JOURNAL OF BACTERIOLOGY, June 1991, p. 3615–3621
0021-9193/91/123615-07$02.00/0

Vol. 173, No. 12

Cyclic AMP Inhibits and Putrescine Represses Expression of the *speA* Gene Encoding Biosynthetic Arginine Decarboxylase in *Escherichia coli*

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Received 17 December 1990/Accepted 25 March 1991

The *speA* gene of *Escherichia coli* encodes biosynthetic arginine decarboxylase (ADC), the first of two enzymes in a putrescine biosynthetic pathway. The activity of ADC is negatively regulated by mechanisms requiring cyclic AMP (cAMP) and cAMP receptor protein (CRP) or putrescine. A 2.1-kb *Bam*HI fragment containing the *speA-metK* intergenic region, *speA* promoter, and 1,389 bp of the 5′ end of the *speA* coding sequence was used to construct transcriptional and translational *speA-lacZ* fusion plasmids. A single copy of either type of *speA-lacZ* fusion was transferred into the chromosomes of *Escherichia coli* KC14-1, CB806, and MC4100, using bacteriophage lambda. The *speA* gene in lysogenized strains remained intact and served as a control. Addition of 5 mM cAMP to lysogenic strains resulted in 10 to 37% inhibition of ADC activity, depending on the strain used. In contrast, the addition of 5 or 10 mM cAMP to these strains did not inhibit the activity of β-galactosidase (i.e., ADC::β-galactosidase). Addition of 10 mM putrescine to lysogenized strains resulted in 24 to 31% repression of ADC activity and 41 to 47% repression of β-galactosidase activity. *E. coli* strains grown in 5 mM cAMP and 10 mM putrescine produced 46 to 61% less ADC activity and 41 to 52% less β-galactosidase activity. cAMP (0.1 to 10 mM) did not inhibit ADC activity assayed in vitro. The effects of cAMP and putrescine on ADC activity were additive, indicating the use of independent regulatory mechanisms. These results show that cAMP acts indirectly to inhibit ADC activity and that putrescine causes repression of *speA* transcription.

Putrescine (diaminobutane) is an aliphatic cation that is required for optimum growth of all cells. Putrescine and other polyamines are involved in many biological processes, but their physiological role(s) remains unclear. Most eucaryotes possess a single putrescine biosynthetic pathway (pathway I), in which ornithine decarboxylase (ODC), encoded by *speC* in *Escherichia coli*, converts ornithine to putrescine. Bacteria and plants also have a second putrescine biosynthetic pathway (pathway II). In *E. coli*, arginine is converted to agmatine by biosynthetic arginine decarboxylase (ADC), encoded by the *speA* gene. Agmatine is hydrolyzed to putrescine and urea by agmatine ureohydrolase (AUH), encoded by the *speB* gene (25). The presence of two parallel putrescine biosynthetic pathways is necessary in *E. coli* because the bacterium lacks arginase and therefore cannot convert arginine to ornithine. In the presence of exogenous arginine, as in the intestine, putrescine synthesis from pathway I would also decrease as the intracellular pool of ornithine becomes limiting as a result of arginine feedback repressing arginine biosynthesis (9, 14). Thus, pathway II ensures that putrescine is produced as nutritional conditions change (26).

Native ADC is a 280,000-Da tetramer that requires magnesium and pyridoxal phosphate for activity (24, 43). Monomers of ADC are synthesized as 74,000-Da precursor polypeptides that are posttranslationally processed to a 70,000-Da form during translocation to the inner periplasmic space (6). This compartmentalization of ADC explains the finding that exogenous arginine is preferentially channeled into putrescine synthesis; approximately 20% of exogenous arginine is converted to putrescine, with the remainder being incorporated into protein (39). In the periplasm, ADC is able to decarboxylate arginine to agmatine as arginine is imported into the cell. Agmatine induces AUH activity in the cytoplasm, resulting in increased putrescine production from pathway II.

Plasmids bearing the putrescine biosynthetic genes (*speA*, *speB*, and *speC*) were isolated from the Clarke-Carbon *E. coli* genomic library by assaying for overexpression of the enzymes (8). The *speA* and *speB* genes have been subcloned (4, 38) and sequenced (23, 36). A polycistronic mRNA is produced from the *speA* promoter, but the *speB* gene also produces a monocistronic mRNA (36). The open reading frames (ORF) for ADC and AUH are separated by 140 bp that contains a rho-independent terminator (23). It is not clear how a polycistronic mRNA containing *speB* is transcribed in the presence of this structure.

