2016

The catabolite repressor protein-cyclic AMP complex regulates csgD and biofilm formation in uropathogenic Escherichia coli

David A. Hufnagel
University of Michigan - Ann Arbor

Margery L. Evans
University of Michigan - Ann Arbor

Sarah E. Greene
Washington University School of Medicine in St. Louis

Jerome S. Pinkner
Washington University School of Medicine in St. Louis

Scott J. Hultgren
Washington University School of Medicine in St. Louis

See next page for additional authors

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology

The Catabolite Repressor Protein-Cyclic AMP Complex Regulates *csgD* and Biofilm Formation in Uropathogenic *Escherichia coli*

David A. Hufnagel,[a] Margery L. Evans,[a] Sarah E. Greene,[b] Jerome S. Pinkner,[b] Scott J. Hultgren,[b] Matthew R. Chapman[a]

Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, Michigan, USA[a]; Department of Molecular Microbiology and Center for Women's Infectious Disease Research, Washington University School of Medicine, St. Louis, Missouri, USA[b]

ABSTRACT

The extracellular matrix protects *Escherichia coli* from immune cells, oxidative stress, predation, and other environmental stresses. Production of the *E. coli* extracellular matrix is regulated by transcription factors that are tuned to environmental conditions. The biofilm master regulator protein CsgD upregulates curli and cellulose, the two major polymers in the extracellular matrix of uropathogenic *E. coli* (UPEC) biofilms. We found that cyclic AMP (cAMP) regulates curli, cellulose, and UPEC biofilms through *csgD*. The alarmone cAMP is produced by adenylate cyclase (CyaA), and deletion of *cyaA* resulted in reduced extracellular matrix production and biofilm formation. The catabolite repressor protein (CRP) positively regulated *csgD* transcription, leading to curli and cellulose production in the UPEC isolate, UTI89. Glucose, a known inhibitor of CyaA activity, blocked extracellular matrix formation when added to the growth medium. The mutant strains Δ*cyaA* and Δ*crp* did not produce rugose biofilms, pellicles, curli, cellulose, or CsgD. Three putative CRP binding sites were identified within the *csgD-csgB* intergenic region, and purified CRP could gel shift the *csgD-csgB* intergenic region. Additionally, we found that CRP binded upstream of *kpsMT*, which encodes machinery for K1 capsule production. Together our work shows that cAMP and CRP influence *E. coli* biofilms through transcriptional regulation of *csgD*.

IMPORTANCE

The catabolite repressor protein (CRP)-cyclic AMP (cAMP) complex influences the transcription of ~7% of genes on the *Escherichia coli* chromosome (D. Zheng, C. Constantinidou, J. L. Hobman, and S. D. Minchin, Nucleic Acids Res 32:5874–5893, 2004, https://dx.doi.org/10.1093/nar/gkh908). Glucose inhibits *E. coli* biofilm formation, and Δ*cyaA* and Δ*crp* mutants show impaired biofilm formation (D. W. Jackson, J.W. Simecka, and T. Romeo, J Bacteriol 184:3406–3410, 2002, https://dx.doi.org/10.1128/JB.184.12.3406-3410.2002). We determined that the cAMP-CRP complex regulates curli and cellulose production and the formation of rugose and pellicle biofilms through *csgD*. Additionally, we propose that cAMP may work as a signaling compound for uropathogenic *E. coli* (UPEC) to transition from the bladder lumen to inside epithelial cells for intracellular bacterial community formation through K1 capsule regulation.

Escherichia coli is a multifaceted Gram-negative bacterium that is equipped for survival in disparate environments ranging from the gastrointestinal and urinary tracts to waterways and soil (1). Growth of *E. coli* can occur with either planktonic or biofilm lifestyles, the transition between which requires a highly organized shift in gene expression. The biofilm lifestyle is characterized by adherence, slow growth, resistance to environmental insults, and production of an extracellular matrix (ECM) (2). CsgD is the major biofilm regulator of *E. coli* and of many other bacteria of the *Enterobacteriaceae* family (3–5), and it promotes the production of two *E. coli* surface appendages, the amyloid fiber curli and the fibrous glycan chain cellulose (2). CsgD represses the transcription of motility-associated genes, while mediating self and surface attachment (6–8).

E. coli is found in ~80% of urinary tract infections (UTI), more than any other causative agent, and has the ability to thrive in the bladder and urinary tract environments (9). During UTI, uropathogenic *E. coli* (UPEC) uses various means to evade and colonize the host. Curli and other factors function in establishing an initial bacterial titer (10). Once in the bladder, *E. coli* utilizes type 1 pili to invade bladder epithelial cells (11). *E. coli* bacteria then form a biofilm-like structure known as an intracellular bacterial community (IBC), and cells are coated by the ECM component K1 for protection from host cell recognition (12). The ability of *E. coli* to recognize multiple environments is critical for production of various UTI-related appendages at the various stages of UTI and IBC formation.

The catabolite repressor protein (CRP) is activated by the alarmone cyclic AMP (cAMP) (13). cAMP is produced by adenylate cyclase (CyaA) in low-glucose environments, leading to activation of CRP (13). Glucose inhibits *E. coli* biofilm formation, and Δ*cyaA* and Δ*crp* laboratory strains have a decreased propensity for biofilm formation, suggesting that cAMP and CRP are important for regulating biofilm development (14, 15). A genome-wide CRP-cAMP analysis revealed that 7% of *E. coli*'s transcriptome is altered

Received 31 August 2016 **Accepted** 27 September 2016

Accepted manuscript posted online 3 October 2016

Citation Hufnagel DA, Evans ML, Greene SE, Pinkner JS, Hultgren SJ, Chapman MR. 2016. The catabolite repressor protein-cyclic AMP complex regulates *csgD* and biofilm formation in uropathogenic *Escherichia coli*. J Bacteriol 198:3329–3334. doi:10.1128/JB.00652-16.

