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A Heterologous Membrane Protein Domain Fused to the C-Terminal ATP-Binding Domain of HlyB Can Export *Escherichia coli* Hemolysin

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The hydrophobic-rich NH_2-terminal 34 amino acids of a tetracycline resistance determinant (TetC) were fused to the COOH-terminal 240 amino acids of the hemolysin transporter, HlyB, which contains a putative ATP-binding domain. This hybrid protein replaced the NH_2-terminal 467-amino-acid portion of HlyB and could still export the *Escherichia coli* hemolysin (HlyA). Export by the hybrid protein was approximately 10% as efficient as transport by HlyB. Extracellular secretion of HlyA by the TetC-HlyB hybrid required HlyD and TolC. The extracellular and periplasmic levels of β-galactosidase and β-lactamase in strains that produced the hybrid were similar to the levels in controls. Thus, HlyA transport was specific and did not appear to be due to leakage of cytoplasmic contents alone. Antibodies raised against the COOH terminus of HlyB reacted with the hybrid protein, as well as HlyB. HlyB was associated with membrane fractions, while the hybrid protein was found mainly in soluble extracts. Cellular fractionation studies were performed to determine whether transport by the hybrid occurred simultaneously across both membranes like wild-type HlyA secretion. However, we found that HlyA was present in the periplasm of strains that expressed the TetC-HlyB hybrid. HlyA remained in the periplasm unless the *hlyD* and *tolC* gene products were present in addition to the hybrid.

The *Escherichia coli* hemolysin is the best-characterized member of a gene family (the RTX family) of cytotoxins and proteases which all share a common domain containing repeated glycine-rich amino acid sequences and a requirement for Ca^{2+} for activity (35). An important feature of this group of related proteins is their mechanism of extracellular secretion. Most of the basic characteristics of secretion have been determined for the *E. coli* hemolysin. This protein is exported by a mechanism that does not require an amino-terminal signal sequence and is independent of the *sec* transport system (6). The recognition signal for transport of the toxin resides in the COOH-terminal 60 amino acids (8, 11, 20). Hemolysin is rapidly secreted directly into the environment without a periplasmic intermediate (8). This process is unusual in that most other extracellular proteins secreted by gram-negative bacteria enter the periplasm first and then cross the outer membrane in a second step (14, 16). The transport process requires a minimum of three gene products. The *hlyB* and *hlyD* genes are absolutely required for export of the toxin and encode 79.9- and 55-kDa proteins, respectively. These genes are linked to the structural hemolysin gene (*hlyA*) and to a third gene (*hlyC*), which encodes a protein involved in activation of the toxin (Fig. 1) (7). It has been demonstrated that at least one other unlinked gene product, TolC (52 kDa), is also required for export of the hemolysin (32). Similar aspects of the secretion process have been confirmed for other RTX family members (3, 4, 19).

Although the basic characteristics of the transport system have been identified, detailed biochemical studies have been limited because of the low levels of expression of transport components and the lack of immunologic reagents. However, there have been studies that address the cellular location of the transport proteins. Several lines of evidence have demonstrated that the three proteins required for the transport of hemolysin are associated with the membranes of *E. coli*. Hydropathy analyses of both HlyB and HlyD have revealed hydrophobic regions that could potentially provide membrane-spanning domains (7). Genetic analysis in which gene fusions to β-lactamase, β-galactosidase, and alkaline phosphatase were used have identified potential periplasmic and cytoplasmic domains of HlyB. When the fusion data are combined with the hydropathy profiles, a picture of HlyB topology with at least six transmembrane domains in the NH_2-terminal portion of the protein emerges (10, 33). Workers have performed cellular fractionation studies to localize HlyB and HlyD to the inner membrane of *E. coli* minicells (33). TolC was first identified by mutations that mediate tolerance to colicin E1 and other pleiotropic outer membrane effects. The cloned *tolC* gene was used to demonstrate an outer membrane location for the protein (25). An analogous RTX transport system has been investigated for secretion of the *Erwinia chrysanthemi* metalloprotease. Antibodies have been raised to the *Erwinia chrysanthemi* secretion system proteins that are similar to HlyB, HlyD, and TolC (PrtD, PrtE, and PrtF, respectively). These antibodies have been used to demonstrate that both PrtD and PrtE are located in the inner membrane and PrtF is associated mainly with the outer membrane when it is expressed in *E. coli*. Furthermore, assays of protease susceptibility on the various membrane surfaces have shown that PrtD has a major hydrophilic domain in the cytoplasm, while the COOH-terminal hydrophilic domain of PrtE is exposed to the periplasm (5). From these data it seems clear that HlyB and HlyD are located in the cytoplasmic membrane. Furthermore, the NH_2-terminal portion of HlyB appears to span the membrane at least six times, with its COOH terminus located in the cytoplasm. It has been proposed that HlyD has its NH_2 terminus anchored in the cytoplasmic membrane and its COOH terminus located in the periplasm.

