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Compensatory mutations in receptor function: A reevaluation of the role of methylation in bacterial chemotaxis

(*Salmonella typhimurium/cheR/cheB*)

JEFF STOCK, ALAIN BORCZUK, FAYE CHIOU, AND J. E. B. BURCHENAL

Department of Molecular Biology, Princeton, NJ 08544

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ABSTRACT **During bacterial chemotaxis membrane receptor proteins are methylated and demethylated at glutamate residues. The generally accepted view is that these reactions play an essential role in the chemosensing mechanism. Strains may be isolated, however, that exhibit chemotaxis in the complete absence of methylation. These are readily obtained by selecting for chemotactic variants of a mutant that completely lacks the methylating enzyme. Methyltransferase activity is not restored; instead, the sensory–motor apparatus is genetically restructured to compensate for the methylation defect. Genetic and biochemical analyses show that the compensatory mutational locus is the structural gene for the demethylating enzyme. Thus, although mutants lacking either the methylating or demethylating enzymes are nonchemotactic, strains defective in both activities exhibit almost-wild-type chemotactic ability.**

Escherichia coli and *Salmonella typhimurium* effect chemotaxis by controlled fluctuations between two modes of motor behavior, smooth swimming and tumbling (1, 2). If a cell swims towards attractants its tendency to tumble is suppressed and movement continues. This response is determined by rates of change in attractant concentration, rather than absolute levels (2, 3). It has been hypothesized that receptor methylation plays a central role in this process. According to this hypothesis, methylated receptors modulate motor behavior in response to imbalances between their levels of modification and their degree of occupancy by stimulatory ligands (4–6). Responses occur whenever the rate of change in receptor occupancy exceeds the rate of change in methylation.

Each methylated receptor consists of two domains separated by a transmembrane sequence (7–10). Stimulatory ligands bind to the extracellular domain, and methylation occurs at glutamate residues within the cytosol. Methylation increases with attractants and decreases with repellents. The addition reaction is catalyzed by a specific methyltransferase, the product of the *cheR* gene (11), and groups are removed by an esterase that is the product of *cheB* (12). Each receptor contains at least four potential sites of glutamate modification (13, 14), some of which are genetically encoded as glutamines. The esterase acts initially as an amidase to convert these glutamines to glutamates, which can then be methylated by the transferase. *cheR* and *cheB* mutants are locked into smooth and tumbly extremes, consistent with the predicted output from unmethylated or fully methylated receptors (15–17). The fact that these strains do not exhibit chemotaxis lends further support to the role of methylation in sensory motor behavior.

A caveat to the methylation-temporal response paradigm was raised by the finding that second-site suppressor mutations could compensate for the transferase defect in null *cheR* mutants (18). If cells use methylation as a memory function to detect temporal environmental changes, how can methylation mutants effect chemotaxis? An investigation of *cheR* suppression shows that mutations in *cheB* compensate for mutations in *cheR*: cells lacking both transferase and esterase activity respond chemotactically much better than cells defective in one of these enzymes. Receptors in transferase–esterase double mutants differ from those in strains lacking only the transferase in that their glutamine residues are not converted to glutamates. Apparently, the resulting intermediate levels of modification maintain the receptors in a sufficiently balanced state to allow chemotaxis.

MATERIALS AND METHODS

Materials. L-[*methyl*-^{3}H]Methionine (15 Ci/mmol; 1 Ci = 37 GBq), *S*-adenosyl-L-[*methyl*-^{3}H]methionine (15 Ci/mmol), and Na^{125}I were obtained from Amersham. Chloramine-T, *S*-adenosyl-L-methionine, amino acids (all in L configuration), and sugars (all in D configuration) were from Sigma. Tryptone, yeast extract, and Bacto-agar were from Difco. Membrane receptors used as substrate for methyltransferase and methylesterase assays were prepared as described previously (11, 12) from sonic extracts of RP4080-p3-55.2, an *E. coli cheR* mutant containing the *Salmonella tar* gene on a multicopy plasmid (19).

