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Inversion of Thermosensing Property of the Bacterial Receptor Tar by Mutations in the Second Transmembrane Region

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The aspartate chemoreceptor Tar of *Escherichia coli* serves as a warm sensor that produces attractant and repellent signals upon increases and decreases in temperature, respectively. However, increased levels of methylation of the cytoplasmic domain of Tar resulting from aspartate binding convert Tar to a cold sensor with the opposite signaling behavior. Detailed analyses of the methylation sites, which are located in two separate α-helices (MH1 and MH2), have suggested that intra- and/or intersubunit interactions of MH1 and MH2 play a critical role in thermosensing. These interactions may be influenced by binding of aspartate, which could trigger some displacement of MH1 through the second transmembrane region (TM2). As an initial step toward understanding the role of TM2 in thermosensing, we have examined the thermosensing properties of 43 mutant Tar receptors with randomized TM2 sequences (residues 190-210). Among them, we identified one mutant receptor (Tar-I2) that functioned as a cold sensor in the absence of aspartate. This is the first example of attractant-independent inversion of thermosensing in Tar. Further analyses identified the minimal essential divergence from the wild-type Tar sequence (Q191V-W192R-Q193C) required for the inverted response. Thus, displacements of TM2 seem to influence the thermosensing function of Tar.

Keywords: aspartate receptor; chemotaxis; methylation; temperature; thermotaxis

Thermotaxis of *Escherichia coli* is one of the best model systems available to study the molecular mechanisms underlying thermosensing. The relevant receptors have been identified to be closely related transmembrane receptors, which also serve as chemoreceptors (Manson, 1992; Parkinson, 1993; Stock & Surette, 1996; Falke *et al*., 1997). These receptors can be divided into the following two classes (Maeda & Imae, 1979; Mizuno & Imae, 1984; Nara *et al*., 1991). The ''warm sensors'' (Tsr, Tar, and Trg) mediate attractant and repellent responses upon increases and decreases of temperature, respectively, and the ''cold sensor'' (Tap) mediates the opposite responses to the same stimuli.

Tar is a warm sensor in the absence of an attractant, but it is converted to a cold sensor after it has adapted to an attractant such as aspartate (Mizuno & Imae, 1984). Covalent modification of the methylation sites (Gln295, Glu302, Gln309, and Glu491; collectively referred to as QEQE) is involved in this inversion of thermosensing (Nara *et al*., 1996). The two glutamine residues are converted to methylatable glutamate residues irreversibly by the deamidase activity of CheB. Methylation and demethylation are catalyzed by the methyltransferase CheR and the methylesterase CheB, respectively. In a $CheB^-$ $CheR^-$ strain, the unmodified form of Tar (QEQE) mediates a chemoresponse but not a thermoresponse. It acquires thermosensing ability when it is deamidated either by CheB or by site-directed mutagenesis: the unmethylated form

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(EEEE) functions as a warm sensor, and the fully methylated form (EmEmEmEm; Em standing for a methylated glutamate residue) functions as a cold sensor in the presence of aspartate. Mutational analyses of the methylation sites of Tar suggested that methylation of any single residue is sufficient for inversion of thermosensing in the presence of aspartate (Nishiyama *et al*., 1999). Methylation has also been implicated in the inverted responses observed in other types of taxis, such as responses to oxygen and weak acids (Taylor & Johnson, 1998). In addition to methylation, binding of aspartate to Tar may be required for its function as a cold sensor (Nara *et al*., 1996; Nishiyama *et al*., 1997).

The first three methylation sites are located in a single putative α-helix (MH1), whereas the fourth site is located in a separate helix (MH2) (Le Moual & Koshland, 1996; Danielson *et al*., 1997; Figure 1). Nishiyama *et al*. (1997) proposed that temperature-dependent changes in intra- and/or intersubunit interactions of MH1 and MH2 may play a critical role in thermosensing. These interactions could be influenced not only by the covalent modification of the methylation sites but also by binding of an attractant to Tar. A bound attractant may trigger a displacement of the second transmembrane region (TM2), which connects the periplasmic and cytoplasmic domains, and hence a displacement of MH1. Indeed, it has been suggested that during a signaling cycle TM2 moves relative to the first transmembrane region (TM1), which pairs stably with TM1′ of the partner subunit in the Tar homodimer (Chervitz & Falke, 1995, 1996; Chervitz *et al*., 1995; Lee *et al*., 1995; Maruyama *et al*., 1995; Hughson & Hazelbauer, 1996). Such movement may be small (Chervitz & Falke, 1996; Umemura *et al*., 1998), but it may still alter interaction(s) between the cytoplasmic domains of the two subunits within a dimer. Studies using cytoplasmic fragments of Tar (Long & Weis, 1992; Cochran & Kim, 1996; Surette & Stock, 1996; Liu *et al*., 1997) and cysteine-scanned mutant Tar receptors (Chen & Koshland, 1997; Danielson *et al*., 1997; Le Moual

