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Reconstitution of Signaling in Bacterial Chemotaxis

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Strains missing several genes required for chemotaxis toward amino acids, peptides, and certain sugars were tethered and their rotational behavior was analyzed. Null strains (called gutted) were deleted for genes that code for the transducers Tsr, Tar, Tap, and Trg and for the cytoplasmic proteins CheA, CheW, CheR, CheB, CheY, and CheZ. Motor switch components were wild type, *flaAII(cheC)*, or *flaBII(cheV)*. Gutted cells with wild-type motors spun exclusively counterclockwise, while those with mutant motors changed their directions of rotation. CheY reduced the bias (the fraction of time that cells spun counterclockwise) in either case. CheZ offset the effect of CheY to an extent that varied with switch allele but did not change the bias when tested alone. Transducers also increased the bias in the presence of CheY but not when tested alone. However, cells containing transducers and CheY failed to respond to attractants or repellents normally detected in the periplasm. This sensitivity was restored by addition of CheA and CheW. Thus, CheY both enhances clockwise rotation and couples the transducers to the flagella. CheZ acts, at the level of the motor, as a CheY antagonist. CheA or CheW or both are required to complete the signal pathway. A model is presented that explains these results and is consistent with other data found in the literature.

Sensory transduction in bacterial chemotaxis involves receipt of information about the external environment, passage of this information across the cytoplasmic membrane, generation of signals that converge on the flagellar motors, and activation of mechanisms that permit adaptation. The concentrations of certain chemicals are sensed by transmembrane receptors, also called transducers or methyl-accepting chemotaxis proteins (1, 11, 39; for a review, see reference 10). Four transducers are known, one sensitive to aspartate, maltose, and certain repellents (Tar), a second sensitive to serine and certain other repellents (Tsr), a third sensitive to galactose and ribose (Trg), and a fourth sensitive to dipeptides (Tap [17]). Changes in receptor occupancy, through a series of intermediate events that we hope to understand, alter the probability that the flagella spin clockwise (CW) or counterclockwise (CCW). If they spin CW, the cells move erratically with little net displacement (they tumble); if they spin CCW, the cells swim smoothly (they run) (15, 16). If a run happens to carry the cell up a spatial gradient of an attractant (e.g., of aspartate), the probability of CW rotation decreases and the probability of CCW rotation increases (4, 5), extending the run. This enables the cell to move toward a more favorable environment (3).

How is sensory information transferred from the receptors to the flagella? From measurements of signal propagation in filamentous cells, Segall et al. (31) showed that there is an internal signal but that its range is short, only a few micrometers. To explain this limited range, they suggested that the signal is a small protein or ligand that is inactivated as it diffuses through the cytoplasm. CheY was considered to be the most likely candidate (see also reference 27). According to this hypothesis, CheY is activated at the transducers at a rate that is depressed by the binding of attractant. Active CheY enhances CW rotation. A second protein, CheZ, inactivates CheY as it diffuses through the cytoplasm. This hypothesis was consistent with the following facts: (i) strains defective in *cheY* have a large bias (tend to rotate their flagella CCW [21, 22]); (ii) cell envelope preparations have a large bias (27); (iii) strains lacking transducers have a large bias (29); (iv) addition of attractant increases the bias (15); (v) strains defective in *cheZ* have a small bias (tend to rotate their flagella CW [25]); (vi) strains defective in *cheZ* have a slow response time (4) and a long signal decay length (31); and (vii) analyses of second-site revertants show that CheY and CheZ interact with components of the flagella motor (with FlaAII and FlaBII [26]).

Recent work has directly implicated CheY in the signaling process. Clegg and Koshland (8) overproduced CheY in a strain deleted for the transducers Tar and Tap and all the chemotaxis genes known to code for cytoplasmic products: *cheA*, *cheW*, *cheR*, *cheB*, *cheY*, and *cheZ* (strain RP1091 [24]). In the absence of CheY, the flagella spun CCW; in its presence, they spun CW and could not be switched back by the addition of serine, an attractant that acts through one of the remaining transducers, Tsr. Ravid et al. (28) added various amounts of purified CheY to the medium used to prepare envelopes from cells wild type for chemotaxis. The more they added, the larger the fraction of reconstituted envelopes that spun their flagella CW. However, once a flagellum spun CW, it did so indefinitely. The reconstituted envelopes failed to respond to chemotactic stimuli.

The work described here follows a similar strategy. We constructed a strain that we call "gutted" that is deleted for the four known transducers, as well as for *cheA*, *cheW*, *cheR*, *cheB*, *cheY*, and *cheZ*. We added back subsets of these genes to the gutted strain, or to strains less extensively deleted, via multicopy plasmids or lambda hybrids. We also effected variable control with inducible promoters. We tethered these cells and measured the fraction of time that they spin CCW and their response to certain attractants and repellents. We conclude that CheY is a diffusible signal; its activity is modulated by the transducers and it interacts with the motors. CheZ counteracts the effects of CheY, but it does not appear to be a CCW signal. The transducer and CheY alone are not capable of transmitting information

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TABLE 1. Bacterial strains used for constructions

