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Mutations Specifically Affecting Ligand Interaction of the Trg Chemosensory Transducer

CHANKYU PARK[1] AND GERALD L. HAZELBAUER[1,2]*

Programs in Genetics and Cell Biology[1] and in Biochemistry/Biophysics,[2] Washington State University, Pullman, Washington 99164-4660

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The Trg transducer mediates chemotactic response to galactose and ribose by interacting, respectively, with sugar-occupied galactose- and ribose-binding proteins. Adaptation is linked to methylation of specific glutamyl residues of the Trg protein. This study characterized two *trg* mutations that affect interaction with binding protein ligands but do not affect methylation or adaptation. The mutant phenotypes indicated that the steady-state activity of methyl-accepting sites is independent of ligand-binding activity. The mutation *trg-8* changed arginine 85 to histidine, and *trg-19* changed glycine 151 to aspartate. The locations of the mutational changes provided direct evidence for functioning of the amino-terminal domain of Trg in ligand recognition. Cross-inhibition of tactic sensitivity by the two Trg-linked attractants implies competition for a common site on Trg. However, the single amino acid substitution caused by *trg-19* greatly reduced the response to galactose but left unperturbed the response to ribose. Thus Trg must recognize the two sugar-binding proteins at nonidentical sites, and the complementary sites on the respective binding proteins should differ. *trg-8* mutants were substantially defective in the response to both galactose and ribose. An increase in cellular content of Trg-8 protein improved the response to galactose but not to ribose. It appears that Trg-8 protein is defective in the generation of the putative conformational change induced by ligand interaction. The asymmetry of the mutational defect implies that functional separation of interaction sites could persist beyond the initial stage of ligand binding.

Sensory transducers are transmembrane signaling proteins that play a central role in bacterial chemotaxis (18). The proteins detect fluctuations in the extracellular concentration of chemical compounds and generate an intracellular sensory signal that modulates the binary mode of flagellar rotation. Adaptation to continued stimulation is linked to methylation of carboxyl groups of specific glutamyl residues in the transducer. These bacterial proteins are functionally analogous to receptors found on the surface of most eucaryotic cells and share certain structural motifs with receptors for polypeptide hormones (9, 42). In *Escherichia coli*, each of three well-characterized transducers, Tsr, Tar, and Trg, recognizes specific ligands. Trg generates sensory information about external concentrations of ribose and galactose by interacting with ligand-occupied ribose- and galactose-binding proteins, respectively (16, 24). Competition experiments have shown that the presence of one sugar inhibits tactic response to the other sugar (2, 10, 39). A simple model for the competition is that the transducer has a single, common interaction site for both ligand-occupied binding proteins (39).

The nucleotide sequences of transducer genes and the respective deduced sequences of amino acids (5, 6, 26, 35) reveal a transducer gene family that codes for homologous proteins of approximately 60,000 daltons. A number of lines of genetic (5, 25) and biochemical (20–22, 31, 40, 41) evidence lend support to a simple model for disposition of the transducer polypeptide chain across the cytoplasmic membrane (26). The model suggests that the proteins span the membrane twice, once with a short hydrophobic sequence near the aminoterminus and again with a short hydrophobic region 40% of the way along the polypeptide chain. This places an amino-terminal, ligand-binding domain on the extracytoplasmic face of the membrane and a carboxy-terminal, covalent modification domain on the cytoplasmic face.

In this report, we focus on the recognition function of the Trg protein by analyzing two *trg* mutations that confer specific defects in the interaction of binding protein and transducer. Our findings provide direct evidence that ligand binding occurs in the amino-terminal domain of Trg and establish that the sites on Trg at which galactose- and ribose-binding proteins interact are separable.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in this work (Table 1) are all derivatives of *E. coli* K-12, except the *E. coli* C2107, which was used as a source of the *polAts* allele. The deletion *tsr-7028* was from RP5700, obtained from J. S. Parkinson. The deletion Δ(*tar-tap*)*5201* (37) inactivates *tar* and *tap* (7) but does not affect expression of the *che* genes. The transposon insertion *zdb*::Tn*5* was described by Harayama et al. (12). The 7.4-kilobase (kb) plasmid pCP31 (34) contains the wild-type *trg* gene. It was obtained by *Bal*31 digestion of the 8.5-kb plasmid pTH105 (5). The plasmids containing deletions of segments of *trg*, pCP25 and pCP27, were derived from pTH105 and pTH51 (11), respectively.

Chemicals and enzymes. Sugars used in growth media were from E. Merck AG, Darmstadt, Federal Republic of Germany. Compounds used as attractants in assays of chemotaxis were of the highest purity available (significantly glucose-free galactose was from Sigma Chemical Co., St. Louis, Mo.; all others were from Merck) and were used without further purification. All sugars were of the D form, and amino acids were of the L form.

L-[*methyl*-^{3}H]methionine (10 to 15 Ci/mmol), L-[^{35}S]methionine (1,000 Ci/mmol), [α-^{32}P]dATP (800 Ci/

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
E. coli		
AW708	F⁻ *trg-8 his lac rpsL*	J. Adler (4)
AW719	F⁻ *trg-19 supE*(λ)	J. Adler (4)
C2107	F⁻ *polA12*(Ts) *rha*	M. Kahn
CP105	F⁻ *zdb*::Tn*5*	(12)
CP177	OW1 Δ(*trg-100*)	This work
CP344	OW1 *zdb*::Tn*5 trg-8*	This work
CP347	OW1 *zdb*::Tn*5 trg-19*	This work
CP362	OW1Δ(*tsr-7028*) Δ(*tar-tap*)*5201* Δ(*trg-100*)	This work This work
CP365	CP362(pCP31)	This work
CP367	OW1 *polA12*(Ts)	This work
CP375	CP362(pCP32)	This work
CP376	CP177(pCP33)	This work
CP454	CP362(pCP33)	This work
CP463	CP177(pCP32)	This work
CP469	OW1 Δ(*tsr-7028*) Δ(*tar-tap*)*5201 lacZ*::*trg zdb*::Tn*5 trg-19*	
CP474	CP177(pCP31)	This work
CP478	OW1 Δ(*tsr-7028*) Δ(*tar-tap*)*5201 lacZ*::*trg zdb*::Tn*5 trg-8*	This work
ET1214	F⁻ *zig-219*::Tn*10*	B. Bachmann
N1126	F⁻ *polA*	A. Iida
OW1	*thr leu his rpsL*	J. Adler (32)
Plasmids		
pCP25	*ori* (pBR322) Ap^r Δ*trg-103* (6.3 kb)	This work (Fig. 5)
pCP27	*ori* (pBR322) Tc^r Δ*trg-104* (5.5 kb)	This work (Fig. 5)
pCP31	*ori* (pbR322) Ap^r *trg*$^+$ (7.4 kb)	This work (34)
pCP32	pCP31 *trg-19*	This work
pCP33	pCP31 *trg-8*	This work

