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Membrane Topology Analysis of *Escherichia coli* K-12 Mtr Permease by Alkaline Phosphatase and β-Galactosidase Fusions

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The *mtr* gene of *Escherichia coli* K-12 encodes an inner membrane protein which is responsible for the active transport of tryptophan into the cell. It has been proposed that the Mtr permease has a novel structure consisting of 11 hydrophobic transmembrane spans, with a cytoplasmically disposed amino terminus and a carboxyl terminus located in the periplasmic space (J. P. Sarsero, P. J. Wookey, P. Gollnick, C. Yanofsky, and A. J. Pittard, J. Bacteriol. 173:3231–3234, 1991). The validity of this model was examined by the construction of fusion proteins between the Mtr permease and alkaline phosphatase or β-galactosidase. In addition to the conventional methods, in which the reporter enzyme replaces a carboxyl-terminal portion of the membrane protein, the recently developed alkaline phosphatase sandwich fusion technique was utilized, in which alkaline phosphatase is inserted into an otherwise intact membrane protein. A cluster of alkaline phosphatase fusions to the carboxyl-terminal end of the Mtr permease exhibited high levels of alkaline phosphatase activity, giving support to the proposition of a periplasmically located carboxyl terminus. The majority of fusion proteins produced enzymatic activities which were in agreement with the positions of the fusion sites on the proposed topological model of the permease. The synthesis of a small cluster of hybrid proteins, whose enzymatic activity did not agree with the location of their fusion sites within putative transmembrane span VIII or the preceding periplasmic loop, was not detected by immunological techniques and did not necessitate modification of the proposed model in this region. Slight alterations may need to be made in the positioning of the carboxyl-terminal end of transmembrane span X.

The *mtr* gene encodes a hydrophobic protein of 414 amino acid residues which constitutes the so-called high-affinity tryptophan transport system of *Escherichia coli*. With the low-affinity tryptophan transporter TnaB and the tyrosine-specific transporter TyrP, Mtr permease forms a separate family of amino acid transport proteins (47, 48). On the basis of hydropathy profiles, Heatwole and Somerville (19) suggested that the Mtr permease was a polytopic protein with 12 transmembrane spans, as has been found for various other integral membrane transport proteins of *E. coli* (19, 28). However, consideration of the distribution of charged amino acid residues, as described by von Heijne (56, 57) and Nilsson (38), and the occurrence and distribution of possible turns, in addition to hydropathy data, led us to a model for Mtr permease which involved only 11 spans, with the carboxyl terminus of the protein in the periplasm (47). Similar models were developed for the TnaB and TyrP permeases (46).

In this report, the validity of our proposed topological model is examined by a genetic approach involving the construction of fusions between the Mtr permease and sensor enzymes which reflect the subcellular disposition of the membrane protein fusion site. A series of gene fusions between the *mtr* gene and *phoA* (encoding alkaline phosphatase) and *lacZ* (encoding β-galactosidase) were constructed to determine the membrane topology of the permease. The signal sequence of alkaline phosphatase can be replaced by export signals derived from other proteins, including those found in integral membrane proteins (14, 53). Alkaline phosphatase-specific activity of a fusion protein is closely correlated with the cellular location, in the native protein, of the domain to which the fusion is made (7, 31). Alkaline phosphatase is only enzymatically active when it is translocated to the periplasm. Fusion of alkaline phosphatase to a periplasmic site of a membrane protein yields a hybrid protein with the alkaline phosphatase moiety in the periplasm, where it is enzymatically active. Fusion of alkaline phosphatase to a cytoplasmic domain yields an inactive, cytoplasmically disposed enzyme (32). In contrast, hybrid proteins containing β-galactosidase show reciprocal behavior to alkaline phosphatase fusions, where attachment of β-galactosidase to cytoplasmic stretches yields hybrid proteins displaying high levels of enzymatic activity (29, 30). Fusions to periplasmic domains are believed not to mediate export of β-galactosidase across the membrane; rather, the enzyme remains embedded within the membrane, preventing proper folding or tetramerization and enzymatic activity (16). Lee et al. suggested that multiple sites throughout β-galactosidase block export of the enzyme across the membrane (25).

In the methods normally used to generate these gene fusions, various lengths of the amino-terminal end of the membrane protein are attached to alkaline phosphatase or β-galactosidase, which replaces a carboxyl-terminal portion of the protein. However, it is possible that the final topology of the permease is dependent upon interactions between the amino- and carboxyl-terminal sequences of the protein. Beckwith and coworkers were the first to construct membrane protein-alkaline phosphatase sandwich fusions, in which alkaline phosphatase is inserted into an otherwise intact membrane protein, and showed that alkaline phosphatase still exhibits normal enzymatic activity, even though it is tethered at both its amino and carboxyl termini (14). By using a series of MalF-alkaline phosphatase sandwich fusions, it was shown that this method provides results that are better correlated with the actual topology of the membrane protein than those obtained with

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TABLE 1. *E. coli* K-12 strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
JM101	Δ(*lac-pro*) *supE thi* (F′ *traD36 proA*$^+$B$^+$ *lacI*^{q} Δ*lacZM15*)	34
JP7742	W3110 Δ*lacU169 tsx*	This study
JP10029	*thr-1 leuB6 supE44 tonA21 gal351 thi-1 rpoB365* Δ*phoA8 tsx*	This study
JP10054	W3110 *aroP523 mtr24 tnaA2* Δ*phoA8 tsx*	This study
W3110	Prototroph	4
Plasmids		
pLG339	Tc^r Km^r, low-copy-number vector, pSC101 *ori*	50
pMU2386	Tp^r, ′*lacZ* low-copy-number gene fusion vector, IncW replicon	24
pMU5190	Km^r, derivative of pLG339 with 234-bp *Bgl*I fragment in Tc^r determinant deleted	This study
pMU5191	Km^r, 2.6-kb *Eco*RI-*Bam*HI fragment containing *mtr* gene in pMU5190	This study
pMU5193	Km^r, derivative of pLG339 containing 2.6-kb *mtr* fragment and 1.4-kb ′*phoA* fragment (Fig. 1)	This study
pMU5194	Tp^r, derivative of pMU2386 containing 2.6-kb *mtr* fragment and 3.0-kb ′*lacZ* fragment (Fig. 1)	This study
pSWFII	Ap^r, *phoA* sandwich fusion construction vector	14

[a] The genetic nomenclature is that described by Bachmann (5). Allele numbers are indicated where known. Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Tp^r, trimethoprim resistance; Ap^r, ampicillin resistance.

carboxyl-terminal replacement fusions, because the entire amino acid sequence of the target protein is present in the fusion product. It was concluded that sandwich fusions provided a more sensitive monitor of the cellular location of the domain of the target protein to which the enzyme is fused (14). All of these techniques were utilized in the examination of the topological structure of the Mtr permease.