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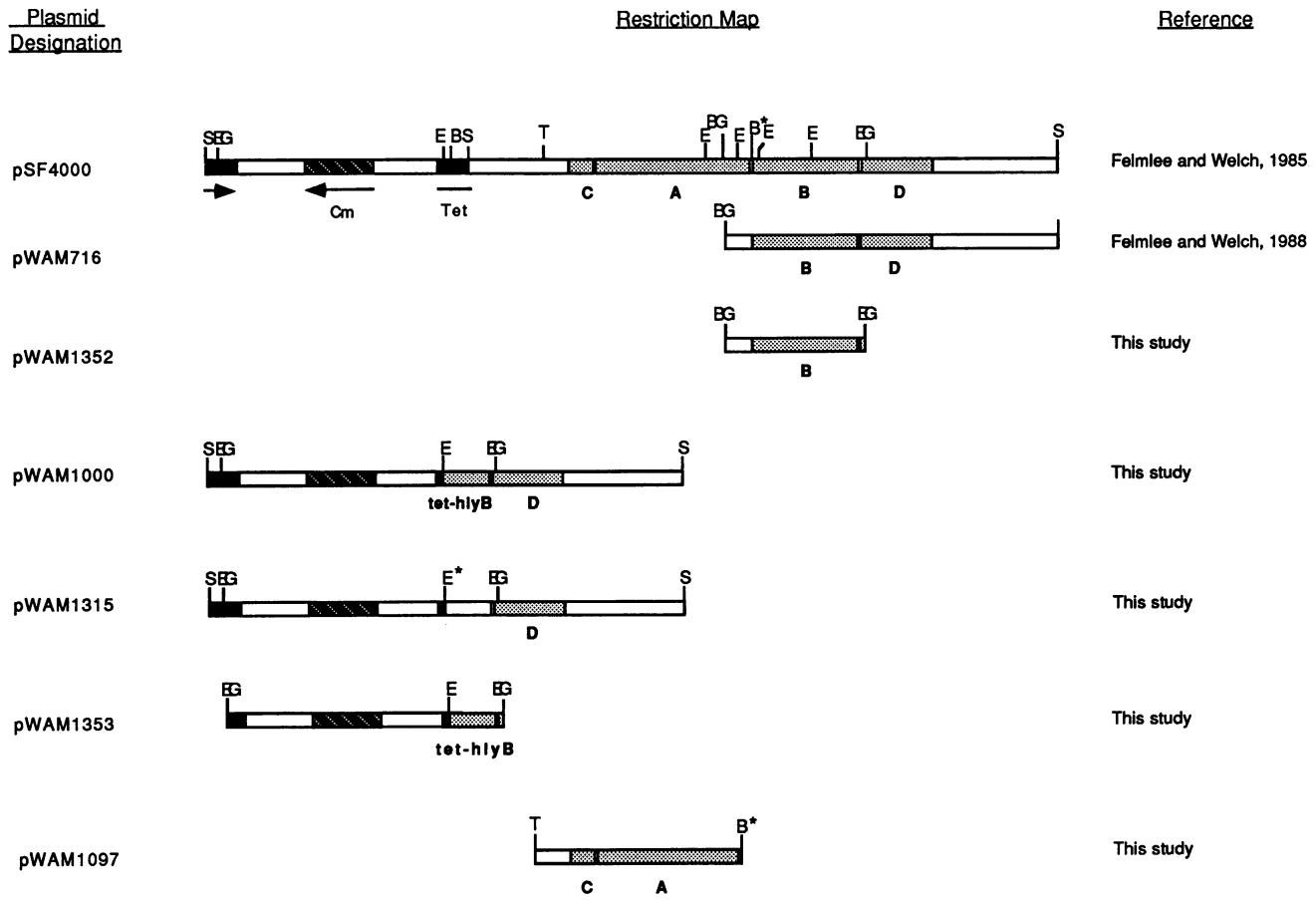

FIG. 1. Plasmid construction. Plasmids containing hemolysin transport genes were derived from the hemolysin operon carried on pSF4000. The genes of the hemolysin operon are indicated below the lightly shaded areas. The antibiotic resistance determinants of pACYC184 are shown as darker shaded areas. The relevant restriction endonuclease sites used are shown above the linear map. Transport genes were expressed from pACYC184-based plasmids. The *hlyC* and *hlyA* structural genes were provided on pWAM1097, a compatible plasmid based on pUC19 (see Materials and Methods). pWAM716 encodes the wild-type transport genes. Vector sequences were included in maps for plasmids containing hybrids for clarity. Plasmid pWAM1000 was created by digesting pSF4000 with *Eco*RV, followed by religation. This ligation generated a hybrid between *tetC* of pACYC184 and *hlyB* (TetC-HlyB). Plasmid pWAM1315 was constructed by inserting an *Xba*I linker containing stop codons in all three reading frames into the *Eco*RV site of pWAM1000. This plasmid was made for use as a negative control since translation of the COOH terminus of *hlyB* was prevented. The *hlyD* gene was removed from pWAM1000 and pWAM716 by digestion with *Eag*I, followed by religation to create pWAM1352 and pWAM1353, which encode HlyB and the hybrid protein alone, respectively. Abbreviations: B, *Bam*HI; B*, *Bam*HI site created by site-directed mutagenesis; BG, *Bgl*II; E*, *Xba*I linker into *Eco*RV; E, *Eco*RV; EG, *Eag*I; S, *Sal*I; T, *Taq*I; Cm, chloramphenicol; Tet, tetracycline.

While the location of the transporters has been studied preliminarily, the function of these proteins is less apparent. Insight into the function of HlyB has come from its amino acid sequence, which exhibits significant homology to a wide variety of procaryotic and eucaryotic membrane transport proteins. These proteins are involved in the import and export of a variety of substrates, including polypeptides (HlyA, LktA, STE6), carbohydrates (ChvA, NdvA), small metabolites and ions (MalK, PstB, CFTR), and therapeutic agents (MDR, CQR) (13). All of these proteins contain membrane-spanning domains and one or more cytoplasmic nucleotide-binding domains, which are thought to hydrolyze ATP, providing the energy for translocation. Therefore, nucleotide binding and hydrolysis may be functions of HlyB that are necessary to energize hemolysin export. The highest level of homology between transporters normally lies within the nucleotide-binding domains, whereas the amino-terminal domains are more divergent. This has led to speculation that the specificity for the substrate transported resides within the amino-terminal domains (2). Considering the similarities of this group of proteins, investigation of the structure-function relationships of HlyB should provide insights into the other transporters.

In this paper we describe the generation of a chimeric protein that could still transport hemolysin. In this hybrid protein a small membrane-spanning domain of TetC was exchanged for the NH_2-terminal hydrophobic domains of HlyB. The resulting transport protein exported hemolysin through a periplasmic intermediate, providing insight into the mechanism of simultaneous export through both inner and outer membranes.