Expression of the putrescine biosynthetic genes is subject to metabolic controls. All three genes are negatively regulated by a mechanism involving cyclic AMP (cAMP) and cAMP receptor protein (CRP) (42). As the growth rate of *E. coli* decreases, so does the bacterium's production of putrescine (3). In *E. coli*, the intracellular concentration of cAMP increases as a carbon source becomes limiting (41). Thus, the level of cAMP acts as a signal to coordinate putrescine production with the growth rate of the bacterium. The concentration of putrescine in *E. coli* controls both the amount and activity of ADC and ODC, suggesting both inhibition and repression of the enzymes (39). The experiments in this report show that cAMP-CRP controls ADC expression by inhibiting the activity of ADC indirectly and that putrescine represses the *speA* gene at the level of transcription. The inhibition of ADC activity by cAMP-CRP

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TABLE 1. *E. coli* strains, plasmids, and bacteriophages

Strain, plasmid, or bacteriophage	Description	Source
Strains		
BD1412	Δ*lac ara thi strA sup*	This study
CB806	F⁻ Δ*lacZ lacY*⁺ *galK rpsL thi recA56 phoA8*	K. Schneider
λCBRM161	CB806 (λRM161)	This study
λCBRM65	CB806 (λRM65)	This study
KC13	*cya* derivative of KC14	E. L. Kline
KC14	*gal*; control strain	E. L. Kline
KC14-1	Δ(*argF-lac*)*U169 zah-735*:Tn*10* derivative of KC14	This study
λKCRM161	KC14-1 (λRM161)	This study
λKCRM65	KC14-1 (λRM65)	This study
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	M. J. Casadaban
λMCRM65	MC4100 (λRM65)	This study
SH205	Hfr Δ(*argF-lac*)*U169* (*zah-735*::Tn*10*)	H. Schweizer
SH305	MC4100 Δ*glpD102 recA1 srl*::Tn*10*	H. Schweizer
Plasmids		
pCB267	*lacZ* transcriptional fusion vector	K. Schneider
pMC1403	*lacZ* translational fusion vector	M. J. Casadaban
pRM15	pGEM-3Z with a 3.2-kb *Acc*I-*Bal*I fragment	R. C. Moore
pRM65	pMC1403 with a 2.1-kb *Bam*HI fragment	R. C. Moore
pRM160	*lacZ* transcriptional fusion vector	This study
pRM161	pRM160 with a 2.1-kb *Bam*HI fragment	This study
pRM162	pCB267 with a 2.1-kb *Bam*HI fragment	This study
Bacteriophages		
λRZ5	λ *lacZYA* transcriptional fusion vector for recombination with plasmids pRM161 and pMC1403	R. P. Gunsalus
λRM65	λRZ5 × pRM65 recombinant; Amp^r Lac$^+$	This study
λRM161	λRZ5 × pRM161 recombinant; Amp^r Lac$^+$	This study
P1::Tn*5*	Kan^r Amp^s	This study

and putrescine is additive, indicating these effectors regulate ADC by independent regulatory mechanisms.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are described in Table 1. *E. coli* strains were grown in either 3-(*N*-morpholino)-propanesulfonic acid (MOPS) medium (27) supplemented with 0.2% glucose, thiamine (1 μg/ml) and amino acids (50 μg/ml) except arginine (MOPS-glucose), Luria-Bertani broth (LB; 1% tryptone, 0.5% yeast extract, 1% NaCl) (21), or Terrific broth (TB; 1.4% tryptone, 2.7% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) (40) at 37°C in a reciprocating water bath. *E. coli* used for lysogen construction were grown in λYM medium (1% tryptone, 1% yeast extract, 0.25% NaCl, 0.2% maltose) (35) and plated on MacConkey's agar supplemented with ampicillin. Lambda infections used λTB top agar (1% tryptone, 0.5% NaCl, 10 mM $MgSO_4$, and 0.7% agar) and TB plates (34). *E. coli* cultures used for P1 infections were grown in LGC broth (1% tryptone, 0.5% yeast extract, 1% NaCl, 0.2% glucose, 5 mM $CaCl_2$, 10 mM $MgCl_2$) and LGC plates. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; and tetracycline, 12 μg/ml. Plasmids were isolated by a modified alkaline lysis procedure (15).