Bacterial Strains and Culture Conditions. Strains used in this study are listed in Table 1. PS1 was isolated from the edge of a swarm of *S. typhimurium* ST1 in 0.35% Bacto-agar containing L broth (23). *che* mutations were transduced into PS1 with P22 phage (*HTint3*, obtained from D. Botstein, Massachusetts Institute of Technology) according to the method of Roth (24) using, as a selectable marker, the tetracycline-resistance gene in a Tn*10* transposon, *zea-2*::Tn*10* (21), located roughly 5 kilobases to the *cheZ* side of the *che* operon just beyond *flaM* and *flaC* (Fig. 1). For instance, the *cheR* mutation in ST1038 was transduced into PS1 by first transducing Tn*10* from a chemotactic (Che$^+$) strain, ST314*zea-2*::Tn*10*, into ST1038. A *cheR*$^-$ tetracycline-resistant recombinant from this cross was then used to transduce the *cheR* mutation into PS1. At each stage recombinants were first selected for tetracycline resistance and then screened for chemotactic ability, using semisolid tryptone swarm plates (0.35% Bacto-agar/1.3% tryptone/0.7% NaCl). *recA*$^-$ strains for complementation tests were constructed by P22 transduction using as donor a strain containing a Tn*10* insert in the *recA* gene (TT521*srl-202*::Tn*10*, obtained from G. Ames, University of California, Berkeley). In all transductions green agar indicator plates were used to ensure that recombinant cultures were free of contaminating P22 phage (27). For some constructions it was necessary to

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Abbreviations: Tet^s, tetracycline-sensitive; Che$^+$ or Che$^-$, chemotactic or nonchemotactic; CheR$^-$, methyltransferase-negative; Crs, *cheR* suppressor.

Table 1. Strains used in this study

Strain	Relevant genotype	Ref. or source
ST1	*che*$^+$	2
ST1038	*cheR*$^-$	20
ST112	*cheY*$^-$	20
ST314	*che*$^+$ *zea-2*::Tn*10*	21
SL4041	*cheB111*	22
TT521	*recA*$^-$ *srl-202*::Tn*10*	G. Ames
PS1	*che*$^+$	ST1 swarm
PS14	*cheB111 zea-2*::Tn*10*	P22:ST314→SL4041→PS1
PS22	*cheY*$^-$ *zea-2*::Tn*10*	P22:ST314→ST112→PS1
PS79	*cheR*$^-$ *zea-2*::Tn*10*	P22:ST314→ST1038→PS1
PS80	*che*$^+$ *zea-2*::Tn*10*	P22:ST314→PS1
PS81-PS90	*crs*$^-$ *cheR*$^-$ *zea-2*::Tn*10*	PS79 swarm
PS220	*cheR*$^-$ *recA*$^-$ *srl-202*::Tn*10*	P22:TT521→Tet^s PS79
PS221	Δ(*flaC–flaM*)*1100* *srl-202*::Tn*10*	P22:TT521→Tet^s PS1100
PS257	*cheY*$^-$ *recA*$^-$ *srl-202*::Tn*10*	P22:TT521→Tet^s PS18
PS430	*cheB*$^-$ *recA*$^-$ *srl-202*::Tn*10*	P22:TT521→Tet^s PS14
PS1051	Δ(*tar–flaM*)*1051*	Tet^s PS80
PS1100	Δ(*flaC–flaM*)*1100*	Tet^s PS80
PS1103	Δ(*cheZ–flaM*)*1103*	Tet^s PS80
PS1109	Δ(*cheB–flaM*)*1109*	Tet^s PS80
PS1250	Δ(*cheB–flaM*)*1250*	Tet^s PS80
PS1259	Δ(*cheY–flaM*)*1259*	Tet^s PS80
PS1275	Δ(*cheB—flaM*)*1109* *recA*$^-$ *srl-202*::Tn*10*	P22:TT521→PS1109
PS1276	Δ(*cheB–flaM*)*1250* *recA*$^-$ *srl-202*::Tn*10*	P22:TT521→PS1250
PS1277	Δ(*tar–flaM*)*1051* *recA*$^-$ *srl-202*::Tn*10*	P22:TT521→PS1051
PS1278	Δ(*cheZ—flaM*)*1103* *recA*$^-$ *srl-202*::Tn*10*	P22:TT521→PS1103
PS1280	Δ(*cheY–flaM*)*1259* *recA*$^-$ *srl-202*::Tn*10*	P22:TT521→PS1259
PS1281	*cheR*$^-$ *cheB111 zea-2*::Tn*10*	P22:PS14→Tet^s PS79

Tet^s, tetracycline-sensitive.

excise the *zea-2*::Tn*10* insert. This was done by selecting for Tet^s variants, using a modification of the method of Bochner *et al.* (28). Strains obtained by this procedure were screened for chemotactic ability on tryptone swarm plates to ensure that Tn*10* excision did not extend into *che* genes. Unless indicated otherwise, cells were grown at 37°C. All strains were maintained as frozen permanent cultures at −80°C in 15% glycerol in L broth.

