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Sensing of Cytoplasmic pH by Bacterial Chemoreceptors Involves the Linker Region That Connects the Membrane-spanning and the Signal-modulating Helices*

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The two major chemoreceptors of *Escherichia coli*, Tsr and Tar, mediate opposite responses to the same changes in cytoplasmic pH (pH_i). We set out to identify residues involved in pH_i sensing to gain insight into the general mechanisms of signaling employed by the chemoreceptors. Characterization of various chimeras of Tsr and Tar localized the pH_i-sensing region to Arg^{259}–His^{267} of Tar and Gly^{261}–Asp^{269} of Tsr. This region of Tar contains three charged residues (Arg^{259}–Ser^{261}, Asp^{263}, and His^{267}) that have counterparts of opposite charge in Tsr (Gly^{261}–Glu^{262}, Arg^{265}, and Asp^{269}). The replacement of all of the three charged residues in Tar or Arg^{259}–Ser^{260} alone by the corresponding residues of Tsr reversed the polarity of pH_i response, whereas the replacement of Asp^{263} or His^{267} did not change the polarity but altered the time course of pH_i response. These results suggest that the electrostatic properties of a short cytoplasmic region within the linker region that connects the second transmembrane helix to the first methylation helix is critical for switching the signaling state of the chemoreceptors during pH sensing. Similar conformational changes of this region in response to external ligands may be critical components of transmembrane signaling.

Many biological processes, such as enzyme reactions and interactions between proteins, are influenced by pH. Therefore, cells have to sense and adapt to changes in extracellular and intracellular pH. Despite the accumulated knowledge about pH-dependent regulation in a wide variety of organisms, the molecular mechanisms of pH sensing are still poorly understood.

Behavioral responses of *Escherichia coli* and *Salmonella typhimurium* to changes in pH provide a convenient system for studying the pH-sensing mechanism. These bacteria show repellent responses to weak acids and attractant responses to weak bases (1, 2). These responses are generated by decreases and increases of cytoplasmic pH (pH_i).[1] The changes in pH_i were documented by ^{31}P nuclear magnetic resonance spectroscopy (3, 4). Usually, pH_i in *E. coli* is maintained at around 7.5 over a range of extracellular pH (pH_o) values from 5.0 to 9.0 (3, 5). However, this strong pH_i homeostasis can be disrupted by the addition of weak acids or weak bases to the culture medium. When pH_o is lower than pH_i, weak acids can traverse the membrane in their protonated (uncharged) form and release protons in the cytoplasm to decrease pH_i. Similarly, when pH_o is higher than pH_i, weak bases can traverse the membrane in deprotonated (uncharged) forms and capture protons in the cytoplasm to increase pH_i. These changes in pH_i correlate well with tactic responses to weak acids and weak bases (4).

The signal transduction pathway for chemotaxis in *E. coli* and *S. typhimurium* has been extensively studied at the molecular level (for reviews, see Refs. 6–9). These organisms have a set of related methyl-accepting chemoreceptors that includes the serine receptor Tsr and the aspartate receptor Tar. These receptors have a remarkable ability to sense a variety of stimuli, including chemoattractants, chemorepellents, temperature, and pH.

Tar and, presumably, the other chemoreceptors exist as a homodimer of about 60-kDa subunits (10). The dimeric cytoplasmic domains form stable complexes with the histidine kinase CheA and the adaptor protein CheW (11, 12). Furthermore, the receptors, together with the CheA and CheW proteins, cluster at a cell pole (13).

CheA phosphorylates itself and then serves as a phosphodonor for the response regulator CheY. The phosphorylated form of CheY interacts with the flagellar motor to promote 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.

In principle, any step in this signal transduction pathway can be influenced by pH. In fact, the activity of the histidine kinase CheA depends sharply on pH (14). However, the chemoreceptors have been considered to be the primary sensors for pH_i, because the two major chemoreceptors, Tsr and Tar, have opposite pH_i-sensing properties (15). When expressed as a sole chemoreceptor, Tsr mediates repellent and attractant responses to decreases and increases in pH_i, respectively, whereas under these conditions Tar mediates the responses

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[1] The abbreviations used are: pH_i, cytoplasmic pH; pH_o, extracellular pH; MGM, motility medium containing glycerol; MH, methylation helix; TM, transmembrane helix.

with opposite polarity. These receptors also sense changes in pH_o (15). Moreover, the minor chemoreceptors Trg and Tap mediate Tar- and Tsr-type responses, respectively, to changes in pH_i when they are expressed as the sole chemoreceptor (16). In a wild-type *E. coli* cell, pH_i responses mediated by Tsr predominate, presumably because Tsr is the most abundant chemoreceptor. In *Vibrio cholerae*, the related TcpI receptor is responsible for the pH_o-dependent regulation of the expression of the toxin-coregulated pilus (17). Therefore, the ability to sense pH might be an intrinsic property of this receptor family.