Editor: T. J. Silhavy, Princeton University

Address correspondence to Matthew R. Chapman, chapmanm@umich.edu.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.00652-16.

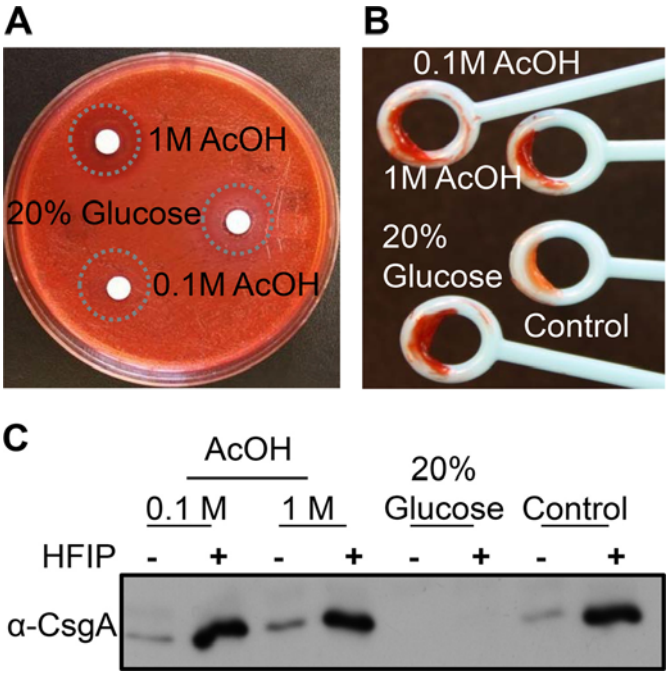

FIG 1 Glucose inhibits Congo red binding and CsgA production. (A) *E. coli* K-12 BW25113 was evenly spread on plates with YESCA and CR and incubated for 48 h at 26°C. Acetic acid or 20% glucose were added to a sterile filter paper disk that was placed on the plate immediately after the bacteria were spread. (B) After growth, bacteria adjacent to the filter paper disk were scraped off the plate using an inoculating loop and imaged. (C) A representative Western blot from cells isolated from the plates with YESCA and CR that were grown for 48 h at 26°C shows that CsgA levels are decreased in the presence of glucose. HFIP was added to some samples to depolymerize curli fibers and allow CsgA monomers to migrate into the gel. AcOH, acetic acid.

by CRP activation, including *csgD* transcription (16). These studies used a laboratory strain of *E. coli* and hence did not investigate UPEC-specific virulence factors, such as type 1 pili and the K1 capsule (11, 16). Interestingly, CRP was later found to be an inhibitor of type 1 pili formation in UPEC strains (17). Additionally, Zheng et al. (16) identified three divergent and putative CRP binding cites upstream of *csgD*. We found that glucose inhibits biofilm formation independent of pH by decreasing *csgD* expression in a CRP-cAMP-dependent pathway. We also show that CRP binds to the *csgD* promoter region. Additionally, CRP binds upstream of the UPEC *kps* operon, which encodes the secretion pore for the K1 capsule. This work provides a better mechanistic understanding of a cAMP-dependent regulon for UPEC strains.

MATERIALS AND METHODS

Strains and growth conditions. Overnight cultures were grown while shaking at 37°C overnight. All *E. coli* strain UTI89 mutants were made via lambda red recombination (18). All primers and strain names can be found in the supplemental material. Strains in the manuscript are referred to by their mutation.

For the glucose addition plates (Fig. 1), wild-type (WT) *E. coli* K-12 strain BW25113 cells with an optical density at 600 nm (OD_{600}) of 0.1 were spread with glass beads. Sterile filter paper disks saturated with 15 μl of 0.1 M acetic acid, 1 M acetic acid, 20% (wt/vol) D-glucose, or H_2O were added to the cells. The plates were incubated at 26°C for 48 h. Blue inoculating loops were used to collect cells around the sterile filter paper disks and were imaged (Fig. 1A). Cells were normalized by optical density and serially diluted, and then 4-μl dots were plated on LB medium and incubated at 37°C overnight (see Fig. S1 in the supplemental material).

Rugose biofilms were grown as previously described (19) with a few modifications. Overnight cultures were diluted to an OD_{600} of 1.0 and 4 μl were dotted and incubated at 26°C for 48 h on YESCA (1 g yeast extract-sucrose and 10 g Casamino Acids per liter) with Congo red (CR; 50 μg per liter) plates supplemented with 15 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and 0.1% (wt/vol) galactose (pH 6.7). Images were captured on an Olympus SZX16 microscope with an Olympus DP72 camera. Pellicles were prepared by adding 2 μl of the overnight culture into 2 ml of YESCA medium and grown statically at 26°C for 48 h (20). Pellicles were stained with 2 ml 0.1% (wt/vol) crystal violet for 5 min, followed by 3 washes with 2 ml H_2O, and then were imaged on a Fluorchem 8900 (Alpha Innotech, San Leandro, CA).

