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Synthesis, Inactivation, and Localization of Extracellular and Intracellular *Escherichia coli* Hemolysins

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Extra- and intracellular *Escherichia coli* hemolysin expressed by two cloned *hly* determinants, both under the control of the activator element *hlyR*, were analyzed. One determinant carried all four *hly* genes (*hlyC*, *hlyA*, *hlyB*, and *hlyD*), whereas the other carried only the two genes (*hlyC* and *hlyA*) required for synthesis of active hemolysin but not those essential for its secretion. It was shown that the total amounts of HlyA protein and of hemolytic activity are similar in both cases in logarithmically growing cultures. The *E. coli* strain carrying the complete *hly* determinant released most hemolysin into the media and accumulated very little HlyA intracellularly. The active extracellular hemolysin (HlyA*) was inactivated in the stationary phase without degradation of the HlyA protein. In contrast, the hemolysin which accumulated intracellularly in the *E. coli* strain carrying *hlyA* and *hlyC* only was proteolytically degraded at the end of the logarithmic growth phase. Immunogold labeling indicates that active intracellular HlyA bound preferentially to the inner membrane, whereas that part of the extracellular HlyA which remained cell-bound was located exclusively at the cell surface. It was shown by fluorescence-activated cell sorter analysis that active extra- and intracellular HlyA* bound with similar efficiency to erythrocytes, whereas hemolytically inactive HlyA protein did not bind to these target cells.

Alpha-hemolysin of *Escherichia coli* is one of the few proteins which are transported across the cytoplasmic and outer membranes of gram-negative bacteria. The formation of active extracellular hemolysin requires the products of four *hly* genes (*hlyA*, *hlyB*, *hlyC*, and *hlyD*) that can be located on the chromosome or on plasmids in *E. coli* (3, 7, 14, 15). HlyA is a protein of 110 kilodaltons which is hemolytically inactive in the absence of HlyC. The mechanism by which HlyC converts HlyA to a pore-forming cytolysin (10) is still unknown, but is has been shown that hemolytically active and inactive extracellular HlyA proteins differ in various properties (18). The specific hemolysin transport system consisting of at least the two membrane proteins HlyB and HlyD (19) allows the translocation of HlyA in the presence or absence of HlyC (11, 12), indicating that HlyC is not essential for hemolysin transport. The secretion of HlyA does not require cleavage of an N-terminal leader peptide (2) but rather depends on an amino acid sequence at the C terminus (11, 12; J. Hess and W. Goebel, unpublished results). There are conflicting published results as to whether active hemolysin accumulates inside the cell in the absence of the two transport genes *hlyB* and *hlyD* and where this intracellular hemolysin is localized (6, 13). We show here that a large pool of intracellular active hemolysin could accumulate in the cell during active growth when transport was blocked. The intracellular hemolysin appears to be fixed to the inner membrane. Intracellular hemolysin was rapidly inactivated in the late growth phase. Inactivation of the intracellular hemolysin occurred by proteolytic degradation of HlyA, in contrast to the inactivation of extracellular hemolysin, which did not alter the size of the HlyA protein. The inactivated form of extracellular HlyA did not bind to erythrocytes.

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MATERIALS AND METHODS

Bacterial strains. *E. coli* 5K(pANN202-812) and *E. coli* 5K(pANN202-8127) have been described previously (11, 17). The plasmid pANN202-812/17 was constructed by K. Geuder (Ph.D. thesis, Universität Würzburg, 1987).

Isolation of extra- and intracellular hemolysin. An overnight culture of *E. coli* 5K carrying pANN202-812 was inoculated in 20 ml of 2× YT medium (4) at a dilution of 1:100 and incubated with shaking at 37°C. Samples were taken at various time points in the logarithmic growth phase, and cells were pelleted by centrifugation and discarded. The cell-free supernatants were mixed with 75% ammonium sulfate. Complete precipitation was reached by incubating the mixture on ice for 1 h. The pellets were suspended in 10 ml of TCU buffer (20 mM Tris, 150 mM NaCl, 6 M urea, pH 7.0) and dialyzed overnight against TCU buffer. After 24 h, the hemolytic activity was measured, and the preparations were stored at −70°C. Samples were suspended in TCU buffer, and HlyA was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (7 to 15% gradient in the presence of 6 M urea) essentially as described by Laemmli (9). Internal (cell-bound) hemolysin from strains *E. coli* 5K(pANN202-812), *E. coli* 5K(pANN202-812/17), and *E. coli* 5K(pANN202-8127) was isolated by lysis of the cells by the method of Koshland and Botstein (8) and was suspended in sample buffer.

