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Evidence against Direct Involvement of Cyclic GMP or Cyclic AMP in Bacterial Chemotactic Signaling

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Defects in phosphotransferase chemotaxis in *cya* and *cpd* mutants previously cited as evidence of a cyclic GMP or cyclic AMP intermediate in signal transduction were not reproduced in a study of chemotaxis in *Escherichia coli* and *Salmonella typhimurium*. In *cya* mutants, which lack adenylate cyclase, the addition of cyclic AMP was required for synthesis of proteins that were necessary for phosphotransferase transport and chemotaxis. However, the induced cells retained normal phosphotransferase chemotaxis after cyclic AMP was removed. Phosphotransferase chemotaxis was normal in a *cpd* mutant of *S. typhimurium* that has elevated levels of cyclic GMP and cyclic AMP. *S. typhimurium crr* mutants are deficient in enzyme III^{glucose}, which is a component of the glucose transport system, and a regulator of adenylate cyclase. After preincubation with cyclic AMP, the *crr* mutants were deficient in enzyme II^{glucose}-mediated transport and chemotaxis, but other chemotactic responses were normal. It is concluded that cyclic GMP does not determine the frequency of tumbling and is probably not a component of the transduction pathway. The only known role of cyclic AMP is in the synthesis of some proteins that are subject to catabolite repression.

Sugars transported by the phosphoenolpyruvate-energized phosphotransferase system (30, 32) are chemoattractants for *Escherichia coli*. The sugar-specific enzymes II of the phosphotransferase system are the receptors for both transport and chemotaxis, and other components of the phosphotransferase transport system are also required for chemotaxis (2, 15, 27). In contrast, chemotaxis to most other attractants can function in the absence of a transport system for the attractant (1). The mechanism of adaptation in phosphotransferase chemotaxis is also exceptional. Adaptation to other known chemoeffectors, with the exception of oxygen, is mediated by changes in the degree of methylation of a chemotactic transducing protein, but adaptation to phosphotransferase sugars is independent of methylation (23, 42).

The basic mechanism of transport via the phosphotransferase system has been elucidated (30, 32). After a soluble enzyme I is phosphorylated by phosphoenolpyruvate, the phosphoryl residue is transferred from enzyme I to another soluble protein, the heat-stable protein (HPr). A majority of the enzymes II are directly phosphorylated by HPr, but the enzymes II that are specific for glucose, glucitol, and mannose are phosphorylated by soluble enzymes III that are phosphorylated by HPr (4, 9, 12, 16, 30, 45). The sugar-specific enzymes II are integral membrane proteins that bind, phosphorylate, and translocate the phosphotransferase carbohydrates.

Enzyme III^{glucose}, the most extensively studied enzyme III, is involved not only in glucose transport but also in regulation of adenylate cyclase, certain nonphosphotransferase transport systems, and glycerol kinase (10, 24, 29). The phosphorylated enzyme III^{glucose} is thought to bind to adenylate cyclase and thereby activate the cyclase (10, 20, 33). The dephosphorylated enzyme III^{glucose} binds to and inactivates nonphosphotransferase uptake systems, such as those for lactose and glycerol (8, 22, 25, 29, 36). In the presence of high concentrations of glucose, the rapid transport of glucose dephosphorylates enzyme III^{glucose} and thereby deactivates adenylate cyclase (catabolite repression) and inhibits certain nonphosphotransferase pathways for the uptake of other carbon sources. In *E. coli* and in *Salmonella typhimurium* LT2, the decrease in cyclic AMP in cells grown on glucose also results in repression of flagellar synthesis (46).

The transduction mechanism for chemotaxis to phosphotransferase substrates has not been determined. Cyclic GMP has been proposed as a potential intermediate in signal transduction on the basis of the following evidence. Intracellular cyclic GMP levels in *E. coli* were observed to increase transiently after addition of an amino acid attractant and to decrease transiently after addition of a repellent (5). Certain *E. coli cya* strains, notably CA8306, are defective in phosphotransferase chemotaxis (6). Adenylate cyclase, the *cya* product, has been reported previously to have guanylate cyclase activity in *E. coli* (40, 41). At high concentrations (33 mM), exogenous cyclic GMP induces a smooth-swimming response in *E. coli* and increases the level of methylation of all the transducing proteins (5). A specific scheme involving cyclic GMP and cyclic AMP in bacterial chemotaxis has been proposed by Glagolev and his collaborators (11, 19).

