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Transport of Antibiotics and Metabolite Analogs by Systems Under Cyclic AMP Control: Positive Selection of *Salmonella typhimurium cya* and *crp* Mutants

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Mutants in the cyclic AMP (cAMP) control system in *Salmonella typhimurium* (*cya* = adenyl cyclase, *crp* = cAMP receptor protein) were partially resistant to growth inhibition by 22 antibiotics (including fosfomycin, nalidixic acid, and streptomycin) and 29 inhibitory analogs of normal bacterial fuel/carbon sources. This resistance was used as the basis for an efficient positive selection of *cya* and *crp* mutants. We propose that these antibiotics and analogs enter the bacteria through transport systems normally used for transporting fuel/carbon sources and that this is accomplished because of a structural similarity between the antibiotic and the natural substrate of the particular transport system involved. We propose that these transport systems are all under positive control by cAMP and that cAMP acts as a signal molecule (alarmone) for fuel/carbon deprivation. Evidence is provided for a hierarchy within operons controlled by cAMP. The methodology is shown to be useful for analyzing both antibiotic transport systems and the cAMP super-control system.

The level of cyclic AMP (cAMP) in *Escherichia coli* and *Salmonella typhimurium* appears to be an indicator of, and coupled with, the state of fuel/carbon source availability: the greater the limitation, the greater the cAMP concentration. cAMP, in turn, regulates cell metabolism for the most efficient response to this limitation. The complex of cAMP and the cAMP receptor protein (CRP) activates the promoters of a wide variety of genes concerned with transport and utilization of fuel sources (20, 34, 35, 37, 38, 41, 46). In *E. coli* and *S. typhimurium*, *crp* mutants (lacking functional cAMP receptor protein) and *cya* mutants (lacking functional adenyl cyclase, which synthesizes cAMP from ATP) are unable to grow on most fuel/carbon sources other than glucose or gluconate (6, 9, 21, 24, 37, 43, 46). A large number of laboratories have contributed to our understanding of this area. Their contributions to the field have been extensively and frequently reviewed (12, 33, 35, 36, 42, 55).

The present study utilizes antibiotics and inhibitory analogs of fuel/carbon sources to analyze the cAMP super-control system and antibiotic and analog transport.

MATERIALS AND METHODS

Chemicals. cAMP, glucose-6-phosphate (G6P), and L-α-glycerophosphate were purchased from Sigma Chemical Co., St. Louis, Mo. ICR-372 was a gift of R. Peck and H. Creech of the Institute for Cancer Research, Philadelphia, Pa. Uniformly labeled L-phenylalanine (455 mCi/mmol) was purchased from Schwarz/Mann, Orangeburg, N.Y. Chemicals were obtained as follows: D-α-hydrazinoimidazole propionate (47) was a gift of J. Lever and G. F.-L. Ames; DL-*p*-fluorophenylalanine, 2-deoxy-D-galactose, 1-amino-2-phenylethyl phosphonic acid, fluoropyruvate, 6-hydroxy-DL-tryptophan, and methionine sulfoximine were purchased from Calbiochem, La Jolla, Calif.; azaserine was from Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.; L-azetidine-2-carboxylic acid was from Aldrich Chemical Co., Milwaukee, Wis.; 3,4-DL-dehydroproline was from Regis Chemical Co., Morton Grove, Ill.; L-norleucyl-glycine, L-norvalyl-L-norvaline, norglycyl-norleucine, L-norglycyl-glycyl-glycine, glycyl-L-norleucine, 4-methyltryptophan, 7-aza-DL-tryptophan, β-chloro-L-alanine, L-ethionyl-L-alanine, 2-thiazolyl-DL-alanine, and DL-*o*-fluorophenylalanine were from Cyclo Chemical Co., Los Angeles, Calif.; 5-methyl-DL-tryptophan and D-cycloserine were from Mann Research Laboratories Inc., New York, N.Y.; β-2-thienylalanine, thioproline, L-3-aminotyrosine, and DL-3-thienylalanine were from Nutritional Biochemicals Corp., Cleveland, Ohio; DL-5-fluorotryptophan and DL-serine hydroxamate were from Sigma; 2,2-difluorosuccinate, DL-*m*-fluorophenylalanine, and 5',5',5'-trifluoroleucine were from PCR Research Chemicals, Inc., Gainesville, Fla.; and dichlorodivinylcysteine was a gift of M. O. Schultze, University of Minnesota, St. Paul, Minn. Antibiotics listed in Table 4 were obtained on paper disks from Baltimore Biological Laboratory and Bioquest, Cockeysville, Md., except for streptomycin sul-

fate (from Eli Lilly & Co., Indianapolis, Ind.) and fosfomycin (phosphonomycin) [(−)-1,2-*cis*-epoxypropyl phosphonic acid], which was a gift of F. Kahan, Merck Center for Therapeutic Research, Rahway, N.J.

Bacterial and phage strains. The bacterial strains used in this study are listed in Table 1. Transductions were done with the *cly-2int*-4 mutant (19) of phage P22. The temperature-sensitive *cly*-2 mutation greatly reduces the lytic response of the phage when grown on adenyl cyclase-lacking bacterial mutants at 37°C (24). Nonsense mutants of P22, *amN6* (isolated by D. Botstein), and *amH204* (30) were provided by J. R. Roth.

Media. Nutrient broth (0.8%) (Difco Laboratories, Detroit, Mich.) with 0.5% NaCl added was used. The E medium of Vogel and Bonner (51), which contains 0.2% citric acid, was used as our minimal salts medium. Carbon sources were sterilized independently and added to the medium (0.4%, wt/vol), which is then referred to as minimal citrate, minimal glucose, etc. Minimal medium without a source of carbon or 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; and $MgSO_4 \cdot 7H_2O$, 0.1 g. NH_4Cl (10 mM) was added as a source of nitrogen. Solid medium contained 1.5% agar (Difco). Top agar (0.6% agar and 0.5% NaCl) was kept molten at 45°C for pouring on plates.

