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Stable periplasmic secretion intermediate in the general secretory pathway of *Escherichia coli*

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The secretion of the *Klebsiella oxytoca* cell surface lipoprotein pullulanase involves translocation across the cytoplasmic and outer membranes of the Gram-negative bacterial cell envelope. A variant of pullulanase was created by fusing the signal peptide-encoding 5′ region of the *Escherichia coli* gene for periplasmic MalE protein to the 3′ end of the *pulA* gene encoding almost the entire mature part of pullulanase. When produced in *E.coli* carrying the *malE–pulA* gene fusion on a high copy number plasmid and the complete set of genes specifically required for pullulanase secretion on a second plasmid, the hybrid protein differed from wild-type pullulanase as follows: (i) it was not fatty-acylated; (ii) it was apparently processed by LepB signal peptidase rather than by LspA lipoprotein signal peptidase; (iii) it was released into the periplasm and was only slowly transported across the outer membrane, and (iv) it was released directly into the medium rather than via the usual surface-anchored intermediate. The hybrid protein was secreted more rapidly when *malE–pulA* was expressed from a low copy number plasmid. The two steps in the secretion pathway could be totally uncoupled by expressing first the *malE–pulA* gene fusion and then the cognate secretion genes. These results show that fatty-acylation of wild-type PulA is not essential for secretion but may improve its efficiency when large amounts of the protein are produced, that the two steps in secretion can occur quite independently and that the periplasmic intermediate can persist for long periods under certain circumstances.

Key words: bacterial lipoprotein/general secretory pathway/ protein secretion/signal peptide

Introduction

The general secretory pathway (GSP) of Gram-negative bacteria is the main route for the export and secretion of signal sequence-bearing proteins. In *Escherichia coli*, the first step in the pathway, i.e. translocation across the cytoplasmic membrane, depends on the *sec* gene products (reviewed by Schatz and Beckwith, 1990). Subsequent steps in the GSP include protein folding in the periplasm (Bardwell *et al.*, 1991; Kamitani *et al.*, 1992), insertion into the outer membrane (Nikaido, 1992) and translocation across the outer membrane leading to extracellular release (Pugsley *et al.*, 1990a; Pugsley, 1992) or pilus assembly (Hultgren *et al.*, 1991).

Our aim is to determine how extracellular proteins are translocated across the outer membrane. We are using a model system based on the amylolytic lipoprotein pullulanase (PulA) of *Klebsiella oxytoca*. The entire PulA polypeptide is extracellular, but it remains anchored to the cell surface by fatty acids attached to the *N*-terminal cysteine of the processed precursor, and is slowly and probably spontaneously released into the growth medium only after the end of exponential growth (Pugsley *et al.*, 1986). The secretion mechanism has been studied in *E.coli* carrying the cloned *pulA* structural gene (d'Enfert *et al.*, 1987). The first step in secretion requires the PulA signal peptide, the Sec proteins and lipoprotein signal peptidase (LspA) (Pugsley *et al.*, 1991a). In *E.coli* carrying and expressing only *pulA*, the enzyme remains anchored by its fatty acids to the periplasmic side of the cytoplasmic membrane (Pugsley *et al.*, 1990b, 1991b; Pugsley and Kornacker, 1991). The products of 14 additional genes (*pulS* and the *pulC-O* operon) that were also cloned from *K.oxytoca* are necessary for the protein to reach the outer face of the outer membrane via the second step in the GSP (Pugsley *et al.*, 1990a; Pugsley, 1992; Possot *et al.*, 1992). The second step of the GSP in other Gram-negative bacteria that secrete exclusively non fatty-acylated proteins [e.g. *Erwinia chrysanthemi* (He *et al.*, 1991a), *Xanthomonas campestris* (Dums *et al.*, 1991; Hu *et al.*, 1992), *Pseudomonas aeruginosa* (Filloux *et al.*, 1990; Bally *et al.*, 1992) and *Aeromonas hydrophila* (Jiang and Howard, 1992)] also depends on proteins similar to those identified in the pullulanase system (see Pugsley, 1992 for review). *E.coli* K12 and closely related bacteria seem to lack this terminal GSP branch (Pugsley, 1992).

The role of fatty acids in anchoring PulA to the cell envelope is well established (Pugsley *et al.*, 1986, 1990b), but it is not clear whether they actively participate in the translocation of the enzyme through the cell envelope. In previous studies, site-directed mutagenesis was used to replace the modified cysteine in PulA produced by *Klebsiella aerogenes* (Murooka and Ikeda, 1989) or by *E.coli* expressing the cloned *Klebsiella oxytoca pul* genes (Kornacker *et al.*, 1991). In all cases, replacement of the *N*-terminal cysteine by another amino acid led to aberrant processing within the signal peptide by unidentified peptidase(s), and to very poor secretion (~10% in every case). Furthermore, the *N*-terminal hydrophobic extensions that remain in these PulA variants because of the aberrant processing could mimic the behaviour of the fatty acids (Kornacker *et al.*, 1991).

A naturally non-fatty-acylated PulA, produced by *K. planticola* (formerly *Klebsiella pneumoniae*) strain K21 together with fatty-acylated PulA, was also studied. The non-fatty-acylated PulA is correctly processed by LspA, indicating that it is modified by the addition of a glyceryl

group, and is secreted directly into the medium (Kornacker *et al.*, 1989a,b). The fatty-acylated PulA produced simultaneously by strain K21 becomes exposed at the cell surface in the same way as that produced by other strains. Since the two variants are the products of the same gene (Kornacker *et al.*, 1989a), the secretion of a non-fatty-acylated form of the protein may reflect competition between the modification and the secretion pathways in this strain (Kornacker *et al.*, 1989b).

Because results of studies with the unacylated PulA variants produced by *E.coli* were difficult to interpret, and because strain K21 is not amenable to genetic analysis, we decided to construct a hybrid protein comprising the *N*-terminal part of the precursor of a periplasmic protein, maltose binding protein (MalE), and the mature part of PulA lacking the extreme *N*-terminus including the signal peptide and the fatty-acylated cysteine. We predicted that such a hybrid would be correctly processed by the LepB signal peptidase at the normal cleavage site within the preMalE-derived segment, and would be consequently released into the periplasm. Studies reported here were conducted to test whether the pullulanase secretion factors can translocate the hybrid protein across the outer membrane.

