JOURNAL OF BACTERIOLOGY, July 1989, p. 3673–3679
0021-9193/89/073673-07$02.00/0

Vol. 171, No. 7

Klebsiella pneumoniae pulS Gene Encodes an Outer Membrane Lipoprotein Required for Pullulanase Secretion

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Received 13 January 1989/Accepted 30 March 1989

The product of the *Klebsiella pneumoniae* gene *pulS*, which is located downstream from the pullulanase structural gene (*pulA*), is essential for the cell surface localization and extracellular release of pullulanase in *Escherichia coli* K-12. *pulS* is transcribed in the opposite direction to *pulA*, from which it is separated by a region of 624 nucleotides. Although this latter region contains a new component of the maltose regulon, *pulB*, which is transcribed from the *pulA* promoter, it is not required for pullulanase synthesis or secretion. Unlike *pulA* and all other pullulanase secretion genes characterized so far, the expression of *pulS* is not induced by growth in the presence of maltose and is unaffected by mutations in the maltose regulator gene *malT*. The *pulS* gene product was identified as a ca. 12-kilodalton outer membrane lipoprotein. The characterization of PulS brings to three the number of identified proteins which are known to be required for pullulanase secretion in addition to the components of the signal sequence-dependent general protein export pathway.

We previously reported that *Escherichia coli* K-12 acquired the ability to synthesize the enzyme pullulanase, to localize it to the cell surface, and subsequently to specifically release it into the medium when it carried an 18.8-kilobase (kb) chromosomal DNA fragment from *Klebsiella pneumoniae* UNF5023 (5). Transposon mutagenesis of this cloned fragment (5) and of the chromosome of another strain of *K. pneumoniae* (10) indicated that genes essential for pullulanase secretion flank the pullulanase structural gene, *pulA*. However, it appeared from studies by Chapon and Raibaud (2) and Pugsley et al. (16) that pullulanase is made as a precursor polypeptide with an N-terminal signal peptide. This suggested that the early stages in pullulanase secretion (transport across the cytoplasmic membrane) were likely to be the same as those followed by most other exported proteins and, therefore, that the products of the *pulA*-linked secretion genes would probably be required only for the later stages of the pathway (transport across the outer membrane and possibly extracellular release).

Pullulanase-specific secretion genes located upstream of *pulA* were found to be part of an operon (*pulCDEFG*) which is transcribed in the opposite direction from *pulA* (5; C. d'Enfert, I. Reyss, C. Wandersman, and A. P. Pugsley, submitted for publication). This operon, like *pulA*, is part of the maltose regulon; i.e., its expression is induced by growth in the presence of maltose and is positively regulated by MalT protein (2, 5; see reference 21 for a general review). Preliminary results from transposon mutagenesis experiments also suggested that the pullulanase secretion gene in the region downstream from *pulA* (previously referred to as the B region and shown here to contain a single secretion gene called *pulS*) has its own promoter and is transcribed independently of *pulA* (5). The aims of the study reported here were to identify the *pulS* gene and its product and to determine whether the *pulS* gene is part of the maltose regulon. The results showed that PulS is an outer membrane lipoprotein whose structural gene is expressed independently of the maltose regulon expression and transcribed in the opposite direction to *pulA*.

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MATERIALS AND METHODS

Strains, media, and assays. Strains of *E. coli* K-12 and cloning vehicles are listed in Table 1. The media used were L broth and agar and M63 minimal medium and agar (14). Minimal medium was supplemented with appropriate amino acids (0.01%), Casamino Acids (0.4%, Difco Laboratories, Detroit, Mich.) and glycerol (0.4%). Maltose (0.4%) and isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM) were used to induce the expression of *pulA* and *pulC* promoters or the *lacZ* promoter, respectively. Antibiotics were used at the following concentrations (micrograms per milliliter): ampicillin, 200; tetracycline, 15; kanamycin, 50; chloramphenicol, 25. In most cases, cultures were incubated at 30°C.

To construct an *E. coli* K-12 strain carrying a mutated *pul* region containing *pulA*, *pulS*::Tn*5-4*, and the *pulCDEFG* operon in the chromosome, we first introduced the wild-type *pul* region from the Mal$^+$ strain PAP2443 (5) into the Mal$^-$ strain C600 *malPp*::*prt polA*(Ts) Tn*10* (constructed by C. Wandersman and O. Raibaud; *prt* is an *Erwinia chrysanthemi* chromosomal DNA fragment encoding extracellular proteases) by P1 transduction and selection on minimal medium containing 0.4% maltose and tetracycline. The resulting strain (PAP2509) was transformed by plasmid pCHAP231T4 and subsequently incubated at 42°C in the presence of kanamycin (15 μg/ml). Kanamycin-resistant clones were then spontaneously cured of the plasmid and tested for failure to export pullulanase to the cell surface. The chromosomal *pulS*::Tn*5-4* region from one such clone was transferred into strain MC4100 *malAΔ512* by P1 transduction (14) and selection for the resulting Mal$^+$ strain (PAP2773) on minimal medium supplemented with 0.4% maltose. *recA*::Tn*10* and pAPIP502 (F′ *lacI*^{q1} *lacZM15 pro*$^+$ Tn*10*) were subsequently introduced into this strain, leading to strains PAP2729 and PAP2776, respectively.