MATERIALS AND METHODS

Strains and culture techniques. The strains and plasmids which were used in this study are shown in Table 1. *E. coli*

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant phenotype	Vector	Reference
E. coli strains			
C600	F⁻ *thr-1 leuB6 thi-1 lacY1 supE44 rfbD1 fhuA21 mcrA1*		1
CJ296	F1 *dut-1 ung-1 thi-1 relA1*		18
WAM1383	C600 with *tolC*::Tn*10-48*		This study
P2495	P1700 *tolC210*::Tn*10-48*		25
Plasmids			
pSF4000	Cm^r HlyCABD$^+$	pACYC184	7
pWAM04	Ap^r HlyCABD$^+$	pUC19	36
pWAM716	Cm^r HlyBD$^+$	pACYC184	9
pWAM721	Ap^r HlyBD$^+$	pGEM2	31
pWAM826bis	HlyA$^+$	M13mp18	9
pWAM1000	Cm^r TetC-HlyBD$^+$	pACYC184	This study
pWAM1097	Ap^r HlyCA$^+$	pUC19	This study
pWAM1315	Cm^r TetC-HlyB$^-$ HlyD$^+$	pACYC184	This study
pWAM1350	Ap^r GST-HlyB[a]	pGEX-3X	This study
pWAM1352	Cm^r HlyB$^+$	pACYC184	This study
pWAM1353	Cm^r TetC-HlyB$^+$	pACYC184	This study

[a] GST, glutathione S-transferase.

was grown in Luria broth for liquid cultures or on solid medium containing Luria broth and 1.5% agar. Hemolytic phenotypes were determined on solid medium by using blood agar plates containing blood agar base (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% defibrinated whole sheep blood and 10 mM $CaCl_2$. All of the strains were cultured at 37°C. When appropriate, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; and tetracycline, 20 μg/ml. Chemicals and antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo.

Determination of hemolytic activity. Supernatants and subcellular fractions were assayed for hemolytic activity essentially as described previously (8). We prepared 2% suspensions of sheep erythrocytes in 0.85% NaCl–10 mM $CaCl_2$. Samples were added to the sheep erythrocyte suspensions, and the resulting preparations were incubated at 37°C for 15 min. Intact erythrocytes were removed by centrifugation. Erythrocyte lysis was assayed by measuring the release of hemoglobin, as determined by A_{540}.

Subcellular fractionation. Strains were grown in Luria broth to late logarithmic phase prior to separation into extracellular, periplasmic, and cytoplasmic fractions. Extracellular fractions were obtained by pelleting whole cells by centrifugation at 13,000 × *g* for 30 s and then filtering the supernatant through Acrodisc filters (pore size, 0.45 μm; Gelman Sciences, Inc., Ann Arbor, Mich.). Periplasmic and spheroplast fractions were generated by using cold osmotic shock and lysozyme treatment, respectively (22). The cell pellet was washed twice in a solution containing 10 mM Tris-HCl and 0.3 M NaCl (pH 8.0) and then was resuspended in 400 μl of a solution containing 20% sucrose, 100 mM Tris-HCl, and 0.5 mM EDTA (pH 8.0). After incubation at ambient temperature for 20 min, the cells were pelleted and shocked by resuspending them in 0.5 mM $MgCl_2$ for 10 min on ice to release the periplasmic contents. Spheroplasts were pelleted and resuspended in 50 mM Tris-acetate–2.5 mM EDTA (pH 8.2) containing 40 μg of lysozyme, and the resulting preparations were incubated for 5 min on ice to release the cytoplasmic contents. The presence of cytoplasmic contents was determined by monitoring the hydrolysis of *o*-nitro-*p*-galactopyranoside by β-galactosidase as an increase in A_{420} (24). The presence of periplasmic enzymes was demonstrated by the hydrolysis of a chromogenic substrate of β-lactamase, PADAC (Calbiochem). Fractions were added to a 25 μM solution of PADAC in 20 mM Tris-HCl (pH 8.0) at 25°C, and hydrolysis was determined by a decrease in A_{572} after 1 min (16). Activities were determined as the percentages of the total β-galactosidase or β-lactamase activity present in each fraction. The fractionations were considered successful if 95% of the total β-galactosidase activity was present in the spheroplast fractions and 85% of the total β-lactamase activity was present in fractions enriched for periplasmic contents.

Genetic and recombinant DNA procedures. The Tn*10* insertion mutation in *tolC* (*E. coli* P2495) (25) was introduced into *E. coli* C600 by transduction, using P1vir and standard protocols (24). Recombinant DNA procedures were performed as described by Maniatis et al. or by using the manufacturer's instructions (21). Restriction endonucleases and DNA-modifying enzymes were obtained from New England Biolabs. T7 DNA polymerase was obtained from Pharmacia. The cloning strategies used to generate recombinant plasmids are shown in Fig. 1. A *Bam*HI site was created at position 4790 (7) of the hemolysin operon in order to generate a plasmid containing the *hlyCA* genes and no *hlyB* sequences. A 20-base oligonucleotide (TTATGACAA GGATCCATTAT) was synthesized and used to introduce the mutation into recombinant M13 phages (pWAM826bis) (9) by the method of Kunkel et al. (18). The mutation was confirmed by restriction digestion followed by DNA sequence analysis. The oligonucleotides used for site-directed mutagenesis and DNA sequence analysis were synthesized by using an Applied Biosystems model 391A DNA synthesizer. DNA sequence analysis was performed by the dideoxy method, using a Sequenase enzyme kit (U.S. Biochemical, Cleveland, Ohio) (27).

Immunologic techniques. Antiserum to the COOH terminus of HlyB was generated by using a gene fusion method. The COOH-terminal portion of HlyB was fused to glutathione S-transferase by cloning the *Eco*RV-*Eco*RI fragment of the *hlyB* gene from pWAM721 into the *Sma*I-*Eco*RI sites of pGEX-3X to create pWAM1350 (28). This fragment encodes the COOH-terminal domain of HlyB. The hybrid protein was affinity purified from whole-cell sonicates carrying pWAM 1350 with glutathione-agarose beads (Sigma). Female New Zealand rabbits were immunized with 500-μg portions of hybrid protein supplemented with complete Freund's adjuvant and were boosted twice with 500-μg portions of hybrid protein supplemented with incomplete Freund's adjuvant on days 14 and 28. Antibodies were purified further by precipitation in 45% ammoniun sulfate, followed by affinity purification in which the glutathione S-transferase–HlyB hybrid was used as a ligand. The affinity column was made by cross-linking the hybrid protein to cyanogen bromide-activated Sepharose (Sigma). Antibodies were used in immunoblotting experiments to detect the presence of HlyB or TetC-HlyB hybrids in total membrane preparations or soluble extracts (9).