A preliminary report of this work has been presented (45).

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The bacterial strains used in this study were all derivatives of *E. coli* K-12, and their relevant genotypes are described in Table 1. The plasmids used are also listed in Table 1. The bacteriophages M13mp18 and M13mp19 have been described previously (39).

Media and reagents. The minimal media used were the half-strength buffer 56 of Monod et al. (36) and the 121 salts medium of Torriani (52), supplemented with 0.2% glucose and appropriate growth factors. Trimethoprim was used in nutrient and minimal media at final concentrations of 40 and 10 μg/ml, respectively. Kanamycin was used at a final concentration of 10 μg/ml in all media. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) from Progen, Australia, and 5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt (XP) from Sigma Chemical Co. were used at 25 and 40 μg/ml, respectively, in solid media. All enzymes were purchased from Amrad Pharmacia Biotechnology, Melbourne, Australia, unless otherwise indicated. [α-^{35}S]dATP (1,200 Ci/mmol; 10 mCi/ml) for use in DNA sequencing, [^{35}S]methionine-cysteine (1,175 Ci/mmol; 7.9 mCi/ml) for use in pulse-labeling, and L-[3-^{14}C]tryptophan (52 Ci/mmol; 0.02 mCi/ml) for use in transport assays were obtained from DuPont, NEN. Oligonucleotides were synthesized on a Gene Assembler Plus [^{35}S]methionine and [^{35}S]cysteine (Pharmacia-LKB).

Recombinant DNA techniques. Standard recombinant DNA procedures were used essentially as described by Sambrook et al. (41).

Construction of *mtr′-′phoA* and *mtr′-′lacZ* gene fusions. Two series of fusion plasmids were constructed in which various lengths of the 5′ end of the *mtr* gene were attached to the *phoA* or *lacZ* genes by using a nested-deletion method described by Sugiyama et al. (51). The vector pMU5193 contains both the complete *mtr* gene sequence, including its regulatory region, and the *phoA* gene, lacking its transcription and translation initiation sequences and the region encoding its signal sequence (designated ′*phoA*). This is a derivative of the low-copy-number vector pLG339 (six to eight copies per cell) (50). The vector pMU5194 contains the same *mtr* fragment as pMU5193, upstream of the *lacZ* gene lacking the start points of transcription and translation (′*lacZ*). This IncW replicon, a derivative of pMU2386 (24), has a copy number of one to two per cell (15). Unique sites for restriction endonucleases generating 5′ and 3′ overhangs are present between the two genes on both plasmids (Fig. 1). Plasmid pMU5193 was linearized by cleavage of the unique *Sal*I and *Kpn*I sites located between the genes, and pMU5194 was similarly digested with *Bam*HI and *Pst*I, thereby generating a 5′ overhang just downstream of the *mtr* gene and a 3′ overhang positioned upstream of the ′*phoA* or ′*lacZ* genes. Exonuclease III was used to progressively delete *mtr* from its 3′ end via the 5′ overhang, essentially according to the method of Henikoff (20) as outlined in the commercial kit of Stratagene Cloning Systems. The reporter genes were protected from digestion, because 3′ overhangs are resistant to exonuclease III action. After treatment with Klenow fragment and the four deoxynucleotide triphosphates, the ends were ligated together. Circularized DNA derived from pMU5193 was transformed into JP10029 and plated onto minimal medium containing kanamycin, XP, and 1 mM L-phenylalanine, whereas DNA derived from pMU5194 was transformed into JM101 and plated onto minimal medium containing trimethoprim, X-Gal, and 1 mM L-phenylalanine.

The *mtr′-′phoA* gene fusions were excised on an *Eco*RI fragment and cloned into the same site of M13mp19, and the *mtr′-′lacZ* gene fusions were introduced into M13mp19 on a *Sac*I-*Sph*I fragment. The exact site of the fusion point was determined by DNA sequencing carried out by the dideoxy-chain termination method of Sanger et al. (42) by using T7 DNA polymerase.

Construction of *mtr′-phoA-′mtr* sandwich gene fusions. Unique *Bam*HI restriction endonuclease sites were individually introduced throughout the *mtr* gene present in M13mp18 (48) by oligonucleotide-mediated site-directed mutagenesis by the method of Vandeyar et al. (55) by using a commercial kit (United States Biochemical Corp.). After the isolation of the desired mutations, the entire *mtr* gene was sequenced to ensure that only the desired change was present. The ′*phoA* gene from pSWFII (14) was excised on a *Bam*HI fragment and introduced separately into the *Bam*HI sites in the *mtr* gene. After screening for the correct orientation by restriction analysis, the two junctions between *mtr* and ′*phoA* were sequenced to ensure that the reading frame was maintained. The gene fusions were then cloned into the vector pMU5190. To construct the carboxyl-terminal *phoA* fusion, a *Kpn*I restriction endonuclease site was introduced over the stop codon of the *mtr* gene. The ′*phoA* gene on a *Kpn*I fragment from pSWFII was introduced into this site. An in-frame stop codon is present immediately after the ′*phoA* coding region in this construct.

Enzyme assays. Cultures of JP10054 containing derivatives of pMU5193 and pMU5190 harboring *mtr′-′phoA* and *mtr′-phoA-′mtr* gene fusions, respectively, and cultures of JP7742 containing derivatives of pMU5194 carrying *mtr′-′lacZ* gene fusions were grown in half-strength buffer 56 containing 0.2% glucose, 1 mM L-phenylalanine, and the appropriate antibiotic, at 37°C to mid-exponential phase. Alkaline phosphatase and β-galactosidase activities were assayed in permeabilized cells as described by Manoil (30) and Miller (35), respectively. The units of enzyme activity were calculated according to the references mentioned above. Each assay was performed in duplicate on at least four separate occasions.

Transport assays. Cultures were grown under the same conditions described for enzyme assays. The uptake of [^{14}C]tryptophan was assayed essentially as described by Wookey et al. (59).

