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Translocation and Compartmentalization of *Escherichia coli* Hemolysin (HlyA)

R. L. OROPEZA-WEKERLE,[1]* W. SPETH,[2] B. IMHOF,[1] I. GENTSCHEV,[1] AND W. GOEBEL[1]

Institut für Genetik und Mikrobiologie, Universität Würzburg, 8700 Würzburg,[1] and Institut für Biologie II, Abteilung für Zellbiologie, 7800 Freiburg,[2] Federal Republic of Germany

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Hemolysin plasmids were constructed with mutations in *hlyB*, *hlyD*, or both transport genes. The localization of hemolysin activity and HlyA protein in these mutants was analyzed by biochemical and immunological methods. It was found that mutants defective in *hlyB* accumulated internal hemolysin, part of which was associated with the inner membrane and was degraded in the late logarithmic growth phase. In an HlyB$^+$ HlyD$^-$ mutant, hemolysin was predominantly localized in the membrane compartment. Labeling of these *Escherichia coli* cells with anti-HlyA antibody indicated that part of HlyA, presumably the C-terminal end but not the pore-forming domains, was already transported to the cellular surface. This finding suggests that HlyB is able to recognize the C-terminal signal of the HlyA protein and to initiate its translocation across the membranes.

Escherichia coli has a limited capacity to transport proteins across its double-membrane envelope. The only well-documented examples of secreted proteins in *E. coli* are colicins (1, 19) and hemolysin (9, 24). The immunoglobulin A protease from *Neisseria gonorrhoeae* (18) and pullulanase from *Klebsiella pneumoniae* (3) are also secreted when the genes for the two latter proteins are cloned and expressed in *E. coli*. However, secretion of pullulanase in *E. coli* requires several additional *Klebsiella* genes. Protein transport across the cytoplasmic membrane in procaryotes and eucaryotes follows a rather universal mechanism that has been extensively studied in many biological systems (for a recent review, see reference 21). In contrast, the strategies that have evolved for transporting proteins across the two membranes of the gram-negative bacterial cell (referred to below as protein secretion) appear to be rather diverse (9, 20).

Secretion of hemolysin (HlyA) seems to be independent of the general protein transport pathway of *E. coli* (9; I. Gentschev, unpublished results) and requires a specific translocator system that consists of at least two proteins, HlyB and HlyD. Both proteins are expressed at low levels in *E. coli*, and attempts to overexpress the HlyB and HlyD proteins have been unsuccessful until now because of the instability of the products (T. Jarchau, unpublished results). This circumstance has severely hampered the biochemical characterization of this novel protein translocation system. Nevertheless, the HlyB/HlyD transport system, and the transport of HlyB in particular, recently received considerable attention when it was shown that HlyB is a member of a larger group of proteins involved in a variety of active transport processes in both procaryotic (9) and eucaryotic (6, 15) systems.

Most information on HlyB and HlyD and their possible localization in the cellular envelope is based on the predicted amino acid sequences (5, 8) and putative secondary structures. These data suggest a transmembrane organization for HlyB (6, 9), with at least three pairs of transmembrane regions spanning the cytoplasmic membrane, thereby generating three periplasmic loops. This membrane-integrated portion of HlyB is represented by the N-terminal half of the protein; the C-terminal half of HlyB, which carries a putative mononucleotide-binding fold, probably faces the cytoplasm. HlyD possesses a single transmembrane region which may anchor this protein to the cytoplasmic membrane; however, in minicells a substantial portion of HlyD fractionates with the outer membrane fraction, suggesting that a major part of HlyD is associated with the outer membrane (7).

The possible interaction of HlyB and HlyD in the formation of the hemolysin translocator and the topology of this translocator within the cell envelope remain speculative at present. The signal on HlyA which apparently recognizes the HlyB/HlyD translocator was identified at the C-terminal end of HlyA (7, 9, 13, 14). This signal peptide seems to possess a minimum length of 60 amino acids (9, 10, 14; T. Jarchau and J. Hess, unpublished results), as shown by deletions in HlyA and construction of fusion proteins, and may consist of several functional regions (9, 10; J. Hess, unpublished results). It is unknown whether this C-terminal HlyA signal is recognized by HlyB, HlyD, or a structure consisting of both components.

Here we demonstrate that HlyB alone can translocate HlyA into the cellular envelope. Under these conditions, part of the HlyA molecule is exposed on the cellular surface, while the pore-forming region of HlyA is still buried within the envelope. We also show that HlyD does not directly interact with HlyA but seems to be required for pulling the N-terminal part of HlyA through the envelope and for releasing hemolysin from the cell surface.

MATERIALS AND METHODS

Bacterial strains. *E. coli* 5K(pANN202-812) and construction of the plasmids used in this study have been described before (13, 16) or will be published elsewhere (A. Ludwig, I. Gentschev, and W. Goebel, unpublished data). Plasmid pANN202-812/17 was kindly provided by K. Geuder.