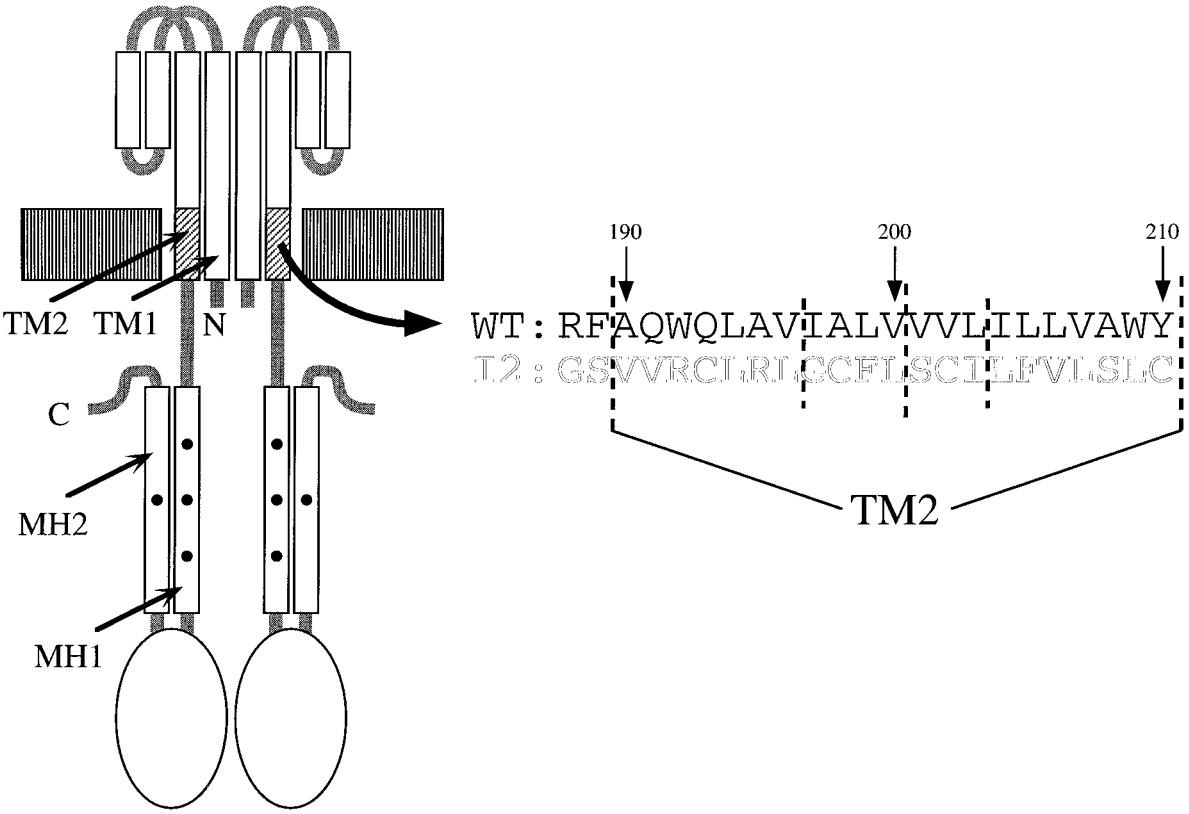

Figure 1. Schematic illustration of Tar. Tar exists as a homodimer of about 60-kDa subunit (Milligan & Koshland, 1988). The dimeric cytoplasmic domains form stable complexes with the histidine kinase CheA and the adaptor protein CheW (Gegner *et al*., 1992; Schuster *et al*., 1993). CheA autophosphorylates and then serves as a phosphodonor for the response regulator CheY. The phosphorylated form of CheY interacts with the flagellar motor to cause clockwise rotation, which results in tumbling behavior by the cell. Without phospho-CheY bound, the motor rotates counterclockwise, which results in smooth swimming of the cell. Binding of an attractant to a receptor inhibits the associated CheA kinase, reducing the level of phospho-CheY and promoting smooth swimming. The hatched boxes indicate the second transmembrane regions (TM2). The TM2 sequences of wild-type Tar (WT) and the mutant Tar-I2 receptor (I2) are aligned. Dotted lines indicate junction points between the wild-type and mutant sequences in the hybrid receptors (see Table 2). N, amino terminus; C, carboxy terminus; MH1 and MH2, first and the second methylation helices; black dots, methylation sites.

Table 1. Chemo- and thermosensing properties of mutant Tar receptors with randomized TM2 sequences

Mutant[a]	Swimming bias[b]	Chemoresponses[c]		Thermoresponses[d]	
		Glyc	Asp (+ Glyc)	−Asp	+Asp
2B	TR	+	10^{-6} (10^{-4})	Warm	Cold
N3	T	NT	10^{-5} (>10^{-3})	None	Cold
N4	T	NT	10^{-5}	None	Cold
Y9	T	NT	10^{-5} (10^{-5})	None	Cold
O14	TR	+	(>10^{-3})	Cold	Cold?
G6	TR	NT	10^{-6}	None	Cold
O13	TR	+	(10^{-5})	None?	Cold
O3	TR	+	(10^{-6})	Warm	Cold
Q12	R	±	(>10^{-3})	None?	None
I3	R	+	10^{-6} (> 10^{-3})	None	Cold
O7	R	+	(10^{-5})	None	None
O2	R	+	(10^{-6})	None	Cold?
O4	R	+	(10^{-6})	Cold?	NT
I2	R	+	(10^{-6})	Cold	Cold?
O5	SR	+	10^{-6}	None	None
O11	SR	+	(>10^{-3})	Warm	NT
R7	SR	+	(>10^{-3})	None	NT
R8	SR	+	(>10^{-3})	None	NT
Y40	SR	+	(10^{-4})	None	NT
O10	SR	+	(10^{-5})	None	None?
M5	SR	+	(10^{-6})	None	None
Q13	SR	+	(10^{-6})	None	None
R9	SR	±	NT	None	NT
U421	SR	±	NT	None	NT
Y5	SR	±	NT	None?	NT
Y37	SR	±	NT	None	NT
Y62	SR	±	NT	None	NT
G1	SR	∓	NT	None	NT
Q4	SR	∓	NT	None	NT
Q7	SR	∓	NT	None	NT
C11	SR	−	NT	None	NT
M1	SR	−	NT	None?	NT
M3	SR	−	NT	None	NT
O6	SR	−	NT	None	NT
Y4	SR	−	NT	None	NT
P3	S	+	(>10^{-3})	None	NT
G7	S	±	(10^{-5})	None	None?
Q11	S	+	(10^{-6})	Cold?	Cold
Y18	S	∓	NT	None	Cold
P4	S	−	NT	None	NT
Y34	S	−	NT	None	NT
Y49	S	−	NT	None	NT
Y50	S	−	NT	None	NT
Y53	S	−	NT	None	NT

[a] Plasmid pTar2B and its derivatives encoding mutant Tar receptors with randomized TM2 sequences are based on the multicopy plasmid vector pBluescriptII KS+ and were described previously (Maruyama *et al*., 1995). These plasmids were introduced into strain HCB339 [Δ*tsr-7021* Δ*(tar-tap)5201 trg*::Tn*10 thr leu his met rpsL136*] (Wolfe *et al*., 1987), which lacks the four chemo/thermoreceptors.