Western blot analysis. Western blotting was performed as previously described (19, 21). Rugose biofilms were collected with blue inoculating loops and resuspended in 1 ml 50 mM potassium phosphate (KPi) buffer (pH 7.2) before being vortexed briefly. From this, 150 μl of cells with an OD_{600} of 1 were spun down and then resuspended in 2× SDS-running buffer for detection of CsgD or in 1,1,1,3,3,3-hexafluo-2-propanol (HFIP) for detection of CsgA on Western blots. HFIP-treated cells were incubated in a Savant SPD SpeedVac at 45°C for 45 min prior to the addition of 2× SDS-running buffer. Samples were heated at 95°C for 10 min, and then 8 μl was separated by SDS-PAGE on a 15% gel for 45 min at 25 mA. For the detection of CsgA, Western blots were transferred onto a polyvinylidene difluoride (PVDF) membrane with a semidry transfer apparatus at 25 V for 10 min at room temperature, whereas CsgD Western blots were wet transferred onto a nitrocellulose membrane in 25 mM *N*-cylclohexyl-3-aminopropanesulfonic acid (CAPS) transfer buffer with 10% methanol at 12 V overnight at 4°C for detection of CsgD. All blots were blocked overnight in Tris-buffered saline with Tween 20 (TBST) with 5% skim milk at 4°C. Primary antibody treatment was for 1 h (1:8,000) dilution for anti-CsgA (rabbit), 1:5,000 dilution for anti-CsgD (rabbit), and 1:10,000 dilution for anti-σ70 (mouse) followed by three 5-min washes in TBST. Secondary antibody treatment was for 1 h (1:15,000 LI-COR secondary anti-rabbit and anti-mouse antibodies with infrared [IR] dyes). Blots were imaged on a an LI-COR Odyssey CLX imager.

β-Galactosidase assays. Miller assays were performed as previously described (19, 22). Rugose biofilms were resuspended in KPi buffer and diluted 1:10. Reaction buffer (90 μl) and 7 μl of diluted cells were incubated at 30°C for 20 min prior to the addition of 20 μl of 4 mg/ml *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG). The reaction was stopped by the addition of 50 μl 1 M Na_2CO_3 when a yellow color developed in the reaction. The absorbance of reactions at 420 and 550 nm on a Tecan Infinite 200 plate reader were measured along with the OD_{600} of the cell dilutions.

Statistics for β-galactosidase assays. All strains were assayed in biological triplicate with pRJ800 (empty vector [EV] with no promoter upstream of *lacZ*), pRJ800-*csgD* (*csgD* promoter upstream of *lacZ*), and pRJ800-16S (16S promoter upstream of *lacZ*). The averages of the EV readings were subtracted from each biological triplicate of the pRJ800-*csgD* and -16S readings in all strains. Averages and standard deviations were obtained from biological triplicates with the EV values subtracted.

CRP purification. CRP was cloned from uropathogenic *E. coli* strain UTI89 into the plasmid pGEM (Promega). A His tag was incorporated into the N terminus of the protein. The construct was sequenced for confirmation. Gene expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG), and the protein was then purified with nickel chromatography and an imidazole gradient.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assay (EMSA) reaction mixtures contained 1 μl 10× EMSA buffer (LI-COR), 1 μl 25 mM dithiothreitol (DTT)–2.5% (vol/vol) Tween 20, 0.5 μl 1 μg/μl poly(dI·dC), 2 μl DNA (30 ng/μl), 4 μl of 0.2 mg/ml CRP-His, 1 μl 2 mM cAMP, 1 μl glycerol, and H_2O to 10 μl. Samples were run on a

1.2% agarose gel or a 6% nondenaturing acrylamide gel at 100 V. Agarose gels were run for 90 min and poststained with ethidium bromide. Acrylamide gels were prerun for 60 min in an ice bath at 100 V and then run for 80 min at 100 V for the fluorescent oligonucleotides or 120 min for the unlabeled intergenic DNA regions. Fluorescently labeled oligonucleotides were ordered from Integrated DNA Technologies. Each oligonucleotide was labeled with the LI-COR IR800 dye. The IR800-labeled oligonucleotides were annealed to unlabeled reverse complements in 10 mM Tris (pH 8.0), 100 mM NaCl, and 100 μM EDTA at a ratio of 1 μM labeled oligonucleotide to 2 μM unlabeled oligonucleotide. The annealed oligonucleotides were used at a final concentration of 10 nM. The IR800-labeled oligonucleotides were visualized by scanning at 800 nm using a LI-COR Odyssey system.

RESULTS AND DISCUSSION

Curli and cellulose production in UPEC is coordinated, so we began our investigation into the role of the CRP-cAMP complex in *E. coli* biofilm development by defining how glucose impacts curli production by using *E. coli* strain K-12 BW25113, which does not produce cellulose. BW25113 was spread evenly on a YESCA plate and grown at 26°C to induce curli expression. The plate was supplemented with Congo red (CR), an amyloid binding dye, to detect curli production. Sterile paper disks saturated with 20% (wt/vol) glucose were then laid on the plate. The plate was incubated for 48 h at 26°C before the cells adjacent to the paper disc (indicated by the dotted circles in Fig. 1A) were removed using a sterile inoculating loop (Fig. 1A and B). CR binding decreased in cells grown near the glucose-containing disk (Fig. 1B). Since glucose metabolism can acidify the media and acidic by-products from glycolysis have been reported to alter *Salmonella* biofilm formation (23), bacteria were also grown in the presence of disks saturated in acetic acid (1.0 or 0.1 M) (Fig. 1A and B). However, acetic acid had no effect on CR binding (Fig. 1A and B). We then asked whether the production of CsgA, the major subunit of curli fibers, was altered by glucose or acetic acid. Western blot analysis demonstrated that CsgA levels decreased in the presence of glucose but not in the presence of acetic acid (Fig. 1C). Therefore, decreased CsgA was the result of glucose-mediated repression and not growth medium acidification. Cellulose is also bound by CR; however, *E. coli* K-12 does not produce cellulose; therefore, CR binding might be attributed exclusively to curli production (24). Neither glucose nor acetic acid negatively affected viability (see Fig. S1 in the supplemental material).

