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Genetic Evidence for Interaction between the CheW and Tsr Proteins during Chemoreceptor Signaling by *Escherichia coli*

JINGDONG LIU AND JOHN S. PARKINSON*

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

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This study presents two lines of genetic evidence consistent with the premise that CheW, a cytoplasmic component of the chemotactic signaling system of *Escherichia coli*, interacts directly with Tsr, the membrane-bound serine chemoreceptor. (i) We demonstrated phenotypic suppression between 10 missense mutant CheW proteins and six missense mutant Tsr proteins. Most of these mutant proteins had leaky chemotaxis defects and were partially dominant, implying relatively minor functional alterations. Their suppression pattern was allele specific, suggesting that the mutant proteins have compensatory conformational changes at sites of interactive contact. (ii) We isolated five partially dominant CheW mutations and found that four of them were similar or identical to the suppressible CheW mutant proteins. This implies that there are only a few ways in which CheW function can be altered to produce dominant defects and that dominance is mediated through interactions of CheW with Tsr. The amino acid replacements in these mutant proteins were inferred from their DNA sequence changes. The CheW mutations were located in five regularly spaced clusters in the first two-thirds of the protein. The Tsr mutations were located in a highly conserved region in the middle of the cytoplasmic signaling domain. The hydrophobic moments, overall hydrophobicities, and predicted secondary structures of the mutant segments were consistent with the possibility that they are located at the surface of the CheW and Tsr molecules and represent the contact sites between these two proteins.

Many chemotactic responses in *Escherichia coli* are mediated by chemoreceptors known as methyl-accepting chemotaxis proteins (MCPs). These inner membrane proteins contain an extracellular ligand-binding domain that monitors attractant levels in the environment and an intracellular signaling domain that controls the direction of rotation of the flagellar motors. *E. coli* possesses four species of MCP molecules, each of which detects a different set of chemical stimuli. Their periplasmic receptor domains differ in structure, whereas their cytoplasmic domains are nearly identical, implying that all MCPs use the same intracellular signaling mechanism (15).

Intracellular signaling appears to proceed via a series of phosphorylation reactions among soluble chemotaxis (Che) proteins (13, 30). The signaling cascade begins with CheA, an autokinase which transfers its phosphoryl groups to two effector proteins, CheB and CheY. Phospho-CheY probably interacts with the switching machinery of the flagellar motors to enhance the probability of clockwise (CW) rotation. Phospho-CheB has an MCP-specific methylesterase activity that produces changes in MCP methylation state leading to sensory adaptation. The MCP chemoreceptors most likely control the flux of phosphate through these signaling components by modulating the autophosphorylation activity of CheA. Another cytoplasmic protein, CheW, plays an essential but poorly understood role in coupling CheA to MCP control.

In vitro work has shown that CheW enables MCPs to stimulate the autokinase activity of CheA (3), which implies that CheW must interact with MCP or CheA molecules, possibly with both. In vivo studies support the notion that CheW interacts with MCP molecules, but the available evidence is mainly circumstantial. Mutants lacking either CheW or all MCPs exhibit exclusively counterclockwise (CCW) flagellar rotation, consistent with an inability to stimulate CheA activity (10, 22). The relative stoichiometry of CheW and MCP molecules is also critical for proper chemotactic signaling. High intracellular levels of either one lead to predominantly CCW rotation (19, 25), but these effects are alleviated by concomitant overexpression of the other component (19). Stoichiometric compensation between CheW and MCP molecules implies that they interact directly with one another.

To explore the possibility that CheW interacts directly with MCP molecules, we looked for examples of conformational suppression between mutant forms of CheW and Tsr, one of the MCPs. If these two proteins make stereospecific contact, a defect in one that disrupts the interaction may be functionally suppressible by a compensating alteration of the other. Several mutually suppressible CheW-Tsr mutant pairs were isolated and characterized. Most of the mutant proteins were partially dominant, consistent with the possibility that suppression takes place through compensatory changes in binding affinity. The locations of the suppressor sites, inferred from DNA sequence analysis, may identify regions of contact between the CheW and Tsr proteins.

MATERIALS AND METHODS

Bacterial strains. All of the strains used in this study were derivatives of *E. coli* K-12. Their relevant properties are listed in Table 1. The following deletion mutations, used to remove various combinations of chemotaxis genes, have been described previously: Δ(*cheW-tap*)*2217* (24); Δ(*tar-tap*)*5201* (26); Δ(*tsr*)*7021* (6). The Δ(*pcnB*)*1*::mini-*kan* deletion was used to confer a PcnB$^-$ phenotype (reduced plasmid copy number) on strains destined to receive high-copy-number plasmids. The deletion was transferred to recipient strains by P1 transduction by using the kanamycin resistance element for selection of recombinants. It was constructed by joining two mini-*kan* insertions in vitro at opposite ends of

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Relevant marker(s)	Source or reference
RP437	Wild type for chemotaxis	24
RP526	*mutD5*	Laboratory collection
RP1078	Δ(*cheW-tap*)*2217*	24
RP5838	Δ(*tsr*)*7021* Δ(*tar-tap*)*5201*	Laboratory collection
RP5927	Δ(*tsr*)*7021* Δ(*tar-tap*)*5201 zjj-101*::Tn*10*	Laboratory collection
RP7947	*pcnB1*	18
RP9006	*recB21 recC22 sbcB15*	JC13146 of Clark et al. (16)
RP9302	Δ(*cheW-tap*)*2217* Δ(*pcnB*)*1*::mini-*kan*	This work
RP9325	Δ(*cheW-tap*)*2217* Δ(*tsr*)*7021* Δ(*pcnB*)*1*::mini-*kan zjj-101*::Tn*10*	This work
RP9327	Δ(*tar-tap*)*5201* Δ(*tsr*)*7021* Δ(*pcnB*)*1*::mini-*kan zjj-101*::Tn*10*	This work

the *pcnB* coding region, one immediately upstream of the *pcnB* promoter and another within the coding region between nucleotides 1202 and 1203 (18). These mini-*kan* insertions are flanked by *Bam*HI sites and were obtained as independent derivatives of pJL89, which has a unique *Eco*RI site outside of the *pcnB* region (18). The two insertion plasmids were treated with *Eco*RI and *Bam*HI enzymes, mixed, and ligated. Transformants resistant to both ampicillin and kanamycin were selected and screened for loss of *pcnB* function as described previously (18). A plasmid with a deletion between the two insertion sites and a mini-*kan* insertion joining the deletion breakpoints was isolated. This plasmid was used to transfer the Δ(*pcnB*)*1*::mini-*kan* construct into the *E. coli* chromosome by linear transformation of RP9006 as previously described (18, 28). The chromosomal deletion marker was then transduced into RP1078, with kanamycin resistance as the selected marker, to create RP9302. This strain was subsequently made Tsr$^-$ by introducing the Δ(*tsr*)*7021* marker by cotransduction with a selectable tetracycline-resistant insertion (*zjj-101*::Tn*10*) to create RP9325. RP9327 was constructed in similar fashion by transducing the Δ(*pcnB*)*1*::mini-*kan* marker into RP5927.

Plasmids. pJL53, a pBR322-derived plasmid carrying the *cheW* gene was constructed as follows. DNA from the *mocha* operon containing *motA*, *motB*, *cheA*, and *cheW* was first cloned into pACYC184 from λche22 phage (24), yielding pJL15. This plasmid was fused with pKK177-3, an expression vector containing the *tac* promoter (8), by using its *Hin*dIII and *Nsi*I sites. The resultant plasmid, pJL52, contains (i) P_{tac} upstream of the *mocha* operon and (ii) a pBR322 origin. pJL53 was then obtained by deleting an *Eco*RI fragment containing the material between P_{tac} and *cheW*. Expression of the *cheW* gene in pJL53 was shown to be under the control of P_{tac} by several criteria, including isopropyl-β-D-thiogalactopyranoside (IPTG)-induced protein overproduction as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and inhibition of chemotaxis. pACYC184-derived *tsr* plasmid pPA144 has already been described (1).