Western blot (immunoblot) analysis of HlyA. Proteins from polyacrylamide gels were transferred to nitrocellulose membranes (2-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.). Electrophoretic transfer was accomplished overnight at 4°C at 70 mA in 0.025 M Tris buffer (pH 8.3, containing 0.19 M glycine), using a transfer unit (model TE 52; Hoefer Scientific Instruments, San Francisco, Calif.). Residual protein-binding sites on the membrane were blocked by incubation in 0.05 M Tris-buffered saline (pH 7.5) containing 5% bovine serum albumin (TBS-BSA) for 60 min at room temperature. The nitrocellulose filter was transferred to a

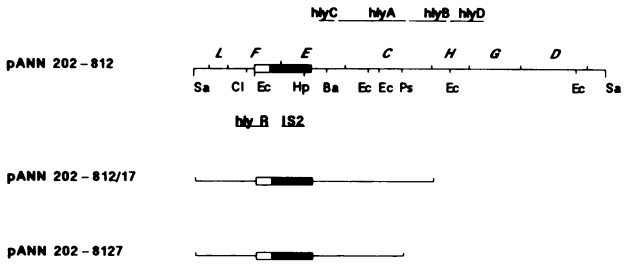

FIG. 1. Physical maps of recombinant plasmids pANN202-812, pANN202-812/17, and pANN202-8127. For a detailed description of the regulation site upstream of *hlyC*, see reference 17. Ba, *Bam*HI; Cl, *Cla*I; Ec, *Eco*RI; Hp, *Hpa*I; Sa, *Sal*I. Only one *Pst*I-site is indicated (Ps), which is the deletion endpoint in pANN202-8127. The single letters indicate the *Hin*dIII fragments (17).

solution consisting of mouse polyclonal antiserum raised against purified HlyA protein. Antibody reaction with hemolysin was detected by incubating the nitrocellulose filter in horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulins (DAKOPATTS A/S, Denmark). After being incubated and washed, the nitrocellulose filter was transferred to a peroxidase substrate solution containing 6 ml of 0.3% of 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo.), 42 μl of 30% H_2O_2 (Fischer Scientific Co., Pittsburgh, Pa.), and 94 ml of TBS for detection of HRP on the membrane.

ELISA assay. For the enzyme-linked immunosorbent assay (ELISA), wells of polyvinyl assay plates (Costar, Cambridge, Mass.) were coated with extra- or intracellular hemolysin from either concentrated culture supernatants or cell lysates. Reaction of hemolysin (HlyA) with polyclonal HlyA antibodies was detected with HRP-conjugated rabbit anti-mouse immunoglobulins. Binding of the conjugated antibodies was determined with ABTS (Serva, Heidelberg, Federal Republic of Germany).

Determination of hemolytic activity. The hemolytic activity of extracellular hemolysin was measured essentially as described elsewhere (16). Incubation mixtures containing 50 μl of cell-free supernatant were added to washed human erythrocytes (HRBC) in 160 mM NaCl–20 mM $CaCl_2$, pH 7.4. Mixtures were incubated at 37°C for 30 min. The unlysed erythrocytes and ghosts were pelleted by centrifugation, and the release of hemoglobin was determined spectrophotometrically at 543 nm. Internal hemolytic activity was determined in a similar assay by using various amounts of cell lysate as the source of internal hemolysin.

Binding of hemolysin to HRBC. The binding of hemolysin to HRBC was determined in a fluorescence-activated cell sorter using mouse-polyclonal antibodies raised against purified HlyA and fluorescein isothiocyanate-conjugated anti-mouse antibodies.