It is not clear why Tar and Tsr are different in their pH-sensing properties. However, this difference is very useful in understanding the mechanisms of receptor signaling. Krikos *et al.* (15) showed that the cytoplasmic regions of Tar (residues 256–468) and Tsr (residues 258–470) are responsible for their differential pH_i-sensing properties. Furthermore, Oosawa *et al.* (18) showed that cells expressing a C-terminal cytoplasmic fragment of Tar (residues 256–553) can mediate responses to a weak acid, indicating that this fragment suffices for modulating the activity of CheA kinase in response to changes in pH_i.

In this study, we constructed a new series of chimeric receptors between Tar and Tsr and identified their pH_i-sensing regions. Subsequent mutational analysis revealed the key residues: When these residues were swapped between Tar and Tsr, the pH_i response was inverted.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—All strains used in this study are derivatives of *E. coli* K-12. Strain HCB339 (Δ(*tar-tap*)*5021* Δ*tsr-7028 trg*::Tn*10 thr leu his met rpsL136* (19)) was used as the plasmid host in chemotaxis assays. Strain DH5α (F^- λ^- *recA1 hsdR17 endA1 gyrA96 supE44 relA1 thi-1* Δ(*argF-lacZYA*)*U169* ϕ80d*lacZ*Δ*M15* (20)) was used for DNA manipulation.

Plasmids pFH2 and pFH5,[2] which carry the *tar* and *tsr* genes, respectively, were constructed by subcloning the *Eco*RI fragments of pLAN931 and pLAN1031 (21) into the *Eco*RI site of pSU18 (22). Plasmid pSU18 was digested with *Pst*I and *Sac*I, blunted with T4 DNA polymerase (Takara Shuzo), and ligated with a *Bgl*II linker to yield plasmid pSU18ΔPS, which lacks the *Kpn*I site in the multicloning site.[2] Plasmid pFH101[2] was constructed by subcloning the *tar*-containing fragment of pLAN931 into the *Eco*RI site of pSU18ΔPS. Plasmids pAB157, pAB160, pTsar-Cla, and pTsar-Nde carrying the chimeric genes encoding Tasr-468, Tasr-256, Tsar-468, and Tsar-256, respectively, were provided by M. I. Simon of the California Institute of Technology (15).[3]

Construction of Chimeric Chemoreceptor Genes—The pUC118-based plasmids, which encode the Tar-Tsr hybrids Tasr-502, Tasr-441, Tasr431, Tasr-375, and Tasr-309 (Tar residue numbering), were constructed by spontaneous homologous recombination between *tar* and *tsr* genes placed in tandem (23). Similarly, the pUC118-based plasmids that encode the Tsr-Tar hybrids Tsar-470, Tsar-441, Tsar-412, Tsar-354, and Tsar-309 were constructed by homologous recombination between tandem *tsr* and *tar* genes. The *Hin*dIII-*Eco*RI fragments containing the chimeric receptor genes from these plasmids were subcloned into the multicloning site of the medium copy number plasmid pSU18.

Oligonucleotide-based Cassette Mutagenesis of tar and tsr—To construct the sandwiched chimeric Tar (Tasar) and the point-mutant Tar receptors, oligonucleotide-based cassette mutagenesis was carried out as follows. We designed pairs of mutagenic oligonucleotides with overlapped regions of about 20 nucleotides at their 3′-ends (synthesized by Sawadi Technology Co., Tokyo). Each pair of oligonucleotides was used for polymerase chain reaction with ExTaq polymerase (Takara Shuzo, Kyoto) to replace the wild-type sequence of the *tar* gene. The resulting fragments were digested with *Nde*I and *Kpn*I and subcloned into the corresponding region of pFH101 to yield plasmids encoding the desired chimeric or mutant receptors. The plasmid encoding Tsasr was constructed in a similar fashion using the *Nde*I and *Bss*HII sites of the *tsr* gene. The DNA sequences were verified by the dideoxy chain-termination method using reagents from Amersham Biosciences, Inc.