Media. Bacteria were grown in tryptone or LB medium (20). Ampicillin and chloramphenicol were purchased from Sigma Chemical Co. and used at final concentrations of 50 and 34 μg/ml, respectively.

Enzymes and chemicals. Restriction enzymes were purchased from Dupont-New England BioLabs and Bethesda Research Laboratories. T4 DNA ligase was from Böhringer Mannheim. Sequenase was from United States Biochemical Corp. All enzymes were used as recommended by the suppliers. [^{35}S]methionine and [^{32}P]dATP were from New England Nuclear Corp. Hydroxylamine was from Sigma Chemical Co.

Mutagenesis of plasmid DNA. For hydroxylamine mutagenesis, plasmid DNA was prepared by the alkaline-lysis method (20). About 10 μg of DNA was mixed in the mutagenesis solution described previously (29) and incubated at 65°C for 100 min. The DNA was dialyzed twice against 4 liters of TE buffer (20) before it was used for transformations. RP526, a *mutD* strain, was used to raise the frequency of spontaneous plasmid mutations. The mutator activities of individual RP526 colonies were first checked by testing for high levels of mutants resistant to nalidixic acid. A suitable clone was then transformed with the plasmid to be mutagenized, and plasmid minipreparations were prepared from individual transformant colonies by the boiling method (20).

Isolation of suppressor mutations in *cheW* and *tsr*. Plasmid-borne suppressors were obtained by transforming nonchemotactic recipients with mutagenized plasmid DNA and selecting for chemotactic revertants on T swarm plates by either of two methods. In the pooling method, about 500 to 1,000 transformant colonies from a single plate were pooled by suspension in 10 ml of LB medium. The bacterial solution was diluted 50×, and 100 μl of the sample was streaked across a swarm plate and incubated for 12 h or more at 35°C. In the multiple-toothpicking method, about five transformant colonies were collected on a single toothpick and transferred to swarm plates. In both methods, revertants could be easily identified among a majority of nonchemotactic cells by virtue of their rapid swarming and were purified by single-colony isolation for further tests.

In the early stages of this study, a chromosomal *tsr* mutation was obtained as a suppressor of *cheW201* in the following manner. RP9302 carrying pJL53-*cheW201* was inoculated onto tryptone swarm agar at 35°C to select spontaneous chemotactic revertants. Many of the revertants arose by second-site mutations in the plasmid, but one proved to have a chromosomal mutation. When this revertant was cured of the pJL53-*cheW201* plasmid and subsequently transformed with wild-type pJL53, it exhibited a leaky nonchemotactic phenotype. Linkage and complementation tests with λtsr72 (5) established that the chemotaxis defect was conferred by an allele of the *tsr* gene. (The cured derivative was subsequently used to obtain additional suppressor mutations in *cheW* by introducing mutagenized pJL53 and selecting for chemotactic revertants.) The *tsr* mutation was transferred by homologous recombination to pPA144 for sequence analysis. This was accomplished by first constructing a deletion derivative of pPA144 lacking a portion of the *tsr* coding region between *Eco*RV sites at codons 157 and 352. The deleted plasmid was introduced into the *tsr* strain and then reextracted. Recombinant plasmids that had acquired the chromosomal *tsr* region by

marker rescue were identified by transformation of RP9325 carrying pJL53-*cheW201* and screening colonies for chemotactic ability.

Isolation of dominant *cheW* mutations. Dominant *cheW* mutations were isolated in pJL63, a plasmid carrying the *lacI*^{q} repressor gene and the *cheW* gene under p_{tac} control (19). pJL63 was mutagenized with hydroxylamine and transformed into RP437, and individual transformant colonies were scored for chemotaxis on tryptone swarm plates containing 20 μM IPTG to induce expression of plasmid-derived CheW to about twice the level found in wild-type cells. Approximately 0.5% of the transformants were scored as partly or fully nonchemotactic. These mutants could arise from either dominant *cheW* mutations or regulatory mutations that lead to overexpression of wild-type CheW from the plasmid. To identify and eliminate *lacI*^{q} mutants, the most frequent type of overexpression mutation, nonchemotactic colonies were transferred to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates. LacI$^-$ plasmids caused the cells to form blue colonies, indicating derepression of the chromosomal *lacZ* gene. (Derepression occurs through titration of the chromosomally encoded LacI repressor by the many copies of the plasmid-borne *lac* operator sequence.) Approximately 55% of the candidates formed blue colonies and were discarded after this test. Plasmids from the remaining mutants that formed white colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were then transformed into RP9302 (Δ*cheW* Δ*pcnB*) and tested for chemotaxis. By lowering the plasmid copy number, the *pcnB* mutation should alleviate any CheW overproduction effects and permit efficient complementation for chemotaxis if the plasmid-encoded CheW protein is functional. Approximately 5% of the candidate plasmids exhibited poor complementation ability and were retained as putative *cheW*-dominant mutants.

DNA sequencing. Mutational changes were identified by double-stranded plasmid sequencing as previously described (18), by using appropriate synthetic oligonucleotide primers.

Measurement of swarm rates and flagellar rotation patterns. Swarm rates were measured at 35°C on semisolid tryptone agar containing appropriate antibiotics. Cells were inoculated by toothpick from a single colony and incubated for 6 to 7 h, after which the diameters of the emerging swarms were measured. Measurements were made at several additional time points (usually at 9.5 and 12.5 h), and the swarm rate was calculated by plotting swarm size against incubation time and extracting the slope by linear regression. All swarm rates were normalized to that of a control strain included on the same plate. Flagellar rotation patterns of antibody-tethered cells were measured as previously described (19).

RESULTS

Identification of chemoreceptor-suppressible *cheW* mutants. We reasoned that if a physical interaction between CheW and the MCP chemoreceptors were important for chemotaxis, then a collection of *cheW* mutants with chemotaxis defects might include some that were specifically defective in that interaction. Further, it might be possible to correct the chemotaxis defect of such mutants through a compensatory change in a chemoreceptor. To look for mutant CheW proteins that might be defective in interacting with wild-type chemoreceptors, we isolated a series of *cheW* mutants and tested the ability of each to be phenotypically suppressed through a mutational alteration of Tsr, the serine chemoreceptor.

To simplify subsequent manipulations, particularly DNA sequence analyses and double-mutant constructions, the *cheW* and *tsr* genes were carried on compatible multicopy plasmids rather than in single copy on the chromosome: pJL53 is a pBR322 derivative containing the *cheW* gene under *tac* promoter control; pPA144 is a pACYC184 derivative carrying the *tsr* gene controlled by its native promoter. At their normal copy numbers, these plasmids express high levels of CheW or Tsr, which causes inhibition of chemotactic ability (19). To alleviate such dosage effects as much as possible, all host strains carried a *pcnB* mutation, which reduces plasmid copy numbers about 15-fold (18). In a *pcnB* background, both plasmids exhibited good complementation of mutants defective in the *cheW* or *tsr* function (data not shown), indicating approximately normal expression levels. More sensitive tests of chemotactic ability, for example, flagellar rotation patterns (see below), showed that Tsr expression from pPA144 was severalfold in excess of the wild-type level but still suitable for this study.

