Membrane Topology of the L-Rhamnose-H+ Transport Protein (RhaT) from Enterobacteria*

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The L-rhamnose-H+ symporter (RhaT) is a 344-amino acid integral membrane protein, found in many Enterobacteria, which couples the uptake of the sugar L-rhamnose with the inward movement of protons. Based on its hydropathy profile and the application of von Heijne's "positive inside" rule (von Heijne, G. (1992) J. Mol. Biol. 225, 487-494), a model of the L-rhamnose-H+ symporter protein (RhaT) is proposed containing 10 transmembrane helices with the NH2 and COOH termini in the periplasm. This model was tested by the creation of random β-lactamase (Bla) fusions. The data from 33 unique, randomly generated, RhaT-Bla fusions and from 5 site-specific fusions supported the proposed topology between transmembrane helices 2-10. However, the localization of the putative first hydrophilic loop and the NH2 terminus was not possible because the β-lactamase fusions in this region were shown to be unreliable indicators of the topology of RhaT.

The L-rhamnose-H+ symporter (RhaT) couples the uptake of the sugar L-rhamnose into Enterobacteria with the inward movement of protons down their concentration gradient (Muir, 1988; Muir et al., 1993). The rhaT gene from Escherichia coli and Salmonella typhimurium has been cloned and sequenced (Tate et al., 1992); the RhaT proteins were predicted to contain 344 amino acid residues and were 91% identical. A hydropathy plot of RhaT (see Fig. 1) showed 10 clearly defined regions of hydrophobic amino acid residues that could form transmembrane α-helices. The "positive inside" rule (von Heijne, 1986) suggested that the orientation of RhaT placed the NH2 and COOH termini in the periplasm (Lewis et al., 1990; Lewis and Simoni, 1992). An alternative structure for RhaT was proposed by Garcia-Martín et al. (1992) from their DNA sequence of the E. coli rhaT gene. However, a sequencing error at the end of the rhaT gene was made, so they predicted a shorter open reading frame than was actually present. Thus, the eight-stranded β-barrel model they proposed was based on erroneous data and will not be considered further.

This paper describes the testing of the 10-helix model of RhaT by constructing gene fusions between rhaT and the blaM gene, encoding β-lactamase (Bla). A truncated form of β-lactamase (Bla') which lacks its NH2-terminal leader sequence can act as a topological reporter molecule when fused to portions of a membrane protein (Broome-Smith et al., 1990). If the β-lactamase is fused to part of a membrane protein that is normally located in the periplasm, then the β-lactamase is translocated to the periplasm where it confers resistance to high concentrations of ampicillin. If the β-lactamase is fused to part of a membrane protein that is in the cytoplasm, then the β-lactamase remains in the cytoplasm, making the cells resistant only to low concentrations of ampicillin. The topology of a number of membrane proteins has been deduced from β-lactamase fusions (see for example, Edelman et al., 1987; Bowler and Spratt, 1989; Wang et al., 1991; Wu et al., 1992; Kampfenkel and Braun, 1993; Weiner et al., 1993). In summary, we find that the β-lactamase fusion data support a model of RhaT comprising 10 transmembrane regions with the NH2 and COOH termini in the periplasm, different from the 12-helix model with NH2 and COOH termini in the cytoplasm proposed for most sugar-H+ symporter proteins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth of Bacteria**—The genotypes of the bacteria used in this work are as follows: E. coli strain TG1, Δ(lac-pro), supE, thi, hsdD5 [F'traD36, proA+B', lacI, lacZD15]; E. coli strain TG2, Δ(lac-pro), supE, thi, hsdD5 D(ori- recA306::Tn10(tetR)] [F'traD36, proA+B', lacI, lacZD15] (T. Gibson, Laboratory of Molecular Biology, Cambridge); E. coli strain BB4, hsdR514, hsdM, supE44, supF58, lacV1 and Δ(lacIV)5; galK2, galT22, metB1, trpR55, Δ(argF-lac) U169, [F'lacI, lacZDM15, proAB, Tn10(tetR)]. Bacterial strains were routinely grown in either 2TY or LB media (Maniatis et al., 1982).

**Preparation of Bacterial Membranes**—Spheroplasts were prepared by the method of Witholt et al. (1976) and lysed by osmotic shock in ice-cold deionized water (Henderson and Macpherson, 1986). Membranes were recovered by centrifugation (40,000 × g for 20 min at 4°C) and resuspended in deionized water by brief sonication (10 s, setting 2) and then resedimented by centrifugation (100,000 × g for 90 min at 4°C). The membrane pellet was resuspended in deionized water and the protein concentration determined by the method of Schaffner and Weissman (1973).

**The abbreviations used are:** Bla, β-lactamase; PCR, polymerase chain reaction.

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DNA Manipulations—Plasmid DNA was prepared by the alkaline lysis method (Maniatis et al., 1982). Restriction digests were carried out according to the manufacturer's recommendations (Amersham Corp., Pharmacia LKB Biotechnology Inc., or New England Biolabs), and ligations were performed according to Maniatis et al. (1982). The CaCl₂ method for preparing competent cells was used routinely. Isolated restriction fragments were isolated from agarose gels by excising the desired band and isolating the DNA by the "glass milk" method (Vogelstein and Gillespie, 1979).

DNA Sequencing—Single-stranded DNA was produced from derivatives of plasmids pJBS633 and pY24 using the helper phage R408 (Russell et al., 1986) and sequenced using Sequenase (U.S. Biological Corp.). The gene fusion point between the rhaT gene and the β-lactamase gene (bla') was sequenced using oligonucleotide BLA1 (5'-CTCGTGACCCACCACTGA-3'), which is complementary to the coding strand of the β-lactamase gene 40 base pairs from the fusion point. Each fusion point was sequenced on only one strand.

