Tandem Duplication and Multiple Functions of a Receptor Gene in Bacterial Chemotaxis*

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The aspartate receptor in bacterial sensing, previously identified with the tar gene, has been shown to be duplicated in tandem in Escherichia coli. Each gene, which we refer to as tar and tap, respectively, codes for a 60,000-dalton protein. By genetic engineering experiments in which each gene is introduced separately into E. coli strains, it is shown that each transmembrane receptor can respond to the small molecule aspartate, to the maltose-protein-chemoeffector complex, and to repellents.

Receptors are molecules which receive stimuli from the environment and transmit that information to direct a change in the cellular behavior. The information is passed on by a conformational change in the receptor, but the understanding of this process is advancing slowly because most receptors are membrane-bound and usually present in small amounts. Bacterial receptors are convenient to study since bacteria can be grown in large quantities and production of individual proteins can be amplified by genetic engineering techniques.

In the chemotaxis system, two basic types of bacterial receptors have been identified so far. The first is the periplasmic binding proteins which serve as primary receptors for galactose (1), ribose (2), and maltose (3). Springer et al. (4) and Silverman and Simon (5) have identified two 60,000-dalton membrane-bound proteins as the products of the tar and tar genes, and we have previously shown them to be the primary receptors for aspartate and serine by genetic and biochemical techniques (6). Mutants in the tar and tsr proteins showed loss of aspartate and serine binding (7, 8).

Genetic studies have suggested that the aspartate receptor is involved in the response to maltose. A single mutation in the tar gene of Escherichia coli eliminated behavioral responses to aspartate as well as maltose and repellents (9). Since the maltose response operates through the periplasmic maltose-binding protein (3, 10), this would mean that the transmembrane signal through tar could be elicited either by direct binding of aspartate or through the maltose-binding protein interaction (6). Such dual behavior has not so far been demonstrated in mammalian and other receptor systems, and therefore, it became important to establish whether, in fact, these two stimuli are processed through the same protein.

* amusement of the genetics suggested the possibility that polar mutations operating on two genes in the same operon, one coding for an aspartate receptor and the second for a maltose receptor, might explain both the bacterial genetics and the unusual receptor behavior. We therefore proceeded to examine the DNA coding for the aspartate receptor in E. coli. We find that there are in fact two genes in tandem coding for separate 60,000 molecular weight proteins. However, most surprisingly, we find that each protein is a receptor that mediates responses to aspartate, maltose, and repellents.

EXPERIMENTAL PROCEDURES

RP strains of E. coli were obtained from J. S. Parkinson (University of Utah). RP437 (leu his thr thi met eda strA) is wild type for chemotaxis; RP4324 (leu his thr thi met rif strA tar52) is a tar deletion mutant with random swimming basal behavior; RP4372 (leu his thr thi met strA tar52 tsr518) is a tar tsr double mutant with smooth swimming behavior. E. coli X1488 was a gift from H. Beyer, Univ. of California. Cells were grown at 30 °C in Vogel-Bonner citrate medium (11) with 1% glycerol, required supplements at 100 µg/ml, and ampicillin at 25 µg/ml where appropriate, and were harvested in mid-to-late logarithmic phase. The behavior of individual bacteria was analyzed by tethering (12), flagella were sheared from the bacteria by brief (12 s) mixing in a Waring blender and were allowed to grow for short times (10–30 min) directly on antiflagellin coated coverslip. Potassium morpholinopropanesulfonate medium without trace metals (13) and with the appropriate supplements and 100 µg/ml chloramphenicol was used as tethering buffer.

Cell labeling (14), sample preparation (15, 16), cell membrane preparation (7), mammalian transferrase assays (6, 14, 17), immunoprecipitation (18), membrane protein solubilization procedure (6), electrophoresis methods (15, 16), analysis of radioactivity in gels (14, 19), and protein determination (20) have been described. Minicell preparation and labeling methods were followed (21) except that [35S]methionine (1149.9 Ci/mol) and Difco methionine assay medium were used.