Culture conditions. Cells were grown at 37°C in a New Brunswick gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.), and growth of the culture was followed by observing the increase in absorbance at 650 nm in a Zeiss model PMQ II spectrophotometer. Inocula were 1 to 2%.

Growth of phage. An overnight culture of the donor strain was diluted 1:100 into nutrient broth supplemented with 1.3% (wt/vol) glucose and 0.67% (vol/vol) medium E. The culture was shaken for 1.5 h at 25°C before phage were added at a ratio of 1 per 100 bacterial cells. Growth was continued overnight, chloroform was added, and the mixed suspension was centrifuged. The precipitated debris was discarded, and the phage (ca. 10^{10}/ml) were stored at 4°C over chloroform.

TABLE 1. *Bacterial strains*

Strain	Genotype	Source and comments
LT2	Wild type	*S. typhimurium*
LT2A	Wild type	Chlorate-resistant variant used only where specified
TA926	*aroP701 hisP1661*	G. F.-L. Ames, this laboratory
TA2301	*cya-408*	J. S. Hong (24) in LT2A (mutagenized background)
TA2305	*crp-403*	J. S. Hong (24) in LT2A (mutagenized background)
TA3301	*cya-408*	LT2 by transduction to fosfomycin resistance with TA2301 as donor
TA3302	*crp-403*	LT2 by transduction to fosfomycin resistance with TA2305 as donor
TA3336	*cya-407*	Spontaneous nonsense mutation to fosfomycin and streptomycin resistance in LT2
TA3339	*crp-401*	Spontaneous nonsense mutation to fosfomycin and streptomycin resistance in LT2
SB3184	*metB36 purG302 F'14*	P. Hartman, by L. Kops and B. Ely
SL4040	*met trp H1b H2enx fla str gal F'gal sup-812*^{och}	B. A. D. Stocker, courtesy of P. E. Hartman

Antibiotic and analog screening. A 0.1-ml amount of 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 solidified, prepared antibiotic-impregnated disks were placed on the plate. Solutions of those compounds for which prepared disks were not available were absorbed into sterile blank paper disks. Results were recorded after 24 h of incubation unless significant changes occurred in the 48 h after the initial reading. The diameter of the paper disk was 6 mm, and lack of inhibition is indicated by <6 mm. The zone of inhibition is defined as the area through which the outline of a 15-watt lit fluorescent bulb could be distinctly seen at a distance of 6 inches (ca. 152.4 mm). All comparisons were made in at least triplicate, and the results were averaged.

Transductions. A 0.15-ml amount of phage preparation was spread on selective medium plates, which were then warmed to 37°C. A 0.1-ml amount of a full grown culture of recipient bacteria was then spread, and the incubation continued until colonies appeared, usually 2 days later.

Centrifugations. Bacterial cultures were sedimented in a Sorvall RC2B Superspeed centrifuge (SS34 or GSA rotors) at 4,000 rpm for 15 min.

Mutagenesis with ICR-372. A 0.01-ml amount of an overnight culture of LT2 in nutrient broth was diluted into 1.0 ml of medium E containing 0.4% glucose and 0.01 mg of ICR-372. After 18 h of incubation at 37°C, 0.1 ml was diluted into 6 ml of nutrient broth and grown to stationary phase.

Reversion of mutants with ICR-372. A 0.1-ml amount of a fully grown culture of the strain to be tested was suspended in 2 ml of top agar and poured on a minimal ribose plate. A paper disk impregnated with 0.005 ml of ICR-372 (1 mg/ml) was placed at the center of the plate, which was then incubated at 37°C for about 4 days or until colonies were visible. The number of colonies within a 1.5-cm radius of the center of the disk was compared to the number outside the area. Since the inner area represented 12% of the area of the plate, a positive result was claimed if more than 50% of the colonies on the plate were within the small circle.

Episome transfer. Donor male strains were diluted 1:20 from an overnight selective medium culture into nutrient broth containing 0.4% glucose and grown without shaking to log phase at 37°C. They were then centrifuged, and the pellet was suspended in one-half the original volume of 0.9% saline 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 suspended in one-half the original volume of saline. When the donor streak had dried, recipients were streaked perpendicularly to it. After several days, colonies appeared at the intersection of the two streaks and along the path of the

recipient after it had crossed the donor streak. Colonies were picked and restreaked on selective medium. Episome-containing strains, which transferred at high efficiency, were crossed directly from washed overnight cultures.

Episome mapping of *cya* mutants. Strain SB3184 carried the M episome F'14, which covers the region of the *E. coli* chromosome including the *cya* gene. When this strain was crossed with our cAMP mutants on minimal ribose plates, colonies appeared at the intersection when the recipient was *cya*, but not when it was *crp*.

Mapping of *cya* and *crp* mutants by transduction. Phage P22*cly-2int-4* was grown on the mutant to be tested, and the resulting phage were used to transduce recipient strains *metE338* and *cysG439* to prototrophy on minimal glucose plates. Small colonies were picked and tested for inability to ferment various sugars, indicating contransduction of the mutant *cya* or *crp* gene with the *metE* or *cysG* gene.

Assay of uptake of amino acids in growing cells. The method of G. F.-L. Ames (4) was used with the modification of filtering the trichloroacetic acid-treated samples without boiling. Cell densities at the time of assay were determined as described (4) and also by determining the number of cells directly (by plating) at the time of the experiment.