Recently, the evidence for a signaling role of cyclic nucleotides in bacterial chemotaxis has been called into question. Armitage measured intracellular cyclic GMP in *E. coli* after the addition of serine and rapid injection of the cells into cold trichloroacetic acid or ethanol; the concentration of cyclic GMP was constant over the 100-s time course of the experiment (J. Armitage, personal communication). Since the earliest measurement was at 20 ms after the addition of attractant, it is unlikely that any transient change in cyclic GMP concentration was missed. Black and co-workers (6) were unable to reproduce the previously reported changes (5) in cyclic GMP concentration during amino acid chemotaxis. In preliminary studies in this laboratory, normal phosphotransferase chemotaxis was observed in an *S. typhimurium cya* mutant grown in the presence of cyclic AMP and then washed free of cyclic AMP (43).

The present investigation of phosphotransferase chemotaxis in *S. typhimurium* is a reexamination of the require-

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TABLE 1. *S. typhimurium* strains

Strain	Relevant genotype	Construction or source
LT2	Wild type	N. Zinder via B. N. Ames
LJ45	*che*$^+$ (originally ST1)	D. E. Koshland, Jr., via M. H. Saier, Jr.
LJ128	LJ45 *ptsI17* (leaky; 1%)	M. H. Saier, Jr.
LJ130	LJ45 *ptsI18* (tight; ochre)	M. H. Saier, Jr.
LJ62	LT2 *cpd-401*	M. H. Saier, Jr.
LJ114	LJ62 *ptsH15*	M. H. Saier, Jr.
NK186	*cysA1539*::Tn*10*	J. Roth via P. W. Postma
TT2104	*cya-961 argI539 proAB47 amtA1* Δ*trp-130 zid-62*::Tn*10*	J. Roth
PP994	*crr-307*::Tn*10*	P. W. Postma
PP888	*crr-3*	P. W. Postma
PP780	*crr-303*	P. W. Postma
PP782	*crr-306*	P. W. Postma
BT51	LT2 *crr-307*::Tn*10*	P22 (PP994) × LT2
BT52	LT2 *cysA1539*::Tn*10*	P22 (NK186) × LT2
BT53	LT2 *crr-3*	P22 (PP888) × BT52
BT54	LT2 *crr-303*	P22 (PP780) × BT52
BT55	LT2 *crr-306*	P22 (PP782) × BT52
BT56	LT2 *cya-961 zid-62*::Tn*10*	P22 (TT2104) × LT2
BT57	LJ45 *cya-961 zid-62*::Tn*10*	P22 (TT2104) × LJ45

ment for adenylate cyclase, cyclic GMP, and cyclic AMP in phosphotransferase chemotaxis. The study also examines the requirement for the *crr* gene, which codes for enzyme III^{glucose}.

MATERIALS AND METHODS

Bacteria. The strains of *S. typhimurium* used in this study are described in Table 1. *S. typhimurium* BT51 was constructed by infecting strain LT2 with a P22 phage lysate grown on strain PP994 (*crr-307*::Tn*10*) and selecting for colonies that were resistant to tetracycline (Tet^r) and failed to grow on citrate (Cit$^-$) or succinate (Suc$^-$) as the sole carbon source (39). The recombinants were subsequently screened for active swarming in tryptone semisoft agar. Strain BT52 (*cysA*::Tn*10*) was obtained by crossing P22 (NK186) and LT2 and selecting for Tet^r Cys$^-$ (cysteine) recombinants. Subsequently, BT52 was used as the recipient for transductional crosses with PP888 (*crr-3*), PP780 (*crr-303*), and PP782 (*crr-306*) to yield BT53, BT54, and BT55, respectively. The colonies selected were Glu$^+$ (glucose) Cit$^-$ Suc$^-$ Tet^s for BT54 and BT55 and grew slowly on citrate and succinate for BT53 (39). Mutants BT56 and BT57 were obtained by infecting LT2 and LJ45, respectively, with a P22 phage lysate grown on strain TT2104 (*cya-961 zid-62*::Tn*10*) and selecting for Tet^r Mal$^-$ (maltose) colonies that were negative for fermentation on MacConkey glycerol agar (Difco Laboratories). The recombinants (*cya*) did not swarm in tryptone semisoft agar unless cyclic AMP (5 mM) was present. The P22 phage lysates from the *crr* mutants that were used in these transductional crosses were kindly donated by P. W. Postma, University of Amsterdam.

