JOURNAL OF BACTERIOLOGY, Nov. 1985, p. 633–638
0021-9193/85/110633-06$02.00/0
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Vol. 164, No. 2

Characterization and Expression of the Structural Gene for Pullulanase, a Maltose-Inducible Secreted Protein of *Klebsiella pneumoniae*

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Received 25 March 1985/Accepted 7 August 1985

Some strains of *Klebsiella pneumoniae* secrete pullulanase, a debranching enzyme which produces linear molecules (maltodextrins, amylose) from amylopectin and glycogen. *pulA*, the structural gene for pullulanase, was introduced into *Escherichia coli*, either on a multiple-copy-number plasmid or as a single copy in the chromosome. When in *E. coli*, *pulA* was controlled by *malT*, the positive regulatory gene of the maltose regulon. Indeed, *pulA* expression was undetectable in a *malT*-negative mutant and constitutive in a *malT*^{c} strain. Furthermore, the plasmid carrying *pulA* titrated the MalT protein. When produced in *E. coli*, pullulanase was not localized in the same way as in *K. pneumoniae*. In the latter case it was first exported to the outer membrane, with which it remained loosely associated, and was then released into the growth medium. In *E. coli* the enzyme was distributed both in the inner and the outer membranes and was never released into the growth medium.

Pullulanase is a starch-debranching enzyme which cleaves glucose polymers at α(1-6) glucosidic linkages (4). One such polymer is pullulan, a linear polysaccharide composed of several hundred maltotriose units linked to one another by α(1-6) bonds. When pullulanase cleaves this substrate, maltotriose is liberated (3, 4, 11, 12, 16, 17, 22, 33, 35).

Klebsiella pneumoniae (previously called *Aerobacter aerogenes* or *Klebsiella aerogenes*) produces a pullulanase which allows this bacterium to use pullulan as a carbon source (4). This property is not found in other enteric bacteria including *Escherichia coli* (9, 17; M. A. Bloch, unpublished data). Production of pullulanase is induced by growth in the presence of pullulan, maltotriose, or maltose and is repressed in the presence of glucose (16, 22, 32, 35). The ca. 145,000-dalton pullulanase polypeptide (12, 33, 35) is initially exported to the *K. pneumoniae* outer membrane (35) and is then quantitatively released into the growth medium at the end of the exponential growth phase (12, 35). The position of pullulanase in either of these locations could account for the growth on pullulan. This polysaccharide, which is probably too large to diffuse across the outer membrane, could be hydrolyzed outside the cell, thereby liberating maltotriose which could then be transported via the outer membrane LamB porin (34).

We have initiated studies on the pullulanase of *K. pneumoniae* as an extension of our interest in the regulation and export of gene products involved in maltose metabolism in *E. coli*. The maltose regulon of *E. coli* K-12 comprises at least seven genes grouped in three operons coordinately regulated by maltose, which acts via the positive regulatory protein MalT (1, 2, 7, 10, 14, 26, 27). We are interested in determining whether an analogous regulon exists in *K. pneumoniae*, and, if so, whether the gene encoding pullulanase is part of it. In addition, pullulanase seems to be a good system for studying protein secretion in gram-negative bacteria, a poorly understood process at this time (25).

pulA, the structural gene for *K. pneumoniae* pullulanase, was cloned into a λ phage derivative by P. Cornelis, C. Digneffe, and K. Willemot (details of the cloning were presented at the 15th FEBS Meeting, July 1983). In this report, we describe our studies on the expression of *pulA* in *K. pneumoniae* and in *E. coli* K-12 carrying the cloned *pulA* gene. Pullulanase production in *E. coli* is maltose inducible and regulated by MalT, suggesting that the equivalent of MalT also exists in *K. pneumoniae* and that the two proteins are interchangeable. However, when produced by *E. coli*, pullulanase is not secreted and may not even be properly localized to the outer membrane.

(A preliminary account of this work was presented by C. Chapon at the 35th Cold Spring Harbor Meeting on phage and bacterial regulatory mechanisms, August 1984.)

MATERIALS AND METHODS

Strains and media. The strains used are listed in Table 1. Unless stated otherwise, minimal medium was M63, rich medium was L broth, and indicator medium was MacConkey agar (20). Carbohydrates, when added, were at a final concentration of 0.4%. Tetracycline was used at 10 μg/ml in solid media and at 5 μg/ml in liquid media.