RESULTS

Isolation and Genetic Characterization of *cheR* Suppressor (*crs*) Mutations. Crs mutants were selected from PS79, an *S. typhimurium* strain that contains a null mutation in the *cheR* gene (18). Approximately 10^5 of these cells were inoculated into the center of a tryptone swarm plate. After 10 hr, mutants with restored chemotactic ability began to emerge. Ten of these strains, PS81–90, each from a different plate, were isolated and characterized. The methyltransferase-negative (CheR$^-$) parent, PS79, contains a Tn*10* promoter distal to the *che* operon, *zea-2*::Tn*10*. The tetracycline-resistance gene within this element was used as a selective marker to transduce *che* from each of the Crs mutants into a wild-type background. In each case the major class of tetracycline-resistant recombinants exhibited the same swarming phenotype as the Crs donor used in its construction. The *crs* mutations are therefore closely linked to the *che* operon. Complementation and recombination analyses using tester strains with deletions extending various distances into the *che* operon (Fig. 1) indicate that the Crs strains are *cheR cheB* double mutants.

Is the Crs phenotype a general consequence of esterase deficiency in a CheR$^-$ background? One approach to this question was to determine the frequency of occurrence of these mutations. Crs variants usually emerge as budding swarms from the perimeter of a CheR$^-$ colony. Sometimes, however, there is a symmetrical transformation from CheR$^-$ to Crs. This occurs when a single Crs cell is added to the initial CheR$^-$ inoculum. The frequency of symmetrical Crs swarms as a function of inoculum size could therefore be used to estimate the rate of *crs* mutagenesis. The value obtained, about 1 mutation every 2×10^6 generations, is typical for the generation of spontaneous forward mutations in *S. typhimurium* (29). The Crs mutants generated in this study (over 50 strains) were subjected to the same genetic analysis as PS81–90. Although most of these strains were *cheR cheB* double mutants, a minor class was identified (10 strains) with *crs* mutations mapping outside the *che* region. When the latter were transduced to *cheR*$^+$, all the recombinants showed a Che$^+$ phenotype. Parkinson has reported that *E. coli* serine receptor mutations can confer Crs phenotype (30). Perhaps the minor class of *Salmonella crs* mutations corresponds to this category. This class swarmed at 2.8 ± 1.0 times the rate of the CheR$^-$ parent strain, while the *cheB* class swarmed 4.5 ± 1.8 times the CheR$^-$ rate.

The high frequency of *crs* mutagenesis indicates that *cheR* suppression is simply a consequence of esterase deficiency. To test this conclusion, a null *cheB* mutation, *cheB111* (22), was used to construct a *cheR cheB* double mutant. The resulting strain, PS1281, swarmed at approximately 4 times the rate of the *cheR* or *cheB* mutants from which it was derived. Block *et al.* have reported that an *E. coli* strain containing a *cheR–cheB* deletion has similarly enhanced swarming ability (31). Thus, strains that lack either the transferase or the esterase are much more defective in chemotaxis than strains that completely lack both enzymes.

Biochemical Characterization of Crs Strains. Crs mutants were analyzed in terms of the following parameters: (*i*) levels of receptor methylation *in vivo*, (*ii*) transferase and esterase activities in cell extracts, (*iii*) levels of CheB protein, and (*iv*) levels of the protein products of genes that are promoter distal to *cheB* in the *che* operon, *cheY* and *cheZ*. The results (Table 2) are consistent with the genetics of these strains. All the mutants are deficient in protein methylation and transferase activity; all are deficient in esterase activity and in most cases there is no detectable CheB protein; and the other genes of the *che* operon are expressed at essentially wild-type levels.