Isolation of extra- and intracellular hemolysin. Extra- and intracellular hemolysin was isolated as previously described (16). Briefly, strains were grown overnight in 50 ml of 2 × YT medium. Overnight cultures were diluted 1:100 and further grown at 37°C. Samples were taken every hour

* Corresponding author.

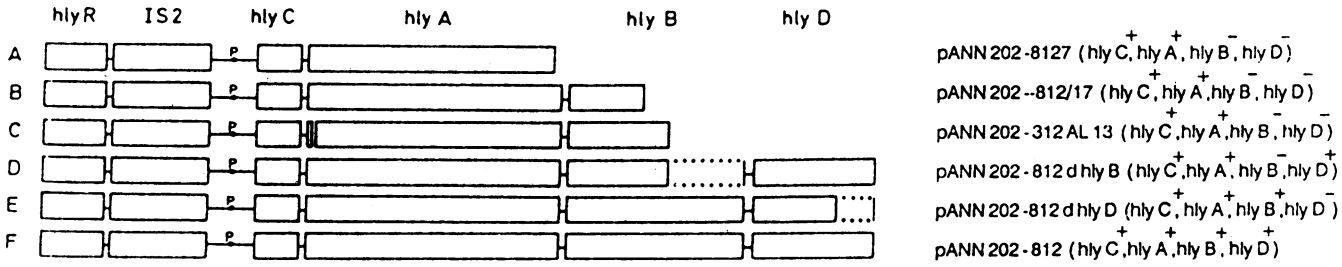

FIG. 1. Construction of hemolysin plasmids with defects in *hlyB*, *hlyD*, or both. The intact *hly* determinant consisting of the four structural *hly* genes (*hlyC*, *-A*, *-B*, and *-D*), the regulatory element *hlyR* (23), and the promoter (P) is carried on plasmid pANN202-812 (construct F); *hlyR* is separated from the transcriptional start site by an IS2 element (23). Plasmid pANN202-8127 (A) carries a deletion that removes the C-terminal 111 nucleotides of *hlyA* and the entire *hlyB* and *hlyD* genes, whereas plasmid pANN202-812/17 (B) and pANN202-312AL13*hlyB,D* (C) carry an active *hlyA* gene (with a deletion at the 3' end from amino acids 9 to 37) in pANN202-312AL13*hlyB,D* (12) and a different truncated portion of *hlyB* but no *hlyD*. Plasmids pANN202-812d*hlyB* (D) and pANN202-812d*hlyD* (E) carry mutant *hlyB* and *hlyD* genes, respectively. In the latter two plasmids, frameshift mutations were introduced at the sites where the dotted lines start. The thick lines indicate the vectors used for construction of the plasmids. Plasmids A and C were constructed by using pACYC184; the others were pBR322 recombinant plasmids.

unless otherwise indicated. Cells were pelleted by centrifugation. The supernatants were used as the source of extracellular hemolysin, and the pellets were used as the source of intracellular hemolysin. The samples were processed as described below. Internal hemolysin from each strain was isolated by lysis of the cells by the modified method of Koshland and Botstein (11), which yields complete cell lysates. Determination of the hemolytic activity in these lysates and the enzyme-linked immunosorbent assay (ELISA) for measuring the HlyA protein concentration were performed as described previously (16). For the isolation of outer and inner membrane fractions, the cells were grown in 2 × YT medium with vigorous aeration at 37°C. After 4 h, the cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. The cell pellet was suspended in cold 0.75 M sucrose–10 mM Tris hydrochloride buffer (pH 7.8). Lysozyme was immediately added at a final concentration of 100 μg/ml, and the mixture was incubated for 2 min at 4°C. The suspension was then slowly diluted with 2 volumes of cold 1.5 mM sodium EDTA (pH 7.5). Lysates were centrifuged in the cold for 20 min at 1,200 × *g* to remove intact cells, and the supernatant fraction was centrifuged at 4°C for 2 h at 360,000 × *g*, using a 60Ti rotor. The membrane pellets were carefully suspended in a small volume of cold 0.25 M sucrose–3.3 mM Tris–1 mM EDTA. The membrane fraction was collected by centrifugation and suspended in 1 ml of cold 25% (wt/wt) sucrose containing 5 mM EDTA (pH 7.5). Sucrose density gradients were run on an SW40 rotor, using step gradients prepared by layering 2.1 ml each of 50, 45, 40, 35, and 30% (wt/wt) sucrose solution on a cushion (0.5 ml) of 55% sucrose. All sucrose solutions contained 5 mM EDTA (pH 7.5). A 1-ml sample of the membrane suspension (containing about 5 mg of protein) was layered on top of the gradient, and centrifugation was carried out at 180,000 × *g* for 15 h at 4°C. Gradients were fractionated. The typical L, M, and H bands representing the inner membrane (L), a mixture of inner membrane and outer membrane (M), and the outer membrane (H) were obtained. A total of 27 fractions were collected and further analyzed for hemolytic activity. The HlyA protein concentration was determined by ELISA.