Strain	Relevant genotype	Source or reference and comments
CP177	Δ(*trg*)*100 zbd*::Tn*5*	G.L. Hazelbauer; *zbd*::Tn*5* approx. 50% linked to Δ(*trg*)*100*
D158*recA*	Δ(*cheY*)*m60-21 recA*	F. W. Dahlquist
RP1091	Δ(*cheA-cheZ*)*2209*	24
RP1131	*trg*::Tn*10*	J. S. Parkinson
RP1616	Δ(*cheZ*)*6725*	J. S. Parkinson
RP2893	Δ(*tap-cheZ*)*2206*	24
RP3000	Δ(*cheY-cheZ*)*m43-13 scyA2 supD metF159*(Am)	26; *scyA2* = *flaAII* allele
RP3001	Δ(*cheY-cheZ*)*m43-13 scyA3 supD metF159*(Am)	26; *scyA3* = *flaAII* allele
RP3068	Δ(*cheY-cheZ*)*m43-13 scyB10 supD metF159*(Am)	26; *scyB10* = *flaBII* allele
RP4979	Δ(*cheY*)*m43-10*	J. S. Parkinson
RP5046	Δ(*cheA-cheB*)*2229*	24
RP5714	Δ(*tsr*)*7021 mal* (λ^r) *eda-50*	J. S. Parkinson
RP5838	Δ(*tar-tap*)*5201* Δ(*tsr*)7021	J. S. Parkinson
RP5854	Δ(*tar-tap*)*5201*	J. S. Parkinson

originating in the periplasm. This requires the addition of CheA or CheW or both.

MATERIALS AND METHODS

Chemicals. Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs. Synthetic amino acids were used for chemotaxis assays (12): sodium L-aspartate was purchased from ICN Pharmaceuticals Inc.; L-serine and L-leucine were purchased from K & K Laboratories, Inc. Ampicillin, tetracycline hydrochloride, and kanamycin sulfate were purchased from Sigma Chemical Co. L-Arabinose was purchased from Fisher Scientific Co. Tryptone and agar were from Difco Laboratories. Other chemicals were reagent grade.

Bacterial strains, plasmids, and bacteriophages. All strains were derivatives of *Escherichia coli* K-12 and are listed in Tables 1 and 2. Generalized transductions involved the use of phage P1kc (33). To verify all constructions, complementation was performed as described by Parkinson, except that the strains tested were not lysogenic for λ (22; see method I). A set of λ*che22* mutants that carry missense mutations in each of the chemotaxis genes of interest (generously supplied by J. S. Parkinson) were used for this purpose.

λgt*4-tar101* was described previously (14). All plasmids were derivatives of pBR322 and are listed in Table 3. Transformants were constructed as described by Silhavy et al. (33) and are listed in Table 4.

The plasmid pBB1 expresses CheZ under the control of the tryptophan promoter of *Serratia marcescens*. It was constructed by ligation of the *Pvu*II fragment of pRL22 (18), which carries most of the *cheZ* gene, to the *Pvu*II site of the plasmid pRL22Δ*cheY*Δ*pvu*II. The latter plasmid contains an approximately 50-base-pair deletion centered about the *Sal*I site of *cheY* and thus does not express a functional chemotaxis gene product. Expression of CheZ by pBB1 was verified via complementation with a strain deleted for *cheZ* (RP1616).

CheY assay. Estimates were made of the amount of CheY produced in strains containing the plasmid pRL22Δ*pvu*II (Table 3), in which transcription of *cheY* was promoted by p*trp*, e.g., HCB328 (Table 4), and in strains containing *cheY* in single copy, in which transcription was promoted by p*mocha*, e.g., HCB350 (Table 2), as compared with the wild type, in which transcription was promoted by p*meche*, e.g., RP437 (22). Cells were grown in tryptone broth (1 liter), containing an antibiotic when appropriate, and harvested at mid-exponential phase. The following steps were carried out at 4°C. Cells were washed three times in 10 ml of 10 mM

TABLE 2. Bacterial strains constructed for this study

Strain[a]	Relevant genotype	Chemotaxis genes present[b]	P1 construction (parents; relevant selection/screen)
HCB314	Δ(*tsr*)*7021* Δ(*cheA-cheZ*)*2209 mal* (λ^r)	*trg*	RP1091 × RP5714; Eda$^+$/Che$^-$
HCB315	Δ(*tsr*)*7021* Δ(*cheA-cheZ*)*2209 mal*$^+$ (λ^s)	*trg*	RP5838 × HCB314; Mal$^+$/λ^s
HCB316	Δ(*tsr*)*7021* Δ(*tar-tap*)*5201*	*trg cheAWRBYZ*	RP5854 × HCB317; Eda$^+$/Che$^-$
HCB317	Δ(*tsr*)*7021 mal*$^+$ (λ^s)	*trg tar tap cheAWRBYZ*	RP5838 × RP5714; Mal$^+$/λ^s
HCB326	Δ(*tsr*)*7021* Δ(*cheA-cheZ*)*2209 trg*::Tn*10*	None	RP1131 × HCB315; Tc^r
HCB339	Δ(*tsr*)*7021* Δ(*tar-tap*)*5201 trg*::Tn*10*	*cheAWRBYZ*	RP1131 × HCB316; Tc^r
HCB349	Δ(*tsr*)*7021* Δ(*tap-cheZ*)*2206*	*trg tar cheAW*	RP2893 × HCB317; Eda$^+$/Che$^-$
HCB350	Δ(*tsr*)*7021* Δ(*cheA-cheB*)*2229*	*trg cheYZ*	RP5046 × HCB317; Eda$^+$/Che$^-$
HCB429	Δ(*tsr*)*7021* Δ(*tar-tap*)*5201* Δ(*tar*)*100 zbd*::Tn*5*	*cheAWRBYZ*	CP177 × HCB339; Kn^r/Tc^s
HCB433	Δ(*tsr*)*7021* Δ(*trg*)*100 zbd*::Tn*5*	*tar tap cheAWRBYZ*	CP177 × HCB317; Kn^r/galatose-blind
HCB437	Δ(*tsr*)*7021* Δ(*trg*)*100 zbd*::Tn*5* Δ(*cheA-cheZ*)*2209 metF159*(Am)	None	RP1091 × HCB433; Eda$^+$/Che$^-$ and Kn^r
HCB482	Δ(*tsr*)*7021* Δ(*trg*)*100 zbd*::Tn*5* Δ(*cheA-cheZ*)*2209 scyA2 supD metF159*(Am)	*flaAII* (*scyA2*)	RP3000 × HCB437; Met$^+$/tumbly
HCB483	As HCB482 except *flaAII*(*scyA3*)	*flaAII* (*scyA3*)	RP3001 × HCB437; Met$^+$/tumbly
HCB484	As HCB482 except *flaBII*(*scyB10*)	*flaAII* (*scyB10*)	RP3068 × HCB437; Met$^+$/tumbly
HCB527	Δ(*tsr*)*7021* Δ(*tap-cheZ*)*2206 flaAII*(*scyA2*)	*trg tar flaAII* (*scyA2*) *cheAW*	RP3000 × HCB349; Met$^+$/tumbly

