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Extracellular Pullulanase of *Klebsiella pneumoniae* Is a Lipoprotein

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Pullulanase is a starch-debranching enzyme produced by the gram-negative bacterium *Klebsiella pneumoniae*. In this organism, the enzyme is first exported to the outer membrane and is subsequently released into the growth medium. Evidence reported here indicates that pullulanase is a lipoprotein. It is apparently synthesized as a precursor with a 19-residue-long signal sequence and modified by the covalent attachment of palmitate to the cysteine residue which becomes the amino terminus after cleavage of the signal sequence. In this respect, pullulanase is similar to some penicillinases produced by gram-positive bacteria which are initially exported to the cell surface and subsequently released into the medium. However, pullulanase and the penicillinases differ in one important aspect, namely, that the extracellular pullulanase still carries the covalently attached fatty acyls, whereas extracellular penicillinases lack the modified amino-terminal cysteine together with a limited number of other residues from the amino terminus.

The ability to secrete proteins into the growth medium has long been recognized as a major and exploitable feature of gram-positive bacteria (19, 22). Gram-negative bacteria also release proteins into the growth medium, but relatively few of these proteins have been characterized in any detail, and little is known about the ways in which most of them are secreted (2, 3, 6, 11, 21). One of the most interesting aspects of secretion in gram-negative bacteria is the way in which the protein crosses the outer membrane, since this implies an additional step which does not exist in gram-positive bacteria. One example of a protein secreted by a gram-negative bacterium, described in this study, is pullulanase, a starch-debranching enzyme produced by some strains of *Klebsiella pneumoniae* (2). Pullulanase, a ca. 145,000-dalton polypeptide, is initially localized to the outer membrane and is released into the medium when the cells have completed exponential growth (12, 27). *pulA*, the structural gene for pullulanase, was recently cloned and introduced into *Escherichia coli* (12, 25). The production of pullulanase, in both *K. pneumoniae* and *E. coli* K-12 carrying the cloned *pulA* gene, is induced by growth in the presence of maltose and is positively regulated by the MalT protein, activator of the maltose regulon (1, 12). Sequencing of part of the *pulA* gene revealed the presence of a potential 19-residue-long signal sequence followed by a cysteine residue which would become the amino terminus of the mature protein (1) (Fig. 1). This feature has hitherto only been found in procaryotic lipoproteins in which the cysteine residue is modified by the addition of two fatty acyl groups through ester linkage involving a glyceride residue and by the addition of a third fatty acyl group via an amide linkage (7, 14, 15, 18). Among these lipoproteins are some of the penicillinases of gram-positive bacteria. These enzymes, like pullulanase, can exist as both cell-bound and extracellular forms (10, 16, 24). Proteolytic removal of the modified amino terminus is assumed to be responsible for the release of the penicillinases into the medium. In this study, we demonstrate that pullulanase, like the penicillinases, is indeed a lipoprotein but that it is released into the medium without removal of the covalently attached lipids.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The *K. pneumoniae* strain used for most experiments was ATCC 15050, obtained from the American Type Culture Collection. This strain produces a thick, slimy capsule, part of which remains in suspension when cultures are centrifuged for the removal of cells. Noncapsulated derivates were obtained after mutagenesis with ethyl methane sulfonate as described by Miller (13). Mutagenized cells were plated out on L broth agar, and colonies displaying an unusual, nonmucoid appearance were selected for purification. Auxotrophs were screened out by testing of their growth requirements on minimal M63 glucose agar medium (13), and the remaining 11 mutants were tested for their ability to produce and secrete pullulanase (12). Three of the mutants released substantial amounts of several other proteins and were not tested further. All other mutants produced and secreted pullulanase normally but did not produce capsular slime.

The *E. coli* K-12 strains were all derivatives of MC4100 (*lac ara rpsL*) and carried pACYC184::*pulA* as described previously (12). Strains MM18 {MC4100 λ72-47 [φ(*malE-lacZ*)]} and MM7 (a derivative of MM18, carrying the mutant strain 19-1 signal sequence mutation in the *malE-lacZ* gene fusion) were described by Ito et al. (9) and were supplied by C. Lee and J. Beckwith.

