Characterization of the Hemolysin Transporter, HlyB, Using an Epitope Insertion*

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Peter Juranka‡‡, Fang Zhang‡, Janus Kulpa‡, Jane Endicott‡‡, Mark Blight¶ **, I. Barry Holland¶, and Victor Ling‡‡

From the Ontario Cancer Institute and the Department of Medical Biophysics, University of Toronto, Ontario M4X 1K9, Canada and the Institut de Génétique et Microbiologie, URA, Centre National de la Recherche Scientifique, D1354, Université Paris-Sud, 91405 Orsay, Cedex, France

The prokaryotic hlyB gene product is a member of a superfAMILY of ATP-binding transport proteins that include the eukaryotic multidrug-resistance P-glycoprotein, the yeast STE6, and the cystic fibrosis CFTR gene products (Juranka, P. F., Zastawny, R. L., and Ling, V. (1989) FASEB J. 3, 2583–2592). Previous genetic studies have indicated that HlyB is involved in the transport of the 107-kDa HlyA protein from Escherichia coli; however, the HlyB protein has not been purified for biochemical studies due to its low abundance. In this study, we have engineered a monoclonal antibody epitope into the C-terminal end of HlyB that did not destroy its function. This has allowed us to use immunological methods to identify and localize various molecular forms of the HlyB protein present in vivo.

The original finding that P-glycoprotein is structurally very similar to the Escherichia coli HlyB protein formed the basis for a proposed model for multidrug resistance in mammalian cells (1, 2). It was envisioned that, analogous to the role of HlyB in the export of α-hemolysin (HlyA) protein out of the bacterium, P-glycoprotein functions in mammalian cells as an energy-dependent pump for the efflux of various anticancer drugs (3). Subsequently, it was demonstrated that both these proteins are, in fact, part of a large superfamily of ATP-dependent transport proteins involved in the translocation of diverse substrates across biological membranes in prokaryotic and eukaryotic cells. These include ions, peptides, large proteins, and sugar polymers (4). The structure of P-glycoprotein can be regarded as a tandem duplication of HlyB. HlyB is composed of two domains, a hydrophobic N-terminal half presumably comprising multiple membrane-spanning sequences and a hydrophilic C-terminal half that exhibits amino acid motifs typical of ATP-binding proteins. P-glycoprotein and HlyB have extensive sequence conservation in the hydrophilic domains. Although they show limited sequence homology in the hydrophobic domain, the proposed number and placement of putative transmembrane sequences are similar (1, 2).

The current model for secretion of HlyA in E. coli proposes that a complex of HlyB and HlyD molecules span the inner and outer E. coli membranes, possibly at junctions between these membranes (4). Energy derived from ATP hydrolysis by HlyB is likely necessary for transport (5). It has been suggested that the hlyB gene may code for two forms of the protein, a 66- and a 46-kDa polypeptide (7, 12). The relative contributions of each of these proteins to the transport process remains to be determined. HlyA does not have a classical N-terminal leader sequence (6). A recognition signal necessary for secretion has been localized to the 27–55 amino acids at the C terminus (8, 9). Other members of the superfamily of ATP-dependent peptide transporters may be similar in mechanism, recognizing and subsequently transporting molecules without using a N-terminal leader sequence. For example, the yeast P-glycoprotein homologue STE6 transports the yeast a-type mating pheromone (a 12-amino acid peptide that does not have a N-terminal leader sequence) from MAT-a type cells (10). Structural and mechanistic precedents suggest that HlyB may be an excellent model system for studying P-glycoprotein and this superfamily of transport proteins.