[a] The initials used to identify the strains constructed in this laboratory have been changed from HB to HCB.
[b] Out of the set *tsr*, *trg*, *tap*, *cheAWRBYZ*, unless otherwise noted.

TABLE 3. Plasmids

Plasmid	Relevant genotype[a]	Source and comments
pRL22	p*trp-cheYZ*	18
pRL22Δ*pvu*II	p*trp-cheY*	P. Matsumura
pRL22Δ*cheY* Δ*pvu*II	p*trp*-Δ(*cheY*)	P. Matsumura
pBB1	p*trp*-Δ(*cheY*) *cheZ*	This study
pJH120	p*ara-cheY*	F. W. Dahlquist; inducible by arabinose
pAK101RI	*tar*	14

[a] p*trp* refers to the tryptophan promoter of *S. marcescens*; *para* refers to the regulatory region of the arabinose operon of *E. coli* B; *tar* is promoted by its native promoter.

$MgSO_4$, resuspended in 10 ml of the same medium, treated for 10 min with lysozyme (10 μg/ml), and repeatedly sonicated; large cell fragments were removed by centrifugation at ca. 8,000 × *g* for 5 min, and smaller material was removed by centrifugation at ca. 300,000 × *g* for 1 h. Samples of supernatant fractions were run on 15% polyacrylamide gels in the presence of sodium dodecyl sulfate, stained with Coomassie blue, and scanned with a densitometer (LKB 2202 run at 633 nm). The integrated optical densities of the CheY peaks for strains HCB328 and HCB350 (less the background owing to a small amount of other protein observed for the gutted strain HCB326) were about 3.8 and 0.7 times larger, respectively, than that for strain RP437.

Swarm plates. Cells (5 μl of a stationary culture) were inoculated at the center of a plate containing tryptone broth in 0.3% agar. Antibiotics and L-arabinose were added when appropriate. The plates were incubated at 30°C in a moist environment.

Tethered cells. Cells were grown in tryptone broth at 30°C. When strains contained plasmids, inocula were taken from stocks stored in 10% dimethyl sulfoxide at −70°C, and the broth was supplemented with the appropriate antibiotic. When strains did not contain plasmids, inocula also were taken from saturated cultures; the behavior of the cells was the same in either case. In experiments involving induction, cultures were grown to saturation in the absence of arabinose and then diluted 1:100 into fresh broth containing the desired concentration of L-arabinose; both media contained ampicillin. All cultures were harvested at mid-exponential phase, washed, and tethered as described by Block et al. (4), except that the motility buffer was 10 mM potassium phosphate (pH 7)–67 mM NaCl–10 mM sodium lactate–0.1 mM disodium EDTA–0.001 mM L-methionine. Neither antibiotics nor arabinose was added to the motility buffer.

Data acquisition and analysis. The cover slip to which cells were tethered was mounted on a flow cell (2) on the stage of a phase-contrast microscope. The behavior of the cells was recorded at room temperature before and after shifts to motility buffer containing attractants or repellents. The data were recorded on videotape and analyzed by computer, as described elsewhere (2a). This system allowed us to detect essentially every transition between CW and CCW states and to carry out a complete statistical analysis. Estimates of bias (the fraction of time that a cell spins CCW, i.e., that it turns its flagella CCW) and of reversal rate (the number of CW-to-CCW and CCW-to-CW transitions per second) for a given culture were based on averages of data spanning about 180 s for each of 10 to 34 cells.