[b] Cells were grown at 30 °C with vigorous shaking in tryptone-glycerol medium (1% (w/v) Bacto-tryptone; Difco Laboratories, Detroit, MI, 0.5% (w/v) NaCl, and 0.5% (v/v) glycerol) supplemented with ampicillin (50 μg/ml). After four hours of cultivation, cells were harvested by centrifugation at room temperature and washed with motility medium, which consists of 10 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 10 mM sodium DL-lactate (pH 7.0), and 0.1 mM methionine. Swimming patterns of cells were assayed at room temperature without adding chemoeffector. S, smooth swimming; SR, smooth to random swimming; R, random swimming; T, tumbly swimming; TR, tumbly to random swimming.

[c] Chemoresponses to glycerol (Glyc) and aspartate (Asp) were examined by temporal stimulation assays essentially as described previously (Nishiyama *et al*., 1997). Cells suspended in motility medium were kept at room temperature. Immediately after adding an attractant or a repellent to the cell suspension the swimming pattern of the cells was observed with a dark-field microscope and recorded on videotape. In an image integrated for one second using an Argus-10 image processor (Hamamatsu Photonics K.K., Hamamatsu, Shizuoka), smooth-swimming and tumbling cells gave linear and blurred-dot traces, respectively. The smooth-swimming fraction was defined as the percent fraction of smooth-swimming cells per total swimming cells. Glycerol was added at 10%. +, Responded; −, did not respond; NT, not tested. Aspartate was added at various concentrations. The concentrations giving half-maximal attractant responses are shown without parentheses when assayed in the absence of glycerol or in parentheses when assayed in the presence of 10% glycerol.

[d] Thermoresponses were examined as described previously (Nishiyama *et al*., 1997). Without adding a chemoeffector (−Asp) or 30 minutes after 0.1 mM aspartate was added (+ Asp), a drop of cell suspension in motility medium was placed on a glass slide mounted on a temperature-control apparatus (Maeda & Imae, 1979). The temperature was changed from 16 to 27 °C and returned to 16 °C. Temperature changes were monitored by a constantan-chromel thermocouple inserted into the suspension. Cold, cold-sensor response; Warm, warm-sensor response; None, no thermoresponse was observed. Question marks indicate weak or no responses, which were difficult to judge.

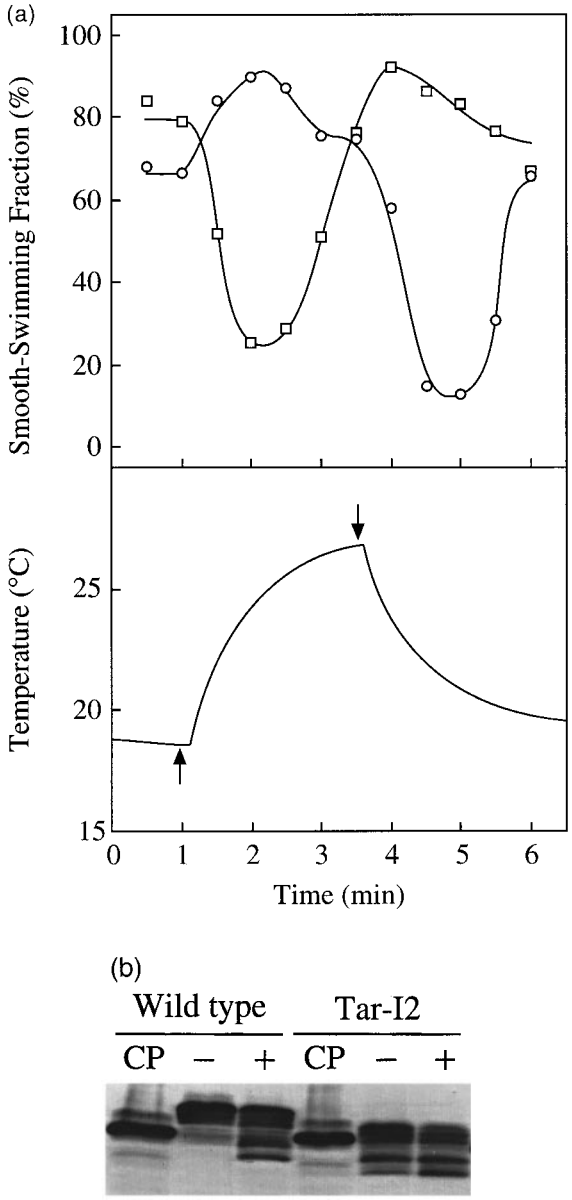

Figure 2. (a) Thermoresponses of cells expressing wild-type Tar (○) or Tar-I2 (□) in the absence of chemoattractant. To remove additional mutations from the *tar* genes of pTarI2 and pTarO14, the 0.3 kb *Eco*RV-*Kpn*I fragments of the relevant derivatives of pTar2B were subcloned into the corresponding region of pNI101, the pBR322-based plasmid carrying the wild-type *tar* gene, which will be described elsewhere. The smooth-swimming fraction of the cells (upper panel) was measured as the temperature was increased and then decreased as shown in the lower panel (at one minute, 16 to 27 °C; at 3.5 min, 27 to 16 °C). Arrows indicate the onset of the temperature ramps. (b) Methylation patterns of wild-type Tar and Tar-I2. The wild-type or mutant receptors were expressed in the $CheB^-$ $CheR^-$ strain CP553 [Δ*trg-100 zab*::Tn*5* ((*tar-cheB)2234* Δ*tsr-7028*] (Burrows *et al.*, 1989) (lanes CP) or in the $CheB^+$ $CheR^+$ strain HCB339 (lanes − and +) and detected by immunoblotting analysis essentially as described previously (Okumura *et al.*, 1998). Cells were preincubated in the absence of attractant (lanes CP and −) or with 10 mM aspartate (lanes +) at room temperature for 20 minutes. The cells were collected by centrifugation and resuspended in loading buffer (67 mM Tris-HCl (pH 6.8), 8 % glycerol, 1 % sodium dodecyl sulfate (SDS), 0.003 % bromophenol blue) supplemented with 7.7 % 2-mercaptoethanol. Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with anti-Tsr serum (Iwama *et al.*, 1997), which cross-reacts with Tar. It should be noted that multiple methylation by the CheR methyltransferase causes stepwise increases in the mobility of a chemoreceptor during SDS-PAGE (Boyd & Simon, 1980; Chelsky & Dahlquist, 1980; DeFranco *et al.*, 1980; Engström & Hazelbauer, 1980). Among the four methylation sites, two glutamine residues are deamidated by the CheB methylesterase/deamidase to glutamate residues, a change which decreases the mobility of Tar.

et al., 1998; Trammell & Falke, 1999) suggest that altered interactions between the cytoplasmic domains are associated with altered signaling states. Changes of interactions within a single cytoplasmic domain or between dimers within a receptor cluster (Maddock & Shapiro, 1993) may also be involved (Milligan & Koshland, 1991; Gardina & Manson, 1996; Tatsuno *et al.*, 1996). In any case, attractant-induced movement of TM2 might impose some physical constraint on the receptor that influences the thermosensing property of Tar.