Site-specific rhaT-bla gene fusions were created using PCR amplification of a portion of the rhaT gene; because of the possibility of errors introduced by the Vent DNA polymerase (oligonucleotides RHA3 and RHA7, which were used for PCR amplification (oligonucleotides RHA7, RHA8, RHA9, see below).

Separation of Proteins and Western Blotting—SDS-polyacrylamide gel electrophoresis was performed as described by Henderson and Macpherson (1986) and Western blotting as described by Burnette (1981). A polyclonal antibody to the β-lactamase (Boehringer Mannheim) was used at a final concentration of 0.1 µg/ml and incubated with the blot for 2 h at room temperature. The anti-β-lactamase antibody (5 Prime 3 Prime Inc) was used at a final dilution of 1:1,000. Blots were autoradiographed for 5-30 s.

Construction of a rhaT-bla COOH-terminal Gene Fusion—Initially, an rhaT-lacZ gene fusion was constructed, which was subsequently used to probe a rhaT-bla gene fusion. The rhaT gene was amplified by PCR to a virtually full-length rhaT gene by using a BalI restriction site at the penultimate codon (Ala[54]) of the S. typhimurium rhaT gene. Plasmid pJG200 (Germino and Bastia, 1984) was used to make the rhaT-lacZ gene fusion. This plasmid contained a unique BamHI site that allows the in-frame fusion of genes with lacZ via a collagenase-sensitive linker. Upstream from the BamHI site is a λ P 1 promoter and the clcr gene; thus, expression of the fusion gene is repressed at 33 °C but not at 42 °C. A BstEII/Ball restriction fragment from plasmid pJR18 (Tate et al., 1992) which encompassed the S. typhimurium rhaT gene was ligated into BamHI-Ball restriction site at the lacZ gene. The BstEII site was blunt ended using DNA polymerase Klenow fragment, which allowed the ligation of BamHI linkers (CGGATCCG, Pharmacia) onto both ends of the DNA fragment. After digestion with BamHI, the DNA fragment was separated from linkers on a Sepharose CL-6B 200 spin column (Sambrook et al., 1989). The BamHI-linkered DNA was ligated into BamHI-digested plasmid pJG200 to produce plasmid pPG17 (see Fig. 2), which contained the rhaT-lacZ gene fusion under the control of the λ P 1 promoter. The correct orientation of the rhaT gene in the vector was checked by measuring the rate of uptake of L-[^14]C]rhamnose in heat-induced TG2(pG17).

The lacZ gene in the rhaT-lacZ gene fusion expressed from plasmid pPG17 was replaced with the bla' gene to create an rhaT-bla fusion at codon Ala[54] in the S. typhimurium rhaT gene. A BamHI restriction fragment that contained the rhaT gene in plasmid pPG17 was ligated into BamHI-digested plasmid pYZ2 (Broome-Smith et al., 1990). The orientation of the gene was determined by an HinclI/HindIII digestion; plasmid pGCT33 contained the rhaT gene in the correct orientation downstream from the lacUV5 promoter for gene expression. Restriction sites proximal to the lacUV5 promoter was then destroyed; gel-purified plasmid pGCT33 that had been partially digested with BamHI was treated with DNA polymerase Klenow fragment to blunt end the DNA and was then religated and transformed into E. coli strain BB8. Plasmid pGCT36 contained a partial insert at the end of the rhaT gene.

The rhaT-bla gene fusion was made by a procedure analogous to that described by Broome-Smith et al. (1990). Plasmid pGCT36 was digested with BamHI, and the single-stranded DNA ends were treated with DNA polymerase Klenow fragment. The single-stranded DNA ends were re-annealed with S1 nuclease (Pharmacia Nested Deletion Kit). The DNA was then digested with EcoRI and ligated with a PoulI/EcoRI restriction fragment from plasmid pY25 (Broome-Smith et al., 1990) which contained the truncated bla' gene. The ligation mix was transformed into strain TG1 and plated out on LB plates that contained kanamycin. The orientation of the gene fusion on plasmid pGCT38 (see Fig. 2) was determined by sequencing using oligonucleotide primer BLA1 (5'-CTCGTGACCCACCACTGA-3') which is complementary to the coding strand of the β-lactamase gene.

Construction of Plasmid pGCT15 and the Generation of Random rhaT-bla Fusions—Plasmid pGCT15 was constructed from plasmid pJBS633 (Broome-Smith and Spratt, 1986) and plasmid pGCT15, which expresses two S. typhimurium rhaT genes under the control of the λ P 1 promoter. To provide tight control of the λ P 1 promoter, the clcr gene was ligated into plasmid pJBS633; a 900-base pair EcoRI/BamHI restriction fragment from plasmid pG200 (Germino and Bastia, 1986) which carried the clcr gene was ligated into EcoRI/BamHI-digested plasmid pJBS653, to form plasmid pGCT14. A 3.4-kilobase R681/BamHI restriction fragment from plasmid pGCT13, which carried the two rhaT genes under the control of the λ P 1 promoter, was then ligated into BamHI-digested plasmid pGCT14. Two orientations of the insert in plasmid pGCT14 were obtained as determined by restriction enzyme digests using HinclI, EcoRI. Plasmid pGCT14 was transformed into CaCl₂ competent TG1 cells and plated out onto LB plates that contained 50 µg/ml kanamycin and grown at 33 °C.