RESULTS

The null phenotype. The gutted strains (HCB326 [Fig. 1] and HCB437 [Table 5]) rotated exclusively CCW. In both strains the bias was 1.0 and the reversal rate was zero. No reversals were detected in 20 cells monitored for periods averaging 180 s. No reversals occurred in response to

TABLE 4. Bacterial strains containing plasmids or phage or both

Strain	Parent[a]	Plasmid or phage[b]	Chemotaxis genes present[c]
D158*recA*(pJH120)	D158*recA*	pJH120	*trg tsr tar tap cheAWRBZ para-cheY*
HCB328	HCB326	pRL22Δ*pvu*II	p*trp-cheY*
HCB330	HCB315	pRL22Δ*pvu*II	*trg* p*trp-cheY*
HCB332	HCB315	λgt*4-tar101*	*trg tar*
HCB334	HCB332	pRL22Δ*pvu*II	*trg tar* p*trp-cheY*
HCB338	RP1091	pRL22Δ*pvu*II	*trg tsr* p*trp-cheY*
HCB351	HCB350	pRL22Δ*pvu*II	*trg cheYZ* p*trp-cheY*
HCB449	HCB437	pRL22	p*trp-cheYZ*
HCB450	HCB437	pRL22Δ*pvu*II	p*trp-cheY*
HCB465	HCB437	pJH120	p*ara-cheY*
HCB475	HCB465	λgt*4-tar101*	*tar* p*ara-cheY*
HCB488	HCB483	pRL22	p*trp-cheYZ*
HCB490	HCB482	pRL22Δ*pvu*II	*flaAII*(*scyA2*) p*trp-cheY*
HCB491	HCB483	pRL22Δ*pvu*II	*flaAII*(*scyA3*) p*trp-cheY*
HCB492	HCB484	pRL22Δ*pvu*II	*flaBII*(*scyB10*) p*trp-cheY*
HCB493	HCB482	pBB1	*flaAII*(*scyA2*) p*trp-cheZ*
HCB494	HCB483	pBB1	*flaAII*(*scyA3*) p*trp-cheZ*
HCB496	HCB482	pRL22Δ*cheY*Δ*pvu*II	*flaAII*(*scyA2*)
HCB497	HCB483	pRL22Δ*cheY*Δ*pvu*II	*flaAII*(*scyA3*)
HCB499	HCB482	pAK101RI	*flaAII*(*scyA2*) *tar*
HCB500	HCB483	pAK101RI	*flaAII*(*scyA3*) *tar*
HCB501	HCB484	pAK101RI	*flaAII*(*scyB10*) *tar*
HCB525	HCB349	pJH120	*trg tar cheAW para-cheY*
HCB526	HCB429	λgt*4-tar101*	*tar cheAWRBYZ*
HCB529	HCB527	pJH120	*flaAII*(*scyA2*) *trg tar cheAW para-cheY*

[a] See column 1 or Table 2.
[b] See Table 3.
[c] See Table 2, footnote *b*.

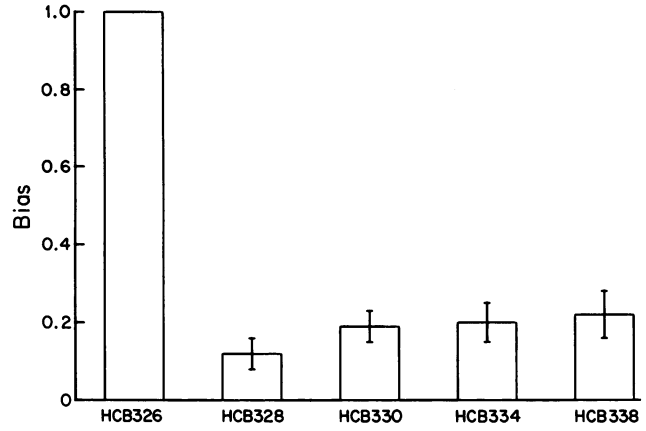

FIG. 1. The bias of a gutted strain (HCB326), that of the same strain containing a plasmid in which CheY is expressed via the tryptophan promoter of *S. marcescens* (HCB328), and those of strains containing this plasmid but not deleted for *trg* (HCB330), *trg* and *tar* (HCB334), or *trg* and *tsr* (HCB338). The error bars represent the standard error of the mean for the cell population.

repellents (1 mM Ni^{2+}, 10 mM L-leucine, shifts from pH 7 to 6, or 10 mM sodium acetate at pH 6).

CheY. CheY was required for CW rotation in both the wild type and gutted background. Strain RP4979, deleted only for *cheY*, exhibited the null phenotype; its bias was 1.0, its reversal rate was zero, and it did not respond to repellents (Table 5). CW rotation was restored to the gutted strain upon addition of a multicopy plasmid in which *cheY* was under the control of the tryptophan promoter (Fig. 1, HCB328; Table 5, HCB450). These cells spent most, but not all, of their time spinning CW. Unlike the situation for reconstituted cell envelopes (28), reversals still occurred. In general, CheY decreased the CW-to-CCW transition rates (lengthened tumbles) and increased the CCW-to-CW transition rates (shortened runs), as noted in the last two columns of Table 5. Both HCB328 and HCB450 failed to respond to attractant (100 μM aspartate) or repellent (1 mM Ni^{2+}). A small decrease in bias was observed following shifts from pH 7 to 6 and upon addition of 10 mM sodium acetate at pH 6. However, this decrease was delayed, developing gradually over a few minutes.

Expression of CheY over a wide range was obtained by using a plasmid (pJH120) in which *cheY* is under the control of the arabinose promoter. The bias and reversal rates of the resultant strain (HCB465) are plotted as a function of arabinose concentration in Fig. 2A and B. As the concentration of arabinose increased, the bias and reversal rate approached the values obtained with strain HCB450. As before, CW intervals lengthened and CCW intervals shortened (data not shown). In the absence of arabinose, the cells spun exclusively CCW but responded to the addition of 10 mM sodium acetate at pH 6, indicating that some CheY must have been present. Additional evidence for the presence of CheY in cells grown without arabinose was obtained by using strain D158*recA*(pJH120), which contains the same plasmid and which is deleted only for *cheY*. These cells, when grown in swarm plates in the absence of arabinose, produced well-formed chemotactic rings approximately 80% as large as those found with a wild-type control (HCB526; data not shown).