Sensory Motor Behavior. Swarming ability was examined in a variety of defined media (Fig. 2). In pyruvate and proline some *cheR cheB* mutants swarmed better than wild type; in maltose, melibiose, glucose, fructose, mannitol, and galactose they swarmed at up to 70–90% the wild-type rate. The two major methylated transducers in *S. typhimurium* and *E. coli* act directly as receptors for serine and aspartate (34, 35), but *cheR cheB* mutants swarm towards these amino acids as well as towards attractants such as fructose, glucose, mannose, galactose, and ribose, whose receptors are not methylated proteins. Moreover, studies of the behavior of individual cells in response to addition and withdrawal of serine and aspartate have shown that *cheR cheB* mutants retain an ability to respond and adapt to these stimuli (36).

cheR cheB mutants exhibited a range of swimming behavior (Table 3). In general, strains which are more tumbly than wild type (i.e., PS81, 82, 88, 89, and 1281) swarm slower than smooth swimming variants (cf. Table 3 and Fig. 2). Since tumbling produces no net motion, any tumbly behavior in excess of that required for reorientation will tend to retard

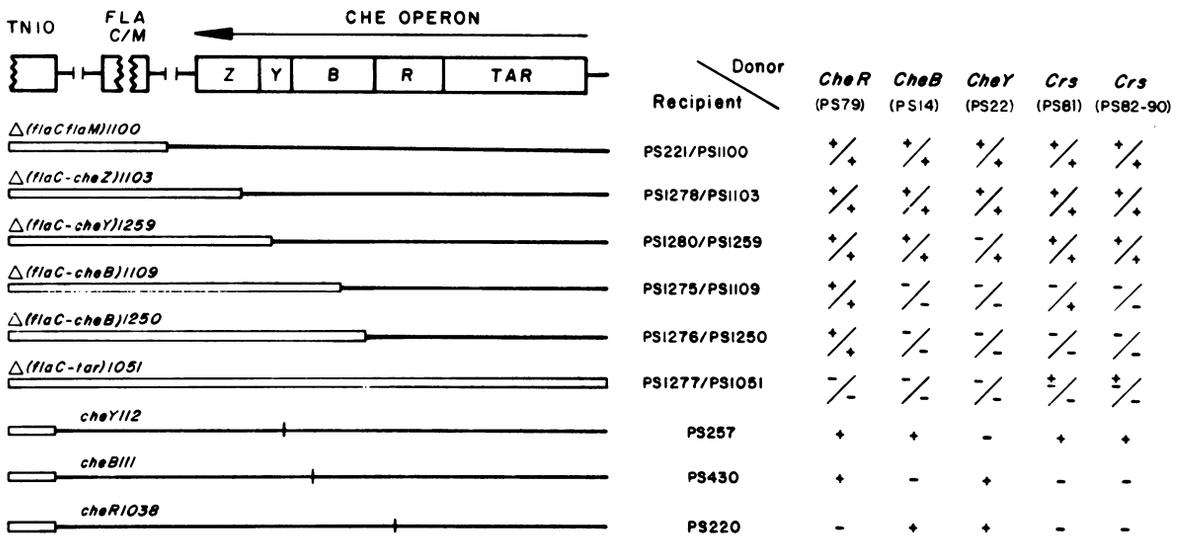

FIG. 1. Deletion mapping of *crs* mutations. A set of deletions extending variable distances from beyond *flaC* into the *che* operon were constructed by Tn*10* excision, and they were mapped by complementation and recombination with defined *che* mutations and by Southern analysis (25) using DNA probes constructed from a plasmid containing the cloned *che* operon [pDK1, obtained from DeFranco and Koshland (21)]. Complementation tests were performed by streaking *recA*$^-$ tester cells with P22*HT* phage grown on the indicated nonchemotactic (Che$^-$) or *cheR* suppressor (Crs) strains across a swarm plate according to the procedure of Aswad and Koshland (26). Recombination analyses were performed by transducing each of the nonflagellated (Fla$^-$) Tet^s deletion mutants to tetracycline resistance, using P22*HT* phage grown on Che$^-$ or Crs strains that contain the *zea-2*::Tn*10* insert. In each case over 200 recombinants were individually screened for swarming ability. Each entry in the table gives complementation/recombination results. Recombination tests were not performed with *cheR*, *cheB*, and *cheY* tester mutations.

chemotactic migration. Of all the strains, wild type exhibits the briefest tumbly intervals.

