Conformational suppression of inter-receptor signaling defects

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Motile bacteria follow gradients of attractant and repellent chemicals with high sensitivity. Their chemoreceptors are physically clustered, which may enable them to function as a cooperative array. Although native chemoreceptor molecules are typically transmembrane homodimers, they appear to associate through their cytoplasmic tips to form trimers of dimers, which may be an important architectural element in the assembly and operation of receptor clusters. The five receptors of *Escherichia coli* that mediate most of its chemotactic and aerotactic behaviors have identical trimer contact residues and have been shown by *in vivo* crosslinking methods to form mixed trimers of dimers. Mutations at the trimer contact sites of Tsr, the serine chemoreceptor, invariably abrogate Tsr function, but some of those lesions (designated Tsr*) are epistatic and block the function of heterologous chemoreceptors. We isolated and characterized mutations (designated Tar^) in the aspartate chemoreceptor that restored function to Tsr* receptors. The suppressors arose at or near the Tar trimer contact sites and acted in an allele-specific fashion on Tsr* partners. Alone, many Tar^ receptors were unable to mediate chemotactic responses to aspartate, but all formed clusters with varying efficiencies. Most of those Tar^ receptors were epistatic to WT Tsr, but some regained Tar function in combination with a suppressible Tsr* partner. Tar^–Tsr* suppression most likely occurs through compensatory changes in the conformation or dynamics of a mixed receptor signaling complex, presumably based on trimer-of-dimer interactions. These collaborative teams may be responsible for the high-gain signaling properties of bacterial chemoreceptors.

chemotaxis | epistasis | receptor clustering | signaling teams | trimers of dimers

Motile bacteria such as *Escherichia coli* track chemical gradients with extraordinary sensitivity. Their chemotactic behaviors provide good models for exploring the molecular mechanisms of stimulus detection and signal amplification in biological systems. The principal chemoreceptors in bacteria are known as methyl-accepting chemotaxis proteins (MCPs). *E. coli* has four transmembrane MCPs that monitor attractant and repellent concentrations by means of external ligand-binding domains and communicate with the flagellar motors through highly conserved cytoplasmic signaling domains (1). MCPs form signaling complexes with CheA, a histidine autokinase, and CheW, which couples CheA to chemoreceptor control. Changes in receptor ligand occupancy modulate CheA activity to control the phosphorylation states of two response regulators: CheY, which modulates motor rotation, and CheB, which modulates MCP methylation state to adjust the receptor's detection range to match ambient chemoeffector levels (see refs. 2 and 3 for recent reviews). In the micromolar attractant range, MCPs can sense concentration changes as small as 0.1% and trigger large fractional changes in motor rotational bias, corresponding to a signal gain of ≈50-fold (4, 5). Much of this amplification occurs at the receptor signaling complex (6), which behaves as an ensemble of allosteric signaling units containing ≈25 receptors each (7).

Physical clustering of the receptor molecules may underlie their cooperative behavior and high-gain signaling properties. In *E. coli*, the MCP signaling complexes are clustered at the cell pole(s) (8). CheA and CheW contribute to cluster integrity (8, 9), but the architecture of receptor networks and their collaborative mechanism of kinase regulation are still poorly understood. However, several lines of evidence indicate that receptor signaling teams based on a trimer-of-dimers organization may be important structural and functional components of chemoreceptor networks. Soluble signaling fragments of the *E. coli* serine receptor (Tsr) crystallized as trimers of dimers, and the principal interdimer contact residues ("trimer contacts") were identical in other *E. coli* MCPs (10), raising the possibility that mixed receptor trimers might form *in vivo*. Indeed, *in vivo* crosslinking studies have provided support for both the trimer-of-dimers organization and the formation of mixed receptor trimers (11, 12). Moreover, amino acid replacements at any of the Tsr trimer contact residues abrogate receptor signaling (11). Some contact site lesions disrupt trimer-based crosslinking, whereas others do not (12). The trimer-competent Tsr mutants exhibit unusual signaling behaviors in the presence of heterologous WT receptors, such as Tar, the aspartate receptor. Some Tsr trimer contact mutants regain serine-sensing and signaling ability in the presence of WT Tar receptors, an effect termed functional rescue (11). Other mutants, designated Tsr*, exert an epistatic effect on WT Tar function, blocking chemotactic responses to aspartate and serine (11). The simplest explanation for