Transducers and CheY. To test the effect of transducer upon the behavior of CheY-containing cells, the plasmid pRL22Δ*pvu*II was transformed into strains which carried single copies of the transducer genes *trg*, *trg* and *tar*, or *trg* and *tsr*, to yield strains HCB330, HCB334, and HCB338, respectively. All three strains had a bias greater than that exhibited by HCB328 (Fig. 1). This difference was small but appeared to be related to the amount of transducer present. To verify this effect, a λgt*4-tar101* lysogen of strain HCB465 was constructed (strain HCB475). The bias and reversal rate of HCB475 are plotted as a function of arabinose concentration in Fig. 2A and B. The presence of Tar resulted in an increase in bias and a decrease in reversal rate (compare strains HCB465 and HCB475). While the standard errors of the means for the aggregate data were relatively large (not shown), the mean bias of HCB475 cells was always greater than that of HCB465 cells tethered on the same day. The difference between these means was generally larger than the sum of their standard errors. All of the above strains decreased their bias when the pH was shifted from 7 to 6 or when 10 mM acetate was added at pH 6 (data not shown). As before, this change in bias developed gradually.

Tar. To rule out the possibility that the shifts in bias effected by Tar were due to an interaction of Tar and the motor not involving CheY, we repeated some of the above experiments in gutted strains that contained either the *flaAII*(*cheC*) allele *scyA2* or *scyA3* or the *flaBII*(*cheV*) allele *scyB10*. These alleles were isolated as second-site mutations from different pseudorevertants of a single strain defective in *cheY* (26). They are thought to be cause defects in components of the motor that control its direction of rotation (so-called switch components). Even in the gutted background (HCB482, HCB483, and HCB484; Table 6), these alleles enabled the cells to spin alternately CW and CCW and thus permitted us to test for CCW shifts in the absence of CheY. CheY significantly decreased the bias in all three constructs (HCB490, HCB491, and HCB492; Table 6). This is not surprising, given that the pseudorevertants retain some chemotactic activity in the wild type background (26). That activity, and the bias in the wild type and p*trp-cheY* backgrounds, fall in the same order: *scyA3* > *scyA2* > *scyB10*. In the absence of CheY, Tar (carried by pAK101RI)

TABLE 5. Behavior of reconstituted strains with wild-type flagellar motors[a]

Strain	Chemotaxis genes present[b]	Bias	Reversal rate (s^{-1})	k_f (s^{-1})	k_r (s^{-1})
HCB437	None	1	0	>180	<.01
RP4979	All but *cheY*	1	0	>180	<.01
HCB450	p*trp-cheY*	0.17 ± 0.14	1.27 ± 0.96	1.29 ± 1.11	46.8 ± 25.6
HCB449	p*trp-cheYZ*	>0.99 ± 0.00	0.04 ± 0.01	167.4 ± 8.7	0.02 ± 0.01
HCB351	*trg cheYZ* p*trp-cheY*	0.98 ± 0.01	0.25 ± 0.12	134.4 ± 17.8	0.15 ± 0.08

[a] Bias = fraction of time spinning CCW; k_f = CW-to-CCW transition rate; k_r = CCW-to-CW transition rate; reversal rate = number of CW-to-CCW and CCW-to-CW transitions per second = $2k_fk_r/(k_r + k_f)$. The numbers given are the means and standard errors of the means for the cell population.

[b] See Table 2, footnote *b*.

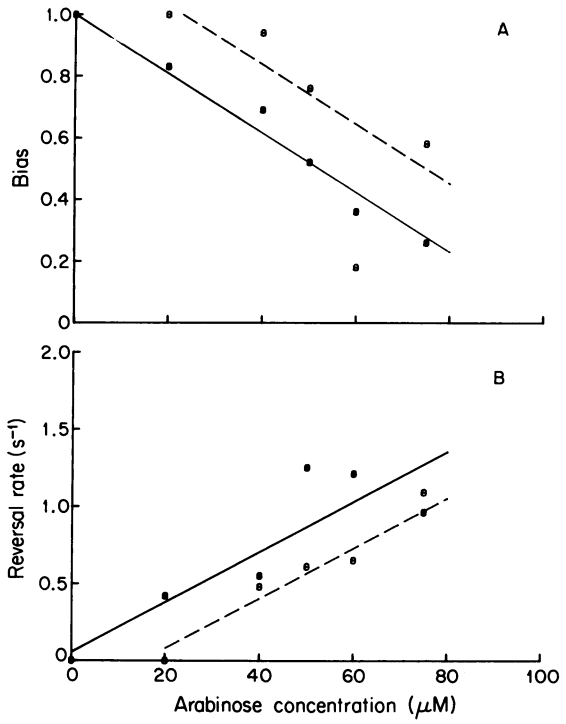

FIG. 2. The bias (A) and reversal rate (B) of a gutted strain expressing CheY (HCB465; ●, ——) or a gutted strain expressing Tar and CheY (HCB475; ○, -----) as a function of the concentration of the inducer for *para-cheY*, L-arabinose. The two strains were identical, except that the second is a λgt*4-tar101* lysogen (Table 4). Each point represents the mean of one to four experiments, each involving 8 to 24 cells. The lines were drawn by eye.

did not alter the behavior of the strains containing *scyA3* or *scyB10* (HCB500 and HCB501, respectively). The presence of Tar in the *scyA2* background did appear to have a significant effect upon behavior (HCB499; Table 6), but this effect proved to be independent of Tar, since a plasmid that expressed no functional chemotaxis gene product (pRL22Δ*cheY*Δ*pvu*II) also generated a similar shift in this background (HCB496). Therefore, the CCW shift produced by Tar in strains wild type for *flaAII* and *flaBII* cannot be accounted for by a direct interaction between the transducer and the flagellar motors.