mmol), and [α-^{32}P]dCTP (800 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. All other chemicals were of reagent grade.

Enzymes and DNA for recombinant DNA techniques including restriction endonucleases, T4 ligase, DNA polymerase large fragment, nuclease *Bal*31, and phage DNA were purchased from New England BioLabs, Inc., Beverly, Mass., and Bethesda Research Laboratories, Inc., Gaithersburg, Md.

Media. Luria broth, tryptone broth, and M9 minimal salts medium were prepared as described by Miller (30), and H1 minimal salts were prepared as described by Adler (1). Sugars (0.2%), sodium succinate (0.4%), thiamine (10 μM), and amino acids (1 mM) were added to minimal medium as required. Solid media for plates contained 1.5% agar.

Semisolid agar swarm plates contained 0.25% agar (Difco Laboratories, Detroit, Mich.) and either 1% tryptone (Difco) and 0.25% NaCl or minimal salts plus required amino acids, thiamine, and a single sugar at 0.05 mM (galactose) or 0.1 mM (ribose or maltose) (19).

Chemotaxis assays. Semisolid agar swarm plates were inoculated from single colonies and scored after 8 h at 35°C for tryptone plates (response to serine and aspartate) or 17 to 24 h for minimal salts plates (response to sugars). For capillary assays (1), bacteria were grown and prepared as described previously (17) and suspended at 5×10^6 cells per ml in chemotaxis buffer (0.1 mM EDTA, 10 mM potassium phosphate, pH 7.0). A capillary (1-μl disposable micropipette) sealed at one end and containing a solution of attractant was placed in 0.4 ml of a bacterial suspension in a 0.5-ml microcentrifuge tube. After 60 min at 30°C, the capillary was removed, and the number of bacteria inside was determined by plating the contents. For competition experiments, both bacterial suspension and capillary solutions contained the competing sugar. For behavioral assays with tethered cells, flagella were sheared from the cells by treatment in a Waring blender. Deflagellated cells were pelleted by centrifugation, suspended in a 1:1,000 final dilution of antiflagellin serum (30 to 40% ammonium sulfate fraction) in chemotaxis buffer, and placed in a chamber made by bridged cover slips. Tethered rotating cells were observed on videotape recordings. Adaptation time was defined as the period between stimulation and the first complete clockwise rotation.

Genetic manipulations. Transduction with bacteriophage P1 and other routine genetic manipulations were carried out as described by Miller (30). The deletion Δ(*trg-100*) was constructed in vitro in pTH51 (11) by excision with *Sal*I of the 4.8-kb fragment containing the entire *trg* sequence (1.6 kb). High concentrations of the resulting plasmid were used to transform the *polA* strain N1126, in which the plasmid could not replicate. Plating the transformation mixture on tetracycline-containing plates selected for cells in which the plasmid had integrated into the chromosome, presumably by homologous recombination. Tetracycline-susceptible derivatives of such cells were selected by growth in the presence of fusaric acid (27). After enrichment, 29% of the cells were tetracycline susceptible and 25% of those were Trg⁻, presumably because recombinational loss of the plasmid occurred in such a way that the deletion remained in the chromosome. Deletion of *trg* DNA was confirmed for one particular isolate by appropriate hybridization experiments (38). The deletion, named Δ(*trg-100*), was transferred to other strains by cotransduction with *zdb*::Tn*5* (12). The *polA12*(Ts) mutation was transduced by utilizing either *zig-219*::Tn*10* or the *rha* marker.

For mapping chromosomal *trg* mutations with plasmid deletions, strains containing *polA12*(Ts) were transformed with plasmids in the presence of antibiotic (10 μg of tetracycline per ml or 50 μg of ampicillin per ml) and grown at the permissive temperature (30°C) to increase the number of plasmids. Cultures were diluted, spread on plates containing the same antibiotic, and incubated at the restrictive temperature (42°C); 30 to 80 independent plasmid integrates were tested for their Trg phenotype on minimal chemotactic swarm plates.

With a strain containing Δ(*trg-100*) and *polA12*(Ts), it was possible to introduce *trg*, carried on a derivative of pUC13 (43), into the *lac* operon in the chromosome. The plasmid carried in its multiple cloning region a 2.2-kb *Bgl*II-*Hin*cII chromosomal fragment including *trg* that is completely eliminated by the 4.8-kb deletion, Δ(*trg-100*). Thus the only regions of homology between plasmid and chromosome were the segments of *lac* DNA that bracket the cloned *trg* gene. Derivatives in which the plasmid had integrated into the chromosome were selected by growth at 42°C in the presence of ampicillin. Integration in the *lac* region, which would inactivate *lacZ*, was identified by using lactose indicator plates. It occurred at a relatively low frequency (10^{-4} to 10^{-5}), probably reflecting the limited size (ca. 400 base pairs) of the homologous DNA sequences. Ampicillin-susceptible segregants of an Lac⁻ Trg⁺ isolate were identified after more than 30 doublings in antibiotic-free medium. Approximately 10% of the segregants remained Lac⁻ Trg⁺. Analysis of one of these strains, CP378, by hybridization (38) with radiolabeled pTH105 revealed that *trg* DNA was con-