Media and growth conditions. Precautions were taken in growing strains with a *cya* or *crr* mutation to reduce the possibility of selecting spontaneous revertants. To prepare an inoculum, 2 or 3 drops from a permanent culture stored at −90°C were added to nutrient broth and grown overnight. Bacteria were routinely grown with constant shaking at 30°C in iron-free minimal medium 63 (37) supplemented with sodium lactate (0.1%, wt/vol), inducing sugar (0.4%), thiamine (0.3 μg/ml), and thymine (17 μg/ml) after inoculation from a culture grown in nutrient broth overnight. The medium 63 was inoculated to an optical density (600 nm) of 0.05. Cells were harvested when the optical density reached 0.3. Samples removed from the culture of *cya* strains before and after each experiment were plated on maltose minimal agar (6) and incubated for 1 week to determine whether *cya*$^+$ revertants were present. The *crr* strains were similarly plated on citrate minimal agar (39).

Tryptone semisoft agar contained (per liter): tryptone (Difco) (13 g), NaCl (7 g), agar (Difco) (2.5 g), thiamine (0.3 mg), and thymine (17 mg).

Sugar transport assay. The procedure used to measure sugar uptake in whole cells was an adaptation of previously published procedures (6, 28, 37). Bacteria were grown as described above in medium 63 containing lactate and the sugar whose uptake was to be measured. Cells were harvested when the optical density at 600 nm was approximately 0.3, washed three times with medium 63 without added carbon source or supplement, and resuspended in the same medium at an optical density (600 nm) of 0.2 (10^8 cells per ml). A 0.4-ml portion of cell suspension was added to a test tube and incubated at 37°C for 5 min. Radioactively labeled sugar solution was added to a final concentration of 25 μM for methyl-α-D-glucoside and 5 μM for other sugars. The mixture was quickly vortexed. Samples (50 μl) withdrawn at 10-s intervals were filtered under suction on a cellulose acetate filter (25-mm diameter, 0.5-μm pore size; Millipore Corp.). The cells were rapidly washed three times at room temperature with 1.0 ml of medium 63 without added carbon source. The filters were dried under infrared illumination and transferred to 20-ml vials. After the addition of 8.5 ml of a toluene-based scintillation fluid, radioactivity was determined in a scintillation counter (Beckman LS7500). The initial rate of uptake was determined from a plot of the time course.

Chemotaxis assay. Bacteria grown to an optical density at 600 nm of 0.3, as described above, were centrifuged (8,000 × *g*, 10 min) and washed twice in chemotaxis buffer containing 10 mM potassium phosphate, pH 7.1, 0.1 mM EDTA, 10 mM sodium lactate, and 1.0 mM $MgSO_4$. The cells were gently suspended in chemotaxis buffer to an optical density between 0.05 and 0.1. Bacterial suspension (8 μl) was added to a microscope slide and mixed with 2 μl of attractant. The smooth-swimming response was observed under a microscope, and the time at which 50% of the cells returned to the prestimulus tumbling frequency was recorded (44). Low concentrations of attractants were used in these temporal assays to ensure the specificity of the response (30).

Chemicals. D-Mannitol, α-aminoisobutyrate, α-methyl aspartate, cyclic adenosine 3′,5′-phosphate (sodium salt), and chloramphenicol were obtained from Sigma Chemical Co. D-Mannose was from J. T. Baker Chemical Co. (Ultrex grade), and methyl-α-D-glucopyranoside was from ICN Nutritional Biochemicals. Sodium L-aspartate and L-serine were from ICN Pharmaceuticals, Inc. D-[1-^{14}C]mannitol (45.0 mCi/mmol), D-[1-^{14}C]mannose (57.0 mCi/mmol), and [U-^{14}C]glycerol (152.0 mCi/mmol) were obtained from New England Nuclear Corp. Methyl-α-D-[U-^{14}C]glucopyranoside (150.0 mCi/mmol) was supplied by Amersham/Searle. D-[2-^{3}H]mannitol (25 Ci/mmol) was obtained from ICN Radiochemicals. The purity of the radiolabeled mannitol preparations was examined on thin-layer plates (Silica Gel 60

TABLE 2. Chemotactic response to mannitol in *S. typhimurium* mutants deficient in phosphotransferase transport[a]

Strain	Response time (s)	Mannitol uptake (nmol/min per 10^8 cells)
LJ45 (*pts*$^+$)	27.2	2.4
LT2 (*pts*$^+$)	25.6	2.09
LJ130 (*ptsI*)	0	0
LJ128 (*ptsI*; leaky)	10.0	ND[b]
LJ62 (*cpd*)	24.3	1.34
LJ114 (*cpd ptsH*)	0	0.028

[a] Bacteria were grown in medium 63 containing 0.4% mannitol and 0.1% sodium lactate. The cells were harvested and assayed for transport of and chemotaxis to D-mannitol (5 μM) as described in Material and Methods.
[b] ND, Not determined.