Results

Construction and characterization of a malE-pulA *gene fusion*

In order to test the role of fatty acids in PulA secretion, we constructed a *malE−pulA* gene fusion encoding a hybrid protein in which 23 residues from the normal amino terminus of prePulA (including the 19 residue signal peptide and the Cys^1 that is fatty-acylated) were replaced by 28 amino acids from the amino terminus of preMalE (including the 26 amino acid signal peptide) and 13 amino acids encoded by linkers (Figure 1; pCHAP1106 and pCHAP1112, see Materials and methods). The MalE−PulA hybrid encoded by this gene fusion hydrolysed pullulan at least as efficiently as the wild-type enzyme. It could not be labelled with [^{3}H]palmitate (not shown), indicating the expected absence of fatty-acylation due to the lack of the recognition site for lipoprotein glyceryl- and fatty acyl transferases in the preMalE fragment of the hybrid protein.

Extracellular secretion of the hybrid protein

When the *malE−pulA* gene fusion carried by the high copy number pBGS18$^-$-derived plasmid pCHAP1106 (>50 copies per cell) was co-expressed in *E.coli* K12 with the pullulanase secretion genes on pCHAP710 (a pACYC184 derivative, 20−30 copies per cell), the hybrid protein was specifically secreted into the medium (Figure 2A), whereas it remained entirely intracellular when pCHAP710 was absent (not shown). The hybrid protein comigrated with the wild-type protein on SDS−PAGE [the predicted sizes of MalE−PulA and PulA are 116 and 118 kDA, respectively, but they appear somewhat larger on SDS−PAGE due to aberrant migration (Michaelis *et al.*, 1985)]. The extracellular enzyme was sometimes resolved into two bands, both of which reacted with anti-PulA serum in immunoblots (not shown).

The enzymatic activity of cellular and extracellular MalE−PulA was measured to determine the efficiency of its secretion. For this experiment we used strain pop3325 F′*lacI*^{q}, which produces a constitutively active MalT protein (the activator of the *pulC−O* operon carried by pCHAP710) (Débarbouillé *et al.*, 1978). This allowed us to measure enzymatic activity in the absence of maltose (which is converted into maltotriose, the activator of MalT), which produced a high background in the assay. Between 30 and 70% of the MalE−PulA activity was found in the medium during exponential growth (not shown). In contrast, <10% of wild-type (fatty-acylated) pullulanase produced by *E.coli* carrying the full set of specific secretion genes is found in the medium during exponential growth (d'Enfert *et al.*, 1987). The remaining cell-associated activity of MalE−PulA in *E.coli* (pCHAP1106, pCHAP710) could only be detected when the cells were lysed (not shown), which is again in contrast to the wild-type enzyme which is entirely exposed on the cell surface (d'Enfert *et al.*, 1987).

The kinetics of MalE−PulA secretion were determined by pulse−chase experiments using either maltose-induced

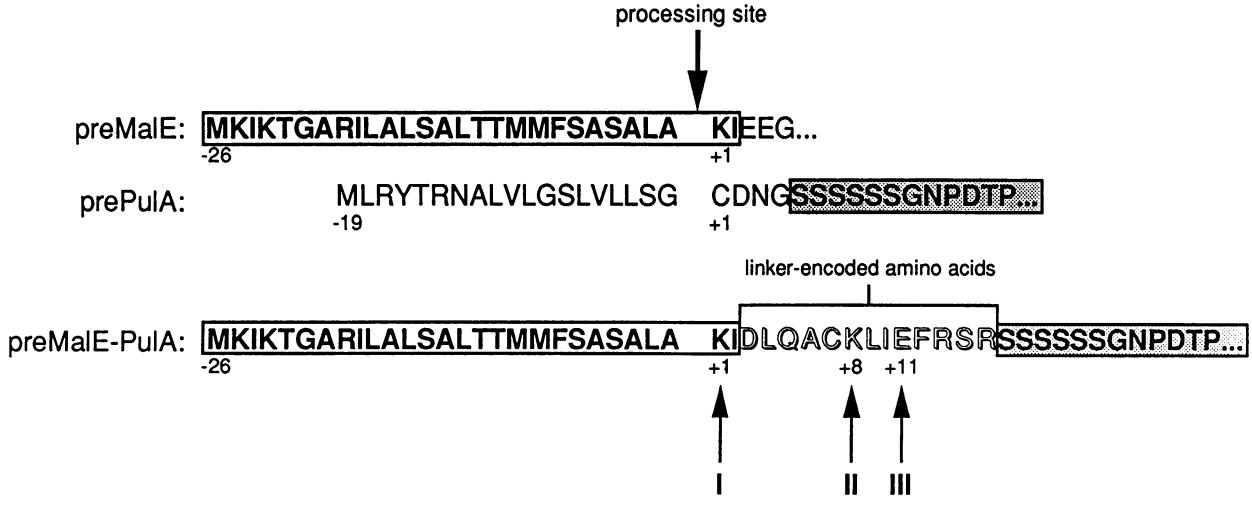

Fig. 1. N-terminal amino acid sequences of preMalE, prePulA and preMalE−PulA aligned at their known or predicted signal peptidase processing sites. The segments of preMalE−PulA derived from preMalE or PulA are in bold characters and boxed with white or shaded backgrounds respectively. Linker-encoded amino acids (see Materials and methods) are shadowed. The first amino acid of the mature part of each protein is numbered +1. The arrows at the bottom of the figure indicate starting points of the three forms of MalE−PulA (named I to III) identified by amino acid sequencing (see Table II).

wild-type *E.coli* strain PAP105 or the MalT^c strain pop3325F′, each carrying pCHAP1106 and pCHAP710. The extracellular protein could be resolved as two bands by SDS−PAGE, whereas only a single band was detected by immunoprecipitation of cell-associated protein in PAP105 (Figure 3). Extracellular release was again shown to be absolutely dependent on the presence of pCHAP710 (not shown). Secretion of MalE−PulA was slow in both strains. In PAP105, secretion was maximum after 4−8 h, but 50% of the labelled protein was extracellular after only 20−30 min (Figure 4A). Moreover, ~20% of the protein was never secreted (Figure 4B). It should be noted that, for an unknown reason, secretion of the more slowly migrating form of MalE−PulA appears to be faster than that of the more rapidly migrating form (50% of maximum after 11 min for the former, compared with >60 min for the latter; not shown). The secretion kinetics suggest that the efficiency of MalE−PulA secretion could be determined, at least in part, by the amount of hybrid protein synthesized.