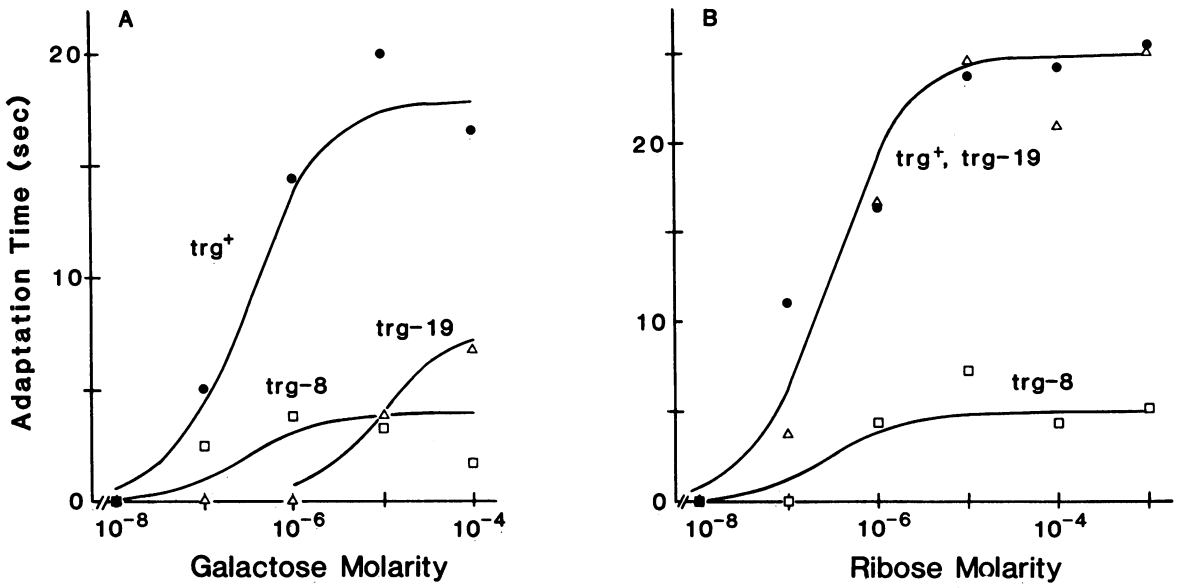

FIG. 1. Response of mutant and wild-type cells to temporal gradients. Cells grown in minimal salts plus galactose (A) or ribose (B) were tethered and tested at 35°C for response to a temporal gradient from no sugar to the concentration indicated. Cells respond by inhibition of clockwise rotation of the flagellar motor to produce exclusively counterclockwise rotation and are adapted when the usual pattern of alternation between directions of rotation is reestablished. A minimum of 10 cells was observed, and an average adaptation time was determined. Standard deviations ranged from 2 to 8 s, depending upon the magnitude of the response. Responses by a Δ(*trg-100*) strain were negligible for stimulation by 10^{-8} to 10^{-5} M ribose and 10^{-8} to 10^{-6} M galactose; averaged approximately 1 s for 10^{-4} and 10^{-3} M ribose, and were 2.4 and 5.6 s for 10^{-5} and 10^{-4} M galactose, respectively. Above 10^{-4} M galactose, the deletion strain responded for period in excess of 25 s; the corresponding increase in response of wild-type cells indicated that the Trg-mediated response and the response observed in the deletion strain were not completely additive. Average response times exhibited by the deletion strain were subtracted from the response times of the other strains to yield the values shown. The curves are calculated assuming the following maximal responses and apparent K_ds: 18 s and K_d 0.3 μM (A, *trg*$^+$); 4 s and K_d 0.3 μM (A, *trg-8*); 8 s and K_d 10 μM (A, *trg-19*); 25 s and K_d 0.3 μM (B, *trg*$^+$ and *trg-19*); and 5 s and K_d 0.3 M (B, *trg-8*). The strains tested were OW1 (*trg*$^+$, ●), CP344 (trg-8, □), and CP347 (*trg-19*, △).

tained in a 12-kb *Pst*I fragment, in contrast to the wild-type strain in which *trg* at its normal location at 31 min is contained in an 8.5-kb *Pst*I fragment (11). The position of *Pst*I sites near *lac* is not known, but since no *Pst*I sites occur in the *lac* operon (8), a *Pst*I fragment containing the operon must be larger than 7 kb. Transduction experiments revealed that the integrated *trg* was 100% linked to *lacZ* and 65% linked with *aroL*::Tn*10*, located at 8 min on the linkage map of *E. coli*. The sum of these observations established that the *trg* gene had been integrated into *lacZ*, and thus we designate the insertion as *lacZ*::*trg*.

Recombinant DNA techniques. Isolation of DNA, enzymatic digestions, and agarose gel electrophoresis were done as described by Maniatis et al. (28). Fragments of wild-type or mutant *trg* were cloned into M13mp11 (29) for subsequent sequencing. The presence and size of inserts was monitored by direct agarose gel electrophoresis of DNA from sodium dodecyl sulfate-disrupted recombinant phage particles. The orientation of inserts was determined by hybridization of candidates at 65°C for 1 h with previously characterized recombinant phages. The presence of hybridized viral DNAs was visualized by agarose gel electrophoresis. Sequencing was done by the dideoxy chain termination method (36) with a kit from New England BioLabs.

Methylation of transducer protein. Methylation experiments and analysis of *methyl*-^{3}H-labeled protein were performed as described by Hazelbauer and Engström (15).

