with *cya fimC* and *crp fimC* double mutants [not shown]). All experiments were done with triplicate independent cultures on at least three different days, and the representative data from 1 day's experiment are shown. Error bars represent 1 standard deviation.

ysis because it provides higher throughput than a screen for suppressors of the biofilm phenotype on glass test tubes. Random mutations were made in a *crp-1* background by using a mariner-based transposon. Approximately 30 suppressors of this dense-colony phenotype were identified from 35,000 candidates. These *crp*(Su) (*s*uppressor of *crp-1*) mutants generate smooth and pliable colonies similar to those of the wild type. Thus far, multiple *crp*(Su) mutations have been mapped to genes in the *fimABCD* operon, which is required for generation of type 1 fimbriae in *S. marcescens*. One *crp*(Su) mutation has been mapped to *fimB*, four independent *crp*(Su) mutations were found in *fimC*, and two independent *crp*(Su) mutations were mapped to *fimD*.

Previous studies have indicated that type 1 fimbrial surface adhesins coded by the *fimABCD* operon are important for *S. marcescens* biofilm formation (31, 58). Additionally, several studies indicate that cAMP levels regulate fimbria production in other species of bacteria (15, 57). Directed mutations in the *fimC* usher protein tested the contribution of fimbriae to biofilm formation in cells grown in glucose and the *cya*, *crr*, and *crp* mutants (Fig. 3A and Tables 1 and 2). Fimbriae were found to be necessary for the hyperbiofilm phenotype on glass test tubes in all cases (Fig. 3A and Table 2). Consistently, downregulation of *fimABCD* expression by placement of the chromosomal *fimABCD* operon under the control of P_{lac} from *E. coli* also eliminated the hyperbiofilm effect caused by *cyaA* mutation and growth in glucose (Table 2). We have previously shown that integration of the pRMQS169 plasmid into the *S. marcescens* chromosome places *fimABCD* under the control of P_{lac} and supports fimbria production at levels indistinguishable from those for the wild-type in LB medium (58). We note that P_{lac} is positively regulated by cAMP-CRP in *E. coli* and consistently see lower levels of fimbriae in the *cyaA* mutant with P_{lac} driving *fimABCD* expression than in the wild type (data not shown).

S. marcescens type 1 fimbriae are inhibited by mannose (41, 54). It follows that if type 1 fimbriae are important for the biofilm phenotype of *cyaA* mutants then exogenous mannose should inhibit biofilm formation. This assumes that the mannose-binding portion of the fimbriae is also responsible for attachment to glass. Two percent mannose and 2% nonhydrolyzable mannose (methyl α-D-mannopyranoside) both severely inhibited *cyaA2* mutant biofilm-forming capacity (Table 2).

Since the wild-type strain does not form robust biofilms on glass test tubes and because fimbriae are required for the hyperbiofilm phenotype, we predicted that the reduced ability to produce cAMP (high glucose and *cya* and *crr* mutations) or respond to cAMP (*crp* mutation) would lead to increased fimbria production, leading to increased biofilm formation. To test this prediction, we used yeast agglutination as a functional assay of fimbria production. Agglutination of *Saccharomyces cerevisiae* by *S. marcescens* has been shown to require type 1 fimbriae (Fig. 3B) (58). The *cyaA2*, *crr-1*, and *crp-1* strains all produced significant increases in the amount of yeast agglutination at ≥1 min ($P < 0.03$) (Fig. 3B and C). The *cyaA fimC*, *crp fimC*, *crr fimC*, and *fimC* mutants completely lose their abilities to agglutinate yeast cells over the course of the experiment, confirming that hyperagglutination of yeast cells is *fimABCD* dependent (Fig. 3B and C and data not shown). As a complementation control, we found that the amount of *cyaA* mutant agglutination could be restored to wild-type levels by the wild-type *S. marcescens cyaA* gene on a plasmid (Fig. 3B). Similarly, the *crr* revertant and *crp* revertant strains established wild-type levels of agglutination (Fig. 3C).

TEM indicates that *cyaA* and *crp* mutants have elevated levels of type 1 fimbriae. Transmission electron microscopy (TEM) was used to directly visualize the extents of fimbriation of cells with *cyaA* and *crp* mutations. Negatively stained *cyaA2* mutant cells exhibited a dramatic, hyperpiliated phenotype compared to wild-type cells (Fig. 4A versus B). The majority of the *cyaA2* culture population had numerous fimbriae (92.1% ± 3.2% of cells; $n = 279$) (Fig. 4B). Wild-type cells were more sparsely decorated with fimbriae, with only 55% ($n = 140$) exhibiting surface pili (Fig. 4A), yet when grown with 2% glucose, the wild type exhibited an intermediate phenotype, with 80% ($n = 147$) appearing to be hyperfimbriate like the *cyaA* mutant. FimC is required to produce type 1 fimbriae in our strain background (58). Directed mutation of *fimC* conferred an absence of obvious fimbriae (<1% Fim^+; $n = 140$). A *cyaA2 fimC4* double mutant was generated and was largely devoid of surface pili (3.3%; $n = 272$), confirming that CyaA negatively regulates the production of type 1 fimbriae rather