In order to test the effect of plasmid copy number, we constructed pCHAP1112, a low copy number pHSG575 derivative (3−8 copies per cell) carrying the same *malE−pulA* gene fusion as pCHAP1106. The pulse−chase experiment was repeated using maltose-induced *E.coli* strain PAP105 carrying pCHAP1112 and pCHAP231T2, a pBR322 derivative which carries all of the pullulanase secretion genes and a Tn*5*-inactivated copy of *pulA* (20−30 copies per cell; d'Enfert *et al.*, 1987). Specific, maltose-dependent secretion of the pCHAP1112-encoded hybrid protein was maximal within 11 min (Figure 5), and was already >50% of maximal at the start of the chase period (3 min pulse; not shown). However, immunoprecipitation of cell-associated protein revealed that between 24 and 28% of the protein remained intracellular even after 60 min (not shown). Then, under these conditions, the secretion kinetics are much faster than when MalE−PulA is produced at high levels.

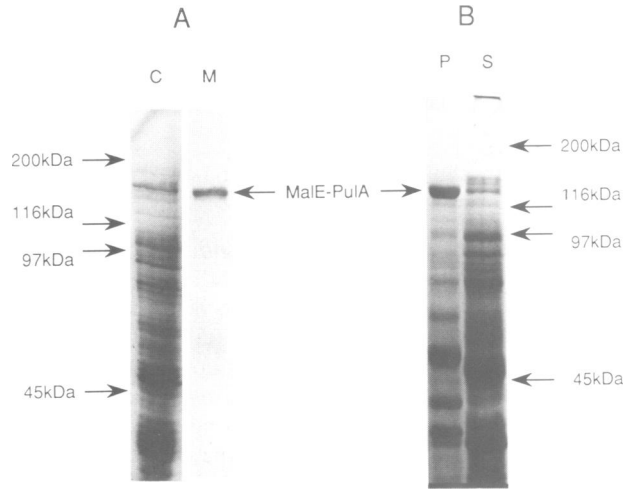

Fig. 2. Extracellular and periplasmic forms of MalE−PulA. (**A**) Strain pop3325F′ (pCHAP1106, pCHAP710) was grown in the presence of IPTG for 1 h to an A_{600} of 0.9. Medium (M) was separated from cells (C) by centrifugation and filtration through a Millex-GV filter (Millipore). Extracellular proteins were precipitated with 16% trichloroacetic acid in the presence of 1% Triton X-100. The sample of proteins from the medium is four times more concentrated than that of the cellular proteins. (**B**) A culture of strain PAP105 (pCHAP1106) was induced by IPTG (A_{600} = 0.7) and the periplasmic content of cells was released by spheroplasting. The sample of periplasmic proteins (P) is 10 times more concentrated than that of spheroplast proteins (S). Samples were electrophoresed on 8% acrylamide−0.21% bisacrylamide gels, which were stained with Coomassie brilliant blue. Molecular size standards are indicated.

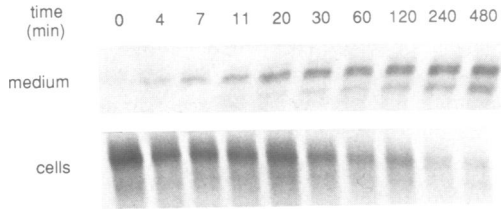

Fig. 3. [^{35}S]methionine pulse−chase labelling of overproduced MalE−PulA. Synthesis of MalE−PulA by *E.coli* PAP105 (pCHAP1106, pCHAP710) (A_{600} = 0.7) was induced for 40 min by IPTG. Proteins were labelled with 70 μCi/ml [^{35}S]methionine for 2 min and then chased (t_0). Samples were removed at time intervals (indicated at the top of the figure) and centrifuged. MalE−PulA was immunoprecipitated from the cells, and medium was prepared for SDS−PAGE as described in Materials and methods. Samples of cells and medium (both equivalent to 10 μl of culture) were electrophoresed on a 7% acrylamide−0.09% bisacrylamide gel, which was then dried and autoradiographed. Only that part of the radiograph displaying MalE−PulA is shown.

Location of overproduced, cell-associated MalE−PulA hybrid

Overproduced MalE−PulA hybrid was efficiently released from cells by osmotic shock, together with periplasmic

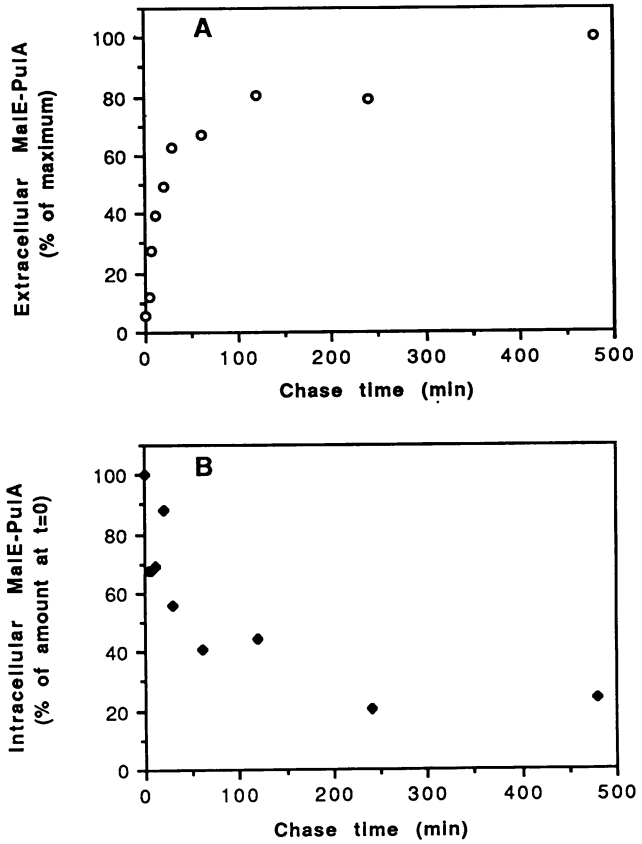

Fig. 4. Quantification of the secretion kinetics of overproduced MalE−PulA. The autoradiographs shown in Figure 3 were scanned. The amounts of extracellular and intracellular MalE−PulA are expressed respectively as the percentage of maximum secretion obtained at t = 480 min, and as the percentage of value at t = 0, which corresponds to 3 min of pulse.

