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Cloning and characterization of the *Salmonella typhimurium*-specific chemoreceptor Tcp for taxis to citrate and from phenol

(citrate taxis/DNA sequence/methylation)

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ABSTRACT **_Salmonella typhimurium_ shows an attractant response to citrate and a repellent response to phenol, and a chemoreceptor mediating these responses has been identified and named Tcp (taxis to citrate and away from phenol). Tcp is one of the methyl-accepting chemotaxis proteins that have a molecular mass of ≈60 kDa estimated by SDS/PAGE, and its methylation level is increased by citrate and decreased by phenol. Tcp also mediates an attractant response to metal–citrate complexes. The complete nucleotide sequence of the _tcp_ coding region has been determined. The deduced amino acid sequence of Tcp, consisting of 547-amino acid residues, is homologous with that of the aspartate chemoreceptor of _S. typhimurium_. Thus, Tcp is another member of the bacterial transmembrane chemoreceptor family. Because citrate is a good carbon source for _S. typhimurium_ but is not a carbon source for the closely related species _Escherichia coli_ and because citrate utilization is used as a key diagnostic character to distinguish these species, it is reasonable to assume that Tcp is specific to _S. typhimurium_.**

In the chemotactic signal-transduction pathway of bacteria the transmembrane chemoreceptors have a role in the first step; they detect the chemoeffectors in the environment, transmit their information through the cytoplasmic membrane, and trigger the production of the signal that controls chemotactic behavior. In *Escherichia coli* and the closely related species *Salmonella typhimurium*, such chemoreceptors are homologous and constitute a receptor family. Their molecular masses are ≈60 kDa, and they are organized in the membrane with a short N-terminal cytoplasmic region, a transmembrane segment, a periplasmic ligand-binding domain, a second transmembrane segment, and a cytoplasmic domain for signal production (for reviews, see refs. 1 and 2). In the cytoplasmic domain, four or five methyl-accepting glutamic acid residues mediate behavioral adaptation (2).

Three such chemoreceptors (Tar for aspartate, Tsr for serine, and Trg for ribose and galactose) are found in both species. However, responses to some chemoeffectors differ in the two species; *S. typhimurium* is attracted by citrate and repelled by phenol but shows no response to dipeptides and maltose, whereas *E. coli* is attracted by dipeptides, maltose, and phenol but shows no response to citrate (3). This evidence suggests that there should be species-specific chemoreceptors in these bacteria. Tap, which mediates the dipeptide response, has previously been identified as an *E. coli*-specific chemoreceptor (4). In *S. typhimurium*, a receptor-like protein, Tip, has been reported (5), but its function is obscure; it has no role in the citrate and phenol response (ref. 5; unpublished data). Differences in Tar chemoreceptor protein are responsible for the presence or absence of maltose sensing in these species (6, 7).

We have been interested in the complicated chemoresponses to phenol in these bacteria. In *E. coli*, the attractant response to phenol is mediated by Tar, and this response dominates the Trg- and Tap-mediated repellent responses to phenol (8, 9). In *S. typhimurium*, however, Tar-mediated attraction to phenol is overcome by the function of an unidentified receptor that mediates a repellent response to phenol (8). To characterize the unidentified receptor in *S. typhimurium*, we have cloned a gene responsible for a repellent response to phenol.† In this paper, we describe that the cloned gene encodes a chemoreceptor mediating not only a repellent response to phenol but also an attractant response to citrate.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *S. typhimurium* ST1 [*his thy*], a derivative of LT2 (10), and pRK41, a pBR322 derivative containing *tar* gene of *S. typhimurium* (11), were obtained from D. E. Koshland, Jr., University of California at Berkeley. *E. coli* strain HCB339 [*Δtsr-7021 Δ(tar-tap)5201 trg*::Tn*10 thr leu his met rpsL136*] was obtained from H. C. Berg, Harvard University (12).

Cloning and Sequencing of *tcp* Gene. Routine DNA manipulations were done as described (13). Southern hybridization was done by using the enhanced chemiluminescence gene detection system (Amersham-Japan, Tokyo), and the probe was a 0.8-kilobase (kb) *Cla* I–*Stu* I fragment of the 3′ half of the *tar* gene from pRK41. For the cloning of *tcp*, genomic DNA of strain ST1 was digested with *Pst* I and ligated into pUC119.