F-254; EM Reagents) with *n*-butanol–glacial acetic acid–diethyl ether–distilled water (9:6:3:1) as the developer (13). Both mannitol isotopes migrated as one major peak and were judged to be >95% pure.

RESULTS

Relationship of transport and chemotaxis. As described above, an intact phosphotransferase transport system is essential for phosphotransferase chemotaxis in *E. coli*. Phosphotransferase chemotaxis was measured in various *S. typhimurium* mutants that were motile but deficient in phosphotransferase transport (Table 2). Mutants with defects in *ptsI* (enzyme I) or *ptsH* (heat-stable protein, HPr) were deficient in chemotaxis to D-mannitol (Table 2) and methyl-α-D-glucoside (data not shown), even though the cells had been preinduced in D-mannitol or glucose, respectively. Methylation-dependent chemotaxis to serine was normal in the *ptsI* and *ptsH* mutants (data not shown). This suggested that in *S. typhimurium*, as in *E. coli* (2), phosphotransferase chemotaxis but not methylation-dependent chemotaxis required all components of the transport system and therefore may be dependent on phosphorylation and vectorial translocation of the attractant. However, a definitive demonstration of the requirement for transport in phosphotransferase chemotaxis would require measurement of the enzyme II receptors and demonstration of the absence of pleiotropic effects on chemotaxis (18).

The intracellular concentration of cyclic AMP is lower in *ptsI* and *ptsH* mutants than in *pts*$^+$ strains (26, 34, 38). Consequently, the experiments reported in Table 2 were repeated in medium 63 supplemented with 5 mM cyclic AMP. Growth in cyclic AMP did not improve either mannitol transport or mannitol chemotaxis.

Adenylate cyclase and phosphotransferase chemotaxis. Adenylate cyclase is the product of the *cya* gene and has been reported to have guanylate cyclase activity in addition to adenylate cyclase activity (40, 41). It has been claimed that *E. coli cya* mutants are defective in chemotaxis to phosphotransferase sugars, even when grown in the presence of cyclic AMP (6, 19).

To investigate the effect of the *cya* mutation on phosphotransferase chemotaxis in *S. typhimurium*, we transferred the *cya-961* allele into strains LT2 and LJ45 by transductional crosses. Flagellar synthesis in *S. typhimurium* LT2 is known to be dependent on cyclic AMP, whereas flagellar synthesis in *S. typhimurium* LJ45 (ST1) is constitutive and independent of cyclic AMP (14). The transductant BT56 (LT2 *cya*) was dependent on cyclic AMP for flagellar synthesis, and BT57 (LJ45 *cya*) was independent of cyclic AMP for flagellar synthesis. Care was taken in the construction and maintenance of these strains to minimize the possibility of selecting second-site suppressors (see Materials and Methods).

Bacteria grown in minimal salts medium with and without cyclic AMP (5 mM) were harvested in the early exponential phase of growth, washed, and suspended in chemotaxis buffer. Chemotaxis was measured by a temporal assay, and the transport of radiolabeled sugar was measured in a filter assay (see Materials and Methods). Cyclic AMP was required for transport of D-mannitol by both BT57 (Fig. 1) and BT56 (data not shown). This presumably reflects the requirement for cyclic AMP for induction of synthesis of enzyme $II^{mannitol}$. Transport of the phosphotransferase substrates D-mannose and methyl-α-D-glucoside was similarly dependent on cyclic AMP in *cya* mutants (Table 3). Synthesis of enzyme $II^{glucose}$, which binds methyl-α-D-glucoside, and enzyme $II^{mannose}$ is under the control of cyclic AMP. Growth of *cya*$^+$ strains in the presence of cyclic AMP resulted in elevated transport of mannose and methyl-α-D-glucoside.

For the phosphotransferase substrates tested, chemotaxis was defective in BT57 grown in the absence of cyclic AMP (Table 3). The BT56 cells were nonmotile when grown without cyclic AMP, and chemotaxis could not be examined. When BT56 and BT57 were grown in cyclic AMP, the rate of transport and the chemotactic response were normal even after the cells were washed. This is consistent with the correlation between transport and chemotaxis observed in Table 2. Chemotaxis to serine and aspartate did not require cyclic AMP, since chemotaxis to these substrates was normal in BT57 cells grown both in the presence and absence of cyclic AMP.

