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# Cyclic 3',5'-Adenosine Monophosphate Phosphodiesterase Mutants of *Salmonella typhimurium*

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Positive selection procedures for mutants of *Salmonella typhimurium* lacking cyclic 3',5'-adenosine monophosphate (cAMP) phosphodiesterase have been devised. The gene (*cpd*) coding for this enzyme has been located on the chromosome and shown to be 25% co-transducible with *metC* using phage P22. The mutants have been used to investigate the role of the enzyme in the control of genes whose expression is known to be dependent on cAMP. Significant alterations in the regulation of some but not others of these genes have been observed in these mutants. Mutants lacking the cAMP phosphodiesterase are more sensitive than their parents to a variety of antibiotics that appear to enter the cell through cAMP-dependent transport systems. They grow faster than the wild type on succinate-ammonia-salts, and glucose-proline-salts media and are inhibited by added cAMP on glucose, citrate, or glycerol-ammonia salts media whereas the wild type is unaffected. Neither the growth of *Salmonella typhimurium* on glycerol or citrate media nor the level of acid hexose phosphatase in the strain is affected by the loss of cAMP phosphodiesterase. In addition, the mutant strains are extremely sensitive to high levels of cAMP. Loss of the cAMP phosphodiesterase in strains unable to synthesize cAMP (adenyl cyclase negative) reduces by 10-fold the requirement for exogenous cAMP for expression of catabolite-sensitive phenotypes. These results suggest that through its control of cAMP levels in the cell the phosphodiesterase may be involved in the regulation of certain classes of catabolite-sensitive operons and also in protecting the cell against high levels of cAMP.

Expression of a large number of bacterial genes has been shown to be dependent on the presence of sufficient intracellular levels of cyclic 3',5'-adenosine monophosphate (cAMP) (5, 25). Although three biological activities have been identified that appear to be capable of influencing the levels of this nucleotide, little is known of the precise mechanism by which they interact to achieve the observed cAMP concentrations. The first activity, adenyl cyclase, was isolated by Tao and Lipmann (29) from *Escherichia coli* and shown to synthesize cAMP from adenosine triphosphate. Makman and Sutherland (21) described the apparent excretion of cAMP into the medium through an as yet undefined mechanism. Our work involves genetic and physiological studies of the third activity, the cAMP phosphodiesterase (EC 3.1.4D), which was first observed in bacteria by Braná and Chytil (4).

cAMP phosphodiesterase catalyzes the cleavage of cAMP to 5'-adenosine monophosphate (5'AMP). The enzyme has been purified 1,000-fold from *Serratia marcescens* (23) and about 100-fold from *E. coli* (22). The *E. coli* enzyme has an apparent molecular weight of 30,000 and a $K_m$ for cAMP of 0.5 mM. The partially purified enzyme has been reported to require either iron or an iron-containing protein for full activity (22). The *S. marcescens* enzyme has an apparent molecular weight of 50,000 and a $K_m$ for cAMP of 0.52 mM. It is stimulated by $Fe^{2+}$, $Ca^{2+}$, and $Ba^{2+}$. It is similar to cAMP phosphodiesterases from mammalian sources but unlike the enzyme from *E. coli* and *Salmonella typhimurium*, it is inhibited by theophylline (23). Recent evidence (A. Peterkofsky, personal communication) suggests, however, that the common belief that cAMP phosphodiesterases from *E. coli* and *S. typhimurium* are not sensitive to theophylline may be mistaken. Two strains of *E. coli*, Crooke's strain and a mutant of AB257, have been shown to lack cAMP phosphodiesterase (15, 25).

In this study we have devised selection procedures for *S. typhimurium* mutants lacking cAMP phosphodiesterase activity and mapped the gene for this enzyme (*cpd*). In addition, we

have shown that the loss of the enzyme results in significant alteration of several growth characteristics of the bacterium. On the basis of these characteristics, we suggest a role for cAMP phosphodiesterase in cell function. (cAMP phosphodiesterase mutants have been recently isolated in *S. marcescens*, by a different procedure, by U. Winkler [personal communication]).

## MATERIALS AND METHODS

**Chemicals.** cAMP, 5'-nucleotidase (EC 3.1.3.5) from *Crotalus adamanteus* venom (16.7 U/mg), 5'-adenosine monophosphate (5'AMP), and glucose-6-phosphate were purchased from Sigma Chemical Company, St. Louis, Mo. Fosfomycin (phosphonomycin) (L-1,2-cis-epoxypropylphosphonic acid) was the gift of F. Kahan at the Merck Center for Therapeutic Research, Rahway, N.J. β-Chloro-L-alanine was obtained from Cyclo Chemical Co., Los Angeles, Calif. Dithiothreitol was obtained from Calbiochem, San Diego, Calif. Difluorosuccinic acid was obtained from PCR, Inc., Gainesville, Fla. Other antibiotics listed (see Table 5) were obtained on paper disks from BBL-Bioquest, Cockeysville, Md.

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

**Bacteriophage.** An integration defective (*int*) mutant of phage P22, *cly-2 int-4*, was used for transduction analysis (12). The temperature-sensitive *cly-2* mutation greatly reduces the lytic response of the phage when grown on *cya* mutants at 37 C (14).