Swarm Assay for Chemotaxis—Swarming ability was determined as described previously (24) using Tryptone semisolid agar (1% Tryptone, 0.5% NaCl, 0.25% agar) or minimal semisolid agar (50 mM potassium phosphate buffer (pH 7.0), 1 mM $MgSO_4$, 1 mM glycerol, 0.1 mM each of threonine, leucine, histidine, and methionine, 1 mg/ml thiamine, 1 mM $(NH_4)_2SO_4$, 0.25% agar) supplemented with 0.1 mM aspartic acid. Chloramphenicol (25 μg/ml) was added as required. Semisolid agar was inoculated with aliquots of overnight cultures (about 4×10^6 cells) and incubated at 30 °C.

Temporal Stimulation Assay of Chemotactic Responses—The temporal stimulation assay was carried out essentially as described previously (25) with some modification. Changes in cytoplasmic pH (pH_i) were elicited by the addition of sodium benzoate or acetate (2). Cells were grown at 30 °C in TG broth (1% Tryptone, 0.5% NaCl, 0.5% (w/v) glycerol) with chloramphenicol. Cells were harvested at late exponential phase, washed twice with motility medium (MGM) adjusted to pH 6.0 or pH 7.4 (10 mM potassium phosphate buffer, 0.1 mM EDTA, 0.1 mM methionine, 1 mM glycerol), and resuspended in MGM at room temperature. Serine, sodium aspartate, or sodium benzoate (pH 6.0) was added to the cell suspension, and aliquots were taken at intervals for microscopic observation. The swimming pattern of the cells was observed with a dark-field microscope and recorded on videotape. The smooth-swimming fraction of the cells was determined by analysis of the video recording with an Argus-10 image processor (Hamamatsu Photonics K. K., Shizuoka).

Immunoblot Analysis of Chemoreceptor Proteins—Receptor expression and methylation were examined by immunoblot analysis as described previously (26) with slight modification.

RESULTS

Characterization of the pH_i-sensing Properties of Tar/Tsr and Tsr/Tar Chimeras—To narrow down the pH_i-sensing regions, we created a new series of chimeric receptors by spontaneous homologous recombination between the *tar* and *tsr* genes placed in tandem on linearized plasmids (23). The resulting chimeric receptors, of which the N- and C-terminal regions were derived either from Tar and Tsr, were named Tasr, using the Tar residue at the junction point to differentiate different hybrids. Similarly, the chimeric receptors with the N-terminal Tsr sequences and the C-terminal Tar sequences were named Tsar, using the same nomenclature with Tar residue numbers.[4]

The chimeric plasmid-borne genes were introduced into strain HCB339, which lacks the four chemoreceptors Tsr, Tar, Trg, and Tap. The resulting transformants were tested for their swarming ability. In Tryptone semisolid agar, cells expressing any Tasr or Tsar protein formed swarm rings, suggesting that the chimeric receptors were expressed and supported chemotaxis.

We then examined their pH_i-sensing properties in the temporal stimulation assay. Cells expressing wild-type Tar, or Tasr-309, -431, -441, or -502, showed attractant responses to a decrease in pH_i (Fig. 1). Cells expressing Tasr-256 showed a repellent response, like cells expressing wild-type Tsr. On the other hand, cells expressing wild-type Tsr or Tsar-309, -354, -412, or -441 showed repellent responses to a decrease in pH_i. Cells expressing Tsar-256 showed an attractant response to the same stimulation. These results suggest that residues 256–309 of Tar and residues 258–311 of Tsr are responsible for pH_i sensing.

Replacement of Short Cytoplasmic Sequences of Tar by the Corresponding Tsr Sequence Inverts the Polarity of pH_i Sensing—To confirm that the identified region is responsible for pH_i sensing, we constructed two "sandwich-type" chimeric receptors, Tasar-256–278 and Tasar-256–267, in which short stretches of the cytoplasmic domain of Tar were replaced by the corresponding Tsr sequences. Immunoblotting analysis demonstrated that these sandwiched chimeras were expressed at levels comparable to wild-type Tar (Fig. 2). HCB339 cells expressing these mutant proteins were tested for their swarming

[2] F. Hattori, M. Homma, and I. Kawagishi, unpublished.
[3] M. I. Simon, personal communication.
[4] In this report, we adopt this system to rename the published and unpublished chimeric receptors obtained from M. I. Simon.

