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Adenylate Cyclase Mutations Rescue the *degP* Temperature-Sensitive Phenotype and Induce the Sigma E and Cpx Extracytoplasmic Stress Regulons in *Escherichia coli*

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Inactivation of the gene encoding the periplasmic protease DegP confers a high-temperature-sensitive phenotype in *Escherichia coli*. We have previously demonstrated that a *degP* mutant of *E. coli* strain CBM (W3110 *pldA1*) is not temperature sensitive and showed that this was most likely due to constitutive activation of the sigma E and Cpx extracytoplasmic stress regulons in the parent strain. In this study, further characterization of this strain revealed a previously unknown cryptic mutation that rescued the *degP* temperature-sensitive phenotype by inducing the extracytoplasmic stress regulons. We identified the cryptic mutation as an 11-bp deletion of nucleotides 1884 to 1894 of the adenylate cyclase-encoding *cyaA* gene (*cyaAΔ11*). The mechanism in which *cyaAΔ11* induces the sigma E and Cpx regulons involves decreased activity of the mutant adenylate cyclase. Addition of exogenous cyclic AMP (cAMP) to the growth medium of a *cyaAΔ11* mutant strain that contains a Cpx- and sigma E-inducible *degP-lacZ* reporter fusion decreased β-galactosidase expression to levels observed in a *cyaA*$^+$ strain. We also found that a *cyaA* null mutant displayed even higher levels of extracytoplasmic stress regulon activation compared to a *cyaAΔ11* mutant. Thus, we conclude that the lowered concentration of cAMP in *cyaA* mutants induces both sigma E and Cpx extracytoplasmic stress regulons and thereby rescues the *degP* temperature-sensitive phenotype.

Gram-negative bacteria such as *Escherichia coli* have developed a set of response regulons designed to maintain cell viability under a variety of stressful environmental conditions. Under conditions that increase the amount of misfolded or aggregated proteins in the periplasm, such as those generated under conditions of heat shock or the overproduction of outer membrane porins, a pair of regulons known as sigma E and Cpx are induced. These regulons prevent cell death by inducing the expression of genes that encode chaperones and proteases that function to refold or degrade misfolded proteins in the periplasm (36).

The sigma E extracytoplasmic stress response regulon initially described by Erickson and Gross (16) utilizes a regulated intramembrane proteolytic pathway (5) to transduce a stress signal from the periplasm to the cytoplasm, whereupon genes in the sigma E regulon are induced. A cascade of events initiating with detection of aberrant proteins in the periplasm results in the release of sigma E normally sequestered by the inner-membrane-spanning protein RseA to the cytoplasm. Release allows members of the sigma E regulon to be upregulated, including; *rpoE*, *rseA*, *rseB*, and *rseC* (14). Other sigma E-controlled genes include *degP*, which encodes the protease/chaperone DegP (46); *fkpA*, which encodes the peptidyl/prolyl isomerase FkpA (31); *rpoH*, which encodes σ^H of the cytoplasmic heat shock response regulon (16); and many others involved in performing basic cellular functions such as metabolism and phospholipid biosynthesis (37).

The Cpx regulon is a three-component regulatory system composed of an inner-membrane-spanning histidine kinase, CpxA; a periplasmic CpxA repressor, CpxP; and a cytoplasmic response regulator, CpxR. Under conditions that induce the Cpx regulon, such as elevated pH (33), absence of phosphatidylethanolamine in the cell envelope (29), or overexpression of envelope proteins such as outer membrane lipoprotein NlpE (43), inner membrane lipoprotein YafY (32), or pilus subunits (19, 21), induction of the Cpx regulon is initiated by activation of CpxA via removal of the periplasmic inhibitory protein CpxP (35). Once activated, CpxA phosphorylates and thereby activates the cytoplasmic transcription factor CpxR, which in turn directs the transcription of genes in the Cpx regulon. These genes include *cpxP*, *cpxA*, and *cpxR* (10, 15, 35) in addition to *degP*, *ppiA*, and *ppiD*, which encode periplasmic peptidyl-prolyl isomerases (12, 34), and *dsbA*, which encodes a periplasmic disulfide oxidoreductase (9).

DegP relieves the deleterious effects of misfolded proteins in the periplasm by acting as both a protease (27) and a chaperone, a dual function dependent upon the environmental temperature (44). The importance of DegP in *E. coli* is confirmed by the finding that *degP* transcription is induced by both the Cpx and sigma E regulons and that, in the absence of DegP, the cell exhibits a temperature-sensitive (ts) phenotype whereby it cannot grow at a temperature of 42°C or greater (26). Rescue of the ts phenotype can be accomplished by overexpression of the non-heat shock-inducible DegP homologue DegQ (47). This finding suggests that an overlapping function of proteases exists in the *E. coli* periplasm; however, *degQ* is not part of either extracytoplasmic stress regulon. The *degP* ts phenotype has also been shown to be rescued by extragenic expression of the *sohA* gene (1), which encodes a putative

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transcriptional regulator protein, by multicopy expression of *sohB*, which encodes a putative periplasmic serine protease (2), and by overproduction of membrane proteins such as NlpE (43) and certain outer membrane proteins, including OmpX, OmpC, and OmpF (28).

We previously reported that *E. coli* W3110 *degP* mutant cells were rescued from the ts phenotype when transduced with the *pldA1* allele from strain CBM (7), which encodes inactive outer membrane phospholipase A (OMPLA) (25). We further showed that these transductants were induced for both sigma E and Cpx extracytoplasmic stress regulons. This result suggested that rescue of the ts phenotype was caused by a compensating effect of Cpx and sigma E regulon induction, a mechanism also involved in the rescue of the *degP* ts phenotype by overexpression of NlpE (11, 43) and YafY (32).