**Media.** Nutrient broth was 0.8% nutrient broth (Difco) with 0.5% NaCl. Minimal salts medium was the E medium of Vogel and Bonner (28) with 0.2% citric acid. Carbon sources were sterilized independently and added to this medium to 0.4%, which is then referred to as "minimal citrate," "minimal glucose," etc. Minimal medium containing neither a source of carbon nor of nitrogen ($N^-C^-$) contained, per liter of distilled water: $K_2SO_4$, 1.0 g; $K_2HPO_4 \cdot 3H_2O$, 17.7 g; $KH_2PO_4$, 4.7 g; $MgSO_4 \cdot 7H_2O$, 0.1 g. Except where noted, $NH_4Cl$ was added as a source of nitrogen in this medium at 10 mM. Solid medium contained 1.5% agar (Difco). "Top agar" contained 0.6% agar and 0.5% NaCl.

**Growth of bacteria.** Cells were grown at 37 C in a New Brunswick gyrotory shaker and were monitored by observing the increase in absorbance at 650 nm ($A_{650}$) in a Zeiss model PMQ II spectrophotometer.

**Growth of phage.** An overnight culture of the

TABLE 1. *Bacterial strains*

| *Salmonella typhimurium* | | Source and comments |
|---|---|---|
| Strain | Genotype | |
| TA63 | *hisC2194 hisO1242 serA821* | This laboratory. |
| TA2301 | *cya-408* | Mutagenized background, reference 14. |
| TA3301 | *cya-408* | cya-408 transduced from TA2301 into unmutagenized LT2. |
| TA3303 | *cpd-401 cya-408* | Spontaneous mutation in TA2301. |
| TA3309 | *cpd-401 cya-408* | TA3301 by transduction to growth on citrate with 0.1 mM cAMP with TA3303 as donor. |
| TA3310 | HfrK5 *cpd-401 serA13* | SA535 as TA3336 from LT2 and TA3303 from TA2301 and TA3311 from TA3309. |
| TA3311 | *cpd-401* | TA3309 by transduction to citrate growth with LT2 as donor. |
| TA3315 | *cpd-402 cya-405 metC30*/F'116 *met*$^+$ | TA3323 × TA3332 for *met*$^+$. |
| TA3319 | *cpd-405 cya-408* | Spontaneous temperature-sensitive mutation in TA3301. |
| TA3323 | *hisC2194 hisO1242 serA821*/F'116 *ser*$^+$ | KLF16/KL110 × TA63 for *ser*$^+$ (*E. coli* episome). |
| TA3324 | *cpd-404 cya-407* | Spontaneous mutation in TA3336. |
| TA3330 | *cya-405 metC30* | Spontaneous mutation to fosfomycin resistance in *metC30*. (Manuscript in preparation.) |
| TA3332 | *cpd-402 cya-405 metC30* | Spontaneous mutation in TA3330. |
| TA3333 | *cpd-404* | TA3324 transduced to citrate growth with LT2 as donor. |
| TA3334 | *cpd-407* | Spontaneous mutation in LT2 to succinate fast growth. |
| TA3336 | *cya-407* | Spontaneous nonsense mutation to fosfomycin and streptomycin resistance in LT2. (Manuscript in preparation.) |
| CGSC 4254 | *argG6 metB1 his-1 thy-23 leu-6 recA1 mtl-2 xyl-7 malA1 gal-6 lacY1* or *lacZ4 str-104 sup-48*?/F'116 | Coli genetic stock center. |
| SA535 | HfrK5 *serA13* | K. E. Sanderson, University of Alberta Calgary, Alberta, Canada. |

donor strain was diluted into nutrient broth supplemented with 1.3% (wt/vol) glucose and 0.67% (vol/vol) medium E and shaken for 1.5 h at 25 C. Phage were added at a multiplicity of infection of 0.01 and growth was continued overnight. Chloroform was added and the suspension was agitated vigorously (on a Vortex) and then centrifuged. The precipitated debris was discarded and the phage were stored at 4 C over chloroform. Titers ranged from 4 to 8 $\times$ $10^9$ plaque-forming units/ml.

**Transductions.** Phage preparation (0.15 ml) was spread on selective medium plates which were then warmed to 37 C. A 0.1-ml sample of recipient bacteria was then spread and the incubation continued until colonies appeared, usually 2 days later. All transductants were screened fro sensitivity to phage P22 to allow isolation of phage-free clones.

**Centrifugations.** Bacteria were centrifuged in a Sorvall RC2B Superspeed Centrifuge (SS34 or GSA rotors) at 4,000 rpm for 15 min at 4 C.

**Episome transfer.** Donor strains were grown overnight in medium selecting for retention of the episome and then diluted into nutrient broth with 0.4% glucose and grown to log phase at 37 C. They were then centrifuged and resuspended in one-half volume of medium E and streaked across the selective medium plate with a 0.1-ml pipette. Recipient bacteria were taken from an overnight culture and also centrifuged and resuspended in one-half volume of medium E. When the donor streak had dried, recipients were streaked at right angles to the original streak. After several days, colonies appeared at the intersection of the two streaks. Episome-containing strains that transferred with high efficiency were streaked directly from washed overnight cultures.