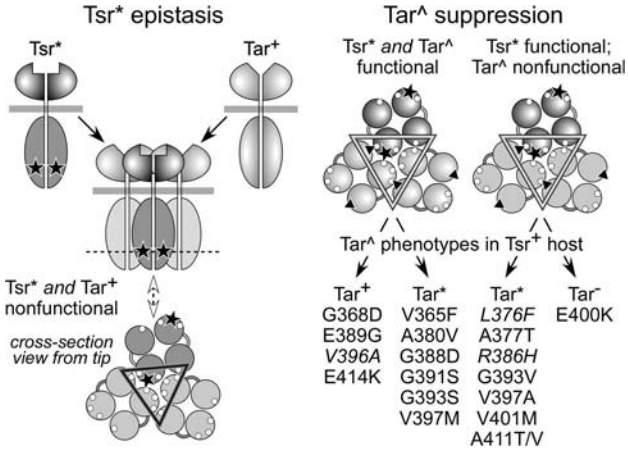

Fig. 1. Working model of inter-receptor epistasis and suppression. Tsr receptors with epistatic lesions (Tsr*) block the function of WT Tar receptors by forming defective mixed trimers of dimers. The Tsr* amino acid replacement (star) affects one of the trimer contact residues (small open circles). Tar^ mutations (small triangles) may impart a compensatory conformational change to mixed trimers. Tar^ mutations listed in italics affect trimer contact residues. Some Tar^ mutants retain signaling function in the presence of their Tsr* partner, whereas others do not. When tested alone, most of the nonfunctional Tar^ receptors are themselves epistatic (Tar*) to WT Tsr receptors.

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Abbreviations: MCP, methyl-accepting chemotaxis protein; IPTG, isopropyl-β-D-thiogalactopyranoside; CW, clockwise.

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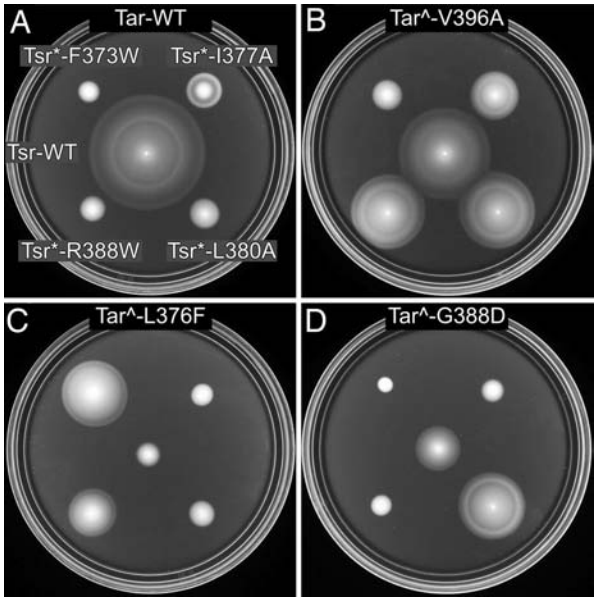

Fig. 2. Chemotaxis phenotypes of Tsr* and Tar^ mutants. Strain UU1250 with various combinations of mutant Tsr and Tar plasmids was tested for chemotactic ability on tryptone soft agar. Plates were photographed after 10-h incubation at 32.5°C. (*A*) Epistatic behavior of Tsr*. The doubly WT control colony (center) forms two cell bands or rings reflecting serine chemotaxis (Tsr function; outer ring) and aspartate chemotaxis (Tar function; inner ring). WT Tar fails to promote aspartate taxis in cells that also carry an epistatic Tsr receptor (Tsr*). (*B*) Suppression by Tar^-V396A. This Tar^ mutant functions well with WT Tsr and restores partial function to Tsr*-I377A and full function to Tsr*-R388W and L380A. Note that the three largest colonies also contain an aspartate taxis ring. (*C*) Epistasis and suppression by Tar^-L376F. This Tar^ mutant is fully epistatic to WT Tsr but suppresses Tsr*-F373W and (less well) R388W. The suppressed colonies have a serine ring but lack an aspartate ring. (*D*) Epistasis and suppression by Tar^-G388D. This Tar^ mutant is moderately epistatic to WT Tsr but specifically suppresses Tsr*-L380A. The suppressed colony has both a serine ring and an aspartate ring.

functional rescue and epistasis effects invokes direct physical interactions between the mutant Tsr and WT Tar molecules, possibly in mixed trimers of dimers (Fig. 1). Rescuable Tsr lesions may adopt normal trimer geometry when paired with Tar team members, whereas epistatic Tsr* mutants may impose an aberrant geometry on the entire team. This model predicts that compensatory structural alterations in the Tar team members could lead to conformational rescue of function in epistatic Tsr* receptors (Fig. 1). Moreover, if Tsr and Tar molecules are in direct contact with one another in signaling complexes, suppression of the Tsr* defect should be allele-specific. (See ref. 13 for a review of allele-specific suppression.) This article documents such suppression effects.