Pulse-labeling and immunoprecipitation. Cultures were grown under the same conditions described for enzyme assays to an optical density at 500 nm reading of 0.5. Strain W3110 was grown in 121 salts medium containing 0.2% glucose, 1 mM L-phenylalanine, and 0.1 mM KH_2PO_4, conditions under which the expression of the chromosomal *phoA* gene is derepressed (9). Aliquots of 0.5 ml of cell culture were pulse-labeled with 50 μCi of [^{35}S]methionine-cysteine for 1 min at 37°C. Preparation of cell extracts and setting up the immunoprecipitation conditions were performed according to the method of Ito and Akiyama (21), except that the alkaline fractionation step was omitted. Specific proteins were precipitated with antisera against bacterial alkaline phosphatase and β-galactosidase (5Prime→3Prime, Inc.). Gels were fixed and processed for fluorography (Amplify; Amersham). Broad-range protein molecular weight standards were purchased from Bio-Rad.

Western immunoblotting analysis. Cultures were grown under the same conditions described for pulse-labeling experiments and adjusted to an optical density at 600 nm of 0.5. Cells were concentrated 50-fold, and 20-μl samples were

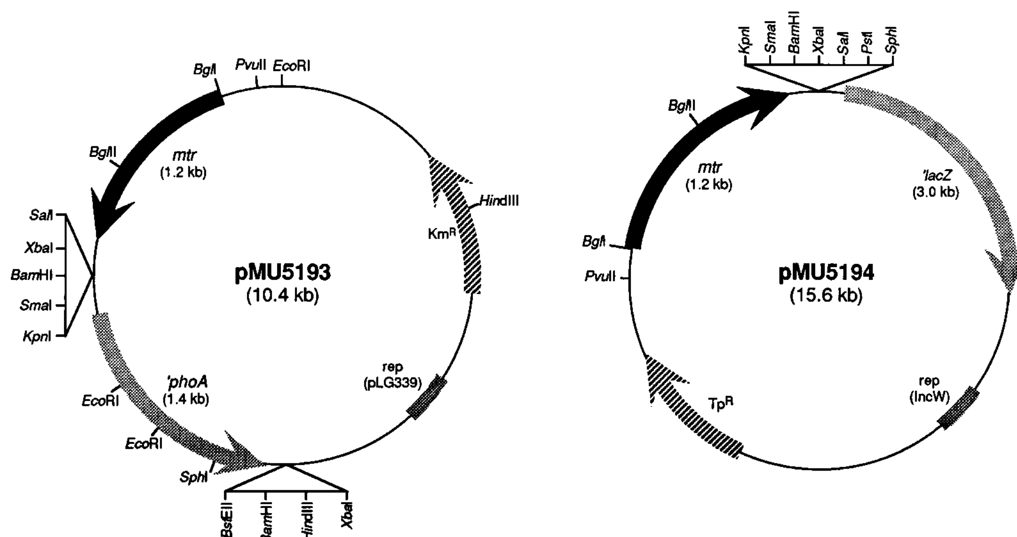

FIG. 1. Structure of vectors used in the generation of *mtr*′-′*phoA* and *mtr*′-′*lacZ* gene fusions. pMU5193 is a derivative of pLG339 (50), which contains both the *mtr* gene and the ′*phoA* gene of pSWFII (14) separated by unique *Sal*I and *Kpn*I restriction endonuclease sites. pMU5194 is an IncW vector which contains both the *mtr* gene and the ′*lacZ* gene of pMU2386 (24) separated by unique *Bam*HI and *Pst*I restriction endonuclease sites. Cleavage of the *Sal*I site in pMU5193 and the *Bam*HI site in pMU5194 produced substrates for exonuclease III digestion. Km^R, kanamycin resistance; rep, replicon.

electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (23), followed by electrophoretic transfer to a nitrocellulose membrane (Schleicher and Schuell [BA85]). Immunoblotting was performed as previously described (11) with antisera against bacterial alkaline phosphatase and β-galactosidase. Bound antibody was visualized by using horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin (DAKO Corp.) and the ECL Western blotting detection reagents (Amersham).

RESULTS

Isolation of Mtr permease-alkaline phosphatase (*mtr*′-′*phoA*) fusions. The nested-deletion method of Sugiyama et al. (51) was used to generate gene fusions encoding hybrid proteins with different lengths of the Mtr permease at their amino-termini joined to alkaline phosphatase, which replaced a carboxyl-terminal portion of the permease. A vector with a low copy number was chosen to harbor the gene fusions, because high-level expression of membrane proteins is often deleterious to cell viability. Exonuclease III was used to digest plasmid DNA from the 3′ end of the *mtr* gene, and the truncated gene was ligated to the ′*phoA* gene sequence. Transformed cells were grown on media containing phenylalanine, conditions under which the TyrR regulatory protein is able to activate expression of the *mtr* gene four- to fivefold (44, 48). This level of expression is not deleterious to cellular growth and facilitated the differentiation of isolates with different activities. In-frame fusions between *mtr* and ′*phoA* resulted in the formation of blue transformants of various intensities on media containing the chromogenic substrate XP. Plasmid DNA from 117 such transformants was analyzed by restriction digestion. Forty-six fusions falling within the coding region of the *mtr* gene were cloned into M13mp19, and the exact fusion point was determined by nucleotide sequencing. A total of 32 unique in-frame *mtr*′-′*phoA* gene fusions were isolated. The derivatives of pMU5193 harboring the gene fusions were then introduced into the strain JP10054, and alkaline phosphatase activity was measured. Plasmid DNA produced after exonuclease III digestion had not been initially transformed into this strain because of its low efficiency of transformation.

With the exception of the region between putative transmembrane spans VII and VIII, the pattern of activity of most

TABLE 2. Properties of Mtr permease-alkaline phosphatase fusion proteins

Isolate (fusion no.)	Fusion position (amino acid)[a]	Alkaline phosphatase activity[b]
1	29	124
2	40	76
3	52	47
4	132	81
5	134	56
6	136	45
7	175	89
8	183	87
9	189	88
10	205	5.3
11	207	13
12	208	10
13	213	17
14	231	8.3
15	233	8.7
16	246	5.3
17	248	16
18	276	1.5
19	281	1.7
20	283	0.7
21	339	77
22	343	68
23	344	78
24	349	69
25	352	88
26	364	37
27	365	25
28	399	87
29	402	72
30	403	101
31	408	64
32	412	69

[a] Amino acid in Mtr permease after which alkaline phosphatase is fused.
[b] Units of alkaline phosphatase activity calculated according to the method of Manoil (30).