The best direct measure of chemotaxis is the net migration of cells from a dilute suspension into a capillary that contains an attractant chemical (Fig. 3). PS1281 was attracted into capillaries containing 10^{-5} M aspartate to about 50% the extent of wild type, and into capillaries containing 10^{-4} M aspartate to about 25% the wild-type level. At higher aspartate concentrations there was a dramatic decline in the ability of the double mutant to accumulate. Responses to ribose were similar to those obtained with aspartate, with the double mutant accumulating up to about 30% the extent of wild type. In contrast to aspartate and ribose, the *cheR cheB* mutant showed little attraction to serine. This was surprising in view of the ability of the double mutant to swarm toward serine. The serine receptor appears to have two serine binding sites, a specific high-affinity site, K_d = 5 μM; and a relatively nonspecific low-affinity site, K_d = 0.1 mM (40). The aspartate and ribose receptors each have only a single specific site, K_d = 6 and 0.3 μM, respectively (40, 41). Perhaps in the absence of methylation changes, cells have difficulty integrating information from the two serine receptor sites. This problem may be overcome in very steep attractant gradients such as those generated at the periphery of a swarming population.

Another difference between chemotaxis into capillaries and swarming was evidenced by the behavior of the *cheR*$^-$

Table 2. Biochemical characterization of Crs strains

Strain	Relevant genotype	% of wild type					
		Receptor methylation	Enzymatic activities		Protein levels		
			Transferase	Esterase	CheB	CheY	CheZ
PS79	*cheR*$^-$	<5	<0.2	90	83	124	122
PS80	Wild type	100	100	100	100	100	100
PS81	*cheR*$^-$ *crs*$^-$	<5	<0.2	<2	<5	132	92
PS82	*cheR*$^-$ *crs*$^-$	<5	<0.2	<2	<5	68	72
PS83	*cheR*$^-$ *crs*$^-$	<5	<0.2	<2	<5	90	120
PS84	*cheR*$^-$ *crs*$^-$	<5	<0.2	21	33	95	124
PS85	*cheR*$^-$ *crs*$^-$	<5	<0.2	<2	<5	131	213
PS86	*cheR*$^-$ *crs*$^-$	<5	<0.2	<2	<5	98	111
PS87	*cheR*$^-$ *crs*$^-$	<5	<0.2	<2	11	136	105
PS88	*cheR*$^-$ *crs*$^-$	<5	<0.2	<2	<5	77	46
PS89	*cheR*$^-$ *crs*$^-$	<5	<0.2	<2	<5	100	141
PS90	*cheR*$^-$ *crs*$^-$	<5	<0.2	<2	59	95	94

Levels of receptor methylation *in vivo* and transferase and esterase activities in cell extracts were measured as described previously (11, 12). Levels of CheB protein were assayed by immunoprecipitation with rabbit antiserum to esterase (12). Radioimmunoassays were used to measure levels of the CheY and CheZ proteins. Purified *S. typhimurium* CheY and CheZ (32) were ^{125}I-labeled (50–100 mCi/mg of protein) by using chloramine-T and were mixed with the same cell extracts used to assay transferase and esterase activity (see above) plus rabbit anti-CheY or anti-CheZ antisera. After 1 hr at 25°C, washed IgGSorb (The Enzyme Center, Boston) was added, and 30 min later the samples were centrifuged. The pellets were washed and dried, then assayed in a Nuclear Enterprises NE1600 γ counter. CheY and CheZ protein levels were estimated by linear regression analysis with a standard curve obtained with extract prepared from PS80, wild-type cells.