Strain RP9325 (Δ*cheW* Δ*tsr*) carrying pJL53 and pPA144 was used as the "wild-type" parent for the suppression studies. Its ability to form chemotactic swarms on semisolid agar media depends on both CheW and Tsr functions, because the *cheW* deletion in RP9325 also removes two MCP genes, *tar* and *tap*. In the absence of these other MCPs, Tsr is primarily responsible for generating the spontaneous flagellar reversals needed for chemotactic behavior. The swarm rate of this strain is probably influenced by several factors, i.e., slight overexpression of Tsr, slower growth caused by the *pcnB* mutation and by the antibiotics (ampicillin and chloramphenicol) included in the medium to select for retention of the plasmids, and possibly by increased plasmid segregation rates owing to low copy numbers in the *pcnB* background. Despite these potential complications, RP9325 carrying both plasmids swarmed on tryptone semisolid agar at 35°C at three-fourths of the speed of RP437, our standard wild-type strain.

A set of *cheW* mutations was isolated following hydroxylamine mutagenesis of pJL53 and screening for nonchemotactic transformants of strain RP9302 (Δ*cheW*). Each mutant *cheW* plasmid was transferred into RP9325, and mutagenized pPA144 DNA was introduced by transformation. Transformants were then tested for restoration of swarming ability to identify putative suppressors in pPA144. Among 22 independent *cheW* mutations examined, we found 2 (*cheW201* and *cheW207*) that could be suppressed by mutagenized *tsr* DNA. The nonsuppressible mutations, which may have had nonsense defects or other drastic coding changes not ordinarily subject to conformational suppression, were not studied further.

Isolation of other suppressible alleles of *cheW* and *tsr*. Starting with the *cheW201* and *cheW207* alleles, we isolated a set of *tsr* mutations that would suppress one or the other of them. Some of those *tsr* mutations, in turn, were sufficiently defective for chemotaxis in a *cheW*$^+$ genetic background to permit isolation of *cheW* mutations that suppressed them. This cycle of suppressor selection was repeated several times to obtain additional alleles of both genes. The mutational changes in 23 *cheW* and 8 *tsr* isolates were then determined by DNA sequence analysis. Although each suppressor was an independent isolate, there were several duplicates in the *cheW* sample, implying that the variety of *cheW* mutations possible with this selection scheme was nearly exhausted. The *tsr* collection is probably less com-

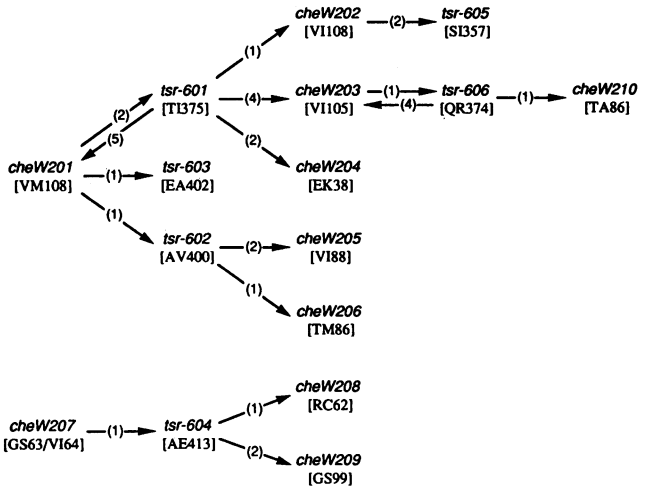

FIG. 1. Pedigrees of CheW* and Tsr* mutants. The amino acid replacements in the mutants, inferred from their DNA sequence changes, are shown in brackets below their allele numbers. The values in parentheses are the numbers of independent isolates of the mutants. All mutants were obtained as hydroxylamine-induced, plasmid-borne suppressor mutations, with the following exceptions. (i) One revertant of *cheW201* contained a chromosomal *tsr* mutation which, upon subcloning, proved to be *tsr-601*. The plasmid-borne version of this mutation was leaky, whereas the chromosomal version was not, presumably owing to the difference in expression levels. The *cheW202*, *cheW203*, and *cheW204* suppressors were obtained from chemotactic revertants of the chromosomal *tsr-601* mutation. (ii) The *cheW210* mutation arose in a *mutD* strain. It has an A/T-to-G/C transition, whereas all other mutations arose through G/C-to-A/T transitions, characteristic of hydroxylamine mutagenesis.

plete, although duplicate isolates of two suppressors were obtained. In all, 10 different *cheW* and 6 different *tsr* alleles were found. Their lineages and sequence changes are summarized in the pedigree chart of Fig. 1.

Suppressor terminology. To simplify discussion of these suppressor mutations and their phenotypic effects, we will refer to their mutational changes in terms of their gene products instead of their isolate or allele numbers. The generic designations CheW* and Tsr* will be used to denote suppressible alterations of the CheW and Tsr proteins. DNA sequence analyses established that all of the CheW* and Tsr* mutant proteins contained missense mutations (Fig. 1). Their inferred amino acid replacements are designated with one-letter symbols to show the wild-type and mutant amino acid residues and a number indicating the position of the change in the protein. For example, the original *cheW201* mutant has a valine-to-methionine change at residue 108 (designated VM108); the original *cheW207* mutant has two mutational changes: glycine to serine at residue 63 and valine to isoleucine at residue 64 (designated GS63/VI64).

Properties of CheW* mutants. The plasmid-borne *cheW* mutations were transferred to RP9325 carrying pPA144 and tested for swarming ability to assess their levels of CheW function in a tsr^+ background. All of the mutant plasmids produced faster swarms than did a pBR322 control plasmid but slower ones than did pJL53, the parental $cheW^+$ plasmid (Table 2). Thus, the CheW* mutants are partially defective in CheW function, with various degrees of residual activity. Six of them (EK38, RC62, TA86, TM86, VI88, and GS99) swarmed at over 45% of the wild-type rate and were too

TABLE 2. Properties of suppressible CheW and Tsr mutants

Strain	Swarm rate (% of wild type)[a]	
	Δ*cheW* Δ*tsr* background	$cheW^+$ tsr^+ background
CheW* mutants		
EK38	46	71
RC62	46	75
GS63/VI64	21	63
TA86	59	100
TM86	76	88
VI88	64	79
GS99	65	83
VI105	34	88
VI108	21	42
VM108	15	50
Tsr* mutants		
SI357	16	61
QR374	13	43
TI375	66	79
AV400	30	61
EA402	25	89
AE413	38	64

[a] Mutant derivatives of pJL53 (*cheW*) and pPA144 (*tsr*) were transferred to RP9325 (Δ*cheW* Δ*tsr*) or RP7947 ($cheW^+$ tsr^+), and swarm rates were measured on semisolid tryptone agar at 35°C. In the RP9325 tests, the cells also carried the complementary plasmid, either pJL53 or pPA144, to furnish a wild-type copy of one of the missing functions. The wild-type controls for normalization purposes were the same recipient strains carrying one (in the case of RP7947) or both (in the case of RP9325) of the parental plasmids.

leaky to use for revertant selections. The other four (GS63/VI64, VI105, VI108, and VM108) were sufficiently defective to permit isolation of chemotactic revertants and engendered all of the Tsr* mutations (Fig. 1).

When transferred to RP7947 ($PcnB^-$ but otherwise wild type for chemotaxis), most of the CheW* plasmids caused a reduction in chemotactic ability compared with pJL53 (Table 2). The partially dominant nature of these defects indicates that the mutant gene products have qualitatively altered function rather than merely reduced activity. Consistent with this conclusion, we found that the steady-state amount of CheW protein in the mutants, assessed by Western blots (immunoblots), ranged from about 50 to 100% of the wild-type level (data not shown).