ELISA. Polyvinyl assay plates (Costar, Cambridge, Mass.) were coated with extracellular hemolysin or intracellular protein extracts. In the assays that determined the hemolytic activity in the outer and inner membrane fractions, the same protein concentration was used. The reaction of HlyA protein with the anti-HlyA antibody was quantified with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (16).

Determination of hemolytic activity. The hemolytic activity of extracellular, intracellular, and membrane-bound hemolysin was measured as described before (16). The amount of released hemoglobin was measured at 543 nm.

Proteinase K digestion. Cells were grown for 4 h, washed with 10 mM Tris (pH 7.6) to eliminate the residual medium, and resuspended in 2 ml of the same buffer. The cell suspension was divided equally in two tubes, one with and the other without 50 μg of proteinase K. The cells were incubated with the protease for 30 min. Digestion was stopped by dilution with buffer. Cells were extensively washed to eliminate residual protease, and the hemolytic activity was tested.

Immunoblot analysis of HlyA. Protein of *E. coli* 5K carrying one of the six plasmids used in this study was isolated as described above and separated on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate; 8 μg of protein was applied in each case. The separated proteins were transferred onto nitrocellulose membranes as described by Towbin et al. (22). The nitrocellulose membranes were then allowed to bind polyclonal anti-HlyA antibody and visualized by using horseradish peroxidase (16).

Electron microscopy and immunogold labeling of HlyA-transporting *E. coli* cells. *E. coli* 5K, *E. coli* 5K(pANN202-812), and *E. coli* 5K(pANN202-812d*hlyD*) were allowed to grow for 4 h. Then 1 ml of the culture was deposited in chamber slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). The cells were fixed with glutaraldehyde (0.25%) and paraformaldehyde (4%) in 0.1 M phosphate buffer (pH 7.4) for 60 min. Cells were washed with 9% NaCl solution and incubated with polyclonal anti-HlyA antibodies for 1 h. After washing of the cells, rabbit anti-mouse immunoglobulin G coated with colloidal gold G15 (Jansen Life Science Products, Div. Jansen Pharmaceutica N.V., Beerse, Belgium) was applied for another hour. Cells were washed again and processed for freeze-fracturing as described previously (2).

RESULTS

Construction of Hly determinants deficient in HlyB, HlyD, or both. Figure 1 shows the physical maps of the plasmids used. Most plasmids are pBR322 derivatives, but pANN202-