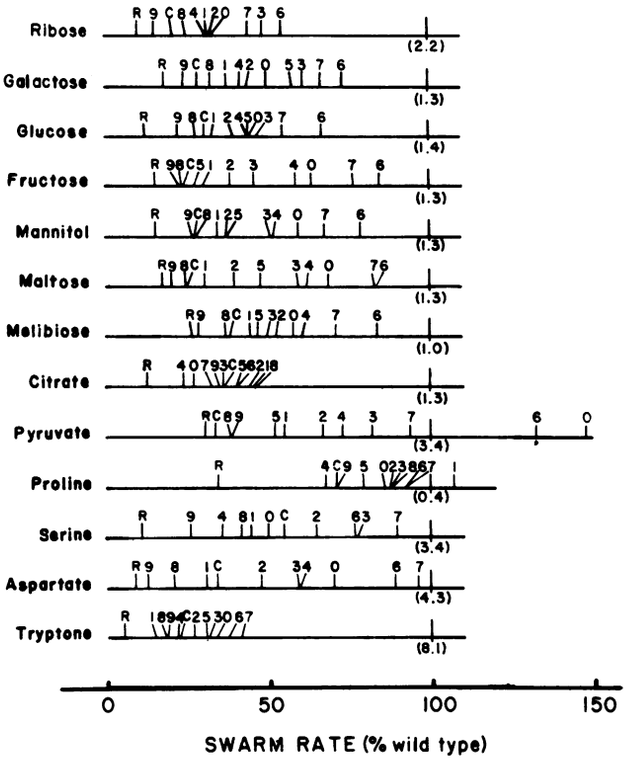

FIG. 2. Swarming ability in various media. Swarm rates were measured in 0.3% Bacto-agar containing modified medium 63 (33) plus the indicated attractant at 0.5% as the sole carbon source except in the case of aspartate and serine, which were present at 0.010 and 0.10 mM, respectively, with 0.5% melibiose added as carbon source. Tryptone swarm plates were prepared using 0.3% Bacto-agar, 1.3% tryptone, and 0.7% NaCl. Swarm rates were determined by linear regression analyses of at least four measurements of swarm diameter taken over at least a 5-hr period. Values for the *cheR*$^-$ parent strain (PS79) are designated R; for the *cheR*$^-$ *cheB111* construct (PS1281), C; and for the *cheR*$^-$ *crs*$^-$ strains (PS81–90), 0–9, respectively. Absolute rates for the corresponding wild-type strain (PS80) are given in parentheses in mm/hr.

cheB$^-$ strains that were selected as CheR revertants. Whereas some of these mutants tended to swarm better than the constructed *cheR*$^-$ *cheB111* null mutant (cf. strains 0–9 and C in Fig. 2), they were generally less efficient in their ability to accumulate into capillaries. In view of the range of swimming and swarming behaviors exhibited by these strains (Fig. 2 and Table 3), it seems likely that many are leaky with respect to CheB expression (e.g., PS84; Table 2). In a swarm, small amounts of esterase activity might provide a range of amidation levels within the population. Individuals with different states of deamidation might respond optimally at different positions within the gradient. In capillary assays each individual must migrate up the entire gradient, so there is no advantage to diversity within the population.

Table 3. Swimming behavior in minimal salts medium

Strain	Genotype	Smooth swimming, %	Duration of intervals, sec	
			Smooth	Tumbly
PS79	*cheR*$^-$	93	>10	1.2
PS14	*cheB*$^-$	0	—	>10
PS80	Wild type	78	2.1	0.6
PS81	*cheR*$^-$ *crs*$^-$	7	0.5	7.3
PS82	*cheR*$^-$ *crs*$^-$	21	1.0	3.7
PS83	*cheR*$^-$ *crs*$^-$	67	2.9	1.4
PS84	*cheR*$^-$ *crs*$^-$	79	5.3	1.4
PS85	*cheR*$^-$ *crs*$^-$	64	3.2	1.8
PS86	*cheR*$^-$ *crs*$^-$	85	5.7	1.0
PS87	*cheR*$^-$ *crs*$^-$	65	4.1	2.2
PS88	*cheR*$^-$ *crs*$^-$	4	0.3	7.0
PS89	*cheR*$^-$ *crs*$^-$	6	0.4	6.4
PS90	*cheR*$^-$ *crs*$^-$	98	>10	0.7
PS1281	*cheR*$^-$ *cheB*$^-$	12	0.8	6.1

Cells were grown to a density of approximately 10^9 cells per ml at 25°C in modified medium 63 (33) containing 0.5% glucose. These cultures were then diluted 1:50 into growth medium, and after about 1 hr of continued incubation, swimming behavior was recorded at ×400 magnification, using a Leitz Dialux microscope equipped with dark-field optics, a Venus model DV2 video camera, and a Panasonic NV8950 video cassette recorder. Samples were placed under the microscope by using the coverslip bridge procedure of Spudich and Koshland (37). Steady-state swimming was analyzed by following at least 50 bacteria of each strain.