Cloning of *pulA*. Derivatives of bacteriophage λNM781 (21) containing *Eco*RI fragments of chromosomal DNA from *K. pneumoniae* ATCC 15050 (see Results) prepared by the method of Hohn and Murray (15) were generously provided by P. Cornelis. Further subcloning and analyses were performed by the methods of Maniatis et al. (19). Mutagenesis of plasmids carrying the cloned pullulanase gene with transposon Tn*5* was essentially as described previously (24).

Lysogenization of *E. coli* K-12 with λNM781 derivatives. To lysogenize the different *E. coli* strains (pop3, pop3325, and pop3971), it was necessary to provide homology on the chromosome for integration of the phage DNA. To do this, we first constructed λ *imm*^{21} lysogens of the different strains as described by Miller (20). These lysogens were then infected with λNM781::*pulA*. Phage λ *c*I *h*80 was used to

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TABLE 1. Bacterial strains

Strain	Relevant genotype	Origin or reference
E. coli K-12		
HB101	*hsd-20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	Maniatis et al. (19)
pop3 (MC4100)	*araD139 lacU169 thiA rpsL*	Casadaban (6)
pop3325	pop3 *malT^c1*	Débarbouillé et al. (10)
pop3971	pop3 *malTp1 malTp7*	Chapon (7)
pop2243[a]	*thr leu fhuA lacY sup malPp::pulA1*	This work
pop4101[b]	pop3 *malPp::pulA1*	This work
pop4102[c]	as pop4101 but *malT*	This work
pop3741	pop3(pACYC184::*pulA*)	This work
pop3742	pop3325(pACYC184::*pulA*)	This work
pop3743	pop3971(pACYC184::*pulA*)	This work
pop3746	pop3(λ *imm*^{21} λNM781::*pulA*)	This work
pop3748	pop3325(λ *imm*^{21} λNM781::*pulA*)	This work
pop4103	pop3971(λ *imm*^{21} λNM781::*pulA*)	This work
pop4104	pop3(pACYC184)	This work
pop4105	pop3971(pACYC184)	This work
AR1062	*thr leu ara azi fhuA lacY tsx min gal xyl mtl thi rpsL hsdR* (minicell producer)	G. Rapoport
K. pneumoniae ATCC 15050		American Type Culture Collection

[a] Insertion of the ca. 6-kilobase fragment containing the *pulA* gene into the *malP* promoter was performed as described by Raibaud et al. (28); the number 1 after *pulA* indicates that the orientation of the fragment is such that gene *pulA* and the *malPQ* operon are transcribed in the same direction (26–28).

[b] pop4101 was constructed by infecting pop3 *malT131* (26) with a P1 phage stock (20) grown on pop2243 and selecting for a Mal$^+$ phenotype. Mal$^+$ clones were screened for a Pul$^+$ phenotype.

[c] pop4102 is a spontaneous phage λ-resistant Mal$^-$ mutant (14) of strain pop4101.

select λ *imm*^{21}, λNM781::*pulA* double lysogens, which had acquired λ immunity.

Assays. Pullulanase was assayed as described by Hope and Dean (16) with slight modifications. Exponentially growing bacteria were concentrated to an A_{600} of about 30. The final reaction volume was 1.4 ml. At intervals, 0.2-ml samples were removed, added to 0.6 ml of dinitrosalicylic acid reagent prepared by the method of Hope and Dean (16), and heated in a boiling water bath for 10 min before final quenching and cooling by adding 3.2 ml of cold water. Pullulanase specific activity was expressed as nanomoles of maltotriose liberated per minute per milligram of protein. Protein was determined by the method of Lowry et al. (18).

