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The Smaller of Two Overlapping *cheA* Gene Products Is Not Essential for Chemotaxis in *Escherichia coli*

HAMIDREZA SANATINIA, ERIC C. KOFOID, TOM B. MORRISON, AND JOHN S. PARKINSON*

Biology Department, University of Utah, Salt Lake City, Utah 84112

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The *cheA* locus of *Escherichia coli* encodes two similar proteins, $CheA_L$ (654 amino acids) and $CheA_S$ (557 amino acids), which are made by initiating translation from different in-frame start sites [start(L) and start(S)]. $CheA_L$ plays an essential role in chemotactic signaling. It autophosphorylates at a histidine residue (His-48) and then donates this phosphate to response regulator proteins that modulate flagellar rotation and sensory adaptation. $CheA_S$ lacks the first 97 amino acids of $CheA_L$, including the phosphorylation site at His-48. Although it is unable to autophosphorylate, $CheA_S$ can form heterodimers with mutant $CheA_L$ subunits to restore kinase function and chemoreceptor control of autophosphorylation activity. To determine whether these or other activities of $CheA_S$ are important for chemotaxis, we constructed *cheA* lesions that abrogated $CheA_S$ expression. Mutants in which the $CheA_S$ start codon was changed from methionine to isoleucine (M98I) or glutamine (M98Q) retained chemotactic ability, ranging from 50% (M98Q) to 80% (M98I) of wild-type function. These partial defects could not be alleviated by supplying $CheA_S$ from a specialized transducing phage, indicating that the lesions in $CheA_L$—not the lack of $CheA_S$—were responsible for the reduced chemotactic ability. In other respects, the behavior of the M98I mutant was essentially normal. Its flagellar rotation pattern was indistinguishable from wild type, and it exhibited wild-type detection thresholds and peak positions in capillary chemotaxis assays. The lack of any substantive defect in this start(S) mutant argues that $CheA_S$ makes a negligible contribution to chemotactic ability in the laboratory. Whether it has functional significance in other settings remains to be seen.

The chemotactic behavior of *Escherichia coli* provides a model system for investigating signal transduction mechanisms at the molecular level (see reference 23 for a recent review). As they swim about, these cells use transmembrane chemoreceptors to monitor concentrations of beneficial or harmful chemicals in their environment. Changes in attractant or repellent levels trigger changes in flagellar rotation that promote migration in favorable directions: counterclockwise (CCW) rotation produces forward movement; clockwise (CW) rotation causes abrupt turns or tumbling episodes. The chemoreceptors communicate with the flagellar motors through a series of intracellular protein phosphorylation reactions, much like the signal transduction pathways of eukaryotes.

The *cheA* locus plays a central role in this signaling cascade. It encodes a histidine kinase that autophosphorylates, using ATP as the phosphate donor (8). CheA donates its phosphate groups to two proteins, CheY and CheB, which control flagellar responses and sensory adaptation, respectively (9). Phospho-CheY interacts with the switching machinery of the flagellar motors to enhance the probability of CW rotation (2, 35). Phospho-CheB covalently modifies stimulated chemoreceptors to attenuate their signaling activity as part of a negative-feedback circuit (17). The phosphorylated forms of CheY and CheB are very short-lived, enabling the chemoreceptors to modulate their steady-state levels in response to chemical stimuli by controlling the autophosphorylation activity of CheA (3). An increase in attractant level prolongs forward swimming by inhibiting CheA, thereby reducing the level of phospho-CheY. An increase in repellent level enhances the likelihood of a change in swimming direction by stimulating CheA autophosphorylation, which in turn raises phospho-CheY levels. The CheW protein plays an important role in coupling CheA to chemoreceptor control (6, 16, 28).

The *cheA* gene of *E. coli* encodes two similar proteins, $CheA_L$ (654 amino acids) and $CheA_S$ (557 amino acids), which are made by initiating translation from different in-frame start sites, designated start(L) and start(S), respectively (11, 30). $CheA_L$ is identical to $CheA_S$ but contains an additional 97 amino acids at its N terminus, including His-48, the site of autophosphorylation. Mutations at His-48 and other sites between start(L) and start(S) eliminate chemotactic ability, demonstrating that $CheA_L$—presumably through its autophosphorylation ability—is essential for chemotaxis (8, 30). To investigate what signaling role $CheA_S$ might play, we constructed and characterized several *cheA* mutants with lesions in start(S) that abrogated $CheA_S$ expression. Two such mutants, despite concomitant amino acid replacements in $CheA_L$, retained chemotactic ability, proving that $CheA_S$ is not essential for chemotaxis. Thus, the functional significance of the overlapping gene organization at the *E. coli cheA* locus is not yet apparent.

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial strains used in this study are listed in Table 1. All are derivatives of *E. coli* K-12; the RP strains are closely related to the chemotactically wild-type strain RP437 (24). λfla52 is a specialized transducing phage that carries the *motA-cheW* operon (29). Deletion and point mutant derivatives of λfla52 that express different combinations of CheA proteins were used for complementation analyses (30).

Plasmids. Plasmids used in this study are listed in Table 2; the positions of restriction sites used in some of the constructions are shown in Fig. 1. Complementation studies of pEK46 and its derivatives, which have very high copy numbers, were performed with host strains containing a *pcnB* defect to reduce plasmid copy levels (15).

Site-directed mutagenesis. Single stranded, dU-substituted pEK46 DNA was prepared in BW313 (12). The template was mixed with a mutagenic primer at 80°C in mutagenesis buffer (10 mM $MgCl_2$, 10 mM Tris [pH 8.0]) and slowly cooled to room temperature (~1°C/min). Extension and ligation of the primed

* Corresponding author. Phone: (801) 581-7639. Fax: (801) 581-4668. Electronic mail address: Parkinson@Biology.Utah.Edu.