Electron microscopy and immunogold labeling of HlyA. (i) Preembedding procedure. Samples of 0.5 ml of *E. coli* 5K(pANN202-812) were taken at a cell density of 5×10^8 cells per ml and placed in chamber slides (Lab-Tek Products, Div. Miles Scientific Laboratories Inc., Westmont, Ill.). Cells were fixed with glutaraldehyde (0.25 %) and paraformaldehyde (4%) in 0.1 M phosphate buffer, pH 7.4, for 60 min. After fixation, the cells were washed three times with 25 mM TBS (pH 7.5), and mouse anti-HlyA polyclonal antibody was applied and incubated for 30 min. The chamber slides were washed and incubated with goat anti-mouse immunoglobulin G antibody coated with colloidal gold G15 (15 nm; Janssen Life Sciences Products, Div. Janssen Pharmaceutica N.V., Beerse, Belgium) for 60 min. Excess gold-labeled antibody was removed by washing with phosphate-buffered saline, and the immunogold-labeled bacteria were treated with 2% OsO_4 before dehydration for 2 h. Chambers were dehydrated with ethanol. Araldite was used as the embedding medium.

(ii) Postembedding procedure. The postembedding procedure was used for intracellular HlyA. *E. coli* 5K and *E. coli* 5K(pANN202-812/17) were harvested at a cell density of 5×10^8 cells per ml, and samples were placed in chamber slides (Lab-Tek). Cells were fixed by the same mixture as described above and processed as follows. After being washed with 25 mM TBS, pH 7.5, the glass slides were dehydrated by an ethanol gradient and embedded in methacrylate resin

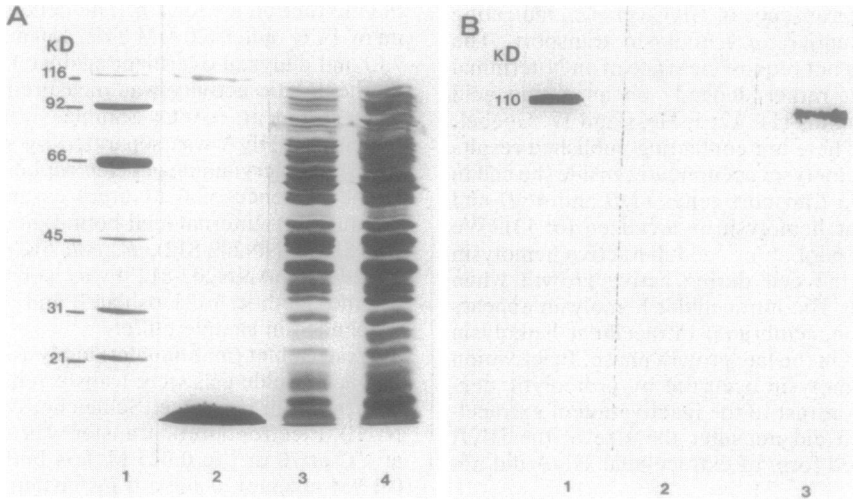

FIG. 2. Identification by SDS-polyacrylamide gel electrophoresis. (A) External and internal HlyA protein from *E. coli* strains carrying either pANN202-812 (lanes 2 and 3) or pANN202-812/17 (lane 4). Lane 2 represents external proteins, and lanes 3 and 4 represent internal proteins. (B) Western blots. Lanes: 1, external hemolysin; 2 and 3, internal hemolysin. Proteins were visualized by staining with coomassie blue in panel A or by immunoblotting with polyclonal anti-HlyA antibodies in panel B. In panel A, lane 1 contains molecular size markers (sizes in kilodaltons [kD]). The 110-kilodalton position indicates HlyA protein.

TABLE 1. Total hemolytic activity of *E. coli* strains carrying pANN202-812, pANN202-812/17, or pANN202-8127

Plasmid carried by *E. coli* 5K	Hemolytic activity (U/ml)[a]		
	Extracellular	Intracellular	Total[b]
pANN202-812	27.3	0.5	27.8
pANN202-812/17	0	17.3	17.3
pANN202-8127	0	12.5	12.5

[a] One unit is defined as 10^3 × the optical density at 543 nm of released hemoglobin.