Fig. 5. [^{35}S]methionine pulse–chase of MalE–PulA encoded by the low copy number plasmid pCHAP1112. Strains were derivatives of PAP105 bearing pCHAP231T2, which carries the *pul* secretion genes, together with pCHAP1112 (+, expressing *malE–pulA*) or pHSG575 (–, not expressing *malE–pulA*). Cells were grown overnight in the presence of maltose to induce expression of *pulC-O* and then diluted into fresh medium containing maltose to an A_{600} of 0.15. IPTG was added to induce the expression of *malE–pulA* for 135 min (A_{600} = 0.5). Proteins were labelled with [^{35}S]methionine for 3 min and then chased (t_0). Other details are as in Figure 3, except that an 8% acrylamide–0.21% bisacrylamide gel was used, and 24 μl of medium were loaded onto the gel. The two samples at the right represent total cell proteins at t_0.

Table I. Enzymatic activities recovered in the periplasm of *E.coli* strains producing MalE–PulA

Enzyme	Pul secretion factors	Cellular activity (units[a])	Activity in periplasm (% of total)[b]
Amylomaltase	–	538.0	6
	+	118.0	12
Alkaline phosphatase	–	111.0	85
	+	103.0	81
Acid phosphatase	–	195.0	64
	+	163.0	76
Pullulanase (MalE–PulA)	–	1273.0	64
	+	1139.5	61

The strain used was PAP105. *malE–pulA* was carried by pCHAP1106, and specific pullulanase secretion genes *pulS* and *pulC-O* by pCHAP710. The periplasmic fraction of cells expressing (+) or not expressing (–) the *pul* secretion genes was recovered by osmotic shock. Amylomaltase was used as a cytoplasmic marker, and alkaline phosphatase and acid phosphatase as periplasmic markers.
[a]Total enzymatic activities (lysed cells) are shown as arbitrary units per minute and per mg of protein, except for pullulanase activity which is expressed as nmol of glucose liberated per minute and per mg of protein. Note that amylomaltase activity is lower in cells expressing the *pul* secretion genes because of titration of MalT (the activator of the *malQ* gene) by the *pulC-O* promoter.
[b]Activity recovered from the periplasmic fraction is shown as the percentage of total activity.

alkaline and acid phosphatases (Table I). The proportion of the cell-associated enzyme that could be released in this way was quantitatively the same irrespective of the expression of the pCHAP710-encoded pullulanase secretion genes (Table I), implying that the enzyme accumulates in the periplasm prior to its translocation across the outer membrane. When the cells were converted into spheroplasts, most of the intracellular MalE–PulA was again released together with periplasmic proteins (Figure 2B). The enzyme that was released with the periplasmic contents remained in the supernatant fraction after centrifugation at 130 000 *g* for 1 h, implying that it is not aggregated or in a large complex (not shown). We observed previously (Pugsley *et al.*, 1991a) that pullulanase that is not exported out of the cytoplasm is inhibited by carboxymethylation with iodioacetamide. The activity of the pCHAP1106-encoded MalE–PulA hybrid that remained cell-associated was unaffected by iodoacetamide (not shown), suggesting that little or none of the enzyme accumulates in the cytoplasm.

Table II. Determined amino-termini of periplasmic and extracellular MalE–PulA

Form	Amino acid sequence	% of total sequenced	
		Periplasm	Medium
I	K I D L Q A - K L I E F R S (+1)	20	60
II	K L I E F R S (+8)	60	30
III	E F R S (+11)	20	10

The pCHAP1106-encoded hybrid protein from the periplasm of a strain lacking the Pul secretion factors or from the medium of a strain carrying pCHAP710 and producing these factors was purified as indicated in Materials and methods. Three major forms of the hybrid protein were revealed by micro-sequencing (see Figure 1). The ratios of each of them are indicated. Note that the seventh amino acid of form I, which is predicted to be a cysteine, could not be identified.

Processing of the MalE–PulA hybrid

The fact that pCHAP1106-encoded MalE–PulA was mainly recovered from the periplasm suggests that the MalE-signal peptide had been processed (Fikes *et al.*, 1990). However, the precursor form produced under conditions known to prevent export (and hence cleavage by signal peptidase) co-migrated with the mature form during SDS–PAGE (not shown). We therefore sequenced the amino termini of periplasmic MalE–PulA from a strain lacking pCHAP710 and of extracellular MalE–PulA from a maltose-induced strain carrying pCHAP710. In both cases, some of the sequenced material began at Lys[1] (named form I, see Figure 1 and Table II), the amino terminus of mature MalE (Fikes *et al.*, 1990). Two other forms, starting at Lys[8] (form II) and at Glu[11] (form III, see Figure 1), were also present (Table II). Neither of these two positions corresponds to alternative LepB signal peptidase cleavage sites (Fikes *et al.*, 1990) and they probably both result from non-specific cleavage. Forms II and III were more abundant in MalE–PulA extracted from the periplasm (Table II), either because they result from slow degradation by periplasmic proteases, or because of differences in their secretion kinetics. It should be noted that form II of MalE–PulA probably does not correspond to the more rapidly migrating band that can be separated by SDS–PAGE (Figure 3), since proteins that differ by only 7 amino acids would not be expected to be resolved while the precursor and mature forms of the protein, which differ by 26 amino acids, cannot be resolved under identical conditions. Rather, we believe that extracellular MalE–PulA is proteolytically cleaved near the carboxy terminus (Kornacker *et al.*, 1991).