The results indicate that cyclic AMP and not the *cya* gene product per se is essential for phosphotransferase chemotaxis. Evidence presented below indicates that the role of cyclic AMP is in the induction of synthesis of proteins that are required for phosphotransferase chemotaxis and transport. It has been suggested that a product of the adenylate cyclase reaction, either cyclic AMP (11) or cyclic GMP (6, 11), has a role in signaling in phosphotransferase chemotaxis. If such were the case, the intracellular level of cyclic nucleotide and changes in the level of cyclic nucleotide would be critical for normal phosphotransferase chemotaxis. This possibility was investigated in *S. typhimurium*. It was possible, although unlikely, that after growth in the presence of cyclic AMP, the washed *cya* mutants retained the precise intracellular concentration of cyclic AMP for normal phosphotransferase chemotaxis. Incubation of washed cells in the absence of cyclic AMP would deplete, in time, the intracellular pools of cyclic AMP, and impairment of chemotaxis should be observed if cyclic AMP is directly involved in signaling. The chemotactic response in washed BT56 and BT57 cells was observed over 4 to 5 h. No change in chemotaxis to D-mannose, methyl-α-D-glucoside, or D-mannitol was observed (data not shown). In an alternative approach, the levels of cyclic nucleotides were raised by introducing a *cpd* mutation. Cyclic AMP phosphodiesterase, the *cpd* product, hydrolyzes both cyclic AMP and cyclic GMP, and in *cpd* mutants the concentration of these nucleotides is increased (3, 7, 26). If cyclic AMP or cyclic GMP was directly involved in signal transduction, an increase in the intracellular concentrations of these cyclic nucleotides would be expected to perturb the chemotactic system. Cells of LJ62 (*cpd*) were grown on medium 63 in the absence of cyclic AMP, and mannitol transport and chemotaxis were measured. The presumed increase in cyclic nucleotide con-

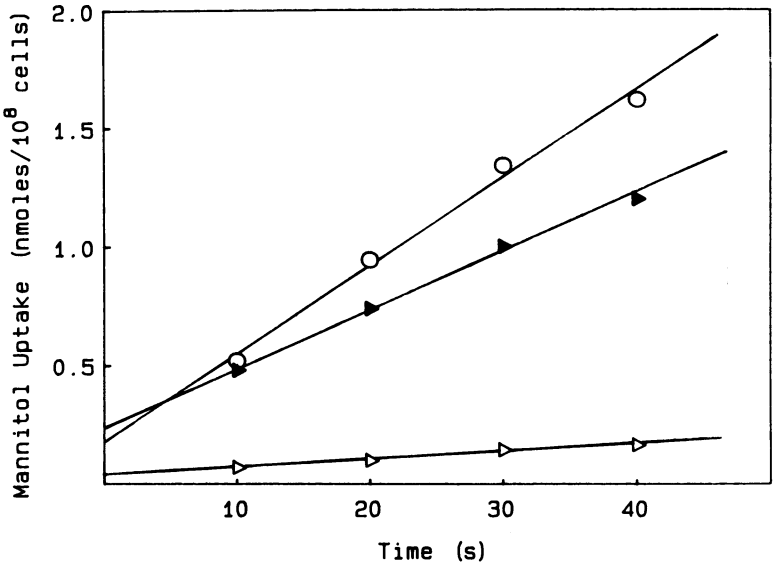

FIG 1. Effect of growth with cyclic AMP on uptake of [^{14}C]mannitol by a *cya* mutant of *S. typhimurium*. Bacteria were grown in medium 63 supplemented with 0.1% sodium lactate and 0.4% mannitol, with or without cyclic AMP (5 mM). The cells were washed, and mannitol uptake was measured as described in Materials and Methods. Symbols: ○, LJ45 (wild type) grown without cyclic AMP; △, BT57 (*cya*) grown without cyclic AMP; ▲, BT57 grown with cyclic AMP.

centration did not impair chemotaxis to D-mannitol (Table 2).

The results obtained with both *cya* and *cpd* mutants of *S. typhimurium* in this investigation are divergent from those reported for *E. coli* (6, 19). We have investigated chemotaxis in *E. coli* AW729 (*cya*) (6), and the results were consistent with those reported above for *S. typhimurium cya* mutants. Black et al. (6) also observed chemotaxis in AW729, but found it to be deficient relative to that in the wild type. We did not observe such a deficiency. On the other hand, *E. coli* CA8306 (*cya*) was defective in phosphotransferase chemotaxis, as reported by Black et al. (6) (see Discussion).