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype[a]	Source or reference
BW313	*dut ung*/F′ *lysA*	12
DH5α	*recA1 endA1 gyrA96*	7
MV1193	Δ(*lac-proAB*) F′ *proAB lacI*^{q} Δ(*lacZ*)*M15*	34
RP437	Wild type for motility and chemotaxis	24
RP1791	Δ(*cheA*)*m40022*	21
RP5045	Δ(*motA-tar*)*2211*	24
RP6663	Δ(*motB-cheA*)*m1111 eda-50*	5
RP6894	*recA1*	5
RP9005	Δ(*motB-cheA*)*m1111 recD*::miniTn*10*	This work
RP9009	Δ(*motA-tar*)*2211 pcnB1*	This work
RP9023	Δ(*cheA*)*m40022 supF*	This work
RP9037	*cheA*(M98am)	This work
RP9038	*cheA*(M98Q)	This work
RP9194	*cheA*(M98I)	This work
RP9195	*cheA*$^+$	This work
RP9535	Δ(*cheA*)*1643*	14

[a] Deletions with *m* allele numbers were obtained by selecting for loss of a heat-inducible, defective Mu prophage (Mu*d1*) (21).

template were done overnight at 15°C in mutagenesis buffer containing 1 U of DNA polymerase Klenow fragment, 0.25 mM each of the four deoxyribonucleoside phosphates (dATP, dGTP, dCTP, and dCTP), 5 mM dithiothreitol, and 1 mM ATP. Treated DNA was introduced by transformation into RP5045 [Δ(*motA-tar*)] to preclude recombination between the plasmid insert and the host chromosome. Plasmid DNA was prepared from individual transformants and tested for the desired mutation by DNA sequencing. Between 5 and 50% of the sequenced plasmids carried the correct mutational change.

DNA sequencing. Sequence determinations were done by the dideoxy-chain termination method, using either single-stranded DNA prepared in strain MV1193 or double-stranded, supercoiled DNA prepared in strain DH5α. Sequenase was purchased from U.S. Biochemicals Corp.

Maxicell analysis of plasmid-encoded proteins. Plasmids were transferred to strain RP6894 (*recA*) for radiolabeling of plasmid-encoded proteins by the maxicell technique (27). Cultures were grown at 35°C in M9 medium (18) to a density of about 2×10^8 cells per ml and irradiated for 30 s with 200-μW/cm^2 UV light. The cells were returned to 35°C and incubated for 1 h, at which time cycloserine was added to a final concentration of 400 μg/ml to kill any dividing cells. After further incubation overnight, the cells were washed twice in M9 buffer and resuspended in 0.4 ml of M9 medium. Cells were incubated at 37°C for 1 h, 5 μl of [^{35}S]methionine (1 μCi/ml) was added, and the mixture was incubated for another 1 h at 37°C. Labeled cells were then pelleted and resuspended in protein sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (13).

Western blots (immunoblots). Cultures were grown in tryptone broth at 30°C to a density of ~2×10^8 cells per ml. Cells from 5 ml of culture were concentrated by centrifugation, resuspended in 75 μl of SDS-PAGE loading buffer, and lysed by boiling for 15 min. Proteins in the cell extracts were separated by SDS-PAGE (10% polyacrylamide) and transferred to 0.2-μm-pore-size polyvinylidene difluoride PVDF membranes with a Semiphor Semi-Dry Transfer Unit (Hoefer Scientific Instruments). Membranes were treated with polyclonal rabbit anti-CheA antibody kindly provided by J. Stock, Princeton University. Antibody-reactive bands were tagged with ^{35}S-protein A (Amersham) and quantified with a Molecular Dynamics PhosphorImager.

Chemotaxis methods. Swarm rates were measured on semisolid tryptone agar (19). Flagellar rotation patterns were assessed with antibody-tethered cells (16). Capillary assays were essentially as described previously (1), with synthetic serine kindly provided by J. Adler, University of Wisconsin, Madison.

General genetic methods. Phage P1kc was used for transductional crosses as described previously (22). Plasmid transformations were done by the $CaCl_2$ heat shock method (7). Lysogens of λfla52 and its derivatives were identified by testing cells from plaque centers for immunity to homoimmune λ phages and sensitivity to heteroimmune λ phages. Because λfla52 has a thermolabile repressor, candidate lysogens could be confirmed by growth tests at 42°C.

Transfer of plasmid-borne *cheA* mutations to the host chromosome. The scheme we used is diagrammed in Fig. 2A. Mutant derivatives of plasmid pEK46 were digested with *Dra*I, and the linearized DNA molecules were transferred into RP9005 [Δ(*motB-cheA*) *recD*]. Cells from the transformation mix were placed in a streak on semisolid tryptone agar plates and incubated overnight at 35°C to identify motile recombinants that had acquired the *motB-cheA* region from the plasmid. Because the recipient deletion extended through the start(S) portion of *cheA*, all motile recombinants in any particular cross had the same chemotaxis phenotype. Some donor *cheA* alleles yielded chemotactic recombinants, whereas others did not. For subsequent characterization, the *cheA* region in these strains was transferred into RP6663, a *recD*$^+$ recipient, by P1 cotransduction with the *eda* locus (22).

Transfer of chromosomal *cheA* mutations to plasmids. The logic of the scheme we used (outlined in Fig. 2B) is similar to the one discussed above. A *motB* deletion derivative of plasmid pEK46 was constructed by digestion with *Bsu*36I and S1 nuclease religation. This plasmid (pHS21) was transferred into *cheA* mutants of interest by selecting for ampicillin-resistant transformants. Plasmid DNA was extracted from transformant colonies and transferred to RP9009 [Δ(*motA-tar*) *pcnB*], again selecting for ampicillin-resistant transformants. Colonies were tested on motility plates to identify those containing recombinant plasmids that had acquired the functional *motB* region from the *cheA* mutant host. Most such recombinants also acquired the mutant *cheA* allele and were readily recognized by their subnormal chemotaxis phenotypes.