Properties of Tsr* mutants. The plasmid-borne *tsr* mutations were transferred to RP9325 carrying pJL53 to assess their levels of Tsr function in a $cheW^+$ background. All six Tsr* mutants swarmed faster than a pACYC184-negative control, with rates ranging from 13 to 66% of that of pPA144, the tsr^+ parental plasmid (Table 2). Coincidentally, TI375, the leakiest mutant, was also isolated as a chromosomal suppressor and was substantially less leaky in single copy (data not shown), enabling us to isolate revertants from it (Fig. 1). This behavioral difference suggests that Tsr expression from pPA144, even in a *pcnB* background, exceeds wild-type levels. Presumably, the phenotypes of the other Tsr* mutants would also be less leaky at normal expression levels.

Excess levels of wild-type Tsr inhibit chemotaxis, presumably by titrating other signaling components (19). We compared the abilities of the mutant and wild-type Tsr proteins to inhibit chemotaxis by transferring them to RP7947 ($PcnB^-$ but otherwise wild type for chemotaxis) (Table 2). The Tsr* mutants inhibited chemotaxis to a greater extent than did pPA144, with swarms ranging in size from 43 to 89% of that

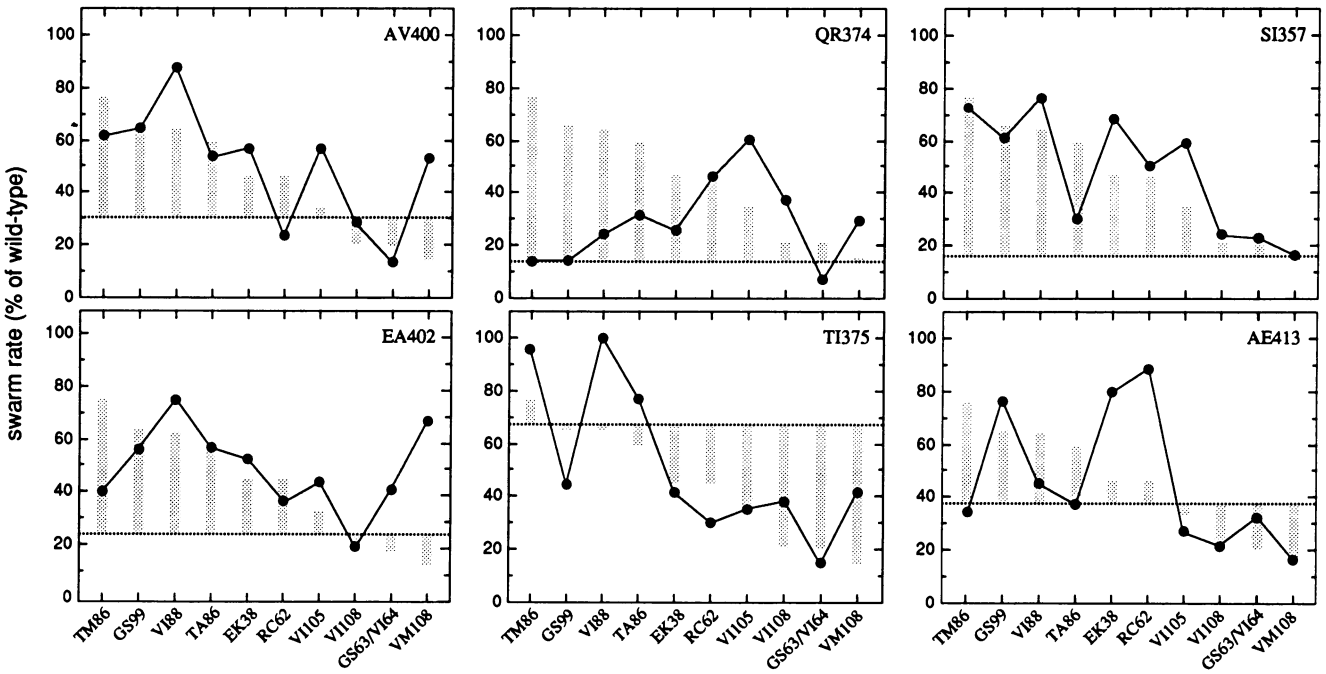

FIG. 2. Suppression patterns of CheW* and Tsr* mutations. Double mutants were constructed by transforming RP9325 with pPA144 (*tsr*) and pJL63 (*cheW*) derivatives. Their chemotactic abilities were assessed by measuring swarm rates on semisolid tryptone agar at 35°C. Each panel presents the swarm rate of one Tsr* mutation in combination with the various CheW* mutations. The lines connecting the points have no meaning and are intended solely to facilitate visual comparison of the patterns. The dotted horizontal lines show the residual chemotactic behavior of the Tsr* mutants in a *cheW*$^+$ background. The shaded bars show the difference between the residual Tsr* behavior and that of the CheW* mutations in a *tsr*$^+$ background. Thus, the shaded region represents the range of swarm rates that fall between those of the two-component mutations in an otherwise wild-type background.

of the wild-type control (Table 2). These findings indicate that Tsr* proteins, like their CheW* counterparts, have altered activities that interfere with wild-type function. Since in a wild-type cell Tsr function is required only for serine responses, the general inhibitory effect of these Tsr* mutants is most likely exerted at some common step in the signaling pathway, perhaps in the postulated interaction with CheW.

Allele specificity of CheW*-Tsr* suppression. Conformational suppression should be highly allele specific, reflecting a precise stereospecific fit between the interacting gene products. To assess the spectrum of suppressor action, each CheW* mutation was combined with each Tsr* mutation by transferring the mutant pJL53 and pPA144 derivatives to RP9325. The chemotactic abilities of the resultant double mutants were measured on swarm plates (Fig. 2). Each panel in Fig. 2 shows the behavior of the CheW* mutations with a different Tsr* allele. To assist comparisons between panels, the order of the CheW* mutations is the same throughout, from most to least chemotactic when combined with the parental (*tsr*$^+$) pPA144 plasmid. The substantial variability seen within each panel shows that the CheW* alleles responded with different efficiencies to the same Tsr* mutation. Similarly, the variation between panels shows that the Tsr* alleles responded differently to the CheW* mutations. The allele-specific nature of the suppression pattern is evident. For example, CheW* VM108 was most efficiently suppressed by Tsr* EA402 and AV400, whereas CheW* RC62 was best suppressed by Tsr* AE413. However, CheW* VM108 was not suppressed by Tsr* AE413, nor was CheW* RC62 suppressed by Tsr* EA402 or AV400.

The quality of suppression in each double mutant can be viewed from the perspective of either component mutation. On the one hand, the chemotactic abilities of five of the six Tsr* mutants were improved by nearly all of the CheW* mutations. The one exception, Tsr* TI375, was the least defective in a wild-type CheW background. On the other hand, the various Tsr* alleles improved the chemotactic abilities of only about half of the CheW* mutants compared with the wild-type Tsr control. Considering both viewpoints, the chemotactic abilities of most double mutants fell between the two parental extremes (i.e., within the shaded bars in Fig. 2). The performance of the less functional component was improved, whereas that of the more functional member was impaired. Additive behavior of this sort is consistent with a variety of mechanistic explanations and neither precludes nor supports direct interaction between the CheW and Tsr proteins. Still, some mutants performed better in combination than either component mutation did with a wild-type partner (e.g., CheW* VM108 with Tsr* EA402 or CheW* VI105 with Tsr* QR374). These examples show that two poorly functional CheW and Tsr proteins can cooperate to alleviate one another's defects. The simplest mechanism that accounts for such suppression is direct interaction of the mutant proteins. This point is addressed further in the Discussion.