Effect of attractants for methylation-dependent chemotaxis on phosphotransferase chemotaxis. A recent report (19) proposes that phosphotransferase chemotaxis in *cya* mutants of *E. coli* utilizes an alternate signal pathway via the methylation-dependent transduction system. Although no direct evidence for methylation dependence of adaptation was presented, it was reported that phosphotransferase chemotaxis was abolished by preincubating *cya* cells in α-aminoisobutyrate and α-methylaspartate to saturate the Tsr and Tar methylation-dependent pathways. In an attempt to verify these findings, *S. typhimurium* BT56 (*cya*) and the isogenic *cya*$^+$ strain (LT2) were harvested, washed, and suspended in chemotaxis buffer. After addition of α-aminoisobutyrate (1 mM) and α-methylaspartate (1 mM), the cells were incubated with shaking at 30°C for 20 min, and the chemotactic response to 5 μM mannitol was determined by the temporal assay. The response of LT2 to mannitol was similar in the absence (25.7 s) and presence (23.7 s) of α-aminoisobutyrate and α-methylaspartate. In contrast to the results reported for *E. coli* (19), the *S. typhimurium cya* cells (BT56) also showed no significant difference in mannitol chemotaxis before (25.2 s) and after (25.3 s) preincubation. We know of no other evidence that would indicate that the mechanism of phosphotransferase chemotaxis is different in *S. typhimurium cya* mutants and *cya*$^+$ strains.

Role of enzyme III^{glucose}. Enzyme III^{glucose} is a phosphocarrier protein which phosphorylates enzyme II^{glucose} and is essential for the transport of methyl-α-D-glucoside and glu-

TABLE 3. Phosphotransferase transport and chemotaxis in *cya* mutants of *S. typhimurium*[a]

Compound tested (μM)	Strain	Response				Uptake, nmol/min per 10^8 cells (% of control)[b]	
		Growth without cAMP		Growth with cAMP		Growth without cAMP	Growth with cAMP
		Time(s)	% of control[b]	Time(s)	% of control[b]		
D-Mannose (5)	LJ45	24.5	100	24.0	98	0.484 (100)	0.609 (125)
	BT57 (*cya*)	0	0	23.1	94	0.113 (23)	0.65 (135)
Methyl-α-D glucoside (25)	LJ45	28.0	100	27.2	96	0.5 (100)	0.794 (158)
	BT57 (*cya*)	0	0	25.0	89	0.15 (29)	0.567 (113)
D-Mannitol (5)	LJ45	28.2	100	28.8	102	2.47 (100)	2.04 (83)
	BT57 (*cya*)	0	0	25.4	90	0.29 (12)	1.83 (74)
	LT2	26.0	100	26.4	102	2.09 (100)	2.2 (107)
	BT56 (*cya*)	NM[c]		25.5	98	0.30 (14)	1.52 (72)

[a] Bacteria were grown in medium 63 containing 0.1% sodium lactate, the compound to be tested, and cyclic AMP (cAMP, 5 mM) where indicated. The harvested cells were washed free of cyclic AMP and carbohydrate and assayed for transport and chemotaxis as described in Materials and Methods.

[b] Control values are those of the wild type grown without cAMP.

[c] NM, Nonmotile.

TABLE 4. Phosphotransferase transport and chemotaxis in *crr* mutants of *S. typhimurium*[a]

Compound tested (μM)	Strain	Response				Uptake, nmol/min per 10^8 cells (% of control)[b]	
		Growth without cAMP		Growth with cAMP		Growth without cAMP	Growth with cAMP
		Time (s)	% of control[b]	Time (s)	% of control[b]		
D-Mannose (5)	LT2	25.9	100	24.5	95	0.394 (100)	0.657 (166)
	BT53 (*crr*)	15.6	60	27.6	106	0.19 (48)	0.47 (120)
	BT55 (*crr*)	5.0	19	25.5	98	0.107 (27)	0.495 (125)
D-Mannitol (5)	LT2	25.5	100	25.0	98	2.11 (100)	2.26 (107)
	BT53 (*crr*)	18.6	72	27.3	107	1.90 (90)	2.15 (102)
	BT55 (*crr*)	5.3	21	26.8	105	1.29 (61)	1.92 (91)

[a] The experimental procedures were similar to those described in Table 3, footnote *a*.
[b] Control values are those of the wild type grown without cAMP.

cose via enzyme $II^{glucose}$. The structural gene for enzyme $III^{glucose}$ is *crr* in *S. typhimurium* and *E. coli* (17, 21, 35). In addition, enzyme $III^{glucose}$ regulates adenylate cyclase and certain uptake systems. For example, in the presence of methyl-α-D-glucoside or D-glucose, there is a reduction in the phosphorylated form of enzyme $III^{glucose}$, which is the activator of adenylate cyclase, and an increase in dephosphoenzyme $III^{glucose}$, which inhibits glycerol kinase (29). In a *crr* mutant, adenylate cyclase is not activated and the glycerol kinase is not inhibited by incubation of the cells in methyl-α-D-glucoside or glucose. Relief of glucose (methyl-α-D-glucoside) inhibition of glycerol uptake is a diagnostic test for *crr* mutations (36).