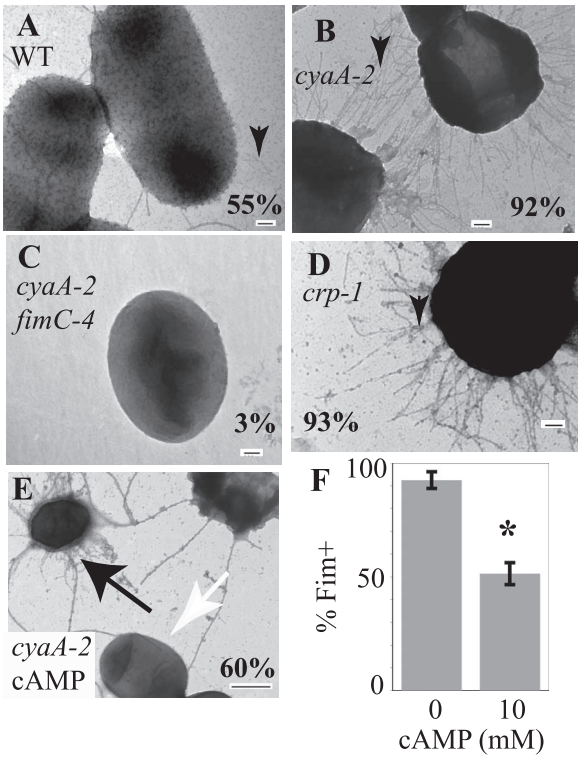

FIG. 4. TEM analysis of fimbria production. TEM micrographs of negatively stained bacteria grown under shaking conditions for 15 h in LB medium at 30°C. The percentage value represents the percentage of cells that exhibit fimbriae ($n \geq 140$ cells per strain) from multiple independent cultures. Black arrowheads indicate fimbriae. (A) Two wild-type (WT) cells with moderate levels of type 1 fimbriae are shown. (B) Most cells in the *cyaA2* mutant culture exhibit high levels of fimbriae. (C) *cyaA2 fimC4* double mutant cell devoid of fimbriae. (D) Bacteria from *crp-1* mutant cultures are highly fimbriate. (E) TEM micrograph of the *cyaA2* mutant grown in the presence of 10 mM cAMP. Note typical *cyaA* mutant morphology (black arrow) on one cell and lack of obvious fimbriae (white arrow) on another cell. The bar represents 100 nm, except in panel E, where the bar represents 500 nm. (F) Quantitation of TEM images from triplicate independent *cya-2* cultures showing the percentage of cells with fimbriae grown in medium supplemented with (10 mM) or without cAMP. The asterisk represents a significant reduction in the percentage of cells with surface fimbriae ($P < 0.02$).

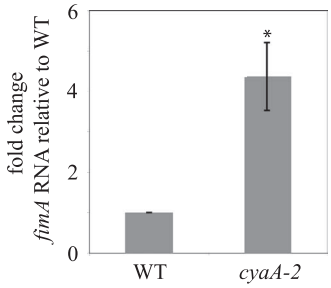

FIG. 5. Transcription of *fimA* is negatively regulated by adenylate cyclase. Real-time qPCR was used to assess transcription of type 1 fimbria subunit coding gene *fimA*. RNA levels relative to those for the wild type (WT) are shown. The difference is significant ($P < 0.03$). The experiment shows an average of three independent cultures made on different days. Error bars indicate standard errors.

than some other surface pili (Fig. 4C). Cells deficient in Crp were also tested for fimbria production by TEM, and a *cya*-like phenotype was observed (Fig. 4D). The percentage of the *crp-1* mutant culture exhibiting fimbriae was found to be 93.2% ± 0.5% ($n = 148$ cells), while a *crp-1 fimC2* mutant exhibited no fimbriae ($n = 28$).

To test the possibility that the mannose-associated reduction in biofilm formation (Table 2) was due to a reduction in fimbria production rather than an inhibition of fimbria binding activity, we assessed the *cyaA* mutant incubated with 2% mannose by TEM and found that >90% of the cells were hyperfimbriate ($n > 100$) and were indistinguishable from the *cyaA* mutant grown without mannose (data not shown).