Enzymes and reagents. Restriction endonucleases, T4 DNA ligase, isopropylthio-β-galactopyranoside (IPTG), halogenated indolyl-β-D-galactopyranoside (Bluo-Gal), and NACS columns were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) and hexamethyltrimethylammonium bromide were purchased from Sigma Chemical Co. (St. Louis, Mo.); L-[1-^{14}C]arginine was purchased from Dupont, NEN Research Products (Boston, Mass.); and Nu-Sieve low-melt agarose was purchased from FMC BioProducts (Rockland, Maine). Southern hybridizations used the Genius hybridization kit from Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

Construction of a *lacZ* transcriptional fusion plasmid. A transcriptional fusion vector (pMR160) for producing lambda lysogens was constructed (Fig. 1) by removing a *Sma*I-*Sac*I restriction fragment from the translational *lacZ* fusion plasmid pMC1403 (7) and replacing it with a *Sma*I-*Sac*I fragment from pCB267 (31). Plasmids pMC1403 and pCB267 were digested with *Sma*I and *Sac*I, producing approximately 7,900- and 2,000-bp and 6,800- and 2,000-bp *Sma*I-*Sac*I restriction fragments, respectively. The 2,000-bp fragment from pCB267 carries a multiple cloning site, the *lacZ* ribosome binding site, and 1,951 nucleotides of the *lacZ* ORF. Restriction fragments were separated on a 1.0% Nu-Sieve gel, and the gel bands containing the 7,970-bp fragment from pMC1403 and the 2,000-bp fragment from pCB267 were excised, melted, ligated, and transformed into *E. coli* DH5α. Clones were selected that produced white, ampicillin-resistant colonies on LB plates supplemented with ampicillin and spread with 100 μl of Bluo-Gal (20 mg/ml). Recombinant plasmids were isolated, and restriction sites were mapped to confirm the identity of the plasmids.

Construction of transcriptional and translational *speA-lacZ* fusions. A transcriptional *speA-lacZ* fusion (pRM161) was constructed in plasmid pRM160 and transferred into the *E. coli* chromosome as a lambda lysogen. A 2,119-bp *Bam*HI fragment containing the *speA* promoter and 1,389 bp of the 5′

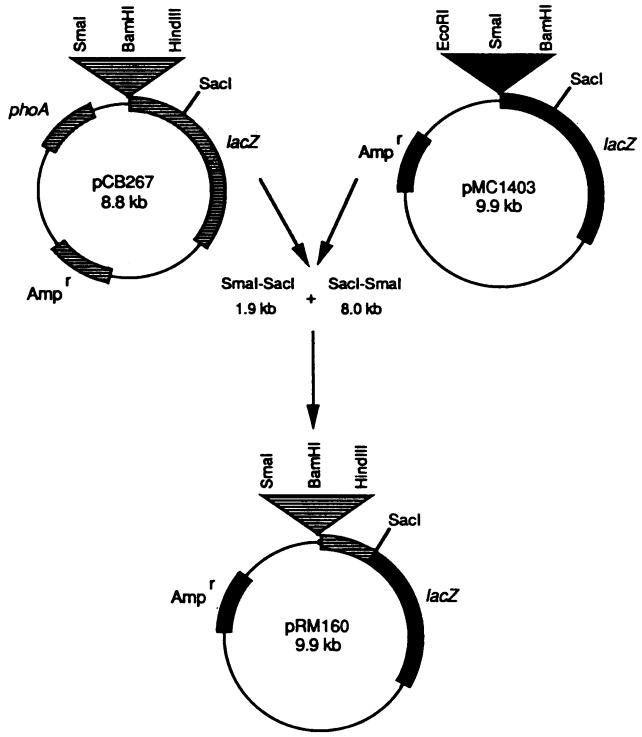

FIG. 1. Construction of the transcriptional *lacZ* fusion plasmid pRM160. Plasmids pCB267 and pMC1403 were digested with *Sma*I and *Sac*I. A 1.9-kb *Sma*I-*Sac*I restriction fragment was removed from pMC1403 and replaced by a 1.9-kb *Sma*I-*Sac*I fragment from pCB267. Insertion of the *lacZ* ribosome binding site and exchange of 1,951 bp of the *lacZ* ORF in pMC1403 produced the transcriptional *lacZ* fusion plasmid pRM160.

end of the *speA* gene was isolated by digesting plasmid pRM15 (Fig. 2) with *Bam*HI and separating the products in a 1.0% Nu-Sieve gel. The band containing the 2,119-bp fragment was excised, melted, ligated into the *Bam*HI site of pRM160, and transformed into *E. coli* DH5α. Recombinant clones were selected on LB plates supplemented with ampicillin and spread with 100 μl of Bluo-Gal (20 mg/ml). Plasmids were isolated from clones which produced blue, ampicillin-resistant colonies. Plasmid pRM162 was constructed to determine whether an antisense RNA regulated *speA* transcription or translation. The same 2,119-bp *Bam*HI fragment was ligated into pCB267 to produce a *speA-lacZ* fusion.