HlyB is present in very low abundance in E. coli, and it has not been possible to purify this protein for biochemical studies. Attempts at raising antisera to HlyB have met with only limited success. We have overcome this problem by using linker insertion and site-directed mutagenesis to engineer a monoclonal antibody epitope into the HlyB protein. This HlyB protein is functional and can be detected by immunological assays. We report here that this epitope tagging (modified form) of HlyB has allowed us to identify and localize various molecular forms of the HlyB protein in E. coli that have not been detected previously. These findings should be important for future functional studies.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—E. coli strains BW318 (dut ung) and JM109 (dut ung) were used to grow M13 phage for the mutagenesis experiments. JM33 was used for cell fractionation and hemolytic assays. The complete hemolysin system is present on two plasmids. The plasmid pLG579 expresses the HlyB protein from a 3-kilobase EcoRI fragment that is present on the pACYC184 vector, which is selectable with tetracycline (11). The other transport protein (HlyD), the structural α-hemolysin molecule (HlyA), and an activator protein of α-hemolysin (HlyC) are expressed from the yeast a-type mating pheromone (a 12-amino acid peptide that does not have a N-terminal leader sequence) from MAT-a type cells (10). Structural and mechanistic precedents suggest that HlyB may be an excellent model system for studying P-glycoprotein and this superfamily of transport proteins.

HlyB is present in very low abundance in E. coli, and it has not been possible to purify this protein for biochemical studies. Attempts at raising antisera to HlyB have met with only limited success. We have overcome this problem by using linker insertion and site-directed mutagenesis to engineer a monoclonal antibody epitope into the HlyB protein. This HlyB protein is functional and can be detected by immunological assays. We report here that this epitope tagging (modified form) of HlyB has allowed us to identify and localize various molecular forms of the HlyB protein in E. coli that have not been detected previously. These findings should be important for future functional studies.
Sequencing hlyB Gene—The hlyB gene, present on an EcoRI fragment in plasmid pLG579, was recloned into M13mp9 to yield the construct M13-E8, in which the gene is antisense to the lacZ promoter. Fourteen 18-mer oligonucleotides complementary to M13-E8 were synthesized on the basis of a previously published hlyB sequence (12). These synthetic oligonucleotides, spaced ~200 base pairs apart, were used as primers for sequencing the entire EcoRI fragment in M13-E8 by the dideoxy chain termination method of Sanger et al. (13).

**Linker Insertion Mutagenesis**—Plasmid pLG579 was randomly linearized with HpaII and inserted into competent DH1 cells. Mutagenic oligonucleotides (Table I) were synthesized, phosphorylated, and annealed to ligate oligonucleotides into vector pACYC184 in the same orientation as pLG579.

**Epitope Linker Insertion**—Two complementary oligonucleotides (5'CGCTGGTACCAG-3') anneals to itself and forms a linker with HpaII restriction site. The presence and relative location of the linker were determined by restriction enzyme analysis, and the number of copies of the linker and its orientation were determined by dideoxy double strand sequencing.

**Oligonucleotide-directed Mutagenesis**—Site-directed mutagenesis was undertaken according to the method of Kunkel (15). Briefly, M13-E8 phage was passaged twice through E. coli BW313 cells grown in uridine to generate uracil-containing phage DNA. Oligonucleotides with desired mismatches (shown in Fig. 1) were phosphorylated and annealed to the uracil-containing template. The linearized plasmid was synthesized in vitro by extension of the primers with Klenow polymerase, and the circle was covalently closed with T4 DNA ligase. Transfection into E. coli JM109 cells results in specific degradation of the wild-type strand, and mutant plasmids were routinely found at ~1-2% frequency. The HBK oligonucleotide (5'-CGCTGGTACCAG-3') anneals to itself and forms a linker with HpaII end sites and a HpaI restriction site. The presence and relative location of the mutagenesis were determined by dideoxy double strand sequencing.

**Results**

Sequence of hlyB gene from E. coli LE2001 Cells—E. coli LE2001 cells were originally isolated from a human urinary tract infection. The complete Hly determinant was cloned from the chromosome onto plasmid pLG570 (20). We have sequenced the hlyB gene of this determinant (see "Materials and Methods" and Fig. 1) and compared it to the sequences of three other isolates of HlyB (data not shown). Two of the HlyB isolates were isolated from E. coli. The hlyB gene of pHy152 is a plasmid-encoded determinant, whereas pSF4000, like the present pLG570, was chromosomal (12, 21). The third hlyB sequence was derived from Proteus vulgaris (22). All four HlyB polypeptides have a high degree of amino acid conservation; however, the Proteus sequence is significantly different from the other three E. coli genes, with a total of 59 amino acid differences compared to pLG573 HlyB. However, the majority of these differences, 51 of 59, were conserved substitutions as determined by the mutational data matrix (25).

