

## *Escherichia coli* Hemolysin Is Released Extracellularly without Cleavage of a Signal Peptide

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**A 110-kilodalton polypeptide isolated from cell-free culture supernatants of hemolytic *Escherichia coli* is shown to be associated with hemolytic activity. The relative amount of the extracellular 110-kilodalton species detected directly reflects the extracellular hemolysin activity associated with *Escherichia coli* strains harboring different hemolysin recombinant plasmids. The predicted molecular mass of the hemolysin structural gene (*hlyA*) based on DNA sequence analysis was 109,858 daltons. Amino-terminal amino acid sequence analysis of the 110-kilodalton polypeptide provided direct evidence that it was encoded by *hlyA*. Based on this information, it was also demonstrated that the HlyA polypeptide was released extracellularly without signal peptidase-like cleavage. An examination of hemolysin-specific polypeptides detected by use of recombinant plasmids in a minicell-producing strain of *Escherichia coli* was performed. These studies demonstrated how hemolysin-associated 110- and 58-kilodalton polypeptides detected in the minicell background could be misinterpreted as a precursor-product relationship.**

Certain *Escherichia coli* strains synthesize extracellular proteins which are lytic or toxic to other cells. It is not always evident where in the cell these proteins are translated and by what mechanism(s) they are released. Evidence suggests that enterotoxins contain amino-terminal (N-terminal) signal sequences, and that their export involves leader peptidase processing during passage through the membrane (3, 18, 22, 23). In contrast, certain colicins do not contain signal sequences and are released by lysis of the producing cell (9, 27). A different export event appears to occur with the biosynthesis of the *E. coli* hemolysin. Four cistrons are necessary for the hemolytic phenotype (4, 16, 26). Hemolysin synthesis is predicted to be in the cytoplasm (24), and a model for the extracellular secretion of active hemolysin (*hlyA* gene product) suggests that three proteins (HlyC and two transport-associated gene products) are required (16, 26). The exported protein is believed to be a 58-kilodalton (kd) active species derived from proteolysis of a 107-kd precursor during the secretion process (7, 8).

Williams (31) previously provided evidence based on exclusion chromatography that the *E. coli* hemolysin protein was ca. 120 kd. Recently, Mackman and Holland (13) showed that a 107-kd polypeptide, the putative hemolysin precursor of the Goebel model, was efficiently released from hemolytic *E. coli*. These investigators provided indirect evidence that this polypeptide was the active hemolysin encoded by the *hlyA* cistron (13, 14). Therefore, these studies provided evidence contradicting the proteolytic processing model of Goebel. In this report, we present evidence supporting the findings of Williams and of Mackman and Holland. DNA and amino acid sequence data for hemolysin show that the *hlyA* gene product was released from the cell without N-terminal processing analogous to signal peptide cleavage; only the formylated methionine was missing.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1.

**Culture growth.** All strains were grown in M63 minimal medium supplemented with glucose, thiamine, and MgSO<sub>4</sub> as described by Miller (15). Chloramphenicol (8 to 9 µg/ml) was added to maintain selection for the recombinant plasmids. Cultures were incubated at 37°C in a shaking water bath. Growth of the broth cultures was monitored by measuring changes in optical density at a wave length of 600 nm by using a Spectronic 20 (Bausch & Lomb, Inc.).