Experiment. All cultures were grown in minimal M63 medium (13) containing 0.4% glycerol. Maltose (0.4%) was added to induce *pulA* gene expression, and Casamino Acids (0.5%; Difco Laboratories, Detroit, Mich.) were added to cultures to be labeled with palmitate or glycerol. Tetracycline (10 μg/ml) was used to maintain pACYC184::*pulA*. All cultures were grown at 30°C with good aeration. Proteins were labeled with ^{14}C-labeled amino acids (50 μCi/ml; CEA, Saclay, France), [^{3}H]glycerol (100 μCi/ml), [^{3}H]palmitate (100 μCi/ml), or [^{14}C]palmitate (50 μCi/ml), all of which were purchased from Amersham Corp., Amersham, United Kingdom. [^{3}H]palmitate was redissolved in *n*-propanol. Where appropriate, labeling was arrested by precipitation of proteins with 10% ice-cold trichloroacetic acid. Triton X-100 (0.5%) was added before trichloroacetic acid to facilitate the precipitation of proteins in cell-free medium, and the pellets were rinsed with 90% ethanol to remove the Triton X-100

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                                                    -4  -3  -2  -1 ↓ +1
Pullulanase. fMet-Leu-Arg-Tyr-Thr-Cys-Asn-Ala-Leu-Phe-Leu-Gly-Ser-Leu-Ile-Leu-Leu-Ser-Gly-Cys-Asp---
Major OM lipoprotein. fMet-Lys-Ala-Thr-Lys-Leu-Val-Leu-Gly-Ala-Val-Ile-Leu-Gly-Ser-Thr-Leu-Leu-Ala-Gly-Cys-Ser---
Penicillinase. fMet-Lys-Leu-Trp-Phe-Ser-Thr-Leu-Lys-Leu-Lys-Lys-Ala-Ala-Ala-Val-Leu-Leu-Phe-Ser-Cys-Val-Ala-Leu-Ala-Gly-Cys-Ala---
                                                Consensus. -Leu-Leu-Val-Ala-Cys----
                                                          (Ala)   Ser Gly
                                                                  Ala
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FIG. 1. Amino acid sequences of the amino termini of the precursors of pullulanase, of *E. coli* K-12 outer membrane Lpp lipoprotein, and of the penicillinase of *Bacillus licheniformis*, as deduced from the DNA sequences of the corresponding genes. The sequences are aligned at the Cys residue which should become the amino-terminal amino acid after cleavage by signal peptidase. The sequences are taken from references 1, 8, and 15. The consensus sequence for the residues immediately preceding Cys+1 in known or putative bacterial lipoproteins is modified from that of Pugsley and Schwartz (22) and is based on 19 known or putative lipoprotein box sequences. The arrow indicates the known or putative signal peptidase cleavage site.

before the precipitates were suspended in sample buffer for electrophoresis. Hydrolysis with NaOH was performed as described by Perumal and Minkley, Jr. (18). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was done essentially as described previously (20), by use of Tris-glycine buffers. After electrophoresis, proteins were fixed in the gel with 10% acetic acid in 40% ethanol and either stained with Coomassie blue or treated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.) or with Amplify (Amersham Corp.) and exposed to Kodak X-Omat AR film at −70°C.

For peptide mapping of pullulanase, a culture of a noncapsulated mutant of *K. pneumoniae* ATCC 15050 was grown in minimal maltose medium and labeled with [^{14}C]palmitate. The cells were harvested in mid-exponential growth phase, broken by sonication, and run on a 9% acrylamide gel. Acrylamide containing the labeled pullulanase was cut from the gel and broken by passage through a 1-ml-volume syringe. The gel fragments were then incubated in 50 mM ammonium carbonate, containing 50 μg of trypsin per ml, at 37°C for 16 h. Acrylamide fragments were then removed by filtration through glass wool, and the filtrate was dried. The residue was dissolved in distilled water, applied to a cellulose thin-layer-chromatography plate (Camag, Multenz, Switzerland), and subjected to two-dimensional chromatography as described by Tsai et al. (26). The plates were then exposed to Kodak X-Omat AR film to detect the [^{14}C]palmitate-labeled peptide(s).