To determine which of the kanamycin-resistant transformants expressed β-lactamase activity, colonies were toothpicked onto LB-kanamycin plates that contained 20 and 200 µg/ml ampicillin; colonies that expressed an RhaT-Bla fusion where the β-lactamase was in the cytoplasm grew only on plates that contained 20 µg/ml ampicillin, whereas colonies that expressed an RhaT-Bla fusion with the β-lactamase in the periplasm also grew on the plates that contained 200 µg/ml ampicillin (Broome-Smith and Spratt, 1986). The premalinacin activity was studied to determine the concentration of ampicillin which was required to kill single cells that expressed the RhaT-Bla fusions. Overnight cultures of strains expressing the fusion proteins were grown at 33 °C in 2TY that contained 50 µg/ml kanamycin. 5 µl of a 10⁻¹ dilution of the overnight cultures was spotted onto LB plates that contained increasing amounts of ampicillin (2, 3, 4, 5, 7.5, 10, 12.5, 15, 20, 25, 30, 55, and 50 µg/ml). To ensure reproducible results, the ampicillin plates were prepared freshly on the day of use and dried for 1 h at 37 °C before use. Ampicillin solutions were always made freshly from the solid and added to molten LB-agar that had been cooled to 45 °C. Expression of the fusion proteins was induced by growing the cells at 39 °C; full induction at 42 °C was lethal.

Generation of Site-specific rhaT-bla Fusions—Three site-specific rhaT-bla fusions were created in the S. typhimurium rhaT gene, one at each specific fusion point. As was created in the E. coli rhaT gene, using PCR-amplified DNA. Oligonucleotide primer RH3 was homologous to the region downstream from the Ncol site at the initiator Met codon but introduced an Ncol site at the initiator Met codon: this oligonucleotide was used in the construction of all of the site-specific rhaT-bla fusions. Oligonucleotides RHA7, RHA8, and RHA9 were used to construct RhaT-bla fusion at amino acid residues Thr[65], Ser[66], and Thr[67] in the S. typhimurium RhaT, respectively. Each of these PCR primers introduced a PoulI restriction site after the codon of interest. In addition, oligonucleotide primers RHA3 and RHA7 were used to create an rhaT-bla fusion at the codon Thr[65] in the E. coli rhaT gene. The sequences of the oligonucleotides (5'-3') were as follows.

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PCR was performed with Vent DNA polymerase using the manufacturer's protocol. Plasmid pAJR18 was used as a template for the *S. typhimurium* gene, and plasmid pCGT12 was used as a template for the *E. coli* *rhaT* gene (Tate et al., 1992). After 15 rounds of amplification of 25 ng of template DNA, the PCR products were purified as described by Crowe et al. (1991). The DNA was then digested with *NcoI* and *PvuII*, repurified, and ligated into *NcoI/*EcoRI-digested plasmid pYZ4 in the presence of a gel-purified *PvuII/*EcoRI fragment from plasmid pXYZ which contained the *bla* gene (Broome-Smith et al., 1990). The ligation mixture was transformed into CaCl2-sensitive TG1 cells and plated out onto LB plates that contained 50 μg/ml kanamycin. Transformants were toothpicked onto LB-kanamycin plates that contained 20 μg/ml ampicillin to identify the transformants that expressed RhaT-β-lactamase fusions. Although the site-specific rhaT-β-lactamase gene fusions were expressed from a lacUV5 promoter under the control of the LacI repressor, it was found that sufficient expression occurred in the absence of inducer (isopropyl-β-D-galactopyranoside) for characterization of the fusions. The concentration of ampicillin required to kill single cells that expressed the RhaT-β-lactamase fusions was determined by spotting 5 μl of a 10−6 dilution of overnight culture (grown in 2TY at 37°C) onto plates that contained increasing concentrations of ampicillin, prepared as described above. Plates were incubated overnight at 37°C. The DNA encoding rhaT from colonies that expressed RhaT-β-lactamase fusions was completely sequenced on at least one strand.

**RESULTS**

**Construction and Analysis of the COOH-terminal RhaT-β-galactosidase and RhaT-β-lactamase Fusion Proteins**—A model of the RhaT protein derived from a hydrophathy plot and the location of positively charged amino acid residues in the cytoplasm suggested that the RhaT protein contained 10 transmembrane regions with both the NH2 and COOH termini in the periplasm (Fig. 1). To provide initial evidence for this model, the location of the COOH terminus of RhaT in either the periplasm or cytoplasm was determined using gene fusions. A unique *BalI* restriction site in the penultimate codon of the *S. typhimurium* *rhaT* gene was used to construct an *rhaT-β-galactosidase* gene fusion and an *rhaT-β-lactamase* gene fusion (see "Experimental Procedures").

The initial gene fusion constructed with the β-galactosidase gene *lacZ* was expressed under the control of the λ *P*1 promoter in plasmid pCGT17 (Fig. 2), but the levels of *LacZ* activity were intermediate between levels expected if the fusion point was in a periplasmic domain or a cytoplasmic domain (results not shown). In addition, the rhamnose uptake activity expressed in heat-induced strain TG2(pCGT17) was low. This could have been caused by either poor expression of the fusion protein or a perturbation in the structure of RhaT by *LacZ*, rendering the transporter less active. Western blot analysis of membranes derived from heat-induced strain TG2(pCGT17) showed that an RhaT-β-Gal fusion protein (Mr, 140,000) was expressed and that only a small proportion of it was degraded (results not shown). These results did not conclusively locate the COOH terminus of RhaT to either the periplasm or cytoplasm, so an RhaT-β-lactamase fusion was constructed.