RESULTS

Initial characterization. Strains carrying the mutations *trg-8* and *trg-19*, analyzed in this work, were isolated in the laboratory of J. Adler. Initial characterization by C. B. Ball (Ph.D. thesis, University of Wisconsin, Madison, 1979) indicated that the mutations were located close to 30 min on the linkage map of *E. coli* and that the mutant strains contained Trg protein able to accept methyl groups. The *trg-19* isolate showed a normal response to ribose but no response to galactose on semisolid agar swarm plates (Ball, Ph.D. thesis). The *trg-8* mutant was originally classified as completely defective in response to both sugars, but we found that *trg-8* strains exhibited weak but distinct responses (see below). Both mutations were linked to *zdb*::Tn*5* (12) to about the same extent (50% cotransduction by P1) as the wild-type *trg* gene, confirming and refining the locations deduced from time of entry measurements with Hfr strains (Ball, Ph.D. thesis). For further study, the mutations were moved by transduction from the original mutagenized strains, AW708 and AW719, to OW1, creating CP344 and CP347, respectively (Table 1).

Behavioral phenotypes. Tactic responses of mutant strains to temporal gradients of galactose and ribose were determined by using tethered cells (Fig. 1). The *trg-19* mutant exhibited responses to ribose that were indistinguishable from those of the wild type. In contrast, sensitivity of the *trg-19* strain to galactose was shifted almost 2 orders of magnitude relative to the wild type. It was not possible with this assay to determine the maximal response of the *trg-19* mutant to galactose, since responses to concentrations ≥1 mM included a substantial, nonadditive component independent of Trg (see legend to Fig. 1). However, changes in levels of methylation after stimulation with 10 mM galactose (see below) indicated that the maximal response of a *trg-19*

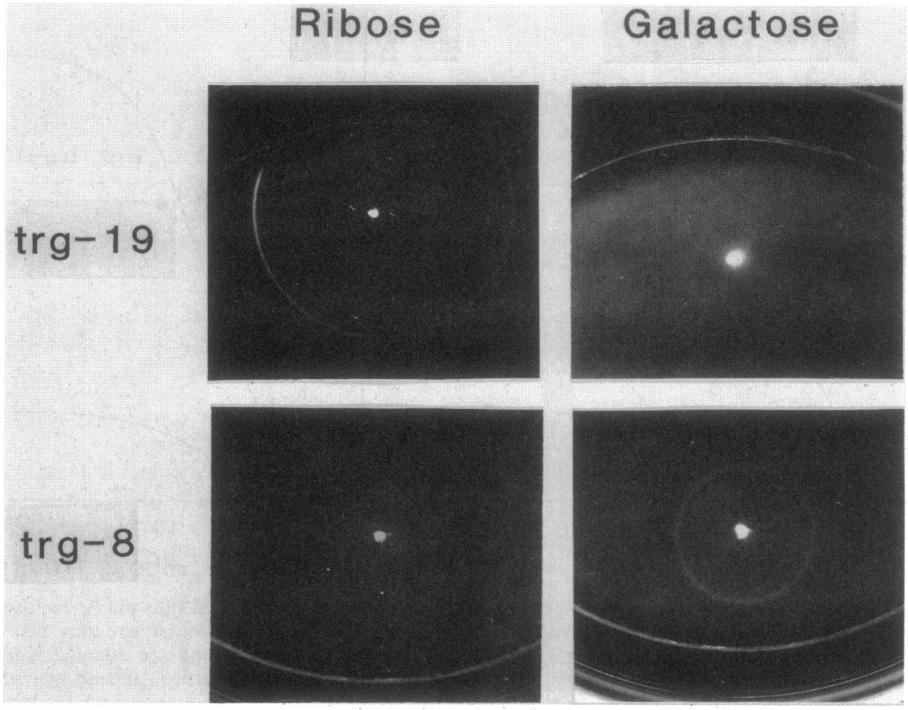

FIG. 2. Chemotactic responses on semisolid agar plates of strains containing multiple copies of *trg-8* or *trg-19*. The photographs show rings formed 20 h after semisolid agar plates containing minimal salts and the indicated sugar were inoculated with a single colony isolate of CP376 (*trg-8*) or CP463 (*trg-19*) and placed at 35°C.

strain was substantially lower than for the wild type. For the *trg-8* mutant, the maximal response to a temporal gradient of galactose or ribose was reduced to a marginally detectable value of approximately 5 s, but the concentration dependence of the responses was not substantially different than that of the wild type. The pattern of response of the mutant strains to temporal gradients paralleled behavior in spatial gradients on chemotactic swarm plates. The *trg-19* strain formed a wild-type ring on a plate containing ribose and no ring at all on a plate containing galactose. The *trg-8* mutant exhibited a weak galactose response that was usually detectable as a faint ring of cells. Response to ribose was only sometimes observed as a faint ring.

We obtained plasmids carrying the *trg-8* and *trg-19* mutant genes by utilizing conduction of a hybrid plasmid carrying wild-type *trg* to select for plasmids that had undergone recombination with chromosomal DNA containing the *trg* mutation. This procedure is described in detail elsewhere (34). Briefly, the mutations were transduced into an Hfr strain (KL16-21), and the resulting *trg* mutants were transformed with plasmid pCP31. Selection for transfer by conduction of the ampicillin resistance property of pCP31 to a *trg* deletion strain resulted in the isolation of phenotypically mutant plasmids: pCP32, which carries the *trg-19* mutation, and pCP33, which carries *trg-8*. As expected (11), cells harboring these multicopy plasmids contained substantially more Trg protein than did cells carrying a single, chromosomal copy of *trg* (data not shown). A *trg* deletion strain harboring pCP32 exhibited the same tactic behavior on swarm plates as the original *trg-19* mutant: a wild-type response to ribose and no detectable response to galactose (Fig. 2). It was not possible to quantify response to temporal gradients by tethered cells harboring *trg* plasmids since the increased content of transducer resulted in a low tumble frequency and very long adaptation times. However, the lack of any detectable response of a pCP32-containing strain on a galactose swarm plate indicated that an increased dosage of the Trg-19 protein does not improve sensitivity to galactose significantly. Response to ribose as measured by the less sensitive capillary assay was the same for strains carrying pCP31 (*trg*$^+$) or pCP32 (*trg-19*) (Fig. 3C). The *trg-19* mutation was recessive, that is, the defect in galactose taxis was corrected by introduction of a single copy of wild-type *trg* into the *lac* region of a *trg-19* strain. Construction of this partial diploid strain is described in Materials and Methods. The sum of these phenotypic observations suggests that *trg-19* alters the Trg protein to create a specific defect in interaction with the galactose-binding protein without affecting other features of the transducer, including interaction with the ribose-binding protein.