Results

Isolation of Tar Mutations That Suppress Epistatic Tsr Defects. Strains carrying a WT aspartate receptor (Tar) and a mutant serine receptor with an epistatic defect (Tsr*) show no chemotactic responses to either attractant (Figs. 1 and 2*A*). We looked for altered forms of Tar (hereafter designated Tar^) that could restore chemotactic ability to Tsr*-containing strains by inducing random mutations in a WT Tar expression plasmid (pLC113) and transferring the mutant plasmids en masse into receptor-less recipient cells (UU1250) that carried a compatible Tsr* plasmid (derivatives of plasmid pJC3). Chemotactic transformants were selected on tryptone soft agar, as detailed in *Materials and Methods*, and their pLC113 plasmids were characterized with a series of additional tests to confirm the presence of a Tar^ mutation. In all, we surveyed 14 different epistatic lesions at Tsr trimer contact sites: four of them

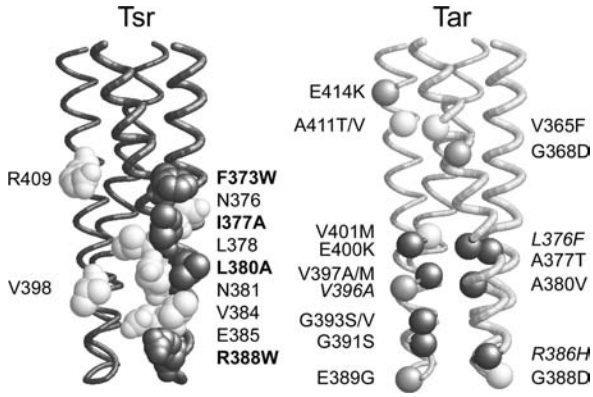

Fig. 3. Trimer contact regions of Tsr and Tar^ receptors. Both structures depict backbone traces of the cytoplasmic tip of a receptor dimer (Tsr residues 361–420), showing residues at the trimer interface. The two subunits are identical but given different thicknesses to indicate their different structural environments in the trimer of dimers. The thicker subunit contributes most of the residue contacts at the trimer interface. Those same residues (atoms not shown) in the other subunit are arrayed on the outside of the trimer. For Tsr, the 11 space-filled residues comprise the principal trimer contact sites. Residues on the right define the major trimer contact helix. Amino acid replacements at any of the trimer contact sites can create epistatic behavior, but only the four with dark shading were suppressible by Tar^ receptors. For Tar^, space-filled alpha carbons denote the residues at which Tar^ mutations were obtained. Residues on the right define the major trimer contact helix. Tar^ amino acid replacements with WT signal output are shaded light gray; counter-clockwise-biased mutations are shaded white; CW-biased mutations are shaded dark gray. Tar^ mutations listed in italics affect trimer contact residues.

(F373W, I377A, L380A, and R388W) produced a total of 19 Tar^ isolates (listed in Fig. 1); the other 10 (N376W, L378A, L378W, N381A, N381P, N381W, V384W, E385W, V398W, and R409W) yielded no Tar^ mutants, despite repeated attempts with independently mutagenized pLC113 pools.

Tsr*/Tar^ strains exhibited several different colony morphologies on soft agar plates (Fig. 2). Some showed a single chemotactic ring characteristic of a serine response (e.g., Tsr*-F373W/Tar^-L376F; Fig. 2*C*). Others showed two chemotactic rings indicative of both serine and aspartate responses (e.g., Tsr*-L380A/Tar^-V396A; Fig. 2*B*). Notably, all of the Tsr*/Tar^ isolates were chemotactic to serine; none were chemotactic to aspartate only. These findings suggest that single-step mutational changes cannot create "epistasis-resistant" Tar proteins, whereas they can create Tar alterations that correct Tsr* functional defects, often with a concomitant loss of Tar function.

Amino Acid Changes in Tar^ Proteins. DNA sequence determinations of the entire *tar* coding region in the Tar^ plasmids revealed that each mutant had a single base-pair change that produced a missense mutation. Tar^-G393V, V397M, and E400K were isolated as suppressors of Tsr*-F373W; Tar^-V365F, E389G, G393S, and E414K as suppressors of Tsr*-I377A; Tar^-G368D, R386H, G388D, G391S, and V396A as suppressors of Tsr*-L380A; and Tar^-L376F, A377T, A380V, V397A, A401M, A411T, and A411V as suppressors of Tsr*-R388W. All Tar^ mutations occurred within or near the trimer contact region of Tar (Fig. 3). Three Tar^ mutations (L376F, R386H, and V396A) correspond to actual trimer contact sites in Tsr (L378, R388, and V398). (Note that the Tar residue numbers in this region are two less than their Tsr counterparts.) These findings suggested that the mechanism of Tar^ suppression of Tsr* defects might involve direct interactions and compensatory conformational changes between the two types of receptor (cf. ref. 13). To explore this possibility, we characterized the functional properties of the