Culture medium for gel filtration was similarly obtained from cultures of a nonmucoid derivative of ATCC 15050. The medium was concentrated by filtration through Amicon Diaflo PM10 filters and was applied to Bio-Rad A5 or A50 agarose columns equilibrated with 50 mM phosphate buffer (pH 7.1). Pullulanase was assayed with the previously described modifications (12) of the procedure of Hope and Dean (5), and proteins in the fractions eluting from the columns were precipitated with trichloroacetic acid and examined by SDS-polyacrylamide gel electrophoresis as described above.

RESULTS

E. coli strains which harbor the *pulA* gene of *K. pneumoniae* synthesize a pullulanase which is identical in size to that produced by *K. pneumoniae* (12). The first piece of evidence for a precursor of pullulanase was obtained with *E. coli* K-12 MM18, which carries a maltose-inducible *malE-lacZ* gene fusion (see Materials and Methods). Expression of this particular gene fusion is known to provoke the accumulation of precursors of many exported proteins, including that of the major outer membrane lipoprotein (4, 9). When strain MM18, carrying a derivative of pACYC184 into which the *pulA* gene had been cloned together with its promoter (12), was grown in the presence of maltose, a new, stable protein with a higher molecular weight than that of pullulanase was detected in addition to the mature form of pullulanase (data not shown). This larger protein was assumed to be the precursor form of pullulanase. This putative pullulanase precursor was not observed with strain MM7, which carries a signal peptide mutation in the *malE-lacZ* gene fusion known to eliminate the adverse effects of *malE-lacZ* expression (9) (data not shown).

Considering that pullulanase might be a lipoprotein, we then investigated whether processing of the precursor was carried out by the lipoprotein signal peptidase. This enzyme is specifically inhibited by the antibiotic globomycin (14). Therefore, we treated *E. coli* K-12 (pACYC184::*pulA*) with globomycin at the same time that we labeled proteins with ^{14}C-labeled amino acids. Globomycin treatment caused the accumulation of pullulanase precursor, which comigrated with the precursor detected after induction of *malE-lacZ*. Accumulation of pullulanase precursor was also observed when *K. pneumoniae* ATCC 15050 was labeled in the presence of globomycin (Fig. 2). In both strains, the precursor could to some extent be chased into the mature form after the removal of the globomycin (Fig. 2).

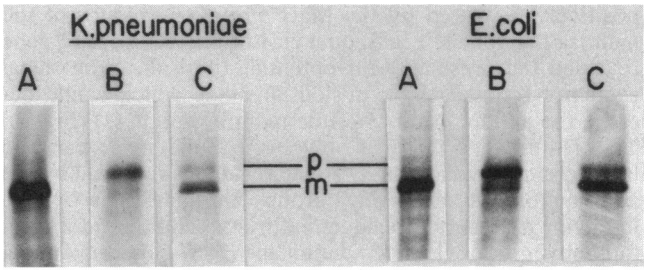

FIG. 2. Production of pullulanase by *K. pneumoniae* ATCC 15050 or *E. coli* K-12 MC4100(pACYC184::*pulA*) in the presence of globomycin. Proteins were labeled with ^{14}C-labeled amino acids, and cells were grown in minimal maltose medium for 3 h before being labeled. Lanes: A, control cells labeled for 10 min in the absence of globomycin; B, cells labeled as described for panel A but in the presence of 150 μg of globomycin per ml; C, cells labeled as described for lane B but then centrifuged, washed, and suspended in fresh medium containing 1% Casamino Acids and 50 μg of chloramphenicol per ml and then incubated for 30 min more. Only that part of the autoradiograph displaying pullulanase is shown. The pullulanase precursor (p) is ca. 3,000 daltons larger than the mature form (m).