Strains containing a single copy of *trg-8* exhibited only a weak galactose response that was just detectable as a faint ring of cells on a swarm plate containing galactose, but that was not detectable in the capillary assay (Fig. 3A). An increased copy number of *trg-8* resulted in a strain with an improved galactose response such that the tactic ring formed on a galactose plate was as sharp and almost as rapidly moving as the ring formed by a wild-type strain (Fig. 2). Similarly, a distinct response to galactose was evident in the capillary assay (Fig. 3B). In contrast, an increased dosage of *trg-8* did not improve the very weak response to ribose observed for the initial mutant strain on a swarm plate (Fig. 2), nor was a response detected in the capillary assay (Fig. 3C). Introduction of a single copy of wild-type *trg* into the *lac* region of a *trg-8* strain restored response to both galactose and ribose. Thus *trg-8* is a recessive mutation that substantially reduces sensitivity to both Trg-linked attractants. However, the effect is not equivalent for the two sugars since an increased dosage of the mutant protein resulted in improved response to galactose but not to ribose.

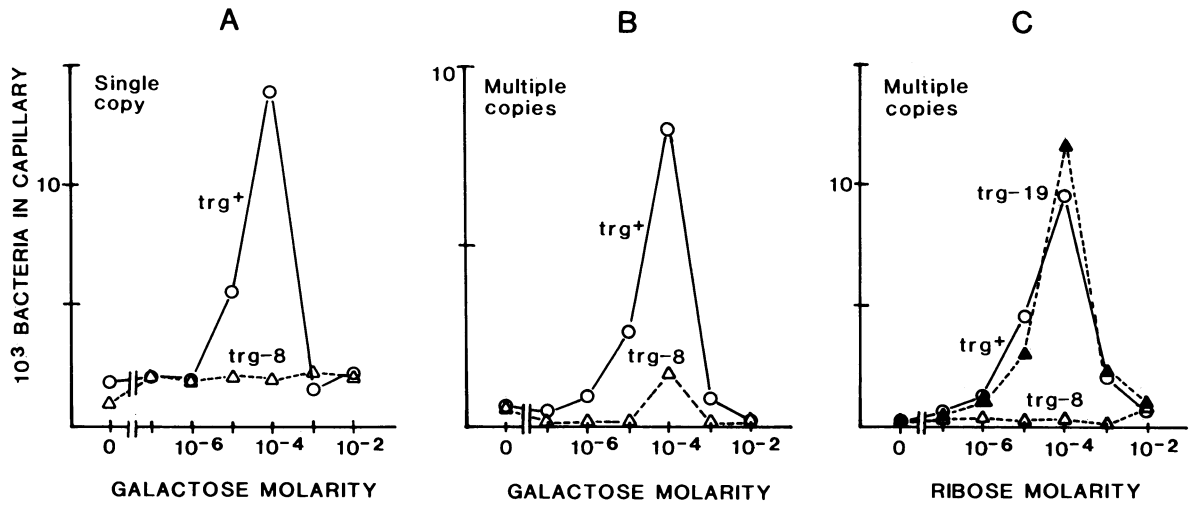

FIG. 3. Chemotactic responses in the capillary assay of strains containing mutant or wild-type *trg* genes. Accumulation of bacteria in microcapillaries from cell suspensions at 3×10^7 (A) or 5×10^6 (B and C) cells per ml is plotted versus the concentration of sugar initially in the capillary. Cultures were grown in H1 minimal salts medium plus the sugar to which taxis was tested. Strains: A, OW1 (○) and CP344 (△); B, CP474 (○) and CP376 (△); and C, CP474 (○), CP375 (▲), and CP376 (△).

Thus in strains with multiple copies of *trg-8*, the Trg^- phenotype is essentially the converse of the phenotype observed for *trg-19* mutants.

Methylation and sensory adaptation. Methylation of the mutant Trg proteins was investigated in strains carrying deletions in all four chromosomal genes for methyl-accepting transducer proteins and harboring a hybrid plasmid containing a wild-type or mutant *trg*. Thus the only methyl-accepting transducer in those cells was the product of the plasmid-carried gene, and the pattern of *methyl*-^{3}H-labeled forms of Trg displayed on fluorograms of SDS-polyacrylamide gels could be examined without the usual interference from *methyl*-^{3}H-labeled forms of other transducers. No significant difference between mutant and wild-type was detected in the pattern of *methyl*-^{3}H-labeled forms resolved by electrophoresis (Fig. 4A). These forms correspond to different extents of deamidation of glutamines and methylation of glutamates at multiple sites on Trg (11, 22). In a wild-type cell, adaptation to stimulation by galactose or ribose is linked to an increase in the level of methylation of Trg (14, 24). The change is observed as both a general increase in intensity of *methyl*-^{3}H-labeled bands of Trg and a relative increase in intensity of the fastest-migrating electrophoretic forms, corresponding to highly methylated species. In the mutant strains, increases in Trg methylation (Fig. 4B) corresponded to the specific tactic phenotype, indicating that the process of adaptive covalent modification was possible on both mutant proteins. In a *trg-19* strain, there was a distinct change in methylation level, comparable to the change observed for the wild type, after stimulation with 10 mM ribose, but only a slight change after stimulation with 10 mM galactose. This concentration of galactose was 100-fold higher than the highest level tested in the temporal assay (Fig. 1). The minimal change observed implies that the *trg-19* mutation affects the magnitude of response as well as the sensitivity to stimulation by galactose. In a *trg-8* strain little change in methylation level was detected after stimulation with either sugar. A minimal increase in intensity of the fastest-migrating bands after galactose stimulation was just discernible on the original fluorographs, consistent with a detectable response to galactose in behavioral assays (Fig. 2 and 3).