RESULTS

Selection of mutants. The antibiotic fosfomycin (phosphonomycin) is an inhibitor of bacterial cell wall biosynthesis (26). It is actively transported into wild-type *E. coli* and *S. typhimurium* by two independent transport systems; that for L-α-glycerophosphate (26, 28) defective in strains with mutations in the gene *glpT* and that for hexose phosphates (18, 25, 26) defective in strains with mutations in the gene *uhp*. Both of these transport systems are under cAMP control; they are lacking in *cya* and *crp* mutants and are repressed by glucose (28, 53). Thus by selecting for fosfomycin-resistant mutants in wild-type bacteria (Table 2), we required simultaneous elimination of the expression of both transport systems and therefore select for *cya* or *crp* mutations. Table 2 shows the selection of these mutants when wild-type (LT2) bacteria (induced for *uhp*) were plated on MacConkey indicator plates with glycerol, ribose, and fosfomycin. Roughly 10 colonies appeared per plate after 2 days of incubation: almost all were gold colored (nonfermenters of both sugars). Of the 37 fosfomycin-resistant nonfermenters analyzed (see above), about half were *cya* (adenyl cyclase) mutants: the addition of 1.0 mM cAMP restored the wild-type phenotype, and the mutation involved was 8% cotransducible (by phage P22) with *metE338* (24, 54). The other half were *crp*

TABLE 2. *Classes of mutants selected on the basis of fosfomycin resistance*

Presumed mutation[a]	Parent bacteria[b]	Selection medium[c]		Colony observed		Phenotype[d]					
		Fosfomycin (μg)	Additions	Color[e]	Frequency	Glc	G6P	Rib	Gly	αGP	Cit
cya/crp	LT2[f]	200	Rib	Gold	~5×10^{-8}[g]	+	−	−	−	−	−
cya/crp	LT2[h]	140	Rib, str	Gold	~5×10^{-8}[g]	+	−	−	−	−	−
glpT	LT2	800		Red	10^{-6}	+	+	+	+	−	+
glpR[c]	LT2	800		Gold	5×10^{-8}	+	+	+	−	−	+
fm[r]	LT2	800		Red	10^{-7}	+	+	+	+	+	+
uhp	*glpT*	200[i]		Red	10^{-6}	+	−	+	+	−	+

[a] *glpT* is deficient in L-α-glycerophosphate transport (26, 28); *glpR*[c] is noninducible for the entire glycerol operon (15); *uhp* is deficient in transport of hexose phosphates (18, 25, 26); *fm*[r] has a presumptive alteration in phosphoenolpyruvate, uridine diphospho-*N*-acetylglucosamine enolpyruvyl transferase (50). Only *cya* and *crp* mutants were verified beyond the phenotypic analysis shown. Mutants in the *pts* operon have also been shown to be resistant to fosfomycin (11, 14).

[b] Bacterial cultures were grown on minimal glucose medium. A 0.1-ml amount was spread on selection plates.

[c] Indicator plates were MacConkey agar with 1% glycerol. Ribose (rib) (1%) was added as indicated. Fosfomycin and 1.25 mg of streptomycin (str) sulfate were spread as indicated.

[d] Phenotype was determined by ability to grow on N^-C^- plate with ammonia and the indicated carbon source (0.6%). Glc, Glucose; G6P, glucose-6-phosphate; rib, ribose; gly, glycerol; αGP, L-α-glycerophosphate; cit, citrate.

[e] Red indicates fermentation of carbon source on selection plates, whereas gold indicates no fermentation.

[f] Cells were grown with the addition of 20 mM glucose-6-phosphate to induce *uhp* before plating.

[g] This selection isolates only "tight" mutants, those unable to ferment ribose. If leaky mutants were included in the calculations, the listed frequencies would be greater. Data from controls (reconstruction experiments) indicate that roughly half the *cya* or *crp* cells plated survived this selection. Thus the actual frequency is double the observed value.

[h] When ICR-372 mutagenized bacteria were used (see text), the frequency of *cya/crp* mutants was increased to 2×10^{-5}.

[i] When the bacteria were not preinduced for *uhp*, the same results were obtained with 1,200 μg of fosfomycin.

(CAMP receptor protein) mutants: cAMP had no effect on their phenotype, and the mutation involved was 16% cotransducible with *cysG439* (7, 24). Other classes of mutants could be obtained by modifying the conditions (Table 2): in particular, if the *uhp* system was not induced, mutants lacking only the *glpT* transport system were fosfomycin resistant.

In the process of characterizing the mutants, it was noted that the *cya* and *crp* mutants were also more resistant than was the wild type to the aminoglycoside antibiotic streptomycin. The *cya* and *crp* mutants could be selected from a population of wild-type cells not induced for *uhp* (not pregrown on G6P) if streptomycin was added along with the fosfomycin (Table 2). It seems likely that streptomycin was transported by a cAMP-controlled system other than that coded for by *glpT* (see below). We found that the procedure using fosfomycin alone with bacteria pregrown on G6P is the simpler and more efficient of the two. (Artman and Werthamer [6] have independently reported a somewhat more complex procedure for selection of *crp* mutants also using streptomycin, and Kumar [29] used phage lambda followed by nalidixic acid.)

Nonsense and frameshift mutations in the *cya* and *crp* genes. The isolation of nonsense or frameshift mutants in the *cya* and *crp* genes indicates that their function is not essential for cell growth on glucose. Among the nine independent *cya* mutants selected, three (TA3336, TA3337, and TA3338) were identified as nonsense mutants. Among the nine independent *crp* mutants, two (TA3339 and TA3340) were shown to be nonsense mutants. They were suppressed (unlike the other mutants) in merodiploids carrying an F′*gal* with a known ochre suppressor, and the mutant phenotype returned with the segregation of the episome. Suppression was also verified by the ability of the suppressed strains to support the growth of nonsense mutants of phage P22 (30) and by the method of Berkowitz et al. (8). The suppressed nonsense *cya* and *crp* mutants grew on ribose, but were still unable to grow on citrate (see below).

Mutagenesis with ICR-372, a potent frameshift mutagen (3, 23), was performed as described above, and a significant increase in the frequencies of both *cya* and *crp* mutations was observed (Table 2). To be certain that the mutants carried frameshift mutations, 25 suspect strains of each type were shown to revert at high frequency in the presence of ICR-372, but not in its absence: TA3325 (*cya-415*) and TA3335 (*crp-404*).