In the studies reported here, we identified a mutation closely linked with *pldA1* in strain CBM. The mutation is an 11-bp deletion of the *cyaA* gene (*cyaAΔ11*) encoding the enzyme adenylate cyclase. We provide evidence that the lowered concentration of cyclic AMP (cAMP) resulting from the partial activity of the truncated adenylate cyclase encoded by *cyaAΔ11* is responsible for induction of the sigma E and Cpx extracytoplasmic regulons and consequent rescue of the *degP* ts phenotype. Our results indicate that the altered expression of one or more cAMP receptor protein (CRP)-regulated genes likely induces the sigma E and Cpx regulons and serve to explain previous evidence obtained by Delaney (13) that describes the increased thermotolerance of *cyaA* mutants relative to *cyaA*$^+$ strains.

MATERIALS AND METHODS

Media, growth conditions, and antibiotics. All *E. coli* strains were grown in Luria-Bertani (LB) broth or agar at 30°C, 37°C, 41°C, or 42°C as specified. When required, medium was supplemented with kanamycin (50 μg/ml) and/or tetracycline (10 μg/ml). Strains were also grown on MacConkey agar and on MacConkey agar base supplemented with rhamnose (0.2%).

Bacterial strains. The bacterial strains used in this study are listed in Table 1.

β-Galactosidase assays. Cultures were grown overnight at 30°C in LB broth with the appropriate antibiotic and then subcultured 1:125 into LB broth and grown until an optical density at 600 nm (OD_{600}) between 0.3 and 0.5 was reached. Assays were performed according to Slauch et al. (42) whereby a 1-ml culture volume was permeabilized with 20 μl chloroform and 10 μl 0.1% sodium dodecyl sulfate. The resultant mixture was assayed for β-galactosidase activity (nanomoles per minute per milliliter per OD_{600} unit) by recording the change in OD_{420} over time upon addition of 100 μl of a permeabilized cell suspension to a reaction solution containing 10-mg/ml *o*-nitrophenyl-β-D-galactopyranoside. β-Galactosidase assay results are presented as the average activity of three or four transductants under the growth conditions specified. Error bars represent the standard deviation of these averages.

Measurement of growth under heat shock conditions. Strains were grown overnight at 30°C in LB broth containing an appropriate antibiotic. They were then subcultured to an OD_{600} of 0.01 and incubated for 1 h at 30°C. Next, half of the volume of each culture was placed in a second Erlenmeyer flask. The duplicate cultures were incubated at 30°C and 42°C and the OD_{600} was assessed over time. The effect of exogenous cAMP on growth was determined as described above, except that following 1 h of incubation at 30°C, each culture was subcultured 1:2 into four flasks containing LB broth supplemented with 0, 2, 4, and 8 mM cAMP. Each culture was then incubated at 42°C, and the OD_{600} was monitored for 6 h.

Marker exchange mutagenesis. Transfer of alleles by marker exchange was performed using the suicide vector pMRS101 (40). Alleles *pldA1*, *pldA*::*kan*, and *cyaAΔ11* were amplified by PCR and cloned into pBluescript II SK(+). Following verification of the correct sequence, the alleles were subcloned into pMRS101 and electroporated into *E. coli* XL-1 Blue cells. The recombinant plasmids were digested with NotI to remove the pBR322 origin of replication of the plasmid, self-ligated, and transformed into *E. coli* SM10λ*pir* by selecting for streptomycin resistance. Donor (SM10λ*pir* containing the recombinant pMRS101) and recipient strains for conjugation were grown overnight, subcultured 1:10 in brain heart infusion (BHI) broth without antibiotic, and incubated at 37°C for 1 h prior to conjugation. The conjugation reaction was initiated by mixing a 500-μl volume of donor and recipient cells. Cells in the mixture were collected by centrifugation, and the pellet resuspended in 100 μl was applied to a prewarmed BHI plate and incubated for 3.5 h at 32°C. Half of the cells were scraped off the BHI conjugation pool plate and streaked onto LB agar containing streptomycin and tetracycline to select for potential integrants, which were then streak purified on the same medium. Selection of integrants in which the pMRS101 plasmid has been lost due to a second recombination event was accomplished by growth on LB agar containing 10% sucrose. Verification of allelic exchange was accomplished by PCR.

Transduction protocol. Transductions were performed according to Miller (30).

RESULTS

Induction of the extracytoplasmic stress regulons in *E. coli* strain CBM is caused by a mutation near *pldA*. In a previous paper by Langen et al. (25), extracytoplasmic stress regulons sigma E and Cpx were shown to be induced in *E. coli* strain CBM, which contains the *pldA1* allele encoding a mutated form of OMPLA. In order to further examine the extracytoplasmic stress regulon-inducing effect of *pldA1*, the *pldA* null allele *pldA20*::*kan* (4) was used. We expected that the *pldA* null allele would induce the sigma E and Cpx extracytoplasmic stress regulons in a manner similar to that of nonfunctional OMPLA encoded by *pldA1*.