Localization of pullulanase. Envelopes were prepared from cells growing exponentially (A_{600} = 1.0) in minimal maltose medium containing 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.). The cells were harvested, washed once with 50 mM Tris hydrochloride (pH 7.4), and suspended in the same buffer supplemented with 0.1 mM $MgSO_4$. The cells were then disrupted by sonication at 4°C. Unbroken cells and debris were removed by centrifugation at 5,000 × *g* for 5 min. Envelope fractions were then separated from soluble proteins by centrifugation at 130,000 × *g* for 90 min. The pellets were resuspended in 50 mM Tris hydrochloride–0.1 mM $MgSO_4$ (pH 7.4) and either treated with Triton X-100 or subjected to isopycnic sucrose gradient centrifugation as specified by Schnaitman (31). Fractions from the sucrose gradients were analyzed for protein content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Tris glycine buffers (23) and for the presence of 2-keto-3-deoxyoctanoic acid (13). Triton-soluble envelope proteins were precipitated with 2 volumes of acetone at −20°C and then compared with total envelope and Triton-insoluble proteins by SDS-PAGE.

Proteins produced by minicells. Minicells were prepared from derivatives of strain AR1062 carrying different plasmids and labeled with ^{14}C-amino acids (50 μCi/ml) by the method of Reeve (29). Labeled proteins were resolved by SDS-PAGE, and the gels were treated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.), dried, and exposed to Kodak X-Omat AR film at −70°C.

RESULTS

Cloning of *pulA*. A derivative of λNM781 carrying the structural gene (*pulA*) from *K. pneumoniae* ATCC 15050 was generously provided by P. Cornelis. This phage (λNM781::*pulA*) was shown by assay to produce pullulanase during lytic growth on *E. coli* HB101 and was found to contain a 6.1-kilobase *Eco*RI insert (P. Cornelis, personal communication). The same fragment was also subcloned into the *Eco*RI site of pACYC184 (19). *E. coli* K-12 strains harboring this plasmid, pACYC184::*pulA*, synthesized pullulanase when grown in the presence of maltose but failed to grow on pullulan (see below). Minicells carrying the *pulA* plasmid produced a 145,000-dalton polypeptide which comigrated with authentic pullulanase from *K. pneumoniae* ATCC 15050 (data not shown). Derivatives of these plasmids in which the pullulanase gene was inactivated either by transposon Tn*5* mutagenesis (Fig. 1) or by in vitro-constructed deletions (9) failed to elicit production of this polypeptide by minicells. This allowed us to localize the *pulA* gene, which must be approximately 3 to 4 kilobases to encode a polypeptide with an apparent molecular weight of 145,000 (Fig. 1).

When present in *E. coli*, the *pulA* gene is controlled by *malT*. Pullulanase synthesis was maltose inducible in *E. coli* cells harboring pACYC184::*pulA* (Table 2). Therefore, we asked whether *pulA* gene expression is controlled by *malT*, the positive regulator gene of the maltose regulon. In a standard *malT*-negative host, no detectable pullulanase was produced (data not shown). This, in itself, did not prove that *malT* controlled *pulA* expression, since *malT* is known to control the formation of the maltose transport system and hence the entry of the inducer (7, 10). More convincing evidence that *malT* controls *pulA* expression was obtained by using hosts which express the maltose regulon constitutively. Two such hosts were used. One carried *malT^c1*, a mutation which results in the synthesis of a modified MalT protein, active in the absence of added inducer (10). The other carried *malTp1* and *malTp7*, two mutations in the control region of gene *malT* which, when combined, lead to a ca. 30-fold overproduction of MalT protein (7). In both instances the synthesis of pullulanase was constitutive (Table 2).

We observed that when a *malT*$^+$ strain of *E. coli* K-12 harbored the *pulA* plasmid, the synthesis of LamB, MalE, and MalP proteins, which are the only maltose-inducible proteins easily detected by SDS-PAGE, was considerably reduced. To examine this phenomenon more quantitatively,