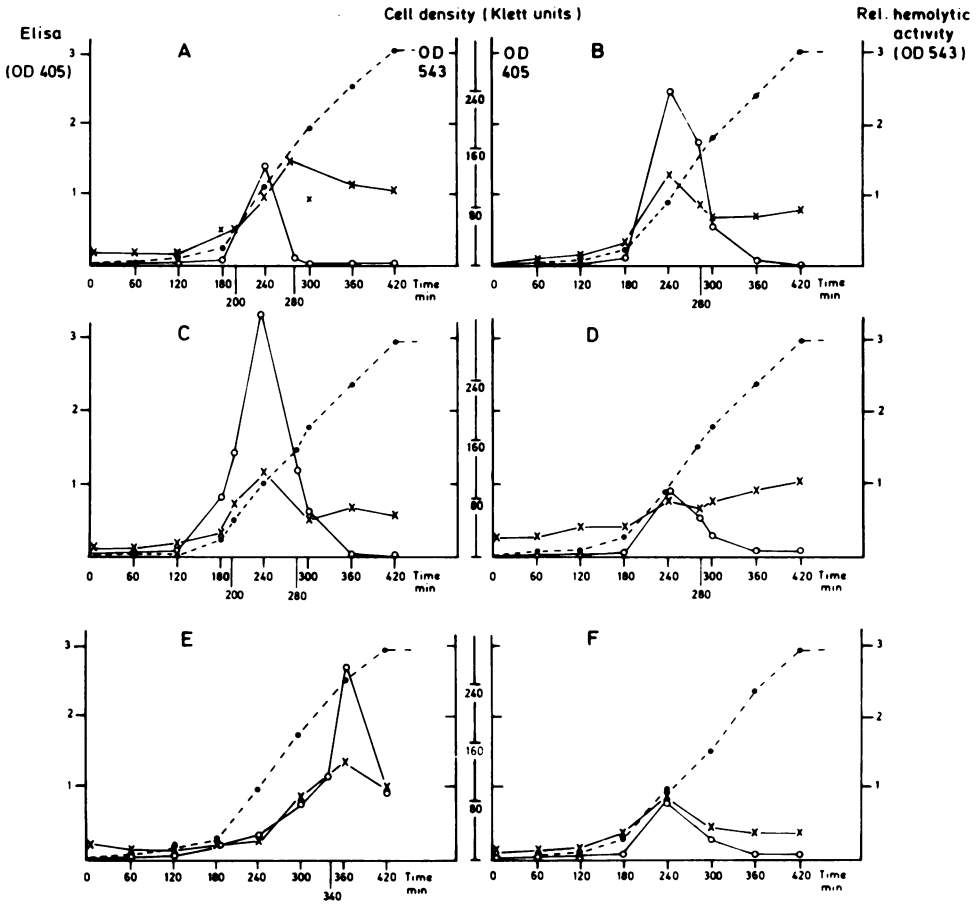

FIG. 2. Intracellular pools of hemolysin in *E. coli* strains carrying the *hly* plasmid pANN202-812 (F) or *hly* plasmids with mutations or deletions in *hlyB* (D), *hlyD* (E), or *hlyB* and *hlyD* (A to C). Plasmids in panels A to F correspond to plasmids A to F in Fig. 1. Growth of the cultures (– – –) was determined by measuring the cell density (given in Klett units). Equal amounts of cells were taken every hour from the growing cultures, which were lysed with EDTA and lysozyme. Hemolytic activity of the cell lysates was determined by the release of hemoglobin (optical density at 543 nm [OD 543]) and is given in relative units of optical density at 543 nm (○). The HlyA protein concentration in the lysates was measured by ELISA, using polyclonal anti-HlyA antibodies and rabbit anti-mouse IgG coupled to horseradish peroxidase (×). Enzymate activity was measured at an optical density of 405 nm (OD 405).

8127 and pANN202-312Al13*hlyB,C* carry the pACYC184 replicon. The regulation site upstream of *hlyC* is identical in all plasmids and includes the entire activator sequence *hlyR* (23). Plasmids pANN202-8127 and pANN202-812/17 were previously described (16), pANN202-312Al13Δ*hlyB,D* was obtained by deleting a region between two *BclI* sites which includes part of the *hlyB* gene and the entire *hlyD* gene. This plasmid carries in addition a short deletion at the 5′ end of *hlyA* (13) which does not affect the transport efficiency or the hemolytic activity of the mutant hemolysin (13). Plasmids pANN202-812d*hlyB* and pANN202-812d*hlyD* were obtained by filling in or deleting the protruding ends of the single *BclI* (in *hlyB*) and *ApaI* (in *hlyD*) sites in plasmid pANN202-812 and religation of the blunt ends. The introduced frameshift mutations in *hlyB* and *hlyD* were confirmed by determination of the DNA sequence around the manipulated restriction sites. *E. coli* 5K transformants carrying the mutant plasmids formed nonhemolytic colonies on blood agar plates, in contrast to the wild-type plasmid pANN202-812, which yields large hemolytic zones (12). None of the strains carrying the mutant plasmids showed extracellular hemolytic activity, but substantial internal hemolytic activity was detected when this activity was determined under optimal growth conditions. In the strains carrying HlyB$^-$ mutant plasmids, the internal hemolytic activity reached a maximal value in the middle of the logarithmic growth phase and dropped sharply thereafter (Fig. 2A to D). Determination of the amount of cellular HlyA protein by ELISA, using polyclonal antibodies directed against purified HlyA protein (16), indicated that the HlyA concentration inside the cell coincided with the peak of internal hemolytic activity. The decline in cellular HlyA protein concentration in the late logarithmic growth phase suggested proteolytic degradation of HlyA. As expected, this degradation proceeded slower than the loss of hemolytic activity. Interestingly, hemolytic activity and internal HlyA protein were still detected in the late logarithmic growth phase in a strain carrying an HlyB$^+$ HlyD$^-$ plasmid (Fig. 2E), suggesting that hemolysin was better protected from proteolytic degradation in this strain. The small amount of the internal hemolysin (about 10% of total activity) that remained associated with *E. coli* 5K cells carrying the complete plasmid pANN202-812 was degraded at a similar rate as the internal hemolysin of the HlyB$^-$ mutants (Fig. 2F).

Western blot (immunoblot) analysis of internal HlyA (Fig. 3) indicated that the amounts of HlyA accumulated intracellularly at the time when the hemolytic activity reached its maximum were similar in all strains. This saturation level of