To investigate the effect of a *crr* mutation, *crr* alleles were transduced into the *S. typhimurium* LT2 background (Table 1). In the transductant BT53 (*crr-3*), glycerol uptake was inhibited 60% by the presence of methyl-α-D-glucoside, compared with an inhibition of 93% in LT2 (crr^+). In BT55 (*crr-306*), glycerol uptake was inhibited only 15% by methyl-α-D-glucoside. A similar lack of methyl-α-D-glucoside inhibition of glycerol uptake was observed in BT51 (*crr-307*::Tn*10*) and BT54 (*crr-303*). These results are consistent with the previous finding that mutants of *S. typhimurium* with the *crr-3* allele contained 20% of normal enzyme $III^{glucose}$ and mutants with the *crr-303*, *crr-306*, or *crr-307* allele had no detectable enzyme $III^{glucose}$ (39).

Mutants carrying *crr* are known to have low intracellular concentrations of cyclic AMP (10, 20). However, the transductants used in these investigations were flagellated when grown in the absence of cyclic AMP. This indicates that the internal cyclic AMP levels in *crr* mutants were higher than the cyclic AMP levels in the *cya* strains used above. Phosphotransferase transport of mannose was more deficient in BT55 (*crr*) than in BT53 (leaky *crr*). Growth of the *crr* mutants in cyclic AMP increased mannose transport above the rate of transport in LT2 (crr^+) grown in the absence of cyclic AMP (Table 4). Transport and chemotaxis were also measured in BT51 (*crr-307*::Tn*10*) and in BT54 (*crr-303*). Since the results were similar to those reported for BT55 (Table 4), they are not shown. Chemotaxis to D-mannose in *crr* mutants correlated closely with the rate of mannose transport. The normal chemotaxis and transport observed in *crr* mutants grown in cyclic AMP indicate that cyclic AMP and not the *crr* gene per se is essential for mannose transport and chemotaxis.

The endogenous levels of cyclic AMP in BT53 and BT55 were apparently sufficient for induction of mannitol transport (Table 4), but mannitol chemotaxis was markedly impaired in BT55 grown without cyclic AMP. Chemotaxis in BT55 was induced by added cyclic AMP.

Cyclic AMP-dependent protein synthesis. The dependence of phosphotransferase transport on cyclic AMP (Table 3) reflects the known role of cyclic AMP in regulation of specific enzyme II receptor proteins (31). The requirement for cyclic AMP-dependent protein synthesis for mannitol chemotaxis (Table 4) was also investigated. BT55 (*crr*) and LT2 (crr^+) cells were grown in medium 63 plus mannitol and were divided into three portions. Cyclic AMP (5 mM) was added to one portion, cyclic AMP and chloramphenicol (150 μg/ml) were added to a second portion, and the cells were incubated at 30°C for 1.25 h. The cells were washed, and chemotaxis to D-mannitol was measured by the temporal assay. Cyclic AMP increased mannitol chemotaxis in BT55 (*crr*) from 5.5 to 25.6 s in cells incubated in the absence but not in the presence of chloramphenicol (5.0 s). Chemotaxis to mannitol was normal (25.6 s) in LT2 (crr^+) in the absence of cyclic AMP and was not affected by incubation with cyclic AMP (25.8 s) or chloramphenicol (25.0 s). Chemotaxis to serine was normal in BT55 (*crr*) grown in the presence and absence of cyclic AMP.

DISCUSSION

The results obtained in the present investigation indicate that all components for transport of the attractant are essential for phosphotransferase chemotaxis in *S. typhimurium* (Tables 2 and 4). This has previously been established for *E. coli* (2). The requirement for an intact transport system might reflect a requirement for transport of the attractant. Since translocation via the phosphotransferase transport system is tightly coupled to substrate phosphorylation, the signal for phosphotransferase chemotaxis may be the phosphorylated form of the attractant and the phosphotransferase sensor for chemotaxis may face the cytoplasm rather than the external environment. However, at present there is no definitive evidence to support this hypothesis.