Plasmid and phage DNA were prepared as described (22). Phage Φla3326 (23) was obtained from M. Simon (University of California, San Diego). Restriction endonuclease digestions were executed as recommended by the manufacturers (New England Biolabs or Bethesda Research Laboratories), and DNA was analyzed on 0.7–1.0% agarose gels run in Tris-borate-EDTA buffer (22). The chemotaxis insert located on an Eco RI fragment in Φla3326 contained the tar, cheR, cheB, cheY, and cheZ genes (23); this fragment was inserted into the Eco RI site of plasmid pBR322 (24) to produce plasmid pWK51 (Fig. 1). Deletions of pWK51 were constructed by removing restriction fragments then recircularizing with T4 ligase (25); plasmid pWK53 was constructed with HindIII, pWK54 with Pst II, and pWK56 with Ava I. Plasmid pCK57 was constructed by ligating pWK51 DNA bracketed by Kpn I and Eco RI sites (Fig. 1) into a pBR322 derivative containing a small SV40 insertion (26) containing a Kpn I site. Plasmids were transformed into E. coli as described (27).

RESULTS

Plasmid pWK51 was constructed by inserting an Eco RI fragment containing the tar, cheR, cheB, cheY, and cheZ genes of E. coli from the previously described (23) Φla3326 into the Eco RI site of the plasmid pBR322. Expression of the cloned genes was then analyzed by (a) production of proteins in minicells, (b) enzyme assay for the levels of methyl transferase and (c) levels of in vivo carboxymethylation in the 60,000-dalton receptor protein. The results shown in Table 1 indicate synthesis of tar, cheR, cheB, cheY, and cheZ from the plasmid and quantitate increased levels of the 60,000-dalton tar protein and of the methyl transferase in plasmid containing cells.

Derivatives of pWK51 were obtained by subcloning restric-
Multiple Functions of Receptor

Table I

| Strain | Plasmid | Proteins encoded by plasmid | Transferase | Methylation level |
|--------|---------|-----------------------------|-------------|------------------|
| RP437  | -       | -                           | -           | 0.9 pmol/mg of protein |
| RP437  | -       | -                           | -           | -                |
| RP437  | pWK51   | tar, tap, cheR, cheB, cheY, cheZ | 3.9 pmol/mg of protein | 1024.3 |
| RP437  | pWK53   | tar, tap, cheR              | 5.4 pmol/mg of protein | 1864.2 |
| RP437  | pWK54   | tar (fragment of 57,000 daltons) | 6.2 pmol/mg of protein | 2678.5 |
| RP437  | pWK56   | tar                         | 12.5 pmol/mg of protein | 5024.6 |
| RP437  | pCK57   | tap, cheR, cheB, cheY, cheZ  | 1778.5 pmol/mg of protein | 6024.6 |

a No plasmid added.

b Also identified by immunoprecipitation.

tion fragments. By restriction mapping and plasmid-directed protein synthesis in minicells from the deletions, the position of the genes can be defined as shown in Fig. 1. Plasmid pWK54, which is missing insert DNA after the Pvu II site 2.1 kilobases from the Eco RI site, and plasmid pWK56 allowed us to define the C-terminal end of the tar gene; plasmid pWK53 localized the HindIII restriction site in the cheB gene; another plasmid, pWK55, placed a Pvu II site in the cheZ gene. This map indicated that there existed between tar and cheB enough DNA to code for both the cheR gene product of 28,000 daltons and another protein of approximately 60,000 daltons.