Selection of leaky mutants. *cya* or *crp* mutants with nonsense or frameshift mutations have a "tight" phenotype and do not grow on ribose, glycerol, citrate, or succinate. We have also been able to isolate strains carrying leaky mutations in both the *cya* and *crp* genes. These leaky mutants fall into three phenotypic classes: (I) ability to grow on ribose but not on the other three carbon sources; (II) ability to grow on ribose and glycerol but not on the other two; or (III) ability to grow on all carbon sources but succinate. (Class I mutants were isolated on MacConkey plates with glycerol, and those in classes II and III were isolated on minimal glucose agar plates containing 400 μg of streptomycin [prepared in the agar] per ml with 800 μg of fosfomycin spread on the agar surface.)

Hierarchy in the cAMP system. The existence of a series of leaky mutants able to grow on an increasing number of carbon sources and the fact that suppressed nonsense mutants grow on ribose but not on citrate suggested that as the intracellular cAMP level increases the bacteria can utilize, in turn, the carbon sources in the hierarchy: ribose > glycerol > citrate > succinate. We found (Table 3) that the concentration of cAMP required for growth of a tight *cya* mutant increased as the carbon source was changed from one that supports a fast growth rate to one that supports a slower one.

Differential antibiotic and amino acid analog sensitivities of mutants. The observation that *cya* and *crp* mutants display marked resistance to fosfomycin and streptomycin

TABLE 3. *Growth rate of LT2 and response to cAMP of TA3301 on various carbon sources*

Carbon source	Diameter (mm) of zone[a] of TA3301 (*cya*) growth	Doubling time (min)[b] of LT2
Glucose		48
Galactose	30	61
Ribose	27	56
Glycerol	26	65
α-Glycerophosphate	22	105
Citrate	15	83
Acetate	15	120
Succinate	10	284

[a] A 0.1-ml amount of an overnight minimal glucose culture of TA3301 was suspended in 2 ml of top agar and poured on an N^-C^- plate with ammonia (10 mM) and indicated carbon source (0.6%). A paper disk containing 2 μmol of cAMP was placed at the center of the plate, and the diameter of the circle of growth around it was measured after 18 h of incubation. Relative growth remained constant during further incubation, except that galactose supported growth throughout the plate after several days.

[b] Growth curve procedure is described in the text. Liquid medium was identical to plate medium with the omission of agar.

prompted us to examine their sensitivity to other antibiotics. In Table 4 we compare antibiotic inhibition of a *cya* or *crp* mutant on glucose (no cAMP-CRP complex) to that of the wild type on glucose (a low level of cAMP-CRP complex) to that of the wild type on citrate (a high level of cAMP-CRP complex). As seen in Table 4, a significant number of antibiotics were more inhibitory at higher intracellular levels of the cAMP-CRP complex. We divided the antibiotics into three general classes: those (class I) showing the maximum difference between the first two columns, those (class II) showing the maximum difference between the second and third columns, and those (class 0) showing no difference. Two of the antibiotics, fosfomycin and nalidixic acid, have been previously shown (1) to be more inhibitory against strains lacking cAMP phosphodiesterase, which presumably accumulate higher cAMP levels than the wild type (1, 10). In interpreting the inhibition data shown, it should be kept in mind that small differences in zones of inhibition actually represent much larger differences in concentration of inhibitor at the point of inhibition, since the square of the radius of the zone of inhibition is proportional to the log of the initial concentration of the inhibitor on the disk (13).

A variety of amino acids, peptides, tricarboxylic acid cycle intermediates, and sugars can be transported into *Salmonella* for use as fuel and carbon. We would expect transport systems for these compounds to be under cAMP control. A variety of inhibitory analogs of these substances, which might be expected to enter the cell through these transport systems, have been examined for differential inhibition of *cya* and *crp* mutants (Table 5). The data in the table represent increasing intracellular cAMP levels. Demonstrably greater inhibition, in parallel with higher cAMP levels, is shown for 29 analogs.

Catabolite sensitivity of aromatic amino acid uptake. Through the use of competition studies, Ames and Roth (4, 5) have been able to correlate inhibitory analogs with the natural product they mimic. On the basis of this work and that of others, we identified the 29 compounds giving positive results in Table 5: 14 are aromatic amino acid analogs, 2 are proline analogs, 4 are analogs of other amino acids, 6 are analogs of peptides of naturally occurring amino acids, 1 is a sugar analog, and 2 are pyruvate and succinate analogs. The work of Ames and Roth demonstrated that the aromatic amino acid analogs are transported into *Salmonella* through the aromatic amino acid transport system. To determine whether the aromatic permease is under cAMP control, transport of phenylalanine and tyrosine through this system was studied in these mutants. Table 6 shows that at the concentration of phenylalanine used (30 nM), about 85% of its uptake was due to the aromatic permease; the *aroP* mutant, completely lacking the system, had only a 15% residual uptake, presumably due to other transport systems (4, 5). Table 6 also shows that mutants unable to produce adenyl cyclase (*cya-408*) or cAMP receptor protein (*crp-403*) had phenylalanine (or tyrosine) uptake activities about half that of the wild-type parent, or less after correction for the 15% residual activity.