Here, we screened for anomalous thermosensing phenotypes among a series of mutant Tar receptors with randomized TM2 sequences (Maruyama *et al.*, 1995). We employed this approach because selection of mutants with altered thermotactic phenotypes is generally very difficult. A total of 43 mutant Tar receptors as well as the parental receptor (2B) were expressed in the receptorless strain HCB339, and their chemo- and thermosensing properties were examined (Table 1). In temporal stimulation assays of chemotaxis, cells expressing 2B or 29 mutant receptors showed attractant responses to aspartate and/or repellent responses to glycerol. Cells expressing the remaining 14 mutant receptors did not show any chemoresponse to glycerol or aspartate. In temporal stimulation assays of thermotaxis, two strains (2B, O3) produced essentially normal warm-sensor responses in the absence of attractant and cold-sensor responses in the presence of aspartate. Six more strains did not give warm-sensor responses in the absence of aspartate but still exhibited cold-sensor responses in the presence of aspartate. Other strains showed inverted thermoresponses. Cells expressing the I2 or O14 mutant proteins gave cold-sensor responses in the absence of aspartate, whereas cells expressing Q11 or O4 gave similar, but weaker, responses. These mutants provide the first examples of Tar receptors that serve as cold sensors in the absence of attractants. In the presence of aspartate, cells expressing I2 still showed a cold-sensor response, albeit a weaker one.

More than half of the mutant receptors showed altered sensitivities to glycerol: 14 mutant receptors mediated no or little response to 10 % glycerol, and

eight mediated no or little response to aspartate in the presence of 10% glycerol (unlike wild-type Tar). These altered sensitivities may reflect the ability of glycerol to act on the transmembrane regions of the receptors, probably by altering their packing (Oosawa & Imae, 1983).

We found additional mutations (D142G and V433A) in I2 and O14 as well as their parental Tar. These substitutions may have occurred during the initial cloning of the *tar* gene (Maruyama *et al.*, 1995). We removed these extra mutations by recombination, using the medium-copy-number plasmid (pBR322) rather than the original high-copy-number plasmid (pBluescript). Of the resulting ''cured'' receptors (named Tar-I2 and Tar-O14), the former still showed an attractant-independent cold-sensor phenotype (Figure 2(a)), whereas the latter no longer did. Therefore, we concluded that the altered TM2 sequence of I2 is responsible for converting Tar from a warm sensor to a cold sensor in an attractant-independent fashion.

The attractant-independent inversion of thermosensing associated with Tar-I2 might be correlated with abnormally enhanced methylation. We examined the methylation pattern of Tar-I2 by immunoblotting (Figure 2(b)) and radiolabeling (not shown) analyses. In strain CP553, which lacks CheB and CheR, Tar cannot be deamidated nor methylated and therefore it produced a single band in SDS-PAGE, whose mobility is presumed to be similar to that of doubly methylated species (Figure 2(b), lanes WT·CP and I2·CP). In strain HCB339 ($CheB^+$ $CheR^+$), Tar-I2 was more heavily methylated than wild-type Tar in the absence of aspartate (Figure 2(b) compare lanes WT·− and I2·−). In the presence of aspartate, Tar-I2 became still further methylated (Figure 2(b), compare lanes I2·+ and I2·−), demonstrating that the methylation level of Tar-I2 can still be regulated in response to aspartate.

To examine whether enhanced methylation of Tar-I2 is required for the attractant-independent inversion of thermosensing, we introduced the plasmid expressing Tar-I2 into two methyltransferase-defective strains, CP553 ($CheB^-$, $CheR^-$) and CP553 carrying the CheB-overproducing plasmid pKB24 (Borkovich *et al.*, 1992). Both strains showed extremely smooth-biased swimming and did not produce any thermoresponse. Moreover, neither of these strains responded to the repellent glycerol. Therefore, we could not conclude that methylation of Tar-I2 is essential for the attractant-independent inversion of thermosensing.

The TM2 sequence of Tar-I2 is extremely different from that of wild-type Tar, differing at 20 of 21 positions (Figure 1). What properties of TM2 cause t-

Table 2. Effects of various wild-type/I2 hybrid TM2 sequences on chemo- and thermosensing properties of Tar

Tar[a]	TM2[b]	Chemoresponses[d] Swimming bias[c]	+Glyc	+Asp + Glyc	Thermoresponse[e]
Wild-type		TR	+	$< 10^{-6}$ (M)	Warm
Tar-I2		SR	+	$< 10^{-6}$	Cold
I2(188-200)		SR	+	$< 10^{-6}$	Cold
I2(201-210)		SR	+	$< 10^{-6}$	Warm?
I2(188-196)		TR	+	$< 10^{-6}$	Cold
I2(197-210)		SR	+	$< 10^{-6}$	Cold
I2(188-203)		S	+	$< 10^{-6}$	Cold?
I2(204-210)		TR	+	$< 10^{-6}$	Warm?
I2(188-196/204-210)		SR	+	$< 10^{-6}$	Cold
I2(197-203)		SR	+	$< 10^{-6}$	Cold
I2(197-200)		SR	+	$< 10^{-6}$	Warm?

[a] The hybrids between the wild-type and I2 *tar* genes were constructed using a two-step polymerase chain reaction (PCR; Landt *et al.*, 1990). Primers used for PCR were synthesized by Sawadi Technology (Tokyo). PCR was carried out using ExTaq polymerase (Takara Shuzo, Kyoto) with 25 cycles of denaturing at 94 °C for 30 seconds, annealing at 50 °C for 1 minute, and extension at 72 °C for 2 minutes. The resulting hybrid genes were subcloned into the corresponding region of pNI101.

[b] The grey and white boxes indicate the sequences of wild-type Tar and Tar-I2, respectively.