Exogenous cAMP inhibits fimbria production. Since mutation of *cyaA* results in an increase in production of fimbriae and reduced levels of cAMP, it follows that cAMP has a negative impact on fimbria production. To test this prediction, the yeast agglutination assay was used. Levels of ≥10 mM but not ≤2 mM significantly reduced the ability of the *cyaA* mutant to agglutinate yeast at 30 seconds or more ($P < 0.05$) (Fig. 3B and data not shown). To confirm data from the indirect yeast agglutination assay (Fig. 3B), exogenous cAMP (up to 20 mM) was added to the *cyaA2* mutant cultures, and the effect upon fimbria production was assessed by TEM (Fig. 4E and F). The presence of cAMP (10 to 20 mM) reduced the percentage of fimbria-positive cells in a dose-dependent manner (98.2% at 0 mM, 59.5% at 10 mM, and 35.2% at 20 mM; $n > 100$ per condition) (Fig. 4F). The addition of 10 to 20 mM of cAMP did not lead to a uniform decrease in the number of fimbriae on all cells; many *cyaA* mutant cells were coated with fimbriae (Fig. 4E), whereas others were devoid of obvious fimbriae (Fig. 4E).

CyaA regulates transcription of fimbria genes. Our data suggest that cAMP has a negative impact on fimbria production. To test this model, real time qRT-PCR was utilized to determine *fimA* transcript levels. A *cyaA2* mutant was used as a strain with reduced cAMP for comparison to the wild-type strain. We found that the absence of Cya leads to a significant (4.4-fold) increase in *fimA* transcription ($P < 0.03$) (Fig. 5).

DISCUSSION

In this report, we demonstrate that biofilm formation by *S. marcescens* is regulated by CRS control of type 1 fimbrial adhesins. Both conditions and mutations that reduced cAMP production (*cyaA* and *crr*) or cAMP-mediated responses (*crp*) stimulated biofilm and attachment phenotypes. Contrary to what has been reported for *E. coli*, we observed that glucose stimulates *S. marcescens* biofilm formation and that this phenotype is sensitive to cAMP (Fig. 1A and B and Table 2), suggesting that biofilm formation is antagonized by cAMP and catabolite repression genes. We concluded that this effect was not a result of increased growth rates due to elevated glucose. Hyperbiofilms were not observed in wild-type cultures grown with both exogenous cAMP and glucose, though they grew at rates indistinguishable from those for cultures supplemented with only glucose (Fig. 1C).

We provide genetic and biochemical evidence that the *S. marcescens cyaA* homolog codes for a functional adenylate cyclase. The *cyaA* mutant was not only defective in production of cAMP but also unable to grow with citrate, glycerol, or

sucrose as a sole carbon source (Fig. 2C and D, Table 2, and data not shown). These growth phenotypes were rescued by exogenous cAMP (≥400 μM) or wild-type *cyaA* on a plasmid. Moreover, the *S. marcescens cyaA* gene, which is highly similar to *cyaA* from *E. coli*, was sufficient to complement cAMP-dependent mutant defects in a Δ*cyaA* mutant of *E. coli*.

The catabolite repression genes *crr* and *crp* were mutated for the first time in *S. marcescens*. We noted that mutation of the CRS component *crr* homolog led to a deficiency in intracellular cAMP levels similar to the *cyaA* mutant. This suggests that the *crr* gene product has a conserved function with the corresponding *E. coli* protein in positive regulation of adenylate cyclase activity. Interestingly, mutation of *crr* reduced, but did not eliminate, the ability of bacteria to grow in minimal media supplemented with citrate, whereas *cyaA* and *crp* mutants were completely unable to grow in citrate medium (Table 2). These data suggest that adenylate cyclase has some activity in a *crr* mutant background sufficient for partial growth in citrate medium and a less extreme hyperbiofilm phenotype; however, no difference between *cyaA* and *crr* mutants was evident when cAMP levels in stationary-phase cells were measured. As predicted, the *crp* mutant of *S. marcescens* was unable to grow on citrate (or sucrose or glycerol) as a sole carbon source, and this effect could not be rescued by exogenous cAMP. Together, mutational analysis of *cyaA*, *crr*, and *crp* and the effects of growth in exogenous glucose and cAMP indicate that the CRS of *S. marcescens* regulates biofilm formation. This suggests that extracellular carbon sources have a profound role in regulation of *S. marcescens* biofilm formation in vivo. Consistently, Rice and colleagues have shown a major role for carbon source type and availability in *S. marcescens* biofilm development (51).