Induction of the sigma E and Cpx extracytoplasmic stress regulons was assessed by *lacZ* reporter fusion analysis whereby the amount of β-galactosidase activity generated from a chromosomal copy of the *lacZ* gene fused to the sigma E- and Cpx-inducible *degP* promoter was quantified. All strains were *degP* mutants and were grown under conditions of heat shock (41°C) because induction of the extracytoplasmic regulons had previously been shown to be the greatest at this temperature (25). The *degP pldA20*::*kan* mutant strain (TS14A) failed to induce the extracytoplasmic regulons to the same level as the *degP pldA1* mutant strain (GL123A) (Fig. 1). Instead, the level of β-galactosidase activity generated by the *pldA20*::*kan* mutant strain was comparable to that of the *pldA*$^+$ strain (GL143), suggesting the presence of a second previously unknown mutation in strain CBM. The existence of a cryptic mutation was verified by construction of two strains by marker exchange. In the first mutant, GL383, *pldA20*::*kan* replaced *pldA1*. In the second mutant, GL393, *pldA1* replaced *pldA20*::*kan*. *degP-lacZ* reporter gene analysis showed that *pldA1* in a non-CBM background (GL393) failed to induce the extracytoplasmic stress regulons, whereas the level of regulon activity in the CBM *pldA20*::*kan* mutant strain (GL383) remained high (Fig. 1).

A second mutation in strain CBM is located in the adenylate cyclase gene *cyaA*. Using a three-factor cross with *metE*::Tn*10* and *pldA20*::*kan* and the expression level of the *degP-lacZ* fusion, the unknown gene was mapped to a position upstream of *pldA*, distal to *metE*. The cryptic allele was then identified as a mutant *cyaA* gene by P1*vir* transduction mapping employing a set of strains containing Tn*5* and Tn*10* insertions in genes close to *pldA*. During this analysis, it was noted that both the cryptic allele and a *cyaA*::Tn*5* allele conferred a white colony phenotype when the strains were grown on MacConkey agar containing various non-phosphotransferase system sugars, in-

TABLE 1. Bacterial strains used in this study

Strain or plasmid	Description	Reference or source
Strains		
XL-1 blue	*recA* (*recA1 lac endA1 gyrA96 thi-1 hsdR17 supE44 relA1* [F′ *proAB lac1^{q}ZΔM15* Tn*10*])	6
W3110	IN(*rrnD-rrnE*)*1 rph-1*	20
SP850	*relA1 spoT* Δ(*cya-1400*)::Km *thi-1* e14$^-$ λ$^-$	41
GE1050	MC4100 Δ*crp*::*cam*	39
CBM	W3110 *pldA1 cyaA*Δ11	7, this study
GL93	CBM *degP*::*kan*	25
GL94	W3110 *degP*::*kan*	25
GL101	W3110 Δ(*argF-lac*)*U169*	25
GL102	CBM Δ(*argF-lac*)*U169*	25
GL104	GL101 *degP*::*kan*	25
GL111	GL101 λRS88 (*porfA-dsbA-lacZ*)	25
GL112	GL101 λRS88 (*fkpA-lacZ*)	25
GL113	GL101 λRS88 (*degP-lacZ*)	25
GL123	GL102 λRS88 (*degP-lacZ*)	25
GL123A	GL123 *degP*::*tet*	This study
GL143	GL104 λRS88 (*degP-lacZ*)	25
GL306	GL93 Δ(*argF-lac*)*U169 nadA*::Tn*10*	This study
GL308	GL94 Δ(*argF-lac*)*U169 pldA*::*kan nadA*::Tn*10*	This study
GL383	GL93 Δ(*argF-lac*)*U169 pldA*::*kan* (ME) *cyaA*Δ*11* λRS88 (*degP-lacZ*)	This study
GL393	GL94 Δ(*argF-lac*)*U169 pldA1* (ME) λRS88 (*degP-lacZ*)	This study
GL111A	GL111 *degP*::*tet*	This study
GL112A	GL112 *degP*::*tet*	This study
TS2	W3110 Δ(*argF-lac*)*U169 metE*::Tn*10* λRS88 (*degP-lacZ*)	This study
TS2-1	W3110 Δ(*argF-lac*)*U169* λRS88 (*degP-lacZ*)	This study
TS2A	TS2 *metE*$^+$ *degP*::*tet*	This study
TS2AB	TS2 *metE*$^+$ *degP*::*tet crp*::*cam*	This study
TS3	W3110 Δ(*argF-lac*)*U169 degP*::*tet* λRS88 (*degP-lacZ*)	This study
TS13	TS2 *cyaA*Δ*11*	This study
TS15	TS2 *metE*$^+$ *cyaA*::Tn*5*	This study
TS16	TS2 *metE*$^+$ *cyaA1400*::*kan*	This study
TS18	TS2 *metE*$^+$ *cyaA*Δ*11* (ME)	This study
TS14A	TS2 *metE*$^+$ *pldA*::*kan degP*::*tet*	This study
TS15A	TS2 *metE*$^+$ *cyaA*::Tn*5 degP*::*tet*	This study
TS16A	TS2 *metE*$^+$ *cyaA1400*::*kan degP*::*tet*	This study
TS18A	TS2 *metE*$^+$ *cyaA*Δ*11* (ME) *degP*::*tet*	This study
TS15AB	TS15A *crp*::*cam*	This study
TS16AB	TS16A *crp*::*cam*	This study
TS18AB	TS18A *crp*::*cam*	This study
TS21A	GL111 *cyaA*::Tn*5 degP*::*tet*	This study
TS22A	GL111 *cyaA1400*::*kan degP*::*tet*	This study
TS23A	GL111 *cyaA*Δ*11 degP*::*tet*	This study
TS31A	GL112 *cyaA*::Tn*5 degP*::*tet*	This study
TS32A	GL112 *cyaA1400*::*kan degP*::*tet*	This study
TS33A	GL112 *cyaA*Δ*11 degP*::*tet*	This study
TS40	GL143 *degP*::*tet*	This study
TS41	GL143 *pldA1 degP*::*tet*	This study
Plasmids		
pBluescript II SK(+)	Cloning vector; Amp^r *lac* promoter (*lacZ*α) f1 ColE1	Stratagene
pMRS101	Suicide vector; Strep^r Amp^r *sacBR oriE1 oriR6K mobRK2*	40

cluding rhamnose (data not shown). The mutant *cyaA* gene (*cyaA*Δ*11*) encoding the enzyme adenylate cyclase contains an 11-bp frameshift deletion of nucleotides 1884 to 1894 (Fig. 2). The protein encoded by *cyaA*Δ*11* is truncated due to the introduction of a stop codon 4 codons downstream of the deletion in the open reading frame (Fig. 2). The resultant adenylate cyclase protein is 631 amino acids in length, 217 amino acids shorter than wild-type adenylate cyclase.