RESULTS

Construction of plasmid-borne *cheA* start(S) mutations. The $CheA_S$ coding region lies entirely within that for $CheA_L$, so any changes made in it will necessarily alter the coding sequence for $CheA_L$ as well. To minimize possible consequences to $CheA_L$ function, we first attempted to eliminate $CheA_S$ expression by introducing genetically silent changes at third-base positions in $CheA_L$ codons that might block translation initiation at start(S). The ribosome-binding (Shine-Dalgarno) site just upstream of the $CheA_S$ initiation codon was an obvious target (Fig. 1). However, the only synonymous codon change possible (AAGGA to AAAGA) actually corresponds to the wild-type *cheA* sequence in *Salmonella typhimurium*, which also makes a $CheA_S$ protein (31). It seemed unlikely that this change would block ribosome binding, so we did not pursue this approach.

The mRNA encompassing start(S) contains a predicted

TABLE 2. Plasmids used in this study

Plasmid	Relevant properties[a]	Selective markers[b]	Derivation, construction or reference
pEK46	P_{mocha} (*motA-cheW*)	*bla*(Amp^r)	pUC118 (11)
pJL15	P_{mocha} (*motA-cheW*)	*cat*(Cam^r)	pACYC184 (14)
pHS7	*motA*$^+$ *motB*$^+$ *cheA*(M98am) *cheW*$^+$	*bla*(Amp^r)	Site-directed mutation of pEK46
pHS9	*motA*$^+$ *motB*$^+$ *cheA*(M98I) *cheW*$^+$	*bla*(Amp^r)	Site-directed mutation of pEK46
pHS16	*motA*$^+$ *motB*$^+$ *cheA*(M98am) *cheW*$^+$	*cat*(Cam^r)	*Mlu*I fragment from pHS7 into pJL15
pHS21	*motA*$^+$ Δ*motB cheW*$^+$	*bla*(Amp^r)	Deletion at *Bsu*36I site in pEK46
pHS25	*motA*$^+$ *motB*$^+$ *cheA*(M98am) Δ*cheW*	*cat*(Cam^r)	Deletion at *Eco*RV site in pHS16
F′402	Inserts serine at UAG Codons	*supD his*$^+$	F (22)
pGFIB series	Inserts alanine, arginine, cysteine, glutamic acid,[c] histidine, phenylalanine, or proline at UAG codons	*bla*(Amp^r)	pBR322 (10)

[a] P_{mocha} is the native promoter for the *motA-cheW* operon.
[b] Selective markers: *bla* (β-lactamase) confers ampicillin resistance (Amp^r); *cat* (chloramphenicol acetyltransferase) confers chloramphenicol resistance (Cat^r); *his*$^+$ (histidine biosynthesis); *supD* (amber suppressor).
[c] The suppressor tRNA that inserts glutamic acid is also charged at lower efficiency with glutamine, tyrosine, and arginine.

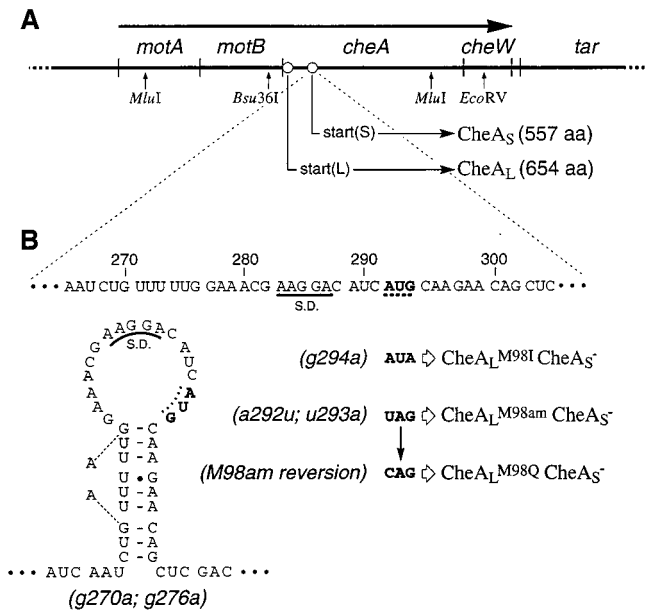

FIG. 1. Physical and genetic features of the *cheA* locus. (A) Genes of the *mocha* operon are drawn to scale, with the positions of restriction sites used in plasmid constructions indicated below the map. (B) Mutational changes created at start(S) of *cheA*. The *cheA* mRNA sequence around start(S) is shown, with nucleotides numbered from the start of the $CheA_L$ coding sequence. S.D. is the Shine-Dalgarno site, and the underlined AUG is the $CheA_S$ start codon.

stem-loop structure that might influence $CheA_S$ expression (11). Both the Shine-Dalgarno sequence and the initiation codon lie within the putative loop (Fig. 1). If this feature is important for $CheA_S$ translation, nucleotide changes that weaken base pairing in the stem might reduce $CheA_S$ expression. Using plasmid pEK46, we introduced two silent changes in $CheA_L$ codons (g270a and g276a) that would disrupt G · C pairs in the stem. The mutant plasmid complemented strain RP9009 [Δ(*motA-tar*)] for chemotaxis, but $CheA_S$ expression relative to $CheA_L$, determined by immunoblotting and by maxicell analysis, was actually severalfold higher in the mutant than in the pEK46 control (data not shown). If anything, the stem-loop arrangement at start(S) appears to retard translation of $CheA_S$, so this plan was also abandoned.