Phenotypic basis of CheW*-Tsr* suppression effects. In wild-type cells, Tar and Tsr, the major chemoreceptors, are mainly responsible for setting the rate of spontaneous flagellar reversals. Either is sufficient to establish a normal swimming pattern, but when both are absent, the cells rotate their flagella almost exclusively in the CCW direction. In RP9325, therefore, both CheW and Tsr are needed to pro-

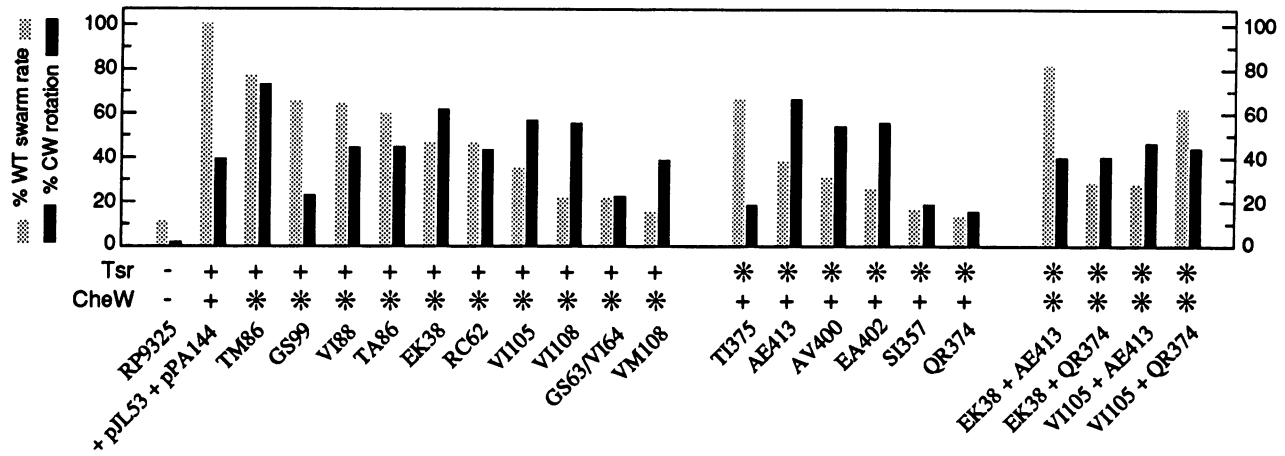

FIG. 3. Comparison of chemotactic abilities and flagellar rotation patterns of CheW* and Tsr* mutants. RP9325 strains with the indicated combinations of CheW* and Tsr* plasmids were examined. Their swarm rates (shaded bars) were taken from the data in Fig. 2. Their flagellar rotation patterns (solid bars) were analyzed by cell tethering. The CW rotation scores represent the proportions of rotating cells that were not exclusively CCW during the observation period. WT, wild type.

duce CW flagellar rotation, so productive interactions between them might be reflected by changes in the rotation pattern. We assessed the abilities of the CheW* and Tsr* proteins to promote CW flagellar rotation by measuring the rotational behavior of antibody-tethered cells (Fig. 3). The different CheW* mutants, in combination with wild-type Tsr, exhibited a variety of rotational behaviors. Some (TM86, VI88, EK38, VI105, and VI108) were more CW biased, and others (GS99 and GS63/VI64) were less CW biased than the wild-type CheW control. There was no obvious correlation between the abilities of the mutant CheW proteins to promote CW rotation and their residual levels of activity as judged by swarm rate (Fig. 3). Similarly, the different Tsr* mutants, in combination with wild-type CheW, also varied in CW ability. AE413, AV400, and EA402 were more CW biased than the wild-type Tsr control, whereas TI375, SI357, and QR374 were less CW biased. Again, we saw no obvious relationship between swarm rate and level of CW rotation (Fig. 3).

Examination of a few selected CheW* Tsr* double mutants showed that establishment of a normal level of CW rotation was not sufficient to restore chemotaxis. Mutant combinations that resulted in good chemotaxis (CheW* EK38 with Tsr* AE413 and CheW* VI105 with Tsr* QR374) had the same level of CW rotation as did combinations with poor chemotaxis (CheW* EK38 with Tsr* QR374 and CheW* VI105 with Tsr* AE413) (Fig. 3). Besides, the level of CW rotation in the double mutants was not merely an additive combination of the rotational effects of the two component mutations, implying that specific functional interactions between the mutant proteins determine the overall chemotaxis phenotype.

Isolation of dominant CheW mutations. Dominance tests indicated that many CheW* mutants possess an altered activity that competes or interferes with wild-type CheW function (Table 2). Dominant mutants are readily understandable in the context of protein-protein interactions and offer an alternate approach to detecting functional contacts between proteins. For example, if CheW interacts with Tsr and other chemoreceptor molecules by binding to them, it should be possible to obtain CheW mutants that are nonfunctional because they bind too tightly. Such mutants should have a dominant chemotaxis defect. Subunit spoiling, a more common mechanism of dominance, is unlikely in the CheW case because the active form of the protein seems to be a monomer (12).

We used plasmid pJL63, which carries the *lacI*^{q} repressor gene and the *cheW* gene under p_{tac} control (19), to look for dominant *cheW* mutations that reduced the swarm size of RP437, a chemotactically wild-type host strain. Following mutagenesis of the plasmid with hydroxylamine, individual transformants were tested on tryptone swarm plates containing 20 μM IPTG to induce CheW expression from the plasmid to about twice the level made by the chromosomal locus. Since overexpression of wild-type CheW also inhibits chemotaxis of RP437, putative dominant mutations were screened to identify and discard any with *lacI* defects that simply led to CheW overexpression. From about 7,500 plasmids tested, we obtained 10 dominant *cheW* isolates, representing five different mutational changes (Table 3).

Two of the dominant mutations (RC62 and VM108) were also found previously as CheW* mutants, and two others (VI36 and DN103) had primary structure changes near those of CheW* mutants (EK38 and VI105). The DN103 and VM108 mutants exhibited a strong dominance effect and were independently isolated several times. The other three mutants produced minor reductions in wild-type swarm size

TABLE 3. Properties of dominant CheW mutants

Amino acid change	No. of independent isolates	Swarm rate (% of wild type)[a]	
		cheW$^+$ background	Δ*cheW* background
EK18	1	67	67
VI36	1	87	73
RC62	1	77	60
DN103	3	53	47
VM108	4	53	33

[a] Derivatives of pJL63 carrying dominant *cheW* mutations were transferred into RP437 (*cheW*$^+$) or RP1078 (Δ*cheW*), and swarm rates were measured at 35°C on semisolid tryptone agar containing 20 μM IPTG. The same recipient strains carrying the parental pJL63 plasmid were used as controls for normalization purposes.