CheZ. In plasmid pRL22, both *cheY* and *cheZ* are expressed by the tryptophan promoter. When this plasmid was transformed into the gutted strain, the resultant strain (HCB449) exhibited a large bias. Of the 25 cells examined, only 2 were able to spin CW; the bias averaged over the cell population was >0.99, and the reversal rate was 0.04 s^{-1} (Table 5). Thus, CheZ counteracts the effect of CheY. Note that this is true even when the expression of CheY greatly exceeds that of CheZ (HCB351; Table 5).

Strain RP1616, whose only defect is a *cheZ* deletion (Table 1), responded to the attractants aspartate and serine. Thus, CheZ is not required for a CCW response. Furthermore, CheZ, introduced via plasmid pBB1, did not change the bias or the reversal rate in the *scyA3* background (HCB494; Table 6). Although the presence of CheZ appeared to shift the bias CCW in the *scyA2* background (HCB493; Table 6), the control plasmid (pRL22Δ*cheY*Δ*pvu*II) also exhibited a similar effect (HCB496; Table 6). Thus, CheZ does not act alone to increase the bias. We conclude that CheZ is not a CCW signal.

When pRL22 was transformed into the *scyA3* background, yielding strain HCB488, the bias was intermediate between that of the null strain, HCB483, and that of the CheY-containing strain, HCB491, while the reversal rate was higher than that of either of these two strains (Table 6). If CheZ acts on CheY only in the cytoplasm, its effects should not depend on the phenotype of the motor; thus, the rotational behavior of HCB488 should mimic that of its gutted parent, HCB483. The sharp increase in reversal rate shows that this is not the case and suggests that CheZ exerts an effect on CheY at the flagellar motors.

Transducer, CheY, CheA, and CheW. No response to the attractant aspartate or the repellent Ni^{2+} was observed in the reconstituted strain that produces CheY and the transducer Tar (HCB475, Table 7). However, a strain that produces CheA, CheW, CheY, and the transducers Tar and Trg (HCB525) did respond to both aspartate and Ni^{2+} (Table 7). It also responded to shifts from pH 7 to 6 and to the addition of 10 mM acetate at pH 6. These responses were inverted, as expected for stains carrying only the Tar transducer (14, 19). All of the responses occurred at a rate expected for *cheZ* mutants (4), and the cells failed to adapt, as expected for *cheR cheB* mutants (4). Despite these differences, it is clear that CheA and CheW restored sensitivity to changes in concentration occurring at the level of the periplasm.

A strain (HCB349) that produces CheA, CheW, Tar, and Trg but not CheY rotated only CCW and did not respond to the repellent Ni^{2+} (Table 7). The *scyA2* allele, when added to this background, enabled the cells to spin alternately CW and CCW and thus permitted us to test for responses to both attractants and repellents. The resultant strain (HCB527) still did not respond to either aspartate or Ni^{2+}. However, when CheY was added back via plasmid pJH120, the responses were restored (HCB529; Table 7).

The presence of CheA and CheW dramatically decreased the bias of a strain containing transducer and CheY (compare HCB475 and HCB525; Table 7). This effect was not significantly diminished by the presence of the *scyA2* allele (compare HCB527 and HCB529; Table 7). However, when

TABLE 6. Behavior of reconstituted strains with flagellar motors defective in FlaAII (CheC) or FlaBII (CheV)[a]

Strain	Allele	Chemotaxis genes present[b]	Bias	Reversal rate (s^{-1})
HCB482	*scyA2*	None	0.56 ± 0.06	3.74 ± 0.45
HCB499	*scyA2*	*tar*[c]	0.80 ± 0.03	3.52 ± 0.28
HCB496	*scyA2*	p*trp*-Δ(*cheY*)	0.75 ± 0.05	2.60 ± 0.34
HCB493	*scyA2*	p*trp*-Δ(*cheY*) *cheZ*	0.82 ± 0.03	2.81 ± 0.32
HCB490	*scyA2*	p*trp-cheY*	0.10 ± 0.05	0.51 ± 0.29
HCB483	*scyA3*	None	0.84 ± 0.02	2.82 ± 0.24
HCB500	*scyA3*	*tar*[c]	0.84 ± 0.02	3.37 ± 0.42
HCB497	*scyA3*	p*trp*-Δ(*cheY*)	0.83 ± 0.02	3.54 ± 0.40
HCB494	*scyA3*	p*trp*-Δ(*cheY*) *cheZ*	0.89 ± 0.01	3.06 ± 0.35
HCB491	*scyA3*	p*trp-cheY*	0.27 ± 0.08	0.95 ± 0.25
HCB488	*scyA3*	p*trp-cheYZ*	0.57 ± 0.07	4.25 ± 0.51
HCB484	*scyB10*	None	0.53 ± 0.08	2.35 ± 0.29
HCB501	*scyB10*	*tar*[c]	0.60 ± 0.06	2.57 ± 0.50
HCB492	*scyB10*	p*trp-cheY*	0.02 ± 0.01	0.20 ± 0.17

[a] See Table 5, footnote *a*.
[b] See Table 2, footnote *b*.
[c] *tar* was carried in multicopy by plasmid pAK101RI.