Several other observations are consistent with the conclusion that the aromatic permease is under cAMP control. Addition of cAMP restored full activity to the *cya* mutant, whereas the *crp* mutant was unaffected by the addition. The wild type (LT2A) grown on citrate (a condition expected to increase internal cAMP) had a markedly increased uptake. We also calculated K_m and V_{max} values for [^{14}C]phenylalanine uptake in TA2301 (*cya*), TA926 (*aroP*), and LT2A (data not shown) (procedure as described above), and these data (K_m, unchanged; V_{max}, de-

TABLE 4. *Inhibition of strains by antibiotics*[a]

Class	Antibiotic	Inhibition zone (mm diameter)		
		cya/crp (glucose)[b]	Wild type (glucose)[b]	Wild type (citrate)[b]
Ia	Fosfomycin (100 μg)	7	24	21
	Sulfathiazole (1 mg)	32	38	38
	Streptomycin (250 μg)	12	15	15
	Colistin (10 μg)	14	20	22
Ib	Polymixin B (300 units)	17	22	28
	Sulfisomidine (1 mg)	28	33	38
Ic	Sulfacetamide (1 mg)	21	30	13
II	Demechlocycline (30 μg)	31	34	44
	Furacin (100 μg)	30	30	36
0	Penicillin (10 units)	17	17	16
	Tetracycline (30 μg)	30	30	31
	Sulfaethiodole (1 mg)	39	41	40

[a] Procedure described in the text. Numbers are averages of three experiments. Readings were taken after 24 h of incubation. The *cya* mutant was TA3336, and the *crp* mutant was TA3339; these gave identical results (within 1 mm) and are averaged. They were isogenic with the wild type, LT2. Class I has been subdivided on the basis of inhibition pattern. Other antibiotics tested were classified as follows. Class Ia: kanamycin, gentamycin, sulfisoxazole, sulfamethoxypyridazine, doxycycline, and nalidixic acid. Class Ib: neomycin. Class Ic: sulfadimethoxine, aureomycin, sulfamerazine, and sulfadiazine. Class II: trichlorbisonium chloride and furadantin/macrodantin. Class 0: ampicillin, carbenicillin, terramycin, methacycline, cephalothin, cephaloglycine, furaltadone, furamazone, sulfachloropyridazine, sulfamethizole, and methenamine mandelate.

[b] Growth medium.

TABLE 5. *Inhibition of strains by analogs*[a]

Class	Analog	Inhibition zone (mm diameter)			
		cya/crp (glucose)[b]	Wild type (glucose)[b]	Wild type (glucose + cAMP)[b]	Wild type (citrate)[b]
I	L-Norleucyl-glycine	<6	24	29	31
	L-Azetidine-2-carboxylic acid	15	23	24	29
	* DL-5-Fluorotryptophan	<6	25	33	39
	* 5-Methyl-DL-tryptophan	<6	15	21	41
IIa	2,2-Difluorosuccinate	<6	<6	12	19
	* β-2-Thienylalanine	<6	<6	27	45
	* DL-*p*-Fluorophenylananine	<6	<6	18	40
IIb	Thioproline	<6	<6	<6	18
	2-Deoxy-D-galactose	<6	<6	<6	25
	L-Norvalyl-L-norvaline	<6	<6	<6	30
	* L-3-Aminotyrosine	<6	<6	<6	23
0	DL-Serine hydroxamate	16	16	17	21
	* Azaserine	38	38	40	46

[a] Procedures and sources of chemicals are described in the text. A 6-mm paper disk containing 400 μg of the analog (800 μg for 2-deoxy-D-galactose) was put on the lawn of bacteria (*cya* = TA3336; *crp* = TA3339; wild type = LT2) on the petri plate containing either minimal-glucose medium (+ 1 mM cAMP where indicated) or minimal-citrate medium. The *cya* and *crp* mutants gave identical results (within 10%) and are averaged. Other analogs tested fell into the following classes: class I: norglycyl-norleucine, L-norglycyl-glycyl-glycine, glycyl-L-norleucine, 4-methyltryptophan, and * 1-amino-2-phenylethyl phosphonic acid; class IIa: fluoropyruvate, * DL-3-thienylalanine, * 7-aza-DL-tryptophan, * DL-*m*-fluorophenylalanine, and β-chloro-L-alanine; class IIb: L-ethionyl-L-alanine, 5′,5′,5′-trifluoroleucine, D-α-hydrazinoimidazole propionate, D-cycloserine, * 2-thiazolyl-DL-alanine, * dichlorodivinylcysteine, * DL-*o*-fluorophenylalanine, and * 6-hydroxy-DL-tryptophan; class 0: methionine sulfoximine, and 3,4-DL-dehydroproline. Asterisk indicates amino acid analogs whose effects are mitigated by the presence of aromatic amino acids.

[b] Growth medium.

TABLE 6. *Uptake of aromatic amino acids by wild-type mutants*[a]

Strain	Growth medium	Phenylalanine		Tyrosine	
		Uptake	%	Uptake	%
LT2A	Glucose	0.302	100	0.289	100
TA926 (*aroP hisP*)	Glucose	0.046	15		
LT2A	Glucose + cAMP	0.389	129		
LT2A	Glucose-6-PO_4	0.300	99		
LT2A	Glycerol	0.350	116		
LT2A	Citrate	0.435	144	0.456	158
TA2301 (*cya*)	Glucose	0.144	48	0.124	43
TA2301 (*cya*)	Glucose + cAMP	0.309	102		
TA2305 (*crp*)	Glucose	0.166	55		
TA2305 (*crp*)	Glucose + cAMP	0.170	55		

[a] Assay performed as described in the text. Uptake is expressed in micromoles per cell (dry weight) per minute. A bacterial density of 1.3×10^8 cells per ml corresponds to 75 μg of cells (dry weight) per ml (4). Amino acid concentration was 30 nM, and cAMP concentration was 1 mM.

creased) also show that almost all the aromatic permease activity was lost in the *cya* mutant.

DISCUSSION

Antibiotic uptake through normal bacterial transport systems. We showed (Tables 2, 4, and 5) that mutants lacking either adenyl cyclase or cAMP receptor protein were partially resistant to 22 antibiotics (including fosfomycin, nalidixic acid, and streptomycin) and 29 inhibitory analogs of normal bacterial fuel/carbon sources. Many of these antibiotics (and analogs) are known to be transported into the bacteria through systems designed to transport fuel/carbon sources, which are under positive control of cAMP. The antibiotic fosfomycin (which inhibits bacterial cell wall synthesis) is transported into *E. coli* and *S. typhimurium* by two independent cAMP-controlled transport systems: that for L-α-glycerophosphate (26, 28), coded at least in part by the gene *glpT*, and that for hexose phosphates (18, 25, 26), coded at least in part by the gene *uhp*. 5-Methyltryptophan and other aromatic amino acid analogs are transported through the aromatic permease (4, 5), which we showed here to be under cAMP control. L-Azetidine carboxylic acid, a natural product that is an analog of proline, and some synthetic proline analogs are most likely transported through the proline permease. Both a proline permease and the proline catabolic enzymes are under cAMP control (16; B. J. Ratzkin, M. Grabnar, and J. R. Roth, manuscript in preparation).