[c] Swimming bias was assayed as described in Table 1.

[d] Chemoresponses to glycerol and aspartate were assayed as described in Table 1.

[e] Thermoresponse in the absence of aspartate was assayed as described in Table 1. Question marks indicate weak responses.

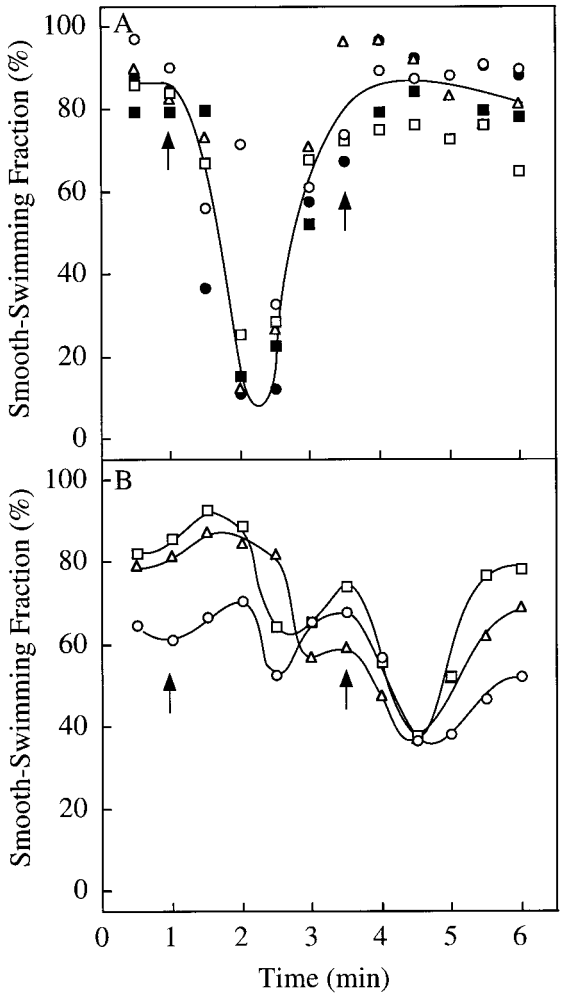

Figure 3. Thermoresponses mediated by receptors with various hybrid TM2 regions. Thermoresponses were measured in the absence of attractant as described in the legend to Figure 2(a). (a) Attractant-independent cold-sensor responses mediated by Tar-I2(188-200) (○), Tar-I2(188-196) (□), Tar-I2(197-210) (△), Tar-I2(188-196/204-210) (●), and Tar-I2(197-203) (■). (b) Weak warm-sensor responses mediated by Tar-I2(201-210) (○), Tar-I2(204-210) (□), or Tar-I2(197-200) (△).

he inversion of thermosensing? To address this question, we constructed various hybrid TM2 sequences that contain different contributions from wild-type Tar and Tar-I2. (The receptors derived from Tar-I2 will be designated as Tar-I2(188-200) etc. with the numbers in the parentheses indicating residues from Tar-I2.)

All of the hybrid receptors had normal aspartate-sensing ability, but their thermosensing properties varied (Table 2). Cells expressing Tar-I2(188-200) showed a cold-sensor response in the absence of aspartate, suggesting that the NH_3-terminal half of the TM2 sequence of Tar-I2 is sufficient for the inversion (Figure 3(a)). Cells expressing the reciprocal hybrid, Tar-I2(201-210) gave a warm-sensor response in the absence of aspartate, but the response was very weak (Figure 3(b)).

Cells expressing Tar-I2(188-196) also showed a cold-sensor response without aspartate (Figure 3(a)), suggesting that the NH_3-terminal third of the TM2 sequence of Tar-I2 is sufficient for the inversion. Surprisingly, cells expressing the reciprocal hybrid (Tar-I2(197-210)) also produced a cold-sensor response without aspartate, suggesting that another inversion determinant exists within the COOH-terminal two thirds of the TM2 sequence. This determinant must be within the middle third of the TM2 sequence, because cells expressing Tar-I2(197-203) exhibited a cold-sensor response without aspartate, whereas cells expressing Tar-I2(204-210) showed a weak warm-sensor response.

Tar-I2(197-203) contains the middle third of TM2 from Tar-I2. Since Tar-I2(201-210) is a weak warm sensor in the absence of aspartate, the last half of TM2 from Tar-I2 is not sufficient for the inverted response. Cells expressing Tar-I2(197-200), which has the first half of the mutant sequence of Tar-I2(197-203), did not give a cold-sensor response, but rather showed a weak warm-sensor response in the absence of aspartate (Figure 3(b)). These results suggest that the seven residues in Tar-I2(197-203) that cause inversion contain elements from both the first and second halves of TM2.

Thus, two distinct regions of TM2 in Tar-I2 lead to attractant-independent inversion of the thermoresponse. However, cells expressing Tar-I2(188-203), which has both regions causing inversion but the last third of wild-type TM2, showed only a very weak cold-sensor response, indicating that the entire TM2 sequence contributes to thermosensing function.

What residues are necessary for attractant-independent inversion of thermosensing in Tar-I2(188-196)? All of the Tar receptors with randomized TM2 sequences had the substitutions R188G and F189S, which were introduced to create a *Bam*HI site for cassette mutagenesis (Maruyama *et al.*, 1995). When we changed these residues back to their wild-type counterparts (Krikos *et al.*, 1983), the resulting receptor (Tar-I2(190-196)) still behaved like Tar-I2, mediating cold-sensor responses in the absence of aspartate (data not shown).

We then reintroduced the wild-type residues one by one into Tar-I2(190-196) starting with the COOH-terminal residue of the mutant sequence (Table 3). Replacement of Cys193 with the wild-type residue Gln changed the thermosensing properties dramatically, whereas replacement of the NH_3-terminal residue (Val190 to Ala) had almost no effect. These results suggest that Cys193 is required for the inversion of thermosensing.