MalT$^-$ derivatives of strain PAP2777 carrying Φ(*pulS-malQ*) were selected by their resistance to bacteriophage λ *vir*. Stable Mal$^-$ mutants were selected and transformed by pOM2, a pBR322 derivative (Tc^r) carrying the cloned *malT* gene of *E. coli* K-12. Clones in which the Mal$^+$ phenotype

TABLE 1. Bacterial strains and cloning vehicles

Strain or vehicle	Genotype	Source or reference
Bacterial strains		
C600	*thr leu fhuA lacY supE*	Laboratory collection
MC4100	Δ(*lac-argF*)*U169 araD139 relA1 rpsL150*	Laboratory collection
PAP105	Δ(*lac-pro*) pAPIP502	Laboratory collection
pop2150	MC4100 Δ*malA510*	18
MCL30	Hfr PO45 *thi* Δ(*srl-recA*)*306*::Tn*10*	11
PAP2443	MC4100 *malPp*::(*pulAB pulS pulCDEFG*)	15
PAP2509	C600 *malPp*::(*pulAB pulS pulCDEFG*) *polA*(Ts) *met*::Tn*10*	This work
PAP2773	MC4100 *malPp*::(*pulAB pulS*::Tn*5-4 pulCDEFG*)	This work
PAP2776	PAP2773 pAPIP502	This work
PAP2729	PAP2773 Δ(*srl-recA*)*306*::Tn*10*	This work
AR1062	*thr leu ara azi fhuA lacY tsx minB gal rpsL xyl mtl hsdR* (minicell producer)	G. Rapoport
Cloning vehicles		
pBGS18$^+$/19$^+$	*neo ori*pMB1 *lacZ*α	22
pEMBL8$^+$/9$^+$	*bla ori*pMB1 *lacZ*α	6
pBR322	*bla tet ori*pMB1	23
pACYC184	*cat tet ori*P15A	1
pOM41	*bla ori*pMB1 *'malT-'malP'-'tet*	24

was restored by pOM2 were assumed to carry a *malT* mutation.

Pullulanase was assayed by the method of Michaelis et al. (13) except that cells were lysed with 0.5% octylpolyoxyethylene. Amylomaltase was assayed as described by Raibaud et al. (19).

Nucleotide sequencing and subcloning. Plasmids carrying clustered deletions were obtained from pCHAP307 (pBGS19$^+$::S1S2), pCHAP339 (pEMBL8$^+$::E_{C40}S1), and pCHAP348 (pBGS19$^+$::S2S1) (Fig. 1) by T4 DNA polymerase action on linearized single-strand derivatives by the method of Dale et al. (3). A unique *Eco*RI site or *Hin*dIII site was generated at each deletion endpoint. These plasmids were used to determine the nucleotide sequence by the dideoxy-chain termination reaction (20) with the Klenow fragment of *E. coli* DNA polymerase I or T7 DNA polymerase. Helper phage M13KO7 was used to obtain phage particles carrying single-stranded plasmid DNA. The entire nucleotide sequence was determined on both strands, and each nucleotide was sequenced at least four times. Restriction endonuclease fragments from plasmids carrying the T4 polymerase-generated deletions were subcloned into pEMBL8/9 vectors, pBR322, or pACYC184 (Table 1) for further analysis.