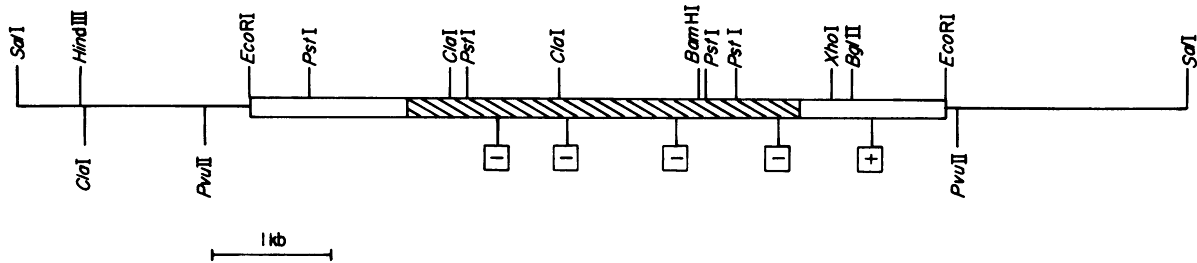

FIG. 1. Restriction endonuclease map of pACYC184::*pulA*. The DNA region corresponding to the *pulA* gene is represented as a hatched bar. The remainder of the *K. pneumoniae* DNA in the cloned fragment is shown as an open bar. pACYC184 DNA is represented by a thin line. Boxes indicate sites of Tn*5* insertions, as determined by restriction endonuclease analyses. Symbols in the boxes indicate the pullulanase phenotype of cells carrying the mutated plasmids (i.e., production of the ca. 145,000-dalton protein by cells grown in the presence of maltose and by minicells carrying the plasmid, and pullulan fermentation on indicator media). More precise localization of the *pulA* gene is based on deletion and DNA sequencing analysis which will be presented elsewhere (9). kb, kilobase.

we assayed amylomaltase, the product of gene *malQ*. Amylomaltase activity was reduced to 25% of the normal level when a *malT*$^+$ strain carried the *pulA* plasmid (Table 3, lines 1 and 3). This effect was suppressed when the strain carried the *malTp1* and *malTp7* mutations, which lead to overproduction of MalT protein (Table 3, lines 2 and 4). A similar situation was reported to occur with a plasmid containing the promoters of the *malEFG* and *malK-lamB* operons and was suggested to indicate titration of MalT protein by multiple copies of the MalT-binding site (2). It seems likely that the same phenomenon occurs with the plasmid carrying *pulA*, thereby providing additional evidence for the regulation of *pulA* by MalT protein.

Comparison of *pulA* expression in *E. coli* K-12 and *K. pneumoniae*. To compare pullulanase synthesis in *E. coli* and *K. pneumoniae* without complications caused by MalT titration, it was necessary to construct strains containing a single copy of *pulA*. This was done both by lysogenizing with λNM781::*pulA* and by inserting the 6.1-kilobase fragment into the chromosome. In the latter case, the insertion was obtained by recombining the fragment into the *malPQ* promoter, in such a way that this promoter was inactivated (26, 28). In both cases the amount of pullulanase activity detected was much lower than that in the original *K. pneumoniae* strain (Table 4). For instance, the constitutive level observed in a *malT*^{c}*1* strain with the *pulA* gene in the *malPQ* promoter was approximately 10% of the maltose-induced

TABLE 2. Pullulanase activity in *E. coli* K-12(pACYC184::*pulA*) strains[a]

Strains	Relevant genotype	Pullulanase activity (U/mg of protein per min)	
		Glycerol	Glycerol + maltose
E. coli K-12			
pop3741	*malT*$^+$(pACYC184::*pulA*)	19	660
pop3742	*malT*^{c}*1*(pACYC184::*pulA*)	590	690
pop3743	*malTp1 malTp7* (pACYC184::*pulA*)	1,200	1,160
K. pneumoniae ATCC 15050		≤2	500

[a] Bacteria were grown at 30°C in minimal glycerol medium with or without maltose. Pullulanase activity is expressed as nanomoles of maltotriose liberated per minute per milligram of protein.

level in *K. pneumoniae*. The λNM781::*pulA* lysogens gave slightly higher values, but it is possible that they contained more than one prophage.

In *E. coli*, as already known in *K. pneumoniae*, pullulanase production was repressed when the cells were grown in the presence of glucose. Hence, no pullulanase activity could be detected in *E. coli* strains pop4101 or pop3746 when they were grown in medium containing maltose and glucose (data not shown).