**Antibiotic and analogue screening.** A sample (0.1 ml) from an overnight nutrient broth culture was suspended in 2 ml of top agar which was then poured on a petri plate containing the desired medium. After the agar had hardened, prepared antibiotic-impregnated paper disks were placed on the surface.

**Preparation of extracts for enzyme assays.** Cells were grown in 0.5 liter of medium in 2 liter flasks at 37 C. All further steps were conducted at 4 C. At an $A_{650}$ of 0.5, cultures were harvested and washed in 200 ml of 0.9% saline, and the pellets were frozen overnight and/or resuspended in 8 ml of 40 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.45, containing 1.6 mM $MgCl_2$ (buffer A). (Freezing of cell pellets for as long as several months had no detectable effect on phosphodiesterase activity.) The $A_{650}$ was determined and the suspensions were then disrupted using a Branson Sonifier Cell Disrupter, model W185D, with eight 20-s bursts of 70 W. Each burst was followed by a cooling period of 1 min. Sonic treatment was considered complete when the $A_{650}$ had been reduced to less than 5% of the original value. The extract was clarified by centrifugation in the Sorvall RC2B at 17,000 rpm for 20 min in the SS34 rotor. For desalting, 1 ml of the supernatant fluid was placed on a 4-ml Sephadex G-50 column equilibrated with buffer A. After the first 0.3-ml fraction was discarded, a second 1.0-ml fraction (containing most of the protein) was collected.

**Assay of cAMP phosphodiesterase.** Levels of cAMP phosphodiesterase activity were determined using a modification of the procedure employed by Butcher and Sutherland (7). A typical assay (0.45 ml) contained 3.5 $\mu$mol of cAMP, 0.1 mg of 5'-nucleotidase, 1 $\mu$mole of dithiothreitol, extract, and 100 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.4, containing 1.6 mM $MgCl_2$. The reaction was begun with the addition of extract. At 4- or 5-min intervals, samples were removed for phosphate determination. Each assay tube was paired with a duplicate containing no cAMP and the absorbance of this blank (due presumably to nuclease activity on nucleic acids) was subtracted from that of the parallel substrate-containing tube.

Desalted extracts exhibited a 6- to 12-min lag before the onset of linear activity. Some of this delay can be attributed to the kinetic properties of coupled reactions (20) and some to the kinetic parameters of the coupling enzyme 5'-nucleotidase (unpublished data). When these desalted extracts were assayed, the first point was not taken until 12 min after the start of the reaction.

**Assay of acid hexose phosphatase.** After the procedure of L. Kier, R. Weppelman, and B. Ames (manuscript in preparation), cells were grown to an $A_{650}$ of 0.5 in 5 ml of medium, harvested, and washed in the original volume of 0.9% saline. The pellet was resuspended in 2.2 ml of 0.1 M sodium acetate, pH 6.0, and the $A_{650}$ was determined for normalization purposes. Cells and buffer were added to 0.45 ml, and the reaction was started with the addition of 5 $\mu$mol of glucose-6-phosphate in 0.05 ml. A control tube containing 5 $\mu$mol of 5'AMP was incubated in parallel. At 0, 15, 30, and 45 min after the start, samples were removed from the reaction mix for phosphate determination. Activity of the acid hexose phosphatase was calculated by subtracting 70% of the observed activity of the extract with 5'AMP as a substrate from the observed activity of the extract with glucose-6-phosphate as the substrate. This correction compensated for the contribution of the non-specific acid phosphatase to the apparent hexose phosphatase activity (Kier et al., manuscript in preparation).

**Phosphate determination.** Phosphate released in the phosphodiesterase assay was determined by a modification of the methods of Chen et al. (8) and Ames (2). Samples (0.1 ml) were added to 0.7 ml of ascorbate-molybdate reagent which consists of 1 part 10% ascorbic and 6 parts 0.42% (wt/vol) ammonium molybdate in 1 N $H_2SO_4$. Tubes were incubated at 45 C for 20 min and absorbance was determined at 820 nm.

**Protein determination.** Protein concentration was determined by the method of Lowry et al. (19) using bovine serum albumin suspended in buffer A as standard.

**Heat sensitivity of phosphodiesterase.** Cells were grown as described with the exception that a temperature of 25 C was maintained. Samples (0.4 ml) of non-desalted extracts from both wild type and mutant were incubated in a water bath at 40 C as indicated, in a manner such that all tubes were withdrawn and placed on ice simultaneously. Ex-

tracts were assayed (at 25 C) within 5 min of their removal from the bath.

## RESULTS

**Selection and identification of cya cpd double mutants.** As shown in Fig. 1, a *Salmonella* mutant lacking adenyl cyclase (*cya*) can grow on minimal citrate medium only in the presence of the cAMP impregnated into the disk. However, in addition to the circle of confluent growth around the disk, and the few revertants to *cya*$^+$ that appear randomly distributed over the plate, a number of large colonies appear in a 1- to 2-cm wide ring beyond the edge of the zone of growth. Use of disk tests allows the study of bacteria in a concentration gradient: bacteria growing in this outer ring thus appear to require less exogenous cAMP for expression of catabolite-sensitive genes involved in growth on citrate than do wild-type bacteria. These colonies were picked and these new strains [among them TA3309 (*cpd-401 cya-408*)] were shown to give larger zones of growth than the parent strain TA3301 (*cya-408*) (38 versus 20 mm) when replated on these citrate plates with a disk of cAMP (Table 2 lines 2 and 3).