The periplasmic hybrid protein is a bona fide secretion intermediate

The results presented so far suggest that preMalE–PulA is first translocated across the cytoplasmic membrane and then cleaved by LepB signal peptidase and released into the periplasm whence it is translocated across the outer membrane. However, the periplasmic pool which accumulates in strains expressing the *pul* secretion genes need not be the same as that which accumulates in their absence, since MalE–PulA may associate with one or more of the

Pul secretion factors until outer membrane translocation occurs. If this were the case, then periplasmic MalE−PulA made in the absence of the pullulanase secretion factors might not be competent for translocation across the outer membrane.

To study this question, we modified a technique developed earlier to uncouple artificially the two steps in PulA secretion (Pugsley *et al.*, 1991b). Exponentially growing cells of strain PAP105 (pCHAP1112, pCHAP231T2) were treated with IPTG to induce expression of *malE−pulA* carried by pCHAP1112. Proteins were labelled with [^{35}S]methionine after 135 min of induction, and the culture was divided into two parts, only one of which was supplemented with maltose to induce expression of the *pulC-O* operon carried by pCHAP231T2. Results in Figure 6 show that labelled MalE−PulA was secreted only by the maltose-induced culture, and that 2 h were necessary for maximum secretion of the labelled hybrid protein. This implies that MalE−PulA that is initially released into the periplasm in the complete absence of the *pulC-O* gene products can be subsequently transported across the outer membrane when these genes are expressed, and thus that the free, periplasmic form of MalE−PulA is a bona fide secretion intermediate.

Extracellular secretion and fatty acylation in K.planticola strain K21

The kinetics of secretion of overproduced MalE−PulA by *E.coli* seem to be similar to those of non-fatty-acylated PulA by *K.planticola* K21 (Kornacker *et al.*, 1989b). However, MalE−PulA is probably processed by LepB signal peptidase (this paper), whereas PulA of strain K21 is processed by LspA lipoprotein signal peptidase (Kornacker *et al.*, 1989b). Since some of the pullulanase produced by strain K21 is fatty-acylated (and consequently becomes cell surface associated), we proposed that translocation across the outer membrane and fatty-acylation were competing events (Kornacker *et al.*, 1989a,b). If this is the case, then more of the protein should be fatty-acylated when the second step in the GSP is blocked. To test this hypothesis, we compared the extent of [^{3}H]palmitate labelling of PulA in strain K21 and in secretion-defective derivatives carrying transposon Tn*10* in the *pul* secretion genes (Kornacker *et al.*, 1989a) (Figure 7). Labelling was increased to 225−265% of the wild-type level in the mutants (see legend of Figure 7 and Materials and methods), while the amount of PulA synthesized remained unaltered (not shown).

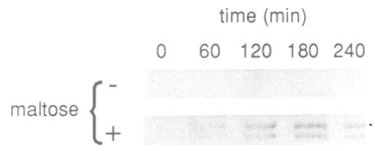

Fig. 6. Uncoupling the two steps in MalE−PulA secretion via the general secretory pathway. PAP105 (pCHAP1112, pCHAP231T2) was grown overnight in the absence of maltose, subcultured in the absence of maltose and then treated as in Figure 5 except that (i) proteins were labelled with 40 μCi/ml [^{35}S]methionine for 10 min, (ii) the culture was divided into two samples at t_0 and maltose (0.4%) was added to only one of them, and (iii) the equivalent of 32 μl of medium were loaded onto the gel. Other details are as in Figure 3.

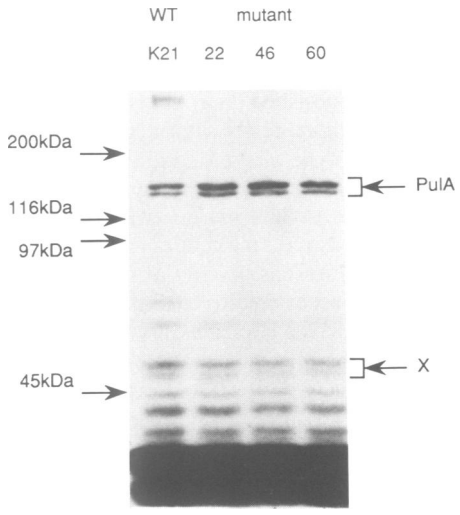

Fig. 7. [^{3}H]palmitate labelling of lipoproteins in *K.planticola* strain K21 and its secretion-defective derivatives. Cultures (A_{600} = 0.15) of strain K21 (WT) and its secretion-defective derivatives 22, 46 and 60 (see Kornacker *et al.*, 1989a) were labelled with [^{3}H]palmitate for 3 h at 30°C (A_{600} = 1.2−1.3). Proteins in 200 μl of the cultures (0.25 A_{600}) were then precipitated with 16% trichloroacetic acid, dissolved in SDS−PAGE sample buffer, heated to 100°C for 5 min and separated on an SDS−PAGE gel (7% acrylamide−0.09% bisacrylamide) which was then subjected to fluorography. The positions of the molecular size standards and of the two forms of palmitate-labelled PulA are indicated respectively on the left and right sides of the figure. The two bands labelled X were used as a standard for quantification. Identical areas of another gel around X and PulA were excised and solubilized and the levels of radioactivity were counted; the ratios PulA:X were then calculated for each sample. The phenotypes of the strains were also verified. As expected, strain K21 was the only one to secrete PulA into the medium, and the extracellular protein present during the exponential growth phase could not be labelled with [^{3}H]palmitate (not shown).

Discussion

We have constructed a non-fatty-acylated variant of pullulanase by fusing the extreme amino terminus of periplasmic maltose binding protein (MalE), including the signal peptide, to the almost entire mature segment of PulA. The hybrid protein is apparently processed by LepB signal peptidase and is exported into the periplasm. In the presence of the Pul secretion factors, it is specifically translocated across the outer membrane and released directly into the extracellular milieu. As expected, the signal peptide does not contain any information for the final localization of the protein. Unlike wild-type PulA, which remains membrane associated via its fatty acids (Pugsley *et al.*, 1990b) and which is rapidly and totally translocated across the outer membrane (Pugsley, 1993), the MalE−PulA hybrid is not membrane associated and is only slowly translocated across the outer membrane when overproduced.