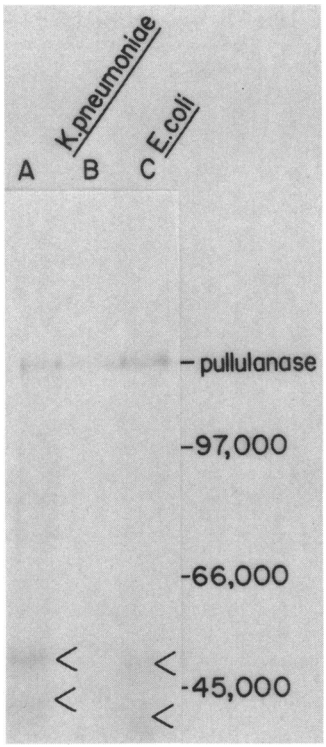

FIG. 3. Incorporation of [^{3}H]glycerol into pullulanase of *K. pneumoniae* (lanes A and B) and *E. coli* K-12(pACYC184::*pulA*) (lane C). The cultures for lanes A and C were grown for 2 h in the presence of [^{3}H]glycerol in minimal maltose medium without added carrier glycerol. Total cell proteins were then separated by SDS-polyacrylamide gel electrophoresis, and [^{3}H]glycerol-labeled proteins were revealed by autoradiography. Note the presence of two other glycerol-containing proteins (indicated by arrowheads). The culture for lane B was incubated under the same conditions for 22 h before proteins in the culture medium supernatant were precipitated with trichloroacetic acid and examined by SDS-polyacrylamide gel electrophoresis and autoradiography.

Further evidence that pullulanase is indeed a lipoprotein was obtained by showing that it could be labeled with [^{3}H]glycerol (Fig. 3) and [^{3}H]palmitate (Fig. 4) in both *K. pneumoniae* and *E. coli* K-12(pACYC184::*pulA*). The possibility that label was being incorporated nonspecifically is ruled out by the fact that pullulanase was the only labeled protein with a molecular weight of 65,000 (we estimate that ca. 10 other proteins with molecular weights between 18,000 and 65,000 were specifically labeled with [^{3}H]palmitate). To test whether only one site in the polypeptide chain was modified by the palmitate, we subjected trypsin-digested, [^{14}C]palmitate-labeled pullulanase to two-dimensional peptide mapping, which revealed the presence of only one labeled peptide (data not shown). Thus, it seems most probable that only the extreme amino-terminal cysteine residue is modified by the attachment of fatty acyl groups. The nature of the palmitate modification was examined by subjecting [^{3}H]palmitate-labeled pullulanase to saponification with NaOH. Approximately 80% of the label was lost after alkali treatment (Fig. 5), suggesting that palmitate is attached by both ester (sensitive to NaOH) and amide (not sensitive to NaOH) linkages.

A surprising feature of the results shown in Fig. 4 is that the extracellular form of pullulanase detected in *K. pneumoniae* cultures also contained labeled palmitate. Moreover, the amount of label recovered from the medium after prolonged incubation relative to that present in the cells at the end of exponential growth was the same, irrespective of whether the pullulanase was labeled with [^{3}H]palmitate or ^{14}C-labeled amino acids (data not shown). These results imply that palmitate-modified pullulanase in the medium is not a minor fraction of the bulk extracellular pullulanase. This, together with the fact that we did not detect any difference in the apparent molecular weights of the cell-associated and free forms of pullulanase (Fig. 6) (12), strongly suggests that the enzyme is released into the medium without proteolysis or removal of attached fatty acyls. It should be noted that [^{3}H]palmitate-labeled pullulanase was detected only in the cells, not in the medium from the *E. coli* K-12(pACYC184::*pulA*) culture (Fig. 4), consistent with previous observations showing that this strain is unable to secrete pullulanase (12, 25).

We next wondered whether pullulanase release from the outer membrane could be explained by the release of pullulanase-enriched vesicles or micelles from the cell surface. Therefore, cell-free medium containing pullulanase was centrifuged at 135,000 × *g* for 4 h, and the supernatant fraction and the pellet were analyzed by SDS-polyacrylamide gel electrophoresis. Approximately 50% of the [^{14}C]palmitate- or ^{14}C-labeled-amino acid-labeled pullulanase was recovered from the pellet, with the rest remaining in the supernatant fraction (Fig. 6 and 7). The