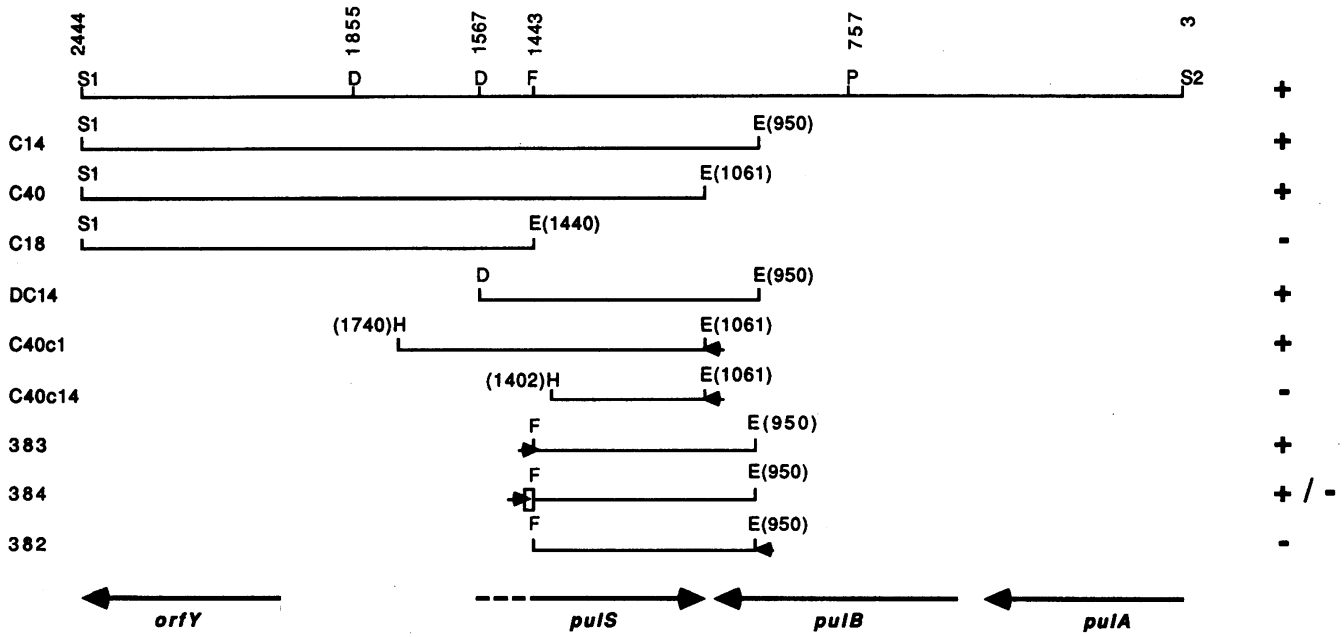

FIG. 1. Map of the DNA fragments used to locate *pulS*. The endpoints of the T4 DNA polymerase-generated deletions are indicated together with the restriction endonuclease site which is created. The position and orientation of the *lac* promoter-operator region in the vector DNA are indicated by a horizontal black arrowhead. The out-of-frame fusion between *lacZ'* and *pulS* in pCHAP384 is indicated by an open box. The four open reading frames present in the *Sma*I fragment are indicated. S, SmaI; P, *Hpa*I; F, *Fsp*I; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII. *Sma*I restriction sites are numbered as done previously (5). The results of the complementation tests involving the different plasmids are shown on the right.

TABLE 2. Organization of *malQ* operon fusions

Strain	Cloned fragment	Genetic organization
PAP2780	*Sac*I[a]-*Sma*I S2	*'pulC* Φ(*pulA-malPQ*)[b]
PAP2781	*Sac*I-*Hpa*I	*'pulC* Φ(*pulAB-malPQ*)
PAP2782	*Sac*I-*Fsp*I	*'pulC pulAB* Φ(*pulS-malPQ*)
PAP2783	*Sac*I-*Sma*I S1	*'pulC pulAB pulS* Φ(*orfY-malPQ*)
PAP2785	*Sma*I S2-*Hpa*I	Φ(*'pulAB-malPQ*)
PAP2784	*Sma*I S2-*Fsp*I	*'pulAB* Φ(*pulS-malPQ*)
PAP2778	*Fsp*I-*Sma*I S1	*pulS'* Φ(*orfY-malPQ*)
PAP2777	*Sma*I S1-*Fsp*I	*orfY* Φ(*pulS-malPQ*)

[a] *Sac*I is a restriction site located inside the *pulC* gene, upstream of the *pulA* promoter.
[b] Φ indicates operon fusion.

Transcriptional analysis. Different restriction fragments listed in Table 2 were subcloned into pOM41 (24). The recombinant plasmids were tested for tetracycline resistance, and insertion of the cloned DNA in front of the chromosomal *malPQ* operon was achieved by homologous recombination in strain pop2150 (for plasmids conferring tetracycline resistance) or in strain MC4100 (for plasmids conferring tetracycline sensitivity) as described by Raibaud et al. (19). The resulting genetic organization is shown in Table 2.

General procedures. Molecular genetic techniques were as described by Maniatis et al. (12). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a highly cross-linked gel system and stained with Coomassie blue by the method of Pugsley (15). Membranes obtained from cells lysed in a French pressure cell or by repeated freeze-thawing were fractionated as described previously (4). Minicells were purified and labeled with [^{35}S] methionine or ^{14}C-amino acids by the method of Pugsley and Oudega (17). Cells were labeled with [^{3}H]palmitate by the method of Pugsley et al. (16).