The *cyaA*Δ*11* mutation induces expression of the sigma E and Cpx extracytoplasmic stress regulons. According to *degP-lacZ* reporter gene fusion analysis, the level of induction of *degP* in *cyaA*Δ*11* mutant strains was greater than twofold higher than in *cyaA*$^+$ strains, indicating that the *cyaA*Δ*11* mutation induces the sigma E and/or Cpx extracytoplasmic stress regulons (Fig. 3A). To verify the sigma E and Cpx regulon-inducing effect of mutant adenylate cyclases, we utilized two additional *cyaA* mutant alleles, *cyaA*::Tn*5* (23) and the *cyaA* null allele *cyaA1400*::*kan* (41) (Fig. 3A). The *cyaA*::Tn*5* strain exhibited induction levels similar to that of *cyaA*Δ*11*, whereas the *cyaA1400*::*kan* null strain generated induction levels greater than five times that of *cyaA*Δ*11*.

The mutant adenylate cyclases cause induction of both the sigma E and Cpx regulons, as shown by the increased β-galactosidase activity in *cyaA* mutant strains that contain *lacZ* re-

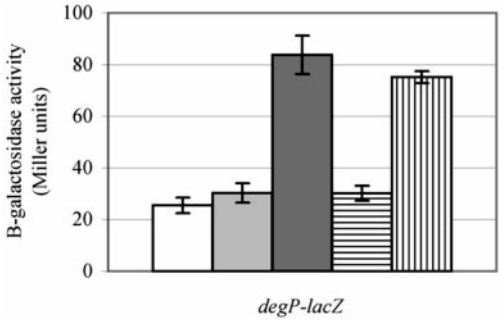

FIG. 1. An uncharacterized mutation is responsible for induction of the sigma E and/or Cpx regulons in strain CBM. *degP* mutant strains GL143 (*pldA*$^+$; white bar), TS14A (*pldA*::*kan*; light gray bar), GL383 (*pldA*::*kan* mutant strain GL306 by marker exchange; dark gray bar), GL393 (*pldA1* mutant strain GL308; horizontally striped bar), and GL123A (*pldA1*; vertically striped bar) were grown in LB broth at 41°C, and the amount of β-galactosidase activity generated from a *degP-lacZ* chromosomal fusion was assayed.

porter gene fusions under the control of either Cpx (*rdoA-dsbA-lacZ*) (3)- or sigma E (*fkpA-lacZ*) (9)-inducible promoters (Fig. 3B and C). As for the *degP-lacZ* fusion, the highest sigma E and Cpx regulon activity was generated in the *cyaA1400*::*kan* null strain.

The sigma E and Cpx regulon-inducing effect of *cyaA* mutations is not only apparent under conditions of high temperature (41°C) in a *degP* background. As shown in Fig. 4, *cyaA* mutations also induce the sigma E and Cpx regulons when cells are grown at 37°C in a *degP*$^+$ background.

Lowered intracellular concentrations of cAMP induce the sigma E and Cpx regulons by altered gene regulation involving CRP. Reporter gene fusion analysis demonstrated that induction of the extracytoplasmic regulons was greatest in the *cyaA* null mutant. Since the *cyaA1400*::*kan* allele is a null mutation (41), this suggested that induction is inversely related to the concentration of cAMP in the cell. Therefore, we measured the relative levels of β-galactosidase activity encoded by the *lacZ* gene in *cyaAΔ11*, *cyaA*::Tn*5*, and *cyaA1400*::*kan* mutant strains compared to that in a *cyaA*$^+$ strain when grown in the presence of high concentrations of the gratuitous inducer isopropyl-β-D-thiogalactopyranoside (IPTG). In the presence of IPTG, *lacZ* expression would be solely dependent upon the amount of CRP-cAMP activator. The β-galactosidase activity in *cyaA*::Tn*5* and *cyaAΔ11* mutant strains was less than that of

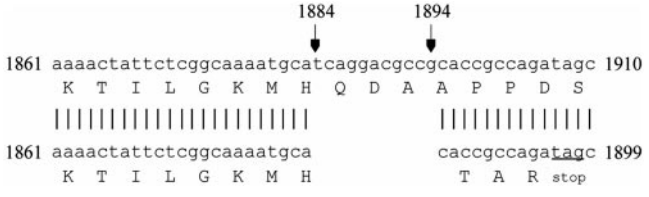

FIG. 2. The cryptic allele in *E. coli* strain CBM is a frameshift mutation of *cyaA*. Comparative nucleotide sequence alignment of nucleotides 1861 to 1910 and the corresponding amino acids encoded by the *cyaA*$^+$ and *cyaAΔ11* ORFs. The *cyaAΔ11* ORF has an 11-bp deletion of nucleotides 1884 to 1894. The resultant frameshift mutation generates a stop codon that truncates the adenylate cyclase by 217 amino acids.