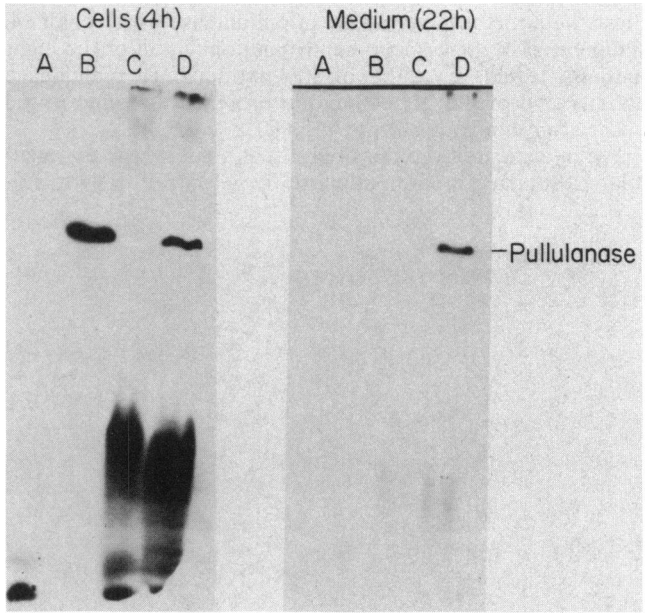

FIG. 4. Incorporation of [^{3}H]palmitate into pullulanase. *E. coli* K-12(pACYC184::*pulA*) (lanes A and B) or *K. pneumoniae* (lanes C and D) cells were labeled with [^{3}H]palmitate which was added during the exponential phase of growth. The cultures for lanes B and D contained maltose to induce pullulanase production. Incubation was continued for 4 h of additional exponential growth before samples of the cells were harvested by centrifugation. The rest of the culture was incubated for 22 h before cell-free proteins were precipitated from the medium with trichloroacetic acid. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The samples were all equivalent to 0.2 ml of the culture. The diffuse, labeled material in lanes C and D is probably lipopolysaccharide; it did not disappear when samples were pretreated with 1 mg of pronase per ml, which completely digested all proteins in these samples.

amount of pullulanase recovered from the pellet was not increased by lengthening the period of centrifugation to 24 h. Therefore, it appeared that extracellular pullulanase consisted of two populations, one possibly free in the medium and the other associated with some sort of vesicles. It should be noted that three other maltose-inducible proteins were also released along with the pullulanase (Fig. 6). One of these proteins is probably the outer membrane LamB maltoporin, the production of which is also maltose inducible, and which is released into the medium in substantial amounts in vesicles, at least by *E. coli* K-12 (C. Braun-Breton, D. Perrin, and M. Hofnung, personal communication). The protein which we identify as maltoporin in Fig. 6 was present only in the pellet after high-speed centrifugation of *K. pneumoniae* culture medium, which is consistent with its being released in vesicles. The identities of the other maltose-inducible extracellular proteins of *K. pneumoniae* ATCC 15050 are unknown at present, but one of them may be the amylase which we detected in very low amounts in these samples. These other two proteins remained entirely in the supernatant fraction after high-speed centrifugation.

The pellets which we obtained after high-speed centrifugation of medium from *K. pneumoniae* ATCC 15050 cultures were large, viscous, and glassy in appearance. This raised the possibility that some pullulanase was being trapped in capsular polysaccharides which were being released by the *K. pneumoniae* cells. Therefore, we selected a series of mutants of this strain which did not produce extracellular polysaccharide and examined culture supernatants from these mutants for the presence of pullulanase which could be sedimented by high-speed centrifugation. In all of the eight mutants tested, ca. 50% of the extracellular pullulanase activity and of the 145,000-dalton protein was found in the pellet after centrifugation at 135,000 × *g* for 4 h.

We investigated the possibility that some of the extracellular pullulanase was monomeric by gel filtration with Bio-