Isolation of membrane proteins. Membrane proteins were generated essentially as described by Delepelaire and Wandersman (5). Bacteria containing various plasmids were grown to an optical density at 600 nm of 1.0 in Luria broth. The cell pellet was washed once in a solution containing 100 mM Tris (pH 8.0), 1 mM EDTA, and 100 μg of phenylmethylsulfonyl fluoride per ml. The cells were concentrated 10-fold in a solution containing 100 mM Tris (pH 8.0), 1 mM

TABLE 2. Hemolytic phenotypes of transport mutants on blood agar

Plasmid[a]	Host strain	Transport proteins expressed	Zone of hemolysis[b]
pWAM716	C600	HlyB, HlyD, TolC	+++
pWAM716	WAM1383	HlyB, HlyD	−
pWAM1315	C600	HlyD, TolC	−
pWAM1352	C600	HlyB, TolC	−
pWAM1000	C600	TetC-HlyB, HlyD, TolC	++
pWAM1000	WAM1383	TetC-HlyB, HlyD	−
pWAM1353	C600	TetC-HlyB, TolC	−

[a] All strains contained pWAM1097 to provide *hlyC* and *hlyA* in *trans* on a plasmid that was compatible with the plasmids producing transport proteins.
[b] The number of plus signs indicates the relative diameter of beta-hemolytic zones surrounding a bacterial colony observed on a sheep red blood agar plate. A minus sign indicates that there is no beta-hemolysis extending beyond the colony margin.

EDTA, and 100 μg of phenylmethylsulfonyl fluoride per ml and were sonicated at 50 W for 10 s in a Branson Sonifier. Whole cells were removed by centrifugation at 1,000 × *g* for 15 min. Sonicates were then centrifuged for 30 min at 13,000 × *g* to pellet the insoluble membrane fraction, while the supernatant was considered the soluble fraction. The insoluble pellet containing the membrane fraction was solubilized with gentle rocking at 37°C by using 1.0% Tween 20 and 1.0% Triton X-100. The soluble proteins in the supernatant that remained after the membranes were pelleted were concentrated by precipitation with trichloroacetic acid prior to electrophoresis. Sample buffer was added, and the proteins were separated by 10% polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes for immunoblotting, as described previously (9).

RESULTS

Construction of plasmids containing HlyB hybrids. In order to facilitate studies of HlyA secretion, the large *Eco*RV fragment of pSF4000 was isolated from agarose gels and religated to generate a pACYC184 plasmid containing only the *hlyD* gene (pWAM1000) (Fig. 1). Surprisingly, colonies harboring this plasmid and a second compatible plasmid (pWAM1097) encoding the *hlyC* and *hlyA* genes were hemolytic on blood agar plates (Table 2). The pWAM1097 *hlyC-hlyA* subclone lacked any *hlyB* sequence because of a deletion endpoint made at a new *Bam*HI site within the first HlyB codon. We felt that construction of this precise deletion was important since we had speculated previously that truncated forms of HlyB may be produced and may be involved in the HlyA secretion process (7). pWAM1097 was used throughout the experiments described below to provide *hlyC* and *hlyA* in *trans* to plasmids containing the transport genes under investigation.

The previously published sequences of *tetC* and *hlyB* were examined more closely, and we found that a hybrid between the two proteins had been created fortuitously. The fusion of the *tetC* and *hlyB* reading frames at the *Eco*RV restriction site was confirmed by a DNA sequence analysis of the hybrid junction of pWAM1000 (data not shown). A 34-amino-acid portion of the NH_2 terminus of TetC had been fused to the COOH-terminal 240 amino acids of HlyB. This stretch of the TetC protein provides a potential membrane-spanning, hydrophobic domain fused to the putative nucleotide-binding domain of HlyB. This hydrophobic portion of

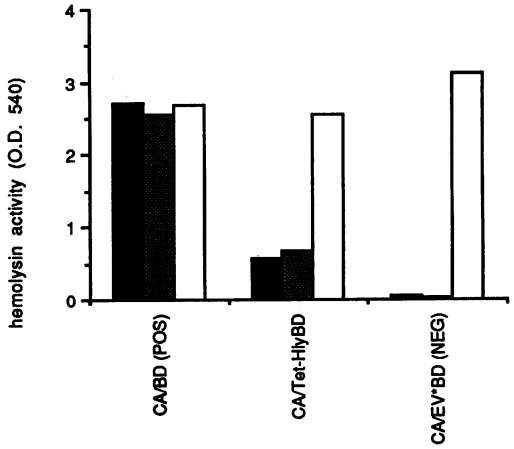

FIG. 2. Hemolysin secretion by the TetC-HlyB hybrid protein. Hemolytic activity was determined for cell-free supernatants (solid bars), whole cultures (shaded bars), and whole-cell sonicates (open bars) by using strains harboring pWAM1097 (HlyCA) and pWAM 716 (HlyBD), pWAM1000 (TetC-HlyBD), or pWAM1315 (HlyD alone). Hemolytic activity was measured by determining optical density at 540 nm ($O.D._{540}$) (see Materials and Methods).

TetC apparently was able to replace a partial function of the NH_2-terminal membrane-spanning domains of HlyB.

To confirm that transport by the hybrid required the TetC domain for functioning and was not the result of a translational restart within the COOH terminus of HlyB, an *Xba*I linker containing stop codons in all three reading frames was introduced into the *Eco*RV site of pWAM1000. No zone of hemolysis was observed when this plasmid (pWAM1315) was placed in *trans* to pWAM1097 (Table 2).