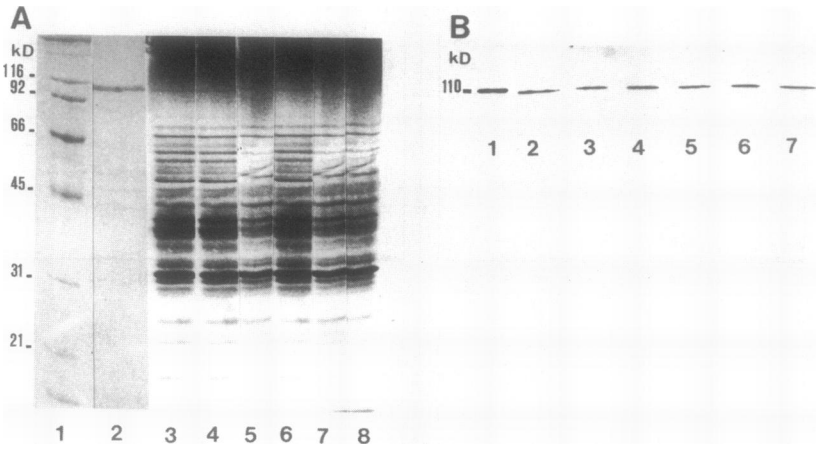

FIG. 3. Identification of hemolysin (HlyA) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (A) Lanes: 1, size markers (positions indicated in kilodaltons (kD); 2 and 8, external (lane 2) and internal (lane 8) protein from *E. coli* 5K carrying pANN202-812; 3 to 7, internal protein from *E. coli* 5K strains with pANN202-8127 (lane 3), pANN202-812/17 (lane 4), pANN202-312AL13*hlyB,D* (lane 5), pANN202-812d*hlyB* (lane 6) and pANN202-812d*hlyD* (lane 7). Protein was visualized by staining with Coomassie blue. (B) Immunoblots of the proteins developed with polyclonal anti-HlyA antibodies. Lanes: 1, external hemolysin from *E. coli* 5K carrying pANN202-812; 2 to 7, internal hemolysin of *E. coli* 5K strains with pANN202-8127 (lane 2), pANN202-812/17 (lane 3), pANN202-312AL13*hlyB,D* (lane 4), pANN202-812d*hlyB* (lane 5), pANN202-812d*hlyD* (lane 6), and pANN202-812 (lane 7).

internal HlyA protein corresponded to about 20% of the amount secreted by the same number of *E. coli* cells in the same period of time in the presence of a functional HlyB/HlyD translocator. This finding suggests that internal HlyA may cause feedback inhibition of HlyA synthesis.

Compartmentalization of internal HlyA. To localize the internal hemolysin (HlyA) in the strains carrying either or both of the $HlyB^-$ and $HlyD^-$ mutant plasmids, total cellular hemolytic activity and membrane-bound hemolysin and HlyA protein were determined in cells harvested at the time point optimal for internal hemolysin accumulation (Fig. 4). The membrane fraction was carefully washed to remove loosely bound hemolysin and fractionated on sucrose gradients by the method of Osborn and Munson (17). Hemolytic activity and HlyA protein concentration were determined in each fraction as described above. It was found that only the H and L bands but not the M band (see Material and Methods) yielded measurable hemolytic activity and HlyA protein. Intracellular hemolytic activity and HlyA protein were detected in all strains (Fig. 4), but the total amount and percentage of membrane-associated material were dependent on the plasmid present in the cell. In strains carrying $HlyB^-$ $HlyD^-$ plasmids (Fig. 4, columns A and B), 20 to 50% of the hemolytic activity and HlyA protein was associated with the inner membrane. This finding suggests again a relatively stable binding of active hemolysin to the inner membrane even in the absence of the HlyB/HlyD translocator, in agreement with our previous data (16). Plasmid pANN202-312A13Δ*hlyB,D*, which is deleted in *hlyB* and *hlyD* similar to pANN202-812/17 but has an addition of 28-amino-acid deletion at the N-terminal end of HlyA, produced higher hemolytic activity and more HlyA protein (Fig. 4, columns C). This mutant hemolysin seemed to be even more tightly bound to the inner membrane than was hemolysin from pANN202-812/17. The strain carrying plasmid pANN202-812d*hlyB*, which has a mutant *hlyB* gene but an intact *hlyD* gene, showed a pattern of distribution of hemolytic activity and HlyA protein similar to that of the $HlyB^-$ $HlyD^-$ strains (Fig. 4, columns D). In contrast, a substantial portion of the hemolytic activity and HlyA protein fractionated to the outer membrane in an $HlyB^+$ $HlyD^-$ strain (carrying plasmid pANN202-812d*hlyD*). In this strain, the hemolytic activity and HlyA protein in the inner and the outer membrane fractions added up to the total cellular hemolytic activity and HlyA protein concentration, respectively (Fig. 4, columns E), indicating that this hemolysin is located entirely in the cellular envelope. Cell-associated hemolysin from an $HlyB^+$ $HlyD^+$ strain (carrying plasmid pANN202-812) fractionated mainly to the outer membrane, with barely detectable activity remaining in the inner membrane (Fig. 4, columns F).

Although similar amounts of hemolytic activity were found in the outer membrane fractions of strains carrying either pANN202-812 or pANN202-812 d*hlyD*, only *E. coli* cells with pANN202-812 were strongly hemolytic when incubated directly with erythrocytes, whereas *E. coli* cells with pANN202-812d*hlyD* were nonhemolytic under the same conditions. This finding suggests that the domains of hemolysin which are required for pore formation (12) are still buried in the cell envelope of the $HlyB^+$ $HlyD^-$ strain but are exposed at the cellular surface in the $HlyB^+$ $HlyD^+$ strain. This assumption was confirmed by protease protection experiments. Treatment of *E. coli* 5K carrying pANN202-812 with proteinase K destroyed the cell-bound hemolytic activity entirely, but almost the total hemolytic activity was recovered from protease-treated cells carrying pANN202-812d*hlyD* (Table 1).