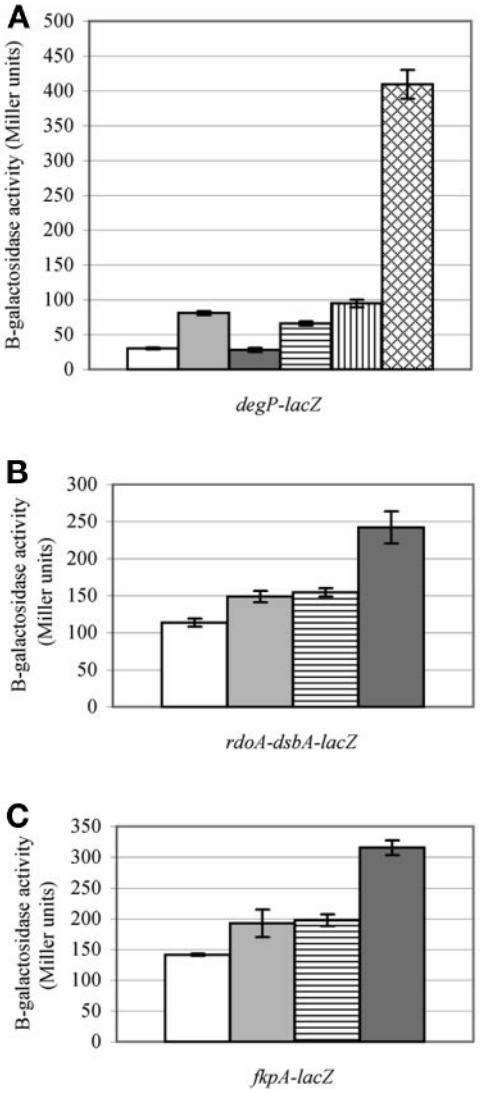

FIG. 3. The *cyaAΔ11* mutation induces both the sigma E and Cpx regulons. *degP* mutant strains that contained (A) a *degP-lacZ* gene reporter fusion, i.e., TS40 (*pldA*$^+$; white bar), GL123A (*pldA1 cyaAΔ11*; light gray bar), TS41 (*pldA1*; dark gray bar), TS18A (*cyaAΔ11*; horizontally striped bar), TS15A (*cyaA*::Tn*5*; vertically striped bar), and TS16A (*cyaA1400*::*kan*; cross-hatched bar); (B) an *rdoA-dsbA-lacZ* gene reporter fusion, i.e., GL111A (*cyaA*$^+$; white bar), TS23A (*cyaAΔ11*; light gray bar), TS23A (*cyaA*::Tn*5*; horizontally striped bar), and TS22A (*cyaA1400*::*kan*; dark gray bar); or (C) an *fkpA-lacZ* reporter fusion, i.e., GL112A (*cyaA*$^+$; white bar), TS33A (*cyaAΔ11*; light gray bar), TS31A (*cyaA*::Tn*5*; horizontally striped bar), and TS32A (*cyaA1400*::*kan*; dark gray bar), were grown at 41°C, and the amount of β-galactosidase activity was assayed.

the *cyaA*$^+$ strain (Fig. 5), suggesting that the adenylate cyclases encoded by *cyaA* mutant alleles have a decreased capacity to produce cAMP compared to wild-type adenylate cyclase. As expected, the *cyaA* null strain exhibited an extremely low level of β-galactosidase activity.

It should be noted that although the results described above indicate that a reduction in the cAMP concentration resulting from the mutant adenylate cyclases induces the extracytoplasmic stress regulons, growth under catabolite repression condi-

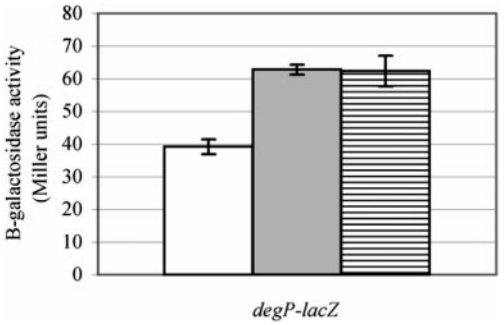

FIG. 4. *cyaA* alleles induce the extracytoplasmic stress regulons in *degP*$^+$ strains. The amount of β-galactosidase produced when bacteria are grown at 37°C was assayed in *degP-lacZ* reporter gene fusion-containing strains TS2-1 (*cyaA*$^+$; white bar), TS18 (*cyaAΔ11*; light gray bar), and TS15 (*cyaA*::Tn*5*; horizontally striped bar).

tions did not induce the regulons. In fact, growth at 37°C in LB medium containing 0.4% glucose did not alter stress levels in *cyaA*$^+$ strains and decreased the induction of the extracytoplasmic stress regulons in *cyaAΔ11* and *cyaA*::Tn*5* mutant strains by approximately 25% (data not shown).

Even though they are apparently partially inactivated, the adenylate cyclases encoded by the *cyaAΔ11* and *cyaA*::Tn*5* alleles remain controlled by catabolite repression, as evidenced by lower levels of β-galactosidase in *cyaA* mutant strains grown in the presence of 1 mM IPTG and 0.4% glucose compared to IPTG alone (Fig. 5). The effect of glucose in the *cyaAΔ11* and *cyaA*::Tn*5* mutant strains was similar to the effect in a *cyaA*$^+$ strain, such that the amount of β-galactosidase activity decreased approximately 64% in each strain when it was grown in the presence of 0.4% glucose.