Next, we tested whether CRP plays a role in regulating CsgD-dependent biofilm formation in the UPEC isolate UTI89. Wild-type UTI89 stained red when grown on plates with YESCA and Congo red (CR) supplemented with 0.1% galactose and buffered with 2-(*N*-morpholino)ethanesulfonic acid (MES) (Fig. 2A). The Δ*crp* and Δ*cyaA* mutants are known to grow inefficiently on peptides and amino acids; therefore, galactose was added to the plates with CR to ensure the presence of a utilizable carbon source (25). However, the addition of galactose to the medium decreased wrinkling by WT UTI89 (Fig. 2A; see also Fig. S2 in the supplemental material) (19). We hypothesize this is due to increased metabolic activity as nutrient limitation was previously shown to increase CsgD activity and biofilm formation (23). The plates were buffered with MES to counter unwanted potential consequences from acidification of the media during the growth of the bacteria (23).

The Δ*cyaA* and Δ*crp* mutant colonies appeared white on plates with YESCA and CR that were supplemented with galactose and buffered with MES (Fig. 2A). The lack of CR binding in Δ*cyaA* and Δ*crp* strains indicates that neither curli nor cellulose was produced as both ECM components bind to CR (3, 26). Exogenous addition of cAMP restored CR binding and partially restored rugose biofilm formation and curli production in the Δ*cyaA* mutant strain but not the Δ*crp* mutant (Fig. 2A). The exogenous addition of cAMP resulted in increased wrinkling in the rugose biofilm and increased curli production in WT strains (Fig. 2A). Consistent with the observed CR binding, the addition of cAMP resulted in

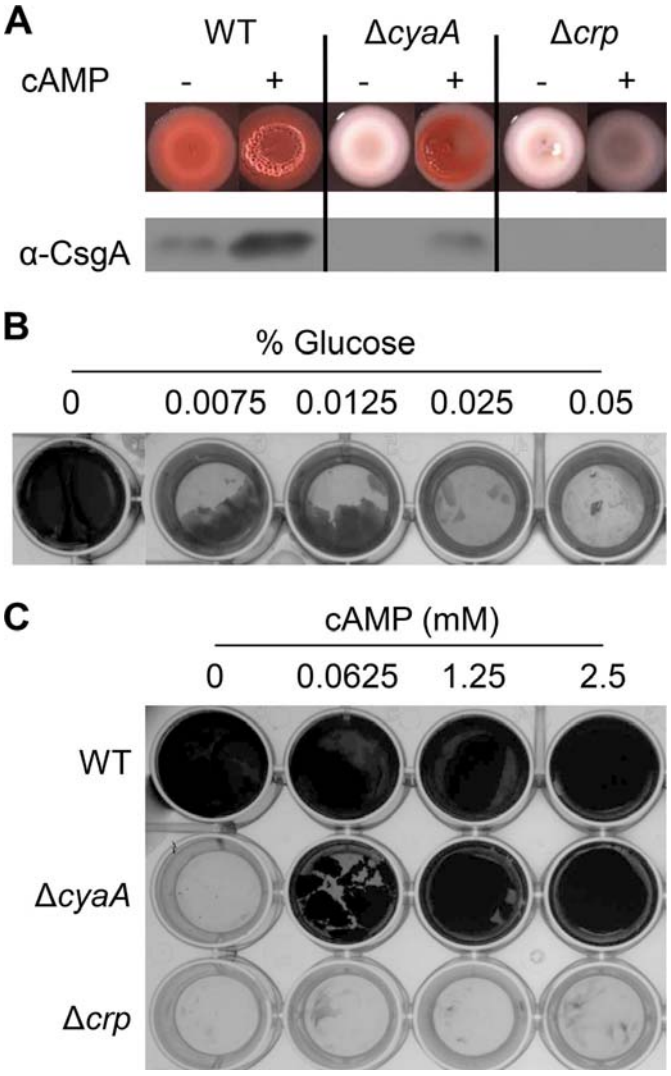

FIG 2 The CRP-cAMP complex regulates UTI89 biofilm formation and *csgD* expression. (A) Overnight UTI89 cultures were spotted 6 mm from a paper disk that was treated with water or cAMP. The paper disk appears to the left of each of the colonies in the pictures. Colonies were grown for 48 h at 26°C before being imaged (top) and then prepared for whole-cell Western analysis using an anti-CsgA antibody (bottom). (B) To assess pellicle formation, WT UTI89 was inoculated into YESCA liquid broth containing the indicated concentrations of glucose and grown statically at 26°C for 48 h. Pellicles were stained with crystal violet and washed with H_2O prior to imaging as described in Materials and Methods. (C) WT, Δ*cyaA*, and Δ*crp* strains of UTI89 were inoculated into YESCA liquid broth containing the indicated concentrations of cAMP. Pellicles did not form without cAMP in the Δ*cyaA* and Δ*crp* strains, but cells were still growing, and after 48 h, the ODs of the liquid broths were similar to the OD of the WT liquid broth under the pellicle.