The most effective way to eliminate $CheA_S$ expression seemed to be through alteration of the start(S) initiation codon. We initially constructed two start codon changes in pEK46 to test this approach (Fig. 1): AUG to AUA, which causes a methionine-to-isoleucine replacement (M98I) in $CheA_L$; and AUG to UAG, which converts the start codon to an amber nonsense codon (M98am). A third start codon mutation (M98Q) was subsequently obtained by reversion of M98am (see below). Plasmids carrying the M98am (pHS7) and M98I (pHS9) mutations were transferred to RP9009 [Δ(*motA-tar*)] to test for CheA function. Although pHS7 failed to support swarming, pHS9 did support it (data not shown), implying that $CheA_L$ alone might be sufficient for chemotaxis. To explore this possibility, we crossed the plasmid-borne start(S) mutations into the *E. coli* chromosome and tested the resultant strains for $CheA_S$ expression and for chemotactic ability.

Isolation of chromosomal *cheA* start(S) mutants. The plasmid-borne M98I and M98am mutations were transferred into the chromosome by linear transformation (see Materials and Methods), using a recipient strain that carried a *motB-cheA* deletion (Fig. 2A). Recombinants that had acquired the *motB-cheA* region from the donor plasmid were selected as motile clones on swarm agar and scored for chemotactic ability. Those derived from pHS9 were chemotactic, indicating that a single chromosomal copy of the M98I allele of *cheA* was sufficient for chemotaxis. Recombinants derived from pHS7 were nonchemotactic, confirming the lack of CheA function in the M98am allele. Both mutations were subsequently transduced into a $recD^+$ recipient to verify that they were located at the chromosomal *cheA* locus (i.e., 20% linkage to the *eda* gene) (22), yielding isogenic strains RP9037 (M98am), RP9194 (M98I), and RP9195 (wild-type control).

Upon prolonged incubation (30 h at 35°C), RP9037 (M98am) gave rise to partially chemotactic revertants (data not shown). The *cheA* regions from different revertants were transferred into plasmid pEK46 by homologous recombination, as outlined in Fig. 2B, to determine the nature of the mutational changes responsible. The recombinant plasmids supported slow chemotactic spreading, indicating that they carried a mutant *cheA* locus (data not shown). Four such plasmids derived from independent revertants were analyzed by DNA sequencing. All four had a UAG-to-CAG change at the start(S) initiation codon, corresponding to a methionine-to-glutamine replacement (M98Q) in $CheA_L$. Subsequent characterization of this *cheA* mutation was done with revertant strain RP9038 (M98Q), which is otherwise isogenic with the M98I and M98am mutants described above.

CheA expression patterns of *cheA* start(S) mutants. The M98I, M98Q, and M98am mutations should prevent synthesis of $CheA_S$. M98am should also truncate $CheA_L$, whereas M98I and M98Q should produce a full-length $CheA_L$ protein. To test these predictions, we measured the levels of $CheA_L$ and

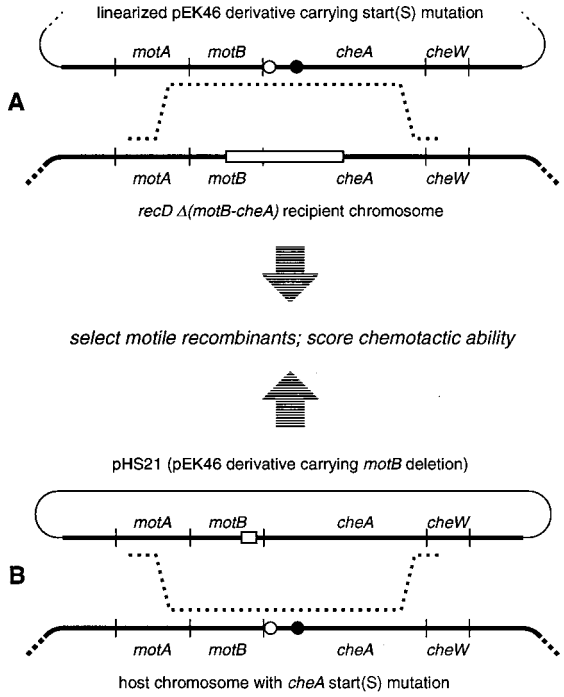

FIG. 2. Methods used to transfer *cheA* start(S) mutations between plasmids and the *E. coli* chromosome. (A) Transfer of plasmid-borne mutations into the chromosome. (B) Transfer of chromosomal mutations into plasmids. Open rectangles indicate deletions; open circles represent the wild-type start(L) sequence; solid circles represents mutant start(S) sequences.

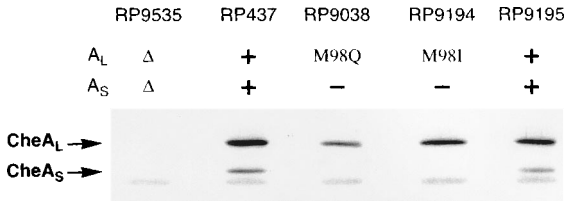

FIG. 3. Immunoblot analysis of *cheA* start(S) mutants. Cell proteins were separated by SDS-PAGE (10% polyacrylamide) and blotted with polyclonal CheA antiserum. The 50- to 80-kDa region of the gel is shown. Band intensities were quantified by volume integration and normalized to the faint band of cross-reacting material below $CheA_S$ to compensate for any variations in sample loading between lanes.