Cells expressing Tar-I2(190-192) tumbled both upon an increase and a decrease in temperature (Figure 4(a)). Tar-I2(190-192) may be an attractant-independent, methylation-dependent cold sensor. Methylated Tar-I2(190-192) may mediate a tumbling response upon an increase in temperature, which should cause the receptor to become

Table 3. Effects of various substitutions in the TM2 sequence on chemo- and thermosensing properties of Tar

Tar[a]	TM2 sequence[b]	Swimming bias[c]	Host: HCB339 ($CheB^-$ $CheR^-$) Chemoresponse[d] +Glyc	+Glyc + Asp	Thermoresponse[e]	Host: CP553 ($CheB^-$ $CheR^-$) Chemoresponse (Glyc)[d]	Thermoresponse[e]
Wild type	AQWQLAV	SR	+	$< 10^{-6}$ (M)	Warm	+	None
Tar-I2	VVRCLRL	SR	+	$< 10^{-6}$	Cold	−	None
I2(190-195)	VVRCLRV	TR	+	$< 10^{-6}$	Cold	−	None
I2(190-193)	VVRCLAV	TR	+	$< 10^{-6}$	Cold	−	None
I2(190-192)	VVRQLAV	TR	+	$< 10^{-6}$	Cold??	+	Cold (5%)
I2(191-193)	AVRCLAV	SR	+	$< 10^{-6}$	Cold	+	Cold (5%)
A190V	VQWQLAV	TR	+	$< 10^{-6}$	Warm	NT	NT
Q191V	AVWQLAV	TR	+	$< 10^{-6}$	Warm	NT	NT
W192R	AQRQLAV	T	ND	2×10^{-6}	Warm	NT	NT
Q193C	AQWCLAV	TR	+	$< 10^{-6}$	Cold?	+	Cold (7%)
W192RA·Q193C	AQRCLAV	TR	+	$< 10^{-6}$	Cold?	+	Cold (7%)

[a] The pBR322-based plasmid encoding Tar-W192R (Oosawa & Simon, 1986) was provided by K. Oosawa. The other site-directed mutant *tar* genes were constructed as described in Table 2.
[b] The first seven residues (190-196) of the TM2 sequences are shown. Underlined letters indicate mutant residues.
[c] Swimming bias was assayed as described in Table 1.
[d] Chemoresponses to glycerol and aspartate were assayed as described in Table 1. ND, not determined because cells swam too tumbly to assay their repellent response.
[e] Thermoresponse in the absence of aspartate was assayed as described in Table 1. Warm, warm-sensor response; Cold, cold-sensor response; Cold?, weak cold-sensor response; Cold??, atypical thermoresponse, which may be regarded as attractant-independent, methylation dependent cold-sensor response (see text for detail); None, no thermoresponse; (5% or 7%), observed in the presence of 5% or 7% glycerol.

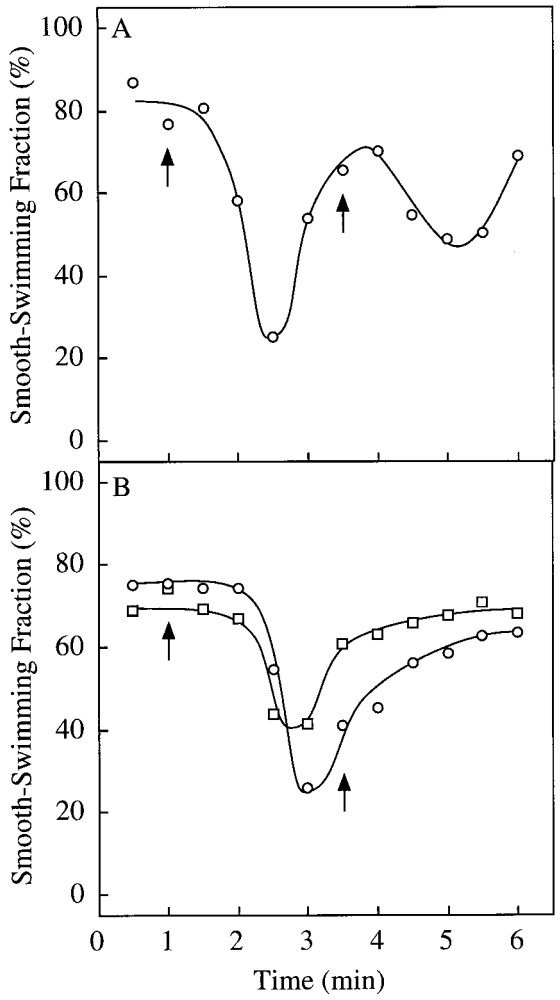

Figure 4. Thermoresponses mediated by Tar receptors with variously substituted TM2 sequences. Thermoresponses were measured in the absence of attractant as described in the legend to Figure 2(a). (a) The atypical thermoresponse (i.e. tumbling both upon an increase and a decrease in temperature) mediated by Tar-I2 (190-192) (○). (b) Attractant-independent cold-sensor responses mediated by Tar-W192R·Q193C (○) or Tar-Q193C (□).

demethylated during adaptation. The unmethylated form of Tar-I2(190-192) may serve as a warm sensor to mediate a tumbling response upon a decrease in temperature and to become methylated again during adaptation. It is consistent with this interpretation that Tar-I2(190-192) meditates a cold-sensor response in strain CP553 ($CheB^-$ $CheR^-$), in which the methylation site of Tar is fixed to QEQE (Table 3), which mimics a methylated state of the receptor.

In a complementary approach, we replaced wild-type residues singly with the corresponding residues from I2 (A190V, Q191V, W192R, and Q193C). HCB339 cells expressing Tar-A190V, Q191V, or W192R showed warm-sensor responses without aspartate. In contrast, cells expressing Tar-Q193C or the double mutant W192R·Q193C gave weak cold-sensor responses in the absence of aspartate (Figure 4(b)). Based on these results, we conclude that the tripeptide sequence Val191-Arg192-Cys193 in Tar-I2(188-196) is sufficient for the full manifestation of the inverted thermoresponse.

Among these substitutions, Q193C might be the most critical one, since Tar with the Q193C substitution alone mediated a weak cold-sensor response without aspartate. Gln193 is predicted to face TM1 of the same subunit in the Tar homodimer (Chervitz & Falke, 1995). Therefore, the mutation might affect the intrasubunit interaction between TM1 and TM2. Intersubunit disulfide cross-linking through Cys193 is unlikely to take place, because such cross-linking has not been detected for Tar-Q193C even in the presence of an oxidant (Pakula & Simon, 1992).