The inverse relationship between the level of cAMP in the cell and the induction level of the sigma E and Cpx regulons was also indicated by the finding that induction of the regulons can be suppressed by addition of cAMP to the growth medium. As shown in Fig. 6, the level of extracytoplasmic regulon activity as measured by the amount of β-galactosidase originating from the *degP-lacZ* reporter fusion was inversely related to the

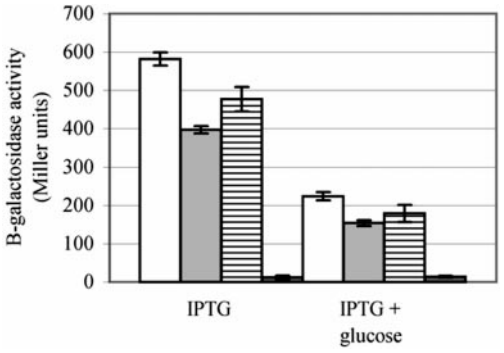

FIG. 5. Induced β-galactosidase levels are lower in strains containing *cyaA* mutant alleles but remain responsive to catabolite repression. W3110 derivative strains W3110 *cyaA*$^+$ (white bars), W3110 *cyaAΔ11* (light gray bars), W3110 *cyaA*::Tn*5* (horizontally striped bars), and W3110 *cyaA1400*::*kan* (dark gray bars) were grown in LB broth containing 1 mM IPTG with or without 0.4% glucose at 37°C, and the amount of β-galactosidase was assayed.

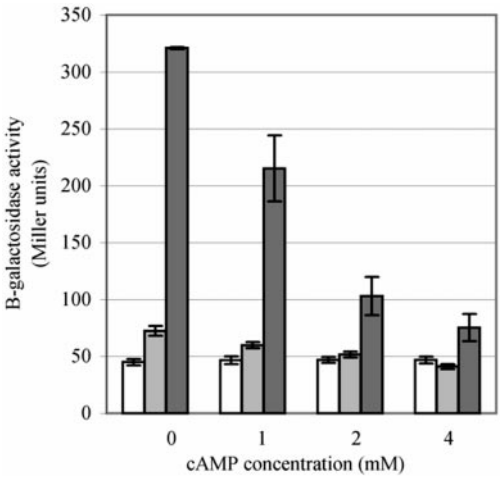

FIG. 6. Extracytoplasmic stress regulon expression is lowered by addition of exogenous cAMP to the growth medium of *cyaA* mutant strains. Strains TS2-1 (*cyaA*$^+$; white bars), TS13 (*cyaAΔ11*; light gray bars), and TS16 (*cyaA1400*::*kan*; dark gray bars) were grown in LB broth supplemented with 0, 1, 2, or 4 mM cAMP as indicated. Cultures were grown at 41°C, and the amount of β-galactosidase activity generated from a *degP-lacZ* chromosomal fusion was assayed.

concentration of exogenous cAMP provided in the growth medium of the *degP cyaAΔ11* and *cyaA1400*::*kan* mutant strains.

If induction of extracytoplasmic stress by the mutant *cyaA* alleles takes place via the CRP-cAMP mechanism of transcriptional regulation, the absence of CRP should also affect the expression of the stress regulons. We found that the absence of CRP uniformly increased the expression of the *degP-lacZ* fusion to a level similar to that displayed by the *cyaA* null strain (Fig. 7). The involvement of CRP-cAMP, not cAMP alone, in induction of the regulons was verified by the finding that induction in a *crp*::*cam* null mutant could not be lowered by the addition of exogenous cAMP (data not shown).

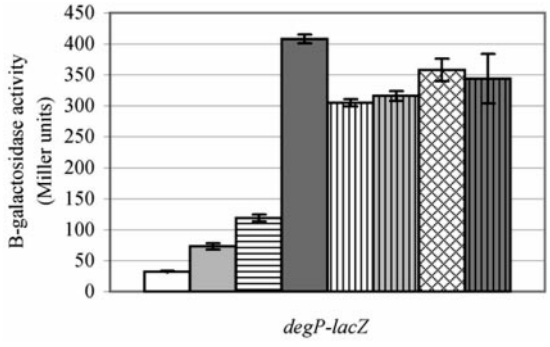

FIG. 7. The extracytoplasmic stress response regulons are strongly induced in the absence of CRP. *degP cyaA crp* mutant derivative strains were grown in LB broth at 41°C, and the amount of β-galactosidase activity was assayed. The levels of β-galactosidase generated in strains TS2A (*cyaA*$^+$; white bar), TS18A (*cyaAΔ11*; light gray bar), TS15A (*cyaA*::Tn*5*; horizontally striped bar), TS16A (*cyaA1400*::*kan*; dark gray bar), TS2AB (*cyaA*$^+$ *crp*::*cam*; white vertically striped bar), TS18AB (*cyaAΔ11 crp*::cam; light gray vertically striped bar), TS15AB (*cyaA*::Tn*5 crp*::*cam*; cross-hatched bar), and TS16AB (*cyaA1400*::*kan crp*::*cam*; dark gray vertically striped bar) are shown.