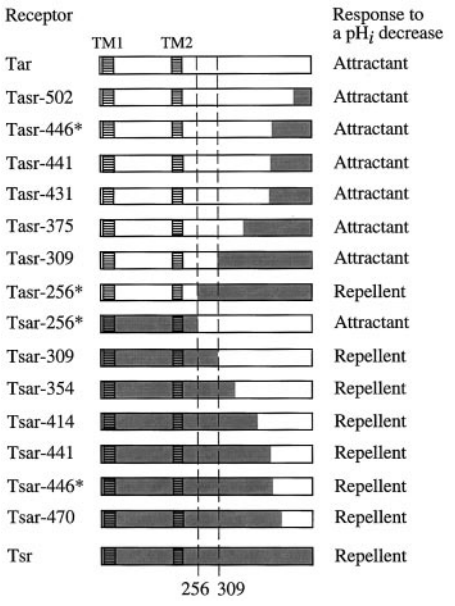

FIG. 1. **Chimeras between the Tsr and Tar chemoreceptors (Tsar and Tasr) and their pH$_i$-sensing properties.** *Gray* and *white portions* indicate the amino acid sequences of Tsr and Tar, respectively. *Hatched boxes* indicate transmembrane regions. The receptors marked with *asterisks* were provided by M. I. Simon (15).[3]

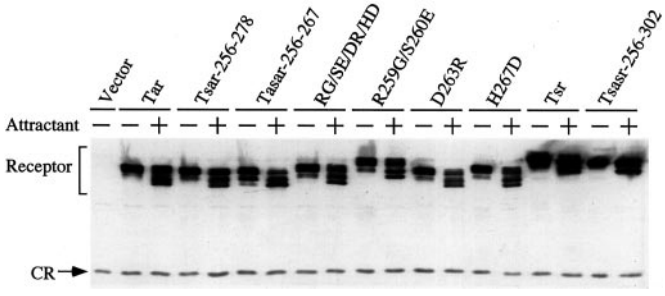

FIG. 2. **Expression levels and methylation patterns of the sandwiched Tar chimeras (Tasar) and the point-mutant Tar receptors.** HCB339 cells expressing wild-type, chimeric, or mutant Tar proteins were incubated in the presence or absence of 10 mM aspartate. Subsequently, their cell lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-Tsr serum. *RG/SE/DR/HD*, Tar-R259G/S260E/D263R/H267D; *R259/S260E*, Tar-R259/S260E; *D263R*, Tar-D263R; *H267D*, Tar-H267D. *CR*, an unspecified protein cross-reacting with the serum.

ability (Fig. 3). Additionally, we tested the attractant responses of these cells to aspartate directly by the temporal stimulation assay (Fig. 4). In minimal semisolid agar containing aspartate, cells expressing Tasar-256–278 produced a swarm ring comparable to that of wild-type Tar. However, Tasar-256–267 did not support swarming. This defect in swarming could be due to a defect in adaptation, because the temporal stimulation assay showed that the chimeric receptor retained aspartate-sensing ability.

We then examined the responses of cells expressing the Tasar receptors to a decrease in pH$_i$ in the temporal stimulation assay. Typical time courses of responses mediated by wild-type Tar, wild-type Tsr, and the two Tasar chimeric receptors are shown in Fig. 4. When the pH$_o$ is 6.0, cells expressing wild-type Tar and Tsr showed attractant and repellent responses, respectively, after the addition of 3 mM sodium benzoate (pH 6.0). Cells expressing either of the two chimeric receptors (Tasar-256–278 and 256–267) showed weak repellent responses (*i.e.* Tsr-type responses) (Fig. 5). Essentially similar responses were observed for another weak acid, acetate (data not shown). These results indicate that the sequence from