**Insertion of Monoclonal Antibody Epitope into HlyB**—To detect HlyB in vivo, we decided to tag the protein with a monoclonal antibody epitope. The epitope sequences for two monoclonal antibodies directed against P-glycoprotein (C219 and C494) have been mapped by Georges et al. (24) and shown to be VQVAALD and PNTLEGN, respectively, located in the cytoplasmatic ATP-binding domain. The small size of these continuous epitopes and the availability of specific monoclonal antibodies made these sequences ideal for the epitope tagging experiments.

The first approach involved an attempt to introduce the C219 epitope into the HlyB protein, encoded by pLG579, by conversion of the amino acid sequence IIMRNMH of HlyB (amino acids 643–649 in Fig. 1) to that of the C219 epitope sequence (VQVAALD) by oligonucleotide-directed mutagenesis using the C219 oligonucleotide shown in Fig. 1. This position in HlyB is equivalent to the position of the C219 epitope in P-glycoprotein (1). The mutant N44A, which contains the C219 epitope (see "Materials and Methods"), was not lytic when transformed into E. coli JM83 cells harboring the pLG570::Tn5-32 plasmid (26). Western blot analysis of membrane and soluble fractions of cells containing the N44A plasmid showed no immunoreactive bands when probed with 125I-labeled C219 antibody (data not shown). The reason for the lack of detectable HlyB in the immunoblots was not investigated further, but this experiment did suggest that HlyB may not easily tolerate mutations in its sequence.

In a second approach, we decided to scan the hlyB gene for any region where insertions of KpnI restriction sites would not disrupt the function of HlyB, reasoning that such sites...
Immunological Detection of HlyB

**Fig. 1. Nucleotide sequence of hlyB gene of E. coli LE2001 cells.**
The amino acid sequence is presented in single-letter code directly above the nucleotide sequence of the hlyB gene. The beginning and end of each line are numbered with the corresponding amino acid or nucleotide position. Oligonucleotides used in this study for mutagenesis and polymerase chain reaction are placed directly below the corresponding position in the nucleotide sequence. *Asterisks* locate the positions in the sequence of HpaI sites where KpnI linkers were inserted.

**Fig. 2. Relative size and intensity of hemolytic zones.** JM83 cells cotransfected with pLG570::Tn5-32 (HlyA/HlyC/HyD) and various mutants of pLG579 (HlyB) were plated on 5% sheep blood agar containing the antibiotics ampicillin (50 mg/ml) and tetracycline (12.5 mg/ml) and incubated at 37 °C overnight. The size of the zone from largest (++) to smallest (+) and no detectable zone (−) correspond to the phenotypes shown in Table 1 A, N57-12A (+++); B, N225-15 (++); C, N225-4 (+); D, N4A (−).

Could then be used to incorporate an epitope sequence. We first tried site-directed linker insertion at HpaI sites within the hlyB gene (see "Materials and Methods"). Of the 10 predicted HpaI sites present within the gene, we were successful in obtaining six linker insertions (see Fig. 1 for locations). The linker insertion mutants were transformed into *E. coli* JM83 cells harboring pLG570::Tn5-32; however, these mutants did not produce hemolytic colonies and were no longer studied.

*KpnI* restriction enzyme sites were also placed at either the 5'-end (N-terminal) or the 3'-end (C-terminal) of the *hlyB* gene by site-directed mutagenesis of M13-EE8 using the oligonucleotides N-KpnI and C-KpnI shown in Fig. 1. The *KpnI*-positive clones were identified by restriction enzyme analysis, and the mutation was transferred to pACYC184 on the EcoRI cassette to yield the plasmids N32 (N-KpnI) and N225 (C-KpnI). Since both these constructs appeared to retain viable HlyB activity (Table I), they were chosen for the C494 epitope addition. The C494 epitope, encoded on complementary oligonucleotides, was inserted into the *KpnI* site of N32 or N225 in two possible orientations (see "Materials and Methods"). The linker was in the sense orientation (coding for the C494 epitope) in 1985 and N32-L5 and in the antisense orientation (coding for the sequence RYLPRTCSV) in N225-4 and N32-L3 (Table I). *E. coli* cells containing (in addition to pLG570::Tn5-32) N225-4 or N225-15 were hemolytic (Fig. 2), whereas those cells with N32-L5 or N32-L3 produced nonhemolytic phenotypes (Table I). All four con-
## Table I