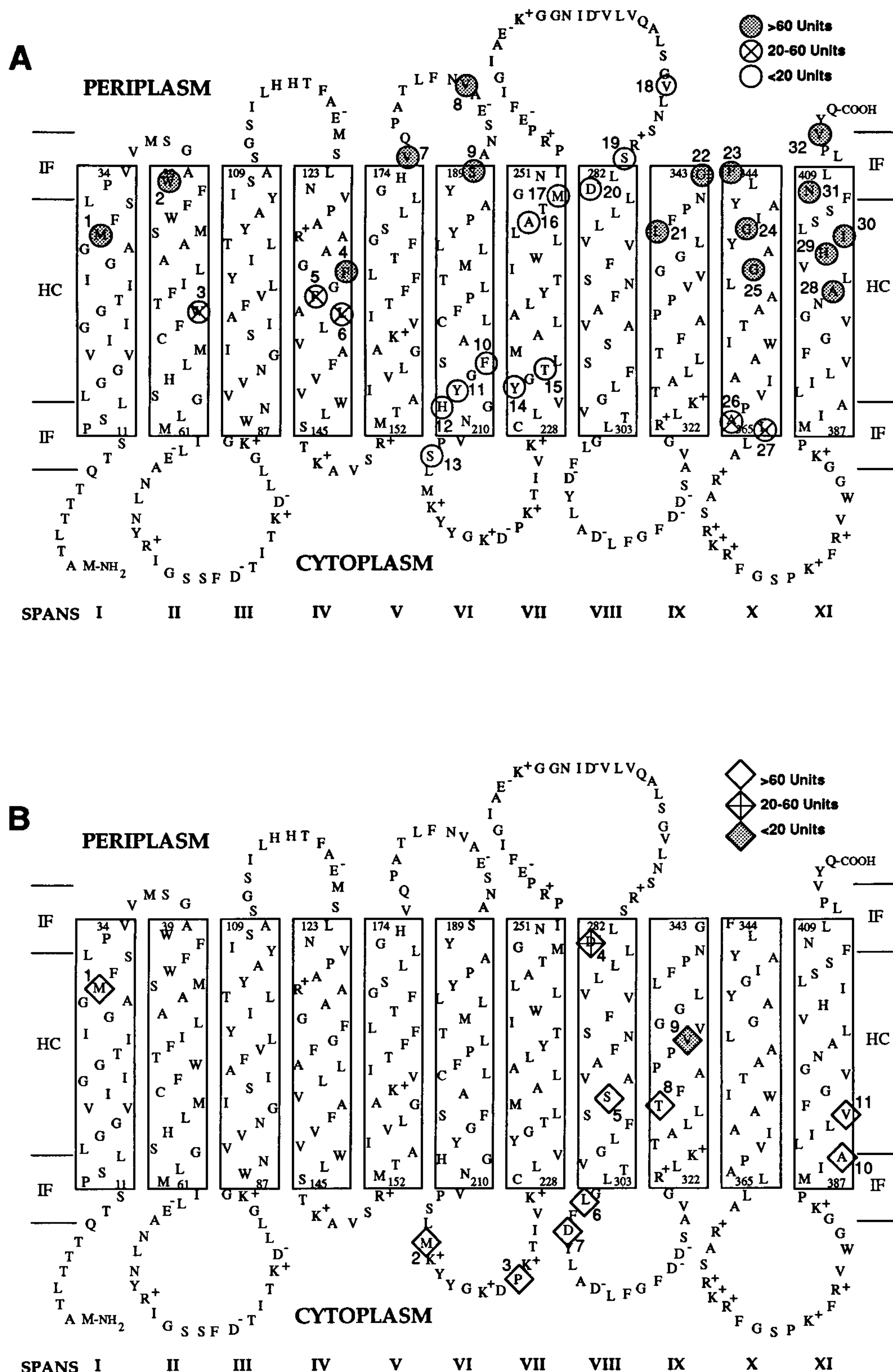

FIG. 2. Proposed topological model of the Mtr permease and properties of gene fusion products. Residues within the 11 membrane-spanning regions are arranged in an α-helical configuration. Charged residues are indicated by + and − symbols. (A) Fusion site and relative alkaline phosphatase activity of Mtr permease-alkaline phosphatase hybrid proteins generated by exonuclease III digestion. (B) Fusion site and relative β-galactosidase activity of Mtr permease–β-galactosidase hybrid proteins generated by exonuclease III digestion. Symbols indicate the amino acid residues after which the alkaline phosphatase or β-galactosidase is fused. (C) Fusion site and relative alkaline phosphatase activity of Mtr permease-alkaline phosphatase sandwich fusion protein constructs. Symbols indicate the amino acid residues into the codons of which the *'phoA* gene was inserted. IF, interface region; HC, hydrocarbon core (3 nm, corresponding to the length of a 20-residue helix).