**Protein gels.** Culture supernatants were obtained by centrifugation of the broth cultures (5,000 × *g*; 12 min; 4°C). Cell-free supernatants were acquired by filtration of the supernatant through a 0.2-µm Acrodisk (Gelman Sciences, Inc.). The proteinaceous material present in the cell-free supernatant was precipitated with either acetone (20% final concentration) or trichloroacetic acid (TCA; 10% final concentration) at 0 to 4°C for >1 h. The precipitated material was harvested by centrifugation (7,800 × *g*; 15 min; 4°C). The pellets were resuspended in either 1 volume of water (acetone precipitates) or 1 M Tris (pH 9.0) (TCA precipitates) and an equal volume of 2× sample buffer (0.15 M Tris-hydrochloride [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol, 0.14% bromophenol blue). The sample was incubated at 95°C for 5 min and then subjected to SDS-polyacrylamide gel electrophoresis. Sample volumes did not exceed 20 µl. The discontinuous buffer system of Laemmli (10) was employed, with 1.0-mm-thick slab gels with a 10% resolving gel and a 4% stacking gel. Samples were subjected to a current of 25 mA until the bromophenol blue dye entered the resolving gel, and electrophoresis was continued at a constant current of 5 mA for ca. 12 h. Gels were fixed and stained by using the silver staining procedure of Wray et al. (33).

**Protein concentration.** When liter volumes of a cell-free culture supernatant were needed (i.e., amino acid sequence analysis), a 0.45-µm filter (Nalgene Labware Div., Nalge/Sybron Corp.) was used to remove cells. The volume of this cell-free supernatant was reduced by use of a pressurized stir cell apparatus (Amicon Corp.). A variety of filter membranes (XM300, YM100, or YM30) were used in ultrafiltration. According to the manufacturer, the nominal molecular weight cut-off for proteins with these filters is 300,

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TABLE 1. Bacterial strains and plasmids

Laboratory strain designation	Host	Plasmid	Reference or Source
J198			(28)
MC4100			(1)
WAF260	J198	pANN202-312	(28)
WAF270	J198	pSF4000	(28)
WAM331	J198	pWAM222	(29)
WAM485	J198	pACYC184	Our laboratory
WAM359	MC4100	pSF4000	Our laboratory
WAM360	MC4100	pANN202-312	Our laboratory
WAM472	MC4100	pACYC184	Our laboratory
DS410			(11)
WAF165	DS410	pACYC184	Our laboratory
WAF167	DS410	pSF4000	Our laboratory
WAF114	DS410	pSF4000 $\Delta$ BamHI	(30)
WAF173	DS410	pSF4000::TnI(47)	Our laboratory

100, and 30 kd, respectively. The stir cell was pressurized with nitrogen gas (15 to 17 lb/in<sup>2</sup>), and ultrafiltration was conducted at 4°C. In some instances, the retentate was subjected to successive 10-ml washes with M63 medium.

**DNA and amino acid sequence analysis.** The DNA sequence of the N-terminal portion of the *hlyA* cistrons from pSF4000 and pANN202-312 was determined by the dideoxy-chain termination method of Sanger (20). The specifics of this analysis are described elsewhere (4). N-terminal amino acid sequence analysis was conducted on the concentrated cell-free supernatant material (YM100 membrane) from 1 liter of WAF270 broth culture. Approximately 0.5 nmol of the 110-kd polypeptide was acquired and then applied to a gas-phase sequencer (Applied Biosystems model 470A). The PTH-amino acid obtained from each Edman degradation was analyzed by reverse-phase high-pressure liquid chromatog-

raphy (R. L. Niece, personal communication). Each sequencing run was done in duplicate, and the results shown (see Fig. 3) were acquired from two preparations of the 110-kd polypeptide.

**Minicell analysis.** The details on the strains and methods employed in minicell analysis of plasmid-encoded polypeptide synthesis are essentially those of Levy (11). The details of our modification of that technique are described elsewhere (4).