RESULTS AND DISCUSSION

A 2.5-kb fragment of DNA located downstream from *pulA* is required in *trans* for pullulanase secretion. Plasmid pCHAP231 is a pBR322 derivative which carries an 18.8-kb chromosomal fragment from *K. pneumoniae* UNF5023. *E. coli* K-12 carrying this plasmid synthesizes pullulanase, translocates it to the cell surface (exposition), and eventually releases it into the medium (5). In a previous study, two Tn*5* insertions (T4 and T13) located downstream from *pulA* were found to abolish pullulanase exposition. These insertions were within a 2.5-kb *Sma*I restriction endonuclease fragment which includes the 3′ end of *pulA*. In the same study, we also identified a Tn*5* insertion (T3) located between *pulA* and T4 and T13 which did not affect pullulanase activity or exposition. Taken together, these results suggested that a gene(s) located downstream from *pulA* was required for pullulanase exposition and was transcribed independently of *pulA* (5).

To study this aspect of pullulanase secretion in greater detail, we subcloned the ca. 2.5-kb *Sma*I fragment into pACYC184 and introduced the recombinant plasmid (pCHAP260) into an *E. coli* K-12 *recA* strain together with plasmid pCHAP231T4, pCHAP231T13, or pCHAP231 T14, the latter carrying a Tn*5* insertion (T14) within the *pulCDEFG* operon (5). The T4 and T13 insertion mutations, but not the T14 insertion, were complemented by pCHAP260, confirming that the 2.5-kb insert carries the gene(s) defined by the T4 and T13 insertions. Furthermore, since the cloning strategy was such that genes in the fragment could not be transcribed from vector promoters, this result indicated that *pulS* is transcribed from its own promoter.

Nucleotide sequencing of 2,447-base-pair *Sma*I fragment. The nucleotide sequence of the cloned *Sma*I fragment was determined as the first step in defining the position of the pullulanase secretion gene(s) (Fig. 2; see Materials and Methods for details). By comparison with the previously reported *K. pneumoniae* W70 *pulA* nucleotide sequence (9), we showed that the sequenced fragment included the last 445 nucleotides of the *pulA* gene. However, despite good DNA homology (81%) between 3′ ends of the two *pulA* genes, it appeared that the UNF5023 and W70 *pulA* stop codons are located at positions 442 and 431, respectively, according to our numbering (Fig. 2) and that the deduced amino acid sequences of the C-terminal regions of the two pullulanases are not highly homologous (54% for 143 amino acids). This suggests that the 3′ ends of the two *pulA* genes have evolved divergently without affecting the properties of pullulanase. We are currently sequencing the entire UNF5023 *pulA* gene; the comparison of the two genes will enable us to determine the extent of sequence divergence.

The *K. pneumoniae* UNF5023 *pulA* gene is followed by a palindromic structure ($\Delta G = -24.6$ kcal; Fig. 2) which is different from the one reported by Katsuragi et al. (9) for the W70 *pulA* gene.

The 2,447-base-pair *Sma*I fragment contains only one gene (*pulS*) required for pullulanase secretion. The sequence of the *Sma*I fragment (see above) was determined in part by using clustered deletion derivatives of plasmids pCHAP307 (pBGS19^{+}::S1S2; Fig. 1) and pCHAP339 (pEMBL8^{+}::E_{C40} S1; Fig. 1). After being subcloned into different vectors, the shortened fragments were tested for their ability to complement the *pulS*::Tn*5-4* mutation and thus define the *pulS* gene. For this purpose, we constructed a derivative of strain PAP2443 (5) in which the wild-type *pul* region (*pulAB pulS pulCDEFG* operon) located in the *E. coli* K-12 chromosome was replaced by the mutated *pul* region of pCHAP231T4 (*pulAB pulS*::Tn*5-4 pulCDEFG* operon) by homologous recombination (see Materials and Methods). A *recA*::Tn*10* mutation was subsequently introduced to avoid recombination between the *pulS* region in the plasmid being tested.

Deletions extending beyond nucleotide 1066 from the right and beyond nucleotide 1567 from the left abolished the ability to complement *pulS*::Tn*5-4* (Fig. 1). *pulS* is therefore contained within a 502-bp region close to the center of the *Sma*I fragment. This region contains a single potential open reading frame extending rightward from nucleotide 1447 to nucleotide 1070 (Fig. 2).