Extracellular transport of *E. coli* hemolysin requires the products of the *tolC* and *hlyD* genes in addition to HlyB. Specific transport of *E. coli* hemolysin by the hybrid protein would necessitate a requirement for these gene products for transport. We constructed isogenic strains which were defective in either HlyD or TolC expression in the presence of the hybrid protein to test this requirement. Plasmid pWAM1353 was constructed to produce the hybrid without HlyD expression (Fig. 1). The *tolC*::Tn*10* allele from *E. coli* P2495 (25) was introduced into *E. coli* C600 by P1vir transduction to create *E. coli* WAM1383. Colonies from bacteria containing pWAM1097 and pWAM1353 in *E. coli* C600 ($HlyC^+$ $HlyA^+$ TetC-$HlyB^+$ $TolC^+$ $HlyD^-$) or pWAM1097 and pWAM1000 in *E. coli* WAM1383 ($HlyC^+$ $HlyA^+$ TetC-$HlyB^+$ $TolC^-$ $HlyD^+$) were nonhemolytic on blood agar plates (Table 2). Extracellular transport by the TetC-HlyB hybrid protein appeared to be specific to hemolysin since HlyD and TolC were still required. Furthermore, the fact that the other transport proteins were required for export demonstrated that the hybrid may have interacted with the export machinery in some fashion.

Hemolytic activity of culture supernatants. Liquid assays for hemolytic activity were performed with culture supernatants to get a more accurate assessment of the secretion of HlyA by the hybrid protein. The TetC-HlyB hybrid protein was able to export HlyA into culture supernatants (Fig. 2). Secretion of HlyA by the hybrid was only approximately 10% as efficient as export by HlyB, but was significantly greater than the background release by the negative controls.

One characteristic of hemolysin secretion is the fact that

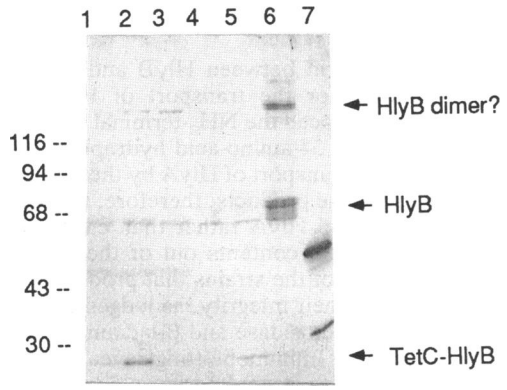

FIG. 3. Immunologic detection of transport proteins. Membrane and soluble proteins were isolated (see Materials and Methods), separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with affinity-purified rabbit antiserum raised against the COOH-terminal one-third of HlyB. The protein obtained from approximately 5×10^8 *E. coli* cells was loaded into each lane. Soluble proteins were prepared from strains containing plasmids pWAM1315 (HlyD on pACYC184) (lane 1), pWAM 1000 (TetC-HlyBD on pACYC184) (lane 2), and pWAM04 (Hly CABD on pUC19) (lane 3); lanes 4 through 6 contained membrane proteins from these three strains, respectively. Lane 7 contained the glutathione S-transferase–HlyB hybrid used to immunize rabbits. The positions of the molecular weight standards (in kilodaltons) are shown on the left. The positions of the HlyB and Tet-HlyB hybrid proteins are indicated by arrows.

the hemolytic activity in culture supernatants varies depending on the stage of growth of the culture at the time that the assay is performed. The maximum levels of HlyA in culture supernatants are achieved in the log phase and decrease rapidly as a culture advances to the stationary phase (26). Therefore, TetC-HlyB transport of HlyA was assayed relative to culture growth (cell density, optical density at 600 nm) and was compared with HlyB-mediated export. The peak extracellular HlyA levels were observed in late-log-phase cells producing the hybrid protein. Supernatant hemolytic activity also decreased as the culture reached the stationary phase. Immunoblotting confirmed that extracellular levels of HlyA protein followed a pattern similar to that observed with hemolytic activity (data not shown).

Immunologic detection of TetC-HlyB hybrid proteins. Immunologic techniques were used to demonstrate that the hybrid protein was produced and to determine its cellular location. HlyB and other related RTX B-like transporters have been shown to be associated with the membrane fraction, specifically the inner membrane. Since TetC-HlyB hybrid proteins mediate the transport of HlyA, we thought that they also might be associated with membrane fractions. We performed immunoblotting experiments with membrane and soluble preparations that were isolated from strains that produced HlyB or the TetC-HlyB hybrid by using polyclonal antibodies raised against the COOH-terminal domain of HlyB. We raised antibodies to this portion of HlyB in rabbits by using a glutathione S-transferase expression system to produce reagent quantities of the domain for immunization. Strains containing pWAM1000 produced a immunoreactive protein band at approximately 27 kDa that was associated mainly with the soluble fraction (Fig. 3, lane 2). This protein migrated to a position consistent with the position predicted for the hybrid from the previously published DNA sequence information. The results of these experiments showed that the TetC-HlyB hybrid protein is detectable mainly in the soluble fraction rather than in the membrane fractions, as might be predicted. The hydrophobic domain provided by TetC may be inefficiently inserted into the membrane of *E. coli* or may provide only a weak peripheral association with the membrane.

TABLE 3. β-Galactosidase and β-lactamase activities in subcellular fractions

Enzyme	Plasmid[a]	% of total enzyme activity in[b]:		
		Supernatant	Periplasm	Spheroplasts
β-Galactosidase	pWAM716 (HlyB, HlyD)	<1	6	94
	pWAM1315 (HlyD)	<1	4	95
	pWAM1000 (TetC-HlyB, HlyD)	<1	6	94
β-Lactamase	pWAM716 (HlyB, HlyD)	11	88	2
	pWAM1315 (HlyD)	8	87	5
	pWAM1000 (TetC-HlyB, HlyD)	10	85	5

[a] All strains contained pWAM1097 to provide *hlyC* and *hlyA* in *trans* on a plasmid compatible with the plasmids producing transport proteins, as well as the *tolC* gene on the chromosome. The transport proteins expressed from the plasmids are indicated in parentheses.
[b] The values are averages from three independent experiments.

It was necessary to isolate membrane fractions containing HlyB from a strain carrying the entire hemolysin operon in a high-copy-number recombinant plasmid, pWAM04 (the vector was pUC19), in order to detect expression of HlyB. The observation that antibodies did not react with membrane preparations produced from strains harboring lower-copy-number plasmids encoding HlyB suggested that the expression of HlyB in *E. coli* must have been low. Strains that produced intact HlyB produced immunoreactive proteins that migrated at approximately 70 and 68 kDa. The lower-molecular-weight protein (68 kDa) became apparent after storage of the membrane preparations and therefore was believed to be a breakdown product of the 70-kDa band. Another higher-molecular-weight band (approximately 150 kDa) also reacted with the anti-HlyB serum, even in the presence of β-mercaptoethanol and sodium dodecyl sulfate (Fig. 3, lane 6). This higher-molecular-weight species did not react with antiserum raised to a β-galactosidase–HlyD hybrid protein (data not shown). Interestingly, the smaller 46- and 25-kDa proteins that previously have been reported to be expressed from the *hlyB* gene in minicells and in in vitro translation assays, as well as the 28- to 33-kDa peptides identified by COOH-terminal epitope tagging, did not react with the serum (7, 17, 33).