Bla, unlike β-galactosidase (*LacZ*), is active when it is fused to a periplasmic domain of a membrane protein (Broome-Smith et al., 1990). The activity of the β-lactamase in vivo is assessed by the amount of ampicillin which is required to kill single cells that express the fusion protein; less than 5 μg/ml ampicillin will kill cells that express β-lactamase fused to a cytoplasmic domain of a membrane protein, but β-lactamase fused to a periplasmic domain confers resistance to much higher levels of ampicillin (Broome-Smith and Spratt, 1986; Edelman et al., 1987).

The RhaT-β-lactamase gene fusion was constructed, using the *rhaT-β-galactosidase* gene fusion as the source of DNA ("Experimental Procedures"), and expressed in plasmid pCGT18 under control of the *lacUV5* promoter; the DNA sequence of the fusion
point was confirmed (Fig. 3). The activity of the $\beta$-lactamase portion of the RhaT-Bla fusion protein was tested by spotting 5 $\mu$L of a 10$^{-5}$ dilution of an overnight culture of strain TG1(pCGT38) onto a plate that contained 5 $\mu$g/ml ampicillin. The cells that contained plasmid pCGT38 grew, but control cells that expressed a fusion gene with $\beta$-lactamase fused to a putatively cytoplasmic portion of RhaT (fusion at Tyr$^{296}$, see below) did not grow. Indeed, strain TG1(pCGT38) grew on plates that contained up to 300 $\mu$g/ml ampicillin in the presence of 0.5 mM isopropyl 1-thio-$\beta$-galactopyranoside to induce gene expression. This confirmed that the COOH terminus of RhaT was in the periplasm.

The transport activity of the RhaT-Bla fusion protein was difficult to assess because of proteolysis of the fusion protein (Fig. 3). Western blot analysis of the fusion protein utilized an anti-$\beta$-lactamase antibody, so that the total amount of potentially functional RhaT (RhaT-Bla fusion and RhaT) could not be determined. A prominent RhaT-Bla degradation product ($M_r$ 27,000) was observed to increase with the length of induction time, whereas the RhaT-Bla fusion protein ($M_r$ 46,000) decreased with time (Fig. 3). In contrast to the decreasing amounts of RhaT-Bla fusion protein in the membrane, the L-rhamnose uptake activity of induced strain TG1(pCGT38) actually increased (Fig. 3). The implication of this observation was that the RhaT-Bla fusion protein was less active than the native protein; removal of the $\beta$-lactamase portion of the fusion protein by proteolysis may be responsible for the regeneration of active RhaT, resulting in the increase in uptake activity observed.

**Isolation and Characterization of Randomly Generated**

**Raht-Bla Fusions**—The localization of the COOH terminus of the RhaT protein to the periplasm was consistent with the orientation of RhaT proposed in Fig. 1; to determine the number of transmembrane regions present in RhaT, $\beta$-lactamase fusions were constructed throughout the *S. typhimurium* *rhaT* gene. All of these gene fusions were expressed under the control of the $\lambda$ P$_I$ promoter.

Random deletions were made in the *rhaT* genes in plasmid pCGT15 with exonuclease III (see “Experimental Procedures”). The plasmid DNA was then blunt ended with S1 nuclease and digested with $Pvu$II. Religation of the partially deleted *rhaT* genes with the bla’ gene (see “Experimental Procedures”) and transformation into TG2 resulted in more than 5,000 kanamycin-resistant colonies. To determine which colonies expressed RhaT-Bla fusion proteins, 2,000 colonies were toothpicked on to LB plates that contained 20 $\mu$g/ml ampicillin and were grown overnight at 37°C. The high inoculum introduced onto the ampicillin plates with the toothpick ensured that cells expressing $\beta$-lactamase fused to a cytoplasmic portion of RhaT could grow, but cells that did not express any RhaT-Bla fusion protein could not grow. At 37°C a small amount of expression from the $\lambda$ P$_I$ promoter, under the control of the cl$_{507}$ repressor, produced sufficient RhaT-Bla fusion protein to give ampicillin resistance to the cells. Of the 175 ampicillin-resistant colonies obtained, 66 were characterized further by sequencing the gene fusion point using the sequencing primer oligonucleotide BLA1. DNA sequencing showed that 49 of the plasmid constructs contained in-frame *rhaT-bla* gene fusions, and 7 of the plasmid constructs expressed *cro-bla* gene fusions; part of the *cro* gene is present downstream from the $\lambda$ P$_I$ promoter in the 900-base pair fragment from plasmid pJG200 which contained the cl$_{507}$ gene (Fig. 2). All of the *cro-bla* fusion genes expressed a protein in which the $\beta$-lactamase activity was predicted to be in the cytoplasm (results not shown). Of the 49 *rhaT-bla* gene fusions obtained, 33 were unique; 14 fusions were sequenced with a fusion point at either the codon for Ser$^{216}$ or Tyr$^{217}$, presumably representing a region of DNA refractory to exonuclease III digestion.

The activity of $\beta$-lactamase in cells that expressed the *rhaT-bla* fusions was determined by spotting 5 $\mu$L of a 10$^{-5}$ dilution of an overnight culture of the cells onto LB plates that contained increasing concentrations of ampicillin (see “Experimental Procedures”). The results shown in Table I were compiled from a single experiment in which all of the plates were poured at the same time. These results were consistent with individual determinations carried out during the sequencing of the fusion points (results not shown). The positions of the RhaT-Bla fusions were identified on the topological model of RhaT (Fig. 4) and were found to be mainly in the COOH-terminal half of the protein; only six fusions were obtained in the region encoding the first six putative transmembrane domains.