Localization of pullulanase in *K. pneumoniae* and in *E. coli*. We confirmed that pullulanase is initially incorporated mainly into the *K. pneumoniae* outer membrane and is then slowly released into the growth medium (35). In contrast, *E. coli* was unable to secrete the pullulanase into the medium, irrespective of whether the *pulA* gene was present in one or several copies (Fig. 2). Furthermore, there were several indications that the location of the cell-bound enzyme was not the same in *E. coli* K-12 as in *K. pneumoniae*. When *E. coli* plasma and outer membranes were separated by isopycnic centrifugation in sucrose gradients, pullulanase was found in both fractions (Fig. 3). In addition, full pullulanase activity was detected with whole cells of *K. pneumoniae*, whereas activity in *E. coli* K-12 could only be detected when the cells were broken or permeabilized (data not shown). These results indicate that pullulanase is probably located on the outer face of the outer membrane in *K. pneumoniae* but not in *E. coli* K-12. In both *K. pneumoniae* and *E. coli* K-12, the association of pullulanase with the envelope was apparently rather loose. Approximately 25% of the pullulanase polypeptide was released by sonication (i.e., failed to sediment when centrifuged at 125,000 × *g* for 90 min). Furthermore, less than 5% of the pullulanase remained insoluble when cell envelopes were treated with Triton X-100 plus Mg^{2+}, i.e., under conditions which failed

TABLE 3. Titration of the MalT protein by pACYC184::*pulA*, as detected by assaying amylomaltase activities[a]

Strain	Relevant genotype	Amylomaltase (U/mg of protein)	
		Glycerol	Glycerol + maltose
pop4104	*malT*$^+$(pACYC184)	7	150
pop4105	*malTp1 malTp7* (pACYC184)	190	370
pop3741	*malT*$^+$(pACYC184::*pulA*)	8	40
pop3743	*malTp1 malTp7*(pACYC184::*pulA*)	235	390

[a] Bacteria were grown in minimal glycerol medium at 30°C in the presence or absence of maltose. Amylomaltase was assayed by the method of Raibaud et al. (28) except that the extracts were not centrifuged.

TABLE 4. Pullulanase activity in *E. coli* K-12 strains carrying the *pulA* gene integrated into the chromosome[a]

Strain	Relevant genotype	Pullulanase (U/mg of protein per min)	
		Glycerol	Glycerol + maltose
E. coli			
pop4101	*malT*$^+$ *malPp*::*pulA*	≤2	50
pop3746	*malT*$^+$(λNM781::*pulA*)	≤2	90
pop3748	*malT*^{c}1(λNM781::*pulA*)	60	40
pop4103	*malTp1 malTp7*(λNM781::*pulA*)	90	40
K. pneumoniae ATCC 15050		≤2	500

[a] Growth conditions and pullulanase assays were as described in Table 2.

to solubilize all other outer membrane proteins (data not shown).

Growth on pullulan. Pullulanase-producing strains of *K. pneumoniae* can use pullulan as a sole carbon source. In contrast, *E. coli malT*$^+$ strains harboring *pulA* either in multiple copies on the plasmid or in single copy in the chromosome failed to grow on pullulan, even if preinduced by growth on maltose. The inability of *E. coli* K-12 to localize pullulanase correctly in the outer membrane (see above) initially seemed sufficient to explain this phenomenon. However, we later found that *E. coli* K-12 strains carrying *pulA* and either *malT*^{c}*1* or *malTp1* plus *malTp7* grew on pullulan, albeit less quickly than *K. pneumoniae*. This must mean that, even if it is incorrectly localized, a small fraction of the fully induced pullulanase of *E. coli* K-12 is accessible to the substrate. Therefore, the inability of the *malT*$^+$ strain to grow on pullulan may be due to a failure in induction. Indeed, pullulan did not induce amylomaltase production in a *malT*$^+$ *E. coli* K-12 strain carrying the *pulA* gene (data not shown). This observation will be discussed in the next section.

DISCUSSION

In this study, we demonstrated that when the *pulA* gene from *K. pneumoniae* is introduced into *E. coli* K-12, its expression is controlled by *malT*, the positive regulatory gene of the maltose regulon. This result indicates both that the equivalent of *malT* exists in *K. pneumoniae*, and that the MalT proteins from the two species are at least to some extent interchangeable, as previously shown for other regulatory proteins (5). Thus, the *pulA* gene which exists in *K. pneumoniae* but probably not in *E. coli* is a new member of the maltose regulon. Evidence presented in the accompanying paper (9) demonstrates that *pulA* is not an additional gene in one of the three maltose-regulated operons known to exist in *E. coli* but actually defines a new *malT*-controlled transcription unit.

The expression of *pulA*, like that of the three previous known *mal* operons, is repressed by glucose. This effect could be mediated directly through control of *pulA* expression via the cyclic AMP-binding protein–cyclic AMP complex or indirectly through inducer exclusion and through the effects of cyclic AMP-binding protein–cyclic AMP on *malT* expression and on the expression of genes encoding components of the maltose permease (8).