Exposure of antigenic epitopes of HlyA on the surface of *E. coli* cells harboring pANN202-812d*hlyD*. To further study the localization of hemolysin in cells with functional HlyB but defective HlyD, we applied the indirect immunogold labeling method (2). The specific antibody applied to the intact *E. coli* cells was a purified polyclonal anti-HlyA antibody that was previously described (16). The immunogold-labeled anti-immunoglobulin G antibody was then added, and the labeled *E. coli* cells were freeze-fractured (2).

E. coli 5K cells carrying pANN202-812 bound polyclonal anti-HlyA antibody on the external surface (ES) (Fig. 5a). Binding of the antibody in the technique used resulted in a rough appearance of the ES. When the same cells were treated with nonimmune serum, the ES remained smooth (Fig. 5b), which demonstrates the absence of unspecific

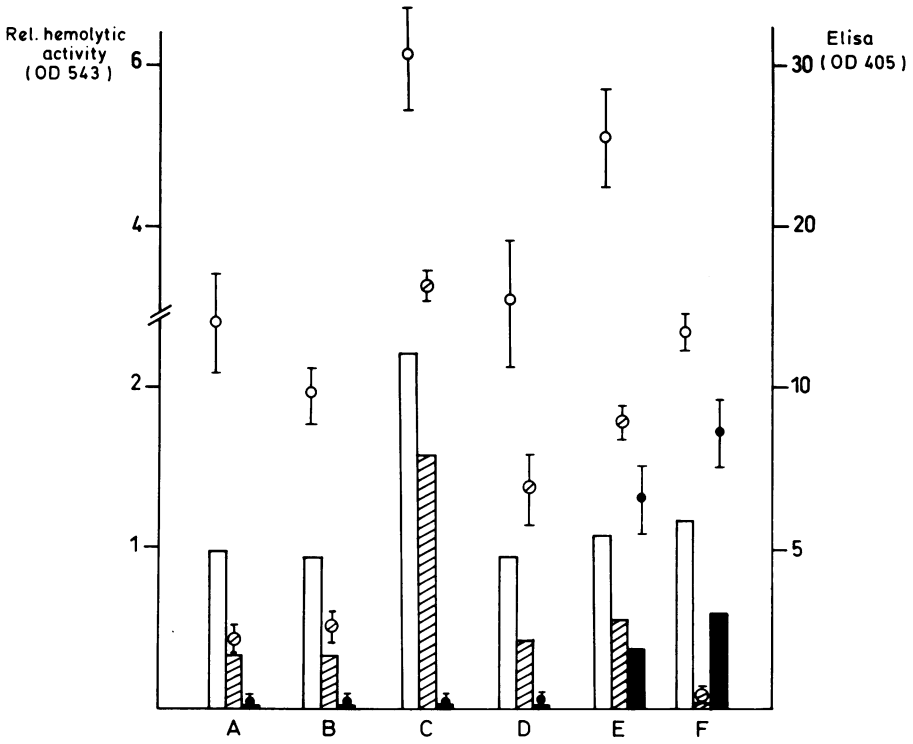

FIG. 4. Cellular localization of hemolytic activity (bars) and HlyA protein (circles). Strains were grown for 4 h under the conditions described in the legend to Fig. 2. Cells were lysed, and the membranes were fractionated by the procedure of Osborn and Munson (17). Hemolytic activity (average of three values) is given in relative units of optical density at 543 nm (OD 543). Symbols: □, hemolytic activity of the total lysates; ▨, hemolytic activity in the inner membrane fractions (L bands); ■, hemolytic activity in the outer membrane fractions (H bands). In parallel, the HlyA protein concentration in these fractions was determined by ELISA as described in the legend to Fig. 2. (A) *E. coli* 5K(pANN202-8127); (B) *E. coli* 5K(pANN202-812/17); (C) *E. coli* 5K(pANN202-312AL13*hlyB,D*); (D) *E. coli* 5K(pANN202-812d*hlyB*); (E) *E. coli* 5K(pANN202-812d*hlyD*); (F) *E. coli* 5K(pANN202-812).

antibody binding. A similar smooth ES was also observed with *E. coli* 5K cells that were treated with polyclonal anti-HlyA (Fig. 5d). Only those sections of the bacterial cell in which the ES was peeled off and the cytoplasmic membrane (protoplasmic fracture) was exposed showed a slightly rough appearance (Fig. 5b and d). The ES of *E. coli* 5K cells carrying pANN202-812d*hlyD* was also decorated by polyclonal anti-HlyA antibody (Fig. 5c) to a similar extent as the ES of *E. coli* cells carrying pANN202-812, indicating the HlyA epitopes were exposed on the cell surface of the $HlyB^+$ $HlyD^-$ mutant. In all cases, the gold particles were removed during washing of the grids with chromic acid (2).