## RESULTS

**Polypeptides identified in cell-free culture supernatants.** The polypeptide profile of mid-log-phase, cell-free culture supernatants from hemolytic strains detected by silver-stained SDS-polyacrylamide gels is shown in Fig. 1. The hemolytic strain WAF270 (lane 2) displayed a major polypeptide band ca. 110 kd in size that could not be seen in the control strain (WAM485; lane 1). These results are comparable with those published by Mackman and Holland (13). A detectable 110-kd polypeptide species was not readily apparent for WAF260 unless a 20-fold greater volume of culture supernatant was prepared (Fig. 1, lane 3). This was consistent with results from hemolysis assays, where WAF270 had a much greater hemolytic activity than WAF260 (29). To strengthen the association of the 110-kd polypeptide with the hemolysin encoded by pANN202-312, a recombinant derivative of pANN202-312 expressing increased hemolytic activity was examined. WAM331 harbors a plasmid in which a 937-base-pair *AvaI* fragment from pSF4000 containing the promoter region for at least *hlyC* and the 48 N-terminal amino acids of *hlyC* has been inserted in frame within the *hlyC* cistron of pANN202-312 (29; R. A. Welch and S. Pellett, manuscript in preparation). This construct not only was more virulent to animals (29), but also produced increased amounts of the extracellular 110-kd polypeptide (Fig. 1, lane 4).

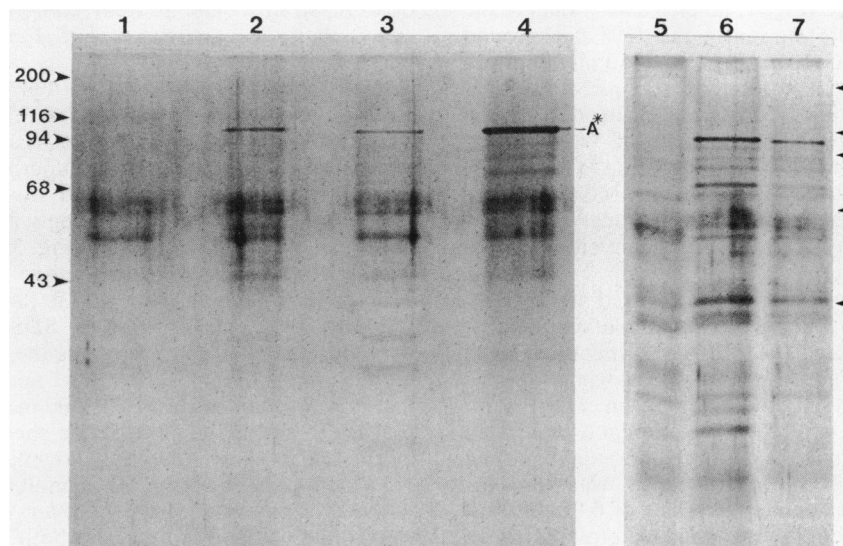


FIG. 1. Polypeptide profiles. Shown are the polypeptide profiles of cell-free supernatants from different hemolytic strains that are detected with silver-stained SDS-polyacrylamide electrophoresis gels. Cultures were harvested (optical density at 600 nm, 0.75 to 0.80), and supernatants were prepared as described in the text. Lanes 1, 2, and 4 contain the acetone-precipitable material from 10 ml of culture supernatant from strains WAM485, WAF270, and WAM331, respectively. Lane 3 contains the acetone-precipitable material from 200 ml of culture supernatant from strain WAF260 initially concentrated 20-fold by using a YM30 filter in a pressurized stir cell (see the text). Lanes 5, 6, and 7 represent the acetone-precipitable material from 10 ml of culture supernatant from strains WAM472, WAM359, and WAM360, respectively. The 110-kd polypeptide is indicated by A\*. The molecular mass standards are indicated by arrows, and the values are expressed in kd.