To determine whether two 60,000-dalton proteins were produced, we used gels with increased resolution to examine the proteins encoded by the plasmids. As seen in Fig. 2, the complete plasmid pWK51 shows 7 bands in the 60,000-dalton region of a sodium dodecyl sulfate-polyacrylamide gel; this banding pattern had been thought to be caused entirely by multiple methylation of tar (28-31). However, plasmid pWK56, missing the last 8 kilobases of the chemotactic insertion, codes for only the lower 4 of the 7 protein bands. Another plasmid, pCK57, missing the initial 2 kilobases of the insertion, showed the missing 2 upper bands. An additional, fast migrating band is produced by minicells containing pWK53, where tar is expected to be more highly methylated because of the absence of cheB, the esterase gene. It is apparent that this region of DNA just upstream of the cheR gene is responsible for two distinct sets of bands seen in sodium dodecyl sulfate gels.

One possible explanation is the existence of two genes, in tandem, coding for the proteins of ~62,000 and 65,000 daltons. A second is the existence of only a single gene, in which elimination of DNA changed the electrophoretic mobility of the single gene product. This would require that the common DNA in plasmids pWK57 and pWK56 must encode a 62,000-dalton protein. However, the common DNA is only 0.9 kilobase and is able to code for a protein of only 30,000 daltons. Therefore, two tandem genes encoding separate proteins must exist in E. coli. These two proteins, one or both of which was previously identified as the tar gene product, will be referred to as tar (62,000 daltons) and tap (65,000 daltons).

It was next necessary to define the relationship between these two proteins. Cell fractionation experiments with minicells showed both tar and tap proteins to be integral membrane components, and both can be solubilized from the membrane in 1.0% Triton X-100. We then used antibody prepared against Salmonella tar (6) as a structural probe with previously identified proteins.

1 While this work was in progress, we learned of the parallel work by Boyd et al. (41) who named the gene tap (taxis protein) because its function was unknown. Although there is some reason to include the information shown here in regard to function in the name, new functions may be found and it will avoid confusion to adopt a common terminology.
the solubilized membrane proteins. This antibody precipitates both tar and tap and a pattern of bands is similar to those in Fig. 2. Since the antibody cross-reacts with Salmonella tar and E. coli tar and tsr (6), at least one region of homology is shared by all of these genes. The homology is not unexpected since (a) there is only one transferase (17) and one esterase (32) in each strain which react with all of these receptors and (b) the Salmonella and E. coli enzymes are similar enough to complement each other (33).

The next functional examination was carboxymethylation. Our in vitro methylation studies (Table I) show that both tar and tap are carboxymethylated, but tap methyl groups are at low concentrations. This is the result of extremely low levels of pCK57 expression since protein synthesis in the pCK57 plasmid is probably dependent on a promoter located in the pBR322 portion of the DNA (35). Assay for methyl transferase, which is coordinately expressed, indicates low plasmid protein expression. Therefore, carboxymethylation was also examined by two-dimensional gel electrophoresis. Tar and tap proteins were identified by minicell labeling and then studied in strains RP437 (wild type) and RP4372 (tar− tsr−) pWK51. As shown in Fig. 3, protein encoded by the tar gene is the previously identified tar protein with an isoelectric point of ~5.8 (31) and both tar and tap show multiple spots. Individual spots of tar and tap from RP4372/pWK51 were analyzed for carboxymethylation in in vitro methylation studies (Table I) show that both tar and tap are carboxymethylated to similar levels, assuming they have about the same number of methionine residues. An aspartate stimulus increases the levels of carboxymethylation in tar and also in tap. An intriguing observation is that tap lacks the electrophoretic mobility change seen with tar (31) upon an aspartate stimulus, despite the increase in the level of carboxymethylation. Comparison of the ratio of radioactivity incorporated in methyl esters and in protein (14); this overproducing strain was used since the background is reduced. Comparison of the ratio of radioactivity incorporated in methylesters to radioactivity incorporated in protein indicates that both tar and tap are carboxymethylated to similar levels, assuming they have about the same number of methionine residues. An aspartate stimulus increases the levels of carboxymethylation in tar and also in tap. An intriguing observation is that tap lacks the electrophoretic mobility change seen with tar (31) upon an aspartate stimulus, despite the increase in the level of carboxymethylation. Comparison of the total methionine incorporated in protein reveals that the level of tar is about 5-7-fold higher than the level of tap, assuming equal amounts of methionine per receptor. A similar ratio of tar/tap expression was also seen in RP437 (wild type).