**Isolation and Characterization of Site-specific RhaT-Bla Fusions**—To improve the coverage of fusions in the NH$_2$-terminal half of RhaT, three site-specific *rhaT-bla* fusions were constructed in plasmid pYZ4 under the control of the lacUV5 promoter (see “Experimental Procedures”). An additional site-specific fusion was constructed at the codon for Thr$^{296}$ in the *E. coli* *rhaT* gene (see “Discussion”). The DNA encoding part of the *rhaT* gene in the site-specific fusion constructs was resequenced on at least one strand to ensure that no mutations had occurred during the PCR amplification that was used to create the fusions. The gene fusions in plasmids pCGT40, pCGT44, and pCGT50 (see Table I) all contained an unchanged DNA sequence, except at the initiator Met
Plasmids pCGT51-83 were made by exonuclease III deletions of pCGT15. Letters after a plasmid name designate individually isolated clones. Plasmids pCGT40, pCGT43, pCGT44, and pCGT50 were site-specific fusions expressed in plasmid pYZ4. Plasmid pCGT43 contained part of the S. typhimurium (St) rhaT gene, and plasmid pCGT50 contained a fusion at the same position but in the E. coli (Ec) rhaT gene. All other constructs were derived from the S. typhimurium rhaT gene. The rhaT-bla fusion in plasmid pCGT38 was constructed using a BglII restriction site in the S. typhimurium rhaT gene. The last amino acid residue of RhaT before the fusion point is given, and the orientation of the β-lactamase moiety is indicated by P (periplasmic) or C (cytoplasmic). The concentration of ampicillin (µg/ml) which causes cell death is given in the final column.

| Plasmid | Fusion point | β-Lactamase location | Ampicillin concentration causing cell death (µg/ml) |
|---------|--------------|----------------------|-----------------------------------------------|
| pCGT51  | Ser2         | C                    | 2                                             |
| pCGT52  | Gly4         | C                    | 3                                             |
| pCGT53  | Gin3'        | P                    | 12.5                                          |
| pCGT54  | Thr2         | P                    | 3                                             |
| pCGT55  | Pro194'      | P                    | 25                                            |
| pCGT56  | Glu46'       | C                    | 2                                             |
| pCGT57  | Gly206'      | P                    | 30                                            |
| pCGT58  | Val200'      | P                    | 7.5                                           |
| pCGT59  | Asp208'      | P                    | 25                                            |
| pCGT60  | Leu210'      | P                    | 25                                            |
| pCGT61  | Val224'      | P                    | 25                                            |
| pCGT62a-h | Ser216'      | P                    | 25                                            |
| pCGT63a-f | Tyr227'     | P                    | 20                                            |
| pCGT64  | Val218'      | P                    | 7.5                                           |
| pCGT65  | Ile201'      | P                    | 2                                             |
| pCGT66  | Asn241'      | C                    | 2                                             |
| pCGT67  | Ser243'      | C                    | 2                                             |
| pCGT68  | Ala246'     | C                    | 3                                             |
| pCGT69  | Phe248'     | C                    | 2                                             |
| pCGT70  | Arg252'      | C                    | 2                                             |
| pCGT71  | Leu234'      | C                    | 2                                             |
| pCGT72  | Asn258'      | C                    | 2                                             |
| pCGT73  | Met268'      | P                    | 12.5                                          |
| pCGT74a,b | Trp286'   | P                    | 15                                            |
| pCGT75a-d | Gln212' | P                    | 20                                            |
| pCGT76  | Tyr227'      | P                    | 30                                            |
| pCGT77  | Asp230'     | P                    | 20                                            |
| pCGT78  | Met240'      | P                    | 30                                            |
| pCGT79  | Met243'      | P                    | 30                                            |
| pCGT80  | Ser250'     | P                    | 30                                            |
| pCGT81  | Tyr259'      | P                    | 12.5                                          |
| pCGT82  | Arg259c'   | C                    | 2                                             |
| pCGT83  | Gly241'      | P                    | 25                                            |
| pCGT84a-f | Tyr266'     | P                    | 25                                            |
| pCGT84a-d | Gly266' | P                    | 12.5                                          |
| pCGT85  | Thr266'     | C                    | 4                                             |

Despite the wide range in levels of expression of the RhaT-Bla fusion proteins, there seemed to be no correlation between the amount of protein that was expressed and the localization of the β-lactamase to either the periplasm or the cytoplasm. β-Lactamase fusions at Thr266, Ser243 and Arg259 (lanes 2, 6, and 7, Fig. 5a) were expressed at relatively high levels, similar to levels of protein expressed by fusions at Gin31 and Ala243 (Fig. 5a, lane 3, and Fig. 5b, lane 2). However, the in vivo assay for β-lactamase activity concluded that the β-lactamase moiety was in the periplasm for fusions at Thr266, Ser243, and Arg259, whereas fusions at Gin31 and Ala243 resulted in the β-lactamase activity in the periplasm (Fig. 4). Problems could potentially arise if the fusion proteins were expressed at very low levels, as observed for fusions at Tyr266, Pro191, and Gly241, because there might be insufficient β-lactamase expressed to protect the cell from lysis, thus giving an erroneous indication of topology. However, the low levels of protein expressed for fusions at Pro191 and Gly241 gave cells resistance to 25 and 30 µg/ml ampicillin, respectively (Fig. 4), indicating that the β-lactamase moiety is in the periplasm. In contrast, the similar level of fusion protein expressed by plasmid pCGT40 (fusion at Tyr266, lane 3) does not give resistance to high levels of ampicillin and was therefore proposed to be in the cytoplasm. We concluded from these Western blots that the amount of fusion protein expressed in this representative sample of fusions did not affect the topological analysis of RhaT.