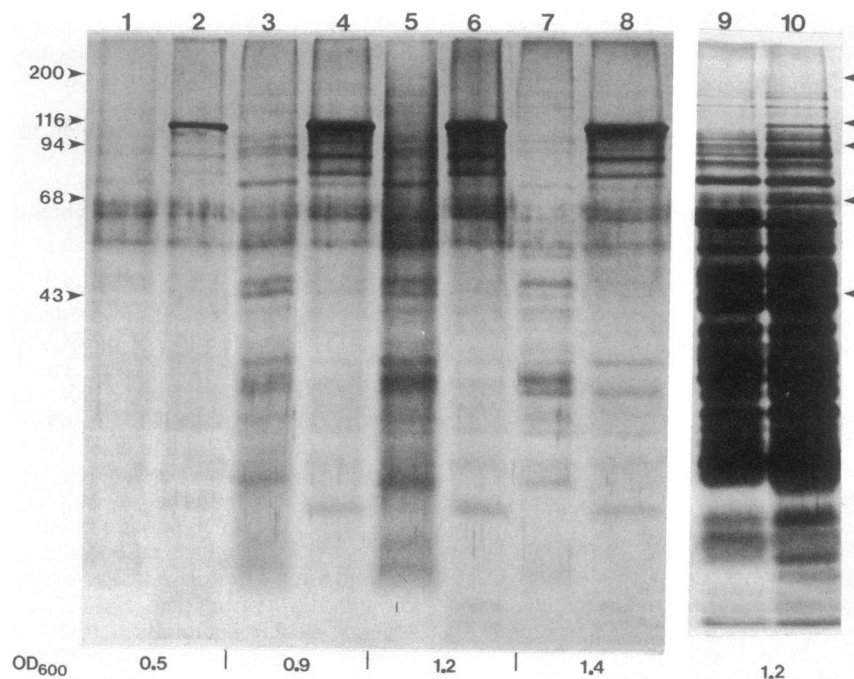


FIG. 2. Accumulation of supernatant protein during growth. This gel depicts the TCA-precipitable material from 10-ml samples of cell-free supernatant from WAM485 (lanes 1, 3, 5, and 7) and WAF270 (lanes 2, 4, 6, and 8) at various points throughout the cell growth curve. TCA-precipitable material from 10-ml samples of WAM472 and WAM359 is shown in lanes 9 and 10, respectively. The optical density at 600 nm ( $OD_{600}$ ) of the cultures is indicated at the bottom of the figure. The optical densities shown for lanes 7, 8, 9, and 10 represent stationary-phase cells which remained at the value for >2 h before being harvested.

We also observed that the amount of the extracellular 110-kD polypeptide detected was related to the strain in which a hemolysin-encoding plasmid resides. We detected a greater amount of the 110-kD polypeptide and an increase in the number of other polypeptide species in culture supernatants of the K12 strain MC4100 transformed with either pSF4000 or pANN202-312 (Fig. 1, lanes 6 and 7) than in the supernatants of hemolytic strains in the J198 background (lanes 2 and 3). In the MC4100 background, 10 ml of culture supernatant was sufficient to detect the pANN202-312-encoded 110-kD polypeptide species (lane 7). In addition, the zones of hemolysis surrounding colonies of WAM360 on blood agar were larger than those surrounding WAF260 (T. Felmlée, unpublished data). Using either the MC4100 or the J198 background, we did not detect an extracellular 58- to 60-kD polypeptide unique to strains harboring either hemolysin recombinant plasmid.

Export of the 110-kD polypeptide occurred without increased cellular breakdown (Fig. 2). We hypothesized that hemolytic strains producing the 110-kD polypeptide would show an increase in total supernatant proteins when the cells were partially or totally lysed during hemolysin export. With J198 as a background strain, little difference was seen between control and hemolytic culture supernatant protein profiles (Fig. 2, lanes 1 through 8). This was true for supernatants harvested at optical densities of 0.9 to 1.0, at which time hemolytic activity has been determined to be maximal (13, 24). Supernatants examined from early (lanes 5 and 6) and late (lanes 7 and 8) stationary-phase cultures appeared not to have had an increase in protein content. Comparison of control and hemolytic supernatant proteins from cultures at the same optical density revealed that some protein bands seen for WAM485 supernatants were absent or inconspicuous for WAF270 samples. At present, we have

no explanation to account for this observation. The principal polypeptide released from the hemolytic strain was the 110-kD species. There were two other bands at 92 and 85 kD which appeared to be associated with the presence of the 110-kD species. Examination of protein obtained from cultures of MC4100 strains revealed a different pattern than that of J198 strains. The protein content of MC4100 culture supernatants was increased (Fig. 1, lanes 5 and 6; Fig. 2, lanes 9 and 10).