The fact that the inhibition by 21 other antibiotics studied is dependent on the levels of the cAMP-CRP complex suggests that they, too, interact in some way with cellular systems under cAMP control. We propose here that: (i) these cellular systems are those involved in the transport of these inhibitors into the cell, (ii) all of these transport systems have natural substrates that serve as fuel/carbon sources for the cell, and (iii) cellular systems involved with fuel/carbon sources are regulated by cAMP. Mutants isolated on the basis of their resistance

to sulfathiazole (52), D-cycloserine (40), or tetracycline (22, 49) have been shown to be related to decreased transport, although the systems involved have not yet been fully characterized. Though the sulfanilic acid moiety of sulfa drugs is the active part of the molecule inside the cell (as a *p*-aminobenzoate analog), the other portion of the molecule is important for transport of the drug into the bacteria. It seems clear that some sulfa drugs are being transported into the bacteria by transport systems under cAMP control (e.g., sulfathiazole), whereas others are not (e.g., sulfaethidole). Clearly, these principles can be exploited in the design of therapeutic drugs.

The same argument can be made about the analogs. Transport of those that mimic the aromatic amino acids and proline have already been discussed. The "illicit" transport of peptide analogs into bacteria through peptide transport systems has been previously investigated (2). We think that a wide variety of peptide antibiotics exist that are likely to be transported into bacteria through these systems (2, 17). The evidence presented in Table 5 on peptide analogs suggest that these peptide transport systems are also under cAMP control. We propose that similar arguments can be made about the analogs of other amino acids.

Many antibiotics (e.g., tetracycline) and analogs (e.g., methionine sulfoximine) are apparently not entering the cell through transport systems that are cAMP dependent (Table 4). We suggest that transport systems for these molecules may be regulated by other super controls. The study of the transport of these antibiotics should be amenable to the methods we have described. Albomycin entry, for example, is through an iron-regulated system that transports natural iron chelators and, in another study, was a useful tool in analyzing the regulation of iron uptake (32). It will be of interest to find out which other super-control systems (e.g., the guanosine 5'-diphosphate 3'-diphosphate system [48]) controls transport of these other antibiotics.

Positive selection of *cya* and *crp* mutants. Many of the antibiotics and analogs we described as showing differential inhibition of *cya* and *crp* mutants could be used for positive selection of *cya* and *crp* mutants. We used fosfomycin, as it enters through two transport systems (both of which are under cAMP control), and thus *cya* and *crp* are the main class of resistant mutants. Other antibiotics and analogs that could also be used for this type of selection would have to be combined in pairs for selection of *cya* and *crp* mutants, rather than specific antibiotic-resistant mutants.

The isolation of nonsense and frameshift mutants in *cya* and *crp* supports the previous work of Brickman et al. and Sabourin and Beckwith (9, 43) that these are not essential genes when *E. coli* or *S. typhimurium* are grown on glucose as the fuel/carbon source.

Hierarchy within the cAMP control system. We have postulated (M. D. Alper, Ph.D. dissertation, University of California, Berkeley, 1973; 1) that there exists a hierarchy of promoters (see also Rickenberg [42]) within the cAMP control system with different affinities for the cAMP-CRP complex. As the cell increases its cAMP concentration in response to fuel/carbon starvation, the concentration of the cAMP-CRP complex rises and new operons can be activated (or inhibited [39]), in turn, as their promoter interacts with the complex. Some sort of control hierarchy is advantageous for the cell's economic efficiency when faced with a variety of alternatives (i.e., choosing between alternative fuel/carbon sources of different quality). This idea is supported by the following lines of evidence: (i) A series of fuel/carbon sources for *Salmonella* can be ordered in a hierarchy based on the maximum rate of growth each supports. In general, a *cya* mutant requires less exogenous cAMP to grow on a higher quality fuel/carbon source than a lower quality source (Table 3). (ii) The fuel/carbon quality hierarchy (ribose > glycerol > citrate > succinate) is also indicated by a series of leaky *cya* and *crp* mutants able to grow on an increasing number of fuel/carbon sources and by partially suppressed nonsense mutants of *cya* and *crp* that grow on ribose, but not citrate. (iii) Arabinose isomerase, β-galactosidase, and tryptophanase are synthesized at maximum rates in response to different levels of cAMP (31, 36). (iv) The intracellular levels of cAMP increase progressively when the cells are grown on lower quality fuel/carbon sources (10, 20, 44). (v) The antibiotics and analogs studied in Tables 4 and 5 fall into several classes based on the extent of inhibition relative to the cAMP level. We interpret these complex patterns as indicating that each specific transport system is induced at a particular cAMP level and that several of the antibiotics are transported by more than one transport system.

Other explanations for hierarchy not involving differences in the promoters of various operons are conceivable, but appear less likely to us. Saier has suggested that the existence of different basal levels of cAMP-dependent enzymes (i.e., in the absence of cAMP) could explain the effect (M. Saier, personal communication; 45).

Alarmones and super controls. Our studies provide additional evidence for the idea that cAMP is a signal molecule that indicates a deficiency in the fuel/carbon supply to the cell.