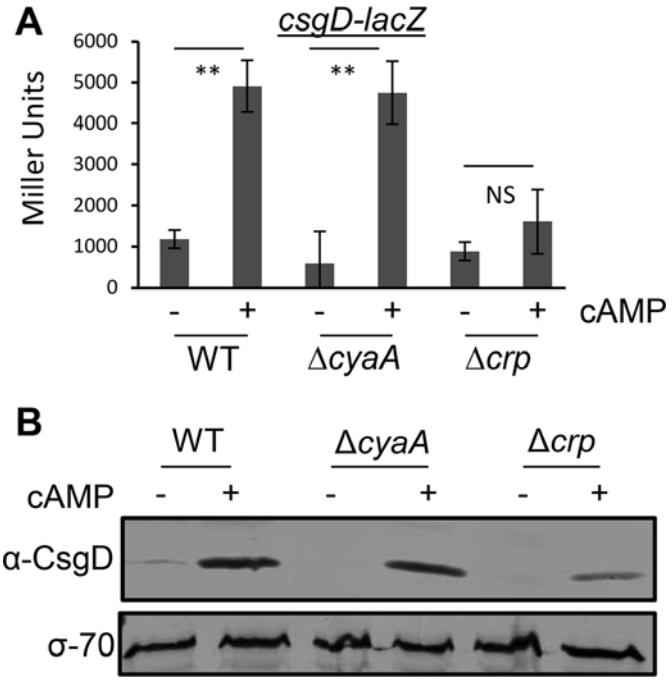

FIG 3 The CRP-cAMP complex controls *csgD* transcription and CsgD levels. (A) β-Galactosidase assays were performed on rugose biofilms of UTI89 WT, Δ*cyaA*, and Δ*crp* strains transformed with pRJ800, pRJ800-*csgD*, and pRJ800-16S (*rrsA*) grown at 26°C for 24 h on MES-buffered YESCA medium with 0.1% galactose in the presence or absence of cAMP (supplemented on a paper disk). (B) Anti-CsgD Western blots of rugose biofilms grown on YESCA media for 24 h at 26°C in the presence or absence of cAMP (supplemented on a paper disk). Anti-σ-70 was used as a loading control. **, $P < 0.02$ as determined by the Student's two-tailed *t* test.

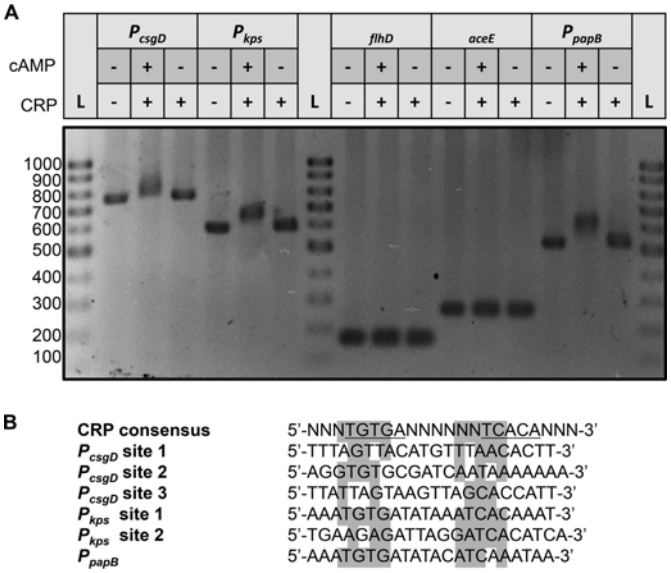

FIG 4 The CRP-cAMP complex binds upstream of *csgD* and *kps*. (A) The *csgD-csgB* intergenic region or the region upstream of *kps* was incubated with or without CRP and/or cAMP, and then was loaded on a 1.2% (wt/vol) agarose gel. Bands were visualized via ethidium bromide staining. cAMP was either not added (−) or added at 200 μM (+). (B) Putative CRP binding sites upstream of *csgD* and *kps* that have similarity to the consensus CRP binding site.

detectable amounts of CsgA produced by the Δ*cyaA* mutant but not the Δ*crp* mutant (Fig. 2A, lower panel).

E. coli pellicles are curli- and cellulose-dependent biofilms that form at the air-liquid interface of static cultures (10, 27). To assess whether glucose inhibits pellicle biofilm formation, UTI89 was grown in liquid cultures in the absence or presence of glucose for 48 h at 26°C. Pellicles were visualized by staining with crystal violet. UTI89 formed robust pellicles in the absence of glucose (Fig. 2B). However, pellicle formation was inhibited by the addition of glucose (Fig. 2B). Also, the Δ*cyaA* and Δ*crp* strains did not form pellicles (Fig. 2C). The addition of cAMP to the media restored pellicle formation in the Δ*cyaA* strain but not in the Δ*crp* strain (Fig. 2C).

The addition of glucose repressed CR binding and CsgA levels in WT BW25113 and WT UTI89 (Fig. 1 and 2). Furthermore, neither the UTI89 Δ*cyaA* nor Δ*crp* mutants formed a biofilm in either the colony or pellicle biofilm model (Fig. 1 and 2). Therefore, we hypothesized that the expression of the master biofilm regulator CsgD was reduced in the Δ*cyaA* and Δ*crp* mutants. To assess the expression of *csgD*, we used a *lacZ* fusion in which *lacZ* was cloned downstream of the *csgD* promoter consisting of the entire *csgD-csgB* intergenic region. β-Galactosidase activity was measured as a proxy for *csgD* expression. The addition of exogenous cAMP increased expression levels of *csgD* in WT and Δ*cyaA* strains but not in Δ*crp* strains (Fig. 3A). Western blot analysis revealed increased CsgD levels in the WT strain compared to those in Δ*crp* and Δ*cyaA* strains and in the presence of cAMP in WT and Δ*cyaA* strains. Unexpectedly, there was a modest increase in CsgD in the Δ*crp* mutant when it was supplemented with cAMP, suggesting there was either a change in physiology in the presence of this nucleotide or a secondary responder to cAMP present in UTI89 (Fig. 3B). The lack of CsgD expression in Δ*cyaA* and Δ*crp* mutants is consistent with the curli and cellulose defects, since CsgD regulates both extracellular matrix components (26, 28). The positive correlation between *csgD* expression and CRP is consistent with previous findings reported from studies in *E. coli* (14–16) but is in contrast with findings reported from studies in *Salmonella enterica* (23). *S. enterica* and *E. coli* reside in different hosts, host niches, and extrahost environments (29, 30), which may influence the evolution of glucose-dependent or -independent *csgD* phenotypes for these two species.