**Partial purification of the 110-kD protein.** A mid-log-phase culture supernatant from strain WAF270 was used to recover microgram quantities of the 110-kD polypeptide. Concentration of the proteins in the supernatant was achieved by ultrafiltration with a pressurized stir cell. A YM100 filter retained the 110-kD species, along with a number of lower-molecular-weight polypeptides (Fig. 3, lane 3). The material passing through the filter membrane was found to contain polypeptides of less than 70 kD (lane 4). Based on the similarity of their migration in SDS-polyacrylamide gels, these polypeptides appeared to be the same as those present in the supernatant of J198 (lanes 1 and 4). It was also found that ultrafiltration with the XM300 membrane did not allow passage of any of the polypeptide species normally retained by the YM100 filter (data not shown).

To determine whether the hemolytic activity was being retained or passed through the filter, hemolysin assays (29) were done at various times on both the retentate and the filtrate during the concentration procedure. At no time was hemolytic activity detectable in the filtrate. We subjected the retained material to washes with M63 medium. The filtrates from these washes still did not have detectable hemolysin activity. All detectable activity was found in the material retained by the filter, but this activity diminished over time. Marked decreases in hemolytic activity occurred with each

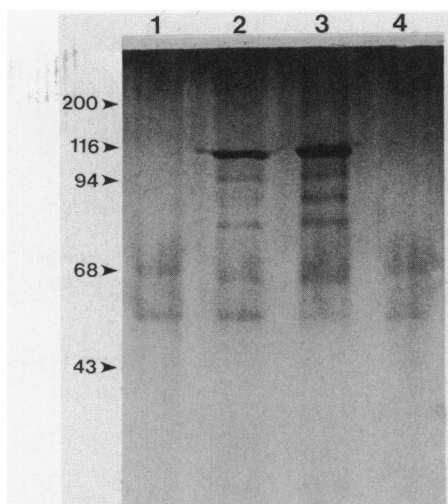


FIG. 3. Concentration and purification of the 110-kd polypeptide from strain WAF270. Cultures were harvested at an optical density at 600 nm of 0.75 to 0.8. Lanes 1 and 2 show acetone-precipitable material from 10 ml of culture supernatant from strains J198 and WAF270, respectively. Lane 3 contains one-ninth of the precipitated material from 100 ml of a WAF270 culture supernatant concentrated 10-fold with a YM100 filter in a pressurized stir cell. Lane 4 shows the precipitated material from 10 ml of filtrate which passed through the YM100 membrane during the concentration of the material in lane 3. The migration pattern of these bands did not change when 2-mercaptoethanol was left out of the electrophoresis sample buffer (data not shown).

successive wash, and the lability of this activity has been noted by others (24). Hemolysin activity has been reported to be partially stabilized by addition of hemoglobin (24). We repeated the concentration procedure with hemoglobin (0.1 mg/ml) present in the growth medium and washes. This modification stabilized the hemolytic activity in the retentate, but still there was no detectable hemolytic activity in the filtrate or washes.

**DNA and amino acid sequence analyses.** The gene (*hlyA*) encoding the structural protein for hemolytic activity has been defined physically and genetically (7, 30). The DNA sequence of the *hlyA* gene of pSF4000 is known, and the predicted molecular mass of the HlyA polypeptide was 109.8 kd (4). The molecular mass of the *hlyA* gene product is similar in size to the 110-kd polypeptide seen in culture supernatants of hemolytic *E. coli*. The DNA sequence of the N termini of pSF4000 and pANN202-312 *hlyA* genes is shown in Fig. 4. Consideration of the first 24 predicted amino acids suggests that in either case the *hlyA* polypeptide lacks a signal sequence similar to that observed for other procaryotic secreted proteins (19, 21, 25). We set out to determine

whether the 110-kd polypeptide seen in culture supernatants was in fact the *hlyA* gene product and to define the extent of N-terminal processing of the *hlyA* gene product by subjecting the polypeptide to automated sequential Edman degradations. The results of this analysis are shown in Fig. 4, row 5. Based on the identity of the first nine N-terminal residues compared with the predicted amino acid sequence, the only detectable N-terminal alteration was the lack of a formylated methionine.