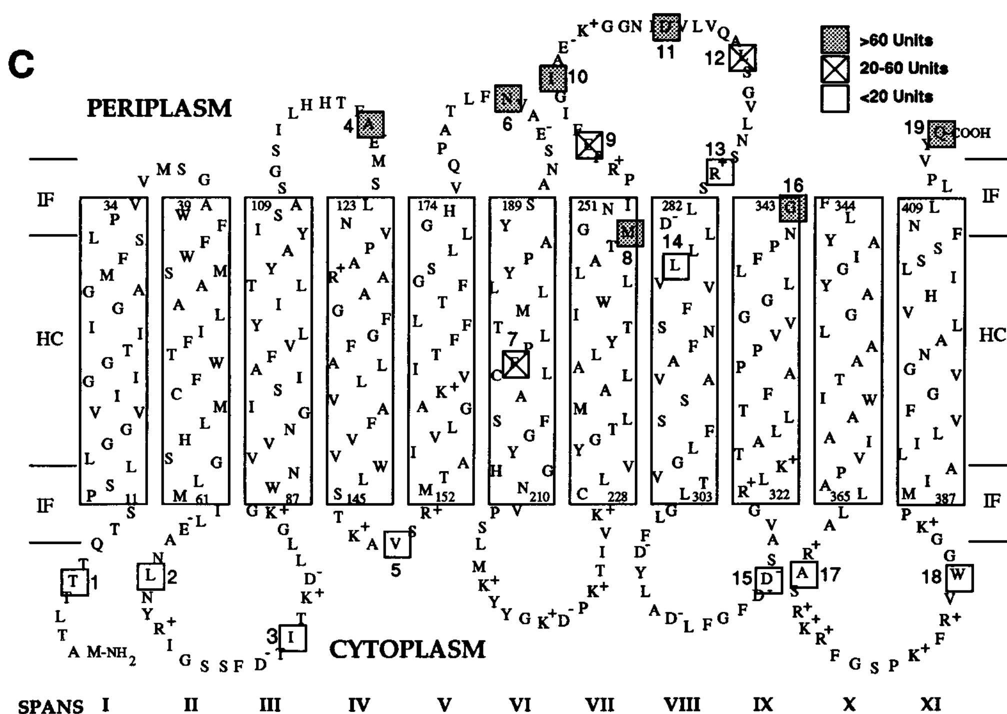

FIG. 2—*Continued.*

of the fusions (Table 2) was compatible with the proposed 11-span topological model of the Mtr permease (Fig. 2A). The range of alkaline phosphatase activity between fusions varied over 100-fold, although in general the difference between cytoplasmically and periplasmically located fusions was closer to 10-fold. The cluster of fusions at the carboxyl end of the protein all exhibited relatively high levels of alkaline phosphatase activity, supporting the prediction that this portion of the permease has a periplasmic location, and fusions 1 to 15 and 21 to 32 exhibited enzyme activities that were compatible with the model. Fusion protein isolates 16 to 20, however, produced results not predicted by the model. Low-level alkaline phosphatase activity is produced by these hybrids, the fusion sites of which are predicted to be situated at the putative periplasmic facing sides of transmembrane spans VII and VIII and in the adjoining hydrophilic periplasmic loop.

Isolation of Mtr permease–β-galactosidase (*mtr′-′lacZ*) fusions. The vector pMU5194 was used to generate a series of *mtr′-′lacZ* gene fusions in a manner similar to that used to construct the *mtr′-′phoA* gene fusions. Blue transformants were obtained on minimal medium containing X-Gal and phenylalanine. After the restriction analysis of plasmid DNA extracted from 100 isolates, the exact locations of the fusion points of 43 *mtr′-′lacZ* gene fusions were determined by nucleotide sequencing. Only 11 unique in-frame fusions were identified.

The β-galactosidase activity of the fusions assayed in strain JP7742 (Table 3) are in general agreement with the proposed model of the Mtr permease (Fig. 2B). The majority of the hybrid proteins exhibited relatively high levels of β-galactosidase activity, and the positions of the fusion sites were located in or near the cytoplasm. Attempts to isolate fusions to periplasmic domains of the Mtr permease by screening plasmid DNA from pale blue transformants almost exclusively resulted in out-of-frame fusions between *mtr* and *′lacZ*. This phenomenon has also been encountered in the analysis of the GlpT, DctA, and HlyB membrane proteins (17, 18, 22), and may result from the reinitiation of translation within the *lacZ* mRNA.

Only one β-galactosidase fusion (isolate 4) was isolated in the region encompassing putative transmembrane spans VII and VIII. The intermediate enzyme activity produced by this fusion protein is in conflict with the proposed model in which the fusion point is expected to be situated adjacent to a periplasmic loop.

One other unexpected result concerns *mtr′-′lacZ* fusion 1. The junction of both *mtr′-′lacZ* fusion 1 and *mtr′-′phoA* fusion 1 is located at the same residue, within transmembrane span I. Both types of hybrid protein exhibit high levels of enzymatic activity, so that whereas the alkaline phosphatase moiety appears to be translocated to the periplasm, β-galactosidase re-

TABLE 3. Properties of Mtr permease–β-galactosidase fusion proteins

Isolate (fusion no.)	Fusion position (amino acid)[a]	β-Galactosidase activity[b]
1	29	7,661
2	215	72
3	222	62
4	283	32
5	296	84
6	305	151
7	307	94
8	329	167
9	334	5.8
10	389	246
11	393	72

[a] Amino acid of Mtr permease after which β-galactosidase is fused.
[b] Units of β-galactosidase activity calculated according to the method of Miller (35).

TABLE 4. Properties of Mtr permease-alkaline phosphatase sandwich fusion proteins

Fusion	Fusion position (amino acid)[a]	Alkaline phosphatase activity[b]	Transport activity (% of wild type activity)[c]
SW1	6	2.2	34
SW2	67	7.9	<1.0
SW3	78	1.2	<1.0
SW4	119	68	23
SW5	149	0.1	<1.0
SW6	182	194	14
SW7	200	42	14
SW8	248	82	<1.0
SW9	255	43	8.8
SW10	259	124	14
SW11	267	77	<1.0
SW12	273	42	38
SW13	280	2.7	2.9
SW14	286	0.7	2.9
SW15	317	1.0	1.6
SW16	344	133	<1.0
SW17	369	1.3	<1.0
SW18	382	<0.1	<1.0
SW19	414	196	78

[a] Amino acid of Mtr permease into the codon of which the *'phoA* gene has been inserted.
[b] Units of alkaline phosphatase activity calculated according to the method of Manoil (30).
[c] The steady-state level of [^{14}C]tryptophan uptake by JP10054 (pMU5191) is 4.2 nmol/mg (dry weight).

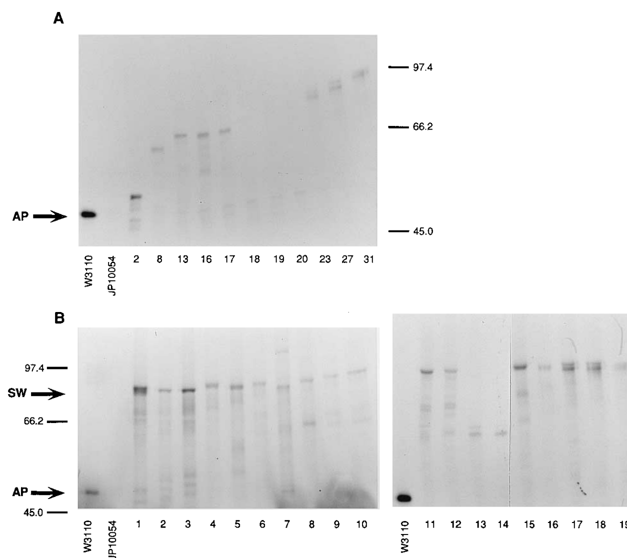

FIG. 3. Synthesis of representative protein fusions between the Mtr permease and alkaline phosphatase (AP). Strains containing the gene fusion plasmids were pulse-labeled for 1 min with [^{35}S]methionine-cysteine and immunoprecipitated with antisera against alkaline phosphatase. (A) Hybrid proteins encoded by *mtr'-'phoA* gene fusions generated by exonuclease III digestion. (B) Mtr permease-alkaline phosphatase sandwich (SW) fusion proteins encoded by *mtr'-phoA-'mtr* gene fusions. Fusion proteins were electrophoresed on SDS–8% polyacrylamide gels (37.5:1 acrylamide/bisacrylamide ratio). The position of native alkaline phosphatase encoded by the chromosomal *phoA* gene of strain W3110 is indicated. Strain JP10054, which does not contain a gene fusion plasmid, was used as a negative control. The positions of molecular weight standards (in thousands) are shown.

mains in the cytoplasm. The extremely high level of activity of this fusion protein will be discussed later.