<table>
<thead>
<tr>
<th>Mutant/Phenotype</th>
<th>Description of mutant</th>
<th>Genotype*</th>
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<tbody>
<tr>
<td>pLG579 ++</td>
<td>Creation of KpnI site at C terminus by oligonucleotide-directed mutagenesis</td>
<td>703 Q L Q S CAGTTACAGTCA</td>
</tr>
<tr>
<td>N225 +++++</td>
<td>Creation of KpnI site at N terminus by oligonucleotide-directed mutagenesis</td>
<td>1 M D S C ATGGATCTTTGT</td>
</tr>
<tr>
<td>N32 + +</td>
<td>Conversion of Met to Val by oligonucleotide-directed mutagenesis</td>
<td>285 V M W GTAAGTG</td>
</tr>
<tr>
<td>N57-12A +</td>
<td>Insertion of C494 epitope DNA sequence in sense orientation into KpnI site of N225</td>
<td>703 Q V V W P S</td>
</tr>
<tr>
<td>N225-15 +</td>
<td>Insertion of C494 epitope DNA sequence in antisense orientation into KpnI site of N225</td>
<td>1 M V P N T L E G N V P S</td>
</tr>
<tr>
<td>N225-4 -</td>
<td>Insertion of C494 epitope DNA sequence into KpnI site of N32</td>
<td>703 Q V V W P S</td>
</tr>
<tr>
<td>N240-S8 +</td>
<td>Combination of N57-12A Met to Val mutant and N225-15 C494 epitope in sense orientation</td>
<td>624 H I I I M R N M H K</td>
</tr>
<tr>
<td>N32-L5 -</td>
<td>Insertion of C494 epitope DNA sequence in sense orientation into KpnI site of N32</td>
<td>624 H I I I M R N M H K</td>
</tr>
<tr>
<td>N32-L3 -</td>
<td>Insertion of C494 epitope DNA sequence in antisense orientation into KpnI site of N32</td>
<td>624 H I I I M R N M H K</td>
</tr>
<tr>
<td>N4A -</td>
<td>Conversion to C219 epitope sequence by oligonucleotide-directed mutagenesis</td>
<td>624 H I I I M R N M H K</td>
</tr>
<tr>
<td>F77 -</td>
<td>Generation of KpnI site by site-directed insertion of HBK linker at HpaII site</td>
<td>423 P E C</td>
</tr>
<tr>
<td>F198 -</td>
<td>Generation of KpnI site by site-directed insertion of HBK linker at HpaII site</td>
<td>462 P E C</td>
</tr>
<tr>
<td>F119 -</td>
<td>Generation of KpnI site by site-directed insertion of HBK linker at HpaII site</td>
<td>473 P E C</td>
</tr>
<tr>
<td>F55 -</td>
<td>Generation of KpnI site by site-directed insertion of two head-to-head HKC linkers at HpaII site</td>
<td>482 P E C</td>
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<tr>
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<td>Generation of KpnI site by site-directed insertion of HBK linker at HpaII site</td>
<td>607 S G C</td>
</tr>
<tr>
<td>F185 -</td>
<td>Generation of KpnI site by site-directed insertion of HBK linker at HpaII site</td>
<td>694 P E C</td>
</tr>
<tr>
<td>F61 -</td>
<td>Generation of KpnI site by site-directed insertion of HKC linker at HpaII site</td>
<td>694 P E C</td>
</tr>
</tbody>
</table>

**The relative size of hemolytic zones around colonies plated on blood agar plates is graded from the largest zone (++++) to the smallest (+) and also no detectable zone (-) as described for Fig. 2. The various HlyB mutant plasmids were transfected into JM83 cells harboring plasmid pLG570:Tn5-32 (HlyA/HlyC/HlyD).**

*The DNA sequence of the mutant and its amino acid translation in single-letter code are given below the corresponding wild-type DNA and amino acid sequences. The position of the amino acid in the wild-type sequence is written to the left of the sequence and follows the numbering system in Fig. 1.*
Structs were studied by immunoblot technique.