DNA sequencing was done by the dideoxynucleotide chain-termination method with the Sequenase version 2.0 DNA sequencing kit (Toyobo, Osaka). [α-^{32}P]dCTP (3000 Ci/mmol; Amersham; 1 Ci = 37 GBq) was used for sequencing. A kit in the Kilo-sequence system (Takara Shuzo, Kyoto) was used to construct a series of deletions for DNA sequencing. The nucleotide sequence of the *tcp* gene was obtained by sequencing both DNA strands.

Measurement of Chemotactic Response. For behavioral assays, cells with plasmids were grown at 30°C in tryptone broth (1% tryptone/0.5% NaCl) supplemented with 0.5% glycerol and ampicillin at 50 μg/ml. When the ribose response was measured, 27 mM ribose was added to the broth. Cells were harvested in the late-exponential phase and washed with motility medium (10 mM potassium phosphate buffer, pH 7.0/0.1 mM EDTA/10 mM sodium DL-lactate/0.1 mM methionine). Temporal stimulation assays for chemoresponses were done as described (9). In some experiments, pH of the motility medium was adjusted to 6.0 or 7.4.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L06029).

For swarm assays, tryptone swarm agar plates (tryptone broth supplemented with 0.35% agar and ampicillin at 50 μg/ml) were incubated at 30°C.

Analysis of Methylation Patterns of Receptors. Methylation of receptors was analyzed as described (9). L-[*methyl*-^{3}H]Methionine (12 Ci/mmol) was obtained from DuPont/NEN.

RESULTS

Cloning of a Phenol-Specific Chemoreceptor Gene. For cloning of the presumed phenol-specific chemoreceptor gene, genomic DNA of *S. typhimurium* ST1 was digested with *Pst* I and ligated into pUC119. The resultant plasmids were introduced into an *E. coli* strain, HCB339, which is nonchemotactic because all four chemoreceptors are absent. The mixture of transformants was streaked on ampicillin-containing tryptone swarm plates at 30°C. Because the introduction of any type of chemoreceptor to this strain is expected to restore the swarming ability to, at least, some extent, all transformants able to swarm were isolated. Among isolates with relatively smaller swarms, we found a transformant that had no chemotactic responses to serine, aspartate, and ribose but gave a clear repellent response to 5 mM phenol. The plasmid in the transformant was isolated and named pKYP2. The plasmid contained a 14-kb *Pst* I fragment of *S. typhimurium* chromosomal DNA (Fig. 1).

To locate the presumed phenol-specific receptor gene in the 14-kb fragment, various deletions were made in pKYP2 with restriction nucleases. The phenol response was observed in HCB339 cells with pKYP21, pKYP24, or pKYP33 but was not observed in cells with pKYP31 or pKYP32 (these data are summarized in Fig. 1). A DNA–DNA hybridization test was done by using as a probe the 3′ half of the *tar* gene of *S. typhimurium*, which encodes a highly conserved cytoplasmic region of the known receptors (2). A cross-reacting band was detected in pKYP21, pKYP24, and pKYP33 but was not detected in pKYP31 and pKYP32 (these data are summarized in Fig. 1). These results indicate that both the presumed phenol-specific receptor gene and a DNA sequence homologous to the 3′ half of *tar* are present in the 2-kb region flanking the *Stu* I site of pKYP2. Because the size of the known receptor genes is ≈1.8 kb, only one receptor gene copy could exist in this 2-kb region.

For behavioral experiments, the 6-kb *Eco*RI–*Sac* I fragment of pKYP2 was ligated into pBR322, and HCB339 cells containing the resulting plasmid pKYP29 were used throughout. We did not use pKYP33 because this plasmid was later found to have a sequence of the receptor gene with a truncation of one codon.

Identification of the Phenol-Specific Chemoreceptor as a Citrate Chemoreceptor. HCB339(pKYP29) cells responded to phenol as a repellent but gave no responses to aspartate, serine, ribose, or dipeptides. These results indicate that the phenol-specific receptor is different from Tar, Tsr, Trg, Tap, and Tip. Because the chemoreceptors identified thus far mediate attractant responses to various nutrients, it is reasonable to expect that the phenol receptor also mediates an attractant response in *S. typhimurium*. Citrate is a good carbon source and attractant for *S. typhimurium* (14), and, therefore, we tested whether the phenol receptor mediates an attractant response to citrate.