Construction of Mtr permease-alkaline phosphatase sandwich fusions. In order to analyze the properties of fusions to regions of the Mtr permease not covered by the previous methods, and particularly to resolve inconsistencies surrounding the region containing putative transmembrane spans VII and VIII, a total of 19 sandwich fusions were constructed by introducing suitable restriction endonuclease sites throughout the *mtr* gene by site-directed mutagenesis, followed by the introduction of the *'phoA* gene from pSWFII (14). An attempt was made to target every putative periplasmic and cytoplasmic loop of the permease, and the region encompassing transmembrane spans VII and VIII. Fusions were not obtained between transmembrane spans I and II and VI and VII. The construct in which alkaline phosphatase is fused to the final amino acid of the Mtr permease (SW19) is itself not a sandwich fusion, but it is included in this class of fusions because it was constructed in a manner similar to that of the sandwich fusions. The alkaline phosphatase activities of the sandwich fusion constructs are shown in Table 4, and their positions in the proposed model of the Mtr permease are presented in Fig. 2C. In most cases, high-level enzymatic activity is exhibited by hybrid proteins containing alkaline phosphatase in a proposed periplasmic location, but only low-level activity is found where the reporter enzyme is situated in a cytoplasmically disposed domain of the permease. The fusion following the final amino acid of the permease (SW19) produces high-level alkaline phosphatase activity, supporting the theory that the carboxyl terminus of the Mtr permease is present in the periplasmic space and that, together with the other sandwich fusions, there is a membrane protein consisting of 11 transmembrane spans. However, the two sandwich fusions SW13 and SW14 are inconsistent with the proposed model. The junction between the Mtr permease and alkaline phosphatase of these isolates is in a region similar to that of the *mtr'-'phoA* fusions 18 to 20, in the putative periplasmic loop between transmembrane spans VII and VIII, but they also exhibit low levels of enzyme activity. The data obtained via the gene fusion methods indicate that this region of the protein may be located closer to the cytoplasm.

The insertion of the alkaline phosphatase moiety into the Mtr permease often severely interfered with the transport activity of the protein (Table 4). A number of sandwich fusions, however, were able to confer both transport activity and alkaline phosphatase activity on the cells in which they were expressed.

Synthesis and stability of fusion proteins. Representative hybrid proteins produced by the gene fusions between *mtr* and *phoA* or *lacZ* were visualized by pulse-labeling of fusion strains with [^{35}S]methionine-cysteine, followed by the immunoprecipitation of the fusion proteins with anti-bacterial alkaline phosphatase or anti-β-galactosidase (21), as appropriate. The results with isolates expressing the alkaline phosphatase fusions are shown in Fig. 3. The majority of isolates produced a polypeptide band approximately the size expected of a fusion protein. Because all sandwich fusions contain the complete Mtr and alkaline phosphatase sequences, all hybrid proteins in this class should be of the same size. The apparent molecular weight of some fusion proteins consisting of a significant portion of the Mtr permease was lower than expected, but hydrophobic proteins have been shown to migrate anomalously through SDS-polyacrylamide gels. In general, there were slight variations in the rate of synthesis of different hybrid proteins. It can be seen that, other than for a few exceptions, the production of low-level alkaline phosphatase enzyme activity appears to be due to the cytoplasmic location of the alkaline phosphatase moiety and not to the low rates of synthesis of hybrid proteins.

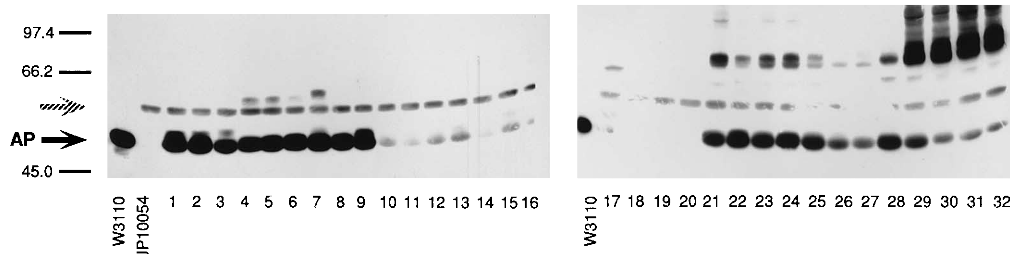

FIG. 4. Immunoblotting of Mtr permease-alkaline phosphatase hybrid proteins. Equal amounts of solubilized cells containing derivatives of pMU5193 carrying *mtr′-′phoA* gene fusions were electrophoresed on SDS–8% polyacrylamide gels, and Mtr permease-alkaline phosphatase fusion proteins were visualized by immunoblotting with anti-alkaline phosphatase serum. The position of native alkaline phosphatase (AP) encoded by the chromosomal *phoA* gene of strain W3110 is indicated. Strain JP10054, which does not contain a gene fusion plasmid, was used as a negative control. A nonspecific band present in all lanes is marked by the hatched arrow. The positions of molecular weight standards (in thousands) are shown.

The steady-state levels of all of the gene fusion products were also analyzed by Western immunoblotting of proteins from whole-cell extracts. The results with isolates expressing the *mtr′-′phoA* gene fusions are shown in Fig. 4. Significant immunoreactivity is only expected with fusion proteins for which the alkaline phosphatase moiety is located in the periplasm. Because cytoplasmically disposed alkaline phosphatase is unable to fold correctly and is rapidly degraded, it is not available for binding by antibody (8, 31, 43). The intensity of the bands present on the immunoblots suggests that there are substantial differences in the stabilities of different fusion proteins, and these generally correlated with the level of enzyme activity produced by the fusion proteins. That is, the most intense bands were apparent for those isolates which produced high levels of enzymatic activity (Fig. 4 and data not shown). The majority of isolates expressing the *mtr′-′phoA* gene fusions generated by the deletion method exhibited an immunoreactive polypeptide at the position corresponding to intact alkaline phosphatase, and many of the isolates which exhibited high-level alkaline phosphatase activity also produced a band corresponding to the size range expected of a full-length fusion protein (Fig. 4).