$CheA_S$ made by start(S) mutants in two ways. CheA expression from mutant plasmids was evaluated in maxicells; expression in chromosomal mutants was analyzed by immunoblotting with polyclonal CheA antiserum. In both cases, the M98am mutant made neither CheA product, whereas M98I and M98Q made only $CheA_L$. The results of Western analysis of RP9038 (M98Q), RP9194 (M98I), and several control strains are shown in Fig. 3. The molar ratio of $CheA_L$ to $CheA_S$ in the two wild-type controls was about 6:1. In contrast, the M98I and M98Q mutants made no detectable $CheA_S$. The apparent $CheA_L$ levels were also reduced in the mutants: 60% of wild type in M98I, and 28% of wild type in M98Q. Assuming that these mutational changes do not greatly alter reactivity with the polyclonal antiserum, the mutant proteins, particularly M98Q, are either translated less efficiently or more susceptible to degradation.

Chemotactic swarming in *cheA* start(S) mutants. On semisolid tryptone agar plates, colonies of RP9038 (M98Q) and RP9194 (M98I) resembled those of chemotactically wild-type strains (Fig. 4). The mutant colonies contained two bands of cells, which in wild-type swarms correspond to chemotactic migrations toward serine and aspartate. Although their ring morphologies were normal, the mutant colonies were consistently smaller than wild-type swarms, which could reflect an impairment in growth, motility, or chemotaxis. The growth rates and swimming speeds of the mutants proved identical to those of the wild type (data not shown), so that chemotaxis defects appear to be responsible for the reduction in swarm size. To evaluate the severity of those defects, we compared the mutant and wild-type swarm rates (Fig. 5). Chemotactic colonies typically exhibit two expansion phases. Expansion is slow when the colony is young but speeds up as the cells exhaust chemoattractant compounds in their vicinity. Rate determinations were made from the latter phase, during which colony diameter increased linearly with time (Fig. 5). The swarm rate for the M98I mutant was 79% of the wild-type rate; the rate for the M98Q mutant was 51% of the wild-type rate.

These results demonstrate that $CheA_S$ is not essential for chemotaxis on swarm plates. However, the absence of $CheA_S$ in the M98I and M98Q mutants might account for their reduced swarm rate. Alternatively, the concomitant mutational changes in $CheA_L$ could be responsible. To distinguish these possibilities, we asked whether restoring $CheA_S$ could alleviate the mutant swarm defect. To avoid differences in gene dosage, which could confound interpretation of the results, we supplied $CheA_S$ by lysogenizing the mutants with λ transducing phages (Table 3). The λfla52 prophage, which carries a wild-type *cheA* locus, restored swarm rates to normal in both mutants, demonstrating that neither defect was dominant. $CheA_S$ alone was supplied by a λfla52 derivative carrying the *cheA169* allele, an amber mutation at codon 10, between start(L) and start(S)

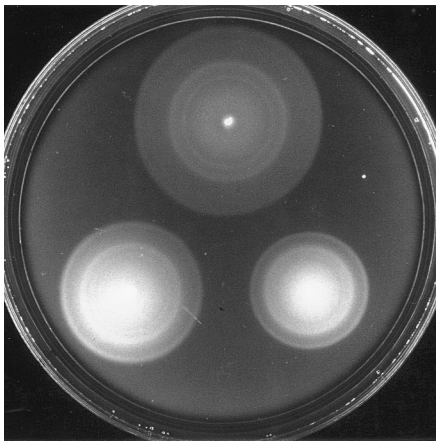

FIG. 4. Colony morphology of *cheA* start(S) mutants on semisolid tryptone agar. Chemotactic swarms were started from a toothpick inoculum and incubated at 35°C for 8 h. The strains shown are, reading clockwise from the top, RP9195 (wild type), RP9038 (M98Q), and RP9195 (M98I).

(11). This prophage produced no improvement in the M98I swarm rate but did increase the M98Q rate to about 75% of the wild-type rate (Table 3). The enhancement seen in M98Q may reflect stabilization of the mutant $CheA_L$ subunits through heterodimer formation with $CheA_S$, which is known to complement other $CheA_L$ defects by this mechanism (32, 38). The fact that $CheA_S$ cannot restore full chemotactic ability to either mutant implicates a functional defect in $CheA_L$ as the principal cause of their aberrant behavior.

Flagellar rotation patterns of *cheA* start(S) mutants. $CheA_L$ is essential for CW flagellar rotation (30), and in unstimulated cells its activity is adjusted by the sensory adaptation machinery to produce frequent episodes of CW rotation. We examined the M98I and M98Q mutants to determine whether their chemotaxis defects were accompanied by a change in unstimulated flagellar rotation pattern (Fig. 6). The rotational behavior of the M98I mutant was not significantly different from that of the wild-type control. In both strains, nearly all of the rotating cells

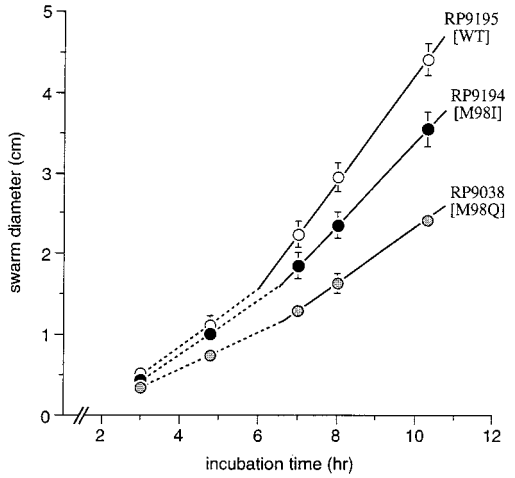

FIG. 5. Swarm sizes of *cheA* start(S) mutants. Colony sizes on semisolid tryptone agar were measured at intervals during incubation at 35°C. Values shown are the mean and standard error of measurements on at least four different colonies for each strain. Swarm rates were determined from the solid-line portions of the plots.