It was also shown that strain TA3309 could grow on minimal citrate medium with as little as 0.1 mM cAMP, one-tenth the level required by the parent. This allowed for a direct selection of these mutants through the plating of samples of a *cya* mutant culture on minimal citrate medium with 0.1 mM cAMP. The frequency of these mutants was 1 to 2 $\times$ $10^{-6}$.

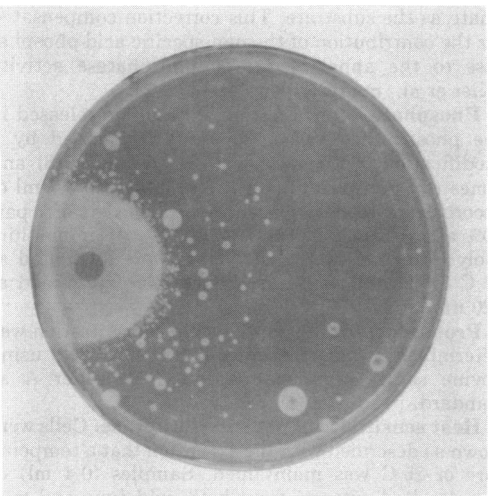

FIG. 1. *Selection of cpd mutants. 0.1 ml of overnight culture of TA3301 (cya-408) was poured on a minimal citrate plate in 2 ml of top agar. Disk contained 2 μmol of cAMP.*

TABLE 2. *Response to cAMP disk on minimal citrate plates, and cAMP phosphodiesterase activity of Salmonella mutants*[a]

| No. | Strain | Diameter of zone of growth | cAMP phosphodiesterase activity |
|---|---|---|---|
| 1 | LT-2 (wild type) | | 37.8 |
| 2 | TA3301 (*cya-408*) | 20 | 39.2 |
| 3 | TA3309 (*cpd-401 cya-408*) | 38 | <0.3 |
| 4 | TA3311 (*cpd-401*) | | <0.3 |
| 5 | TA3332 (*cpd-402 cya-405 metC30*) | 40 | <0.3 |
| 6 | TA3315 (TA3332/F'116 *met*$^+$) | 10 | 116.0 |
| 7 | SA535 (HfrK5 *serA13*) | | 34.4 |
| 8 | TA3310 (HfrK5 *cpd-401 serA13*) | | <0.3 |
| 9 | SA535 + TA3310 | | 36.4 |

[a] Enzyme assay described in Materials and Methods. Cells were pregrown in minimal glucose medium. In disk test, 0.1 ml of cells was poured (in 2 ml of top agar) on minimal citrate plate and zone of growth was measured around disk with 2 μmol of cAMP after 24 h. Enzyme activity is in nanomoles of phosphate released per milligram of protein per minute. Activity of mixed extracts of SA535 and TA3310 was calculated on the basis of half the actual protein added. Equal amounts of extracts were mixed.

**Assays of extracts.** When extracts from several of these mutants (e.g., TA3309, TA3332) were assayed, it was found that they were devoid of cAMP phosphodiesterase activity (less than 1% of wild type) (Table 2, lines 2,3,5). Table 2 (lines 7,8,9) also shows the results of reconstruction experiments in which extracts from wild-type (SA535) and mutant strains (TA3310) are combined. As can be seen, the activity of the wild type is unaffected by the extract from the mutant. Thus, loss of activity in our mutants is not due to the presence of an inhibitor.

**Map positions of cpd gene.** Transduction studies (Table 3) ruled out the possibilities that the ability of the adenyl cyclase-negative strain to grow on citrate with low cAMP was a result of a new mutation in or linked to either the *cya* or *crp* genes. The mutation was not co-transducible with either *metE* or *cysG*, markers linked by phage P22 transduction to *cya* and *crp* with frequencies of 8 and 16%, respectively (4, 15). Time of entry experiments indicated that the gene for the phosphodiesterase lay between 80 and 120 min on the *Salmonella* chromosome. Co-recombination frequency determinations with markers in this region narrowed the range. Finally, *cpd* was shown to be 25% co-transduci-

ble with *metC* using phage P22 (Table 3). Further verification of this position was provided by episome crosses. *E. coli* episome F'116 which covers the region from 95 to 105 min on the *Salmonella* chromosome was crossed into strain TA3332 (*cpd-402 cya-405 metC30*). The resulting merodiploid strain TA3315 has cAMP phosphodiesterase activity (Table 2 lines 6,7) indicating that the gene for the enzyme is carried on this episome. The excess of enzyme activity compared with that of the wild-type presumably results from a gene dosage effect.