TABLE 7. Behavior of reconstituted strains in the presence of transducers and the *cheY* or *cheAW* gene products or both[a]

Strain	Allele	Chemotaxis gene present[b]	Concn of arabinose (μM)	Bias	Reversal rate (s^{-1})	Response to following change[c]: Aspartate (0 to 2.5 μM)	$NiCl_2$ (0 to 0.1 mM)	pH 7 to pH 6	Acetate (pH 6) (0 to 10 mM)
HCB475	*scyA*$^+$	*tar para-cheY*	20	1	0	NR	NR	−	−
HCB349	*scyA*$^+$	*cheAW tar trg*	0	1	0	NR	NR	NR	NR
HCB525	*scyA*$^+$	*cheAW tar trg para-cheY*	0	0.01 ± 0.00	0.12 ± 0.03	+	−	+	+
HCB527	*scyA2*	*cheAW tar trg*	0	0.91 ± 0.04	1.11 ± 0.52	NR	NR	NR	NR
HCB529	*scyA2*	*cheAW tar trg para-cheY*	0	0.08 ± 0.04	0.69 ± 0.35	+	−	+	+

[a] See Table 5, footnote *a*.
[b] See Table 2, footnote *b*.
[c] NR, No response; +, an increase in bias; −, a decrease in bias. The − responses in strain HCB475 developed over a few minutes.

CheY was absent, CheA and CheW had no effect upon bias (HCB349; Table 7). Thus, CheA or CheW or both appear to modulate the effectiveness of CheY. Whether this occurs in the absence of transducer is not known.

DISCUSSION

In summary, we constructed strains of *E. coli* deleted for genes that code for the known transducers Tar, Tsr, Trg, and Tap and the known cytoplasmic *che* gene products CheA, CheW, CheR, CheB, CheY, and CheZ. These strains possessed functional flagellar motors, with either wild type or mutant switch components, so that they could be tethered and their rotational behavior could be analyzed. One or more of the missing components were added back, and changes in rotational bias and sensitivity to attractants and repellents were noted. We established the following facts. (i) In the absence of transducers and cytoplasmic *che* gene products, wild type motors rotate exclusively CCW, while motors containing defective FlaAII or FlaBII components continue to change their directions of rotation. (ii) CheY decreases the bias in either case by lengthening CW intervals and shortening CCW intervals. (iii) The magnitude of the shift observed with wild type motors increases with the concentration of CheY and is greater at lower external and internal pH. (iv) The magnitude of this shift is reduced by transducers; however, transducers do not affect the bias in the absence of CheY. (v) The effect of CheY is offset by CheZ, even when *cheZ* is expressed at a relatively low level; however, CheZ does not affect the bias in the absence of CheY. (vi) Cells containing transducers and CheY fail to respond to attractants or repellents known to interact with periplasmic transducer domains. (vii) This sensitivity is restored by CheA and CheW; however, these components do not affect the bias in the absence of CheY. Whether CheA can restore this sensitivity in the absence of CheW, or whether CheW can do so in the absence of CheA, is not yet known.

We conclude that CheY interacts with both the transducers and with the flagellar motors. Thus, it appears to play a central role in coupling one to the other. Given the small size of the shift in bias as a result of the action of transducers and the lack of genetic evidence indicating a direct interaction between transducers and CheY (in particular, that pseudorevertants of CheD have not been found in CheY [23]), this interaction might well be indirect. However, since there are allele-specific pseudorevertants of *cheY* in *flaAII* and *flaBII* (26) and since CheY can change the direction of flagellar rotation in cell envelopes depleted of other cytoplasmic components (28), the interaction between CheY and the motor must be direct.

CheZ acts as a CheY antagonist. It does not appear to shift flagellar bias in the absence of CheY. Given that its effect on CheY activity differs depending upon the structure of the switch components and that allele-specific pseudorevertants of *cheZ* also occur in *flaAII* and *flaBII* (26), CheZ appears to exert its antagonism at the flagellar motors. However, our evidence does not rule out the possibility that CheZ also acts in the cytoplasm. Note that the filamentous cells studied previously (31) contained many flagellar motors, only a few of which carried external markers, so that CheZ could have shortened the signal decay length by acting either at the flagellar motors or in the cytoplasm or both.

CheA or CheW or both are required to complete the signal pathway. Cells containing transducers, CheA, CheW, and CheY respond to attractants and repellents. CheR, CheB, and CheZ are not necessary. Strain constructions are under way that will allow us to determine whether CheA or CheW act alone or whether both are required.

A model for the signal pathway is given in Fig. 3. It includes a molecule of low molecular weight and its precursor, as explained below. The model is meant to be provocative: we hope that it will stimulate the design of significant experiments.