TABLE 3. Swarm rates of lysogenic *cheA* start(S) mutants

Host CheA products			Prophage CheA products			% Wild-type swarm rate[a]
Strain	$CheA_L$	$CheA_S$	Prophage	$CheA_L$	$CheA_S$	
RP9194	M98I	−	λ^{++}	−	−	75 ± 6
RP9194	M98I	−	λfla52	+	+	94 ± 8
RP9194	M98I	−	λfla52*am169*	−	+	76 ± 1
RP9038	M98Q	−	λ^{++}	−	−	52 ± 3
RP9038	M98Q	−	λfla52	+	+	91 ± 8
RP9038	M98Q	−	λfla52*am169*	−	+	73 ± 1

[a] Swarm rates were measured on tryptone semisolid agar at 30°C, as illustrated in Fig. 5, and normalized to those of RP9195 (*cheA*$^+$) with the same prophage. Values represent the mean and standard error of at least six independent determinations.

exhibited reversals during the observation period. The M98Q mutant exhibited fewer reversing cells and an overall CCW bias in rotational behavior, consistent with its reduced chemotactic ability. Lysogens carrying a λfla52 prophage (*cheA*$^+$ *cheW*$^+$) exhibited a moderate CW shift in all three strains, but similar shifts were produced by a λfla52Δ107 prophage (Δ*cheA* *cheW*$^+$), suggesting that excess CheW is responsible for this rotational effect, as noted in a previous study (16). Although these lysogens had similar rotational patterns, only the λfla52 prophage complemented the chemotaxis defect in the M98I and M98Q mutants (data not shown). The rotation patterns of the λfla52*am169* lysogens were essentially unchanged, consistent with the inability of $CheA_S$ to restore wild-type swarming ability to the M98I and M98Q mutants. This prophage should also produce CheW, but polar effects of the nonsense mutation in *cheA* may reduce its level of expression.

Effects on $CheA_L$ function of amino acid replacements at Met-98. The M98I and M98Q mutants demonstrate that $CheA_L$ alone is sufficient for chemotaxis, but amino acid replacements at residue 98 impair its function. $CheA_L^{M98I}$ supports 75 to 80% of wild-type chemotaxis, and $CheA_L^{M98Q}$ provides about 50% of wild-type ability. To assess the effects of other amino acid replacements at residue 98, we examined the chemotactic ability of strains carrying the M98am mutation in combination with various amber suppressors. Suppression of the amber codon should not alleviate the block to $CheA_S$ initiation but could restore chemotactic ability if the inserted amino acid allows $CheA_L$ to function. We saw no recovery of chemotactic ability when suppressors were introduced into strain RP9037, which carries a chromosomal copy of the *cheA*(M98am) mutation (data not shown). To enhance the sensitivity of the suppression tests, we increased the dosage of the M98am mutant gene by supplying it on a compatible plasmid (pHS25). Seven synthetic suppressors on high-copy-number plasmids were tested (hatched bars in Fig. 7). Those that insert cysteine, alanine, or phenylalanine enhanced chemotactic ability above background levels, whereas those that insert histidine, glutamic acid, proline, or arginine did not. When present in single copy, two natural suppressors that insert tyrosine (*supF*) or serine (*supD*) also improved chemotactic ability (open bars in Fig. 7). Variations in gene dosage and suppression efficiency could also influence the apparent functionality of different amino acids in these tests. However, among the residues examined, isoleucine proved to be the most acceptable substitute for methionine at position 98 of $CheA_L$. Wolfe and coworkers have constructed two additional $CheA_L$ mutants with mutations at position 98 (36). They found that valine worked as well as isoleucine and that leucine was even better, supporting wild-type chemotactic ability. Thus, amino acids with structures and chemical properties similar to methionine are most acceptable as functional substitutes at residue 98 of $CheA_L$.

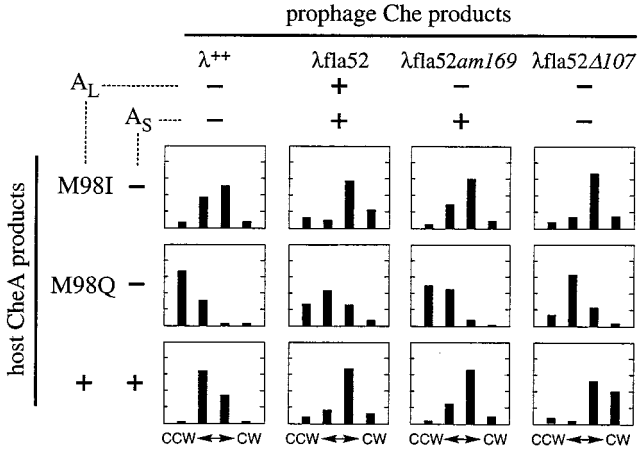

FIG. 6. Flagellar rotation patterns of *cheA* start(S) mutants. Strains RP9038 (M98Q), RP9194 (M98I), and RP9195 (wild type) were made lysogenic for the indicated λ phages and analyzed by cell tethering. For each strain, at least 100 rotating cells were each observed for 15 s and classified into one of four groups (listed from left to right on the figure): exclusively CCW, reversing but CCW biased, reversing but CW biased, and exclusively CW. The solid bars indicate the percentage of cells assigned to each category.