The function of these related proteins was studied by examining the behavior in tar deletion mutants containing plasmids with tar and/or tap. The results of these experiments are shown in Table II. Responses to aspartate, maltose, NI2+, and Co2+ were restored in the tar− deletion strains by addition of plasmids containing either tar or tap. The response times vary because of different chemotactic gene dosages. Cells containing plasmid pWK51, with normal tar, transferase, and esterase ratios, show response times similar to wild type. Cells containing plasmid pWK56, with a high ratio of receptor to methylating enzymes, exhibit extremely long adaptation times. Cells containing plasmid pCK57, with a low ratio of receptor to methylating enzymes, have very short response times. The plasmid pCK57 also shows a distinctive Ni2+ response, the Ni2+ response can be seen only as a suppression of attractant-induced smooth swimming (see Table II). This result could be due to gene dosage or may be a unique characteristic of tap. Thus, at this stage, the two genes appear

![Figure 3](http://www.jbc.org/)
to code for similar but not identical receptor proteins. Responses are dependent on tar and tap levels and the contribution of tap to chemotaxis of wild type levels would seem to be appreciably less than tar. A number of other chemoeffectors were also examined to determine whether some additional receptor specificity differentiated these two genes. However, no response to leucine, acetate, benzoate, glycine, or alanine was seen.

DISCUSSION

The discovery of a tandem gene duplication of tar in E. coli is intriguing in a number of ways. Tandem duplications of large and small segments of DNA occur at a fairly high frequency \((10^{-3}-10^{-4})\) in bacteria (36); however, duplications are generally unstable and the extra DNA is deleted when selective pressure is removed. Gene duplication may be directed at gene amplification in order to increase the resulting gene products (37). The marked difference in tar and tap expression suggests that this is not the reason for the duplication. Gene duplication can also allow the acquisition of new functions in an evolutionary sense. For example, recent DNA sequence studies on the his \(J\) and arg \(T\) genes in Salmonella indicate gene duplication of a basic amino acid-binding protein which then allowed evolution of two proteins with separate binding specificities (38). In the case of the aspartate receptor, each individual gene provides the bacterium with the capability of a behavioral response to aspartate, maltose, \(\text{Ni}^{2+}\), and \(\text{Co}^{2+}\). However, these genes are not identical; the \(\text{Ni}^{2+}\) response, the change in protein migration upon exposure to aspartate, and the levels of each protein in the cell are different, and it is possible that an additional function which distinguishes these two genes will be discovered. Hybridization studies on tar and tap also indicate that the DNA sequences are similar but not identical (41).\(^2\) These studies show that chromosomal DNA from several \(E.\ coli\) strains also contains tar and tap in tandem.\(^2\) Further studies on these genes are underway. Since this is the first example of a behavioral system gene duplication, the reason for the selective advantage of the tandem duplication should be particularly revealing.

The finding that plasmids containing each individual gene product can restore responses to both aspartate and maltose establishes the hypothesis (6) that a single protein receptor can be activated either by a small molecule or a protein-chemoeffector complex. Since aspartate elicits a very strong response and is a good bacterial nutrient, it seems likely that the primary evolutionary function for the tar protein was as an aspartate receptor. Mutations broadened the receptor functions to include \(\text{Ni}^{2+}\), \(\text{Co}^{2+}\), and the periplasmic maltose-binding protein which probably increased the survival capacity of the organism. Since the tap protein shows many of the properties of receptors in higher organisms (39, 40), these results suggest that mammalian receptors may also exhibit the properties shown here, and that responses to different effectors may not necessarily require different transmembrane components.

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