**Minicell preparations.** The analysis of patterns of <sup>35</sup>S-labeled polypeptides encoded by *E. coli* hemolysin recombinant plasmids and their mutant derivatives in *E. coli* minicells is difficult. The predicted encoding capacity of pSF4000 cannot account for all the labeled polypeptides observed. For example, in Fig. 5, lane 2, there are at least 10 major polypeptide species specific for the 11.7-kilobase *SalI* insert of pSF4000 (30). If each polypeptide represents a unique gene, then DNA greater than 15 kilobases in length would be required for their synthesis, assuming there are no overlapping reading frames. Four to five polypeptide species ranging in size between 58 to 110 kd were not produced by a mutant derivative of pSF4000 when the ampicillin resistance transposon *TnI* was inserted in the N-terminal region of *hlyA* (Fig. 5, lane 4) (30). We demonstrated using minicells that when a *TnI* unit is present in the middle of the *hlyA*-encoding region, an apparent truncated form of the HlyA polypeptide, along with an array of polypeptides smaller than the truncated form, is synthesized (4). The array of 58- to 100-kd polypeptides were still observed in a derivative of pSF4000 where *hlyC* was removed by a *Bam*HI deletion (Fig. 5, lane 3). This information indicates that the array of 58- to 110-kd polypeptides is associated with *hlyA* expression and is not dependent on *hlyC* expression.

DISCUSSION

The precipitation of proteins present in supernatants of broth cultures of hemolytic *E. coli* resulted in our detection of a 110-kd polypeptide associated with hemolytic activity. Using DNA and amino acid sequence analyses, we showed the polypeptide to be the gene product of *hlyA*, the gene defined to encode the structural hemolysin protein (4, 7). The amount of this polypeptide detected in culture supernatants was directly related to the age of the culture, since greater amounts of the polypeptide were detected in late-log and stationary-phase cultures (Fig. 2). The number and amount of smaller polypeptides (<80 kd) in the culture supernatant increased with the later stages of growth. This "background" of protein bands was no more prominent in supernatants of hemolytic strains than in those of nonhemolytic strains. We did observe an increase in the higher-molecular-weight species (>80 kd) over the growth phase, which we speculate represents proteolytic breakdown products of the hemolysin. Because hemolysin is not

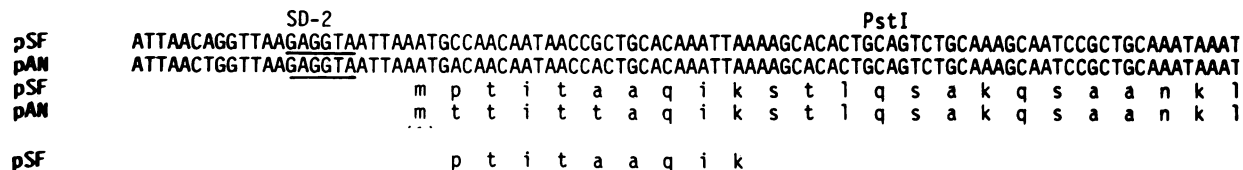


FIG. 4. DNA and amino acid sequence analysis of the *hlyA* gene and the 110-kd protein. The first 72 base pairs of the N-terminal region of *hlyA* from pSF4000 and pANN202-312 are shown in the first two rows. The predicted amino acid sequence (represented by the standard single letter code) from these genes is shown in rows 3 and 4. The N-terminal amino acid sequence of the pSF4000-encoded 110-kd polypeptide is shown in row 5. The SD-2 designation above the pSF4000 DNA sequence indicates the likely ribosomal binding site on the *hlyA* mRNA.

released in conjunction with an increase in other protein species, we conclude that hemolysin export occurs in the absence of cellular breakdown.