Although the *pulA* gene is expressed in *E. coli* K-12, the amount of pullulanase activity detected in cells harboring a single copy of the gene was only 10 to 20% of that found in the original *K. pneumoniae* strain. One explanation may be that the *pulA* gene is inefficiently transcribed in *E. coli* K-12, possibly because the *E. coli* MalT protein is not fully active on the *pulA* promoter. Alternatively, pullulanase may not be as stable in *E. coli* K-12 as in *K. pneumoniae* due to the observed improper localization in the *E. coli* envelope. Even though pullulanase cannot even be assayed in intact *E. coli* cells, it still allows some growth of this bacterium on pullulan, but only if the maltose regulon is expressed constitutively. Our interpretation for the failure of *malT*$^+$ strains to grow on pullulan, even if preinduced with maltose, is that the rate of production of maltotriose (the inducer) from pullulan is much slower than its rate of degradation through the combined action of amylomaltase and maltodextrin phosphorylase (*malQ* and *malP* gene products), such that a concentration of maltotriose sufficient to induce cannot be maintained inside the cells.

In contrast to *K. pneumoniae*, *E. coli* K-12 was unable to secrete pullulanase. This result was rather surprising in view of the fact that these bacteria are closely related. Pullulanase secretion in *K. pneumoniae* may only require the participation of very few gene products. The introduction of these genes into *E. coli* K-12 may enable this organism to also secrete the enzyme.

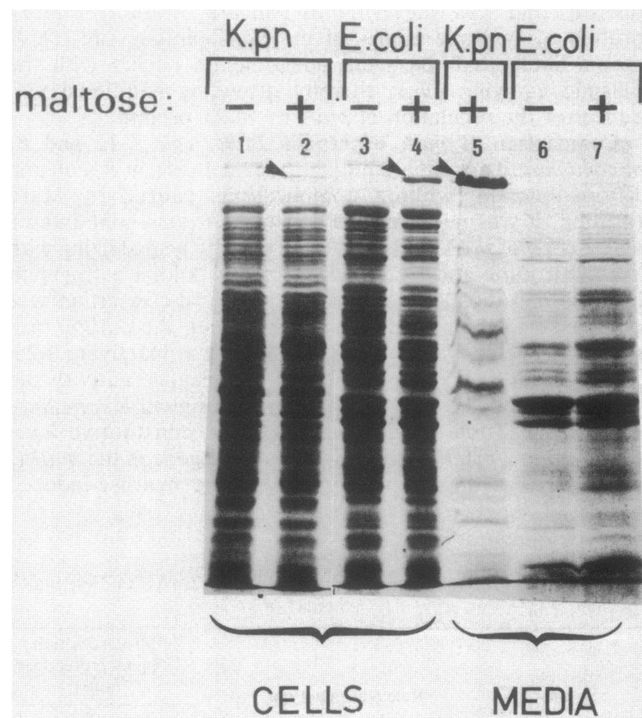

FIG. 2. Pullulanase production and release by *K. pneumoniae* ATCC 15050 (K. pn.) and by *E. coli* K-12(pACYC184::*pulA*). Cells were grown at 30°C in minimal glycerol medium with or without maltose as indicated. Cells (lanes 1 to 4) were harvested in mid-exponential phase, suspended in sample buffer, and heated to 100°C for 5 min before electrophoresis. Samples of media (lanes 5 to 7) were from cultures grown for 24 h. Cells were removed by repeated centrifugation, and the supernatant medium was concentrated by filtration through an Amicon Diaflo PM10 filter. Proteins in the concentrated media were precipitated with 3 volumes of cold acetone, suspended in sample buffer, and heated to 100°C. Samples in lanes 6 and 7 are equivalent to approximately 20 times the volume of culture medium as used to prepare sample 5. Arrows indicate the position of pullulanase.