Mapping and nucleotide sequencing. We determined the region of the gene in which the *trg-8* and *trg-19* mutations were located by recombination experiments in vivo between mutant and truncated *trg* genes and in vitro between fragments of mutant and wild-type *trg* genes generated by digestion with restriction endonucleases. A temperature-sensitive *polA12* strain was used to select for recombinants in vivo between a mutant *trg* gene on the chromosome and two truncated *trg* genes, carried on plasmids with ColE1 replicons, created by digestion with *Pvu*I and *Nru*I, respectively (Fig. 5). Wild-type recombinants were recovered for each combination, indicating that both mutations lay within the *Pvu*I-*Nru*I fragment of *trg*. The single *Sna*BI site in the plasmids containing *trg* is within the *Pvu*I-*Nru*I fragment (Fig. 5). Recombinants made in vitro by joining wild-type and mutant *trg* genes at the *Sna*BI site were tested in appropriate host cells for the Trg phenotype they conferred. The results placed both mutations in the *Pvu*I-*Sna*BI segment of *trg*, probably at some distance from the *Pvu*I site, since recovery of wild-type recombinants in the in vivo experiments required a recombination between the *Pvu*I site and the site of the mutation. The *Bgl*II-*Sna*BI fragments from plasmids carrying *trg-8*, *trg-19*, or wild-type *trg* were cloned into M13mp11 (29) and sequenced by the dideoxy chain termination method (36). The sequence of the wild-type fragment was identical to that previously published (5). For each mutation only a single nucleotide change was detected in the *Pvu*I-*Sna*BI segment shown to contain the mutational alteration. For *trg-8*, a transition of G-C to A-T at position 254 substitutes histidine for arginine at position 85 (Fig. 5). Identification of the mutation as a transition is consistent with its induction by the alkylating agent ethyl methanesulfonate (Ball, Ph.D. thesis). The *trg-19* alteration is a transition of G-C to A-T at nucleotide position 452, resulting in a change of glycine 151 to an aspartyl residue (Fig. 5).

Reciprocal inhibition of taxis by ribose and galactose. Tactic response to a sugar recognized by one of the two binding

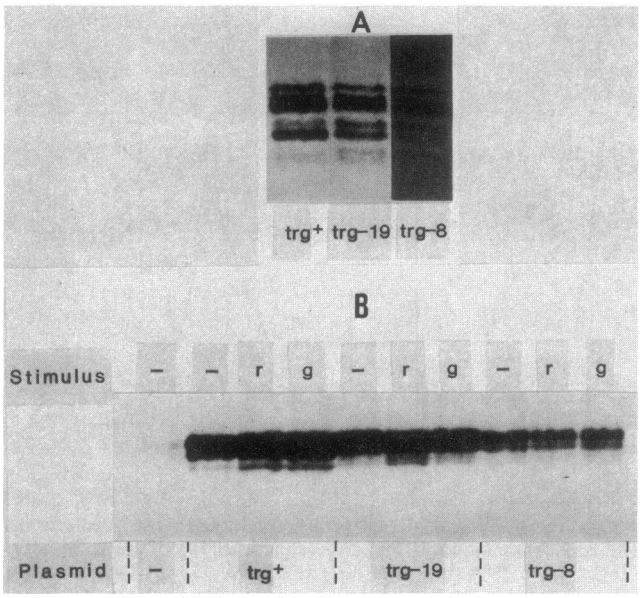

FIG. 4. Patterns of methylation of Trg protein from wild-type and mutant strains. The figures are fluorographs of the 60,000-molecular-weight region of sodium dodecyl sulfate-polyacrylamide gels containing samples of *methyl*-^{3}H-labeled cellular protein. pCP31 (*trg*$^+$), pCP33 (*trg-8*), or pCP32 (*trg-19*) plasmids were carried by CP362, which contains chromosomal deletions in the four known transducer genes. The lower level of radiolabel in the strain harboring pCP33 was not consistently observed and may reflect variation in the number of plasmids per cell. A, High-resolution patterns of the multiple forms of Trg samples from unstimulated cells were analyzed by using gels made with 11% acrylamide and 25% of the usual amount of bisacrylamide and a pH in the separation gel of 8.2 (10). Lanes: 1, CP365 (*trg*$^+$); 2, CP375 (*trg-19*); 3, CP454 (*trg-8*). B, Low-resolution patterns of Trg in stimulated cells. Conventional 9% polyacrylamide gels do not resolve the complete array of Trg form, but display clearly the shift to faster-migrating forms upon adaptation. Strains listed in A were either not stimulated (–) or subjected to a stimulus of 10 mM galactose (g) or ribose (r) and allowed to adapt.

proteins linked to Trg is inhibited by the presence of ligand for the other binding protein (2, 10, 39). This implies that the transducer becomes saturated even though adaptation has occurred and that sugar-occupied binding proteins compete for a limited number of Trg molecules. We investigated the effect on this competition of the *trg* mutations described in this report. In contrast to wild-type cells, for which reciprocal inhibition was evident, no inhibition of taxis by the mutant strains was caused by the sugar to which the cells were insensitive (Fig. 6). As measured in the capillary assay, taxis toward ribose by a *trg-19* mutant was unaffected by galactose, even at concentrations 1,000-fold above the value of the dissociation constant of the galactose-binding protein for its sugar ligand (Fig. 6B). Similarly, the modest accumulation of *trg-8* cells in a gradient of galactose was unchanged by the presence of ribose (Fig. 6A). These observations suggest that each mutant protein has a block in productive interaction with one or the other binding protein at a stage before integration of stimuli from the two binding proteins.

DISCUSSION

We would like to define relationships between particular structural features of the Trg protein and specific aspects of transducer function. If certain amino acid substitutions in Trg were to affect only a subset of transducer functions, then isolation and characterization of such mutants would constitute a powerful genetic approach for investigating structure-function relationships. The *trg* mutants described in this report provide clear examples of function-specific defects and thus document the productivity of this strategy.