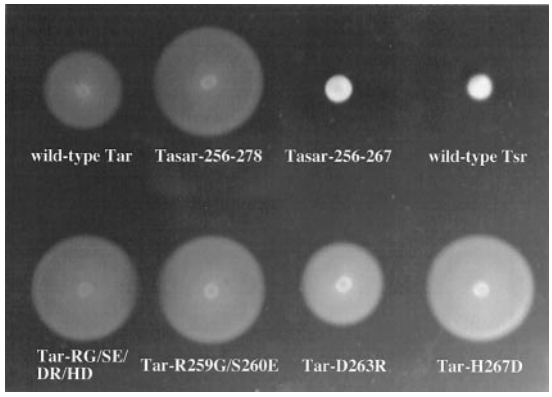

FIG. 3. **Swarming abilities of HCB339 cells expressing the sandwiched chimeric Tar (Tasar) and the point-mutant Tar receptors.** Aliquots (2 μl of each) of overnight cultures were spotted onto minimal semisolid agar containing 0.1 mM aspartate and 25 μg/ml chloramphenicol. The plate was incubated at 30 °C for 20 h.

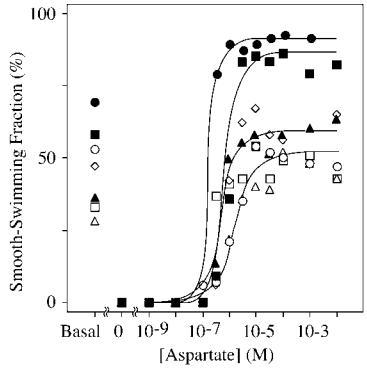

FIG. 4. **Aspartate-sensing ability of the sandwiched Tar chimeras (Tasar) and the point-mutant Tar receptors.** Immediately after the addition of 10% glycerol, various concentrations of aspartate were added to a suspension of HCB339 cells expressing wild-type Tar (*closed circles*), Tasar-256–278 (*closed squares*), Tasar-256–267 (*open squares*), Tar-R259G/S260E (*open circles*), Tar-D263R (*open triangles*), Tar-H267D (*open diamonds*), and Tar-R259G/S260E/D263R/H267D (*closed triangles*). After 20 s, the percentage of smooth-swimming cells was determined. "Basal" indicates the smooth-swimming fraction in the absence of aspartate and glycerol. For simplicity, *lines* are drawn only for wild-type Tar, Tasar-256–278, Tar-R259/S260E, and Tar-R259G/S260E/D263R/H267D.

His^{256} to His^{267} of Tar and the corresponding sequence from His^{258} to Asp^{269} of Tsr are involved in pH$_i$ sensing. Because the tripeptide sequence His^{256}-Met^{257}-Gln^{258} of Tar is perfectly conserved in Tsr, the nonapeptide sequences (Arg^{259}–His^{267} of Tar and Gly^{261}–Asp^{269} of Tsr), in which three residues are conserved between Tar and Tsr, must be relevant (see Fig. 7). However, the responses mediated by the two chimeric receptors were weaker and more transient than that mediated by wild-type Tsr. Some other residues may be required for a complete Tsr-type response, or these chimeric receptors may be somewhat impaired in a general receptor function, such as adaptation.

Replacement of a Short Cytoplasmic Sequences of Tsr by the Corresponding Tar Sequence also Inverts the Polarity of pH$_i$ Sensing—To confirm that this region is responsible for the type of pH$_i$ sensing, we also constructed the complementary sandwiched chimeric receptor, Tsasr-256–319. Expression of this receptor in strain HCB339 and its function as a serine chemoreceptor were confirmed by immunoblotting and in the swarm assay and the temporal stimulation assay, as described for the Tasar receptors (data not shown). As expected, HCB339 cells expressing this receptor showed attractant responses to decreases in pH$_i$ (Fig. 6). Essentially similar responses were

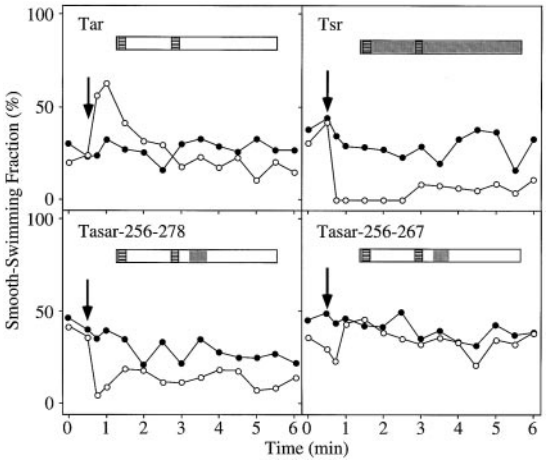

FIG. 5. **Responses of HCB339 cells expressing the sandwiched Tar chimeras (Tasar) to a decrease in pH_i.** Cells expressing an indicated receptor were suspended in MGM at pH 6.0 (*closed circles*) or at pH 7.4 (*open circles*) and incubated at room temperature for 20 min. At the time indicated by an *arrow*, 3 mM sodium benzoate was added.