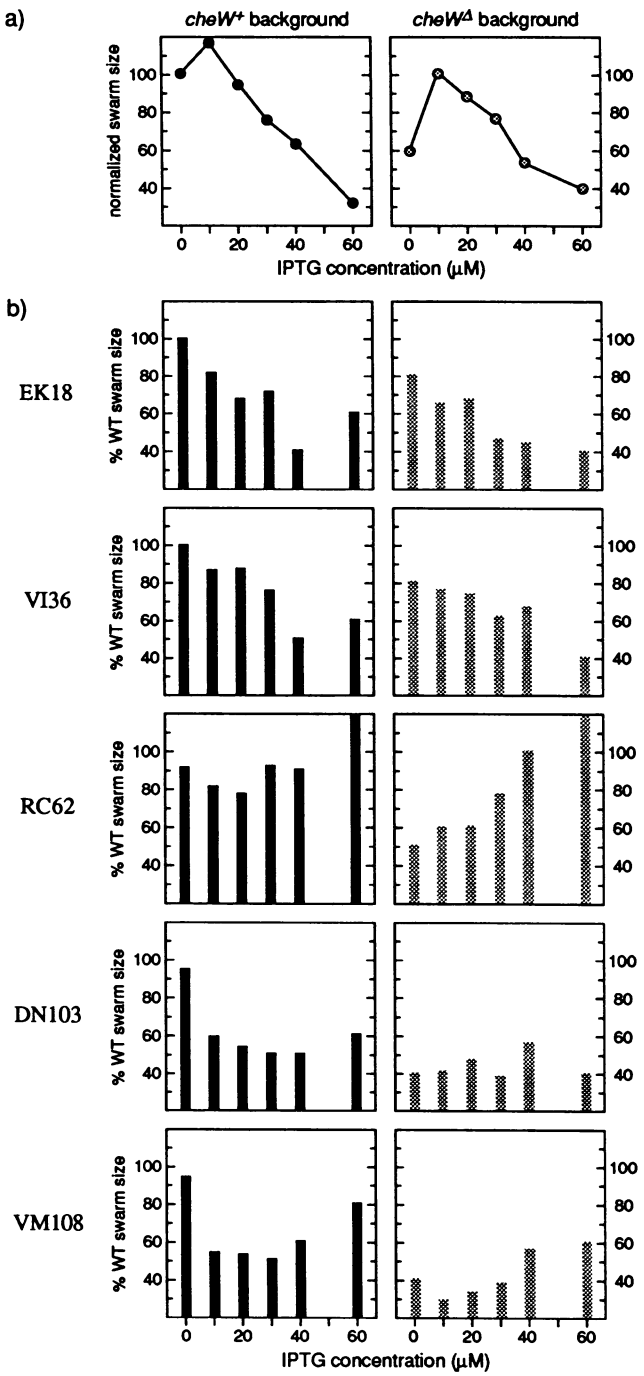

FIG. 4. Complementation properties of dominant *cheW* mutants. (a) Wild-type (WT) CheW plasmid pJL63 was transferred into RP437 (*cheW*$^+$) and RP1078 (Δ*cheW*). Chemotaxis was assessed at 35°C on tryptone swarm plates containing various concentrations of IPTG to induce expression of plasmid-borne *cheW* to different levels. Swarm sizes were measured after 10 h of incubation and then normalized to that of the uninduced RP437 control. The decline in chemotactic ability upon CheW induction is caused by MCP-dependent inhibition of CW flagellar rotation (19). (b) pJL63 derivatives with the CheW mutations shown along the left margin were transferred into RP437 (left column) and RP1078 (right column), and chemotactic ability was measured on tryptone swarm plates at 35°C. Note that in this case, the swarm size produced by the mutant plasmids is expressed as a percentage of that produced by the wild-type control plasmid at each IPTG concentration. Thus, for example, the RC62 mutant is more chemotactic than the corresponding wild-type control at 60 μM IPTG. However, because of the CheW overexpression effect, its overall chemotactic ability is less than it is at lower induction levels. The relative increase in chemotactic ability of the mutants at high expression levels suggests that they are partially defective in eliciting this overexpression effect.

and were each isolated only once, implying that some weakly dominant mutants were overlooked in our initial screenings. Nevertheless, these findings suggest that dominant CheW defects are most easily generated by the same sorts of functional alterations that lead to suppression of Tsr* mutants. However, the EK18, VI36, and DN103 mutants exhibited no suppression activity against any of the available Tsr* mutants (data not shown), suggesting that they differ from CheW* mutants in some important respect.

When tested for ability to complement a Δ*cheW* host (RP1078), all of the mutants were discernibly defective in CheW function, with various extents of leakiness (Table 3). In general, the more dominant mutants were also more defective in complementation. Each mutant exhibited a characteristic pattern of dominance (Fig. 4a) and complementation ability (Fig. 4b) at different CheW expression levels. The dominance of the EK18 and VI36 mutants became more pronounced at higher expression levels, whereas that of RC62 lessened and that of DN103 and VM108 remained essentially constant over the same range of induction conditions (10 to 40 μM IPTG). The complementation ability of the mutants exhibited roughly the same induction profile as did their dominance. At 60 μM IPTG, which corresponds to about 10 times the wild-type expression level, the chemotactic ability of cells containing the mutant CheW proteins improved somewhat compared with that of cells with the same level of wild-type CheW. This apparent reduction in dominance implies that the mutant proteins are partially defective in producing the inhibition of chemotaxis that is caused by overexpression of wild-type CheW.

DISCUSSION

Protein-protein interactions involved in chemoreceptor signaling. Chemosensors of the MCP family appear to mediate flagellar rotational control by modulating the enzymatic activity of CheA, an autokinase that phosphorylates CheB and CheY, the response effector proteins. Both in vivo evidence and in vitro evidence indicate that MCP molecules generate two kinds of flagellar signals, a CCW signal that inhibits CheA activity, and a CW signal that stimulates CheA activity (1, 2, 4). The two signaling modes of MCP molecules are assumed to correspond to different conformational states, with flagellar behavior reflecting the relative number of molecules in each conformation. Changes in ligand occupancy state, and subsequent changes in methylation state, can shift the equilibrium distribution of the two signaling forms, enabling the receptors to trigger a transient flagellar response to changes in chemoeffector concentration (Fig. 5a).

Signal production may depend on the formation of ternary complexes between CheA, CheW, and receptor molecules, mediated by reversible, pairwise protein-protein interactions (Fig. 5b). CheA is probably able to interact with both conformational forms of the receptor, leading to inhibition or stimulation of its activity. The CheW protein has been implicated in the coupling of CheA to chemoreceptor control

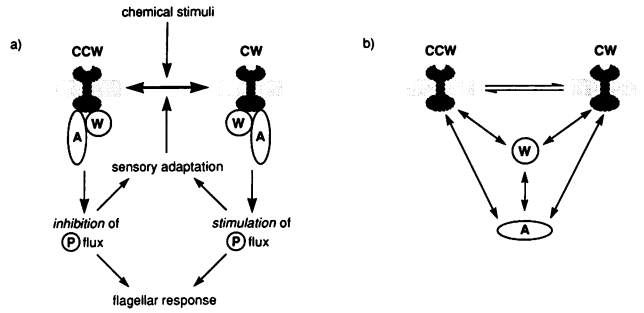

FIG. 5. A working model of receptor coupling and the protein-protein interactions that may be involved. Details of the model and supporting evidence are presented in the text. A, CheA; W, CheW; P, phosphate.

and has been shown to interact directly with CheA molecules (12, 21). Those interactions could effect changes in CheA activity and are probably modulated by interactions between CheW and the MCP receptors. CheW function is essential for production of the CW signal in vivo and for enabling chemoreceptors to stimulate CheA activity in vitro. It also may play a role in CCW signaling. Although CCW responses can be produced in the absence of CheW, for example, by mutant receptors that are locked in the CCW signaling mode or by high levels of wild-type receptors, overproduction of CheW leads to MCP-dependent inhibition of CW rotation, suggesting that a stoichiometric excess of CheW can trap MCP molecules in the CCW signaling state. Thus, it appears that CheW can interact with both signaling forms of MCP molecules and in normal circumstances may play a role in generating both CW and CCW receptor signals by stimulating or inhibiting the enzymatic activity of CheA.

Functional alterations in CheW* and Tsr* mutants. The partial dominance of many CheW* and Tsr* isolates suggests that the mutant proteins can somehow interfere with wild-type function. In principle, dominant effects could occur by either of two mechanisms: spoiling or competition. Dominant spoilers arise from loss-of-function lesions that cause the mutant gene product to inactivate its wild-type counterpart through subunit association. Dominant competitors typically arise from gain-of-function mutations that enable the mutant gene product to block or mask the activity of its wild-type counterpart through interactions with other components. For CheW, it may be difficult to achieve dominance through spoiling because CheW probably functions in monomeric form (12). Tsr, which functions as a dimer, could conceivably cause spoiler effects, but no other dominant mutants of Tsr are known to act by this mechanism. We conclude that the dominant behavior of both CheW* and Tsr* mutants is probably due to competitive effects.