Subcellular location of hemolytic activity. Strains were fractionated into extracellular, periplasmic, and cytoplasmic fractions and assayed for hemolytic, β-galactosidase, and β-lactamase activities to determine the subcellular location of HlyA. Activities other than hemolytic activity were determined in order to confirm normal compartmentalization of cellular components, as well as to monitor fractionation efficiency in strains that produced the hybrid protein. The apparent secretion of HlyA by TetC-HlyB could have been the result of extracellular leakage of cytoplasmic contents. However, the integrity of the cellular envelopes of the strains harboring the hybrid was apparently maintained since β-galactosidase and β-lactamase activities were present in

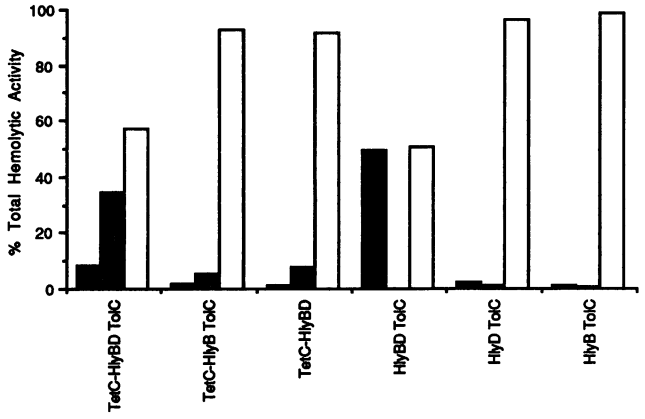

FIG. 4. Subcellular location of hemolytic activity. Hemolytic activities were determined for extracellular fractions (solid bars), periplasmic fractions (shaded bars), and spheroplast fractions (open bars). Fractions were prepared from strains containing pWAM1097 (*hlyCA*) and the various combinations of transport proteins as shown at the bottom. Fractions were prepared and hemolytic activities were determined as described in the text. Results are represented as the percentages of the total hemolytic activity present in each fraction.

supernatant and periplasmic fractions at the same levels as in control strains (Table 3). When TolC, HlyB, and HlyD were all present to transport HlyA, hemolytic activity was detected in the cytoplasmic and extracellular fractions but not in the periplasm (Fig. 4). This is consistent with previous findings that HlyA antigen or hemolytic activity is not present in the periplasm (8). However, when the hemolysin was transported with the TetC-HlyB hybrid in the presence of HlyD and TolC, hemolytic activity was detected in the periplasm, as well as in the extracellular and cytoplasmic fractions. This observation suggests that, in part, transport by the hybrid differs mechanistically from HlyB-mediated transport.

Plasmids with deletions in HlyB, HlyD, or the TetC-HlyB hybrid were placed in *trans* to pWAM1097 (HlyCA), and the resulting recombinant cells were separated into subcellular fractions to test for hemolytic activity. pWAM1315 produced HlyD but prevented any HlyB or TetC-HlyB expression because of the *Xba*I linker inserted into the *Eco*RV site. The HlyD gene was removed from pWAM716 by digestion with restriction endonuclease *Eag*I, followed by religation to generate pWAM1352, which produced HlyB alone. These plasmids were not able to mediate transport of HlyA extracellularly or into the periplasm when they were introduced in *trans* into pWAM1097 (HlyCA). Mutations in TolC also prevent extracellular or periplasmic transport of HlyA (32). Thus, if any of the wild-type transport genes were missing, the hemolysin remained cytoplasmic (Fig. 4). We also performed fractionation experiments with strains that produced the TetC-HlyB hybrid without HlyD or without TolC. Interestingly, periplasmic hemolytic activity was detected in strains that produced the hybrid even in the absence of HlyD or TolC (Fig. 4). Extracellular hemolysin activity could not be demonstrated in either case. However, it should be noted that the periplasmic levels of hemolytic activity were significantly lower in the absence of TolC or HlyD, suggesting that these proteins may also affect crossing of the inner membrane.

DISCUSSION

We created a hybrid between HlyB and TetC that was partially functional for the transport of HlyA. The new transport protein replaced the NH_2-terminal 467 amino acids of HlyB with a short 34-amino-acid hydrophobic stretch of TetC. Extracellular transport of HlyA by the hybrid required the *hlyD* and *tolC* gene products; therefore, transport by the hybrid was specific to HlyA rather than caused by simple leakage of cytoplasmic contents out of the cells. Furthermore, the envelopes of the strains that produced the hybrid apparently retained their integrity, as judged by appropriate localization of β-galactosidase and β-lactamase activities in subcellular fractions. Immunoblotting revealed that the hybrid protein was not as strongly associated with membrane fractions as HlyB. Extracellular secretion of HlyA by the hybrid, in contrast to the wild-type secretion event, led to a HlyA periplasmic intermediate. Finally, the hybrid protein was able to secrete HlyA into the periplasm, regardless of the presence of the other proteins involved in HlyA transport.

The hybrid protein consists of at least two functional domains, a short 34-amino-acid hydrophobic stretch of residues and a putative nucleotide-binding domain. The structure of the hybrid in the membrane can be predicted by considering several properties of TetC and HlyB. This hydrophobic stretch of TetC is not removed by signal peptidase (22). Apparently, this hydrophobic stretch allows the nucleotide-binding region of HlyB to become associated with the inner membrane. It is thought that the COOH-terminal portion of HlyB is on the cytoplasmic side of the inner membrane. Protease accessibility experiments performed with the *Erwinia chrysanthemi* protease secretion system showed that the HlyB homolog PrtD is an integral inner membrane protein that contains a cytoplasmic domain (5). The results of gene fusions with HlyB and a hydropathy analysis predicted that this cytoplasmic region is COOH terminal (10, 33). Therefore, the orientation of the hydrophobic region must allow the putative nucleotide-binding domain to be located normally, since the TetC-HlyB hybrid does transport HlyA.