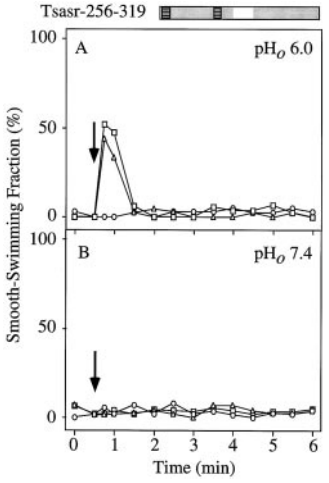

FIG. 6. **Responses of HCB339 cells expressing the sandwiched Tsr chimera (Tsasr) to a decrease in pH_i.** Cells were suspended in MGM at pH 6.0 (*A*) or at pH 7.4 (*B*), and responses to 3 mM (*open circles*), 13.5 mM (*open triangles*), or 22.5 mM (*open squares*) sodium benzoate were determined as described in the legend to Fig. 5. Higher concentrations of sodium benzoate were required for clearer responses probably because the tumble-biased signaling behavior of Tsasr.

observed for another weak acid, acetate (data not shown). Thus, Tsasr mediates a Tar-type (or inverted) response to a decrease in pH_i.

Replacement of Two Consecutive Residues (Arg^{259}-Ser^{260}) of Tar by the Corresponding Tsr Residues (Gly^{261}-Glu^{262}) Inverts the Polarity of pH_i Sensing—Sequence alignment of the pH_i-sensing regions of Tar and Tsr revealed three pairs of residues with opposite charges in Tar and Tsr (Fig. 7). These residues seemed good candidates to be directly involved in pH_i sensing. Therefore, we replaced these three sites in Tar (Arg^{259}/Ser^{260}, Asp^{263}, and His^{267}) by the corresponding residues in Tsr (Gly^{261}/Glu^{262}, Arg^{265}, and Asp^{269}), either individually or in combination. Immunoblotting analysis showed that these proteins were expressed in levels comparable to wild-type Tar (Fig. 2). Their abilities to support chemotaxis were examined in the swarm assay (Fig. 3). The mutant Tar receptors supported formation of swarm rings in minimal semisolid agar containing aspartate.

In the temporal stimulation assay for pH_i taxis, cells expressing the "triple" mutant (Tar-R259G/S260E/D263R/H267D) or one of the "single" mutants (Tar-R259G/S260E) showed repellent responses to a decrease in pH_i, although the duration of these responses was much shorter than that mediated by wild-type Tsr (Fig. 8). Thus, swapping of the two consecutive residues Arg^{259} and Ser^{260} of Tar with the corresponding residues of Tsr inverted the polarity of pH_i sensing. In contrast, cells expressing Tar-D263R or Tar-H267D still gave attractant responses to a decrease in pH_i. However, the response mediated by the H267D mutant Tar was delayed both in its onset and its completion than the response mediated by wild-type Tar. The response mediated by the triple mutant Tar receptor appeared to combine the Tsr-type response of Tar-R259G/S260E and the slow response of Tar-H267D. Essentially similar responses were observed for another weak acid, acetate (data not shown). These results suggest that residues Arg^{259}-Ser^{260} of Tar and Gly^{261}-Glu^{262} of Tsr play important roles in determining the polarity of pH_i sensing and that His^{267} of Tar and Asp^{269} of Tsr might also be involved in modulating the response to changes in pH_i.

DISCUSSION

In this study, we constructed and characterized various Tar/Tsr and Tsr/Tar chimeric receptors and identified the pH_i-sensing region of the chemoreceptors. We also identified the key residue in the receptors that determines the polarity of the pH_i response.