DISCUSSION

The secretion machinery of *E. coli* hemolysin requires HlyB and HlyD (24). The predicted amino acid sequences and the secondary structures of HlyB and HlyD (5, 8) strongly suggest that both proteins are inserted in the cell envelope of *E. coli*. This prediction has been basically confirmed by the biochemical identification of the gene products of *hly*B and *hly*D in the membrane faction (7, 9). However, major questions remain concerning the nature of the HlyB protein, the precise localization of HlyB and HlyD, and in particular their functions in the transport of HlyA. To better understand the functions of HlyB and HlyD, we have undertaken to study the localization of hemolysin in the cell envelope in the presence and absence of HlyB and HlyD, using cell fractionation and immunoelectron microscopic methods to identify the hemolytic activity and the HlyA protein in the respective compartments.

TABLE 1. Hemolytic activity of cellular extracts of *E. coli* strains after treatment of whole cells with protease K

Plasmid carried by *E. coli* 5K	Hemolytic activity[a] of cell extracts	
	Untreated	Treated with protease K
pANN202-812	30.1	0.5
pANN202-312[b]	25.6	0.2
pANN202-812d*hlyD*	29.4	28.5

[a] Expressed as relative units of optical density at 543 nm (measuring released hemoglobin) per microliter of extract. Protein concentration of all cellular extracts was constant.

[b] Carries all four structural *hly* genes (*hlyC*, *-A*, *-B*, and *-D*) but lacks *hlyR* (23).

Our data show that cells without a functional HlyB accumulate similar amounts of hemolysin intracellularly regardless of whether functional HlyD is present in the cell. Under both conditions, active internal hemolysin and a proportional amount of HlyA protein, as measured by ELISA, reached maximal levels in the early logarithmic growth phase. Hemolysin was degraded thereafter, as indicated by the rapid loss of hemolytic activity and by the gradual decline of cellular HlyA protein. Fractionation of the cell envelope into the inner and outer membranes from $HlyB^-$ mutants showed an appreciable percentage of hemolytic activity and a proportional amount of HlyA protein associated with the inner membrane. This finding is in agreement with our previous results (16), which also indicated a close association of hemolysin to the inner membrane in an $HlyB^-$ $HlyD^-$ mutant. We previously speculated that the contact