Plasmid pCB267 contains a pair of divergently oriented indicator genes, *lacZ* and *phoA*, so that divergent promoter activity can be monitored. The identity and orientation of the 2,119-bp *Bam*HI fragment in pRM161 or pRM162 were confirmed by restriction analysis. A translational *speA-lacZ* fusion carrying the same 2,119-bp *Bam*HI fragment in plasmid pMC1403 has been previously described (23).

P1 transduction of *(argF-lac)U169* into *E. coli* KC14. A Lac$^-$ derivative of *E. coli* KC14 was constructed as a host for the *speA-lacZ* fusions. *E. coli* SH205 (*zah-735*::Tn*10*) carries the Δ(*argF-lac*)*U169 lac* mutation tightly linked with transposon Tn*10*, which carries the tetracycline resistance gene (32). The Δ(*argF-lac*)*U169* (*zah-735*::Tn*10*) fragment was transduced to *E. coli* KC14, using a kanamycin-resistant P1 phage (P1::Tn*5*) according to standard methods (34). Transductants were plated onto LB plates containing tetracycline and spread with Bluo-Gal. White, tetracycline-resistant colonies were screened on LB-kanamycin plates spread with 100 μl of Bluo-Gal (20 mg/ml) to ensure that Tn*5* had not integrated into the *E. coli* chromosome. *E. coli* KC14-1 (Lac$^-$, tetracycline resistant, kanamycin sensitive) was chosen for studies of cAMP- and putrescine-mediated regulation of ADC.

Construction of *speA-lacZ* lysogens. *E. coli* MC4100 was transformed with either *speA-lacZ* fusion plasmid pRM161 or pRM65 and infected with lambda RZ5 (17). Phage were harvested by treatment of the culture with chloroform, and the cell debris was removed by centrifugation. Lambda lysates were titered and used to produce lysogens in *E. coli* CB806, KC14-1, and MC4100. Lambda was preadsorbed to the bacteria for 20 min at room temperature and shaken for 2 h at 37°C. Bacteria were plated on MacConkey lactose plates supplemented with ampicillin (100 μg/ml). Ten Lac$^+$, ampicillin-resistant clones were selected and assayed for β-galactosidase activity. Lysogens containing single copies of either transcriptional or translational *speA-lacZ* fusions were chosen on the basis of their levels of β-galactosidase activity; the presence of lambda prophage in the *E. coli* chromosome was confirmed by Southern analysis (2).

Regulation of *speA-lacZ* by cAMP and putrescine. A 5-ml culture of each *E. coli* strain was grown overnight in MOPS-glucose medium. Cultures of *E. coli* KC14 were supplemented with IPTG (5 mM final concentration) to induce the lactose operon. The overnight cultures were diluted into fresh medium that either contained or lacked the appropriate effector: 5 or 10 mM (final concentration) cAMP or putrescine or 5 mM cAMP and 10 mM putrescine. Cells were grown for a minimum of three generations to a density of approximately 80 Klett units and chilled on ice. Aliquots were removed from each culture, the A_{600} was recorded, and β-galactosidase activity was determined. The remaining cells were harvested by centrifugation, washed with saline, sonicated, and assayed for ADC activity.

Effect of cAMP on ADC activity in vitro. A 5-ml culture of *E. coli* KC13 (*cya*) was grown in MOPS-glucose medium supplemented with thiamine and amino acids except arginine. The overnight culture was diluted 1:100 into 100 ml of fresh medium and grown to 90 Klett units. The bacteria were harvested by centrifugation, washed with saline, and resuspended in 1 ml of ADC breakage buffer. Crude extracts were prepared by sonication, and cell debris was removed by centrifugation. Aliquots of the cell extract were preincubated for 10 min at room temperature in the presence of 0.1, 1.0, or 10 mM cAMP and assayed for ADC activity.

Enzymes assays and protein determination. ADC activity was determined by decarboxylation of [^{14}C]arginine (43). β-Galactosidase activity was determined by hydrolysis of ONPG (22), and alkaline phosphatase activity was determined by hydrolysis of *p*-nitrophenyl phosphate (31). Protein concentrations were determined by the Bradford method (5).