Table 1. Properties of Tar^ mutants

Tar^ mutation†	Expression level‡	Tar function§	Dominance¶	Epistasis‖	Cluster formation††	CW time‡‡	Suppression by Tsr*§§
V365F	1.05	0.30	0.65	0.55	0.30	0.09	+
G368D	0.40	0.55	0.30	0.15	0.30	0.22	na
L376F	2.40	0.40	0.65	0.70	0.70	0.97	−
A377T	1.05	0.30	0.65	0.65	0.90	0.71	−
A380V	1.10	0.30	0.65	0.45	0.55	0.37	+ (F373W)
R386H	2.95	0.45	0.50	0.80	0.40	0.61	−
G388D	2.80	0.30	0.65	0.50	0.85	0.04	+(L380A)
E389G	3.25	0.70	0.20	0.35	0.65	0.20	na
G391S	3.00	0.40	0.65	0.60	nd	0.55	+
G393S	0.35	0.40	0.45	0.40	0.40	0.88	+(I377A)
G393V	1.70	0.35	0.65	0.60	0.85	0.79	−
V396A	0.85	0.65	0.25	0.15	0.20	0.26	na
V397A	1.00	0.30	0.60	0.45	0.20	0.84	−
V397M	0.60	0.20	0.70	0.60	0.30	0.71	−
E400K	1.15	0.25	0.45	0.20	0.20	0.59	−
V401M	2.65	0.25	0.75	0.85	0.90	0.00	−
A411T	0.15	0.20	0.70	0.50	0.55	0.09	−
A411V	0.65	0.20	0.75	0.85	0.60	0.01	−
E414K	0.50	0.45	0.40	0.10	0.50	0.26	na
WT	1.00	1.00	0.00	0.00	1.00	0.28	na

Fractional data are rounded to the nearest 5% value. nd, not determined; na, not applicable.

†Tar residue numbers are two less than the corresponding Tsr residues, e.g., E400 of Tar corresponds to E402 of Tsr; mutations in italics are at trimer contact residues.

‡pLC113-Tar^/UU1250: fraction of WT Tar expression level.

§pLC113-Tar^/UU1250, T soft agar, 32.5°C, 10 hr: fraction of WT Tar colony diameter.

¶pLC113-Tar^/UU1624, T soft agar, 32.5°C, 10 hr: (1 − colony diameter normalized to WT).

‖pLC113-Tar^/UU1615, T soft agar, 32.5°C, 10 hr: (1 − colony diameter normalized to WT).

††pLC113-Tar^ + pPA789/UU1638: fraction of cells with one or more receptor clusters, normalized to WT Tar; 85% of Tar^+ cells had clusters.

‡‡Fraction of time that tethered cells spent in CW rotation.

§§pLC113-Tar^ + pJC3-Tsr*/UU1250, T soft agar, 32.5°C, 10 hr. + indicates that the Tar defect was suppressed by the Tsr* partner(s) of the Tar^ mutation. When only one Tsr* partner suppressed the Tar^ defect, the suppressing mutation is shown in parentheses.

Tar^ receptors with a series of tests that are summarized in Table 1 and detailed below.

Chemotaxis Defects of Tar^ Mutants. We tested the ability of Tar^ receptors to mediate aspartate chemotaxis on tryptone soft agar plates in cells lacking other receptors. At the inducer concentration optimal for WT Tar function (0.7 μM salicylate), most of the Tar^ mutants exhibited severe aspartate chemotaxis defects, with colony sizes less than half as large as WT. Four Tar^ mutants with colony sizes 45–70% of WT (G368D, E389G, V396A, and E414K) exhibited partial chemotactic responses, as evidenced by band formation at the colony periphery. The Tar^ chemotaxis defects were not obviously correlated with the steady-state expression levels of the Tar^ proteins, measured in the same host (Table 1). For example, all but three of the nonfunctional mutant proteins were expressed at 50% or more of the WT level, whereas two of the partially functional proteins (G368D and E414K) had relatively low expression levels (40% and 50% of WT, respectively). We conclude that the chemotaxis defects of the Tar^ mutants reflect qualitative changes in Tar receptor function rather than quantitative changes in Tar expression level.

Complementation Behavior of Tar^ Mutants. To explore the nature of the functional defects in Tar^ receptors, we tested their effects on chemotaxis mediated by WT Tar or Tsr. For dominance tests, Tar^ plasmids were transferred to strain UU1624, which lacks all receptors except WT Tar. Tar^ expression was induced with 0.7 μM salicylate, which produces optimal aspartate chemotaxis by pLC113 in an otherwise receptor-less host strain (UU1250). Under these conditions, the Tar^ mutants fell into three general classes based on the dominance tests (Table 1): (*i*) The four partially functional Tar^ mutants (G368D, E389G, V396A, and E414K) and E400K, a nonfunctional mutant, were recessive to WT Tar. Their colony size was reduced by <50%, and the formation and quality of the aspartate ring were not impaired (data not shown). We conclude that these mutant subunits can contribute to Tar function in heterodimers containing WT subunits. (*ii*) The R386H and G393S mutations exhibited partial dominance, reducing colony size by ≈50% and attenuating the aspartate ring (data not shown). (*iii*) The remaining 12 Tar^ mutations exhibited complete dominance to WT Tar, reducing colony size by at least 65% and obliterating the aspartate ring (data not shown). These dominance effects could operate through at least two different mechanisms, which are not mutually exclusive. First, subunits of the dominant Tar^ mutants may spoil the function of WT Tar subunits through heterodimer formation. Second, dimers containing a mutant subunit could spoil the function of WT dimers through formation of mixed trimers of dimers or other higher-order Tar complexes.