Competitive dominance could arise from changes in the relative affinities of any of the protein-protein interactions shown in Fig. 5b. For example, alterations of CheW or Tsr that significantly increase their strength of binding to one another or to CheA could interfere with receptor signaling by shifting the relative levels of CCW and CW complexes, by inhibiting the ability of the receptors to change conformations, or by disabling the generation of flagellar signals. Conversely, lesions that reduce the binding affinity for one target also could cause competitive effects if CheW or Tsr uses different contact sites for its various interactions. For example, a mutant CheW with a defective Tsr interaction site that retains its ability to bind to CheA would effectively reduce the amount of CheA available for interaction with wild-type CheW molecules.

The dominance of CheW* and Tsr* mutants resembles that of other, independently isolated CheW and Tsr mutants. Previous work has described a class of dominant Tsr mutants that behave as though locked in a CCW or CW signaling state (1, 6, 14, 23). These mutants function by competition rather than spoiling because they can produce their aberrant signaling effects in cells lacking all other chemoreceptors (2). Some locked receptor mutants could have structural defects that prevent changes in signaling conformation; others might have affinity changes that favor binding of CheW or CheA to one conformation, effectively trapping the receptor in that signaling mode. Conceivably, the partially dominant Tsr* mutants obtained in the present study could represent less drastic examples of locked receptors. Three of them (SI357, QR374, and TI375) exhibited CCW rotational bias, and three (AV400, EA402, and AE413) exhibited CW bias. In the present study, we isolated five different CheW mutants (EK18, VI36, RC62, DN103, and VM108) that were dominant at slightly elevated expression levels. Two (RC62 and VM108) were also isolated as suppressors of Tsr mutants, and two others (VI36 and DN103) had lesions located near CheW* sites (EK38 and VI105), suggesting that dominant CheW mutants are most easily generated by the same kinds of alterations that lead to suppression of Tsr defects and may, therefore, have the same functional basis.

Functional basis of CheW*-Tsr* suppression. We postulate that CheW* and Tsr* mutants have altered interaction affinities that account for their partial dominance and their chemotaxis defects. Such affinity changes should shift the equilibria pictured in Fig. 5b, leading to an excess of CCW or CW signal in the unstimulated state. Although CheW* and Tsr* mutants do exhibit biased rotation patterns, they are not dramatically different from the wild type, perhaps owing to the action of the sensory adaptation machinery, which is feedback regulated by receptor signals (Fig. 5a). The feedback system is set to obtain an intermediate rate of flagellar reversals corresponding to random-walk swimming and can probably compensate for affinity changes that merely perturb the efficiency of receptor coupling. Thus, defects that lead to an excess of either CW or CCW signal should be appreciably moderated by the adaptation mechanism unless the receptor is refractory to adaptation or altogether incapable of producing one type of signal. Still, such coupling mutants would not be expected to modulate receptor signal output with wild-type efficiency and might be partially defective in chemotaxis, as are many CheW* and Tsr* mutants.

Suppression of these behavioral defects most likely occurs through compensatory changes in affinity. Conceivably, suppression could be mediated indirectly through CheA. For example, a CheW mutant with altered affinity for CheA might be suppressible by a Tsr mutant with similarly altered affinity for CheA. Although this would restore the correct relative affinities, we might expect chemotactic ability to be impaired owing to changes in the overall efficiency of receptor coupling. A more straightforward and easily envisioned suppression mechanism would involve mutually compensatory affinity changes in CheW and Tsr. For example, a CheW mutant with enhanced affinity for Tsr should be suppressible by a Tsr mutant with reduced affinity for CheW. If the compensatory mutations alter the actual residues involved in these contacts, the suppression pattern should be highly

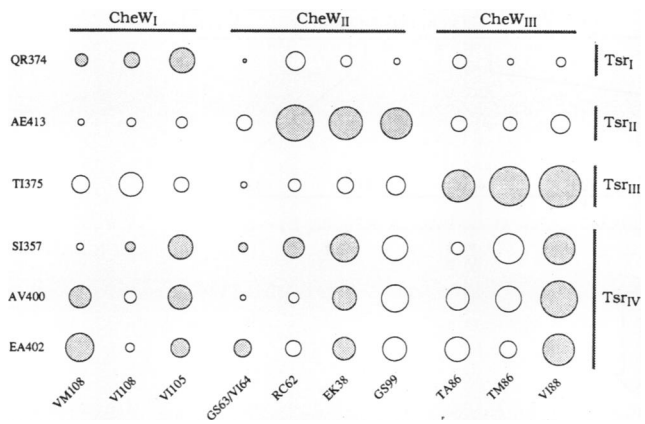

FIG. 6. Summary of CheW*-Tsr* suppression patterns. The swarm rates of the different double mutants are directly proportional to the diameters of the circles. Mutant combinations that exceed the swarming abilities of both component mutations are indicated by shaded circles. The CheW* and Tsr* mutants are arranged in suppression groups based on the considerations described in the text.

allele specific. If, however, they alter the overall conformation of the binding sites, the patterns could be less specific.

The suppression data show that both affinity correction mechanisms may be represented among our mutants. Although there are many examples of allele-specific behavior, the suppressors seem to fall into groups that could be based on similar functional, rather than strictly conformational, properties (Fig. 6). Three of the Tsr* mutants can be distinguished by their responses to three different groups of CheW* mutants. Tsr* QR374 is suppressed only by mutants of the CheW$_I$ group (VI105, VI108, and VM108), Tsr* AE413 is suppressed only by mutants of the CheW$_{II}$ group (EK38, RC62, and GS99), and Tsr* TI375 is suppressed only by mutants of the CheW$_{III}$ group (TA86, TM86, and VI88). Although the CheW* GS63/VI64 mutant did not give significant suppression of Tsr* AE413, it was included with the CheW$_{II}$ group because of its proximity to the CheW* RC62 site. Its two amino acid changes may cause general defects in CheW function that attenuate its suppression activity. The remaining three Tsr* mutants (SI357, AV400, and EA402) are suppressed by members of each CheW* group.

The group-specific suppression effects (i.e., CheW$_I$ by Tsr$_I$, CheW$_{II}$ by Tsr$_{II}$, and CheW$_{III}$ by Tsr$_{III}$) may reflect compensatory changes in binding affinities between CheW and Tsr. For example, Tsr* QR374 causes CCW-biased rotation, so its CCW conformation may have enhanced affinity for CheW. The CheW$_I$ mutants may suppress this defect through enhanced affinity for the CW conformation of Tsr or reduced affinity for the CCW conformation. Similarly, Tsr* AE413 causes a CW rotational bias, so its CW conformation may have enhanced affinity for CheW. The CheW$_{II}$ mutants may suppress this defect through enhanced affinity for the CCW conformation of Tsr or reduced affinity for the CW conformation. Because all of the group I and II mutants are dominant and exhibit pronounced defects in chemotaxis, we conclude that they are more likely to have enhanced, rather than reduced, affinities. The group III mutants, however, are not dominant and retain considerable chemotaxis ability, consistent with a relatively minor reduction in binding affinity. Thus, the CW conformation of Tsr* TI375 may have somewhat reduced affinity for CheW. The CheW$_{III}$ mutants may suppress this defect through reduced affinity for the CCW conformation of Tsr.

The inferences drawn from analysis of the group-specific suppression patterns cannot easily account for the allele-specific behavior of the Tsr$_{IV}$ mutants. Because they can discriminate among the members of each CheW group, the Tsr$_{IV}$ suppression effects may involve compensatory changes in overall affinity that are influenced by direct interactions between the mutant residues at the respective contact sites. Two of the Tsr$_{IV}$ mutants (AV400 and EA402) exhibit CW-biased flagellar rotation, intermediate levels of leakiness, and nearly identical suppression patterns, which differ only with respect to the anomalous GS63/VI64 CheW* mutant. These similarities, besides the proximity of their amino acid replacements, imply that AV400 and EA402 have similar changes in binding affinity or other functional alterations. The other Tsr$_{IV}$ mutant (SI357) is more defective in chemotaxis, causes CCW-biased flagellar rotation, and has a somewhat different suppression pattern. The fact that it suppresses many of the same CheW* mutants as AV400 and EA402 is difficult to reconcile with simplistic models of compensatory affinity changes.