While *crr* has a role in the CRS of *E. coli* and there is a link between the CRS and biofilm formation in several organisms, this is the first report to directly link a *crr* homolog (enzyme IIA^{Glc}) to regulation of biofilm formation. Recent work with other organisms shows that other PTS components play a role in biofilm formation. PTS enzyme I is important in regulation of *Vibrio cholerae* biofilm formation, but mutation of enzyme IIA^{Glc} had no effect on biofilm growth (22). A multicomponent, fructose-specific PTS component, FruI, has been found to positively regulate *Streptococcus gordonii* biofilm formation (35). Mutations in PTS component IIB (glucose specific) and enzyme IIC (cellobiose specific), but not enzyme IIA, of *Klebsiella pneumoniae* were isolated in a signature-tagged mutagenesis screen for genes required for attachment to extracellular matrix components (7).

In gram-negative bacteria, the effects of glucose and mutations in genes that code for catabolite repression functions have a range of effects on biofilm formation. The first evidence that a catabolite repression gene could contribute to biofilm formation was found in *Pseudomonas aeruginosa*, where mutation of the catabolite repression control gene (*crc*) led to a severe reduction in biofilm formation due to a reduction in type IV pilus production (42). Interestingly, Crc controls catabolite repression in a cAMP-independent manner (56, 68). *P. aeruginosa* and *S. marcescens* both control surface attachment through CRSs; however, Crc appears to be a positive regulator of surface adhesins, whereas CRS genes of *S. marcescens* are shown here to negatively regulate type 1 fimbria production. Likewise, *Salmonella enterica* serovar Enteritidis biofilm formation and virulence potential are stimulated by growth in glucose-rich medium, suggesting that lower levels of cAMP could influence these phenotypes (8). In *V. cholerae*, mutations of some CRS genes, including the CRP homolog, have negative impacts on biofilm formation (22, 33); however, these changes are thought to be mediated through alteration of exopolysaccharide production (22, 33). Lastly, a CRP homolog of *Shewanella oneidensis* has been found to regulate biofilm detachment in response to environmental stimuli (63).

Cyclic nucleotides have previously been reported to regulate pilus formation in other organisms (6, 30, 62). In *Salmonella enterica* serovar Typhimurium and *E. coli*, cAMP production was shown to be a positive regulator of type 1 fimbria production (15, 57), K99 pilus production (24), and *pap* pilus production (3). In *P. aeruginosa*, the CRP homolog Vfr is required for positive regulation of type IV pili (5), and cyclic di-GMP levels positively regulate CupA fimbria production (38).

We report here that the addition of exogenous cAMP was correlated with a reduction in the percentage of fimbriate bacterial cells in the *cyaA* mutant background, further supporting the conclusion that cAMP inhibits *S. marcescens* fimbria production (Fig. 4E and F). However, in this case, the extents of fimbriation varied greatly in those cells with fimbriae, with most cells displaying reduced levels similar to those in the wild type and a subset of cells remaining indistinguishable from the parental *cyaA* mutant (Fig. 4E). This pleiotropic effect suggests that either there is variable penetration of cAMP into *Serratia* cells or cAMP can modulate phase variation of fimbria production. Fimbria production in several species of bacteria is controlled in a phase-variable manner (6), though this phenomenon has never been shown to occur in *Serratia* spp.

Evidence presented here indicates that catabolite repression proteins direct fimbria production, but it is unknown whether there are intermediate regulators between CRP and *fimABCD* transcription. We found no obvious CRP consensus binding site in the region upstream of the *fimABCD* operon; however, we were able to find consensus sequences upstream of predicted sugar transporters. This suggests that the CRP consensus binding site is conserved in *S. marcescens*, as would be predicted by the >99% identity between the CRP proteins from *E. coli* and *S. marcescens*. We are currently screening for factors that may be intermediates between CRP and the *fimABCD* operon.

The significance of this report is that a central environmental carbon regulatory circuit, the CRS, plays a profound role in the surface behaviors of the opportunistic pathogen *S. marcescens* through regulation of a large surface adhesin. Carbon source and availability have a large impact on the way *S. marcescens* interacts with its environment and are critical elements in the surface behaviors of this organism. Carbon levels have previously been shown to affect *S. marcescens* biofilm formation (51). The interaction of *S. marcescens* with other organisms can also be regulated by carbon sources. One study showed that *S. marcescens* inhibitory effects on fungi proliferation can be suppressed by added glucose (54). In the human environment, glucose levels are elevated at several sites associated with infections, including the bloodstream, vaginal fluid, synovial fluid, nasopharyngeal tissue, and aqueous humor (16, 61). The presence of sugars in these environments can have a major impact on bacterial adherence (48). Under environmen-

tal conditions, *S. marcescens*-derived, polymer-degrading enzymes, such as cellulase and chitinases, could influence its surface attachment behaviors in niches with high levels of cellulose or chitin through alteration of sugar levels in its microenvironment (49). Future studies will include determining the role of the CRS in the control of biofilm formation and fimbria production in response to environmental cues.

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