[b] Total hemolytic activity is defined as the sum of extracellular and internal (cell bound) hemolytic activity.

(5; London Resin Co.). Following polymerization, ultrathin sections were prepared on an ultramicrotome (Ultratom; C. Reichert Optische Werke AG, Vienna, Austria), and the sections were mounted on nickel grids. These grids were incubated for 15 min in normal goat serum (5% in Tris buffer solution, pH 7.4). After the excess solution was removed, the grids were incubated in mouse antihemolysin polyclonal antibody (1:500) for 1 h. After incubation, the grids were extensively washed in the TBS-BSA (TBS containing 0.1% bovine serum albumin). The excess buffer was removed, and the grids were incubated with goat anti-mouse immunoglobulin G antibody-coated colloidal gold (GAM IgG G15; 15 nm; Janssen) for 30 min. The grids were carefully washed in distilled water, air dried, and examined by a Zeiss 10 CR electron microscope.

RESULTS

Extra- and intracellular pools of hemolysin of *E. coli* strains carrying either the entire *hly* determinant or a transport-deficient *hly* determinant. The physical maps of the three plasmids used in this study, pANN202-812 (*hlyC*$^+$ *hlyA*$^+$ *hlyB*$^+$ *hlyD*$^+$), pANN202-8127 (*hlyC*$^+$ *hlyA*$^+$), and pANN202-812/17 (*hlyC*$^+$ *hlyA*$^+$), are shown in Fig. 1. As indicated in Fig. 1, the two plasmids pANN202-8127 and pANN202-812/17 lack functional *hlyB* and *hlyD* genes; the *hly* determinant of pANN2021-812/17 terminates within *hlyB*, whereas that of pANN202-8127 lacks the last 112 base pairs of *hlyA* in addition to *hlyB* and *hlyD*. This deletion does not affect the hemolytic activity of the truncated HlyA protein (in the presence of HlyC) but abolishes its transport function (11). *E. coli* 5K carrying either of the latter plasmids were nonhemolytic on blood agar plates but showed significant levels of intracellular hemolytic activity when the cells were gently lysed by using the protocol of Koshland and Botstein (8). The hemolytic activities of both strains are similar, and in the active growth phase, the activities reached a level comparable to the total hemolytic activity of extracellular and intracellular hemolysin found in *E. coli* 5K carrying pANN202-812 (Table 1). The internal hemolytic activity of the latter strain was very low. This is in agreement with the observation that internal HlyA protein (110 kilodaltons) can be detected by immunoblotting of cellular proteins with specific anti-HlyA antibody in *E. coli* carrying pANN202-812/17 but not in *E. coli*(pANN202-812) (Fig. 2A and B).

Localization of internal and external HlyA. Immunoelectron microscopy by means of an indirect immunogold labeling method was applied to localize intracellular and extracellular hemolysin. The specificity of the applied technique was controlled with the nonhemolytic *E. coli* 5K strain which exhibited no gold labeling by either the pre- or the postembedding technique. Figure 3a shows *E. coli* 5K cells treated with a mouse polyclonal anti-HlyA antibody developed with goat anti-mouse immunoglobulin G antibody coated with gold according to the preembedding procedure. Similarly, no labeling was obtained when *E. coli* 5K was treated with the postembedding technique (data not shown). Likewise, no immunogold labeling was observed by the preembedding technique with *E. coli* 5K carrying pANN202-812/17. This strain was, however, gold labeled when the postembedding method was applied (Fig. 3b). The gold particles were mainly concentrated along the inner membrane (Fig. 3b). *E. coli* 5K carrying pANN202-812 yielded positive results with both procedures. As expected, the gold particles in these *E. coli* cells were clearly concentrated at the outside of the cell surface when the cells were treated with the preembedding technique (Fig. 3c). The postembedding technique showed similar results.