Fig. 2 shows that HCB339(pKYP29) cells in motility medium tumbled in response to 5 mM phenol for several minutes. The addition of 1 mM citrate quickly restored smooth swimming in almost all cells. However, addition of a mixture of 10 mM each of aspartate, serine, and ribose had no effect. Citrate also induced a smooth-swimming response in the absence of phenol. Like the phenol response summarized in Fig. 1, the citrate response was detected in HCB339 cells containing pKYP21, pKYP24, or pKYP33 but was not detected in cells containing pKYP31 or pKYP32 (data not shown). Thus, the phenol receptor mediates an attractant response to citrate. Hence, we have named this chemoreceptor Tcp (taxis to citrate and away from phenol). HCB339(pKYP29) cells showed no growth in the presence of citrate as a sole carbon source (data not shown), indicating that Tcp has no role in citrate utilization.

The data obtained by the DNA–DNA hybridization analysis suggest that Tcp has an amino acid sequence homologous to the cytoplasmic domain of Tar. We, therefore, examined whether Tcp is a methyl-accepting chemotaxis protein like the known chemoreceptors in *E. coli* and *S. typhimurium*. Methylation analysis of HCB339(pKYP29) cells revealed the presence of several methylated bands at ≈60 kDa in the SDS/polyacrylamide gel electrophoretogram (Fig. 3). Consistent with the behavioral responses, the methylation level was increased by the addition of 10 mM citrate and decreased by the addition of 5 mM phenol. These results clearly indicate that Tcp is one of the methyl-accepting chemotaxis proteins and has a molecular mass of ≈60 kDa.

Other Properties of Tcp. The concentration of citrate required to induce an attractant response in 50% of HCB339(pKYP29) cells was ≈20 μM (Fig. 4). This value is consistent with the concentration of citrate needed to induce an attractant response in a wild-type strain of *S. typhimurium* (14). Isocitrate, which was suggested to be a weak attractant

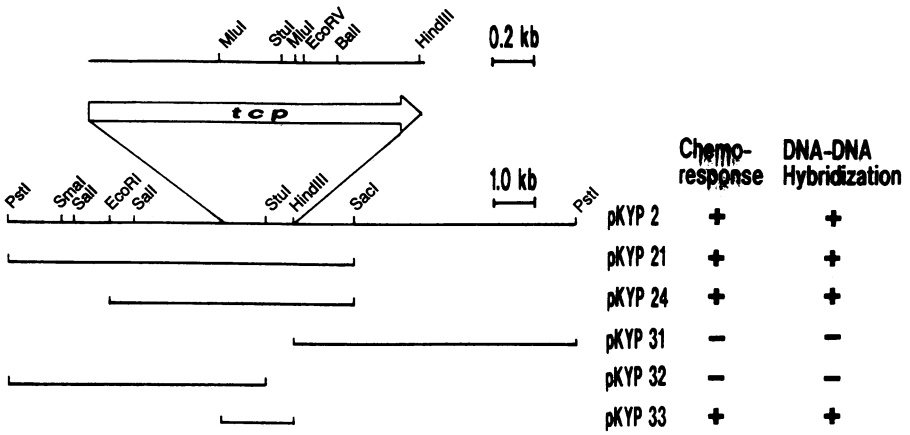

FIG. 1. Restriction nuclease map and phenotypes of pKYP2 and its derivatives. HCB339 cells containing each plasmid were tested for response to 5 mM phenol: +, response occurred; −, no response. For the DNA–DNA hybridization test, a 3′ half of the *tar* gene of *S. typhimurium* was used as a probe: +, hybrid formed; −, hybrid not formed.

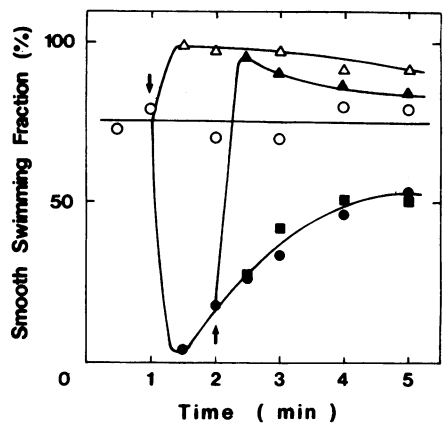

FIG. 2. Responses of HCB339(pKYP29) cells to phenol, citrate, and other effectors. Cells in motility medium at 25°C were exposed to 5 mM phenol or 1 mM citrate at the first arrow, and at the second arrow, 1 mM citrate or a mixture of 10 mM each of aspartate, serine, and ribose was added. The fraction of smooth-swimming cells was determined at regular intervals. ○, No addition; ●, 5 mM phenol only; △, 1 mM citrate only; ▲, 5 mM phenol and then 1 mM citrate; ■, 5 mM phenol and then the aspartate/serine/ribose mixture.