The precise way that the hybrid associates with the membrane is not clear. The NH_2-terminal hydrophobic domain may allow peripheral association of the hybrid with the inner membrane or another membrane protein. Our immunoblotting data suggest that the association of the hybrid with the membrane is weak. An alternative hypothesis is that the TetC-HlyB protein is an integral membrane protein that is inefficiently inserted into the membrane, and thus a cytoplasmic pool accumulates. The 34-amino-acid region (MK*SNNALIVILGTVTLD*AVGIGLVMPVLPGL LR*D) contains two hydrophobic stretches (boldface type) and charged residues (asterisks) that could lead to a structure that traverses the membrane twice (30). Small peptides (more than 97 amino acids long) of TetC alone are capable of increasing gentamicin sensitivity and can complement phosphate transport mutations (12). This suggests that these small hydrophobic TetC peptides are capable of some membrane-associated transport. In the case of the TetC-HlyB hybrid, the 34-amino-acid TetC domain probably provides only a membrane-anchoring function; a more complex role is unlikely considering the short length of this domain and the lack of sequence homology to any portion of HlyB.

The immunoblots of membrane fractions from strains producing HlyB (Fig. 3, lane 6) revealed several interesting features. First, antibodies directed to the COOH-terminal

portion of HlyB do not react with the putative lower-molecular-weight HlyB species observed in minicells or in in vitro translation assays (46 and 25 kDa) (33) and in immunoblots when epitope tagging is used (28 to 33 kDa) (17). In the case of minicell analysis and in vitro translation, this observation could be due to the fact that our antibodies were raised only to the HlyB COOH-terminal antigen or to the fact that these smaller peptides were artifacts of minicell analysis unrelated to HlyB. Differences in the isolation procedures involved in preparing HlyB extracts could explain the detection of the 28- to 33-kDa bands by epitope tagging (17) and the absence of these bands in our study. Second, the finding that HlyB appeared as a pair of polypeptides migrating at 70 and 68 kDa was probably due to the breakdown of the more slowly migrating band in vitro because the lower-molecular-weight band appeared only after freezing and storage of the membrane preparations (29). This may also explain the pair of bands observed by Juranka et al., who used epitope tagging to detect HlyB (17). The large (<140-kDa) high-molecular-weight species which we observed in immunoblots were possible HlyB dimers. These species disappeared in sample buffer-membrane preparations that were heated prior to loading at 65 rather than 37°C (Fig. 3). This phenomenon has been observed in the electrophoresis of other membrane proteins (34). The fact that these antibodies react specifically with HlyB should prove to be valuable in the physical and biochemical dissection of the hemolysin transport system.

We concluded that the NH_2-terminal domain of HlyB may not be as crucial in hemolysin transport as previously believed since the NH_2-terminal 467 amino acids of HlyB can be removed while the protein retains partial function. HlyB is a member of a large family of membrane translocator proteins that have striking amino acid sequence similarities, examples of which can be found in bacteria as well as eucaryotes. The fact that these proteins share greater sequence homology in the nucleotide-binding domains than in other areas has led to speculation that the more divergent protein regions are involved in specificity for the molecule to be translocated. Similarly, it has been proposed that the NH_2-terminal domain of HlyB is involved in recognition of HlyA for transport (2). The observation that this region is missing in a functional hybrid protein that specifically transports hemolysin suggests the hypothesis that at least part of the transport machinery involved in recognition of HlyA is located outside this region. Domains involved in the recognition of the transport signal of HlyA may be found in HlyD or in the COOH-terminal portion of HlyB since both have cytoplasmic regions. Transport of hemolysin into the periplasm by the hybrid in the absence of HlyD or without TolC implies that HlyA is at least partially recognized for transport by the COOH-terminal portion of HlyB. If HlyD and TolC do export periplasmic HlyA across the outer membrane, these proteins may also be involved in HlyA signal recognition. The amino acid sequence divergence outside the nucleotide-binding domains of these homologous transporters may reflect the facts that these regions are not critical for transport and that selection pressures for sequence conservation are not nearly as great as selection pressures for the cytoplasmic domain.

The role of the NH_2-terminal portion of HlyB may be to interact closely with one or both of the members of the transport machinery (HlyD and TolC). When this domain is removed, as in the case of the hybrid, hemolysin transport is inhibited such that HlyA activity may be detected in the periplasm. The presence of hemolytic activity in the periplasmic space could be explained in several ways. First, simultaneous transport across both membranes could occur, but the removal of the NH_2-terminal domain of HlyB prevents normal interaction with TolC or HlyD and thus hemolysin "leaks" into the periplasm. The periplasmic hemolytic activity observed in strains that produce the TetC-HlyB hybrid may not be available to the export pathway. Alternatively, the hemolysin transport process could be a two-step transport mechanism, with the hybrid transporting HlyA across the inner membrane while HlyD and TolC are involved in transporting HlyA through the periplasm and across the outer membrane. This model predicts that the two steps normally occur so quickly that they appear to be simultaneous and that the hybrid slows the process to the point where a periplasmic pool of hemolysin is detectable. If such a two-step process in transport does occur, the apparently simultaneous traversal of both the inner and outer membranes may be independent of other membrane structures, such as Bayer's junctions or zones of adhesion, as previously hypothesized (8). Future pulse-chase transport kinetic experiments will be required to resolve these questions.

The interaction of the NH_2 terminus of HlyB with HlyD and TolC is illustrated by the difference in transport of HlyA by the hybrid and by HlyB when HlyD or TolC is not present. One might predict that HlyB would be able to transport HlyA across the inner membrane in the absence of HlyD or TolC, because the hybrid alone is capable of transporting some HlyA activity into the periplasm. However, in a wild-type HlyB background, null mutations in either *hlyD* or *tolC* prevent extracellular secretion of HlyA and even detection of HlyA activity in the periplasm. If HlyB is responsible for HlyA crossing the inner membrane, apparently HlyB is in a "closed" or inactive conformation when HlyD or TolC is missing, and these proteins are not able to interact with the HlyB NH_2-terminal domain. If the NH_2-terminal domain of HlyB is present, it must interact with HlyD or TolC to be active for transport. When that domain is absent, we found that amino-truncated forms of HlyB alone may still facilitate HlyA movement across the inner membrane.