Degradation products corresponding to the size of native alkaline phosphatase were also evident for a number of isolates examined by pulse-labeling experiments, including *mtr′-′phoA* fusions 18 to 20, for which a full-length hybrid polypeptide was not apparent (Fig. 2A). However, no specific bands were visible in immunoblots of these same fusions, indicative of rapid breakdown. In fact, degradation products corresponding to the size expected of native alkaline phosphatase or β-galactosidase were evident for many of the isolates examined by either immunological technique (Fig. 3 and 4 and data not shown).

Bands of an intermediate size between that of intact alkaline phosphatase and a full-length fusion protein were detected for sandwich fusions 13 and 14 by pulse-labeling experiments (Fig. 3B). A faint full-length hybrid protein band of sandwich fusion 13 was present on immunoblots (data not shown). The product of *mtr′-′lacZ* gene fusion 9 was not detected by either immunological technique; however, isolate 4, which was not visualized by pulse-labeling, did produce a product the size of β-galactosidase detectable by immunoblotting (data not shown). It is not possible to conclude with certainty whether the lack of immunoreactive polypeptide is due to an unusually low rate of synthesis of these isolates or to the very rapid degradation of the fusion proteins. The presence and integrity of the plasmid vectors in those strains exhibiting few or no immunoreactive peptides were reexamined and were found not to be abnormal (data not shown).

DISCUSSION

Knowledge of the structure of membrane transport proteins and their orientation across the membrane is a necessary requirement for determining the molecular mechanism responsible for the phenomenon of active transport. The structure of the Mtr permease was examined experimentally with a genetic approach involving the construction of fusions between the *mtr* gene and the genes encoding alkaline phosphatase (*phoA*) and β-galactosidase (*lacZ*). The use of gene fusions to elucidate the architecture of membrane proteins is a well-established technique (29, 30, 32, 33, 53).

Taken together, the 62 gene fusions cover much of the sequence of the Mtr permease. The theory of an integral membrane protein consisting of 11 transmembrane spans with a cytoplasmically located amino terminus and periplasmically situated carboxyl terminus is well substantiated. The high-level enzyme activities obtained with the alkaline phosphatase fusions to the carboxyl end of the Mtr permease support the previously published model in which the carboxyl terminus was assigned a location in the periplasm (47). The exonuclease III-generated *mtr′-′phoA* fusions provide strong support for the rest of the proposed topological model, except for a small region including the periplasmic side of transmembrane span VII, the beginning of span VIII, and the periplasmic loop connecting these two spans. These fusion proteins (isolates 16 to 20) produced low alkaline phosphatase activity levels, whereas from this model one might have expected higher values. When the same region was examined with sandwich fusions between the Mtr permease and alkaline phosphatase, one such hybrid protein (SW8) in which the fusion point was at the same position as *mtr′-′phoA* fusion 17 exhibited high levels of alkaline phosphatase, suggesting that the low levels of enzyme activity produced by the latter fusion protein and the adjacent *mtr′-′phoA* fusion 16 could be due to the absence of stabilizing effects normally rendered by the missing carboxyl terminus of the Mtr permease. Furthermore, sandwich fusions SW9 to SW12, in all of which the fusion site is located in the putative periplasmic loop joining transmembrane spans VII and VIII, produced intermediate or high levels of alkaline phosphatase activity.

The only protein fusions whose activities did not agree with the position of the fusion site on the proposed model of the Mtr permease are those in putative transmembrane span VIII and the preceding hydrophilic stretch (*mtr′-′phoA* fusions 18 to 20, *mtr′-′lacZ* fusion 4, and sandwich fusions 13 and 14). In the case of *mtr′-′phoA* fusions 18 to 20, pulse-labeling and immunoblot experiments did not reveal the presence of a fusion

protein, although bands corresponding to native alkaline phosphatase were detected by immunoprecipitation (Fig. 3A and 4). A product of *mtr'-'lacZ* fusion 4 was not detected by pulse-labeling analysis, but a band corresponding to the size expected of native β-galactosidase was visualized by immunoblotting (data not shown). Sandwich fusions SW13 and SW14 also failed to produce a fusion protein of the expected molecular weight in immunoprecipitation experiments (Fig. 3B). However, a faint full-length protein band was evident in immunoblots of sandwich fusion SW13 (data not shown). Insufficient full-length protein may have been synthesized during the short 1-min pulse-label reaction to detect via immunoprecipitation. The gene fusions which failed to exhibit an immunoreactive polypeptide were not distributed randomly throughout the *mtr* gene, but rather they all contained the fusion site in a relatively small region of the *mtr* gene which encodes the stretch of amino acids forming putative transmembrane span VIII and the surrounding regions. It is not known why fusions to this region of the gene interfere with the rate of synthesis of the hybrid proteins or result in rapid breakdown of the fusion products. If these isolates are omitted from the analysis of the topological structure of the transport protein, the remaining gene fusions would not favor any alteration to the proposed model of the permease.

It has been shown that polytopic integral membrane proteins from both prokaryotic and eukaryotic organisms generally possess a net positive charge in polar regions of the protein present in the cytoplasm, which connect transmembrane segments, and a net negative charge in extracytoplasmic polar domains (38, 56). The distribution of charged amino acid residues of the Mtr permease is consistent with the "positive-inside" rule. In fact, the only putative periplasmic domain in the original model of the transport protein containing positively charged amino acids is the loop between proposed spans VII and VIII. Studies with the MalF protein and protein leader peptidase indicate that cytoplasmic domains are preeminent topogenic signals because they contain information which determines their cytoplasmic location and thus the orientation of the surrounding membrane-spanning segments (33, 56). An important feature of these cytoplasmic domains has been found to be the density of positively charged amino acids within them (33). In the case of *mtr'-'phoA* fusions 18 to 20 and *mtr'-'lacZ* fusion 4, the concentration of charged residues in this region, especially the uncommon cluster of positively charged amino acids normally associated with the determination of a cytoplasmic location, may interfere with the ability of this portion of the membrane protein fused to the reporter enzyme to assume its native cellular location in the absence of the missing carboxyl-terminal portion of the protein. It is not known if such interference with proper folding can lead to increased instability and degradation of the hybrid protein.

Other fusions for which results are slightly unexpected are the exonuclease III-generated *mtr'-'phoA* fusions 26 and 27 (Fig. 2 and 3). The fusion point of these two isolates has been assigned to the cytoplasmic end of incoming transmembrane span X, and yet they exhibit intermediate values of alkaline phosphatase activity. It is possible that, in the absence of the cluster of positively charged residues which normally follow the fusion point of these isolates, span X is unable to anchor in the membrane and tends to translocate toward the periplasm. However, it is possible that the carboxyl-terminal end of span X may terminate near residue Ser-370 rather than Leu-365. The positively charged Arg-368 could be accommodated in the interface region of the membrane, and such an arrangement would alleviate the extremely tight turn between transmembrane spans IX and X (Fig. 2A).