**Selection of temperature-sensitive mutants.** To verify that our mutations are in the structural gene for phosphodiesterase rather than in a regulatory gene, temperature-sensitive mutants were isolated. Mutants of strain TA3301 were selected as described (on minimal citrate plates containing 0.1 mM cAMP) except that the plates were incubated at 40 C for the first day and at 25 C for second. Small colonies were picked and scored for growth on this same selective medium and on minimal glucose at both 40 and 25 C. Strains that grew under three of these conditions, but not on citrate-cAMP medium at 25 C, were picked and examined for temperature-sensitive phenotype (Table 4). Phosphodiesterase activity in the extract from a temperature-sensitive strain TA3319 (*cpd-405 cya-408*) was lost at a far greater rate than activity in an extract from wild-type cells when both crude extracts were incubated at the nonpermissive temperature of 40 C (Fig. 2). Both strains were grown at 25 C, the temperature at which the mutant produces wild-type levels of the enzyme, so it is extremely unlikely that loss of activity was due to anything other than heat inactivation of the protein.

TABLE 3. *Co-transduction of cpd mutation with known markers*[a]

| Recipient | % of prototrophic transductants that are capable of growth on minimal citrate with 0.1 mM cAMP |
|---|---|
| TA2602 (*cya-408 cysG439*) | 0 (0/479) |
| TA2604 (*cya-408 metE338*) | 0 (0/529) |
| TA3330 (*cya-408 metC30*) | 24.5 (39/159) |

[a] For recipients TA2602 and TA2604 phage were grown on TA3303 (*cpd-401 cya-408*). Crosses were performed selecting for growth on unsupplemented glucose medium and the resulting transductants were scored for growth on minimal citrate medium with 0.1 mM cAMP. For recipient TA3330, phage were grown on TA3309 (*cpd-401 cya-408*) and the same cross was performed. (Genotype of *met*⁻ recipients was verified by nutritional analysis with intermediates in methionine biosynthesis and the location of *metC30* was examined by crosses with episome F'116.)

TABLE 4. *Characteristics of temperature-sensitive cAMP phosphodiesterase mutant*[a]

| Strain | Diameter of zone of growth | | cAMP phosphodiesterase activity (25 C) |
|---|---|---|---|
| | 25 C | 40 C | |
| LT-2 (wild type) | | | 12.1 |
| TA3301 (*cya-408*) | 18 | 20 | |
| TA3309 (*cpd-401 cya-408*) | 36 | 50 | |
| TA3319 (*cpd-405 cya-408*) | 18 | 45 | 11.6 |

[a] Procedures as described in Table 2.

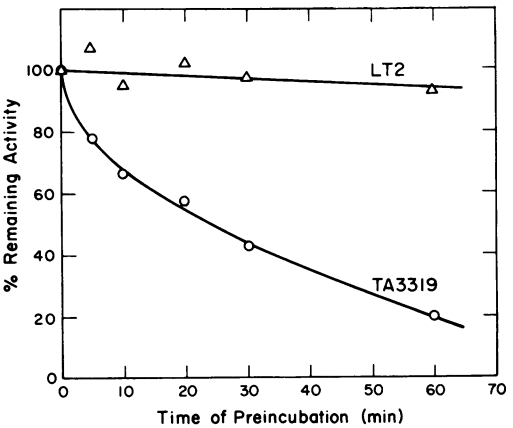


FIG. 2. *Heat sensitivity of cAMP phosphodiesterase activity in extract from temperature-sensitive mutant TA3319 (cpd-405 cya-408). Procedures are as described in Materials and Methods. 100% activity is 12.1 nmol/mg per min for LT2 and 11.6 nmol/mg per min for TA3319, at 25 C.*

**Growth characteristics of cpd⁻ cya⁺ strains.** It was expected that strains lacking cAMP phosphodiesterase activity might have increased intracellular levels of cAMP under certain conditions. If so, such strains might exhibit increased sensitivities to those antibiotics and analogues whose effectiveness against *Salmonella* has been linked to endogenous cAMP levels (Alper and Ames, manuscript in preparation). These expections were realized as shown in Table 5. Three inhibitors, fosfomycin, nalidixic acid, and 2,2-difluorosuccinic acid, all of which are less effective against *cya* and *crp* mutants than against the wild-type (Alper and Ames, manuscript in preparation), were more effective against *cpd* mutants than against the wild type. Two others, penicillin and tetracycline, which affected *cya*, *crp*, and wild-type strains equally, were no more effective against *cpd* mutants than against these other strains.

TABLE 5. *Increased analogue sensitivity of cAMP phosphodiesterase mutant*[a]

| Inhibitor | Diameter of zone of inhibition: LT-2-glucose (mm) | Ratio of diameters of zones of inhibition [TA3333[c]/LT2] × 100) | | |
|---|---|---|---|---|
| | | Glucose | Citrate | Citrate + 1 mM cAMP |
| Fosfomycin (20 μg | 20 | 115 | 155 | 170 |
| Nalidixic acid (30 μg) | 28 | 110 | 133 | 183 |
| 2,2-Difluoro-succinate (400 μg) | NI[b] | NI[b] | 115 | 236 |
| Penicillin (10 U) | 17 | 103 | 111 | 96 |
| Tetracycline (30 μg) | 30 | 97 | 100 | 103 |

[a] Procedure described in Materials and Methods. Zone of inhibition was defined as area through which the outline of a 15-W fluorescent bulb could be clearly seen at a distance of 6 inches. All readings done in at least triplicate and the results averaged.