RESULTS

Strain-specific repression of ADC by cAMP. Because Shaibe et al. (33) had shown that ADC activity in certain strains of *E. coli* was not inhibited by cAMP supplementation, five strains of *E. coli* were tested for their ability to respond to cAMP. The amount of repression of ADC in these *E. coli* strains was found to be strain specific. The specific activities of ADC from *E. coli* KC14, KC14-1, BD1412, CB806, and MC4100 grown in the presence of 5 mM cAMP were approximately 62, 65, 92, 84, and 81%, respectively, of the ADC activity of bacteria grown in the

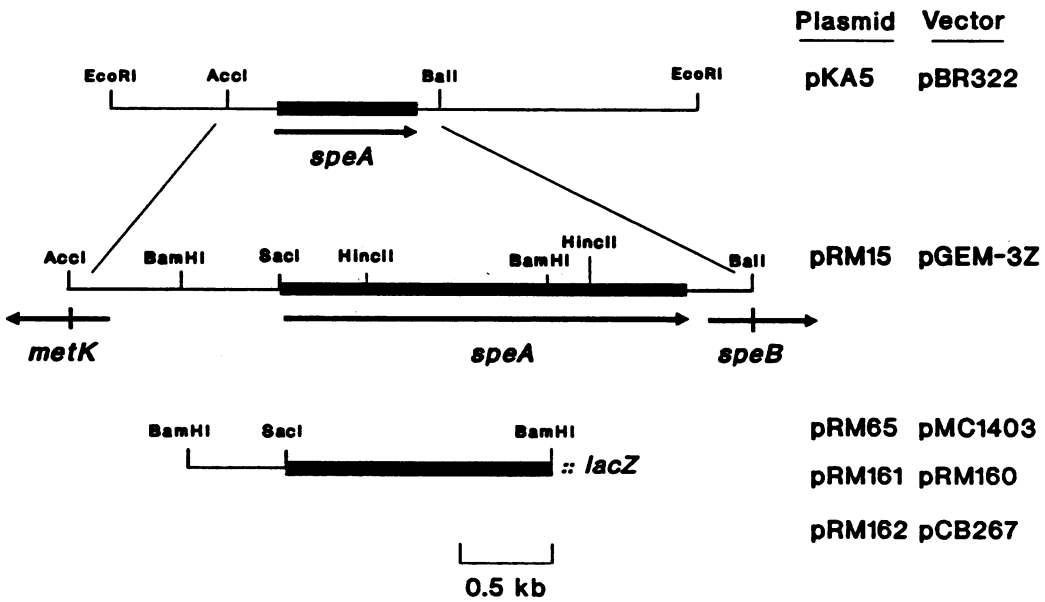

FIG. 2. Physical maps of plasmids. Thick lines indicate the *speA* (ADC) gene; arrows show direction of transcription; arrow with bar shows truncated *speB* (AUH) and *metK* (methionine adenosyltransferase) genes.

absence of cAMP. The growth rate of each strain was unaltered by the presence of cAMP in the media. This reduction in ADC activity was reproducible and did not vary significantly among experiments. The β-galactosidase activity of *E. coli* KC14 (cultured in 5 mM IPTG) increased 1.6- to 2-fold when cultures were supplemented with 5 mM cAMP (final concentration) and 2.4-fold when they were supplemented with 10 mM cAMP.

β-Galactosidase activities of *speA-lacZ* fusions. To determine whether β-galactosidase is being expressed from the *speA* promoter, the β-galactosidase activities of plasmid-borne *speA-lacZ* fusions were assayed. *E. coli* CB806 was transformed with one of the following plasmids: pRM160, pMC1403, pRM161, pRM162, or pRM65 (Table 1). Bacteria were grown to mid-log phase, harvested, and assayed for β-galactosidase activity. The transcriptional *speA-lacZ* fusions in pRM161 and pRM162 produced 124 and 98 U of β-galactosidase, respectively, and the translational *speA-lacZ* fusion pRM65 produced 1,192 U of β-galactosidase. The 10-fold-higher β-galactosidase activity produced by the translational *speA-lacZ* fusion appears to be an artifact of the transcriptional fusion vector pCB267 and its derivative, pRM160. When pRM162 was assayed for promoter activity divergent to *speA*, no alkaline phosphatase activity was found. Therefore, *speA* is not regulated by an antisense RNA.

To accurately examine the influences of effectors on the *speA* promoter, single copies of either transcriptional or translational *speA-lacZ* fusions were transferred to the chromosomes of *E. coli* KC14-1, CB806, and MC4100. The level of β-galactosidase activity expressed by *speA-lacZ* fusions in various *E. coli* hosts was found to be strain dependent (Table 2) and ranged from 1 to 19% of β-galactosidase activity in *E. coli* KC14.