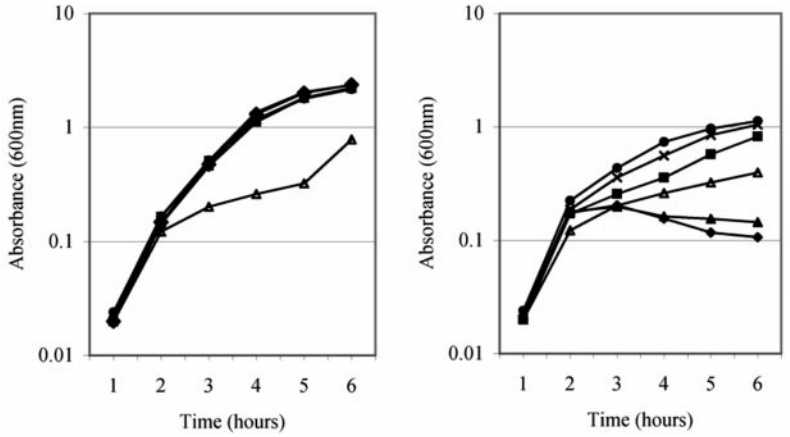

FIG. 8. Partial or complete inactivation of adenylate cyclase rescues the *degP* ts phenotype. Cell densities of *degP* mutant strains TS2A (*pldA*$^+$ *cyaA*$^+$; ◆), TS41 (*pldA1*; ▲), GL123A (*pldA1 cyaAΔ11*; ■), TS18A (*cyaAΔ11*; ●), TS15A (*cyaA*::Tn*5*; ×), and TS16A (*cyaA1400*::*kan*; △) grown in LB broth and incubated at (A) 30°C or (B) 42°C were determined.

***cyaA* mutations rescue the *degP* ts phenotype but are prevented from doing so by the addition of exogenous cAMP.** We confirmed that *cyaAΔ11*-mediated induction of the extracytoplasmic stress regulons was responsible for rescue of the *degP* ts phenotype by a growth assay. In this assay, the ability of *degP* derivatives to grow at both 30°C and the nonpermissive temperature of 42°C was assessed by determining cell density over a 6-h growth period (Fig. 8). Strains which contained the *cyaAΔ11* or *cyaA*::Tn*5* allele grew best at 42°C. In contrast, *pldA1 cyaA*$^+$, *pldA*::*kan cyaA*$^+$, and *pldA*$^+$ *cyaA*$^+$ strains that were *degP* mutants grew very poorly, exhibiting a decrease in cell density after incubation at 42°C for 2 h.

The *degP cyaA1400*::*kan* mutant exhibited a greatly reduced growth rate in comparison with other *cyaA* mutant derivatives when incubated at both 30°C and 42°C. This result can be attributed to a complete lack of cAMP in the cell due to the absence of adenylate cyclase. The null strain has a "sickly" phenotype identifiable by small colony size when grown on various media, including LB agar and MacConkey agar, and a reduced growth rate in liquid medium. However, although it confers a poor-growth phenotype even under nonstress conditions, *cyaA1400*::*kan* rescues the *degP* ts phenotype. This is evident by a comparison of the growth curve of the *cyaA1400*::*kan* mutant strain with that of a *cyaA*$^+$ strain. The *cyaA* null strain grew, albeit at a reduced rate, when incubated at both 30°C (Fig. 8A) and 42°C (Fig. 8B), whereas the *cyaA*$^+$ *degP* mutant strain could not continue to grow following a 2-h incubation at 42°C.

The results shown in Fig. 6 show that addition of exogenous cAMP to the growth medium of *cyaA degP* mutant strains decreases Cpx and sigma E induction levels in a dose-dependent manner. Since rescue of the *degP* ts phenotype is attributed to the induction of these regulons, we determined whether addition of exogenous cAMP to the growth medium would also affect rescue in a dose-dependent manner. *degP cyaAΔ11* mutant strain TS18A exhibits a specific set of growth patterns when grown at 42°C in LB medium supplemented with increasing concentrations of exogenous cAMP (Fig. 9). The best growth was observed when the cells were grown in LB broth containing no or 1 mM cAMP. A decrease in the growth rate was observed in concentrations of exogenous cAMP greater than 1 mM. In fact, addition of 4 mM cAMP decreased the growth rate to a level near that observed for a *degP cyaA*$^+$ mutant strain (Fig. 9). In control experiments, addition of exogenous cAMP did not affect the inability of a *degP cyaA*$^+$ mutant strain to grow at nonpermissive temperature, nor did it alter the growth of a *degP*$^+$ *cyaA*$^+$ strain at 42°C (data not shown).

DISCUSSION

E. coli responds to conditions that increase the amount of misfolded or aggregated proteins in the periplasm such as high temperature and the overproduction of outer membrane proteins, by inducing the sigma E and Cpx extracytoplasmic stress regulons (36). Members of these regulon families act as proteases, chaperones, and isomerases that function to degrade

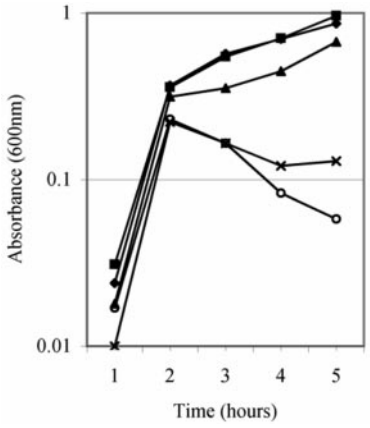

FIG. 9. Addition of exogenous cAMP changes the growth profile of a *degP cyaA* mutant strain grown at 42°C. The cell density of a *degP cyaAΔ11* mutant strain (TS18A) grown in LB broth supplemented with 0 (◆), 1 (■), 2 (▲), or 4 (×) mM cAMP and incubated at 42°C was determined. The growth curve of *cyaA*$^+$ *degP* mutant strain TS2A grown in the absence of exogenous cAMP (○) is shown for comparison.

and refold aberrant periplasmic proteins. DegP is a periplasmic protease/chaperone whose expression is controlled by both the sigma E and Cpx regulons and whose activity is integral to the ability of the cell to remain viable at high temperature, as evidenced by the ts phenotype of a *degP* mutant (26, 27, 44, 45, 46). Rescue of the *degP* ts phenotype has been shown to involve the induction of both the Cpx and sigma E extracytoplasmic regulons, which presumably compensate for the lack of DegP by upregulating the expression of proteins with overlapping functions (25, 43). In this work, we show that rescue of the *degP* ts phenotype in *E. coli* strain CBM by induction of the extracytoplasmic stress regulons is accomplished by a decrease in the cAMP concentration, resulting from the decreased activity of adenylate cyclase encoded by a mutant *cyaA*Δ*11* allele.