It is interesting to note that there are differences in the amount of hemolysin released by different strains harboring the same hemolysin recombinant plasmids. Compared with J198 strains harboring the hemolysin plasmids, the colonies of MC4100 hemolytic strains had larger zones of hemolysis on blood agar, and this is directly associated with greater amounts of the 110-kd hemolysin protein detected in the cell-free culture supernatants. Whether this phenomenon is due to enhanced hemolysin synthesis or release of the hemolysin in the MC4100 background awaits further experimentation.

The observation that the 110-kd polypeptide was unable to pass through an XM300 filter may suggest that the active hemolysin appears larger than a simple 110-kd monomer. If it is a multimer of the 110-kd polypeptide, it is not held together by disulfide bridges, since DNA sequence analysis revealed no cysteine residues in *hlyA* (4). The apparent large size of the active hemolysin may be explained in part by the presence of associated lipid and carbohydrate material that we have not yet identified. Work in the laboratory of I. S. Snyder does in fact suggest that the active extracellular hemolysin is greater than 400 kd in molecular mass and has lipids and carbohydrates noncovalently associated with a 107-kd polypeptide (2). Clearly, further biochemical characterization is needed to understand the structural and functional aspects of the hemolysin.

Our DNA and amino acid sequence data provided direct evidence that the 110-kd protein was being released without cleavage of a signal sequence. It appeared that only the formylated methionine was missing from the N terminus of the hemolysin. The absence of formylated methionine is a common occurrence among *E. coli* proteins (12). Therefore, the *hlyA* gene product passed through the *E. coli* cell envelope without processing of a signal-like sequence. Proteins without N-terminal signal sequences have been shown to insert into the cytoplasmic membrane (32), and others have reported the export of flagellar proteins which also lack N-terminal signal peptides (6). Although the work of Hartlein et al. (8) also suggests the absence of an HlyA signal sequence, they concluded that the active hemolysin protein is a 58-kd protein uniquely processed from a 107-kd precursor. We cannot make the same conclusion based on our work with the isolated extracellular hemolysin produced by vegetative cells. The putative proteolytic processing event that these investigators observed and which we have reproduced appears to be associated with hemolysin recombinant plasmids present in the mini- and maxicell systems. These systems are known to be inefficient in protein secretion (21). The predicted slow export of the *hlyA* gene product in these systems may subject it to the proteolytic breakdown that was observed. From our studies with strains WAF260, WAF270, and WAM331, the major polypeptide apparent in broth cultures at the time of appearance of hemolytic activity was the 110-kd HlyA polypeptide. We observed two additional polypeptides (92 and 85 kd) in the hemolysin-containing cell-free supernatants. These may represent two different proteins exported along with the hemolysin or may be proteolytic products of the hemolysin. The resolution of this question awaits the generation of hemolysin-specific antibody.

It has been hypothesized that the *hlyC* gene acts as a hemolysin-activating protease (7, 16). The examination of different mutant derivatives of pSF4000 in a minicell-produc-