[b] NI, no inhibition of either strain on minimal glucose.

[c] *cpd-404*. Almost identical results were obtained using independently isolated strain TA3311 (*cpd-401*).

Also, as expected, the difference in sensitivities between wild type and strain TA3333 (*cpd-404*) increased when cellular levels of cAMP were increased through growth of the cells on poor carbon sources. Addition of 1.0 mM cAMP further magnified these differences.

*cpd* mutants also exhibited striking differences in growth rate on a variety of media when compared to their isogenic parents. These mutants grew normally on glucose (with and without added nutrient broth or glucose-6-phosphate) on glycerol or on citrate as a carbon source (Table 6, group I). However, they exhibited significantly increased growth rates relative to the wild type on succinate as carbon source, or on glucose with proline rather than ammonia as the source of nitrogen. Use of succinate as a source of carbon and of proline as a source of nitrogen are both dependent on the expression of catabolite-sensitive genes (11, 18, 24; B. J. Ratzkin, M. Grabnar, and J. R. Roth, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1973, G9, p. 27) and, as is evident from the effect of the addition of cAMP to the medium (group III), cAMP levels were limiting under growth conditions used here. Further, relative to the wild type, the mutants showed distinctly decreased growth rates on either glucose or citrate when 1.0 mM cAMP was added and failed to grow at all on glycerol with cAMP (group II).

These differences could also be demonstrated on solid media and were adapted for rapid screening tests for *cpd* mutants. Sensitivity to cAMP can readily distinguish mutant from wild type on citrate medium (Table 7). Response to cAMP for growth on plates with glucose as the source of carbon and proline as a source of nitrogen can also be used to identify mutants (Table 8). High concentrations of cAMP inhibited the *cpd* mutant whereas lower concentrations stimulated its growth more than that of the wild type. Finally, as expected from results on Table 5, cAMP phosphodiesterase mutants can be identified by their failure to grow on medium with glycerol and cAMP. This is perhaps the most useful phenotype since many strains

TABLE 6. *Growth rates of strains retaining and lacking cAMP phosphodiesterase*[a]

| Growth medium | Addition | Doubling time (min) | |
|---|---|---|---|
| | | LT2 | TA3311 (*cpd-401*) |
| Group I | | | |
| Glucose-ammonia | Nutrient broth | 32 | 33 |
| Glucose-ammonia | Glucose-6-$PO_4$ | 45 | 48 |
| Glucose-ammonia | | 53 | 52 |
| Glycerol-ammonia | | 67 | 67 |
| Citrate-ammonia | | 78 | 76 |
| Succinate-ammonia | | 343 | 165 |
| Glucose-proline | | 830 | 346 |
| Group II | | | |
| Glucose-ammonia | 1 mM cAMP | 55 | 90 |
| Glycerol-ammonia | 1 mM cAMP | 66 | ND[b] |
| Citrate-ammonia | 1 mM cAMP | 85 | 250 |
| Group III | | | |
| Glucose-proline | 1 mM cAMP | 322 | 127 |
| Succinate-ammonia | 1 mM cAMP | 240 | 89 |
| Succinate-ammonia | 2 mM cAMP | 176 | 94 |
| Succinate-ammonia | 4 mM cAMP | 105 | 96 |
| Succinate-ammonia | 10 mM cAMP | | 96 |
| Ammonia | 5 mM cAMP | ND[b] | |

[a] Overnight cultures were diluted 1/50 into 6 ml of medium. Incubation and monitoring of growth were as described in Materials and Methods. Cells were pregrown in minimal citrate except for those to be tested in glucose medium which were pregrown in glucose. $N^-C^-$ medium was used throughout except when citrate was added. In those cases, medium E was used. Growth conditions list carbon and nitrogen source present. All determinations were done in at least triplicate. Carbon sources were at 0.4% except nutrient broth (0.8%) and glucose-6-phosphate (0.5%). Ammonia was at 15 mM, proline at 20 mM. Results with TA3311 were duplicated with independently isolated *cpd* mutant TA3333 (*cpd-404*).

[b] ND, no detectable increase in turbidity after 4 days of incubation.

TABLE 7. *Sensitivity of phosphodiesterase mutant to cAMP on citrate medium*[a]

| Carbon source | cAMP on disk (μmol) | Diameter (cm) zone of inhibition | |
|---|---|---|---|
| | | LT-2 | TA3311 (*cpd-401*) |
| Glucose | 1 | <0.6 | <0.6 |
| Citrate | 1 | <0.6 | 1.6 |
| Citrate | 2 | 1.0 | 2.0 |

[a] Phenotype studies performed as described in Materials and Methods. Since paper disks have a diameter of 0.6 cm, zone of inhibition cannot be smaller.