Immunodetection of HlyB Proteins—Conventional membrane purification methods and immunoblotting using monoclonal antibody C494 were used to localize the epitope-tagged hlyB product in E. coli cells harboring plasmid N225-15. The maximum coding capacity of the hlyB gene has been estimated as 79.9 kDa (12). As can be seen in Fig. 3, the inner membrane contains a major polypeptide of ~66 kDa. The size of HlyB detected here is smaller than the predicted 79.9 kDa, but this may be due to the fact that highly hydrophobic membrane proteins can migrate anomalously on SDS-polyacrylamide gels. Other explanations are also possible. The 66-kDa protein, however, is likely the authentic HlyB protein since a Tn5 insertion into hlyB leads to the disappearance of a component of this size (7), and this is also the size of the major gene product detected for pLG579 in minicells (7, 25). Purification of the epitope-tagged HlyB protein and determination of its sequence will ultimately be required to define the exact relationship of this product with the total coding capacity of the hlyB gene.

In addition to the major 66-kDa polypeptide, three minor C494 reactive bands of 33, 32, and 28 kDa are frequently observed in the inner membrane fractions of JM83 cells containing N225-15 (Fig. 3, A, lanes 4-6; and C, lane 1). Densitometry measurements revealed that the relative amount of the 66-kDa polypeptide was twice that of the combination of 33- and 32-kDa bands (Fig. 3A, lane 6). These three polypeptides are HlyB-specific since they are present in E. coli cells carrying N225-15 but absent in the inner membrane fractions of strains with N225-4 (Fig. 3A, lane 7), E. coli cells without a plasmid, and cells containing N32-L5 (data not shown). It should be noted that cells carrying plasmid N225-4 are the negative control for N225-15 since it contains the epitope sequences in the reverse orientation that would not be recognized by monoclonal antibody C494 (see Table I). The stained polypeptide at 54 kDa is not HlyB-specific since it is also present in inner membranes of E. coli cells containing N32, N32-L5, or N225-4 as well as E. coli cells without a plasmid. It is noteworthy that a 46-kDa component observed in previous in vitro and minicell studies (7) is not detected in this system.

The origin of the minor HlyB polypeptides of the inner membrane is unknown. Both the total membrane and inner membrane fractions show the 66-kDa HlyB protein; however, the pattern of the low molecular mass species appears to depend on the extraction procedure. The 33- and 32-kDa polypeptides of the inner membrane are more prominently observed during the Sarkosyl extraction procedure, which involves a 90-min room temperature Vortex step (compare inner membrane (Fig. 3A, lane 5) with total membrane (Fig. 3B, lane 3)). It is possible that these low molecular mass species are degradation products of larger precursors.

Three strongly immunoreactive polypeptides are detected in the soluble fraction of cells with N225-15 and are present in approximately equal concentrations (Fig. 3, A, lanes 8-10; and C, lane 2). These proteins migrate on SDS-polyacrylamide gels with apparent molecular masses of 65, 32, and 31 kDa. It is not clear at present how these proteins relate to the 66-, 33-, and 32-kDa HlyB peptides found in the inner membrane. However, initial pulse-chase experiments using [35S]methionine indicate that radiolabeled immunoprecipitable peptides of approximately these molecular masses can be chased to higher molecular mass forms in the inner membrane. Further studies will be required to elucidate the nature of this post-translational conversion. The other C494 immu