Function-specific mutations. Both mutations described here affect the ability of the Trg protein to generate excitatory signals in response to specific attractant stimuli, but do not block the methyl-accepting activity of the mutant protein. Adaptation appears normal for stimuli that do evoke responses in the mutant cells. Thus the mutational defects are focused in the earliest steps of the sensory cascade, attractant binding and the linked excitatory alteration of the ligand recognition domain. The locations of the two mutational substitutions provide direct evidence that the Trg domain between the two putative membrane-spanning regions is involved in ligand recognition, as suggested by comparison of deduced amino acid sequences of the four transducers (5, 26) and by analogy with Tsr and Tar, for which genetic (25) and biochemical (31) observations have identified the corresponding domains. In a previous report (5), we described mutations, isolated in plasmid-borne *trg*, that conferred a phenotype (ribose taxis positive, galactose taxis negative) similar to that of the *trg-19* mutant. However, when those mutations were present in a single chromosomal copy, the Trg phenotype differed only slightly from the wild type (C. Park, unpublished observations), and thus we have not studied the mutations further.

The mutant phenotypes indicate that the ability of a transducer to accept or to lose methyl groups does not require the activity of ligand-binding sites. The Trg-8 protein became radiolabeled with *methyl*-^{3}H groups to approximately the same extent, over the same time period as did wild-type protein, even though the mutant protein is significantly defective in mediation of excitation by either Trg-linked ligand. The Trg-19 protein was radiolabeled in a manner indistinguishable from that of the wild-type protein in cells induced for the active ligand (ribose-binding protein) or the inactive ligand (galactose-binding protein). Thus, the steady-state turnover of methyl groups must be essentially independent of input from the ligand-binding domain, even though changes in the level of ligand binding certainly do affect methyl-accepting sites by inducing competency for net methylation or demethylation. The converse observation of independent functioning of a transducer domain has been made for a mutant Tar transducer inactive as a methyl acceptor (35). Thus the two postulated transducer domains can each be active independent of the functional competency of the other.

The existence of *trg* mutations that confer ribose taxis-positive, galactose taxis-negative phenotypes or vice versa implies that the intratransducer pathway from sugar-occupied binding protein to excitatory signal is not identical for galactose- and ribose-binding proteins. Identification of the sequence alteration in two such mutations demonstrates that single amino acid substitutions can have drastically asymmetrical effects on the ability of Trg to mediate tactic response to the two sugars. The *trg-19* alteration is almost surgically clean in its specific effect on galactose taxis. A hypothesis consistent with all of the experimental observations is that the mutational defect is in the site on Trg that interacts with galactose-binding protein, either because glycine 151 is a crucial residue in the site or because substitution of an aspartate at that position disrupts the

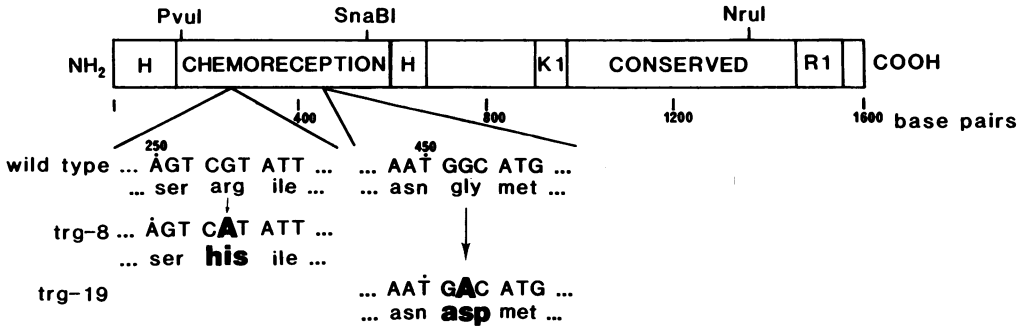

FIG. 5. Mutational changes in the nucleotide sequence and deduced amino acid sequence of *trg*. Important features of the deduced amino acid sequence are indicated: H, hydrophobic regions; chemoreception, proposed ligand-interaction domain; K1 and R1, tryptic peptides containing methyl-accepting sites; conserved, a conserved region of substantial amino acid identity for the four known transducers. The scale is numbered in nucleotides from the beginning of the coding region, and important restriction sites are indicated. The mutational changes that were observed are indicated in boldface type.

structure of the site. In either case, the substitution leaves undisturbed interaction between Trg and ribose-binding protein. Thus the Trg protein must bind the sugar-binding proteins with two nonidentical sets of amino acid residues. This implies that the complementary sites on the two respective binding proteins differ. Amino acid sequences shared specifically by the pair of sugar-binding proteins (4) will not include the complete transducer-interaction sites, and it may be that the sites on the two binding proteins are not substantially related by primary structure. In any case, detailed characterization of the Trg-19 phenotype has eliminated the possibility that the two sugar-binding proteins interact with identical regions at a single site on Trg. Characterization of additional mutants should establish the degree of overlap, if any, between the two binding sites on the Trg protein.

The *trg-8* mutation greatly reduced response to both galactose and ribose. A simple explanation of the phenotype would be that substitution of histidine for arginine at position 85 disrupts Trg in the region of overlap between the binding sites for the two sugar-binding proteins. However, a disrupted binding site should have a lowered affinity for ligand, yet this does not appear to be the case for the Trg-8 protein. The dose-response curves shown in Fig. 1 indicate that the Trg-8 protein has apparent affinities for ligands that are indistinguishable from the affinities of normal Trg, but that the apparent effectiveness of ligand-transducer interaction is substantially reduced. Thus it appears that the substitution at position 85 does not alter ligand binding but rather the ability of the ligand-transducer complex to generate the excitatory cascade. This implies that arginine 85 is not a functional part of the ligand-binding sites, but rather is important in the putative conformational change generated by ligand binding.