If receptor dominance can operate at the level of dimer–dimer interactions, the dominant Tar^ mutants might be expected to block the function of WT Tsr dimers through formation of mixed receptor complexes. To test Tar^ mutants for such epistatic effects, we expressed the mutant plasmids in strain UU1615, which lacks all receptors except WT Tsr, and measured chemotactic ability in tryptone soft agar containing 0.7 μM salicylate. All 12 of the fully dominant Tar^ mutants, and the partially dominant R386H mutant, impaired Tsr function. The colonies were considerably reduced in size (Table 1) and had no apparent serine ring (e.g., Tar^-L376F and

Table 2. Tar^ suppression classes

Tar^ class	Tar^ alleles	Suppression of Tsr* allele			
		F373W	I377A	L380A	R388W
1	V397M, E400K	+	−	−	−
2	*L376F*, A377T, A380V, G393V, V397A	+	−	−	+
3	V401M, A411T, A411V	−	−	−	+
4	G393S, *V396A*	−	+	+	+
5	V365F, E389G, E414K	−	+	+	−
6	G368D, *R386H*, G388D, G391S	−	−	+	−

Tar^ alleles in italics denote trimer contact residues. Suppression was assessed by colony morphology of Tsr*/Tar^ strains on tryptone soft agar. + indicates colony diameter at least 50% that of a Tsr^+/Tar^+ control, with a perimeter band of cells indicative of serine chemotaxis; − indicates colony diameter <50% of the WT control with no serine ring.

G388D; Fig. 2 *C* and *D*). We conclude that these Tar^ mutants have functional lesions comparable in effect to those of epistatic Tsr* mutants. In contrast, the partially dominant Tar^-G393S mutant and the five recessive Tar^ mutants did not significantly impair Tsr function (e.g., V396A; Fig. 2*B*).

CheA Activation by Tar^ Receptors. To test the ability of Tar^ mutants to stimulate CheA kinase activity through formation of ternary signaling complexes, we expressed the mutant plasmids in strain UU1250, which encodes no receptors but contains physiological levels of WT CheA and CheW. In this strain, receptor-activated CheA produces sufficient phospho-CheY to cause episodic clockwise (CW) rotation of the flagellar motors, which we monitored by observing tethered cells (see *Materials and Methods*). Under these conditions, cells containing WT Tar (pLC113) spent 28% of their time in the CW mode. The fraction of time spent in CW rotation reflects the cell's level of CheA activity, and the Tar^ mutants exhibited a wide range of CheA-stimulating activities in this test (Table 1). The four Tar^ mutants that retained partial aspartate chemotaxis exhibited WT levels of CW rotation. In contrast, the nonchemotactic Tar^ mutants displayed aberrant rotation patterns. Five mutants (V365F, G388D, V401M, A411T, and A411V) spent <10% of their time in CW rotation, behavior that presumably reflects defects in CheA activation. The other 10 nonchemotactic mutants exhibited high levels of CW rotation, ranging from 0.37 (A380V) to 0.97 (L376F), behavior consistent with abnormally high levels of CheA activation.

Cluster Formation by Tar^ Receptors. To follow ternary complex formation and clustering by Tar^ receptors, we used a CFP-CheZ reporter that interacts with an alternate form of CheA ($CheA_S$) associated with receptor signaling complexes (14, 15). Coexpression of WT Tar (pLC113) and CFP-CheZ (from a compatible plasmid) in strain UU1638, which lacks all receptors and CheZ, resulted in one or more bright spots of fluorescence in 85% of the cells. These clusters, generally one per cell, were typically located at or near a cell pole and did not form in cells lacking CheA, CheW, or receptors (data not shown), demonstrating that their formation depends on assembly of a receptor/CheW/CheA ternary complex. The Tar^ receptors all formed clusters, but with somewhat reduced proficiency compared with the WT control (Table 1). Surprisingly, we saw no relationship between the signaling characteristics of a Tar^ mutant (described above) and its extent of cluster formation. For example, clustering ability was not correlated with residual function in the Tar^ mutants, their pattern of flagellar rotation, or whether their defects were dominant, recessive, or epistatic. Most importantly, two of the mutant receptors with the greatest residual function (G368D and V396A) had among the lowest clustering efficiencies (20–30% of WT levels) (Table 1). Thus, cluster formation, at least with the CFP-CheZ reporter, seems to be an imprecise measure of receptor function. Nevertheless, these results demonstrate that all of the Tar^ receptors, regardless of their functionality or signal state bias, are capable of ternary complex assembly.