Studies presented here show that the two steps in the secretion pathway can be totally dissociated, either by overproducing MalE−PulA, or by uncoupling *malE−pulA* expression from that of *pulC-O*. This confirms previous observations showing that the two steps in PulA secretion (across the cytoplasmic membrane and then the outer membrane) can occur independently and that the *pulC-O*-encoded secretion factors are only involved in the second step of the GSP (Pugsley *et al.*, 1991b). The results also show that a non-fatty-acylated variant of pullulanase can be secreted, but the efficiency of secretion seems to depend on the amount of protein synthesized (see next paragraph).

Finally, it is highly unlikely that the MalE−PulA that accumulates in the periplasm prior to extracellular secretion could be maintained in a loosely folded conformation, suggesting that it must be translocated across the outer membrane as a folded polypeptide (Pugsley *et al.*, 1991b; Pugsley, 1993).

One of the main interests of this study is that secretion of MalE−PulA occurs very slowly when the hybrid protein is encoded by a high copy number plasmid. Subcellular fractionation, pulse−chase and protein sequencing analyses show that large amounts of signal peptidase-processed MalE−PulA are stockpiled in the periplasm prior to translocation across the outer membrane under these conditions. Such a situation was not observed previously with wild-type PulA, overproduction of which does not saturate late steps in the secretion pathway or prevent it from reaching its normal position on the cell surface (Pugsley *et al.*, 1991a,b and unpublished data). This implies that the secretion pathway is more easily saturated by the hybrid protein than by the wild-type enzyme, and thus that fatty-acylation increases both secretion efficiency and kinetics at high expression levels. When *malE−pulA* is carried by a low copy number plasmid, maximum secretion of the hybrid protein occurs almost as quickly as that of wild-type PulA, which becomes accessible to external proteinase K (while remaining cell surface associated) within 5 min of synthesis when produced by *E.coli* carrying the pullulanase structural and secretion genes on the same pBR322-derived plasmid (Pugsley, 1993). Thus, the kinetics of secretion of MalE−PulA encoded by a low copy number plasmid are virtually indistinguishable from those of wild-type protein encoded by the same vector. However, secretion is never completed; ~25% of the protein remains intracellular even 60 min after its synthesis.

MalE−PulA is similar in several respects to proteins secreted by other Gram-negative bacteria via the GSP (Pugsley, 1992) and which are not fatty acylated. For example, secretion of proaerolysin by *A.hydrophila* (Jiang and Howard, 1992) is almost complete within 4−10 min depending on the experimental conditions (Howard and Buckley, 1985), while PelE pectate lyase of *E.chrysanthemi* is secreted within 1 min of *E.coli* carrying the cloned *pelE* and its cognate secretion genes (*out*; He *et al.*, 1991a,b). Indeed, PelE secretion occurs so quickly that it is unlikely to transit via a free periplasmic intermediate similar to that demonstrated here with overproduced MalE−PulA, although periplasmic accumulation of PelE can be imposed by high level production (He *et al.*, 1991b) or by mutations in the *out* genes (Andro *et al.*, 1984). Similarly, when the *P.aeruginosa* PAK toxin A gene is expressed in the hypotoxigenic *P.aeruginosa* mutant PAO-T1, 80% of the toxin is secreted but the remaining 20% could not be detected in the periplasm (Hamood *et al.*, 1989). Thus, the studies on overproduced MalE−PulA presented here represent the first demonstration of a long-lived periplasmic intermediate in the GSP, although such intermediates probably do not exist, or at most are only very short-lived, under natural conditions.

Several hypotheses can be envisioned for the comparatively slow secretion of overproduced MalE−PulA. The additional amino acids introduced at the extreme *N*-terminus of mature PulA could mask or alter the as yet unidentified secretion signal to render it less effective or prevent the hybrid protein from folding into a conformation compatible with efficient secretion. However, the presence of two different lipoprotein *N*-terminal sequences in front of the same PulA fragment as in MalE−PulA does not have any measurable effect on secretion (not shown). We therefore favour the idea that the fatty acids, though not absolutely required for secretion, might improve its efficiency. For example, they may prolong retention of PulA at a site from which it can be rapidly and efficiently translocated across the outer membrane. The initial interaction between PulA and the PulA-specific part of the GSP might occur very early, perhaps before translocation across the cytoplasmic membrane is complete, but may be less stable in the absence of the fatty acids on PulA. Alternatively, the fatty acids might increase the chances of productive interactions between the enzyme and the secretion machinery.

We previously proposed that one or more of the 14 *pul* gene products that are needed for PulA secretion might be specifically required to transport the covalently attached fatty acids to the cell surface (Pugsley *et al.*, 1990a; Kornacker *et al.*, 1991; Pugsley, 1992). If such a gene exists, then it might not be present in other Gram-negative bacteria that secrete exclusively non-fatty acylated proteins by a similar pathway. Based on the sequence data available to us when this work was carried out, it appeared that homologues of all of the *pul* secretion genes except *pulS* (d'Enfert and Pugsley, 1989) had been identified in other protein-secreting Gram-negative bacteria (Pugsley, 1992). However, we found in the present study that PulS is absolutely required for MalE−PulA secretion (data not shown), in agreement with previous results on the secretion of other non-fatty-acylated variants of PulA (Kornacker *et al.*, 1989a, 1991). Thus, we conclude that PulS is not specifically required for translocation of the PulA fatty acids across the outer membrane. Furthermore, since this work was carried out, we learnt that *E.chrysanthemi* has a PulS homologue which is required for extracellular secretion of non-fatty-acylated proteins (G.Condemine, personal communication).