TABLE 8. *Response of phosphodiesterase mutant to cAMP on glucose-proline medium*[a]

| cAMP on disk (μmol) | LT2 | | TA3311 (*cpd-401*) | |
|---|---|---|---|---|
| | Diameter of zone of inhibition (cm) | Diameter of ring of growth (cm) | Diameter of zone of inhibition (cm) | Diameter of ring of growth (cm) |
| 1 | <0.6 | 0.7 | <0.6 | 1.7 |
| 2 | <0.6 | 1.1 | 0.8 | 2.0 |

[a] Disk tests were performed as described in Materials and Methods. Inhibition phenomenon is described in text, see also Table 6.

can be tested on a single glycerol-ammonia-salts-cAMP plate. This inhibition might be due to the accumulation of toxic levels of methylglyoxal (1).

**Selection of cpd mutants in adenyl cyclase-containing strains.** As mentioned, *cpd* mutants grow faster on succinate-ammonia media than the wild type (Table 6). This difference was exploited to provide a selection procedure for the mutants in wild-type backgrounds. When $10^8$ wild-type cells were plated on this medium, approximately 400 fast-growing colonies appeared after 2 days of incubation, a day before the remaining colonies appeared as a lawn. Roughly one-third of these were distinctly smaller than the others. Among these colonies, 5 of 28 of those tested contained cells that were hypersensitive to cAMP, whereas none of 50 large ones tested did. Both of the cAMP-hypersensitive strains tested further (TA3334*a* and *b*) lacked detectable cAMP phosphodiesterase activity.

**Other classes of cya⁻ mutants responding to low levels of cAMP.** Nielson et al. (22) have reported that activity of the cAMP phosphodiesterase in *E. coli* is dependent on two proteins. We have been unable to find evidence for two genes for this enzyme in *Salmonella*. Thirty-three independent mutants isolated in six different strains were examined. Twenty-seven of these carried *cpd* mutations that were co-transducible with *metC*. Six others from two *cya* mutants (five in one) were not co-transducible with *metC*, but they all appeared to grow slightly on ribose and all were shown to have normal phosphodiesterase activity. These are probably partial revertants of *cya*.

**Activity of acid hexose phosphatase in strains lacking phosphodiesterase.** To study the effect of the loss of the phosphodiesterase on catabolite repressed systems, the levels of acid hexose phosphatase were examined in both the wild-type and the isogenic mutant after growth on glucose and on the poor carbon sources glycerol, citrate, and succinate. Results of these experiments are shown in Table 9. *cpd* mutant TA3311 (*cpd-401*) is clearly not released from the catabolite repression effect of growth on glucose, producing no more hexose phosphatase than the parent strain. In addition, it shows normal derepression when grown on poorer carbon sources. This is, or course, in sharp contrast with the results presented above implicating the cAMP phosphodiesterase directly in the expression of catabolite-sensitive systems.

## DISCUSSION

The involvement of cAMP in the regulation of expression of a wide variety of genes involved in carbon and energy source utilization in the bacterial cell has been amply demonstrated (5, 9, 17, 25). One aspect of this control is catabolite repression: the presence of a good carbon source such as glucose results in a lowering of cAMP levels below that required for the synthesis of enzymes involved in transport and metabolism of other carbohydrates. cAMP phosphodiesterase, which so far as is known is the only bacterial activity capable of degrading cAMP, would be expected to be involved in the regula-

TABLE 9. *Acid hexose phosphatase activities in wild-type and phosphodiesterase-lacking mutant*[a]

| Growth medium | Strain | |
|---|---|---|
| | LT-2 | TA3311 (*cpd-401*) |
| Glucose | 18.0 | 18.6 |
| Glycerol | 22.0 | 23.2 |
| Citrate | 42.0 | 61.6 |
| Succinate | 123.6 | 101.0 |

[a] Procedure as described in Materials and Methods. Activity is expressed in millimolar phosphate released per minute per optical density unit.

tion of cAMP levels, but in fact little evidence exists concerning its role in cell physiology.

To study this question we have devised positive selection techniques for the isolation of *S. typhimurium* mutants lacking cAMP phosphodiesterase. All 27 mutations of this type were mapped at a single locus, *cpd*, which is 25% co-transducible by phage P22 with *metC* at 100 min on the chromosome. Several lines of evidence indicate that the *cpd* gene is the structural gene for the enzyme. One of the mutants is phenotypically temperature sensitive and produces an enzyme that loses activity in extracts incubated at the nonpermissive temperature. In addition, all the mutations tested are recessive in merodiploids made with an *E. coli* episome covering the *metC* region.

Nielson et al. (22) have reported that cAMP phosphodiesterase activity of *E. coli* is dependent on two proteins, one of which contains iron. We have found no genetic evidence for the existence of more than one protein in this enzyme, nor for any effect of iron on enzyme activity in the admittedly crude extracts from wild-type or phosphodiesterase-lacking mutants of *Salmonella*. If, however, a second protein in the phosphodiesterase had additional functions and were essential for cell viability, or if the gene for the "second" protein were closely linked to the first, it would have escaped our detection.

In addition, we assayed extracts of two of our mutants in the presence of added iron (unpublished data), but neither regained any of the lost activity. Nielson et al. (22) had reported that in *E. coli* iron could substitute for the second (iron containing) protein in activation of partially purified extracts containing the first (catalytic) unit. If this is true in *Salmonella*, then neither of these two mutants is lacking the iron-containing protein.