Structural determinants of CheW*-Tsr* suppression. The positions of the mutational changes in the primary structures of CheW and Tsr are summarized in Fig. 7. The Tsr* changes are clustered in the cytoplasmic portion of the receptor molecule in a region that has long been known to play a key role in generating output signals. This signaling domain is flanked, and its activities are presumably modulated, by the K1 and R1 segments that contain the methylation sites involved in sensory adaptation. The amino acid sequences of all four *E. coli* MCP species are highly conserved in the middle of the signaling domain, implying that this is the part of the receptor that interacts with CheA, CheW, or other cytoplasmic components of the flagellar signaling system. All six of the Tsr* mutational changes fall within this highly conserved region, and all affect residues that are identical in the four *E. coli* MCPs. Less is known about structure-function relationships in CheW. The molecule is 167 amino acids long and contains a sequence near its C terminus that resembles a nucleotide-binding motif (27), although no such activity has been demonstrated. The suppressible and dominant CheW mutations are distributed in five clusters throughout the first two-thirds of the molecule, with approximately even spacing between the first four clusters (Fig. 7).

Hydropathy plots of the Tsr and CheW regions containing these mutations are shown in Fig. 8. Two of the Tsr* sites (SI357 and AE413) fall within hydrophilic segments, and the other four (QR374, TI375, AV400, and EA402) fall in hydrophobic segments. Many dominant, locked output mutations (indicated by circles in Fig. 8) occur in the same portion of the Tsr signaling domain, including several (SN357, AV413, and AT413) at the same residues as Tsr* mutations. The 357 and 400-to-413 sites are predicted by the Chou and Fasman (7) and Garnier et al. (11) secondary-structure algorithms to lie in α-helical segments. The calculated hydrophobic moment of those α helices is large, and the ratio of their hydrophobic moments to their overall hydrophobicity is characteristic of amphipathic helices at the surfaces of protein molecules (9). These features are at least consistent with the idea that the Tsr* sites represent positions involved in a contact site for CheW. The CheW* positions are also consistent with a surface location. The first four CheW* clusters fall at transition points between hydrophobic and hydrophilic segments (Fig. 8) and are predicted to lie within

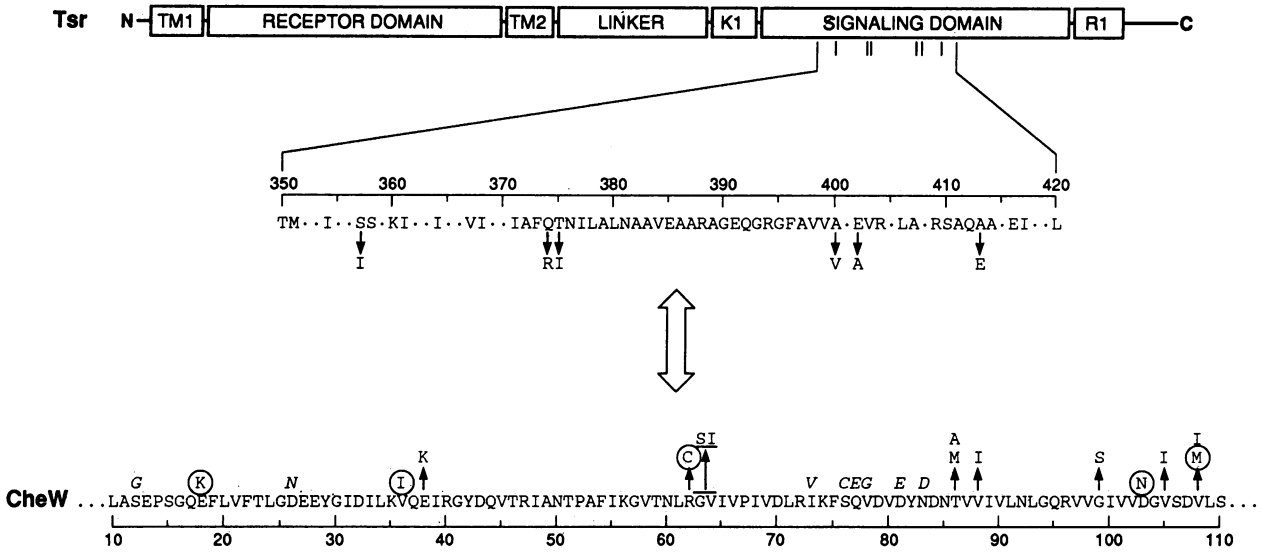

FIG. 7. Primary structure locations of Tsr* and CheW* mutations. The known structure-function organization of the Tsr molecule is shown at the top. TM1 and TM2 are membrane-spanning segments that array the receptor domain on the periplasmic side of the membrane, with the linker, K1, and R1 segments and the signaling domain on the cytoplasmic side. The Tsr* mutations are located within a highly conserved portion of the signaling domain and affect conserved sites. (The residues indicated by letters in the expanded part of the signaling region are found in all MCPs of *E. coli*). The portion of CheW that contains the dominant and suppressible mutations is shown at the bottom. Circled residues indicate dominant mutations; arrows point to suppressible mutations. The italic letters just above the main sequence indicate the residues that differ in the CheW of *Salmonella typhimurium* (27).

or near turns connecting stretches of α helix or β strand. Conceivably, these sites may be located close to one another at the surface of the native molecule and may comprise the interaction site for Tsr. Alternatively, they may function as Tsr suppressors by influencing the general conformation of the molecule, including that of the actual contact site.

In summary, our genetic results support the proposition that CheW interacts directly with chemoreceptors of the MCP family to transmit sensory signals during chemotactic responses. We suggest that mutual suppression between specific pairs of Tsr and CheW mutations involves compensatory changes in their relative affinities for one another or for CheA. We are testing this prediction by examining the behavior of Tsr* and CheW* molecules in coupled in vitro phosphorylation assays.

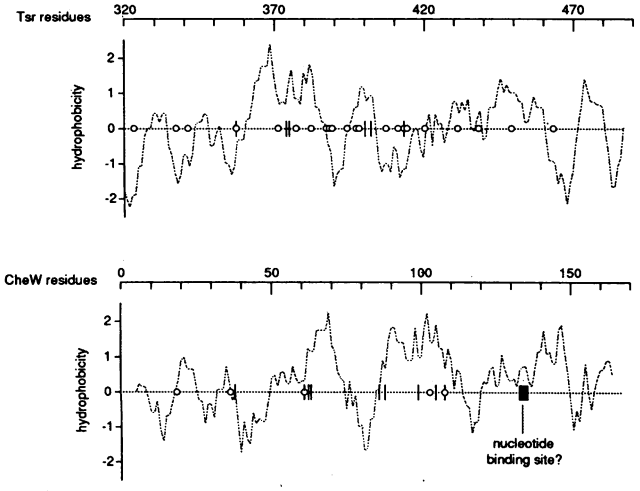

FIG. 8. Hydropathy profiles of Tsr and CheW proteins. The average hydrophobicity of amino acid residues in a sliding window nine residues long is plotted against the midpoint position of the window. The hydropathy scale of Kyte and Doolittle (17) was used. Only a portion of the Tsr signaling domain is shown. The positions of suppressible mutations are indicated by short vertical lines, and those of dominant mutations are indicated by small circles. The dominant *tsr* mutations are described by Ames and Parkinson (1).

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