(14), elicited no response at 10 mM. Other citrate analogs, such as oxalate, α-ketoglutarate, malate, fumarate, and succinate, also gave no response at 2 mM. EDTA at 2 mM also showed no response.

Citrate and metal–citrate complexes are different attractants for *S. typhimurium* (14, 15). We, therefore, tested whether Tcp mediates an attractant response to metal–citrate complexes. For this experiment, HCB339(pKYP29) cells were first adapted to 10 mM citrate by 10-min incubation, and a tumble response was induced by adding 5 mM phenol. Further addition of 1 mM citrate had no effect, confirming adaptation of the cells to citrate. However, when 1 mM $MgCl_2$ was added, a clear smooth-swimming response was induced in most cells (data not shown). Addition of $MgCl_2$ without pretreatment by citrate caused no response. Similar results were obtained with $CaCl_2$, $NiSO_4$, and $ZnSO_4$ but were not obtained with NaCl. These results indicate that Tcp recognizes metal–citrate complexes as attractants distinct from citrate.

Indole at 1 mM caused a weak tumble response in HCB339(pKYP29) cells (data not shown). Glycerol at 0.5 M also caused a tumble response (see Fig. 4). Furthermore, at pH 6.0, but not at pH 7.4, 30 mM acetate and 10 mM benzoate

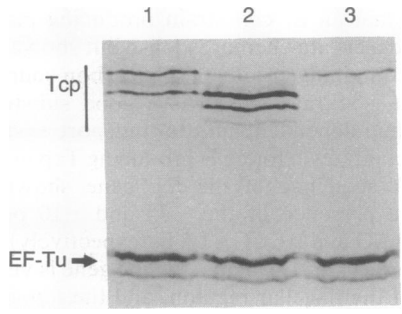

FIG. 3. Methylation banding patterns of the Tcp receptor. HCB339(pKYP29) cells were incubated with 6 μM [*methyl*-^{3}H]methionine at 30°C for 40 min. Then, distilled water (lane 1), 10 mM citrate (lane 2), or 5 mM phenol (lane 3) was added, and incubation was continued for 10 min. Samples were subjected to SDS/PAGE and autoradiography. The Tcp bands migrate at a position corresponding to that of a 60-kDa protein. The bands around the 43-kDa protein contain elongation factor Tu (EF-Tu).

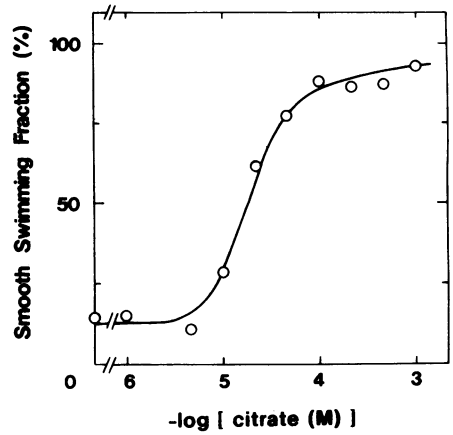

FIG. 4. Relationship between concentration of citrate and fraction of smooth-swimming HCB339(pKYP29) cells. Cells were challenged with 0.5 M glycerol mixed with various concentrations of citrate, as indicated. The smooth-swimming fraction was measured 30 sec after mixture addition. Addition of glycerol alone reduced the smooth-swimming fraction from 70% to 15% or less, and the response lasted for ≈1 min.

caused a repellent response (data not shown), indicating that Tcp is an intracellular pH sensor.

Characterization of the *tcp* Gene. DNA sequencing of the region flanking the *Stu* I site of pKYP2 was done by using plasmids pKYP23, pKYP24, and pKYP26, which contain a 3.9-kb *Sal* I–*Hin*dIII fragment in pUC119, a 6-kb *Eco*RI–*Sac* I fragment in pUC118, and a 1.5-kb *Hin*dIII–*Sac* I fragment in pUC118, respectively. Deletions of the plasmids were prepared by exonuclease III digestion. Because a deletion plasmid, pKYP33, derived from pKYP23 has the smallest insert that supports both citrate and phenol responses, we first sequenced both strands of the whole insert in this plasmid. However, we found that the insert had an incomplete sequence of *tcp* gene, and so, we sequenced both strands for ≈80 more base pairs (bp) downstream of the *Hin*dIII site. The complete 1840-nucleotide sequence is shown in Fig. 5, along with the deduced amino acid sequence of the Tcp protein.