They complement other work that has shown that the lack of sufficient fuel/carbon to support full growth, in addition to the nature of the carbon source, appears to be a factor determining the level of cAMP and catabolite repression. Bacteria grown in a chemostat with glucose concentrations low enough to slow the growth rate show progressively more acid hexose phosphatase as the growth rate decreases (27). cAMP would thus be analogous in function to guanosine 5'-diphosphate 3'-diphosphate, which indicates to the cell a deficiency in amino acid availability (48). This type of signal molecule, which we call an alarmone (48), serves as the key component in a super-control system to reorient the cell's economy in response to stress in a particular area of metabolism. We suggest the existence of a number of alarmones, and that the methods employed in this study are likely to be helpful in identifying and characterizing them.

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LITERATURE CITED

1. **Alper, M. D., and B. N. Ames.** 1975. Cyclic 3',5'-adenosine monophosphate phosphodiesterase mutants of *Salmonella typhimurium*. J. Bacteriol. **122**:1081-1090.
2. **Ames, B. N., G. F. Ames, J. D. Young, D. Tsuchiya, and J. Lecocq.** 1973. Illicit transport: the oligopeptide permease. Proc. Natl. Acad. Sci. U.S.A. **70**:456-458.
3. **Ames, B. N., and H. J. Whitfield, Jr.** 1966. Frameshift mutagenesis in *Salmonella*. Cold Spring Harbor Symp. Quant. Biol. **31**:221-225.
4. **Ames, G. F.-L.** 1964. Uptake of amino acids by *Salmonella typhimurium*. Arch. Biochem. Biophys. **104**:1-18.
5. **Ames, G. F.-L., and J. R. Roth.** 1968. Histidine and aromatic permeases of *Salmonella typhimurium*. J. Bacteriol. **96**:1742-1749.
6. **Artman, M., and S. Werthamer.** 1974. Use of streptomycin and cyclic adenosine 5'-monophosphate in the isolation of mutants deficient in CAP protein. J. Bacteriol. **120**:542-544.
7. **Berkowitz, D.** 1971. D-mannitol utilization in *Salmonella typhimurium*. J. Bacteriol. **105**:232-240.
8. **Berkowitz, D., J. M. Hushon, H. J. Whitfield, Jr., J. Roth, and B. N. Ames.** 1968. Procedure for identifying nonsense mutations. J. Bacteriol. **96**:215-220.
9. **Brickman, E., L. Soll, and J. Beckwith.** 1973. Genetic characterization of mutations which affect catabolite-sensitive operons in *Escherichia coli*, including deletions of the gene for adenyl cyclase. J. Bacteriol. **116**:582-587.
10. **Buettner, M. J., E. Spitz, and H. V. Rickenberg.** 1973. Cyclic adenosine 3',5'-monophosphate in *Escherichia coli*. J. Bacteriol. **114**:1068-73.
11. **Castro, L., B. U. Feucht, M. L. Morse, and M. H. Saier, Jr.** 1976. Regulation of carbohydrate permeases and adenylate cyclase in *E. coli*. J. Biol. Chem. **251**:5522-5527.
12. **Chamberlin, M. J.** 1974. The selectivity of transcription. Annu. Rev. Biochem. **43**:721-775.
13. **Cooper, K. E.** 1963. Theory of antibiotic inhibition zones, p. 1-88. *In* F. Kavanagh (ed.), Analytical microbiology. Academic Press Inc., New York.
14. **Cordaro, J. C., T. Melton, J. P. Stratis, M. Atagun, C. Gladding, P. E. Hartman, and S. Roseman.** 1976. Fosfomycin resistance: selection method for internal and extended deletions of the phosphoenolpyruvate: sugar phosphotransferase genes of *Salmonella typhimurium*. J. Bacteriol. **128**:785-793.
15. **Cozzarelli, N. R., W. B. Freedberg, and E. C. C. Lin.** 1968. Genetic control of L-α-glycerophosphate system. J. Mol. Biol. **31**:371-387.
16. **Dendinger, S., and W. J. Brill.** 1970. Regulation of proline degradation in *Salmonella typhimurium*. J. Bacteriol. **103**:144-152.
17. **Diddens, H., H. Zähner, E. Icrass, W. Göhring, and G. Jung.** 1976. On the transport of tripeptide antibiotics in bacteria. Eur. J. Biochem. **66**:11-23.
18. **Dietz, G. W., and L. A. Heppel.** 1971. Studies on the uptake of hexose phosphates. J. Biol. Chem. **246**:2885-2890.
19. **Ely, B., R. M. Weppelman, H. C. Massey, Jr., and P. E. Hartman.** 1974. Some improved methods in P22 transduction. Genetics **76**:625-631.
20. **Epstein, W., L. B. Rothman-Denes, and J. Hesse.** 1975. Adenosine 3':5'-cyclic monophosphate as mediator of catabolite repression of *in vitro* transcription of the *lac* operon in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **72**:2300-2304.
21. **Eron, L., and R. Block.** 1971. An adenosine 3':5'-cyclic monophosphate-binding protein that acts on the transcription process. Proc. Natl. Acad. Sci. U.S.A. **68**:1828-1832.
22. **Franklin, T. J., and A. Godfrey.** 1965. Resistance of *Escherichia coli* to tetracyclines. Biochem. J. **94**:54-60.
23. **Hartman, P. E., Z. Hartman, R. C. Stahl, and B. N. Ames.** 1971. Classification and mapping of spontaneous and induced mutations in the histidine operon of *Salmonella*. Adv. Genet. **16**:1-34.
24. **Hong, J. S., G. R. Smith, and B. N. Ames.** 1971. Adenosine 3':5'-cyclic monophosphate concentration in the bacterial host regulates the viral decision between lysogeny and lysis. Proc. Natl. Acad. Sci. U.S.A. **61**:2258-2262.
25. **Kadner, R. J., and H. H. Winkler.** 1973. Isolation and characterization of mutations affecting the transport of hexose phosphates in *Escherichia coli*. J. Bacteriol. **113**:895-900.
26. **Kahan, F. M., J. S. Kahan, P. J. Cassidy, and H. Kropp.** 1974. The mechanism of action of fosfomycin (phosphonomycin). Ann. N.Y. Acad. Sci. **235**:364-386.
27. **Kier, L. D., R. Weppelman, and B. N. Ames.** 1977. Regulation of two phosphatases and a cyclic phosphodiesterase of *Salmonella typhimurium*. J. Bacteriol. **130**:420-428.
28. **Koch, J. P., S. Hayashi, and E. C. C. Lin.** 1964. The control of dissimilation of glycerol and L-α-glycerophosphate in *Escherichia coli*. J. Biol. Chem. **239**:3106-3108.
29. **Kumar, S.** 1976. Properties of adenyl cyclase and cyclic adenosine 3',5'-monophosphate receptor protein-deficient mutants of *Escherichia coli*. J. Bacteriol. **125**:545-555.
30. **Lew, K. K., and J. R. Roth.** 1970. Isolation of UGA and UAG nonsense mutants of bacteriophage P22. Virology **40**:1059-1062.
31. **Lis, J. T., and R. Schleif.** 1973. Different cyclic AMP requirements for the induction of the arabinose and lactose operons of *Escherichia coli*. J. Mol. Biol. **79**:149-162.
32. **Luckey, M., J. R. Pollack, R. Wayne, B. N. Ames, and J. B. Neilands.** 1972. Iron uptake in *Salmonella typhimurium*: utilization of exogenous siderochromes