Next, we tested whether CRP can bind directly to the region between *csgD* and *csgB* (*csgD-csgB* intergenic region). Electrophoretic mobility assays were conducted using the *csgD-csgB* intergenic region as the binding target. Binding of CRP to the intergenic region was assessed in the presence or absence of CRP and cAMP. CRP alone had no effect on DNA migration in the gel (Fig. 4A; see also Fig. S3A in the supplemental material). However, a band shift was observed when CRP and cAMP were incubated with the *csgD-csgB* intergenic region and then analyzed on a 1.2% agarose gel (Fig. 4A). Purified CRP was run on an SDS-PAGE gel to verify the purity of CRP (Fig. S3C). Regions upstream of the *flhD* and *aceE* genes, which are not subject to CRP regulation, were used as negative controls. Neither the *flhD* nor the *aceE* DNA targets were shifted by the addition of CRP and cAMP (Fig. 4A; see also Fig. S3A). However, the region upstream of *papB* (which contains a CRP binding site [17]) exhibited slower mobility when incubated with CRP-cAMP, indicating a binding interaction (Fig. 4A; see also Fig. S3A). Interestingly, a smear was observed when binding of CRP to the *csgD-csgB* intergenic region was analyzed on

a 6% nondenaturing acrylamide gel (see Fig. S3A). The *papB* positive control exhibited distinct shifts while the *flhD* and *aceE* negative controls did not shift (see Fig. S3A). Together, our results suggest a binding interaction between CRP and the *csgD-csgB* intergenic region, albeit a likely weaker interaction than occurs between CRP and *papB*.

Using the known CRP binding consensus sequence, we identified three putative CRP binding sites upstream of UTI89 *csgD* (Fig. 4B). Three sites were chosen based on highly conserved nucleotides in the CRP binding consensus sequence (31) (Fig. 4B). We tested the ability of CRP to bind directly to the putative CRP binding sites within the *csgD-csgB* intergenic region using fluorescently labeled oligonucleotides corresponding to each predicted binding site in the intergenic region. Intriguingly, the addition of CRP-cAMP to each of the CRP binding sites in the intergenic region resulted in a slight change in mobility for only the curli site one (*csg*1) (see Fig. S3B in the supplemental material). Combined with our results demonstrating a weak interaction between CRP and the full-length *csgD-csgB* intergenic region (Fig. 4A; see also Fig. S3B), the observance of a weak interaction between CRP and the *csg*1 oligonucleotide suggests that the CRP binding sites within the *csgD-csgB* intergenic region may act synergistically to bind CRP. The complex regulation and the plethora of overlapping transcription factor binding sites upstream of *csgD* (32, 33) make it difficult to rule out the possibility that CRP indirectly regulates expression of the curli operons, despite the observations that CRP binds upstream of *csgD* and transcription of *csgD* is induced by cAMP.

During an infection, host cells recognize *E. coli* lipopolysaccharide and upregulate cAMP production by 600% in exocytic compartments (34, 35). During UTI or bladder infection, UPEC invades bladder epithelial cells in a type 1 pilus-dependent manner (36). While type 1 pili are repressed by CRP, the cAMP concentration is low enough in the urine that UPEC can produce type 1 pili and evade bladder epithelial cells during infection (17, 37). Once inside bladder epithelial cells, UPEC begins a program leading to the formation of intracellular bacterial communities (IBCs), which are biofilm-like communities encased in a type 1 sialic acid (K1) capsule that are resistant to host-immune system detection (11, 12). K1 capsule mutants (Δ*kps*) do not form IBCs and are killed by neutrophils (12). The K1 capsule gene *kpsM* encodes the transporter for the K1 capsule, whereas *kpsT* encodes the ATPase that is necessary for export of the capsule (38). How curli might be involved in IBC development is unknown; however, there is a modest correlation between curli production and initial titers of UPEC during UTI (10). Curli are important for many biofilm models, and cAMP-mediated curli production might potentially play a role during biofilm-like IBC formation (2). If cAMP acts to initiate IBC formation, then cAMP should also upregulate other IBC matrix components, such as the K1 capsule.

The region upstream of *kpsMT* in UTI89 contains two predicted CRP binding sites (Fig. 4B). The region upstream of *kps* that spans both predicted CRP binding sites was amplified by PCR and used in CRP binding assays. The addition of CRP and cAMP resulted in a gel shift of the *kps* promoter target DNA relative to DNA alone (Fig. 4A; see also Fig. S3A in the supplemental material). Furthermore, the addition of CRP-cAMP to oligonucleotides corresponding to one of the predicted CRP binding sites upstream of *kpsM* resulted in a clear slowed mobility indicating a direct interaction between CRP and the upstream region of *kps* (see Fig. S3B). CRP also regulates the K5 *N*-acetylheparosan-type capsule in the probiotic *E. coli* Nissle 1917 (39). During a urinary tract infection, cAMP might modulate attachment to the bladder epithelium, epithelial cell invasion, and IBC formation, all by differentially regulating type 1 pili (17), the K1 capsule, and curli/cellulose.