The *crr* gene product, enzyme $III^{glucose}$, was required for enzyme $II^{glucose}$-mediated chemotaxis but was not essential for other phosphotransferase chemotaxis in *S. typhimurium*, except the known role in activation of adenylate cyclase. Lengeler et al. (15) made a similar observation for *E. coli*. The *S. typhimurium crr* mutants were flagellated but only slightly induced for mannose transport in the absence of exogenous cyclic AMP (Table 4). This suggests that the cyclic AMP levels in BT55 (*crr*) were higher than in BT56 (*cya*) but less than in the wild-type strain, LT2. This finding reflects the relative activity of adenylate cyclase in the strains (20). BT55 (*crr*) could be induced for mannitol transport by growth in the presence of D-mannitol without the addition of cyclic AMP. However, such bacteria were poorly induced for mannitol chemotaxis unless cyclic AMP

was added to the growth medium. Cyclic AMP did not induce the mannitol chemotaxis system when chloramphenicol was also added, confirming a requirement for protein synthesis. In an independent investigation of *E. coli*, Lengeler selected a mutant that is mannitol transport positive and mannitol chemotaxis negative, and those studies suggest that there is a specific phosphotransferase chemotaxis protein (J. Lengeler, personal communication).

Once chemotaxis and the phosphotransferase system were induced, cyclic AMP was no longer required for phosphotransferase chemotaxis. The LJ45 derivative BT57 (*cya*) was motile in the absence of exogenous cyclic AMP and was chemotactic to serine, but lacked phosphotransferase chemotaxis unless cyclic AMP was added to induce phosphotransferase transport. However, once induction was complete, chemotaxis was unaffected by removal of the cyclic AMP. Extended incubation in the absence of cyclic AMP, which would further deplete endogenous pools of cyclic AMP, did not decrease phosphotransferase chemotaxis. It is therefore unlikely that cyclic AMP is a signaling component of the transduction pathway for phosphotransferase chemotaxis. This is in agreement with the findings of Black et al. for *E. coli* (6).

The earlier evidence that the *cya* gene product was essential for phosphotransferase chemotaxis rested heavily on the study of *E. coli cya* mutants by Black et al. (6). The *cya* mutant CA8306 was deficient in cyclic AMP and cyclic GMP synthesis and in phosphotransferase chemotaxis. The latter finding has been confirmed in the present investigation. However, Lengeler and co-workers recently determined that *E. coli* CA8306 has a defect in phosphotransferase chemotaxis in addition to the *cya* mutation (J. Lengeler, personal communication). Another *cya* mutant of *E. coli* (AW729) was found by Black et al. (6) to be partially deficient in phosphotransferase chemotaxis, but appeared to have normal chemotaxis in the present study.

Glagolev and co-workers recently reported that phosphotransferase chemotaxis was present but reduced in *E. coli* CA8056 (*cya*) and that the residual phosphotransferase chemotaxis was mediated by the methylation-dependent transduction pathways rather than the normal methylation-independent pathway for phosphotransferase chemotaxis (19). This hypothesis is based on a reported inhibition of phosphotransferase chemotaxis in CA8056 by preincubation of the bacteria with α-aminoisobutyrate and α-methylaspartate to saturate the methylation-dependent pathways. In the present study, preincubation of *S. typhimurium* BT56 (*cya*) with α-aminoisobutyrate and α-methylaspartate did not inhibit phosphotransferase chemotaxis. At present we are not aware of any unambiguous evidence that the *cya* gene product is directly involved in phosphotransferase chemotaxis.

Although levels of cyclic GMP were not measured, this investigation addressed the validity of evidence that had been presented in support of a role for cyclic GMP in chemotaxis. The defect in phosphotransferase chemotaxis in *E. coli* CA8306 (*cya*) and *cpd* mutants (6) and changes in intracellular levels of cyclic GMP in chemotaxis were the strongest evidence for the cyclic GMP hypothesis (5), but the current investigation demonstrated normal chemotaxis in *cya* and *cpd* mutants of *S. typhimurium* after addition of cyclic AMP. Cyclic GMP excites a smooth-swimming response in *E. coli* (6). This finding has been reproduced in several laboratories, including this one. But purported changes in cyclic GMP with chemotaxis have proved harder to reproduce. The original observation by Black and co-workers (5) was not reproduced by them in a subsequent study (6). Extensive measurements of cyclic GMP during chemotaxis by J. Armitage (personal communication) also failed to detect any changes. Thus, at present there is no unequivocal evidence for a central role of cyclic GMP in any type of bacterial chemotaxis. Until new evidence is presented in support of the hypothesis, cyclic GMP and cyclic AMP should be omitted from schematic representations of the mechanism of sensory transduction in bacteria.

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