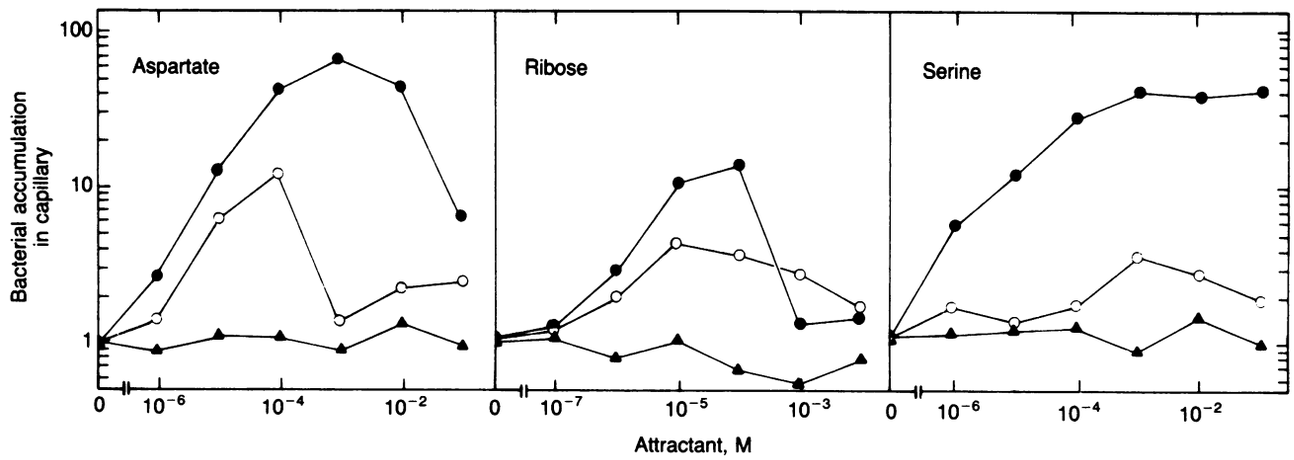

FIG. 3. Chemotaxis into capillary tubes. Cells (wild type, PS80, ●; *cheR*$^-$ *cheB111*, PS1281, ○; and *cheR*$^-$, PS79, ▲) grown to a density of approximately 10^9 cells per ml in Vogel–Bonner citrate medium (38) at 30°C were diluted 1:1000 into this medium, incubated an additional 2 hr at 30°, and placed in a chamber on a slide warmer at 30°C as described previously (39). Capillary tubes (Clay Adams 10-μl Micropet) containing the indicated concentrations of attractants in Vogel–Bonner medium were inserted into the chamber. After 1 hr the capillaries were removed and rinsed with water, and the numbers of bacteria accumulated were determined by spreading appropriate dilutions on nutrient agar plates and counting the colonies that developed. Results are presented as fold accumulation: the number of cells in the capillary divided by the number in control capillaries containing only Vogel–Bonner medium. The latter value was generally about 1000 cells. The results shown are mean values obtained from three independent determinations.

DISCUSSION

During chemotaxis, cells respond to the time rate of change in receptor occupancy. According to the methylation–temporal response hypothesis, the level of receptor methylation is continuously adjusted to provide a reference for temporal environmental comparisons. The finding that transferase–esterase double mutants exhibit chemotaxis at almost wild-type rates argues strongly against this hypothesis. When cells migrate in chemical gradients they must significantly alter their behavior in response to rates of change in receptor occupancy of only about 0.1%/sec (3, 42). All of the experimental results that show a correlation between methylation changes and chemosensing involve stimuli that are 2–3 orders of magnitude greater than those actually encountered during chemotaxis (9, 15–17, 43, 44). It seems likely that methylation functions to modulate transducer activity over these broad ranges of stimulus intensity, while other mechanisms mediate the second-to-second adjustments to small changes that are directly responsible for chemotaxis.

The bacterial chemosensory apparatus is an ultrasensitive switching device (45). The adaptive significance of altered methylation states could be to adjust the threshold of this system so that a very small change in receptor occupancy gives a large response whatever the background level of saturation. When cells are exposed to very large and essentially instantaneous environmental changes, the receptor methylation level is thrown out of alignment, and behavior will be unbalanced until the level of modification has been readjusted. This response is probably analogous to that which occurs when one suddenly goes from darkness into sunlight. The associated processes of disorientation and the mechanisms that serve to reestablish a sensory balance may have only an indirect relationship to the physiology of behavior within the context of a more limited range of stimulus intensities.

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