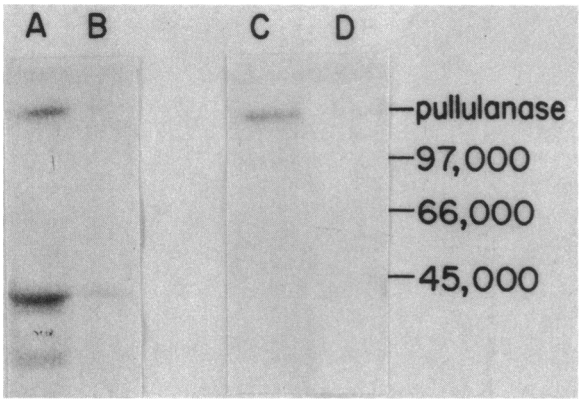

FIG. 5. Saponification of cell-associated and cell-secreted forms of pullulanase. *E. coli* K-12(pACYC184::*pulA*) cells (lanes A and B) were labeled with [^{3}H]palmitate for 4 h in maltose minimal medium as described in the legend to Fig. 4. The harvested cells were then subjected to saponification (B) or parallel treatment without sodium hydroxide (lane A) before the proteins were precipitated with trichloroacetic acid and resolved by SDS-polyacrylamide gel electrophoresis for autoradiography. Samples for lanes C and D were obtained from the culture medium of *K. pneumoniae* grown in maltose minimal medium and labeled with [^{3}H]palmitate for 22 h. Extracellular proteins were precipitated with trichloroacetic acid and then subjected to saponification (lane D) or parallel treatment without sodium hydroxide (lane C). The samples were then treated as described for lanes A and B.

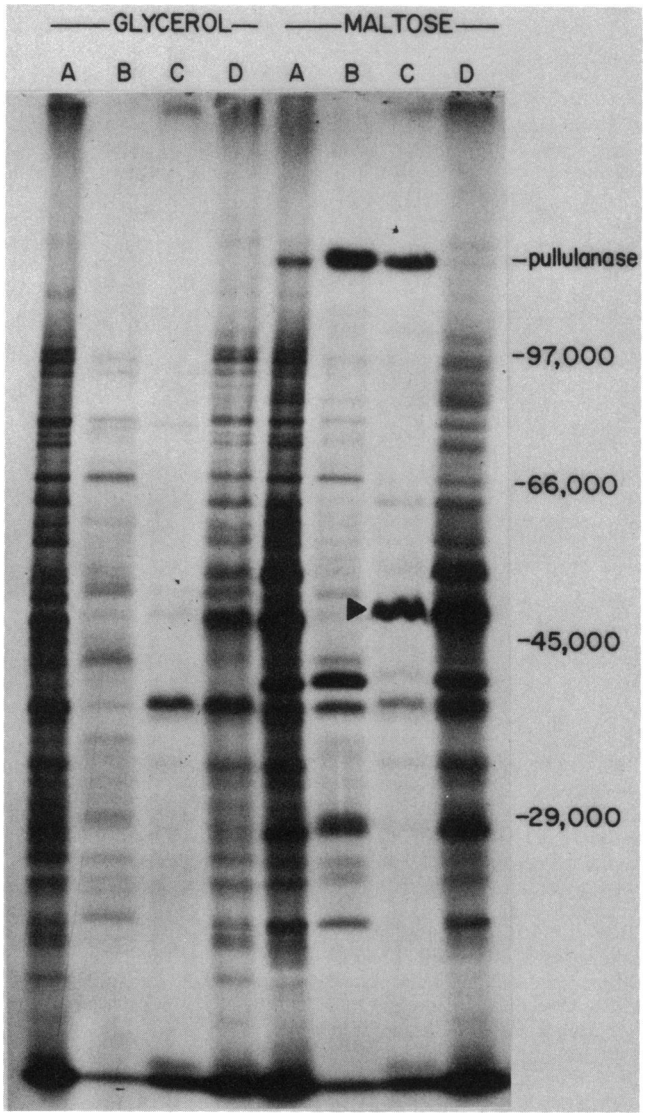

FIG. 6. Analysis of cell-associated and cell-free proteins in cultures of *K. pneumoniae* ATCC 15050 labeled with ^{14}C-labeled amino acids during early exponential growth. Cultures were grown in the presence of glycerol with or without maltose to induce pullulanase production. Lanes: A, cell-associated protein 2 h after addition of label; B, proteins in cell-free medium 22 h after addition of label supernatant of a sample centrifuged at 135,000 × *g* for 4 h; C, proteins in pellet after centrifugation of culture medium to obtain sample for lane B; D, cell-associated proteins 22 h after addition of label. Sample volumes in lanes B and C are equivalent to 20 times the volume in lanes A and D. The positions of pullulanase and molecular weight standard proteins are indicated. The putative maltoporin is the maltose-inducible 49,000-molecular-weight protein in lane C (arrowhead).