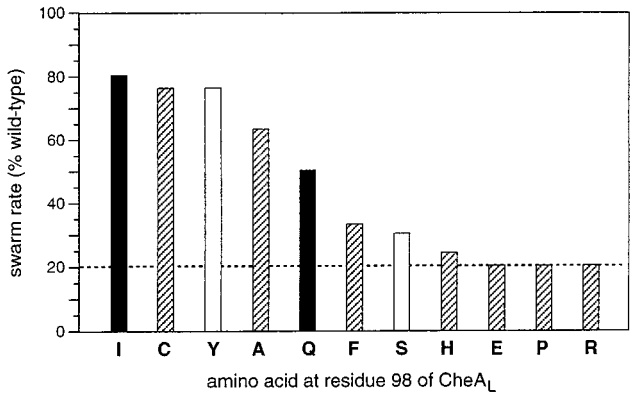

FIG. 7. Chemotactic proficiency of substitutions at Met-98 of $CheA_L$. Swarm rates were measured on semisolid tryptone agar and compared with those of isogenic wild-type control strains. Solid bars indicate strains with chromosomal *cheA* start(S) mutants: RP9194 (M98I) and RP9038 (M98Q). Open bars indicate Δ*cheA* strains carrying pHS25 (M98am) and a single-copy nonsense suppressor: RP9023 [*supF* (Y)] or RP1791/F′402 [*supD* (S)]. Hatched bars indicate Δ*cheA* strains carrying pHS25 (M98am) and a multicopy nonsense suppressor on a compatible plasmid. The dotted line indicates the expansion rate of a nonchemotactic control strain, RP9535 (Δ*cheA*) carrying pHS25 (M98am).

Chemotactic ability of the M98I mutant. The M98I mutant has a normal unstimulated flagellar rotation pattern but swarms about 20% more slowly than the wild type does. Its swarm speed was not enhanced by supplying $CheA_S$, implying that the alteration in $CheA_L$ was responsible. To better evaluate the severity of this defect, we measured the chemotactic ability of the M98I mutant and a wild-type control in capillary assays (1). The responses toward serine are shown in Fig. 8; similar results were obtained for the attractant aspartate (data not shown). The mutant had a wild-type response threshold (Fig. 8, inset) and peak position. However, it showed a somewhat lower maximal response and a more rapid decline in accumulation at high serine concentrations. The decline phase

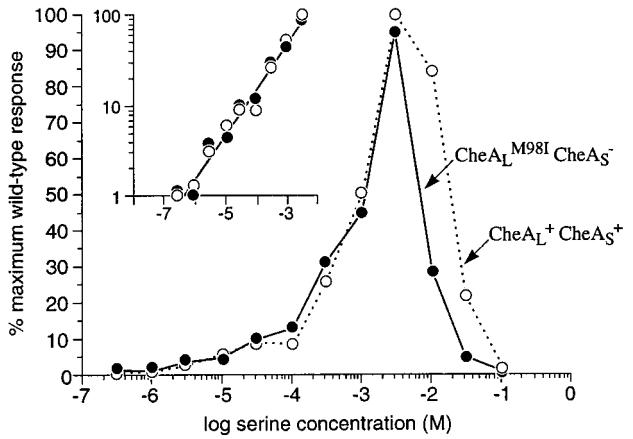

FIG. 8. Capillary chemotaxis assay of the M98I start(S) mutant. The number of cells entering serine-filled capillary tubes was measured at various concentrations and plotted as a percentage of the maximum wild-type response. Each datum point represents the mean of two or more determinations. The strains used were RP9194 (M98I) and RP9195 (wild type). The inset shows a log-log plot for determining response thresholds. The best-fit lines for the wild-type (open circles) and mutant (solid circles) data were virtually superimposable.

of the response curve reflects saturation of the chemosensors, which causes the bacteria to accumulate farther from the mouth of the capillary, reducing their rate of entry. The M98I mutant evidently has a lower signaling capacity, consistent with a reduction in the specific activity of $CheA_L$. In all other respects, its signaling behavior seems normal, demonstrating that the lack of $CheA_S$ is of little or no consequence to chemotactic ability.

DISCUSSION

Signaling functions of $CheA_L$ and $CheA_S$. $CheA_L$ plays an essential role in chemotactic signaling by autophosphorylating and then donating its phosphate groups to CheB and CheY. It has a segmental structural organization, with discrete domains for each of its various functional activities (Fig. 9). $CheA_S$ does not have the phosphorylation site at His-48 and cannot support chemotaxis in mutants lacking functional $CheA_L$ (30). Otherwise, its primary structure is quite similar to that of $CheA_L$ (Fig. 9). Both proteins contain an intact catalytic domain (20), a region needed for control by receptors and the CheW coupling factor (4), and the P2 domain, which may bind CheY during the phosphotransfer reaction (19, 33).

Although unable to autophosphorylate, $CheA_S$ exhibits other signaling-related activities consistent with its structural organization. It complements some *cheA* missense mutants in vivo (30), and in vitro it restores autokinase activity to those with catalytic defects (32, 38). CheA probably autophosphorylates as a dimer, and these complementation effects most probably occur through formation of $CheA_L/CheA_S$ heterodimers. $CheA_S$ can also restore receptor control to $CheA_L$ molecules that lack the coupling region (37). Heterodimer formation may account for this complementation effect as well. Finally, overexpression of $CheA_S$ in a wild-type cell inhibits chemotaxis, suggesting that one or more of its activities can interfere with normal signaling processes (26, 37). For example, $CheA_S$ homodimers might compete with $CheA_L$ homodimers for binding to CheW and receptor molecules.

Behavior of mutants lacking $CheA_S$. Despite the signaling-related functions of $CheA_S$, it is not essential for chemotaxis. To show this, we constructed several different mutations at the initiation codon of start(S). The resulting mutants made no detectable $CheA_S$ but were nevertheless chemotactic. The M98Q strain swarmed at about 50% of the wild-type rate, and the M98I mutant swarmed at nearly 80% of the wild-type rate. The chemotaxis defects of these strains were not alleviated by supplying $CheA_S$ from a λ prophage, implying that the fault lies instead with their alterations in $CheA_L$. Several additional findings indicated that residue 98 might be particularly important to $CheA_L$ function. First, only a few amber suppressors improved the chemotactic ability of the M98am mutant, consistent with the premise that many amino acids at this position are functionally unacceptable. Second, the M98Q protein, although functional, was present at substantially reduced levels, most probably because of reduced intracellular stability.