The activities of some of the cytoplasmic signal-transducing proteins, CheA, CheB, CheR, and CheY (14, 27–29) vary with pH *in vitro*, and the other signal-transducing proteins may also be affected by pH. However, because strain HCB339, which lacks the four chemoreceptors but has all of the cytoplasmic signal-transducing proteins, was used as the plasmid host throughout this study, the differences in pH_i-sensing among the strains carrying the various plasmids can be attributed to the chemoreceptors they produce.

To define the region responsible for pH sensing, we constructed a series of chimeric receptors between Tar and Tsr, using homologous recombination between two tandem receptor genes on linearized plasmids. This method can create a wide variety of chimeras between two homologous proteins, because it does not require restriction sites to be present at the chimeric junctions.

The secondary structure of the cytoplasmic region of Tar has been predicted by the sequence alignment and its close examination of the related receptors (30, 31). The three-dimensional structure of a cytoplasmic fragment (residues 286–526) of Tsr was solved (32), but the fragment does not contain the pH_i-sensing region identified in this study (residues 258–280 of Tsr). However, chemical modification and disulfide cross-linking of a series of mutant receptors generated by site-directed introduction of cysteine residues (31, 33–36) have given us a fairly clear picture of the three-dimensional structure of this region. Cysteine-scanning mutagenesis located Arg^{259} of Tar on the solvent-exposed face and Ser^{260} on the buried face of a short helix connecting the second transmembrane helix (TM2) to the first methylation helix (MH1) (31). More recently, disulfide scanning revealed that Ser^{260} of one subunit faces toward Ser^{260} of the partner subunit of the Tar homodimer (35). This arrangement might be slightly different in Tsr, because its counterparts for Arg^{259} and Ser^{260} of Tar are Gly^{261} and Glu^{262}, respectively (Fig. 7).

Several inverted responses mediated by Tsr, Tar, and other chemoreceptors have been reported. Responses to temperature mediated by Tar are inverted when Tar becomes methylated after the addition of aspartate (25, 37, 38) or when certain mutations are introduced into TM2 (39). Responses of *S. typhi-*

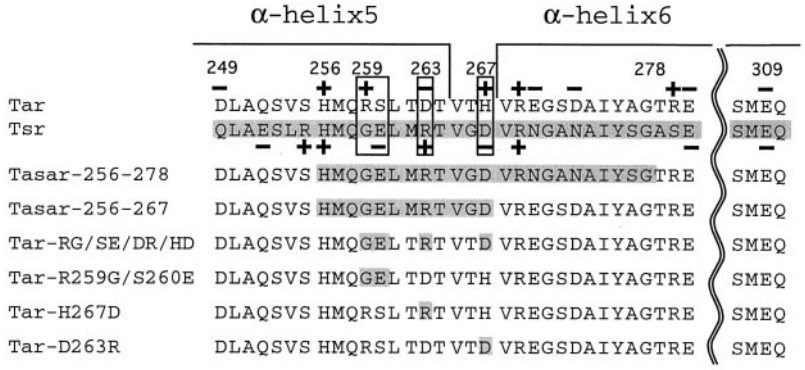

FIG. 7. **Amino acid sequences of the predicted pH_i-sensing regions of Tar and Tsr.** These regions (256–309) were implicated in pH_i taxis based on the results presented in Figs. 5 and 6. The second structures of these regions of Tar have been studied by cysteine-scanning mutagenesis (31). *Numbering* of the residues corresponds to Tar. *Shaded letters* indicate the Tsr sequence. *Boxes* indicate pairs of residues that have opposite charges in Tar and Tsr. The sandwiched chimeras and point-mutant Tar receptors constructed in this study are also shown.

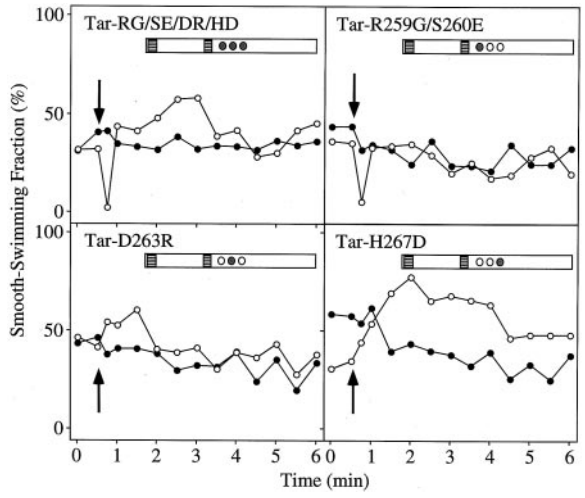

FIG. 8. **Responses of HCB339 cells expressing the point mutant Tar receptors to a decrease in pH_i.** The assays were carried out as described in the legend to Fig. 5.