ACKNOWLEDGMENTS

We thank Blaise Boles, Robert Bender, and William DePas for in-depth discussions on this project. We thank Ann Miller for use of her dissecting scope for colony images. We thank Ute Römling for supplying the anti-CsgD antibody.

This work was supported by a University of Michigan Rackham Dissertation Fellowship (to D.A.H.) and National Institutes of Health grant R01 GM118651 (to M.R.C.).

FUNDING INFORMATION

This work, including the efforts of Matthew R. Chapman, was funded by HHS | National Institutes of Health (NIH) (GM118651).

REFERENCES

1. **Hufnagel DA, Tukel C, Chapman MR.** 2013. Disease to dirt: the biology of microbial amyloids. PLoS Pathog **9:**e1003740. http://dx.doi.org/10.1371/journal.ppat.1003740.
2. **Hufnagel DA, Depas WH, Chapman MR.** 2015. The biology of the *Escherichia coli* extracellular matrix, p 249–267. *In* Ghannoum M, Parsek M, Whiteley M, Mukherjee P (ed), Microbial biofilms, 2nd ed. ASM Press, Washington, DC. http://dx.doi.org/10.1128/microbiolspec.MB-0014-2014.
3. **Hammar M, Arnqvist A, Bian Z, Olsen A, Normark S.** 1995. Expression of two *csg* operons is required for production of fibronectin- and Congo red-binding curli polymers in *Escherichia coli* K-12. Mol Microbiol **18:**661–670. http://dx.doi.org/10.1111/j.1365-2958.1995.mmi_18040661.x.
4. **Evans ML, Chapman MR.** 2014. Curli biogenesis: order out of disorder. Biochim Biophys Acta **1843:**1551–1558. http://dx.doi.org/10.1016/j.bbamcr.2013.09.010.
5. **Romling U, Bian Z, Hammar M, Sierralta WD, Normark S.** 1998. Curli fibers are highly conserved between *Salmonella* typhimurium and *Escherichia coli* with respect to operon structure and regulation. J Bacteriol **180:**722–731.
6. **Ogasawara H, Yamamoto K, Ishihama A.** 2011. Role of the biofilm master regulator CsgD in cross-regulation between biofilm formation and flagellar synthesis. J Bacteriol **193:**2587–2597. http://dx.doi.org/10.1128/JB.01468-10.
7. **Austin JW, Sanders G, Kay WW, Collinson SK.** 1998. Thin aggregative fimbriae enhance *Salmonella enteritidis* biofilm formation. FEMS Microbiol Lett **162:**295–301. http://dx.doi.org/10.1111/j.1574-6968.1998.tb13012.x.
8. **Zhou Y, Smith D, Leong BJ, Brannstrom K, Almqvist F, Chapman MR.** 2012. Promiscuous cross-seeding between bacterial amyloids promotes interspecies biofilms. J Biol Chem **287:**35092–35103. http://dx.doi.org/10.1074/jbc.M112.383737.
9. **Sabir S, Ahmad Anjum A, Ijaz T, Asad Ali M, Ur Rehman Khan M, Nawaz M.** 2014. Isolation and antibiotic susceptibility of *E. coli* from urinary tract infections in a tertiary care hospital. Pak J Med Sci **30:**389–392.
10. **Cegelski L, Pinkner JS, Hammer ND, Cusumano CK, Hung CS, Chorell E, Aberg V, Walker JN, Seed PC, Almqvist F, Chapman MR, Hultgren SJ.** 2009. Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. Nat Chem Biol **5:**913–919. http://dx.doi.org/10.1038/nchembio.242.
11. **Mulvey MA, Schilling JD, Hultgren SJ.** 2001. Establishment of a persistent *Escherichia coli* reservoir during the acute phase of a bladder infection. Infect Immun **69:**4572–4579. http://dx.doi.org/10.1128/IAI.69.7.4572-4579.2001.
12. **Anderson GG, Goller CC, Justice S, Hultgren SJ, Seed PC.** 2010. Polysaccharide capsule and sialic acid-mediated regulation promote biofilm-like intracellular bacterial communities during cystitis. Infect Immun **78:**963–975. http://dx.doi.org/10.1128/IAI.00925-09.