Rad A5 (nominal exclusion, 5,000,000) and A50 (nominal exclusion, 50,000,000) agarose gel matrices. Concentrated medium from a maltose-induced culture of a nonmucoid derivative of *K. pneumoniae* was applied to an A5 gel column, and eluted fractions were assayed for pullulanase and for protein content by SDS-polyacrylamide gel electrophoresis. Pullulanase eluted as a sharp peak in the void volume together with the protein we identified as maltoporin. Less than 5% of the pullulanase was detected in

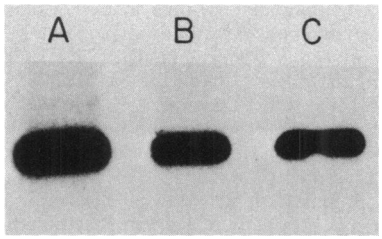

FIG. 7. Extracellular [^{14}C]palmitate-labeled pullulanase from a culture of *K. pneumoniae* ATCC 15050. Cells were grown in medium containing maltose and were labeled with [^{14}C]palmitate during exponential growth. Incubation was continued for 22 h, the cells were removed by centrifugation, and proteins were either precipitated with trichloroacetic acid (lane A) or centrifuged as described in the legend to Fig. 6. Lane B, Proteins remaining in the supernatant fraction after high-speed centrifugation; lane C, proteins in the pellet after high-speed centrifugation. All sample volumes were adjusted so that each contained material from the same amount of spent medium. Only that region of the autoradiograph displaying pullulanase is shown.

the included volume, and none was found in fractions which would be expected to contain pullulanase monomers (i.e., between myosin [205,000 daltons] and *E. coli* alkaline phosphatase [94,000 daltons] [data not shown]). Thus, it seems that extracellular pullulanase is not monomeric.

Heterogeneity of secreted pullulanase was revealed by gel filtration through Bio-Rad A50 agarose. In this case, pullulanase eluted as a broad peak. The leading fractions eluted with the void volume and also contained the maltoporin, whereas trailing fractions in the pullulanase peak were devoid of this protein (Fig. 8). When material in the leading and trailing fractions of the pullulanase peak was centrifuged at 135,000 × *g* for 4 h, pullulanase protein was found almost exclusively in the pellet and supernatant fractions (data not shown). Gel filtration through Bio-Rad A50 agarose was also performed with nonconcentrated cell supernatant. Pullulanase, as shown by its enzymatic activity, eluted in the same way as it did when concentrated supernatant was used.

DISCUSSION

Several lines of evidence suggest that pullulanase is a lipoprotein. The amino-terminal sequence of the *pulA* gene product bears strong similarities to that of precursors of known lipoproteins, the processing of the pullulanase precursor is inhibited by globomycin, and pullulanase contains covalently linked fatty acyls and can be specifically labeled with [^{3}H]glycerol. We assume that the fatty acyl groups are linked by glyceride-ester and amide bonds to the cysteine residue which probably becomes the amino-terminal amino acid of the mature protein, as is the case for all other bacterial lipoproteins which have been studied in detail (14). This is, to our knowledge, the first report of the secretion of a lipoprotein by a gram-negative bacterium, and it may have important implications for our understanding of the ways in which bacteria export and secrete proteins.

The release of pullulanase into the growth medium is probably an extension of the signal-peptide-dependent pathway of protein export, as adapted to lipoproteins. The inhibition of processing of the pullulanase precursor after expression of the *malE-lacZ* gene fusion in *E. coli* K-12 provides support for this idea.

It should be recalled here that *E. coli* K-12 is not only unable to secrete pullulanase but also unable to localize it correctly in its envelope. In *K. pneumoniae*, pullulanase is found mainly in the outer membrane and is accessible to substrate (pullulan) added to the medium (12). This is not the case for *E. coli* K-12 carrying one or more copies of the *pulA* gene; pullulanase protein was found in both inner and outer membranes and was not accessible to pullulan unless the