The *cyaA*Δ*11* allele contains an 11-bp deletion of nucleotides 1884 to 1894 of the 2,544-nucleotide *cyaA* open reading frame (ORF) and generates a stop codon 4 codons from the site of the deletion (Fig. 2). The mutant gene thus encodes a 631-amino-acid protein that contains the entire catalytic domain composed of amino acids 82 to 341 (18) and only 30% of the putative C-terminal regulatory domain normally composed of amino acids 536 to 848 (38).

The adenylate cyclases encoded by *cyaA*Δ*11* and a second mutant *cyaA* allele, *cyaA*::Tn*5* (encodes amino acids 1 to 507), apparently have a reduced ability to produce cAMP compared to the wild-type enzyme but surprisingly retain the ability to be regulated by catabolite repression (Fig. 5). This suggests that the C-terminal domain is not involved in regulation by catabolite repression but instead is required for maximal adenylate cyclase activity. This result is difficult to reconcile with the proposal that the (C-terminal) regulatory domain is inhibitory to the catalytic domain (8) and that the regulatory domain is essential for catabolite repression by glucose (38).

Quantification of β-galactosidase activity originating from *lacZ* reporter gene fusions whose promoters (that do not contain consensus CRP-cAMP binding sites) are controlled by sigma E (*fkpA*, Fig. 3C), Cpx (*rdoA-dsbA*, Fig. 3B), or both (*degP-lacZ*, Fig. 3A) demonstrated the sigma E and Cpx regulon-inducing effect of mutant adenylate cyclase proteins. We propose that an inverse correlation exists between the magnitude of sigma E and Cpx regulon induction and the concentration of cAMP in the cell. That is, a *cyaA*$^+$ strain should contain the highest concentration of cAMP (Fig. 5) and exhibits the lowest induction level of the extracytoplasmic stress regulons (Fig. 3 and 4). *cyaA*Δ*11* and *cyaA*::Tn*5* mutant strains generate higher levels of regulon induction compared to a *cyaA*$^+$ strain. The *cyaA1400*::*kan degP* mutant strain does not produce any cAMP (17) and exhibits a highly elevated sigma E and Cpx induction level (Fig. 3). Therefore, these results suggested that it is a reduction of the cellular cAMP concentration that is responsible for induction of the extracytoplasmic stress response regulons, which in turn rescues the ts phenotype of the *degP* mutants. This conclusion is supported by the finding that the activity level of the regulons in *cyaA* mutant strains was reduced in a dose-dependent manner by addition of exogenous cAMP to the growth medium, while at the same time this addition compromised the ability of the *cyaA* mutant strains to rescue the *degP* ts phenotype (Fig. 6 and 9).

The most likely mechanism by which a decreased cAMP concentration induces the sigma E and Cpx regulons would involve an alteration of gene expression mediated by CRP-cAMP. cAMP acts as a second messenger in the cell whereby it is used to regulate transcription when complexed with CRP. In keeping with this hypothesis, we found that the absence of CRP causes high-level induction of the sigma E and Cpx regulons similar to that observed for the *cyaA* null mutant (Fig. 7).

Because there are a large number of metabolic processes regulated by CRP-cAMP, there are many candidate genes that could be responsible for the induction of the stress regulons in *cyaA* mutants. We do not yet know whether the decreased CRP-cAMP concentrations induce the sigma E and Cpx regulons by actually generating periplasmic stress; however, all other reported cases of suppression of the *degP* ts phenotype involve overexpression of either normal or aberrant envelope proteins (1, 2, 28, 43, 47). It is unlikely that direct effects on the expression of the *cpx* and *rpoE* genes are involved, since CRP sites have not been detected in the *cpxR* and *rpoE* promoters, and in any case increased expression of these regulators would not necessarily increase the expression of their regulons since they are held inactive at the inner membrane in the absence of periplasmic stress.

Changes in the expression of the responsible gene could also be indirectly caused by the decrease in CRP-cAMP concentrations. For example, the transcription levels of the cytoplasmic stress sigma factor genes *rpoS* and *rpoH* are repressed by cAMP (22, 24). It has been reported, however, that cytoplasmic stress responses were not elevated in *cyaA* mutants (13). The same study also found that despite the inability to generate a cytoplasmic heat shock response when grown at 42°C, the *cyaA* null strain exhibited increased thermoresistance compared to a *cyaA*$^+$ strain. The author attributed this finding to the existence of an uncharacterized heat shock pathway, but the results presented here suggest it could have been due to induction of the extracytoplasmic stress response regulons.

It is interesting that in control experiments, growth in the presence of glucose did not cause increases in the extracytoplasmic stress regulon expression level, even though a decrease in cAMP levels in glucose-containing medium is a major mechanism of catabolite repression. This suggests that, in addition to CRP-cAMP, the gene whose altered expression generates periplasmic stress could also be regulated by the catabolite repressor/activator protein, which functions as a cAMP-independent mechanism of gene regulation.

In summary, the studies described here indicate that *cyaA* mutations rescue the ts phenotype of *degP* cells. Our results further indicate that this rescue results from induction of the Cpx and sigma E stress regulons and that this is caused by the effect of decreased cAMP concentrations on the CRP-cAMP regulon. Further experiments are required to identify the genes involved in induction of the stress response and the mechanisms involved in their homeostasis during cellular growth in various nutrient environments.

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