The putative initiator AUG codon of *pulS* is not preceded by a typical ribosome-binding site (Fig. 2) (8). The following experiments were designed to determine whether *pulS* was indeed translated. First, we subcloned the region between the *Fsp*I site at position 1443 and a deletion endpoint (*Eco*RI site) at position 950 in different orientations and in different positions in pEMBL8/9 vectors. The *pulS* gene was devoid of its own ATG codon in all cases and was fused in frame to *lacZ'* in pCHAP383, out of frame with *lacZ'* in pCHAP384, and in the opposite orientation to *lacZp* in pCHAP382 (Fig. 1). All three plasmids were then introduced into PAP2776 [*malPp*::(*pulAB pulS*::Tn*5-4 pulCDEFG*)], and the three resulting strains were tested for complementation of the *pulS*::Tn*5-4* mutation after induction with 0.5 mM IPTG. For pCHAP383, 100% of the pullulanase activity was exposed on the cell surface, whereas only 15% of the activity was

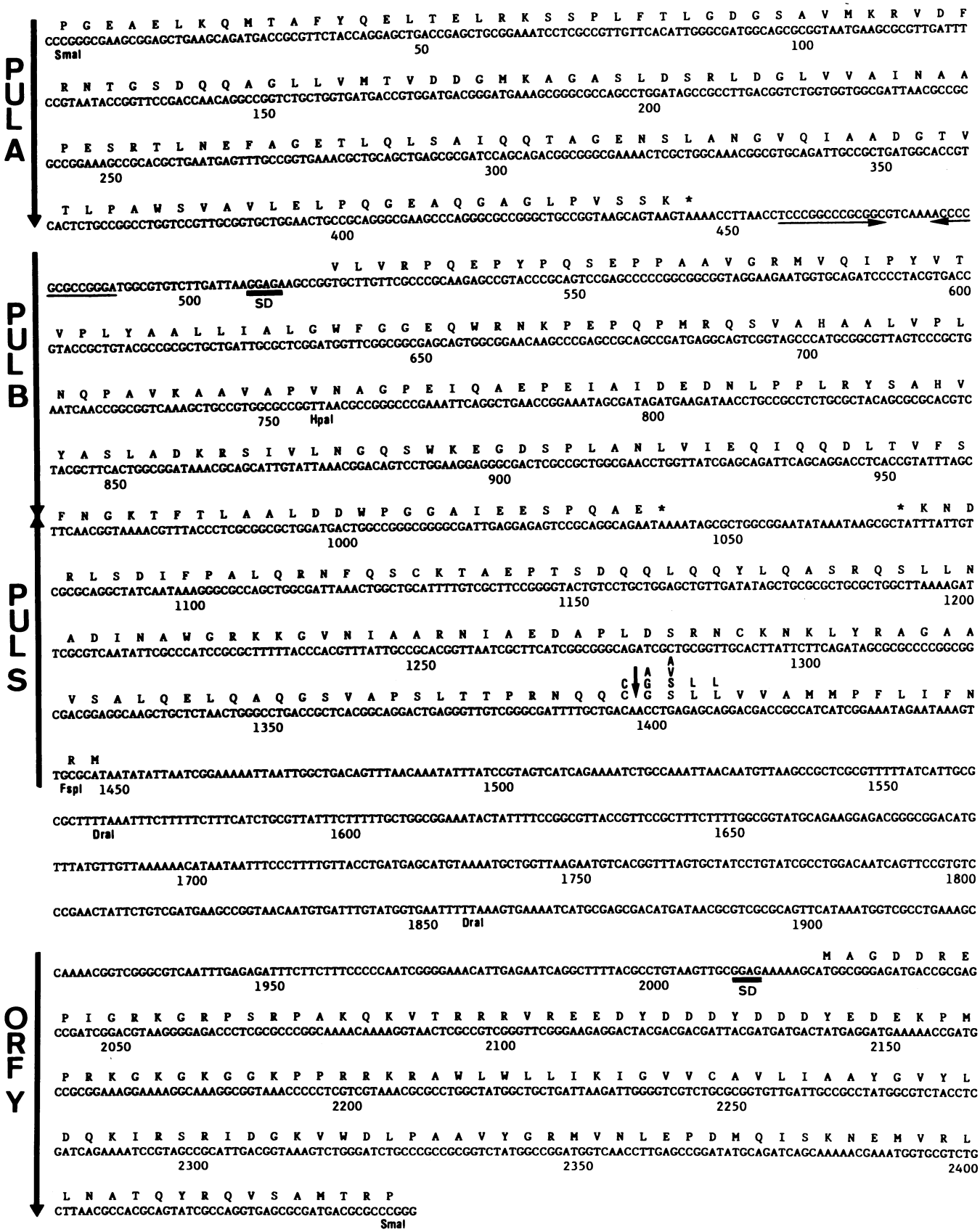

FIG. 2. Nucleotide sequence of the 2,447-base-pair *Sma*I fragment. The sequence shown is the nontranscribed strand for *pulA*, *pulB*, and *orfY* and the transcribed strand for *pulS*. The positions of the putative ribosome-binding sites in *pulB* and *orfY* mRNA are labeled SD. The predicted amino acid sequences of the C terminus of pullulanase, of the PulB and PulS proteins, and of the N terminus of OrfY are shown above the nucleotide sequence. The predicted processing site in prePulS is indicated by a vertical arrow. The consensus lipoprotein signal peptidase cleavage site is shown for comparison. This sequence has been submitted to the GenBank/EMBL data libraries under accession number M24118.