There are two ATG codons in the same correct reading frame. However, the second of these is considered to be the initiation codon, because a Shine–Dalgarno sequence (AGGA) is located at the proper position (16 to 13 bp upstream of the second ATG codon). The termination codon, TAA, occurs 1644 bp from the initiation codon, so that the *tcp* gene encodes 547-amino acid residues with a calculated molecular mass of 58,947 Da, which agrees well with the molecular mass of 60 kDa estimated from the SDS/PAGE shown in Fig. 3. It is noted that the presumed flagella-specific promotor sequences (16, 17), TAAG and ACCGATA, are found at 61 and 39 bp upstream of the initiation codon, respectively.

The *Hin*dIII site, used to construct pKYP33 (Fig. 1), is located 2 bp upstream of the termination codon. Hence, the C-terminal sequence of the altered Tcp encoded in pKYP33 is Ser^{546}-Leu-Ala-COOH, whereas the corresponding wild-type sequence is Ser^{546}-Phe-COOH. As a consequence, it was noted that HCB339 cells with pKYP33 responded to citrate and phenol but showed almost no adaptation (data not shown).

The deduced amino acid sequence of Tcp was significantly homologous to Tar of *S. typhimurium* (Fig. 5). Two hydrophobic segments, presumed to constitute transmembrane segments, are located at positions close to those in Tar. In addition to the presence of especially high identity in the presumed cytoplasmic domains between Tcp and Tar, four