Mutational analyses of the methylation sites of Tar detected three discrete types of thermoresponses among differentially methylated states of the protein; Tar could exist in forms that produce warm-, cold-, or null-thermosensor responses (Nara *et al.*, 1996; Nishiyama *et al.*, 1997). Here we found various intermediate thermoresponses. Tar-I2(201-210) and I2(202-210) appear to be weak warm sensors, whereas I2(197-200), Tar-Q193C, and Tar-W192R·Q193C seem to be weak cold sensors. These weak responses are reminiscent of thermoresponses in the presence of sub-saturating concentrations of aspartate (Mizuno & Imae, 1984).

Thus, the wild-type TM2 sequence appears to be tuned for thermosensing, although it is relatively tolerant for mutations in terms of chemosensing (Jeffery & Koshland, 1994; Tatsuno *et al.*, 1994; Maruyama *et al.*, 1995; Baumgartner & Hazelbauer, 1996). Further investigation of the role of TM2 in thermosensing should also help in elucidating the role of TM2 in chemotactic signaling.

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References

Baumgartner, J. W. & Hazelbauer, G. L. (1996). Mutational analysis of a transmembrane segment in a bacterial chemoreceptor. *J. Bacteriol.* **178**, 4651-4660.

Borkovich, K. A., Alex, L. A. & Simon, M. I. (1992). Attenuation of sensory receptor signaling by covalent modification. *Proc. Natl Acad. Sci. USA*, **89**, 6756-6760.

Boyd, A. & Simon, M. I. (1980). Multiple electrophoretic forms of methyl-accepting chemotaxis proteins gen-

erated by stimulus-elicited methylation in *Escherichia coli*. *J. Bacteriol.* **143**, 809-815.

Burrows, G. G., Newcomer, M. E. & Hazelbauer, G. L. (1989). Purification of receptor protein Trg by exploiting a property common to chemotactic transducers of *Escherichia coli*. *J. Biol. Chem.* **264**, 17309-17315.

Chelsky, D. & Dahlquist, F. W. (1980). Structural studies of methyl-accepting chemotaxis proteins of *Escherichia coli*: evidence for multiple methylation sites. *Proc. Natl Acad. Sci. USA*, **77**, 2434-2438.

Chen, X. & Koshland, D. E., Jr. (1997). Probing the structure of the cytoplasmic domain of the aspartate receptor by targeted disulfide cross-linking. *Biochemistry*, **36**, 11858-11864.

Chervitz, S. A. & Falke, J. J. (1995). Locked on/off disulfides identify the transmembrane signaling helix of the aspartate receptor. *J. Biol. Chem.* **270**, 24043-24053.

Chervitz, S. A. & Falke, J. J. (1996). Molecular mechanism of transmembrane signaling by the aspartate receptor: A model. *Proc. Natl Acad. Sci. USA*, **93**, 2545-2550.

Chervitz, S. A., Lin, C. M. & Falke, J. J. (1995). Transmembrane signaling by the aspartate receptor: engineered disulfides reveal static regions of the subunit interface. *Biochemistry*, **34**, 9722-9733.

Cochran, A. G. & Kim, P. S. (1996). Imitation of *Escherichia coli* aspartate receptor signaling in engineered dimers of the cytoplasmic domain. *Science*, **271**, 1113-1116.

Danielson, M. A., Bass, R. B. & Falke, J. J. (1997). Cysteine and disulfide scanning reveals a regulatory α-helix in the cytoplasmic domain of the aspartate receptor. *J. Biol. Chem.* **272**, 32878-32888.

DeFranco, A. L. & Koshland, D. E., Jr (1980). Multiple methylation in processing of sensory signals during bacterial chemotaxis. *Proc. Natl Acad. Sci. USA*, **77**, 2429-2433.

Engström, P. & Hazelbauer, G. L. (1980). Multiple methylation of methyl-accepting chemotaxis proteins during adaptation of E. coli to chemical stimuli. *Cell*, **20**, 165-171.

Falke, J. J., Bass, R. B., Butler, S. L., Chervitz, S. A. & Danielson, M. A. (1997). The two-component signaling pathway of bacterial chemotaxis: a molecular view of signal transduction by receptors, kinases, and adaptation enzymes. *Annu. Rev. Cell Dev. Biol.* **13**, 457-512.

Gardina, P. J. & Manson, M. D. (1996). Attractant signaling by an aspartate chemoreceptor dimer with a single cytoplasmic domain. *Science*, **274**, 425-426.

Gegner, J. A., Graham, D. R., Roth, A. F. & Dahlquist, F. W. (1992). Assembly of an MCP, CheW, and kinase CheA complex in the bacterial chemotaxis signal transduction pathway. *Cell*, **70**, 975-982.

Hughson, A. G. & Hazelbauer, G. L. (1996). Detecting the conformational change of transmembrane signaling in a bacterial chemoreceptor by measuring effects on disulfide cross-linking *in vivo*. *Proc. Natl Acad. Sci. USA*, **93**, 11546-11551.

Iwama, T., Homma, M. & Kawagishi, I. (1997). Uncoupling of ligand-binding affinity of the bacterial serine chemoreceptor from methylation- and temperature-modulated signaling states. *J. Biol. Chem.* **272**, 13810-13815.

Jeffery, C. J. & Koshland, D. E., Jr. (1994). A single hydrophobic to hydrophobic substitution in the transmembrane domain impairs aspartate receptor function. *Biochemistry*, **33**, 3457-3463.

Krikos, A., Mutoh, N., Boyd, A. & Simon, M. I. (1983). Sensory transducers of *E. coli* are composed of discrete structural and functional domains. *Cell*, **33**, 615-622.

Landt, O., Grunert, H. P. & Hahn, U. (1990). A general method for rapid site-directed mutagenesis using the polymerase chain reaction. *Gene*, **96**, 125-128.

Lee, G. F., Dutton, D. P. & Hazelbauer, G. L. (1995). Identification of functionally important helical faces in transmembrane segments by scanning mutagenesis. *Proc. Natl Acad. Sci. USA*, **92**, 5416-5420.

Le, Moual H. & Koshland, D. E., Jr (1996). Molecular evolution of the C-terminal cytoplasmic domain of a superfamily of bacterial receptors involved in taxis. *J. Mol. Biol.* **261**, 568-585.