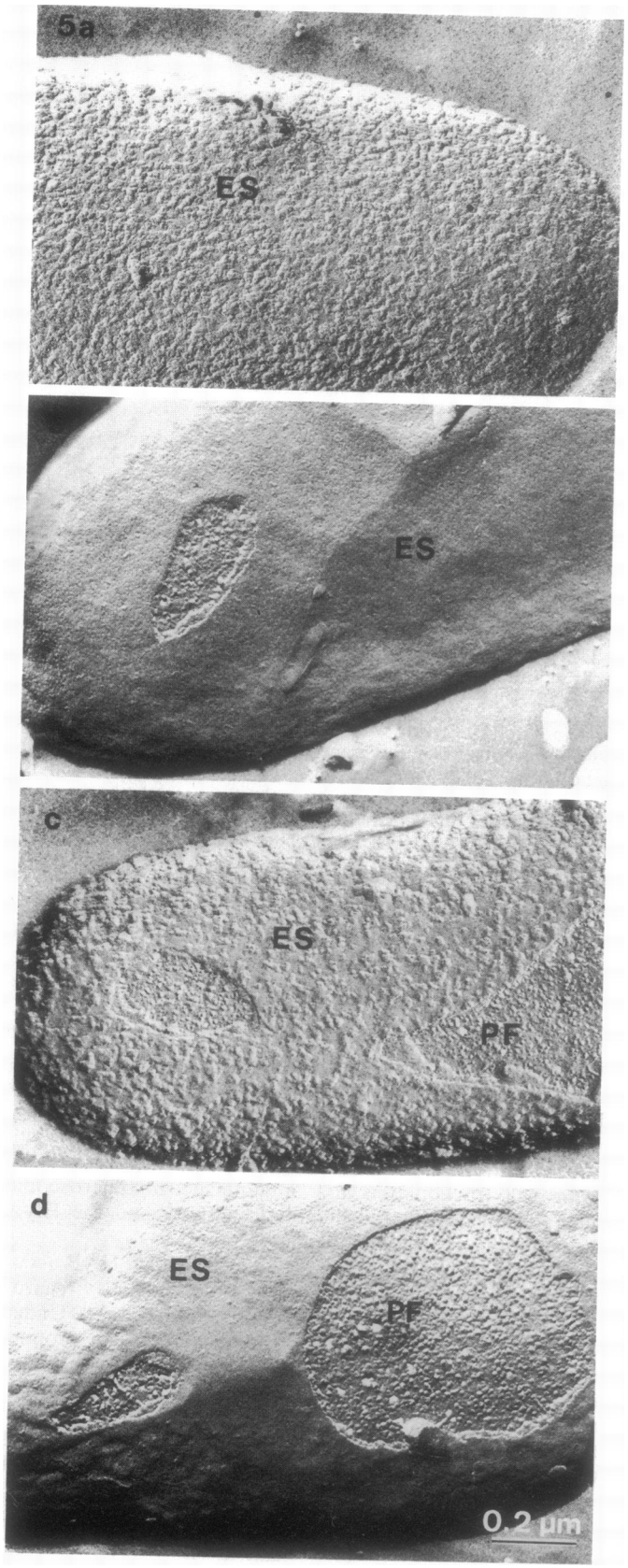

FIG. 5. Electron micrographs of freeze-fractured *E. coli* cells labeled with polyclonal anti-HlyA antibodies. A smooth external ES indicating the absence of antibody binding is observed in panels b and d, which show as controls an *E. coli* 5K cell treated with the polyclonal anti-HlyA antibody (d) and an *E. coli* 5K cell with pANN202-812 treated with preimmune serum (b). Binding of the anti-HlyA antibody to the ES is indicated by the rough appearance of the ES in *E. coli* 5K carrying pANN202-812 (a) and *E. coli* 5K carrying 202-812d*hlyD* (c). PF (protoplasmic fracture) indicates areas where the external layer is peeled off and the inner membrane is exposed with the transmembrane particles. Magnification, ×60,000.

between hemolysin and the inner membrane may be triggered by the amphiphilic α-helical structure of the N-terminal end of HlyA (4). The data presented here rule out this assumption, since an HlyA mutant protein that lacks amino acids 9 to 37 from its N-terminal end still exhibited a tight association with the inner membrane. It is interesting that this mutant not only possessed a higher intracellular hemolytic activity but also accumulated a larger amount of internal HlyA protein (in an $HlyB^-$ background) than did the above-described $HlyB^-$ mutants, all of which carry a wild-type *hlyA* gene. Since the regulatory sites for expression of the hemolysin genes are identical in all strains used, these data suggest that this mutant hemolysin has a higher stability, at least when inside the cell, possibly because of the tighter membrane association.

The $HlyB^-$ $HlyD^+$ mutant accumulated the same amount of internal hemolytic activity and HlyA protein as did the $HlyB^-$ $HlyD^-$ mutant, which indicates that HlyA is not inserted into the membrane by HlyD. The frameshift mutation in *hlyB* used for the construction of this $HlyB^-$ $HlyD^+$ mutant does not alter the first 330 amino acids of HlyB, which include almost the entire putative membrane-anchoring N-terminal portion of the HlyB. The expected truncated HlyB protein would lack the C-terminal portion, but this should not change its compartmentalization in the inner membrane. The truncated HlyB protein, if correctly inserted in the membrane, is apparently unable to channel HlyA into the membrane.

Our data support the view that the intact HlyB itself, e.g., in the absence of a functional HlyD, is able to lead the HlyA protein into the membrane compartment. Hemolytic activity and a proportional amount of HlyA protein fractionated to the inner and the outer membrane. The added pools of hemolytic activity and HlyA protein, respectively, in these two membrane fractions corresponded well to the total cellular hemolytic activity and HlyA concentration, whereas in $HlyB^-$ mutants only a relatively small percentage of cellular hemolytic activity and HlyA protein was detectable in the inner membrane fraction. Moreover, internal hemolysin seems to be much better protected in the $HlyB^+$ $HlyD^-$ mutant than in the $HlyB^-$ mutants and is still present in the late logarithmic growth phase. Accumulation of internal HlyA starts reproducibly later in this mutant. We do not know the reason for this phenomenon. One explanation could be that HlyB in the absence of HlyD causes a change in regulation so that synthesis of HlyA is shifted to a later phase in the growth cycle. The polyclonal anti-HlyA antibody reacts with an epitope(s) on the cell surface of the $HlyB^+$ $HlyD^-$ mutant but not with $HlyB^-$ mutants (data not shown). $HlyB^+$ $HlyD^-$ cells are nonhemolytic even when brought in close contact with erythrocytes, and the hemolysin in these cells is protected against proteolytic degradation, whereas $HlyB^+$ $HlyD^+$ cells are strongly hemolytic and the hemolytic activity is totally destroyed by proteinase K treatment. These data suggest that only a small portion of HlyA, presumably the C-terminal signal sequence of HlyA

which is not required for hemolytic activity (12), is exposed to the cell surface, whereas the major portion of this protein, including the domains of HlyA involved in pore formation (12, 13), are still within the cell envelope.

It thus appears that HlyB alone can form a transmembrane pore that recognizes the C-terminal transport signal sequence of HlyA and allows a partial vectorial transport of HlyA. HlyD is, however, indispensible for the translocation of the entire HlyA to the cellular surface and for the release of HlyA from the surface. Our data do not allow us to differentiate whether these latter steps are distinct functions of HlyD or require a composite HlyB-HlyD pore.

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