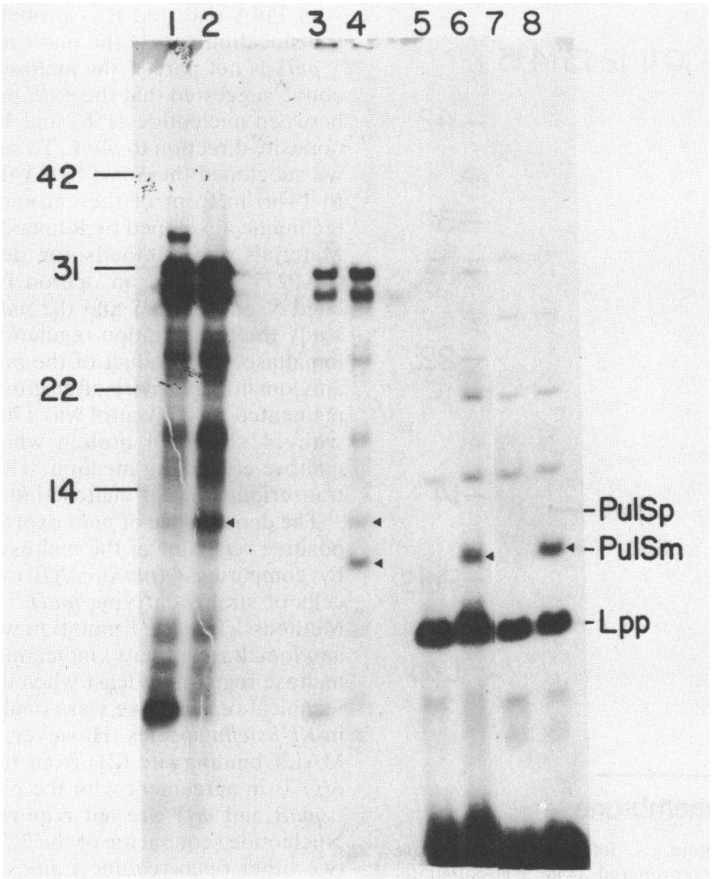

FIG. 3. Identification of precursor and mature forms of PulS protein. Lanes 1 to 4, Proteins produced by minicells carrying pBR322 (lanes 1 and 3) or pCHAP378 (lanes 2 and 4). Proteins were labeled with [^{35}S]methionine (lanes 1 and 2) or ^{14}C-amino acids (lanes 3 and 4). The precursor and mature forms of PulS protein are labeled p and m, respectively. Lanes 5 to 8, [^{3}H]palmitate-labeled proteins from cells carrying pBR322 (lane 1), pCHAP378 (lane 2), or pCHAP384 after growth in the absence (lane 3) or presence (lane 4) of IPTG. Proteins were separated on a highly cross-linked gel which was autoradiographed. Lpp, Major outer membrane lipoprotein. Positions of molecular mass markers (in kilodaltons) are shown at left.

detected at the cell surface with pCHAP384. Pullulanase produced by cells carrying pCHAP382 was not detectable in unlysed cells. Thus, we conclude that *pulS* has to be translated to complement the *pulS*::Tn*5-4* mutation. We presume that the low level of pullulanase detected at the cell surface when *pulS* is not in frame with *lacZ'* is due to the combined effects of translation staggering (to put the ribosome into the correct *pulS* reading frame), the high copy number of the plasmid, and the requirement for only very low amounts of PulS protein. Minicell experiments described below confirmed that the integral *pulS* gene is in fact translated. From the results described in this section, we can also conclude that the *pulS* promoter is located between positions 1567 and 1448 (Fig. 1 and 2).