Several refinements of the model proposed by Holland and his colleagues (15) are suggested by our results (Fig. 5). The cellular locations of the three components of the transport system are as previously reported (5, 33). Secretion of HlyA despite the removal of the hydrophobic and periplasmic regions in the HlyB NH_2-terminal 467 amino acids may have implications for the function of these domains in hemolysin transport. First, the transport apparatus recognizes the COOH terminus of HlyA, at least in part, by means of direct or indirect interaction with the cytoplasmic HlyB or HlyD domains rather than exclusively the HlyB NH_2 terminus. Second, this region of HlyB is not absolutely required for crossing of the inner membrane, periplasm, or outer membrane during HlyA secretion. This domain of HlyB seems to be required for optimal extracellular secretion of HlyA rather than being a critical component of the transport machinery itself. Apparently, it is the nucleotide-binding domain of HlyB that is most critical for HlyA export and the NH_2 terminus may simply optimize the cellular location of this protein.

The periplasmic domains of HlyD and TolC may function as a channel that traverses the periplasmic space. If HlyA extracellular secretion by the TetC-HlyB hybrid depends on interactions of the hybrid with HlyD, the interactions between HlyB and HlyD may not occur exclusively through

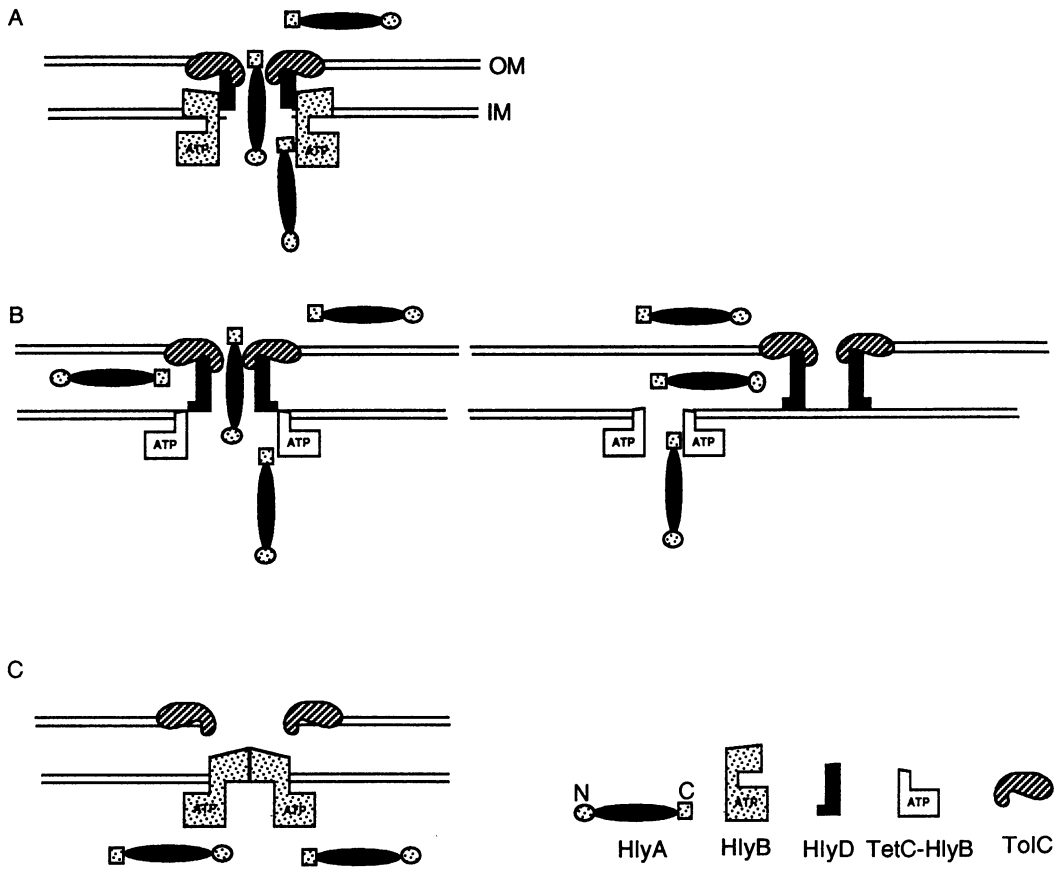

FIG. 5. *E. coli* hemolysin secretion model. (A) Wild-type secretion. (B) Secretion by the TetC-HlyB hybrid and HlyD-TolC. (C) HlyD mutant strain with HlyB. The components of the secretion apparatus are labeled as shown at the lower right. The NH_2 and COOH termini of HlyA are indicated by circles and squares, respectively. Abbreviations: OM, outer membrane; IM, inner membrane; ATP, nucleotide-binding domain.

their respective transmembrane and periplasmic domains. The periplasmic loops of HlyB may be involved in the stabilization or "sealing" of the channel across the periplasm (Fig. 5A) but may not be a critical part of the export channel itself. We believe that this is the case because extracellular transport of HlyA by the TetC-HlyB hybrid does occur (Fig. 5B). The presence of periplasmic HlyA indicates that the HlyB NH_2-terminal domain may improve the interaction between HlyB and HlyD or TolC and thus seal the periplasmic channel. Alternately, removal of the HlyB NH_2-terminal domain may prevent any interaction of HlyB with HlyD or TolC, and thus the TetC-HlyB hybrid can secrete HlyA into the periplasm, where HlyD and TolC can move it across the outer membrane. When either HlyD or TolC is absent, HlyB is incompetent for HlyA transport across the inner membrane. An explanation for this is not readily evident, although appropriate HlyB localization via interaction with HlyD and TolC may dictate HlyB functionality. The models proposed above are consistent with the existing information but are speculative. The precise localization and nature of the interactions proposed in this paper will require further genetic and physical analyses.

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