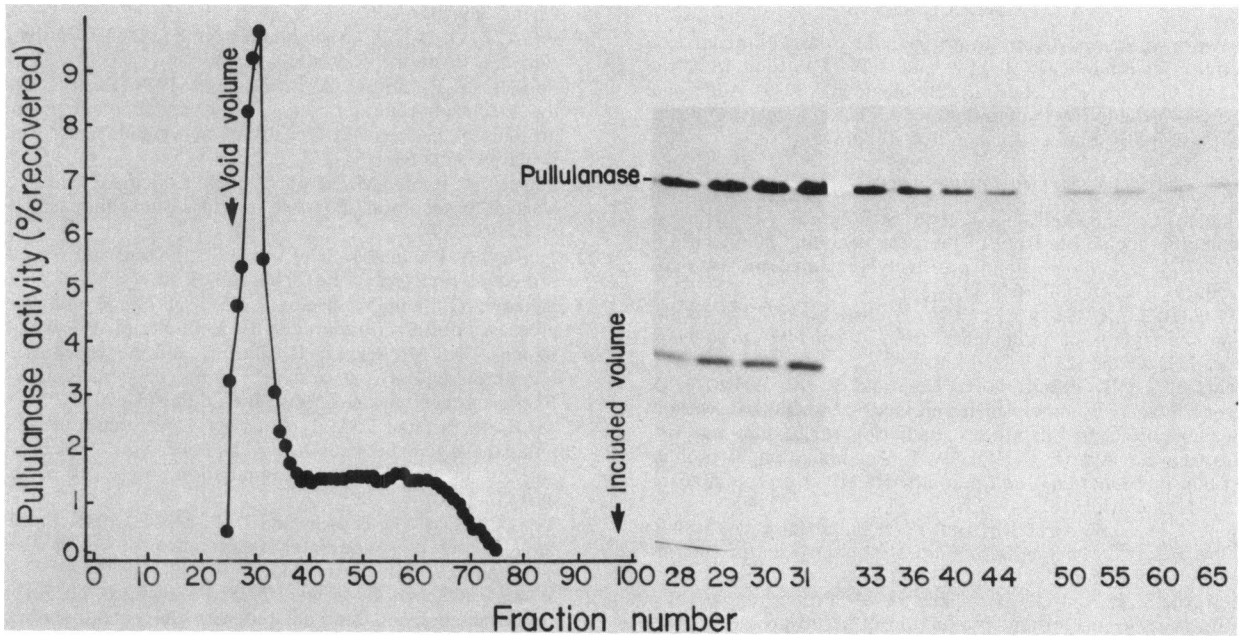

FIG. 8. Gel filtration of extracellular pullulanase on Bio-Rad Biogel A50. Concentrated medium from a culture of a nonmucoid derivative of *K. pneumoniae* (0.5 ml, equivalent to 60 ml of original medium) was applied to the column (1 by 60 cm). Eluted fractions (2 ml each) were assayed for pullulanase and examined by SDS-polyacrylamide gel electrophoresis. Included and excluded volumes were determined with riboflavin and blue dextran, respectively.

cells were permeabilized or disrupted (12). The results presented here suggest that the failure of *E. coli* K-12 to position pullulanase correctly in its envelope and to secrete it into the medium is not caused by differences in processing or modifying activities between *E. coli* K-12 and *K. pneumoniae*.

It might have been anticipated that pullulanase would be anchored to the *K. pneumoniae* outer membrane by its fatty acyl groups and that release (secretion) into the medium would involve proteolytic cleavage near the amino terminus, but this does not appear to be the case. Extracellular pullulanase still contains covalently linked fatty acyls. However, this extracellular enzyme is not present as a free monomer. About half of it is associated with fast-sedimenting particles, which could be some sort of outer membrane vesicles. The other half is present in nonsedimenting aggregates, which could be pullulanase micelles held together by the fatty acyl groups of the protein. Efforts are now under way to determine whether pullulanase is actually released as micelles or vesicles, possibly from regions of the *K. pneumoniae* outer membrane where this enzyme would be present in very large amounts.

The present finding, that a lipoprotein can be released from a membrane in the absence of a proteolytic event, should perhaps lead to a reevaluation of the data suggesting that such an event is required to release penicillinases from the surface of gram-positive bacteria. Extracellular forms of these enzymes lack a limited number of amino acids from the amino terminus (10, 15, 16, 24). However, cultures of these bacteria abound with proteases, and, therefore, it seems equally plausible that the shortened forms of these proteins result from limited proteolysis after their release. Other extracellular proteins of *Bacillus* species also lack a limited number of amino-terminal residues from the region immediately after the signal peptidase cleavage site (22). In one case, there is evidence that these residues do not have any special importance in protein secretion (17).

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