Identification of PulS and subcellular location. As predicted by the nucleotide sequence, the *pulS* gene could encode a 125-amino-acid protein with a molecular weight of 13,792. It appeared from this sequence that PulS has a single major stretch of hydrophobic amino acids close to the N terminus. This sequence bears all the characteristics of a cleavable signal sequence (25), and the cleavage site is reminiscent of the consensus cleavage site for lipoprotein signal peptidase (Fig. 2) (26), indicating that PulS could be a processed lipoprotein with a fatty-acylated N-terminal cysteine (M_r = 11,873). To test this, we first attempted to label the *pulS* gene product in minicells with ^{14}C-amino acids or [^{35}S]methionine. Since methionine was predicted to occur only in the signal peptide of prePulS, only the precursor should be labeled with [^{35}S]methionine whereas both mature and precursor forms should be labeled with ^{14}C-amino acids. This prediction was borne out by results in Fig. 3, which show that two bands (14 and 12 kilodaltons [kDa]) were labeled with ^{14}C-amino acids, whereas only the 14-kDa band was labeled with [^{35}S]methionine. Neither band was detected when *pulS* was absent. The 14-kDa band was therefore assumed to be a PulS precursor and the 12-kDa band to be the mature PulS polypeptide.

We then determined whether [^{3}H]palmitate was incorporated into PulS protein in cells carrying pCHAP378 (fragment DC14 extending from nucleotide 1567 to nucleotide 950 cloned into pBR322; Fig. 1) or pCHAP383 (see above and Fig. 1). In both cases, we detected a [^{3}H]palmitate-labeled 12-kDa polypeptide which was absent from cells lacking a functional *pulS* gene (Fig. 3). Thus, we conclude that PulS is a lipoprotein.

Lipoproteins may be anchored in either of the two *E. coli* membranes, their precise location being determined at least in part by sequences immediately after the signal peptidase processing site (27). PulS protein does not have an acidic residue immediately downstream from the N-terminal cysteine and, according to Yamaguchi et al. (27), should therefore be located in the outer membrane. This was tested

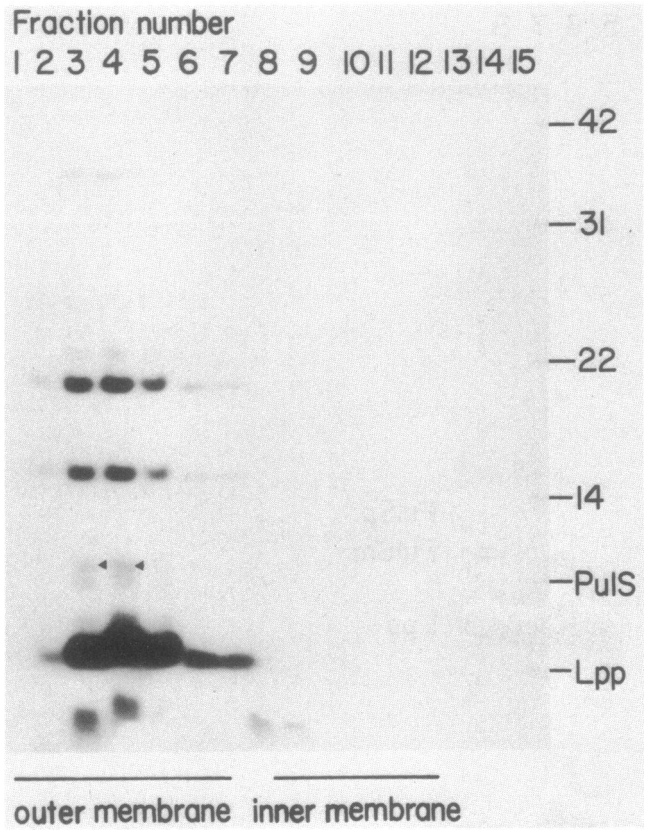

FIG. 4. Location of PulS protein as determined by sucrose gradient centrifugation of vesicles prepared from [^{3}H]palmitate-labeled cells of strain MC4100(pCHAP378) by French press lysis. The band identified as PulS was absent from [^{3}H]palmitate-labeled cells of MC4100(pBR322). The presence of proteins in fractions corresponding to the inner membrane was confirmed by staining the gel with $AgNO_3$. The total protein profile as revealed by this technique was identical to those presented previously (4). Note the apparent absence of lipoproteins from the inner membrane, as observed previously (15). A minor inner membrane-specific lipoprotein (M_r = 28,000, tentatively identified as NLPA) appeared as a faint band in overexposed gels. Lpp, Major outer membrane lipoprotein. The density of the fractions decreased from left to right. Positions of molecular mass markers (in kilodaltons) are shown at right.

directly by analyzing membranes from [^{3}H]palmitate-labeled MC4100(pCHAP378) (see above for structure of this plasmid) cells by isopycnic sucrose gradient centrifugation. PulS protein was found to be mainly associated with outer membrane vesicles (Fig. 4). An identical result was obtained with vesicles obtained from freeze-thaw-lysed cells (data not shown). We therefore propose that PulS is an outer membrane-anchored lipoprotein.