Le, Moual H., Quang, T. & Koshland, D. E., Jr (1998). Conformational changes in the cytoplasmic domain of the *Escherichia coli* aspartate receptor upon adaptive methylation. *Biochemistry*, **37**, 14852-14859.

Liu, Y., Levit, M., Lurz, R., Surette, M. G. & Stock, J. B. (1997). Receptor-mediated protein kinase activation and the mechanism of transmembrane signaling in bacterial chemotaxis. *EMBO J.* **16**, 7231-7240.

Long, D. G. & Weis, R. M. (1992). Oligomerization of the cytoplasmic fragment from the aspartate receptor of *Escherichia coli*. *Biochemistry*, **31**, 9904-9911.

Lynch, B. A. & Koshland, D. E., Jr (1991). Disulfide cross-linking studies of the transmembrane regions of the aspartate sensory receptor of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **88**, 10402-10406.

Maddock, J. R. & Shapiro, L. (1993). Polar location of the chemoreceptor complex in the *Escherichia coli* cell. *Science*, **259**, 1717-1723.

Maeda, K. & Imae, Y. (1979). Thermosensory transduction in *Escherichia coli* inhibition of the thermoresponse by L-serine. *Proc. Natl Acad. Sci. USA*, **76**, 91-95.

Manson, M. D. (1992). Bacterial motility and chemotaxis. *Advan. Microbial Physiol.* **33**, 277-346.

Maruyama, I. N., Mikawa, Y. G. & Maruyama, H. I. (1995). A model for transmembrane signalling by the aspartate receptor based on random-cassette mutagenesis and site-directed disulfide cross-linking. *J. Mol. Biol.* **253**, 530-546.

Milligan, D. L. & Koshland, D. E., Jr. (1988). Site-directed cross linking: establishing the dimeric structure of the aspartate receptor of bacterial chemotaxis. *J. Biol. Chem.* **263**, 6268-6275.

Milligan, D. L. & Koshland, D. E., Jr (1991). Intrasubunit signal transduction by the aspartate chemoreceptor. *Science*, **254**, 1651-1653.

Mizuno, T. & Imae, Y. (1984). Conditional inversion of the thermoresponse in *Escherichia coli*. *J. Bacteriol.* **159**, 360-367.

Nara, T., Lee, L. & Imae, Y. (1991). Thermosensing ability of Trg and Tap chemoreceptors in *Escherichia coli*. *J. Bacteriol.* **173**, 1120-1124.

Nara, T., Kawagishi, I., Nishiyama, S., Homma, M. & Imae, Y. (1996). Modulation of the thermosensing profile of the *Escherichia coli* aspartate receptor Tar by covalent modification of the methyl-accepting site. *J. Biol. Chem.* **271**, 17932-17936.

Nishiyama, S., Nara, T., Imae, Y., Homma, M. & Kawagishi, I. (1997). Thermosensing properties of mutant aspartate receptors with methyl-accepting sites replaced multiply or singly by alanine. *J. Bacteriol.* **179**, 6573-6580.

Nishiyama, S., Umemura, T., Nara, T., Homma, M. & Kawagishi, I. (1999). Conversion of a bacterial warm sensor to a cold sensor by methylation at a single residue in the presence of an attractant. *Mol. Microbiol.* In the press.

Okumura, H., Nishiyama, S., Sasaki, A., Homma, M. & Kawagishi, I. (1998). Chemotactic adaptation is altered by changes in the carboxy-terminal sequence conserved among the major methyl-accepting chemoreceptors. *J. Bacteriol.* **180**, 1862-1868.

Oosawa, K. & Imae, Y. (1983). Glycerol and ethylene glycol: members of a new class of repellents of *Escherichia coli* chemotaxis. *J. Bacteriol.* **154**, 104-112.

Oosawa, K. & Simon, M. I. (1986). Analysis of mutations in the transmembrane region of aspartate chemoreceptor in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **83**, 6930-6934.

Pakula, A. A. & Simon, M. I. (1992). Determination of transmembrane protein structure by disulfide cross-linking: the *Escherichia coli* Tar receptor. *Proc. Natl Acad. Sci. USA*, **89**, 4144-4148.

Parkinson, J. S. (1993). Signal transduction schemes of bacteria. *Cell*, **73**, 857-871.

Schuster, S. C., Swanson, R. V., Alex, L. A., Bourret, R. B. & Simon, M. I. (1993). Assembly and function of a quaternary signal transduction complex monitored by surface plasmon resonance. *Nature*, **365**, 343-347.

Stock, J. B. & Surette, M. G. (1996). Chemotaxis. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Neidhardt, F. C., Curtiss, R., III, Ingram, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. & Umbarger, H. E., eds), 2nd edit., pp. 1103-1129, American Society for Microbiology, Washington, DC.

Surette, M. G. & Stock, J. B. (1996). Role of α-helical coiled-coil interactions in receptor dimerization, signaling, and adaptation during bacterial chemotaxis. *J. Biol. Chem.* **271**, 17966-17973.

Tatsuno, I., Lee, L., Kawagishi, I., Homma, M. & Imae, Y. (1994). Transmembrane signalling by the chimeric chemosensory receptors of *Escherichia coli* Tsr and Tar with heterologous membrane-spanning regions. *Mol. Microbiol.* **14**, 755-762.

Tatsuno, I., Oosawa, K., Homma, M. & Kawagishi, I. (1996). Signaling by the *Escherichia coli* aspartate chemoreceptor Tar with a single cytoplasmic domain per dimer. *Science*, **274**, 423-425.

Taylor, B. L. & Johnson, M. S. (1998). Rewiring a receptor: negative output from positive input. *FEBS Letters*, **425**, 377-381.

Trammell, M. A. & Falke, J. J. (1999). Identification of a site critical for kinase regulation on the central processing unit (CPU) helix of the aspartate receptor. *Biochemistry*, **38**, 329-336.

Umemura, T., Tatsuno, I., Shibasaki, M., Homma, M. & Kawagishi, I. (1998). Intersubunit interaction between transmembrane helices of the bacterial aspartate chemoreceptor homodimer. *J. Biol. Chem.* **273**, 30110-30115.

Wolfe, A. J., Conley, M. P., Kramer, T. J. & Berg, H. C. (1987). Reconstitution of signaling in bacterial chemotaxis. *J. Bacteriol.* **169**, 1878-1885.

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