Allele Specificity of Tar^/Tsr* Suppression. All Tar^ isolates were obtained as suppressors of one of four different Tsr* mutants (see Fig. 3). None were isolated from more than one Tsr* partner, suggesting that the Tar^/Tsr* interaction is allele-specific. To examine suppression specificity, we combined each of the 19 Tar^ mutant plasmids with each of the four Tsr* mutant plasmids in strain UU1250 and tested the resulting double transformants for chemotaxis in tryptone soft agar. Tsr* expression was set at the optimal level for function of WT Tsr [20 μM isopropyl-β-D-thiogalactopyranoside (IPTG)], and suppression was evaluated over a range of Tar^ expression levels by varying its inducer concentration (0, 0.35, 0.7, or 1.4 μM salicylate). Suppression was usually most apparent at 0.7 μM salicylate, the optimal inducer level for Tar WT function, but we scored a Tar^/Tsr* combination as positive if suppression occurred at any Tar^ expression level. The suppression tests divided the 19 Tar^ mutations into six major classes (Table 2). Members of classes 1, 3, and 6 acted allele-specifically; each suppressed only one of the original Tsr* alleles. Members of classes 2 and 5 suppressed complementary pairs of Tsr* alleles [F373W, R388W] or [I377A, L380A]. Finally, members of Tar^ class 4 had the least specific behavior, suppressing all but Tsr*-F373W.

We also tested the Tar^ mutations in combination with the 10 Tsr* mutations that failed to yield Tar^ isolates in the initial suppressor selections. To facilitate the survey, the Tsr* lesions were tested in two pooled sets [(N376W, L378W, N381A, N381P, N381W, E385W, R409W) and (V384W, V398W)] by streaking transformants en masse on soft agar to detect flares of chemotactic cells. Neither Tsr* pool produced any chemotactic isolates, indicating that all 10 Tsr* mutations were recalcitrant to suppression by any of the Tar^ mutations (data not shown). These findings demonstrate that epistatic lesions are suppressible at only a subset of Tsr trimer contact sites and that the suppression pattern at those sites is allele-specific.

Suppression of Tar^ Defects by Tsr* Lesions. Six of the Tar^ receptors that were epistatic to WT Tsr exhibited Tar function in combination with some Tsr* receptors (Fig. 1 and Table 1). Three of those Tar^ alleles could be suppressed by only one (different) Tsr* allele (Table 1), demonstrating that mutual Tar^-Tsr* suppression effects are also allele-specific.

Discussion

Direct vs. Indirect Mechanisms of Receptor Epistasis and Suppression. Epistatic Tsr* receptors could conceivably block the function of Tar and other heterologous receptors indirectly, for example, through titration or inactivation of shared signaling partners, such as the

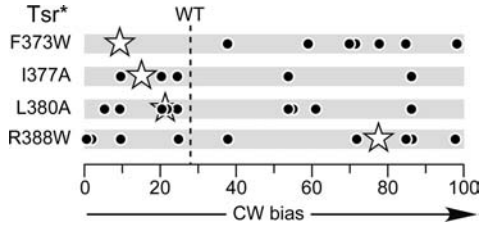

Fig. 4. Nonadditivity of Tsr* and Tar^ flagellar rotation patterns. In each row, the extent of CW rotational bias produced by Tsr* receptors alone is indicated by stars, and the rotational bias produced by each of their Tar^ suppressors alone is indicated by circles. The WT label identifies the rotational bias of cells carrying both WT Tsr and WT Tar receptors.

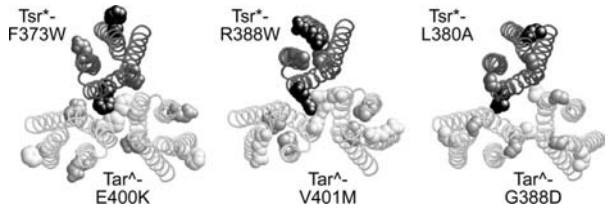

Fig. 5. Examples of conformational suppression mechanisms in receptor trimers of dimers. Backbone traces of the trimer contact region of three different trimers, viewed from the cytoplasmic tip. Each trimer contains one Tsr* dimer (dark gray) and two Tar^ dimers (white). The black space-filled residues in Tsr indicate the trimer contact residue altered by the Tsr* mutation; white atoms indicate the corresponding (WT) residue in the Tar^ receptors. Light-gray space-filled atoms in Tar indicate the residue changed by the Tar^ alteration; dark gray atoms indicate its (WT) counterpart in the Tsr* receptor.

CheW/CheA components of ternary complexes, or by creating locked output signals (16) that jam the flagellar motors. In principle, such epistatic defects should be suppressible by mutations in other receptors that offset the signaling imbalance, through either enhanced competition for common components or an opposing flagellar signal that restores a more moderate rotation pattern. Three lines of evidence argue that Tar^ suppressors need not operate through a balancing or phenotypically additive mechanism. (*i*) The flagellar rotation signals of Tsr* mutants and their Tar^ suppressors were often biased in the same direction (Fig. 4). Some counter-clockwise (CCW)-biased Tar^ receptors suppressed CCW-biased Tsr* defects (e.g., I377A and L380A) and some CW-biased Tar^ receptors suppressed a CW-biased Tsr* defect (R388W). (*ii*) The pattern of productive Tar^/Tsr* interactions was allele-specific (Table 2). If suppression occurred by a phenotypic balancing mechanism, any Tar^ alteration that caused CW-biased output might be expected to compensate for any CCW-biased Tsr* defect. (*iii*) Tar^ mutations only arose in the trimer contact region of Tar, which is known from *in vivo* crosslinking studies (12) to interact directly with the trimer contact region of Tsr, where the Tsr* mutations were located (Fig. 3). Although lesions in other parts of Tar, e.g., the methylation segments, can lead to biased signal output (17, 18), evidently only alterations in the Tar trimer contact region are able to suppress Tsr* defects. These findings strongly suggest that Tsr* receptors block Tar function through a direct, physical interaction and that Tar^ suppressors act on their Tsr* partners through a similar direct interaction.