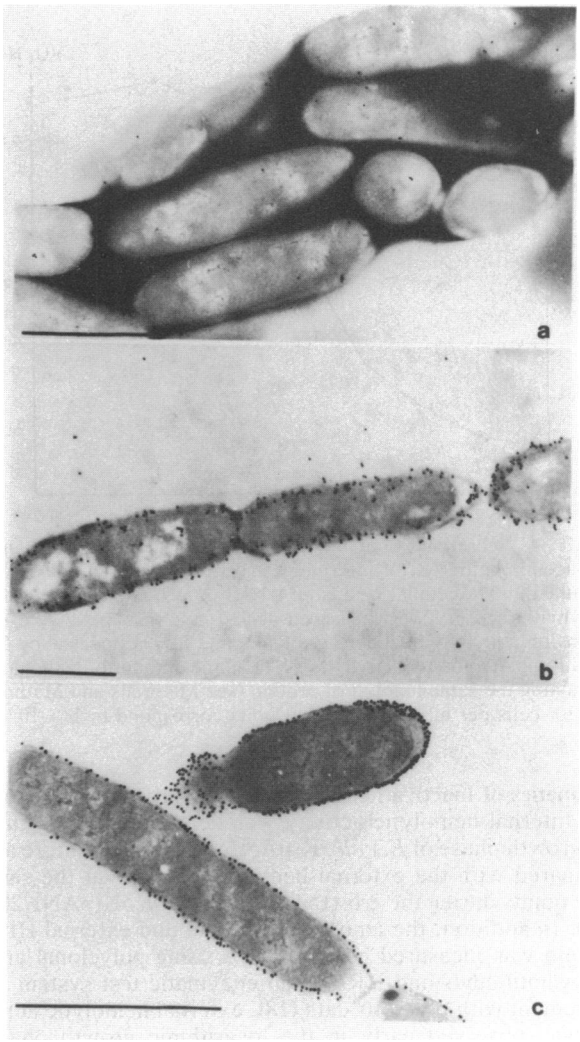

FIG. 3. Localization of hemolysin by immunogold labeling by using polyclonal anti-HlyA antibodies. Neither the preembedding (a) nor the postembedding technique (data not shown) showed any labeling of *E. coli* 5K cells. Positive labeling of *E. coli* 5K carrying pANN202-812/17 was obtained only by the postembedding technique when the cells were thin sectioned (b). *E. coli* cells carrying pANN202-812 were already labeled by the preembedding technique (c). Labeling of HlyA was always performed by the indirect immunogold technique (5). Bars, 1 μm.