The reason why *K.planticola* strain K21 fatty-acylates only some of the PulA polypeptides it produces is not immediately apparent. We think it most likely that reduced fatty-acylation is due to a partial defect in one or both of the fatty acyl transferases in this strain. Some of the PulA polypeptides may therefore be shunted into the second, pullulanase-specific part of the GSP before they are fatty-acylated (Kornacker *et al.*, 1989a,b). Mutations which block the second step in the pullulanase secretion pathway in strain K21 without affecting the level of PulA synthesis (Kornacker *et al.*, 1989a) are shown here to increase fatty-acylation of PulA. This can be explained by the fact that all of these mutations probably block the entry of PulA into the second step of the GSP by preventing the assembly of the different secretion factors into a functional secretion complex (Pugsley, 1992; Possot *et al.*, 1992; Pugsley and Dupuy, 1992). Thus, the mutants are able to complete the fatty-acylation process, albeit more slowly than in other strains, because PulA remains accessible to the fatty acyl transferases.

Materials and methods

Bacterial strains and plasmids

Strains of *E.coli* K12 used were PAP105 (d'Enfert *et al.*, 1987) and pop3325F′, a derivative of strain pop3325 (*malT*^{c}; Débarbouillé *et al.*, 1978), which carries the same F′ (*lacI*^{q1} *lacZM15 pro*$^{+}$ Tn*10*) as PAP105, in order to repress *lac*Zp. pCHAP710 (Kornacker and Pugsley, 1990a) and pCHAP231T2 (d'Enfert *et al.*, 1987) carry the constitutively expressed *pulS* gene and the maltose-inducible *pulC-O* operon.

The *malE–pulA* gene fusion was constructed as follows. pCHAP1000, which carries the *pulA401* mutation, was first constructed by inserting a 12 bp linker (5′-pGATCTAGATCTA-3′) into the first *Bam*HI site of *pulA* in pCHAP656 (Kornacker and Pugsley, 1990a), which creates a unique *Bgl*II restriction site (Pugsley *et al.*, 1991a). The *Eco*RI–*Hin*dIII fragment of pCHAP1000, including the *pulA401* allele, was subcloned into pBGS18$^-$ (Spratt *et al.*, 1986), to give pCHAP1001. pCHAP1001 was digested with *Bgl*II, filled in using the Klenow fragment of DNA polymerase I, and ligated with the linker 5′-pGGAATTCC-3′ to create a second *Eco*RI site (pCHAP1104). The 195 bp *Eco*RI–*Eco*RI fragment of pSB118::Ep36, which carries the 5′ end of *malE* including the first 28 codons (Raibaud *et al.*, 1989), was used to replace the small *Eco*RI fragment of pCHAP1104 that includes the 5′ end of *pulA* and upstream regions, so that sequences derived from *malE* were in-frame with those derived from *pulA*. The resulting plasmid, pCHAP1106, encodes the hybrid protein preMalE–PulA which contains the 28 *N*-terminal amino acids of preMalE, followed by 13 amino acids encoded by linkers, and then by PulA starting at position +4 (see Figure 1). The *Eco*RI–*Hin*dIII and the *Eco*RI–*Eco*RI fragments from pCHAP1106 (3.6 kb and 195 bp, respectively) were successively introduced into pHSG575 (Takeshita *et al.*, 1987). The *Eco*RI–*Eco*RI–*Hin*dIII fragment carrying the gene fusion *malE–pulA* was thereby reconstituted in pHSG575 to create pCHAP1112. All DNA manipulations were essentially as described in Perbal (1988).

Growth conditions

Cultures were incubated at 30°C in L broth (Miller, 1972) or in M63 minimal medium (Miller, 1972) or low-potassium medium (Pugsley *et al.*, 1991a), with vigorous shaking. Minimal media were supplemented with 0.4% glycerol and with 0.4% casamino acids or a methionine-free amino acid mixture (Pugsley *et al.*, 1991a). Maltose (0.4%) was added to induce expression of the *pulC-O* operon, and 200 μM isopropyl-β-D-galactoside (IPTG) was used to induce expression of *lacZ*p-controlled *pulA* or *pulA* derivatives. Antibiotics were used at the following concentrations: ampicillin, 400 μg/ml; tetracycline, 15 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml.

Enzyme assays

Pullulanase (Michaelis *et al.*, 1985; Pugsley *et al.*, 1991a), amylomaltase (Pugsley and Dubreuil, 1988) and alkaline and acid phosphatases (Kornacker and Pugsley, 1990b) were assayed as previously.

Subcellular fractionation

Osmotic shock was performed as described by Brockman and Heppel (1968). Spheroplasts were prepared essentially as described in Pugsley *et al.* (1991a).

Protein sequence determination

Derivatives of strain pop3325F′ expressing *malE–pulA* alone (pCHAP1106) or *malE–pulA* and the *pul* secretion genes (pCHAP1106 and pCHAP710) were grown in 1 litre of L broth containing 200 μM IPTG to an A_{600nm} of 0.8–1.0. The periplasmic fraction of pop3325F′ (pCHAP1106) was recovered by osmotic shock, and the clarified medium from pop3325F′ (pCHAP1106, pCHAP710) was concentrated by filtration through an Amicon Diaflo PM10 filter. Proteins were precipitated with 16% trichloroacetic acid, resuspended in SDS–PAGE sample buffer (see below) and heated to 100°C for 5 min. Approximately 30–60 μg (300–600 pmol) of MalE–PulA protein in each case were electrophoresed, electroblotted onto Pro-Blott membranes (Applied Biosystems) and sequenced in a Blott cartridge using an Applied Biosystems Model 477A Pulsed Liquid Protein Sequencer according to the standard procedure of the manufacturer.

Protein labelling and immunoprecipitation

Lipoproteins were specifically labelled with 20 μCi/ml [9–10(n)-^{3}H]palmitic acid (Amersham) for 3 h. In all other cases, proteins were labelled with [^{35}S]methionine (Amersham) as indicated. [^{35}S]methionine was chased by adding unlabelled methionine to 0.025 or 0.05%. Media and cells were separated by centrifugation. Medium was diluted 4:1 in 50% glycerol, 5% β-mercaptoethanol and 5% SDS and heated to 100°C for 5 min for SDS–PAGE. Cells were resuspended in SDS–PAGE sample buffer (0.1 M Tris–Cl, pH 8.0, 5% SDS, 12.5% glycerol, 1% β-mercaptoethanol) at 100°C for 5 min, or in 25 mM Tris–Cl (pH 7.5) and 0.8% SDS, heated to 100°C for 5 min and then immunoprecipitated as in Pugsley *et al.* (1991a). Treatment of radioactive gels and quantification of radioactivity were as described previously (Pugsley *et al.*, 1991a), except that Solvable™ solution (DuPont) was used.