13. **Fic E, Bonarek P, Gorecki A, Kedracka-Krok S, Mikolajczak J, Polit A, Tworzydlo M, Dziedzicka-Wasylewska M, Wasylewski Z.** 2009. cAMP receptor protein from *Escherichia coli* as a model of signal transduction in proteins—a review. J Mol Microbiol Biotechnol **17:**1–11. http://dx.doi.org/10.1159/000178014.
14. **Jackson DW, Simecka JW, Romeo T.** 2002. Catabolite repression of *Escherichia coli* biofilm formation. J Bacteriol **184:**3406–3410. http://dx.doi.org/10.1128/JB.184.12.3406-3410.2002.
15. **Sutrina SL, Daniel K, Lewis M, Charles NT, Anselm CKE, Thomas N, Holder N.** 2015. Biofilm growth of *Escherichia coli* is subject to cAMP-dependent and cAMP-independent inhibition. J Mol Microbiol Biotechnol **25:**209–225. http://dx.doi.org/10.1159/000375498.
16. **Zheng D, Constantinidou C, Hobman JL, Minchin SD.** 2004. Identification of the CRP regulon using *in vitro* and *in vivo* transcriptional profiling. Nucleic Acids Res **32:**5874–5893. http://dx.doi.org/10.1093/nar/gkh908.
17. **Muller CM, Aberg A, Straseviciene J, Emody L, Uhlin BE, Balsalobre C.** 2009. Type 1 fimbriae, a colonization factor of uropathogenic *Escherichia coli*, are controlled by the metabolic sensor CRP-cAMP. PLoS Pathog **5:**e1000303. http://dx.doi.org/10.1371/journal.ppat.1000303.
18. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
19. **Hufnagel DA, DePas WH, Chapman MR.** 2014. The disulfide bonding system suppresses CsgD-independent cellulose production in *Escherichia coli*. J Bacteriol **196:**3690–3699. http://dx.doi.org/10.1128/JB.02019-14.
20. **Andersson EK, Bengtsson C, Evans ML, Chorell E, Sellstedt M, Lindgren AE, Hufnagel DA, Bhattacharya M, Tessier PM, Wittung-Stafshede P, Almqvist F, Chapman MR.** 2013. Modulation of curli assembly and pellicle biofilm formation by chemical and protein chaperones. Chem Biol **20:**1245–1254. http://dx.doi.org/10.1016/j.chembiol.2013.07.017.
21. **Zhou Y, Smith DR, Hufnagel DA, Chapman MR.** 2013. Experimental manipulation of the microbial functional amyloid called curli. Methods Mol Biol **966:**53–75. http://dx.doi.org/10.1007/978-1-62703-245-2_4.
22. **Miller J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. **Gerstel U, Romling U.** 2001. Oxygen tension and nutrient starvation are major signals that regulate *agfD* promoter activity and expression of the multicellular morphotype in *Salmonella* typhimurium. Environ Microbiol **3:**638–648. http://dx.doi.org/10.1046/j.1462-2920.2001.00235.x.
24. **Serra DO, Richter AM, Hengge R.** 2013. Cellulose as an architectural element in spatially structured *Escherichia coli* biofilms. J Bacteriol **195:**5540–5554. http://dx.doi.org/10.1128/JB.00946-13.
25. **Lengeler J, Drews G, Schlegel H.** 1999. Biology of the prokaryotes. Wiley-Blackwell, Hoboken, NJ.
26. **Zogaj X, Nimtz M, Rohde M, Bokranz W, Romling U.** 2001. The multicellular morphotypes of *Salmonella* typhimurium and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol Microbiol **39:**1452–1463. http://dx.doi.org/10.1046/j.1365-2958.2001.02337.x.
27. **Hung C, Zhou YZ, Pinkner JS, Dodson KW, Crowley JR, Heuser J, Chapman MR, Hadjifrangiskou M, Henderson JP, Hultgren SJ.** 2013. *Escherichia coli* biofilms have an organized and complex extracellular matrix structure. mBio **4:**e00645-13. http://dx.doi.org/10.1128/mBio.00645-13.
28. **DePas WH, Hufnagel DA, Lee JS, Blanco LP, Bernstein HC, Fisher ST, James GA, Stewart PS, Chapman MR.** 2013. Iron induces bimodal population development by *Escherichia coli*. Proc Natl Acad Sci U S A **110:**2629–2634. http://dx.doi.org/10.1073/pnas.1218703110.
29. **van Elsas JD, Semenov AV, Costa R, Trevors JT.** 2011. Survival of *Escherichia coli* in the environment: fundamental and public health aspects. ISME J **5:**173–183. http://dx.doi.org/10.1038/ismej.2010.80.
30. **Winfield MD, Groisman EA.** 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. Appl Environ Microbiol **69:**3687–3694. http://dx.doi.org/10.1128/AEM.69.7.3687-3694.2003.
31. **Munch R, Hiller K, Barg H, Heldt D, Linz S, Wingender E, Jahn D.** 2003. PRODORIC: prokaryotic database of gene regulation. Nucleic Acids Res **31:**266–269. http://dx.doi.org/10.1093/nar/gkg037.
32. **Ogasawara H, Yamada K, Kori A, Yamamoto K, Ishihama A.** 2010. Regulation of the *Escherichia coli csgD* promoter: interplay between five transcription factors. Microbiology **156:**2470–2483. http://dx.doi.org/10.1099/mic.0.039131-0.
33. **Gerstel U, Park C, Romling U.** 2003. Complex regulation of *csgD* promoter activity by global regulatory proteins. Mol Microbiol **49:**639–654.
34. **Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ.** 2015. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol **13:**269–284. http://dx.doi.org/10.1038/nrmicro3432.
35. **Song JM, Bishop BL, Li GJ, Grady R, Stapleton A, Abraham SN.** 2009. TLR4-mediated expulsion of bacteria from infected bladder epithelial cells. Proc Natl Acad Sci U S A **106:**14966–14971. http://dx.doi.org/10.1073/pnas.0900527106.
36. **Martinez JJ, Mulvey MA, Schilling JD, Pinkner JS, Hultgren SJ.** 2000. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. EMBO J **19:**2803–2812. http://dx.doi.org/10.1093/emboj/19.12.2803.
37. **Greene SE, Pinkner JS, Chorell E, Dodson KW, Shaffer CL, Conover MS, Livny J, Hadjifrangiskou M, Almqvist F, Hultgren SJ.** 2014. Pilicide ec240 disrupts virulence circuits in uropathogenic *Escherichia coli*. mBio **5:**e02038. http://dx.doi.org/10.1128/mBio.02038-14.
38. **Whitfield C, Roberts IS.** 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol Microbiol **31:**1307–1319. http://dx.doi.org/10.1046/j.1365-2958.1999.01276.x.
39. **Yan H, Bao F, Zhao L, Yu Y, Tang J, Zhou X.** 2015. Cyclic AMP (cAMP) receptor protein-cAMP complex regulates heparosan production in *Escherichia coli* strain nissle 1917. Appl Environ Microbiol **81:**7687–7696. http://dx.doi.org/10.1128/AEM.01814-15.