murium to pH (2) and of *E. coli* to oxygen (40) are inverted when the *cheB* gene is deleted. These inverted responses might result from hypermethylation of the most abundant chemoreceptor, Tsr, in the absence of the methylesterase CheB. Tsr also plays a role in aerotaxis (41, 42). These examples of inverted responses seem to involve changes in the interaction between the methylation helices (MHs) of the relevant receptors (25). The residues identified in this study are located in the C-terminal part of the linker region, *i.e.* the predicted helix and turn preceding MH1, and, therefore, they may regulate the signaling state by altering the interactions among MHs. However, it should be noted that these residues are not necessarily pH sensor residues, although they are responsible for differential responses between Tar and Tsr.

What is the mechanism of pH_i sensing? Binding of a chemoattractant to a chemoreceptor is believed to trigger a subtle but critical inward movement of a continuous helix consisting of helix 4 of the periplasmic domain and TM2 (Ref. 43 and references therein), which in turn may induce a critical movement of MH, which is the central processing unit for control of the cytoplasmic histidine kinase CheA (44). A simple scenario may be that protonation and deprotonation at one or more residues alter the interactions between MHs. However, this cannot be the whole story. For example, the pK_a value of the guanidino group of arginine is 12.48, which is much higher than the physiological pH. Possible explanations to resolve this difficulty include: (i) The pK_a of Arg^{256} is somehow decreased to a physiological range; (ii) Arg^{256} interacts with one or more other residues that accept a proton to alter its interaction with Arg^{256}; and (iii) the absolute value of pH_i might not be the actual signal that is sensed by the chemoreceptors. Although previous studies indicated that changes in pH_i serve as chemotactic stimuli (4), bacterial cells respond to other signals such as changes in proton motive force, oxidation-reduction potential, and membrane potential (45). Therefore, changes in pH_i may affect one or more of these factors, which are sensed by the chemoreceptors. For example, changes in membrane potential would affect the conformation of the linker region and hence the signaling state of the chemoreceptors without involving protonation or deprotonation of the charged residues in the linker region. The presence of opposite charges between Tar and Tsr would result in their opposite polarity of signaling.

The chemoreceptor forms a ternary complex with CheA and the adaptor protein CheW (11, 12) and is localized to the pole of the rod-shaped cell (13). The cytoplasmic fragment of Tar fused to a leucine-zipper forms a well defined supramolecular structure in association with CheA and CheW, and the degree of methylation of the receptor is critical for the stability of the complex (46). A hexagonal receptor-kinase network has been proposed (47) based on the crystallographic trimer of dimers of the cytoplasmic fragment of Tsr (32). Moreover, the rate of formation of the receptor·CheW·CheA complex is greater than that had been expected and is affected by the ligand, raising the possibility that assembly/disassembly of the ternary complex is involved in signaling and adaptation (48). Therefore, interactions within and/or among receptor-kinase complexes might also play critical roles in pH sensing, as has been suggested for receptor signaling and/or signal amplification (Ref. 9 and references therein).

In any case, the mechanism of pH_i sensing seems to be closely related to general receptor function. In this regard, it is intriguing that the linker region is suggested to be involved in sensing of pH_i. Because of the high degree of sequence similarity of this regions among the related chemoreceptors and its location between TM2 and MH1, it has been speculated that this region may play a critical, but perhaps rather passive, role in transmembrane signaling (*e.g.* Refs. 8, 9), although a model involving two amphipathic helices in this region has been proposed (49). For the first time, this report presents experimental evidence that associates this "linker" region to a particular receptor function. Our results raise the possibility that changes in pH_i, and possibly other stimuli, alter the conformation of this part of the receptor and/or affect the way that it interacts with other polypeptides within or outside of the receptor dimer or with the membrane. Determination, of such changes in conformation and/or interactions involved in the pH_i response in an *in vitro* system that reproduces the *in vivo* responses, should provide a valuable insight into the general mechanism of signaling mediated by chemoreceptors.

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