Expression of *speA-lacZ* fusions. The synthesis of β-galactosidase proteins from *speA-lacZ* fusions was confirmed by using immunoaffinity batch purification. Stained gels of immunoaffinity-purified antigens revealed an approximately 116,000-Da polypeptide produced by *E. coli* strains bearing a transcriptional *speA-lacZ* fusion. *E. coli* strains containing a translational *speA-lacZ* fusion produced an approximately 160,000-Da polypeptide recognized by the antisera (data not shown). The 160,000-Da protein results from the fusion of 44,000 Da of ADC to the 116,000 Da of β-galactosidase (12).

Regulation of ADC and *speA* by cAMP and putrescine. The presence of a second copy of the *speA* promoter in the chromosome of *E. coli* strains containing *speA-lacZ* fusions had no detectable effect on the regulation of ADC by 5 mM cAMP or 10 mM putrescine. The addition of 5 mM cAMP to cultures of *E. coli* strains carrying single copies of *speA-lacZ* fusions resulted in a strain-specific inhibition of ADC activity (Table 3). While the inhibition varied with the strain tested, the β-galactosidase activities of both transcriptional and translational *speA-lacZ* fusions were not repressed. In contrast, the β-galactosidase activity of the lactose operon of strain KC14 was induced 1.6-fold. The generation time of each strain remained unchanged when the bacteria were grown in either the presence of absence of 5 mM cAMP or 10

TABLE 2. ADC and β-galactosidase activities of *E. coli* strains bearing *speA-lacZ* fusions grown in MOPS-glucose medium

Strain	Fusion[a]	Sp act[b]	
		ADC (pmol of CO_2/h/mg of protein)	β-Galactosidase[c]
KC14	None	253	2,235[d]
KC14-1	None	246	0
λKCRM161	TS	264	8
λKCRM65	TL	246	66
CB806	None	141	0
λCBRM161	TS	157	12
λCBRM65	TL	143	433
MC4100	None	280	0
λMCRM65	TL	315	180

[a] TS, transcriptional; TL, translational.
[b] Average of at least three assays.
[c] Calculated as described by Miller (22).

TABLE 3. Relative ADC and β-galactosidase activities of *E. coli* strains grown in MOPS-glucose with cAMP, putrescine, or both

Strain	Fusion[a]	Relative sp act[b]									
		cAMP				5 mM cAMP + 10 mM putrescine		Putrescine			
		5 mM		10 mM				5 mM		10 mM	
		ADC	β-Gal	ADC	β-Gal	ADC	β-Gal	ADC	β-Gal	ADC	β-Gal
KC14	None	0.64	1.56	0.46	2.80	0.48	2.26	0.79	1.05	0.78	1.05
λKCRM161	TS	0.62	0.94	ND	ND	0.49	0.69	ND	ND	0.56	0.75
λKCRM65	TL	0.64	0.90	0.40	0.89	0.39	0.48	0.80	1.08	0.76	0.59
λCBRM161	TS	0.76	1.07	ND	ND	0.51	0.56	ND	ND	0.51	0.37[c]
λCBRM65	TL	0.80	1.08	0.81	1.41	0.54	0.59	0.80	1.22	0.69	0.60
λMCRM65	TL	0.81	1.20	Lysis	Lysis	Lysis	Lysis	0.83	0.96	0.53	0.56

[a] TS, transcriptional; TL, translational.
[b] Specific activity relative to specific activity in cells grown in unsupplemented MOPS-glucose medium (see Table 2). The strains were grown for three to four generations, and extracts were assayed as described in Materials and Methods. Values are averages of three assays from one to four experiments. β-Gal, β-galactosidase; ND, not determined.
[c] Low activity.

mM putrescine (Table 4). Thus, the changes in activity cannot be attributed to a reduction in the growth rate. Increasing the cAMP concentration to 10 mM did not affect the growth of *E. coli* KC14, λKCRM161, or λKCRM65 but increased the induction of the lactose operon of strain KC14. These results indicate that cAMP does not repress the *speA* gene directly, but acts directly or indirectly to inhibit the activity of ADC. To determine whether cAMP was directly inhibiting ADC, cAMP was added to crude extracts of *E. coli* KC13 (*cya*), and ADC activity was assayed. cAMP at 0.1, 1.0, and 10.0 mM did not cause any significant inhibition of ADC (data not shown). Thus, the ability of cAMP to inhibit *speA* expression is the result of cAMP utilizing an indirect mechanism, e.g., via another gene product, since ADC is not directly inhibited by cAMP.