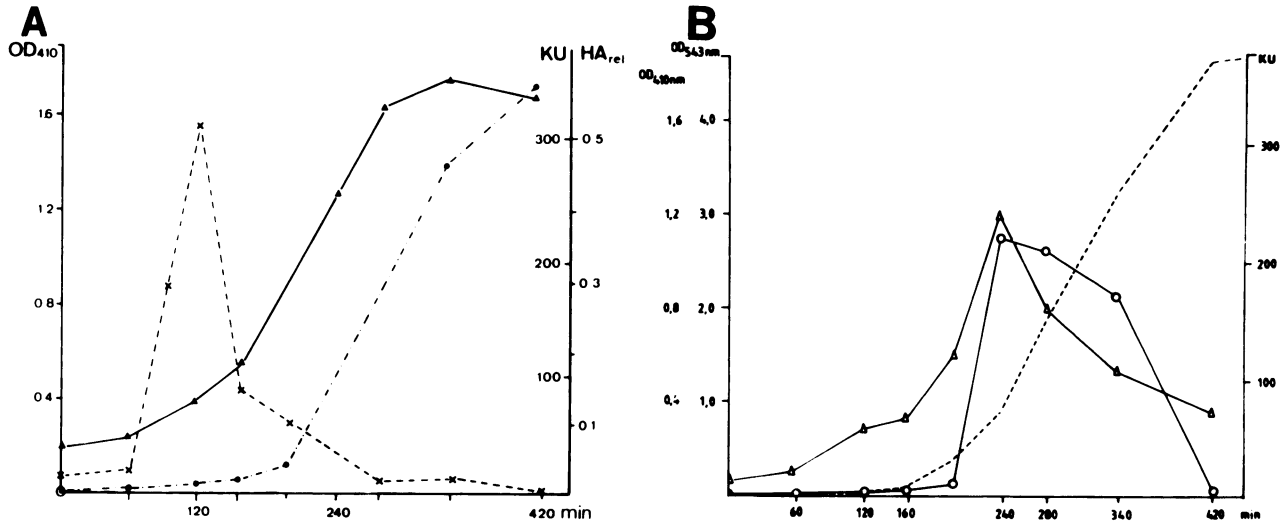

FIG. 4. Secretion (A) of active extracellular hemolysin (×) and HlyA protein (▲) by *E. coli* 5K carrying pANN202-812 during growth (●) and accumulation (B) of internal active hemolysin (○) and HlyA protein (△) in *E. coli* 5K(pANN202-812/17) during growth (- - - -). The amount of extracellular and internal HlyA protein was determined by an ELISA by using polyclonal anti-HlyA antibodies and HRP. Enzymatic activity was measured at 410 nm. In panel A the relative extracellular hemolytic activity (HA_{rel}) is given as the amount of hemoglobin at an optical density of 543 nm (OD_{543}) released by 50 μl of supernatant, normalized to the same number of bacteria. In panel B the internal hemolytic activity is given as the amount of hemoglobin at an optical density of 543 nm released by 50 μl of the cellular extracts containing the same amount of protein (see Materials and Methods). Cell density is given in Klett units (KU); 100 Klett units correspond to 5×10^7 cells per ml, and 300 Klett units correspond to 1×10^9 cells per ml.

Kinetics of inactivation of extra- and intracellular hemolysin. Internal hemolytic activity was determined throughout the growth phase of *E. coli* 5K(pANN202-812/17) culture and compared with the external hemolytic activity at the same time points during the growth phase of *E. coli* 5K(pANN202-812). In addition, the amount of internal and external HlyA protein was measured by an ELISA using polyclonal anti-HlyA antibodies and HRP as an enzymatic test system. In agreement with previous data (18), external hemolytic activity was observed early in the logarithmic growth phase, reached a maximum during logarithmic growth, and then declined (Fig. 4A). The amount of extracellular HlyA protein measured by ELISA indicates, however, that HlyA protein accumulated in the supernatant in parallel with the cell density up to the point when the culture entered the stationary phase (Fig. 4A). From this time point on, the amount of HlyA protein appeared to remain constant for at least 2 h. These data indicate that the amount of HlyA in the supernatant did not parallel the external hemolytic activity, in agreement with our previous results (18). The HlyA protein isolated from cells harvested at the time when the external hemolytic activity reached its peak and the HlyA protein from cells harvested in the stationary growth phase (low level of hemolytic activity) had indistinguishable sizes on SDS-polyacrylamide gels (Fig. 2A).

Internal hemolytic activity in an *E. coli* 5K(pANN202-812/17) culture started after a relatively long lag phase, reached a peak in the middle of the logarithmic growth phase, and declined rapidly in the late log phase (Fig. 4B). The amount of internal HlyA protein measured by ELISA roughly followed the internal hemolytic activity. This suggests that the internal HlyA is either proteolytically degraded or converted to a conformation which no longer binds to the polyclonal anti-HlyA antibody. To determine the fate of the intracellular HlyA more precisely, total cellular protein was isolated from *E. coli* 5K carrying either pANN202-812/17 or pANN202-8127 at various time points during growth, separated by SDS-polyacrylamide gel electrophoresis, and electrotransferred to nitrocellulose filters. HlyA was identified by immunoblotting with anti-HlyA antibodies. Figure 5B indicates the accumulation of HlyA protein up to the mid-log phase and the gradual disappearance of HlyA protein thereafter. These data suggest that HlyA is proteolytically degraded in the cell during the late growth phase, in