Determination of the Level of Expression of RhaT-Bla Fusion Proteins by Western Blot Analysis—To ensure that the levels of fusion protein expression were not affecting the topological analysis, membranes were prepared from some strains expressing RhaT-Bla fusion proteins and analyzed by Western blotting with an anti-β-lactamase antibody. Bacterial membranes were prepared from strains containing plasmids pCGT78, pCGT40, pCGT43, pCGT44, pCGT50, pCGT58, pCGT55, pCGT57, pCGT62, pCGT76, and pCGT82 (see Table I). This is a representative sample of all the rhaT-bla fusions, each plasmid expressing a fusion protein with β-lactamase fused to a different hydrophilic loop in RhaT. Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose (see “Experimental Procedures”). The blots were probed with an anti-β-lactamase antibody (Fig. 5). In each lane there was a single major band and a number of minor bands. The most intense band was assumed to be the intact fusion protein, and the less intense bands were assumed to be either oligomers of the RhaT-Bla fusion protein or proteolytic products. The relative mobilities of the RhaT-Bla fusion proteins on SDS-polyacrylamide gel electrophoresis was consistent with the increasing size of the fusion proteins, but the molecular masses determined from the gels were less than would be expected from the molecular mass of β-lactamase and the portion of RhaT fused to it. The RhaT-Bla fusion protein with β-lactamase fused at Ala243 (encoded by plasmid pCGT38) has an expected molecular mass of 66 kDa but has an apparent molecular mass of 46 kDa on SDS-polyacrylamide gels. This is probably a consequence of the abnormal mobility of the RhaT protein on SDS-polyacrylamide gel electrophoresis; RhaT has a molecular mass of 37 kDa but has an apparent molecular mass of 27 kDa on SDS-polyacrylamide gels (Baldona et al., 1990).

Despite the wide range in levels of expression of the RhaT-Bla fusion proteins, there seemed to be no correlation between the amount of protein that was expressed and the localization of the β-lactamase to either the periplasm or the cytoplasm. β-Lactamase fusions at Thr266, Ser243 and Arg259 (lanes 2, 6, and 7, Fig. 5a) were expressed at relatively high levels, similar to levels of protein expressed by fusions at Gin31 and Ala243 (Fig. 5a, lane 3, and Fig. 5b, lane 2). However, the in vivo assay for β-lactamase activity concluded that the β-lactamase moiety was in the periplasm for fusions at Thr266, Ser243, and Arg259, whereas fusions at Gin31 and Ala243 resulted in the β-lactamase activity in the periplasm (Fig. 4). Problems could potentially arise if the fusion proteins were expressed at very low levels, as observed for fusions at Tyr266, Pro191, and Gly241, because there might be insufficient β-lactamase expressed to protect the cell from lysis, thus giving an erroneous indication of topology. However, the low levels of protein expressed for fusions at Pro191 and Gly241 gave cells resistance to 25 and 30 µg/ml ampicillin, respectively (Fig. 4), indicating that the β-lactamase moiety is in the periplasm. In contrast, the similar level of fusion protein expressed by plasmid pCGT40 (fusion at Tyr266, lane 3) does not give resistance to high levels of ampicillin and was therefore proposed to be in the cytoplasm. We concluded from these Western blots that the amount of fusion protein expressed in this representative sample of fusions did not affect the topological analysis of RhaT.