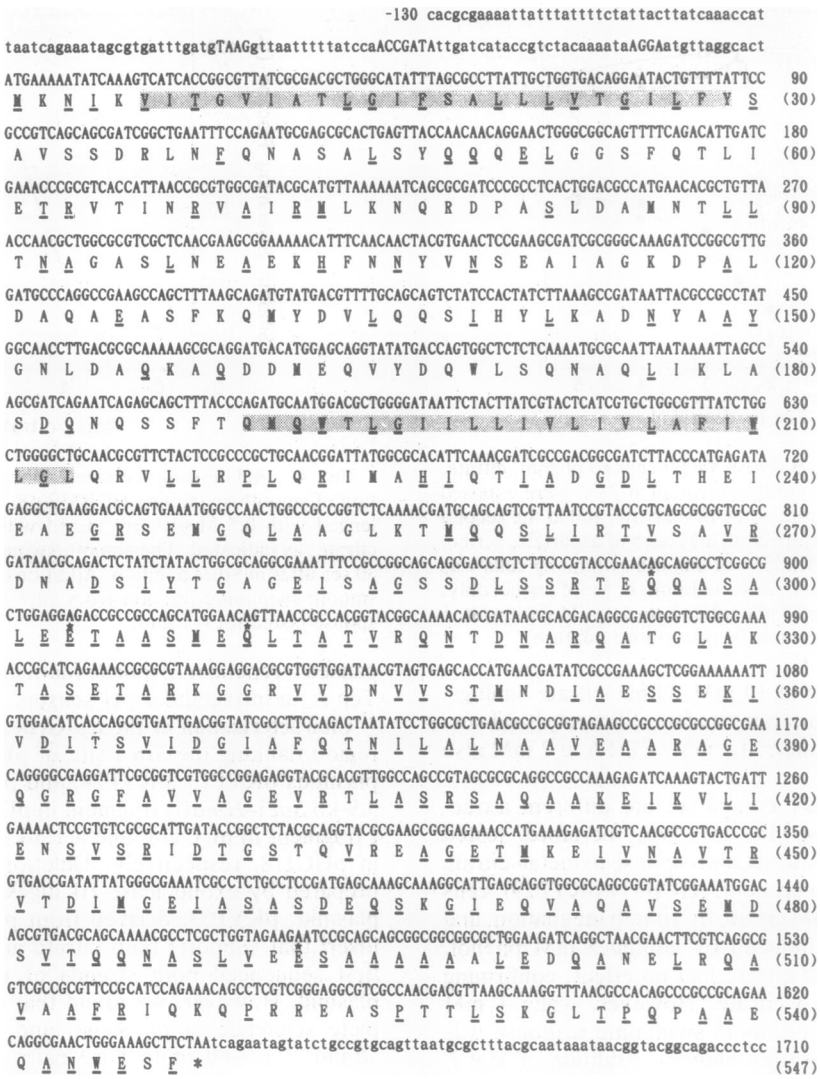

FIG. 5. Nucleotide sequence encompassing the *tcp* gene and deduced amino acid sequence of the Tcp receptor of *S. typhimurium*. The nucleotide sequence was determined from both strands; sequence of the sense strand is shown. A possible Shine–Dalgarno sequence (AGGA) is indicated by capital letters 16–13 bases upstream of the putative initiation ATG. The presumed −35 and −10 promoter sequences specific for flagellar operons are also presented as capital letters. Underlined amino acid residues are identical residues to the Tar receptor of *S. typhimurium*. Putative transmembrane segments are indicated by shading, and possible methylation sites are marked by stars.

presumed methyl-accepting glutamic acid residues in Tar are conserved in Tcp. These results clearly indicate that Tcp is another member of the bacterial transmembrane chemoreceptor family.

DISCUSSION

We report here the characterization of the transmembrane chemoreceptor for citrate and phenol, Tcp, in *S. typhimurium*. Because citrate is a nutrient and attractant for *S. typhimurium* but is neither for *E. coli* (14), it is reasonable that Tcp evolved as a *S. typhimurium*-specific receptor, just as Tap is an *E. coli*-specific dipeptide transducer (4). Although Tcp is likely to be the reported phenol-specific receptor (8), more data are required to clarify this point.

The reason why citrate is utilized by *S. typhimurium* but is not utilized by *E. coli* is that citrate-transport systems are present only in *S. typhimurium* (18–20). However, various mutants of *S. typhimurium* defective in citrate transport have normal citrate chemotaxis (14, 15). Also, citrate-transporting strains of *E. coli* and *E. coli* strains harboring the *S. typhimurium tctI* operon, which encodes a periplasmic binding-protein-dependent citrate-transport system, did not have restored citrate chemotaxis (21, 22), although these strains utilized citrate for their growth. Thus, neither citrate utilization, citrate transport, nor periplasmic citrate-binding proteins have any relation to citrate chemotaxis. Furthermore, we showed that an *E. coli* strain producing plasmid-borne Tcp acquired a citrate chemoresponse but showed no growth in medium containing citrate as sole carbon source. We also showed that isocitrate, which is a good substrate for the binding-protein-dependent citrate-transport system (22), is not an attractant, even for cells producing Tcp in abundance.

The DNA sequence of the *tcp* gene shown in Fig. 5 indicates the presence of the −35 and −10 promoter sequences (TAAG and ACCGATAT, respectively) specific for flagellar operons (16, 17). Thus, the *tcp* gene is very probably a member of the flagellar regulon, and the *tcp* gene is not a simple substitute of *E. coli*-specific *tap* gene, which is located downstream of *tar* gene in the *meche* operon (23).

The restriction map shown in Fig. 1 had no similarity to the map of the *tip*-containing fragment (5). Our preliminary data suggest, instead, that the restriction map of the *tip*-containing fragment is homologous to that of the recently identified *trg*-containing fragment of *S. typhimurium* (Honda, T., K.Y., I. Kawagishi, and Y.I., unpublished work).

At least two amino acid residues, Arg-64 and Thr-154, in the ligand-binding domain of Tar are considered to have a specific function in the recognition of aspartate by the Tar receptor (7, 24, 25). A recent report (26) on the three-dimensional structure of the ligand-binding domain of Tar gives more direct evidence that the α-carboxyl group and the amino group of aspartate are recognized by Arg-64 and Thr-154, respectively. In the deduced amino acid sequence of Tcp shown in Fig. 5, a positional equivalent of Arg-64 is present in Tcp as Arg-63, suggesting that the latter residue may also interact with the α-carboxyl group of citrate. Tcp has no positional equivalent of Thr-154 but has several more basic amino acid residues than Tar, such as Lys-75, Arg-78, and Lys-157 in the presumed ligand-interaction region, which may interact with the extra carboxyl group and the hydroxyl group of citrate.

It was suggested that citrate and metal–citrate complexes were recognized by different receptors (3, 15). However, our results clearly indicate that Tcp mediates attractant responses to both of these classes of compounds. Furthermore, metal–citrate complexes give signals even after the adaptation of Tcp to citrate, indicating that Tcp recognizes citrate and metal–citrate complexes as distinct attractants.

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