Since CheY acts as a tumble generator, it is easier to describe the response of the system to the addition of repellent (or removal of attractant) than it is to the addition of attractant. The steps in the pathway are as follows (with numbers corresponding to those in Fig. 3). (Step 1) An increase in the concentration of repellent changes the configuration of the transducer. (Step 2) This increases the rate of catalytic conversion of the precursor to the low-molecular-weight molecule. This catalysis requires CheA or CheW or both. In addition, the production of these molecules requires ATP. Some molecules (or functional analogs) are present endogenously, even in a gutted strain. (Step 3) The molecule behaves as an activated intermediate; it decays, e.g., by hydrolysis, back to the precursor or to some other inert product. (Step 4) The molecule activates CheY; it also activates CheB (not shown). (Step 5) Activated CheY binds to the switch components of the motor and enhances CW rotation. (Step 6) CheZ inactivates CheY at the motor, releasing the precursor or some other inert product. CheZ might also inactivate CheY in the cytoplasm. To explain the CCW shift effected by transducers in the presence of CheY (Fig. 1 and 2), we assume that the transducer, acting alone, weakly hydrolyzes the molecule; see the discussion of *cheD* mutants given below. The rate of this hydrolysis is not

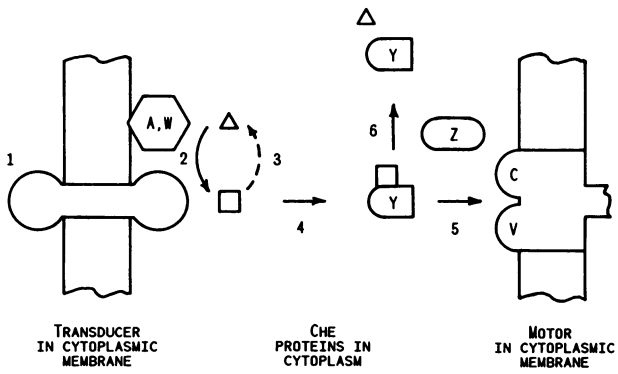

FIG. 3. Schematic representation of a model for the chemotaxis signal pathway. The numbers refer to steps along the path, the letters refer to *che* gene products, the square represents a molecule of low molecular weight, and the triangle represents its precursor. See the discussion in the text.

affected by the addition of attractants or repellents. In the presence of CheA and CheW, on the other hand, the transducer synthesizes the molecule (dramatically reducing the bias [Table 7]) at a rate that is affected by attractants and repellents. CheA or CheW or both might couple the synthesis of this molecule to the hydrolysis of ATP.

In addition to explaining the facts available to Segall et al. (31), outlined in the Introduction, and the set enumerated above, the scheme outlined in Fig. 3 has the following merits. (i) Step 2 above is catalytic and, hence, increases the gain. The gain of the chemotactic system is enormous. For example, a step change in the concentration of L-aspartate that changes the average occupancy of Tar by one molecule (1 part in 600, assuming 600 copies of Tar per cell [7]) increases the bias by about 0.1 (30). Given a stoichiometry of expression of *cheY* that is four to five times larger than that of *tar* (9), a system without amplification would require a change in bias of this magnitude to result from the modification of one molecule of CheY out of a potential pool of 3,000. This seems unlikely. (ii) The assumption that the synthesis of the low-molecular-weight molecule requires ATP would explain why cells deprived of ATP spin their flagella CCW (32, 38) and why ATP exerts this effect at the level of the cytoplasm (27). (iii) *cheD* mutants of *tsr* spin their flagella CCW, and the mutations are dominant; however, cells can respond if the bias of the motor is set low enough (6). Also, in *cheD* mutants, CheB is inhibited (13). These effects could be explained if the *cheD* gene product has an anomalous catalytic site that strongly favors hydrolysis. Then the *cheD* gene product would destroy the molecule, and the mutation would be dominant. (iv) *cheA* mutants spin their flagella CCW (22), and CheA is required for the activation of CheB following the addition of repellent or removal of attractant (36). Thus CheA must be involved in step 2 above. Whether the large or small *cheA* gene product or both are involved is not known (35). (v) CheY binds L- and D-*S*-adenosylmethionine and L-*S*-adenosylhomocysteine (18), and so it would not be unreasonable for CheY to be activated by the low-molecular-weight molecule if this molecule contains adenine. (vi) The N-terminal regulatory subunit of CheB, which suppresses the esterase activity (34), is homologous to CheY (37), and so it is not unreasonable (as Stock et al. [37] argue) for CheY and CheB to be activated by a common transducer-generated signal. (vii) This model cannot readily account for transducer mutants that fail to tumble yet demethylate in response to the addition of repellents or removal of attractants. A mutant of this kind (*tar* mutated at site 372R) has been described by Mutoh et al. (20). However, in our hands, this allele does signal when tested either in the wild type background or in a background deleted for *cheR* and *cheB* (unpublished data). (viii) Finally, we note that diffusion of the low-molecular-weight molecule could link the receptors to the flagella, but that it need not do so, since CheY is small enough for that task (31).

The scheme outlined in Fig. 3 is incomplete, because we have not included the methyltransferase (CheR) or considered feedback from the motor involved in regulation of methylation and demethylation. However, this part of the system does not appear to play an essential role in excitation. In addition, the model can be criticized, because it introduces elements (the low-molecular-weight molecule and its precursor) for which there is no direct experimental evidence. However, such components would not be easily identified. From measurements of impulse responses (4, 30), we know that the lifetime of the activated state of CheY is quite short. In the presence of CheZ, this lifetime is only a few tenths of a second. Even in its absence, it is only a few seconds. Therefore, one would not expect to be able to isolate activated CheY in a trivial manner.

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