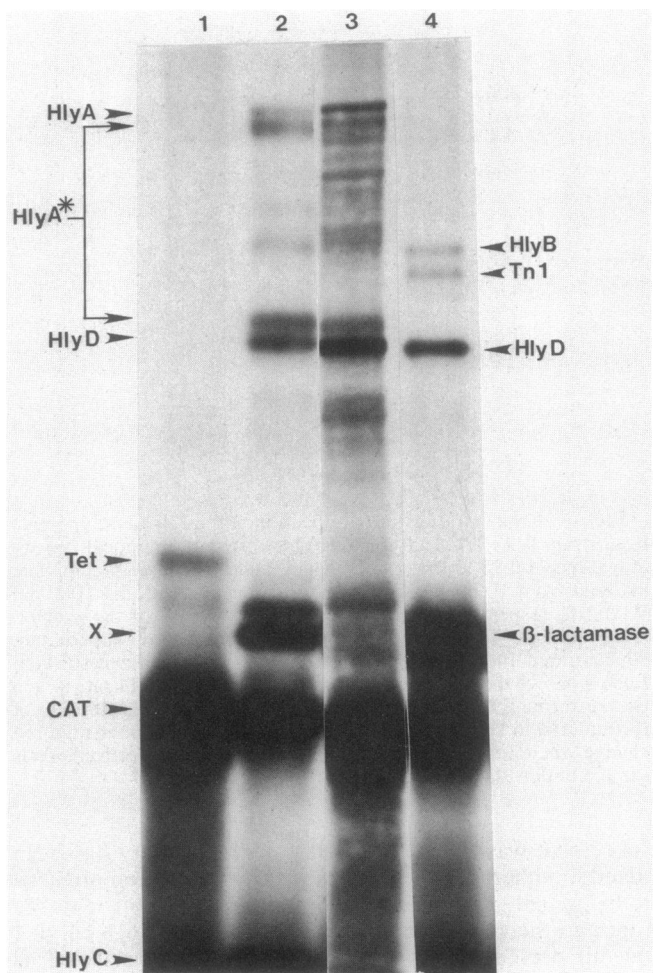


FIG. 5. pSF4000-specific polypeptides detected in *E. coli* minicells. Depicted is a fluorogram of an SDS-polyacrylamide electrophoretic gel containing [<sup>35</sup>S]methionine-labeled polypeptides synthesized by plasmids present in isolated *E. coli* minicells. Shown are the polypeptides synthesized by the vector plasmids pACYC184 (lane 1), pSF4000 (lane 2), pSF4000Δ*Bam*HI (lane 3), and pSF4000::Tn1(47) (lane 4). The arrows designate the following polypeptides: HlyA, 110 kd; HlyB, 79 kd; HlyD, 55 kd; HlyC, 19 kd; Tet, the 34-kd membrane protein involved in tetracycline resistance; X, a pair of 31- and 32-kd polypeptides of unknown function encoded outside the pSF4000 hemolysin region; CAT, the 21-kd chloramphenicol acetyl transferase; Tn1, a 70-kd polypeptide associated with different Tn1 insertions in pSF4000; and β-lactamase, which designates the precursor and mature forms of the Tn1-encoded β-lactamase. The bracketed region designated HlyA\* represents an array of 58- to 110-kd polypeptides that are tentatively identified as proteolytic breakdown products of HlyA. The clarity of the β-lactamase, CAT, and HlyC bands was lost because a long exposure (10 days) was performed to demonstrate clearly the larger-molecular-weight species. All four lanes were from the photograph of a single gel; intervening lanes have been removed.

ing strain of *E. coli* suggests that the *hlyC* gene product could be eliminated and the putative minicell-related proteolytic breakdown of *hlyA* would still occur. This, combined with our N-terminal analysis of *hlyA*, would rule out a functional role of the *hlyC* gene product as an endoproteolytic processing enzyme of the HlyA polypeptide.

The extracellular release of the *E. coli* hemolysin represents an intriguing cellular event. It would appear to be



analogous to the situation in eucaryotic cells with the secretion of ovalbumin in which there is no cleavage of a signal peptide (17). In fact, there is efficient secretion of recombinant-derived ovalbumin across the cytoplasmic membrane of *E. coli*, suggesting that there is no requirement per se in gram-negative bacteria for a cleavable signal sequence (5). The machinery and events leading to the extracellular transport of the *E. coli* hemolysin remain to be elucidated. Whether it is a true extracellular secretion or simply the peeling away of portions of the outer membrane enriched with the hemolysin protein has to be examined.

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