Mechanisms of Conformational Suppression in Receptor Trimers of Dimers. The genetic evidence for conformational suppression is consistent with mechanisms involving stereospecific contacts between different receptor dimers. We propose that the collaborative signaling unit is based on a trimer of receptor dimers (see Fig. 1) and that these structural interactions underlie cooperative signaling between different types of receptors, which has been observed both *in vivo* (6, 7) and *in vitro* (19). It seems unlikely that trimers represent a transitory step in cluster assembly because all of the cell's receptor molecules, not just those newly synthesized, exhibit crosslinking patterns consistent with trimer geometry (12, 20). Moreover, the CheA and CheW proteins stabilize the members of receptor teams against exchanges with other trimers, implying that trimer-based interactions between different receptors persist while they are actively signaling (20). Thus, trimers of dimers may be essential for CheA activation and stimulus control. The trimer interface might provide a relatively static conformational fulcrum for movements in other segments of the receptor dimers. Stimuli might, for example, tighten or relax the coiled-coil interactions within dimer subunits and/or the supercoiling interactions between subunits to alter the signaling conformation or dynamics of the trimer complexes. In the context of this model, functional suppression would occur through altered conformation or dynamic behavior of the Tar^ and Tsr* members of a receptor signaling team.

Some suppressors may adjust dimer–dimer interactions to achieve a suitable trimer geometry. Tsr*-F373W and R388W might exemplify this type of suppression mechanism because they have opposing signal biases but share a number of Tar^ suppressors (Table 2). F373 and R388 lie at opposite ends of the trimer interface, where replacement with a bulky tryptophan residue might be expected to create a bulge that pushes the dimers apart. Their shared suppressors (Tar^-L376F, G393V, A377T, A380V, and V397A) are located near the middle of the trimer interface (Fig. 3); four of them introduce larger side chains that might expand interdimer distances in their vicinity. These suppressors might serve to realign the individual dimers along the trimer interface, producing an arrangement that is more conducive to signaling. Tsr*-F373W, but not R388W, is also suppressed by Tar^-V397M and E400K (see Fig. 3), which might act in a similar fashion (see E400K in Fig. 5).

In contrast, the suppressors specific for Tsr*-R388W (Tar^-V401M, A411T, and A411V) may act by altering the dynamic properties of the trimer. Alone, none of these three Tar^ receptors is able to activate CheA (Table 1), whereas Tsr*-R388W hyperactivates CheA (Fig. 4). CheA activation seems to be associated with reduced dynamic motion of the receptor signaling domain, whereas activation-impaired signaling domains seem to have less stable structures (21–23). The Tar^ residues altered by suppressors of Tsr*-R388W are not located at the trimer interface but rather are buried at the subunit interface of individual dimers (see V401M in Fig. 5). We suggest that the suppressor mutations, which in all cases introduce larger amino acids, destabilize the dimer interface, producing more rapid dynamic motions. Reduced dimer stability could, in turn, offset an enhanced stability of the trimer produced by the bulky hydrophobic tryptophan replacement in Tsr*-R388W.

Suppressors of Tsr*-I377A and L380A may also act through compensatory changes in trimer conformational dynamics (see Fig. 5). These Tsr* residues lie near the middle of the trimer interface in the major trimer contact helix (Fig. 3). The alanine replacements introduce a smaller, less hydrophobic residue that might reduce trimer stability. A number of the Tar^ suppressors have replacements at glycine residues that are located either in the hairpin loop (Tar^-G388D, G391S, and G393S) or just above the trimer contact region (Tar^-G368D) (Fig. 3). Amino acid replacements at these positions might impede relative motions of the dimers and thereby offset the trimer-destabilizing properties of the Tsr* defect.

Constraints on Suppression of Trimer Contact Defects. We were unable to find Tar^ suppressors for most of the epistatic Tsr trimer contact mutants we tested (see Fig. 3). The suppressible sites were all in the major trimer contact helix, which we suggest acts as a fulcrum for modulating structural and dynamic changes in the subunits containing the minor contact helix. Three of the suppressible sites (F373, I377, and L380) also form a contiguous section of

the trimer interface; mutations affecting these residues might be particularly amenable to conformational suppression. However, these commonalities may not be the most critical factor in establishing the pattern of Tar^ suppression effects. The trimer contact residues are highly conserved (10, 11) and might well have multiple functional roles, possibly related to the two very different structural environments in which they can reside. In trimers of dimers, the contact residues in one subunit of each dimer comprise the trimer interface, whereas in the other subunit they lie in solvent-accessible positions on the outside of the trimer. Some Tsr* mutants may be nonsuppressible because residue replacements that perturb interdimer contacts also impair other receptor functions, such as interaction with CheA or CheW to form ternary signaling complexes.