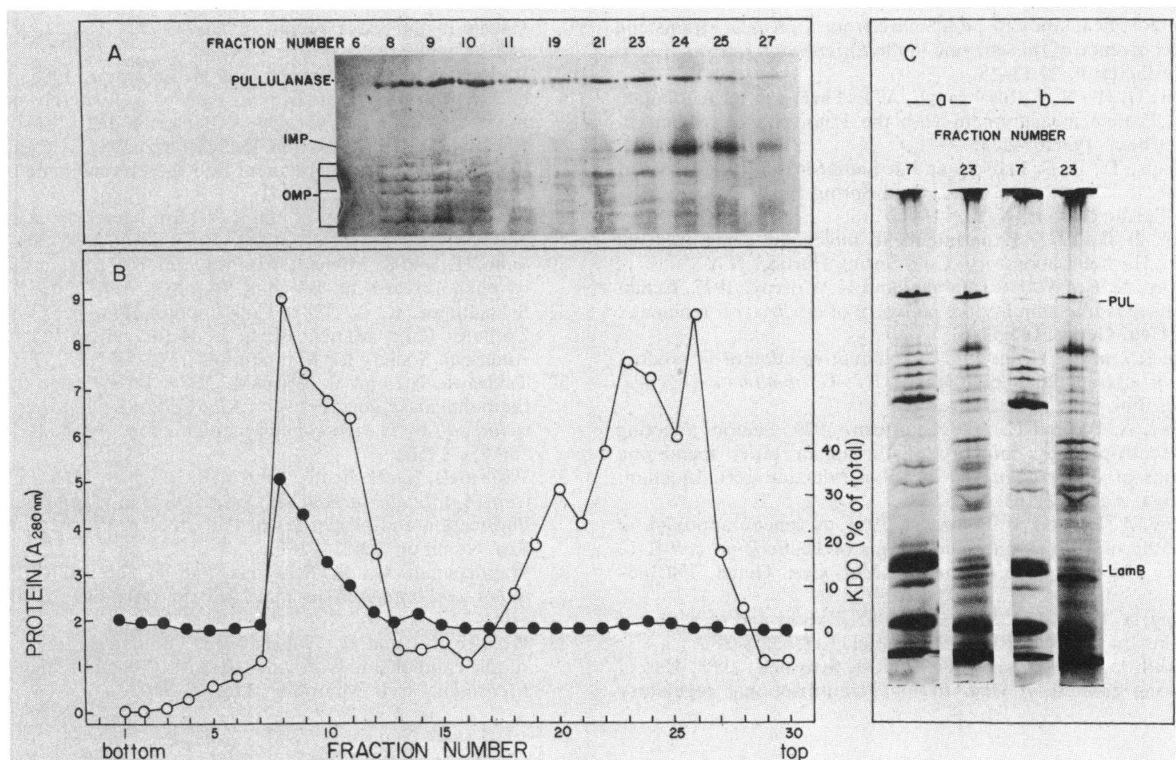

FIG. 3. Localization of membrane-associated pullulanase by isopycnic sucrose density centrifugation. (A and B) Results obtained with strain pop3(pACYC184::*pulA*) (pop3741). The 35 to 55% sucrose gradient was centrifuged at 130,000 × *g* for 48 h. Fractions were collected and analyzed for protein content (A_{280}) (○) and for outer-membrane-specific 2-keto-3-deoxyoctanoic acid (KDO) (B) (●). Samples from each fraction were analyzed by SDS-PAGE; results from selected fractions are shown in panel A, in which proteins of molecular weights between ca. 75,000 and 150,000 are displayed. Note that pullulanase was detected in all fractions, whereas outer membrane proteins involved in siderophore-iron transport (OMP) were only found in the leading fractions (outer membrane), and a presumed inner membrane protein (IMP) was only detected in the trailing fractions. Pullulanase polypeptide was not detected in fractions 13 through 17. (C) Results obtained with fractions 7 (outer membrane) and 23 (inner membrane) obtained from envelopes of strains pop3(pACYC184::*pulA*) (pop3741) (a) and pop2243 (*malPp*::*pulA*) (b). The positions of pullulanase (Pul) and maltose-inducible outer membrane LamB protein are indicated.

ACKNOWLEDGMENTS

We thank P. Cornelis and his colleagues who initiated this work with financial assistance from Corn Products Inc., and O. Raibaud for constructing strains and for many helpful discussions.

This work was supported by grants from the Centre National de la Recherche Scientifique (UA 270) and by the Ministère de la Recherche et de la Technologie (82 V 1279 and ATP Microbiologie CP96.0031).

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