2 M. S. Poruchynsky and V. Ling, unpublished data.

![Fig. 3. Immunoblot analysis of subcellular fractions of E. coli cells carrying pLG579 (HlyB) mutants probed with C494 monoclonal antibody. Subcellular fractions were prepared as described under "Materials and Methods." Samples were run on SDS-polyacrylamides gels, blotted to the nitrocellulose membranes, and probed with 125I-labeled C494 monoclonal antibody. A, immunoblot of 100 ng of protein of the outer membrane (lanes 1-3), inner membrane (lanes 4-7), and cytoplasmic plus periplasmic fraction (lanes 8-11) of N225-4 (lanes 3, 7, and 11) and N225-15 (lanes 1, 2, 4-6, and 8-10). Cells were fractionated at different stages of exponential growth: N225-15 grown to Amn = 0.07 (lanes 4 and 8), 0.47 (lanes 1, 5, and 9), and 1.0 (lanes 2, 6, and 10) and N225-4 cells grown to Amn = 0.47 (lanes 3, 7, and 11). B, immunoblot of 150 ng of total membrane protein (lanes 1, 3, and 5) and 100 ng of cytoplasmic plus periplasmic protein (lanes 2, 4, and 6) from N225-4 (lanes 1 and 2), N225-15 (lanes 3 and 4), and N240-S8 (methionine to valine mutant) (lanes 5 and 6) cells grown to Amn = 0.5 C, immunoblot of 100 ng of inner membrane protein (lane 1) and cytoplasmic plus periplasmic protein (lane 2) of N225-15 cells grown to Amn = 0.5.
the inner membrane since the pattern of polypeptides detected by monoclonal antibody C494 is clearly different in the two fractions. More important, calculations suggest that the total amount of HlyB in the soluble fraction is equal to or greater than the amount in the inner membrane. Equal amounts of protein were analyzed from both fractions; however, the distribution of total cellular protein in E. coli cells was 90% in the cytoplasm plus periplasm, 5% in the inner membrane, and 3% in the outer membrane under the growth conditions used.3

The level of HlyB in this system in both the membrane and soluble fractions varies <2-fold from the early to late logarithmic phase of growth (Fig. 3A). No immunoreactive material was detected in the outer membrane fraction, and epitope-tagged HlyB appears to be localized only to the inner membrane and the soluble fraction of E. coli cells.

**Mutagenesis of Putative Internal Methionine Start Site—** Previous in vitro translation studies of the wild-type hemolysin B plasmid (pLG579) revealed that a 46-kDa protein is the major product (4), whereas minicells carrying this plasmid yield both 66- and 46-kDa peptides (25). Felmlee et al. (12) suggested that the smaller (46 kDa) protein may be derived from an internal translational start site (located at position 286 in Fig. 1) since this internal methionine is preceded by a potential ribosome-binding sequence (GCGG) and it has a predicted molecular mass of 46 kDa (12). As noted above, in our in vivo experiments, a 46-kDa polypeptide was not detected in the Western blots, suggesting that, if this species exists in vivo, it is present at very low levels, or, alternatively, it may be rapidly processed to a molecule of a different molecular size. To test this latter possibility, we converted the internal methionine 286 codon (ATG) to valine (GTG). An oligonucleotide (HMUT1) complementary to this region with a mismatch introduced at base pair 856 (Fig. 1) was used to construct N57-12A, which is a cognate of the original pLG579 plasmid now containing a valine instead of methionine at amino acid 286. This was further converted into N240-S8 carrying the C494 epitope (Table I).

Transfection of N57-12A or N240-S8 into E. coli cells containing pLG570::Tn5-32 resulted in hemolytic colonies on blood agar plates (Fig. 2 and Table I). In the former case, the size of the hemolytic halo was slightly reduced compared to wild type, whereas in the latter case, it was greatly reduced; however, it is only somewhat reduced from the appropriate control N225-15 (see Table I).

Western blot analysis of E. coli cells with N240-S8 reveals the same pattern of HlyB-specific bands in the total membrane and cytoplasmic plus periplasmic fractions as E. coli cells with N225-15 (Fig. 3B, compare lanes 3 and 4 with lanes 5 and 6). This result suggests that, in vivo, the potential internal start site at methionine 286 is not used. The levels of all HlyB polypeptides are 4–5-fold lower in E. coli cells carrying plasmid N240-S8 compared with those carrying N225-15, as determined by densitometry. This indicates that the smaller hemolytic zone observed in cells carrying N240-S8 is due to a lower level of hemolysin B protein.