The possibly multiple functional roles for residues in the trimer contact portion of the signaling domain present a difficult challenge for further elucidating signaling mechanisms through genetic analyses. Nevertheless, the Tar^/Tsr* suppression effects described here, regardless of their detailed mechanism, almost certainly occur through compensatory conformational changes between directly interacting proteins. Those interactions most likely take place in the context of a trimer-of-dimers arrangement of the receptors.

Materials and Methods

Bacterial Strains. Strains and their relevant genotypes were: RP526 (*mutD*) (24); UU1250 [Δ*tsr-7028* Δ(*tar-tap*)*5201* Δ*trg-100* Δ*aer-1*] (25); UU1448 [Δ*tsr-7028* Δ(*tar-tap*)*5201* Δ*trg-100 recA56*] [obtained from B. Bourret (University of North Carolina Medical School, Durham) as KO607 (26)]; UU1615 [*D(tar-tap)5201* Δ*trg-100* Δ*aer-1*]; UU1624 [Δ*tsr-7028* Δ*tap-3654* Δ*trg-100* Δ*aer-1*]; and UU1638 [Δ*tsr-7028* Δ(*tar-tap*)*5201* Δ*trg-100* Δ*aer-1* Δ*cheZ-6725*].

Plasmids. Plasmid pJC3 (11, 27) was the expression vector for WT Tsr. Its derivatives expressing the F373W, N376W, I377A, I377W, L378A, L378W, L380A, N381A, N381P, N381W, V384W, E385W, R388W, V398W, and R409W mutations have been described (11). Other plasmids used were pCJ30 (11, 28); pLC112 (11), a pACYC184-derived plasmid (29) that confers chloramphenicol resistance and carries a salicylate-inducible promoter (30); pLC113, a pLC112 relative that expresses WT Tar (11); and pPA789 (this work), a pCJ30 relative that expresses an IPTG-inducible CFP-CheZ fusion protein.

Behavioral Assays. Chemotactic ability was assessed at 32.5°C in tryptone soft agar plates (10 g of tryptone, 5 g of NaCl, and ≈2.5 g of agar per liter) supplemented as appropriate with ampicillin (50 μg/ml), chloramphenicol (12.5 μg/ml), IPTG (20 μM), and salicylate (0.35, 0.7, or 1.4 μM). Flagellar rotation patterns were analyzed with antibody-tethered cells as described (31).

Isolation of Tar^ Mutants. To identify Tar mutations (Tar^) that suppressed the chemotaxis defects of epistatic Tsr trimer contact mutants (Tsr*), plasmid pLC113 was mutagenized by passage through RP526 and then electrotransformed into UU1448 cells harboring pJC3-Tsr* mutants. Transformants were selected on tryptone soft agar plates containing ampicillin (50 μg/ml), chloramphenicol (12.5 μg/ml), IPTG (20 μM), and salicylate (0.7 μM). After incubation at 30°C for 14–20 h, cells from chemotactic "flares" were picked and colony was purified on selective antibiotic medium. Their pLC113 plasmids were purified and tested for suppressor properties by retransformation of UU1250 cells harboring pJC3-Tsr* mutants.

Expression Levels of Tar^ Proteins. UU1250 strains carrying pLC113-Tar^ plasmids were grown at 30°C to midexponential phase, and total protein was precipitated from 1-ml aliquots of each culture by addition of 10% cold trichloroacetic acid. The precipitated proteins were pelleted by centrifugation (12,000 × *g*, 10 min, 4°C), rinsed with cold acetone, and air-dried. After resuspension in SDS/PAGE sample buffer (32), proteins were separated in 12% SDS-polyacrylamide gels and visualized by Western blotting with a mixture of two antibodies that recognize the cytoplasmic domain of Tar and an unidentified cell protein that served as a convenient reference marker. Tar levels relative to the reference marker were determined by PhosphorImager analysis (Molecular Dynamics model SI).

Complementation Tests of Tar^ Mutants. Tar^ plasmids were transferred to UU1624 (Tar^+) and UU1615 (Tsr^+) to test for dominance and epistasis, respectively. Transformant colonies selected on L plates containing chloramphenicol (25 μg/ml) were picked to tryptone soft agar plates containing chloramphenicol (12.5 μg/ml) and salicylate at 0, 0.35, 0.70, or 1.4 μM, incubated at 32.5°C for 7–8 h, and evaluated for serine or aspartate chemotaxis.

Tar^ Receptor Clustering Assay. pLC113-Tar^ derivatives were transformed into UU1638 cells carrying plasmid pPA789. The resulting strains were grown for 4–5 h at 30°C in tryptone broth containing ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), 0.7 μM salicylate, and 200 μM IPTG. Cell fields were photographed in phase contrast and cyan fluorescence, and 100 cell images were scored for the presence of one or more fluorescent foci (clusters).

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