Addition of 10 mM putrescine to *E. coli* KC14 inhibited ADC activity 22% but did not affect expression of β-galactosidase activity from the lactose operon. In contrast, ADC activities in strains λKCRM65 and λCBRM65 were inhibited 24 and 31% and β-galactosidase activities were repressed 41 and 40%, respectively (Table 3). When cultures were grown in the presence of both 5 mM cAMP and 10 mM putrescine, the lactose operon of *E. coli* KC14 was induced 2.3-fold. Lambda lysogens in strain λMCRM65 were induced to lytic growth when grown under these conditions. ADC activities of *E. coli* λKCRM65 and λCBRM65 were reduced 61 and 46% and β-galactosidase activities were reduced 52 and 41%, respectively (Table 3). These results suggest that cAMP does not directly repress *speA* transcription. In conclusion, cAMP and putrescine regulate ADC activity via independent mechanisms because (i) *speA* but not the *speA-lacZ* fusions respond to cAMP, (ii) both *speA* and the *speA-lacZ* fusions respond to putrescine, and (iii) the effects of cAMP and putrescine on ADC and β-galactosidase activities are additive.

TABLE 4. Generation times of various *E. coli* strains grown for three to four generations in MOPS-glucose medium

Strain	Generation time (min)[a]					
	No addition	cAMP		Putrescine		5 mM cAMP + 10 mM putrescine
		5 mM	10 mM	5 mM	10 mM	
KC14	62	62	64	60	62	64
λKCRM161	66	66	—	—	64	65
λKCRM65	66	66	60	66	60	62
λCBRM161	60	62	—	—	60	58
λCBRM65	55	50	PG	58	60	48
λMCRM65	42	34	Lysis	40	50	Lysis

[a] —, Not tested; PG, poor growth because of inhibited growth rate.

DISCUSSION

The cAMP-CRP complex regulates gene expression in *E. coli* by a number of mechanisms. cAMP-CRP can act directly by binding within or near promoters of regulated genes to alter the rate of transcription (1, 11), or the complex can inhibit the activity of an enzyme, as in the case of adenylate cyclase (*cya*) (20). cAMP-CRP could also control *speA* transcription indirectly by controlling the synthesis of a repressor protein or an antisense (divergent) RNA, as in the *crp* gene (1, 28). As a first step to determine whether transcriptional or posttranslational mechanisms were operative in regulating ADC, we constructed transcriptional and translational *speA-lacZ* fusions and transferred single copies of either type of fusion to the chromosomes of several strains of *E. coli*. The specific activities of ADC in lysogenic strains of *E. coli* were the same as in the parental strain. Lysogenized strains also grew at the same rate as cells without the fusion. The presence of a second copy of the *speA* promoter in the *E. coli* chromosome did not alter either cAMP- or putrescine-mediated regulation of ADC. Therefore, any effector that is required for regulation of *speA* is not limiting in the lysogenized cells which are diploid for the *speA* promoter.

Plasmid pRM160 contains the translational termination codons preceding the *lacZ* gene and the *lacZ* ribosome binding site from pCB267. Both transcriptional *speA-lacZ* fusion plasmids, pRM161 and pRM162, carry identical 2.1-kb *Bam*HI fragments in which the *speA* reading frame uses a translation termination codon (TAG) that overlaps the *lacZ* ribosome binding site (AGAGGG). Apparently a ribosome that stalls at the TAG translation termination codon is hindered in initiation of translation of *lacZ* mRNA, resulting in a lower level of expression of β-galactosidase activity.

The ADC::β-galactosidase hybrid protein produced by the translational *speA-lacZ* fusions contains 70% of the *speA* ORF beginning with the 5′ end (23). These ADC::β-galactosidase fusion proteins do not produce an active ADC in addition to β-galactosidase since the specific activity of ADC

in cells bearing a translational *speA-lacZ* fusion remains the same as in the host cells without the fusion. This finding is not unexpected, as deletion of 355 nucleotides (18% of *speA*) from the 3′ end of the *speA* ORF results in the loss of ADC activity (23). Since the expression of β-galactosidase from *E. coli* strains carrying transcriptional or translational *speA-lacZ* fusions is not affected by cAMP, the regulation of ADC activity by cAMP-CRP does not appear to be at the transcriptional or translational level. It is still possible, however, that the 30% of the 3′ end of the *speA* mRNA missing in the *speA-lacZ* fusion is required for some type of translational regulation of ADC.

In vitro and in vivo studies have shown cAMP-CRP regulates the activity of adenylate cyclase (*cya*) posttranslationally, at the enzyme level (20, 41). It appears that the activity of ADC is also indirectly regulated posttranslationally by the cAMP-CRP complex. This finding was unexpected since the region upstream of *speA* contains a CRP-like binding sequence, 5′-TGTGC-3′ (consensus: 5′-TGTGA-3′), located near the *speA* promoter (23). Gel retardation assays have shown that changing the CRP binding sequence of the lactose operon from 5′-TGTGA-3′ to 5′-TGTGC-3′ reduced, but did not abolish, CRP binding (16). Although this site may bind the cAMP-CRP complex in *speA*, results of *speA-lacZ* fusion experiments indicate that cAMP does not control the expression of ADC at the transcriptional level.