Weiner et al. (1993) used β-lactamase fusions to study the topology of the anchor subunit of E. coli dimethyl sulfoxide reductase (DmsC), and, in addition to using the in vivo β-lactamase assay used here, they also used a colorimetric assay for measuring β-lactamase activity. These colorimetric assays on the whole confirmed the analysis by the in vivo assay, but in some cases the colorimetric assays gave very low values because of proteolysis of the fusion protein. In these cases, the topological information was derived solely from the in vivo assay.
FIG. 4. Positions of the β-lactamase fusions on the topological model of RhaT derived from hydrophathy analysis and charge distribution. The positions of the β-lactamase fusions are indicated by circled or boxed residues. Circled residues indicate that the RhaT-Bla fusion conferred resistance to high concentrations of ampicillin (β-lactamase in the periplasm), whereas cells that expressed RhaT-Bla fusion proteins shown by the boxed residues were killed by low levels of ampicillin (β-lactamase in the cytoplasm). The concentration of ampicillin (µg/ml) required to kill single cells expressing fusion proteins is shown for each fusion in bold figures. The number of each amino acid residue is also shown. All fusions are with the S. typhimurium rhaT gene, except at Thr^{48} where a fusion was also made to the E. coli rhaT gene. The RhaT-Bla fusion at Ala^{453} was constructed from a BalI restriction site and the Xal promoter. To ensure that the levels of fusion protein expression were not affecting the topological analysis, membranes were prepared from cells that expressed RhaT-Bla fusion proteins and analyzed by Western blotting with an antip-lactamase antibody (Fig. 5). The levels of expression of the RhaT-Bla fusion proteins varied enormously, but there seemed to be in the periplasm, there is no NH2-terminal leader peptide sequence. The positions of all of the β-lactamase fusions on this model and the localization of the β-lactamase portion of the fusion proteins are shown in Fig. 4. In addition, the concentrations of ampicillin required to kill single cells that expressed RhaT-Bla fusion proteins are shown in Table I. Two RhaT-Bla fusions were obtained at the beginning of the RhaT protein (fusions at Ser^2 and Gly^4). Since these fusions contained too few amino acid residues of RhaT to provide any means of crossing the membrane, the β-lactamase had to be located in the cytoplasm. Consistent with this conclusion was the observation that single cells that expressed these fusion proteins were killed by 2 or 3 µg/ml ampicillin. All other strains that expressed RhaT-Bla fusion proteins which were killed by less than 5 µg/ml ampicillin were assumed to express the β-lactamase portion of the fusion protein in the cytoplasm (Broome-Smith and Spratt, 1986). The concentration of ampicillin required to kill single cells that expressed the β-lactamase portion of the fusion proteins in the periplasm varied between 7.5 and 30 µg/ml. The generally low value of these figures compared with other fusion data (Broome-Smith and Spratt, 1986; Edelman et al., 1987; Wu et al., 1992) was probably caused by the low levels of expression from the λ promoter or from the lacUV5 promoter. This was done deliberately to prevent high levels of RhaT expression which are known to be lethal to the cell.2 Indeed, cells that expressed RhaT-Bla fusion proteins under the control of the λ promoter and the cλal repressor protein could not grow on LB plates at 42 °C. One reason why very few fusions were obtained in the NH2-terminal half of RhaT might be that fusions in this region are more toxic and therefore would be less likely to be isolated in the initial screening. Certainly the site-specific fusions created at Gly^{48} and Tyr^{48} resulted in cells that could be visibly distinguished from normal E. coli by their poor growth on LB plates, despite the absence of any inducer (i.e. isopropyl 1-thio-β-D-galactopyranoside) for the lacUV5 promoter. To ensure that the levels of fusion protein expression were not affecting the topological analysis, membranes were prepared from some strains expressing RhaT-Bla fusion proteins and analyzed by Western blotting with an anti-β-lactamase antibody (Fig. 5). The levels of expression of the RhaT-Bla fusion proteins varied enormously, but there seemed to

![Image of Western blots of membranes prepared from cells that expressed RhaT-Bla fusion proteins. Each lane contained 30 µg of protein from membranes prepared from E. coli strains by spheroplast lysis (see “Experimental Procedures”). Cells that expressed the rhaT-bla fusions from the lacUV5 promoter in plasmid pYZ4 were grown at 37 °C until they reached an A600 of 3; the cells were then harvested and membranes prepared. Cells that expressed the rhaT-bla fusions from the λ P, promoter under the control of the cλal repressor protein were grown overnight at 39 °C and harvested. Membranes were prepared from the following strains: panel a: lane 1, TG1(pCGT57) [Gly^{48}]; lane 2, TG1(pCGT67) [Ser^{48}]; lane 3, TG1(pCGT53) [Gin^2]; lane 4, TG1(pCGT55) [Pro^{48}]; lane 5, TG1(pCGT76) [Y287]; lane 6, TG1(pCGT82) [Arg^{48}]; lane 7, TG1(pCGT50) [Thr^48], E. coli RhaT]; panel b: lane 1, TG1(pCGT14) [control]; lane 2, TG1(pCGT38) [Ala^{48}]; lane 3, TG1(pCGT40) [Tyr^{48}]; lane 4, TG1(pCGT43) [Thr^{48}, S. typhimurium RhaT]; lane 5, TG1(pCGT44) [Gly^{48}]. The material in square brackets refers to the final amino acid residue in RhaT and its position in the RhaT protein before the fusion point with β-lactamase. The letters underneath the lane numbers in the figure refer to the orientation of the β-lactamase part of the fusion proteins. P, periplasmic; C, cytoplasmic.

DISCUSSION

The topological model for S. typhimurium RhaT depicted in Fig. 4 was derived from the analysis of the hydrophobic profile and the distribution of charged residues (Tate et al., 1992). The E. coli RhaT sequence is 91% identical to the S. typhimurium sequence and was predicted to have an identical topology. Although the NH2 terminus of RhaT is predicted to
be no correlation between the amount of protein that was expressed and the localization of the \(\beta\)-lactamase to either the periplasm or the cytoplasm.

The fusion data in the COOH-terminal half of the protein are consistent with the model proposed from the hydrophathy plot and charge distribution (Fig. 4). In addition, the smaller number of fusions in the hydrophilic loops between transmembrane regions 2, 3, 4, and 5 are consistent with the localization of these loops proposed by the model. The fusion in transmembrane region 3 at Thr\(^{72}\) suggested that this region should be in the cytoplasm; as 9–11 hydrophobic amino acids are thought to be sufficient to anchor the \(\beta\)-lactamase in the membrane, residue Thr\(^{72}\) was placed in the cytoplasmic leaflet of the membrane so that the transmembrane region started at residue 62 rather than 69 (compare Fig. 1a with Fig. 6a). However, such conclusions must be treated with extreme caution as they are based only upon the assumption that 9–11 hydrophobic amino acid residues are required for the translocation of the \(\beta\)-lactamase across the membrane (Calamia and Manoil, 1990) and that no protein-protein interactions occur between transmembrane helices (see below). In addition, it is a tacit assumption that transmembrane helices are perpendicular to the plane of the membrane, although this is clearly not the case for bacteriorhodopsin (Henderson et al., 1990) and a plant light-harvesting complex (Kuhlbrandt and Wang, 1991).