Immunoblot analysis of hybrid protein synthesis revealed that stabilities were not the same for different isolates (Fig. 4). Signs of significant proteolytic degradation were evident for the majority of fusions, as has also been seen in fusions to the lactose permease, signal peptidase II, and glucose transporter of *E. coli* (10, 12, 37). The correlation between enzymatic activity of the fusion proteins and the intensity of bands on the immunoblots suggests that the level of activity depends on sensitivity to proteolysis. Hybrid fusion proteins which exhibited high-level enzymatic activity all presented an immunoreactive polypeptide the size of native alkaline phosphatase. In some cases, a band corresponding to the full-length fusion protein was evident, particularly in those hybrids in which the fusion site was toward the end of the Mtr permease. In those isolates, in which alkaline phosphatase is predicted to be fused to a cytoplasmic domain and little enzymatic activity is produced, only faint bands which also correspond to alkaline phosphatase were detected. Similar observations were made in the analysis of the UhpT permease (27). Presumably, low-level enzymatic activity is the result of the minor amounts of alkaline phosphatase which are able to transverse the membrane, as has been reported in the studies of the MalF protein (54). The presence of a polypeptide band the size of native alkaline phosphatase or β-galactosidase has frequently been reported in the analysis of other membrane proteins by using gene fusion approaches (1, 12, 13, 18, 27).

The junctions of *mtr'-'phoA* fusion 1 and *mtr'-'lacZ* fusion 1 are located at the same residue, within transmembrane span I (Fig. 2A and B). Both types of hybrid protein exhibit high levels of enzymatic activity, so that whereas the alkaline phosphatase moiety appears to be translocated to the periplasm, β-galactosidase remains in the cytoplasm. The short hydrophobic segment serves as a signal sequence for the export of alkaline phosphatase across the membrane but is not sufficient to tether the bulkier β-galactosidase enzyme to the membrane in such a way as to interfere with its activity and certainly does not mobilize it to the periplasm.

The *mtr'-'lacZ* fusion 1 contains only the first 29 amino acid residues of the Mtr permease fused to β-galactosidase. Cells expressing this hybrid protein exhibit approximately 50-fold greater enzymatic activity than any of the other cytoplasmically situated β-galactosidase fusion proteins. A similar phenomenon has been seen after the construction of β-galactosidase fusions to short amino-terminal sequences of the MalF, GlpT, and the pBR325 Tet membrane proteins (16, 18, 29). A speculative explanation for these observations is that factors which limit the translation of the membrane protein portion of the hybrid lead to reduced stability of the *lacZ* mRNA. Recent reports suggest *lacZ* mRNA stability is related to the translation initiation frequency of the message (60) or that ribosomal binding to the Shine-Dalgarno sequence of the *lacZ* transcript (but not translation of the coding region) is necessary to maintain mRNA stability, possibly by ribosomal protection of an endoribonuclease site located at the 5′ end of the transcript (58). The Shine-Dalgarno sequence of the *lacZ* gene is absent in the gene fusion constructs, and the 5′ end of the *lacZ* mRNA is located within a larger transcript, thus not affording it ribosomal protection unless the gene fusion contains only a small portion coding for the membrane protein. Such small sequences may not limit translation before ribosomes encounter *lacZ* mRNA.

A consensus structure for membrane transport proteins has recently been proposed by Maloney (28), suggesting that there are two clusters of six helical segments that span the membrane, often separated by a large cytoplasmic hydrophilic loop. Proteins consisting of 12 transmembrane spans are most com-

mon, but the presence of 10 or 14 α-helical segments has also been observed. In some cases, the transport system takes the form of a homodimer of two subunits consisting of five to seven hydrophobic spans. The Mtr permease would also appear to belong to this large group of proteins, but we believe that this is the first confirmed report of a prokaryotic membrane transport protein composed of 11 transmembrane spans. Membrane proteins consisting of an odd number of transmembrane segments are not uncommon. Examples include the photoreaction center of *Rhodopseudomonas viridis*, of which the L and M subunits have been shown to each consist of five transmembrane α-helices by X-ray crystallography (2, 3); the proton pump of bacteriorhodospin spans the membrane seven times (40), as do the many members of the guanine nucleotide-binding protein-coupled receptor family in eukaryotic organisms (26). Both the ArcD protein of *Pseudomonas aeruginosa* and the LysI protein of *Corynebacterium glutamicam* are postulated to be composed of 13 α-helical transmembrane segments, with the carboxyl terminus present on the outer surface of the cytoplasmic membrane (6, 49). The significance and requirement of the novel structure believed to be shared by the related Mtr, TnaB, and TyrP permeases are currently the subject of further investigation.

The presence of a number of Mtr permease-alkaline phosphatase sandwich fusions retaining some level of transport activity indicates that the permease is able to assume its normal tertiary structure, even with the inclusion of the alkaline phosphatase moiety. Interactions between amino- and carboxyl-terminal segments of the protein can still take place. The bifunctional hybrid protein (SW19) consisting of alkaline phosphatase fused to the final amino acid residue of the Mtr permease exhibited both high levels of alkaline phosphatase activity and tryptophan transport activity approaching wild-type levels. Immunoblot analysis revealed that cells expressing this fusion contained only a relatively small amount of free alkaline phosphatase (data not shown), indicating that the hybrid protein is quite stable and is most likely responsible for both activities. Breakdown of the other sandwich fusions would yield truncated permeases which would not be expected to retain any transport activity. The isolation of a bifunctional Mtr permease-alkaline phosphatase hybrid protein enables a novel approach for the isolation of transport-defective mutants which retain the ability to correctly fold and insert into the membrane. The selection of transport-negative mutants of transport proteins often produces many isolates in which the defect is the result of gross structural alterations and/or the inability of the protein to be localized efficiently into its normal membrane position. However, by simultaneously screening for alkaline phosphatase activity, it should be possible to identify mutants still able to assume their native structure. Such a novel technique has been successfully used to identify specific residues in the Mtr permease believed to have an important functional role and will be described in a future communication (46). The method should apply equally well for other membrane proteins in which alkaline phosphatase fusions have been made to periplasmic loops of a membrane protein and in which full biological activity is retained.

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