Because cAMP supplementation is known to alter the growth rate of *E. coli* (42), a number of *E. coli* strains were tested for their responsiveness to cAMP. The effects on the growth rate and degree of regulation of *speA* expression by cAMP were determined to be strain specific. The decrease in ADC specific activity varied from 9 to 40%, depending on the strain of *E. coli* tested. This finding supports the observation by Shaibe et al. (33) that the activities of ADC and ODC from some *E. coli* strains are not regulated by cAMP. Shaibe et al. also noted that the cAMP-mediated regulation of ADC and ODC occurred in strains with a slow growth rate. The slow growth rate of *E. coli* KC14 and its derivatives may reflect low intracellular levels of cAMP, much as with *cya* mutants, which grow more slowly than wild-type bacteria (10). Thus, the addition of exogenous cAMP to the growth medium would be expected to have a greater effect on the ADC activity of *E. coli* strains containing low levels of cAMP (i.e., strain KC14-1 versus strain MC4100).

Addition of putrescine to the growth medium also results in inhibited ADC activity. Our results support the observation of Tabor and Tabor (39) that putrescine caused inhibition of ADC and repression of *speA*. The specific activity of ADC from *E. coli* grown in a chemostat decreased approximately 45% following the addition of 10 mM putrescine. The specific activity of ADC and β-galactosidase in *E. coli* strains carrying *speA-lacZ* fusions decreased 24 to 31% and 41 to 47%, respectively, in cultures containing 10 mM putrescine. The amount of *speA* gene repression by putrescine was determined to be independent of the strain tested. In vitro studies show that putrescine can also competitively inhibit ADC activity (39). A possible means by which putrescine could negatively regulate ADC activity posttranslationally would be by inducing the synthesis of two basic proteins, called antizymes, that are known to inhibit the activity of ADC. These ADC antizymes have been identified in *E. coli* as ribosomal proteins S20/L26 and L34 (29). Nonspecific inhibition of *E. coli* ODC by ribosomal proteins has also been reported (18). However, there are no published reports in which cAMP-CRP induces the synthesis of ribosomal proteins. Thus, ADC antizymes do not appear to be involved in cAMP-mediated regulation of ADC.

The activities of ADC and ODC decrease in response to putrescine supplementation (26, 30, 42). Data presented in this paper indicate that cAMP and putrescine control ADC activity independently. The decrease in ADC activity in *E. coli* strains supplemented with both 5 mM cAMP and 10 mM putrescine was additive, suggesting independent regulatory mechanisms. The addition of 5 mM cAMP to cultures supplemented with 10 mM putrescine did not further decrease the β-galactosidase activity encoded by *speA-lacZ* fusions, indicating that cAMP does not regulate *speA* transcription. cAMP and putrescine regulate ODC activity via independent regulatory mechanisms (6a); the activity of ODC decreased when *E. coli* KC42, a *cya crp* derivative of strain KC14, was grown in the presence of 10 mM putrescine.

The mechanism by which cAMP regulates the activity of the putrescine biosynthetic enzymes remains unclear. *E. coli* bearing transcriptional or translational *speA-lacZ* fusions in single-copy or multicopy states fail to respond to cAMP supplementation, while the ADC activity of control cells is decreased. Since the addition of cAMP to extracts in vitro did not inhibit ADC activity, cAMP must be inhibiting ADC indirectly and presumably via the synthesis of some regulatory element, e.g., a protein. The activity of AUH may also be regulated by cAMP posttranslationally; transcriptional *speB-lacZ* fusions carried on plasmids do not respond to cAMP but do respond to agmatine (35a). Regulation of ADC activity by cAMP and putrescine at posttranslational and transcriptional levels, respectively, suggests that cellular putrescine levels are strictly regulated in *E. coli*. We are currently investigating whether ODC and AUH are also posttranslationally regulated by cAMP.

ACKNOWLEDGMENTS

We thank M. Szumanski for helpful discussion and J. Kromhout for technical assistance in preparing the immunoblot.

This work was supported by grant DMB-8508917 from the National Science Foundation (S.M.B.), by graduate fellowships from the College of Veterinary Medicine and Virginia Polytechnic Institute and State University (R.C.M.), and by a grant-in-aid from Sigma Xi.

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