There is an asymmetry in the effect of *trg-8* that was clear in strains carrying multiple copies of the gene. The increased dosage improved response to galactose but not to ribose. If the Trg-8 phenotype is the result of defective generation of the excitatory conformational change, then the asymmetry implies that generation of the change by the two binding proteins is not by identical pathways. This would be the case if there were a substantial separation of the two ligand-interaction sites on Trg.

Relative sensitivities of tactic assays. In analyzing the *trg* mutants, we used three different tests for tactic behavior: formation of chemotactic rings on semisolid agar swarm plates, response of tethered cells to temporal gradients, and accumulation of cells in capillaries in response to spatial gradients. Measurement of defective responses with all three of the procedures provided insight into the relative sensitivities of the assays. The most sensitive and informative test was temporal stimulation of tethered cells. However, an altered balance between directions of flagellar rotation and extended adaptation times made the assay unusable for characterization of strains harboring multicopy plasmids carrying *trg*. Both the capillary and swarm plate assays measure response of populations to spatial gradients and were useful for strains with either a normal or an increased dosage of transducer. The capillary assay provides good discrimination for responses that are a substantial proportion of the wild type but is insensitive to marginal responses. In contrast, the swarm plate procedure reveals certain classes of marginal responses as faint rings while providing little discrimination between a more substantial but reduced response and a wild-type response. Thus, certain kinds of transducer mutants with limited defects might well be missed in surveys with swarm plates.

Competition among ligands. In early studies of chemotaxis in *E. coli*, the results of competition experiments grouped chemoattractants into receptor classes, structurally related compounds thought to be recognized by the same receptor site (2). The presence of saturating amounts of glucose did not affect sensitivity to maltose but inhibited response to galactose. The reciprocal experiments provided symmetrical results, and thus it could be concluded that there was an independent maltose receptor and a single receptor that bound galactose and glucose (2). An anomaly occurred for the sugars galactose and ribose. The compounds were relatively unrelated structurally, yet significant competition was observed (2). In addition, parallel observations identified separate receptors for the two sugars (3, 13). An explanation was provided by the discovery that the two independent receptor proteins for galactose and ribose were linked to the chemotactic system by a common component, the Trg transducer (17, 24, 32, 33). The implication was that the competition was not for receptor sites but for interaction with transducer. However, another transducer, Tar, interacted with two independent ligands, aspartic acid (44) and ligand-occupied maltose-binding protein (23), yet no competition could be detected between aspartate and maltose by the usual tactic assays. Why is the situation different for Trg? One difference between the Tar and Trg transducers is cellular concentration. There are approximately 10-fold more Tar molecules than Trg molecules per cell (16), and thus a limiting amount of Trg could be the basis of the

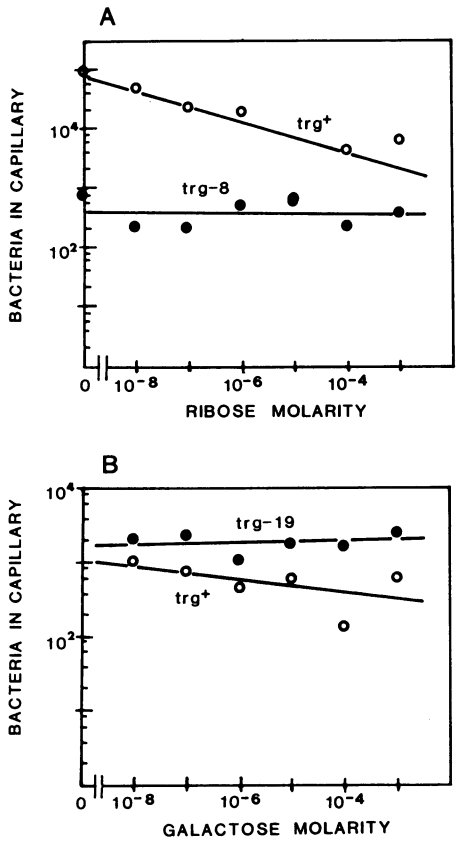

FIG. 6. Cross-inhibition by Trg-linked attractants in wild-type and mutant strains. Accumulation of bacteria in microcapillaries containing 10^{-4} M galactose (A) or 10^{-4} M ribose (B) is plotted versus the concentration of competing sugar (ribose in A, galactose in B) present in both capillary and cell suspension. Cells were grown in H1 minimal salts plus ribose and galactose (A) or only galactose (B). For the assays shown in B, both capillary and cell suspension contained 200 μg of chloramphenicol per ml to prohibit synthesis of additional receptor protein during the time of the assay. The high response shown for the *trg*$^+$ strain in A is probably the result of variation in the number of plasmids per cell. The low response to ribose shown in B reflects a low content of ribose-binding protein in cells not induced for the expression of the corresponding gene. A high cellular content of galactose-binding protein and a low content of ribose-binding protein was required to detect inhibition by galactose of response to ribose. Strains: CP474, harboring a wild-type *trg* plasmid (○ in A and B); CP376, harboring a *trg-8* plasmid (● in A); CP375, harboring a *trg-19* plasmid (● in B).

observed competition. This explanation is made unlikely by the observations documented in Fig. 6. Increasing Trg dosage to a level comparable to that of Tar by introduction of an appropriate multicopy plasmid did not alleviate the reciprocal inhibition characterized for cells with a single chromosomal copy of *trg*. A second difference between Tar and Trg is that the ligands for the former are structurally unrelated, whereas those for the latter are proteins with significant sequence homology (4). This provides the possibility that competition is for a single recognition site that binds both sugar receptors. A refinement of that model is necessary to explain the phenotype of *trg-19* mutants. Interaction of the two sugar-binding proteins with Trg can be mutually exclusive, but the region recognized on the two proteins by Trg cannot be identical. The situation may be analogous to galactose and glucose both fitting in the site of galactose-binding protein (13), or, at the other extreme, competition could be the result of conformational blocking of independent sites. The asymmetrical effect of the *trg-8* mutation argues for a functional separation that persists beyond the stage of initial binding and thus implies a significant degree of physical separation between the two sites for binding protein interaction. These notions can be tested by a combination of genetic and biochemical investigations.

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