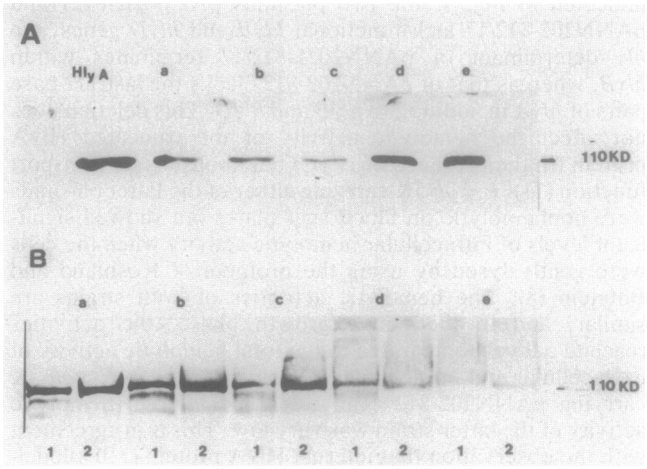

FIG. 5. Accumulation of extracellular HlyA protein in the supernatant (A) and internal HlyA (B) at different time points during growth of the bacteria. In panel A *E. coli* 5K carrying pANN202-812 was used, and in panel B *E. coli* 5K carrying either pANN202-812/17 (1) or pANN202-8127 (3) was used. Samples were taken at cell densities (cells per milliliter) of 2×10^7 (a), 5×10^7 (b), 2×10^8 (c), 6×10^8 (d), and 2×10^9 (e). In panel A equal volumes of concentrated supernatants and in panel B equal amounts of total cellular protein were applied and electrophoresed on SDS-polyacrylamide gels. HlyA protein (110 kilodaltons [kD]) was visualized by immunoblotting with polyclonal anti-HlyA antibodies.

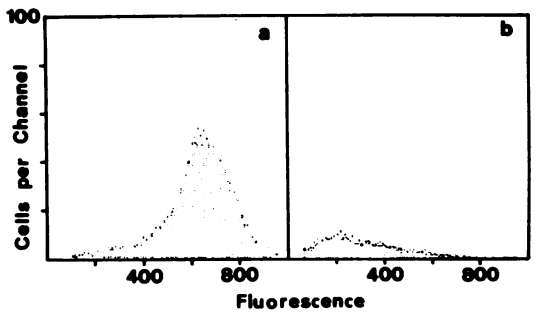

FIG. 6. Measurement of binding of active hemolysin (a) and inactive HlyA (b) to HRBC in a fluorescence-activated cell sorter. Hemolysin was labeled with polyclonal anti-HlyA antibodies and fluorescein-isothiocyanate-coupled anti-mouse antibodies. The HRBC-HlyA complex in panel a shows a high fluorescence intensity of about 680 (relative U) when measured at a excitement wavelength of 488 nm. The low fluorescence intensity in panel b is probably caused by binding of unspecific immunoglobulin to HRBC. The used inactive HlyA was obtained from the culture supernatant of an *E. coli* 5K strain carrying a pANN202-812 derivative from the supernatant of a late-stationary phase culture. An *E. coli* 5K strain carrying a pANN202-812 derivative which lacks functional *hlyC* gave similar results.

contrast to external HlyA from *E. coli* 5K carrying pANN202-812 (Fig. 5A).

Inactive external HlyA did not bind to erythrocytes. As shown above, hemolytically inactive HlyA accumulated in the supernatant of stationary cultures of *E. coli* 5K(pANN202-812) (Fig. 4A). Likewise, hemolytically inactive HlyA is secreted into the medium when HlyC is inactivated (18). Inactive HlyA was isolated from both sources, and its binding affinity to HRBC was determined by binding anti-HlyA antibody and fluorescein isothiocyanate-conjugated anti-mouse antibody to HlyA. The amount of HRBC-HlyA-antibody complex formed was then measured in a fluorescence-activated cell sorter. Figure 6a shows that hemolytically active HlyA bound to HRBC, in contrast to inactive HlyA isolated from the supernatant of stationary cultures of *E. coli* 5K carrying pANN202-812, which did not bind to HRBC (Fig. 6b). Likewise, no binding was obtained with hemolytically inactive HlyA isolated from the supernatant of a *hlyC*-deficient *E. coli* strain.