as iron carriers. J. Bacteriol. **111**:731–738.
33. **Magasanik, B.** 1970. Glucose effects: inducer exclusion and repression, p. 189–219. *In* J. R. Beckwith and D. Zipser (ed.), The lactose operon. Cold Spring Harbor Laboratory, New York.
34. **Makman, R. S. and E. W. Sutherland.** 1965. Adenosine 3′:5′-phosphate in *Escherichia coli*. J. Biol. Chem. **240**:1309–1314.
35. **Pastan, I., and S. Adhya.** 1976. Cyclic adenosine 5′-monophosphate in *Escherichia coli*. Bacteriol. Rev. **40**:527–551.
36. **Pastan, I., and R. Perlman.** 1970. Cyclic adenosine monophosphate in bacteria. Science **169**:339–344.
37. **Perlman, R. L., and I. Pastan.** 1969. Pleiotropic deficiency of carbohydrate utilization in an adenyl cyclase-deficient mutant of *Escherichia coli*. Biochem. Biophys. Res. Commun. **37**:151–157.
38. **Peterkofsky, A., and C. Gazdar.** 1974. Glucose inhibition of adenyl cyclase in intact cells of *Escherichia coli* B. Proc. Natl. Acad. Sci. U.S.A. **69**:2324–2328.
39. **Prusiner, S., R. E. Miller, and R. C. Valentine.** 1972. Adenosine 3′:5′-cyclic monophosphate control of the enzymes of glutamine metabolism in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **69**:2922–2926.
40. **Reitz, R. H., H. D. Slade, and F. C. Neuhaus.** 1967. The biochemical mechanisms of resistance by streptococci to the antibiotic D-cycloserine and O-carbamyl-D-serine. Biochemistry **6**:2561–2570.
41. **Rephaeli, A. W., and M. H. Saier, Jr.** 1976. Effects of *crp* mutations on adenosine 3′,5′-monophosphate metabolism in *Salmonella typhimurium*. J. Bacteriol. **127**:120–127.
42. **Rickenberg, H. V.** 1974. Cyclic AMP in procaryotes. Annu. Rev. Microbiol. **28**:353–369.
43. **Sabourin, D., and J. Beckwith.** 1975. Deletion of the *Escherichia coli crp* gene. J. Bacteriol. **122**:338–340.
44. **Saier, M. H., B. V. Feucht, and M. T. McCaman.** 1975. Regulation of intracellular cAMP in *E. coli* and *S. typhimurium*. J. Biol. Chem. **250**:7593–7601.
45. **Saier, M. H., R. D. Simoni, and S. Roseman.** 1976. Sugar transport. J. Biol. Chem. **251**:6584–6597.
46. **Schwartz, D. O., and J. R. Beckwith.** 1970. Mutants missing a factor necessary for the expression of catabolite-sensitive operons in *E. coli*, p. 417–422. *In* D. Zipser and J. Beckwith (ed.), The *lac* operon. Cold Spring Harbor Laboratory, New York.
47. **Sletzinger, M., R. A. Firestone, D. F. Reingold, C. S. Rooney, and W. H. Nicholson.** 1968. The α-hydrazino analog of histidine. J. Med. Pharm. Chem. **11**:261–263.
48. **Stephens, J. C., S. W. Artz, and B. N. Ames.** 1975. Guanosine 5′-diphosphate 3′-diphosphate (ppGpp): positive effector for histidine operon transcription and general signal for amino acid deficiency. Proc. Natl. Acad. Sci. U.S.A. **72**:4389–4393.
49. **Unowsky, J., and M. Rachmeler.** 1966. Mechanisms of antibiotic resistance determined by resistance-transfer factors. J. Bacteriol. **92**:358–365.
50. **Venkateswaran, P. S., and H. C. Wu.** 1972. Isolation and characterization of a phosphonomycin-resistant mutant of *Escherichia coli* K-12. J. Bacteriol. **110**:935–944.
51. **Vogel, H., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*. J. Biol. Chem. **218**:97–102.
52. **Watanabe, T.** 1963. Infective heredity of multiple drug resistance in bacteria. Bacteriol. Rev. **27**:87–115.
53. **Winkler, H. H.** 1970. Compartmentation in the induction of the hexose-6-phosphate transport system of *Escherichia coli*. J. Bacteriol. **101**:470–475.
54. **Yokota, T., and J. S. Gots.** 1970. Requirement of adenosine 3′,5′-cyclic phosphate for flagella formation in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **103**:513–516.
55. **Zubay, G., D. Schwartz, and J. Beckwith.** 1970. Mechanism of activation of catabolite-sensitive genes: a positive control system. Proc. Natl. Acad. Sci. U.S.A. **66**:104–110.