**DISCUSSION**

In this study, the insertion of an antibody epitope into a functional HlyB protein by genetic engineering has allowed us to identify directly this low abundance protein in E. coli cells. The detection of a 66-kDa form of the HlyB protein in the inner membrane and its absence in the outer membrane provide definitive evidence in support of a recent report of this localization in E. coli cells using minicells (25). Although the soluble fractions were not tested in the above report, we detected the presence of a major proportion of HlyB in the soluble (cytoplasmic plus periplasmic) fraction of normal, exponentially growing cultures of E. coli cells. Interestingly, the major nonmembrane form of HlyB appears to be 1 kDa smaller than the major inner membrane form of this protein. Initial pulse-chase experiments indicate that the newly synthesized HlyB protein may be post-translationally modified to a higher molecular mass form. Further studies will be required to determine the nature of this modification and whether it is involved in HlyB function.

The inability to detect a 46-kDa polypeptide in our system or a modified peptide when methionine 286 is mutated strongly suggests that this potential translational start site is not used in vivo. It is possible that the 46-kDa HlyB protein observed in vitro or in minicells in previous studies is derived from this internal translational start site; alternatively, the 46-kDa peptide may be a specific degradation product. The latter possibility cannot be tested definitively in our system since a 66-kDa molecule cleaved at the C-terminal end would eliminate the inserted monoclonal antibody epitope. Such a molecule would not be detected in our system.

The alteration of the putative methionine 286 to valine, however, did result in a decrease in hemolysin secretion, as demonstrated by the smaller sized hemolytic zones. There was a concomitant 5-fold decrease in all hlyB products in both the inner membrane and the cytoplasm. Evidently, a single base change could affect the in vivo level of HlyB. In this context, the detection of the highest level of HlyB protein in cells containing plasmid N225-15 and a lower level in cells with N240-S8 and the absence of any HlyB protein in the cells with N4A and N32-L5 plasmids correlates well with the relative level of hemolysin secretion as detected by the size of hemolytic zones. This observation indicates that HlyB may be the rate-limiting protein in the hemolysin secretion system. Possibly the number of transport complexes may, in fact, be controlled by the amount of HlyB inserted into the inner membrane.

It is interesting to compare our HlyB mutants with mutants of P-glycoprotein (26). Deletion of the last seven amino acids of P-glycoprotein was shown to have no effect on its function. In HlyB, the C terminus proved to be the only region where we could successfully insert the C494 epitope, suggesting that this region is not critical for function. Since a 23-amino acid deletion at the C terminus of P-glycoprotein reduces function (26), and an insertion within the last 13 amino acids of HlyB is also nonhemolysin (P61 and P135 in Table I), it seems that only the last several amino acids of these proteins are non-essential for function. In contrast, P-glycoprotein can tolerate an insertion of four amino acids into the sequence LSGGQ, a sequence found close to the “B” site required for ATP binding (27) that is highly conserved in all members of this superfamily of transport proteins (28, 29). However, a similar insertion in our HlyB plasmid P100 (Fig. 1) and a point mutation in another E. coli HlyB that converts this sequence in LSQRG (22) result in a nonhemolytic phenotype. However, we are wary of drawing conclusions about the structure/function relationships of the various domains of HlyB from constructs that exhibit a nonhemolytic phenotype because we clearly established that, in at least two of the constructs obtained in this study (N4A and N32-L5) (Table I), the absence of protein rather than mutational inactivation is likely the cause of the negative phenotype. Possibly many of the insertion mutations destabilize HlyB and lead to its rapid degradation or inhibit expression at the translational level.

The present engineering of a monoclonal antibody epitope

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2 P. Juranka, unpublished data.
into a functional hlyB gene will facilitate the purification of this low abundance protein for biochemical studies. At the same time, it should allow us to test, for example, the hypothesis that the hemolysin transport complex is located at zones of adhesion between the inner and outer membranes. The investigation of HlyB will not only help us to understand other homologous protein transport systems, but may also help to elucidate the basic mechanism of transport in the larger superfamily of transport proteins.

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REFERENCES