Studies by Pugsley et al. (16) have shown that pullulanase is itself a lipoprotein and that it is secreted as micelles without cleavage of its fatty-acylated N terminus. We therefore speculated that PulS could be cosecreted with PulA and be present in the pullulanase micelles. However, an analysis of extracellular and cell-associated [^{3}H]palmitate-labeled proteins present in cultures of strain MC4100(pCHAP231) incubated in maltose minimal medium for 24 h showed that, whereas PulA was secreted very efficiently, PulS, like all other lipoproteins, remained entirely cell associated (data not shown). Thus, we conclude that PulS is not cosecreted with PulA and that it is probably required for pullulanase translocation across the outer membrane.

***pulS* is not part of the maltose regulon.** Results described above suggested that the *pulS* promoter region was included between nucleotides 1567 and 1448 and was oriented in the opposite direction to *pulA*. To see whether this was the case, we subcloned the *Sma*I S1-*Fsp*I fragment (nucleotides 2444 to 1444) in front of the chromosomal *malPQ* operon by a technique developed by Raibaud and co-workers (19, 24; see Materials and Methods for details). The resulting strain (PAP2777) carried an operon fusion between the untranslated 5′ end of *pulS* and the *malPQ* operon, allowing us to study the transcription regulation of *pulS* by assaying amylomaltase, the product of the *malQ* gene. In this strain, the amylomaltase activity after growth in minimal medium supplemented with glycerol was 170 U/mg of protein, compared with 142 U/mg of protein when the cells were grown in maltose-containing medium. This result indicated that *pulS* transcription is not maltose inducible.

The dependence of *pulS* expression on MALT protein, the positive regulator of the maltose regulon (21), was assessed by comparing Φ(*pulS-malQ*) expression in glycerol-grown cells of strains carrying *malT*$^+$ or *malT* (see Materials and Methods). The *malT* mutation was entirely without effect on amylomaltase activity, indicating that *pulS* is not part of the maltose regulon, at least when it is expressed in *E. coli*. For technical reasons, we were unable to study *pulS* expression in *Klebsiella* species. However, the absence of a consensus MALT-binding site (21) from the region between *pulS* and *orfY* is in agreement with the results described above.

***pulB* and *orfY* are not required for pullulanase secretion.** Nucleotide sequencing of the 2.5-kb *Sma*I fragment revealed two other open reading frames, here called *pulB* and *orfY* (Fig. 2), each preceded by a typical ribosome-binding site (8). Analyses similar to that described in the previous section showed that *orfY* is constitutively expressed and extends beyond the *Sma*I site S1 (Fig. 2; data not shown) and that *pulB* is cotranscribed with *pulA*. This latter result suggests that the palindromic structure located between *pulA* and *pulB* is a palindromic unit similar to ones described in other bacterial operons (7) rather than a transcription terminator.

During transcriptional analysis of *pulB*, we constructed an *E. coli* K-12 strain, PAP2781, in which a restriction endonuclease fragment including only the entire *pulA* gene was cloned in front of the chromosomal *malPQ* operon. When grown in the presence of maltose, this strain produced pullulanase but could not transport it to the cell surface. Introduction of plasmid pCHAP40 (*pulCDEFG* [5]) and plasmid pCHAP378 (*pulS*) together into this strain was sufficient to allow pullulanase to be exposed. This result showed that neither *pulB* nor *orfY* is required for pullulanase exposition, which is in agreement with results described previously (5). *pulB* is thus a new component of the *K. pneumoniae* maltose regulon whose function remains to be established.

Conclusion. Results presented in this report show that *pulS* encodes a 12-kDa outer membrane lipoprotein required for pullulanase translocation. This is compatible with the idea that pullulanase secretion proteins should be located in the cell envelope and might form a transmembrane complex. The finding that *pulS* is linked to *pulA* and yet transcribed independently of the maltose regulon, and therefore of *pulA*, was somewhat surprising in view of the fact that all other genes required for the late stages of pullulanase secretion are apparently located in a *pulA*-linked operon which is part of the maltose regulon. This could be explained by one of at

least two possibilities. The first is that PulS is required for the secretion of other extracellular proteins. This hypothesis could be tested by studying the effects of a *pulS* mutation in *K. pneumoniae* (10) on protein secretion. However, growth conditions which stimulate the synthesis of novel secreted enzymes have not yet been established. The second possibility is that PulS protein must be synthesized before pullulanase and other pullulanase secretion proteins to ensure the efficient secretion of pullulanase or the correct assembly of a pullulanase secretion complex.

ACKNOWLEDGMENTS

We thank O. Raibaud for pOM2 and M. Schwartz, O. Raibaud, I. Reyss, and M. Kornacker for constant thoughtful comments and encouragement.

This work was financed by the Institut Pasteur and the Centre National de la Recherche Scientifique (URA 152).

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