DISCUSSION

Alpha-hemolysin of *E. coli*, encoded by *hlyA*, requires the action of another gene product, HlyC, in order to become a cytolytically active, i.e., pore-forming, protein (6, 10, 13). This activation of HlyA by HlyC is independent of the transport of hemolysin across the inner and/or the outer membrane (11, 13). The predominant localization of internal active hemolysin (HlyA*) at the inner membrane as visualized by electron microscopy of immunogold-labeled HlyA suggests that this event may take place at the cytoplasmic side of the inner membrane. The binding of HlyA to the membrane may occur via its amphiphilic N terminus (1). From the presented data, it cannot be excluded, however, that binding of HlyA to the membrane occurs only after its activation by HlyC. The accumulation of a relatively large pool of active internal hemolysin in the logarithmic growth phase suggests further that this cytolytic protein does not damage the membrane of the producing bacterial cell.

The data indicate that *E. coli* strains lacking both genes *hlyB* and *hlyD* synthesized similar amounts of HlyA protein during the early logarithmic growth phase as a strain carrying all four *hly* genes. It is more likely that the higher instability of the internal hemolysin protein compared with external hemolysin and not the possible involvement of HlyB and/or HlyD in the transcriptional or posttranscriptional activation of HlyA is responsible for the somewhat lower total hemolytic activity in the absence of HlyB and HlyD. While plasmid pANN202-812/17 still possesses the intergenic region between *hlyA* and *hlyB* and part of *hlyB*, plasmid pANN202-8127 lacks these sites entirely. Yet, the expression of both HlyA proteins was equally efficient in *E. coli* 5K in the early to mid-logarithmic phase. The situation is clearly different in the late logarithmic and the stationary growth phase. Both extracellular and intracellular hemolytic activity declined when the producing *E. coli* cells entered this growth phase. The internal hemolytic activity was lost, however, at a higher rate than the extracellular activity. Inactivation of the two pools of hemolysin appears to occur by different mechanisms. Inactivation of extracellular hemolysin seems to be caused by the decay of a complex which may involve, in addition to HlyA, negatively charged phospholipids (18). Proteolytic degradation of HlyA did not occur. In contrast, internal HlyA was proteolytically degraded during inactivation of internal hemolytic activity. It cannot be excluded from our data that the proteolysis of internal HlyA is also preceded by the decay of a hemolytically active complex, rendering the HlyA protein more accessible to intracellular proteases which are absent in the extracellular environment.

The affinities of internal and external hemolysin for erythrocytes were similar, as shown by binding of hemolysin labeled with fluorescent anti-HlyA antibodies to HRBC and measurement of the formed complexes by flow cytometry. Regardless of whether it derives from a stationary-phase culture supernatant (Fig. 6b) or from an *E. coli* strain which is deficient in *hlyC* (data not shown), hemolytically inactive HlyA protein is unable to bind to HRBC and possesses an altered isoelectric point (13, 19; F. Garcia, T. Jarchau and W. Goebel, unpublished results).

Taken together these data allow the following conclusions. (i) HlyC is required for the formation of the hemolytically active complex (HlyA*), and inactivation of extracellular hemolysin may represent the reversible process. (ii) Binding of HlyA to HRBC is only accomplished if the protein is in the hemolytically active form (HlyA*), as previously suggested (10). (iii) HlyA* is already generated inside the cell, possibly at the cytoplasmic side of the inner membrane, and is subsequently transported by the specific secretion machinery consisting of HlyB and HlyD. The activation of HlyA, however, is not required for the recognition and transport of HlyA by the HlyB/HlyD system, since HlyA not activated by HlyC or fusion proteins which possess the C terminus of HlyA only are transported by HlyB/HlyD with similar efficiency (12; J. Hess, T. Jarchau, and W. Goebel, unpublished results).

ACKNOWLEDGMENTS

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