The chemotactic behavior of the M98I mutant was close to normal in all respects. Its unstimulated flagellar rotation pattern was indistinguishable from the wild-type pattern; it swarmed at near wild-type rates; and it exhibited wild-type detection thresholds and peak positions in capillary chemotaxis assays. The lack of any substantial behavioral deficit in this start(S) mutant argues that $CheA_S$ makes a negligible contribution to chemotactic ability under standard assay conditions. More-sensitive tests with the M98I strain, for example, measuring response latency or duration to small temporal stimuli, might reveal a subtle signaling defect attributable to the lack of $CheA_S$.

Why overlapping *cheA* genes? The *cheA* locus in *S. typhimurium* also contains tandem translation starts in the same reading frame and makes $CheA_L$ and $CheA_S$ products (31). Although the *cheA* sequences from more distantly related bacteria have no obvious internal start sites, the presence of the same overlapping gene arrangement in *E. coli* and *S. typhimurium* suggests that it may have some utility to these organisms. $CheA_S$ might, for example, participate in tactic responses to pH, temperature, repellents, or other attractant stimuli not

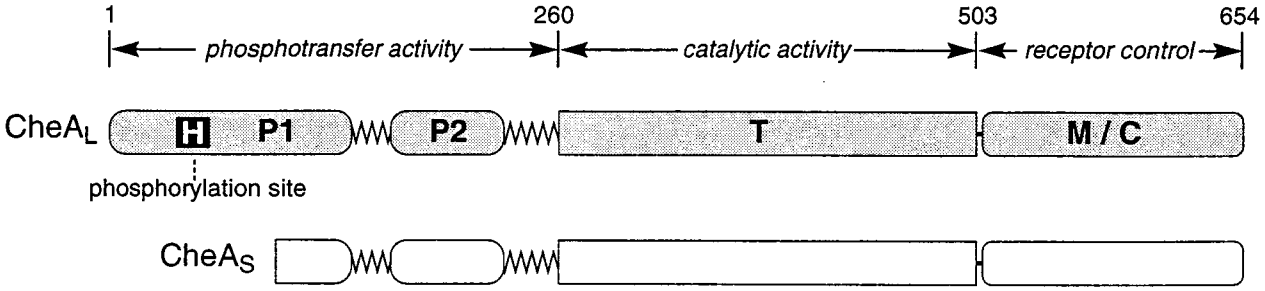

FIG. 9. Structural organization and functional domains in CheA proteins. This figure summarizes information from a number of recent studies (4, 19, 25, 33). The wavy lines on each side of the P2 domain denote flexible linker sequences.

examined in this study. Alternatively, it might confer a selective advantage under conditions not yet reproduced in the laboratory.

The relative positions of start(L) and start(S) and the surrounding sequence features suggest that changes in growth rate might modulate the relative expression levels of $CheA_L$ and $CheA_S$ (11). If the cell in fact adjusts its $CheA_L$-to-$CheA_S$ ratio to fit different environmental conditions, this would lend support to the view that $CheA_S$ serves a useful purpose. Swanson et al. (32) suggested an interesting functional role for $CheA_S$ that should vary with its relative expression level. They pointed out that by forming heterodimers with $CheA_L$ subunits, $CheA_S$ could influence the distribution of available CheA phosphorylation sites across a population of receptor molecules. At low levels of $CheA_S$, most of the $CheA_L$ subunits would be in the form of homodimers and each receptor would effectively control two phosphorylation sites. At very high levels of $CheA_S$, all of the $CheA_L$ subunits would be in heterodimers. Receptors that formed signaling complexes with a heterodimer could control one CheA phosphorylation site, whereas those that complexed with $CheA_S$ homodimers would have no signaling voice. Intermediate $CheA_S$ levels should maximize the number of receptors able to command a CheA phosphorylation site. Thus, if $CheA_L$ were in limiting supply, the $CheA_L$-to-$CheA_S$ ratio could influence the sensitivity, amplification, and other signaling characteristics of the cell. These could well be subtle effects that are undetectable by swarm or capillary assays.

It is also conceivable that the overlapping *cheA* genes of *E. coli* and *S. typhimurium* have no functional significance. In the laboratory at least, *E. coli* is able to carry out chemotaxis quite well without a $CheA_S$ protein. However, if $CheA_S$ really has no adaptive value, why would both *E. coli* and *S. typhimurium* persist in making it? Perhaps the sequence features that define start(S) are constrained by the primary structure of $CheA_L$. The *E. coli* and *S. typhimurium* proteins are identical in sequence throughout the start(S) region of *cheA*, implying that this portion of $CheA_L$ is critical to its function. Indeed, most amino acid replacements at residue 98 of $CheA_L$, encoded by the $CheA_S$ start codon, clearly impair chemotactic ability. Unless $CheA_S$ represents a major drain on the resources of the cell, mutational changes at the start(S) initiation codon would probably not confer an overall selective advantage. Mutations that create synonymous codon changes in $CheA_L$ also fail to reduce start(S) activity. Alterations designed to destabilize the potential stem-loop structure that brackets start(S) (Fig. 1) actually enhanced $CheA_S$ expression, suggesting that this sequence feature normally serves to inhibit translation from start(S). Interestingly, *E. coli* and *S. typhimurium* both maintain complete base complementarity throughout the putative stem, but the adjacent sequences preclude extension of the base-paired region through silent $CheA_L$ mutations. This mRNA secondary structure may represent the only ploy available to the cell for lowering production of $CheA_S$.

The hypothesis that $CheA_S$ has no adaptive value to *E. coli* can be disproved only by finding circumstances in which mutants lacking this protein are at a selective disadvantage. Until such conditions are found, $CheA_S$ must remain a tantalizing enigma.

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