A major discrepancy between the model and the \(\beta\)-lactamase fusion data for the \(S.\ typhimurium\) RhaT protein occurred in the first hydrophilic loop, between putative transmembrane regions 1 and 2. The model predicted that this region was in the cytoplasm, but the \(\beta\)-lactamase fusion data suggest that this region is in the periplasm (Fig. 4). If the data from the \(\beta\)-lactamase fusions are considered alone, then a 9-helix model for RhaT can be produced (Fig. 6), suggesting that the NH\(_2\) terminus is in the cytoplasm, the COOH terminus is in the periplasm, and that there are only nine transmembrane regions. However, this nine-helix model does not conform to the positive inside rule; in a recent analysis of 24 bacterial membrane proteins using this rule, 23 of the predicted topologies were identical to the topologies established by experiment (von Heijne, 1992). This problem with the nine-helix model is exacerbated when the protein sequence of the \(E. coli\) RhaT protein is considered. In the region between putative transmembrane regions 1 and 2 in the \(S.\ typhimurium\) RhaT sequence (Fig. 4) there are 2 Lys residues, whereas in the \(E. coli\) protein there are 4 Lys residues (both Gln\(^{29}\) and Gln\(^{31}\) in the \(S.\ typhimurium\) sequence are Lys in the \(E. coli\) sequence). All of the studies performed on altering the topology of leader peptidase suggest that a group of Arg or Lys residues will alter the topology of the protein so that they will be in the cytoplasm (Andersson et al., 1992), suggesting that the first hydrophilic loop in \(E. coli\) RhaT is most likely to be in the cytoplasm. An additional reason why the nine-helix model seems unfavorable is that a hydrophobic region (helix 2 in Fig. 6) is placed totally in the periplasm.

Can the \(\beta\)-lactamase fusion data in the region of the first hydrophilic loop be reconciled with the 10-helix model for RhaT? As mentioned above, an assumption of the analysis of any gene fusion technique is that the fusion proteins behave in an identical fashion to the same region in the native protein. Alternatively, it is an assumption that there are no interactions between transmembrane helices required to maintain topology. This assumption may not be true for the \(\beta\)-lactamase fusions at Gln\(^{31}\) and Thr\(^{36}\), i.e. the topology of helix 1 may be maintained by an interaction with, for example, helix 2, and, if this were the case, then the \(\beta\)-lactamase fusion data in this region would be an unreliable indicator of the topology of RhaT. The analysis of a \(\beta\)-lactamase fusion made at Thr\(^{36}\) in the \(E. coli\) RhaT protein was performed to see if the localization of Thr\(^{36}\) in the \(E. coli\) RhaT protein was predicted to be the same as for the \(S.\ typhimurium\) protein. The only difference between the \(E. coli\) and \(S.\ typhimurium\) Thr\(^{36}\) \(\beta\)-lactamase fusions is that the \(E. coli\) fusion has 4 Lys residues in the region between the first putative transmembrane region, and the \(\beta\)-lactamase, whereas the \(S.\ typhimurium\) sequence has only 2 Lys residues. The ampicillin concentration required to kill single cells that expressed the \(E. coli\) \(\beta\)-lactamase fusion was 4 \(\mu\)g/ml, compared with 15 \(\mu\)g/ml for the \(S.\ typhimurium\) protein (Table 1). Thus, the \(\beta\)-lactamase fusions at Thr\(^{36}\) predict a different localization of this residue in the RhaT protein sequence depending upon the source of the protein. Therefore, we have concluded that the \(\beta\)-lactamase fusion data in the first hydrophilic loop of RhaT are an unreliable indicator of the topology of RhaT and that they should not influence the final model of RhaT based upon this analysis. However, the RhaT-Bla fusions at Thr\(^{36}\) created from the \(E. coli\) and \(S.\ typhimurium\) rhaT genes are both associated with the membrane (Fig. 5), suggesting that the first hydrophilic region of the RhaT protein could be inserted in the membrane. This would be consistent with the 10-helix model in Fig. 4, with the NH\(_2\) terminus in the periplasm. A different technique, such as chemical labeling, will be needed to assign definitively the location of the NH\(_2\) terminus.

![Diagram of Membrane Topology of RhaT](image-url)
CONCLUSION

A model for the structure of RhaT was tested using $\beta$-lactamase fusions. The fusion data were consistent with the orientation in the model of putative transmembrane helices 2–10, locating the COOH terminus in the periplasm. However, the $\beta$-lactamase fusions were thought to be unreliable for determining the orientation of the first hydrophilic loop of RhaT, predicted to be in the cytoplasm in the model derived from a hydrophathy plot and charge distribution. Two fusions were made in this region at residue Thr10; one fusion was made using the E. coli rhaT gene, and the other fusion was constructed from the S. typhimurium rhaT gene. The $\beta$-lactamase portion of these fusions was found to be in the cytoplasm in the E. coli RhaT-Bla fusion and in the periplasm in the S. typhimurium RhaT-Bla fusion. The only difference between the E. coli and S. typhimurium RhaT sequences in this region is that the E. coli sequence contains 4 Lys residues, whereas the S. typhimurium RhaT protein contains only 2 Lys residues. This suggested that other parts of the RhaT protein were probably required for the maintenance of topology of the first putative transmembrane region in the S. typhimurium RhaT protein and thus precluded the positive assignment of the first putative hydrophilic loop to the cytoplasm or periplasm. However, both fusion proteins at Thr23 were associated with the membrane, suggesting that the first hydrophobic domain could be inserted in the membrane. Therefore, the final model for RhaT, taking into account the $\beta$-lactamase fusion data, the hydrophobic profile, and the charge distribution, has 10 transmembrane regions with the NH$_2$ and COOH termini in the periplasm.

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