We have shown here that there are several catabolite-sensitive growth characteristics of *Salmonella* that are altered in mutants lacking cAMP phosphodiesterase. These results would support a claim that the enzyme is involved at least to some extent in catabolite repression. The mutants exhibit increased sensitivity to a variety of amino acid analogues and antibiotics and they show accelerated growth on glucose-proline and succinate-ammonia media. The most likely explanation for increased antibiotic and analogue sensitivity lies in the increased activity of cAMP-controlled transport of these inhibitors (Alper and Ames, manuscript in preparation). Increased transport of succinate through the cAMP-controlled dicarboxylic acid transport system (18, 24) is the most reasonable explanation of the mutant's faster growth on succinate. Although there is no proof that the limiting factor in proline utilization as a nitrogen source is its transport rather than degradation, both have been shown to be catabolite sensitive (11; B. J. Ratzkin, M. Grabnar, and J. R. Roth, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, G9, p. 27). Inhibition of phosphodiesterase mutants by growth on cAMP and glycerol (and perhaps the other carbon sources noted) might also be a result of increased transport or metabolism resulting in accumulation of the proximate inhibitor (1). On the other hand our results showing that levels of acid hexose phosphatase (a cAMP-controlled enzyme; Kier et al., manuscript in preparation) are equivalent in isogenic strains lacking and retaining the cAMP phosphodiesterase (Table 9) indicate that cAMP phosphodiesterase does not play a role in certain other aspects of catabolite repression. No more clarifying are the results from two other labs; one shows that Crooke's strain and a mutant of AB257, both of which lack cAMP phosphodiesterase, are released from catabolite repression (6, 15) whereas the other shows that, at least with respect to $\beta$-galactosidase and acid hexose phosphatase, loss of phosphodiesterase has no effect on catabolite repression (26). Recent reports from a third group (U. Winkler, personal communication) indicate that cAMP phosphodiesterase mutants of *S. marcescens* are not released from catabolite repression. It should be noted here that parent strain AB257 is itself hypersensitive to catabolite repression and that true isogenic wild-type strains were not available for either of these strains. In fact, it has been shown (M. Saier, personal communication) that the phosphodiesterase mutant of AB257 differs from the parent at at least two loci.

cAMP phosphodiesterase is clearly capable of a role in the fixing of the cAMP levels in the cell. We suggest that the apparent contradiction presented here—some catabolite-sensitive systems being affected by the loss of cAMP phosphodiesterase whereas others are not—can be explained by postulating a "hierarchy" of cAMP-sensitive operons (M. D. Alper, Ph.D. thesis, Univ. of California, Berkeley, Calif., 1974; Alper and Ames, manuscript in preparation). In such a model, operons would be ordered on steps each requiring more cAMP-CRP complex for induction than the one a step lower. Thus, the higher cAMP levels resulting from loss of phosphodiesterase activity might result in increased expression of some operons, but not in others. We have shown that the most significant effects of the loss of the phosphodiesterase occur at high cAMP levels, thus, those operons not affected by its loss would be those

at the bottom of the hierarchy which are fully expressed at the high levels of cAMP existing even in the presence of the enzyme. These would therefore not respond to further increases in the level of cAMP. Evidence from both in vitro and in vivo systems (17, 25) has already documented the requirement of different cAMP concentrations by different systems.

cAMP phosphodiesterase also appears to protect the cell from high, inhibitory levels of cAMP that it might itself synthesize or accumulate from the medium. It is known that high levels of cAMP lead to inhibition of various systems in *E. coli* (9, 10, 13, 17) and, in fact, the growth of the organism itself (1, 16). We have shown this to be true also in wild-type *Salmonella*, and to a much greater extent in strains lacking the phosphodiesterase (Tables 6, 7, 8). It has also been shown in *E. coli* that significant amounts of cAMP accumulate in the growth medium (21, 26). Finally, as seen in Tables 2, 5, 6, 7, 8, the phosphodiesterase is very efficient in cleaving exogenously supplied cAMP, thereby decreasing its influence over cell functions. For example, *cya⁻cpd⁻* strains respond to one-tenth the cAMP required by *cya⁻cpd⁺* strains for growth on citrate-ammonia media. It therefore appears that a very important function of the phosphodiesterase is to protect the bacteria from excess cAMP. Further support of this argument is the reported $D_m$ of the enzyme for cAMP: 0.5 mM (22). This represents a level of substrate at least an order of magnitude above reported physiological, noninhibitory levels (6). Thus, the rate of degradation of cAMP increases in proportion to the increase in its concentration towards toxic levels.

Although, as mentioned above, the cAMP phosphodiesterase is efficient in cleaving exogenously supplied cAMP, it is not a periplasmic enzyme. We were unable to detect any activity in undisrupted cells (unpublished data). This observation is in contrast to the *Salmonella* phosphodiesterase which cleaves 2',3'-cyclic nucleotides. This enzyme is active in whole cell assays (Kier et al., manuscript in preparation).

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## ADDENDUM IN PROOF

H. V. Rickenberg has independently suggested the existence of a "hierarchy" of operons regulated by cAMP (Annu. Rev. Microbiol. **28**:353-369, 1974).

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