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References

Andro,T., Chambost,J.-P., Kotoujansky,A., Cattaneo,J., Bertheau,Y., Barras,F., vanGijsegem,F. and Coleno,A. (1984) *J. Bacteriol.*, **160**, 1199–1203.

Bally,M., Filloux,A., Akrim,M., Ball,G., Lazdunski,A. and Tommassen,J. (1992) *Mol. Microbiol.*, **6**, 1121–1131.

Bardwell,J.C.A., McGovern,K. and Beckwith,J. (1991) *Cell*, **67**, 581–589.

Brockman,R.W. and Heppel,L.A. (1968) *Biochemistry*, **7**, 2554–2562.

Débarbouillé,M., Shuman,H.A., Silhavy,T.J. and Schwartz,M. (1978) *J. Mol. Biol.*, **124**, 359–371.

d'Enfert,C. and Pugsley,A.P. (1989) *J. Bacteriol.*, **171**, 3673–3679.

d'Enfert,C., Ryter,A. and Pugsley,A.P. (1987) *EMBO J.*, **6**, 3531–3538.

Dums,F., Dow,J.M. and Daniels,M.J. (1991) *Mol. Gen. Genet.*, **229**, 357–364.

Fikes,J.D., Barkocy-Gallagher,G.A., Klapper,D.G. and Bassford,P.J.,Jr (1990) *J. Biol. Chem.*, **265**, 3417–3423.

Filloux,A., Bally,M., Ball,G., Akrim,M., Tommassen,J. and Lazdunski,A. (1990) *EMBO J.*, **9**, 4323–4329.

Hamood,A.N., Olson,J.C., Vincent,T.S. and Iglewski,B.H. (1989) *J. Bacteriol.*, **171**, 1817–1824.

He,S.Y., Lindeberg,M., Chatterjee,A.K. and Collmer,A. (1991a) *Proc. Natl Acad. Sci. USA*, **88**, 1079–1083.

He,S.Y., Schoedel,C., Chatterjee,A.K. and Collmer,A. (1991b) *J. Bacteriol.*, **173**, 4310–4317.

Howard,S.P. and Buckley,T.J. (1985) *J. Bacteriol.*, **161**, 1118–1124.

Hu,N.-T., Hung,M.-N., Chiou,S.-J., Tang,F., Chiang,D.-C., Huang,H.-Y. and Wu,C.-Y. (1992) *J. Bacteriol.*, **174**, 2679–2687.

Hultgren,S.J., Normark,S. and Abraham,S.N. (1991) *Annu. Rev. Microbiol.*, **45**, 383–415.

Jiang,B. and Howard,S.P. (1992) *Mol. Microbiol.*, **6**, 1351–1361.

Kamitani,S., Yoshinori,A. and Ito,K. (1992) *EMBO J.*, **11**, 57–62.

Kornacker,M.G. and Pugsley,A.P. (1990a) *Mol. Microbiol.*, **4**, 73–85.

Kornacker,M.G. and Pugsley,A.P. (1990b) *Mol. Microbiol.*, **4**, 1101–1109.

Kornacker,M.G., Boyd,A., Pugsley,A.P. and Plastow,G.S. (1989a) *J. Gen. Microbiol.*, **135**, 397–408.

Kornacker,M.G., Boyd,A., Pugsley,A.P. and Plastow,G.S. (1989b) *Mol. Microbiol.*, **3**, 497–503.

Kornacker,M.G., Faucher,D. and Pugsley,A.P. (1991) *J. Biol. Chem.*, **266**, 13842–13848.

Michaelis,S., Chapon,C., d'Enfert,C., Pugsley,A.P. and Schwartz,M. (1985) *J. Bacteriol.*, **164**, 633–638.

Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Murooka,Y. and Ikeda,R. (1989) *J. Biol. Chem.*, **264**, 17524–17531.

Nikaido,H. (1992) *Mol. Microbiol.*, **6**, 435–442.

Perbal,B. (1988) *A Practical Guide to Molecular Cloning*. Wiley, New York, USA.

Possot,O., d'Enfert,C., Reyss,I. and Pugsley,A.P. (1992) *Mol. Microbiol.*, **6**, 95–105.

Pugsley,A.P. (1992) In Mohan,S.B., Dow,C. and Cole,J.A. (eds), *Prokaryotic Structure and Function: a New Perspective*. Society for General Microbiology Symposium 47, Cambridge University Press, Cambridge, pp. 223–248.

Pugsley,A.P. (1993) *Proc. Natl Acad. Sci. USA*, in press.

Pugsley,A.P. and Dubreuil,C. (1988) *Mol. Microbiol.*, **2**, 473–479.

Pugsley,A.P. and Dupuy,B. (1992) *Mol. Microbiol.*, **6**, 751–760.

Pugsley,A.P. and Kornacker,M.G. (1991) *J. Biol. Chem.*, **266**, 13640–13645.

Pugsley,A.P., Chapon,C. and Schwartz,M. (1986) *J. Bacteriol.*, **166**, 1083–1088.

Pugsley,A.P., d'Enfert,C., Reyss,I. and Kornacker,M.G. (1990a) *Annu. Rev. Genet.*, **24**, 67–90.

Pugsley,A.P., Kornacker,M.G. and Ryter,A. (1990b) *Mol. Microbiol.*, **4**, 59–72.

Pugsley,A.P., Kornacker,M.G. and Poquet,I. (1991a) *Mol. Microbiol.*, **5**, 343–352.

Pugsley,A.P., Poquet,I. and Kornacker,M.G. (1991b) *Mol. Microbiol.*, **5**, 865–873.

Raibaud,O., Vidal-Ingigliardi,D. and Richet,E. (1989) *J.Mol. Biol.*, **205**, 471–485.

Schatz,P.J. and Beckwith,J. (1990) *Annu. Rev. Genet.*, **24**, 215–248.
Spratt,B.G., Hedge,P.J., te Heesen,S., Edelman,A. and Broome-Smith,J.K. (1986) *Gene*, **41**, 337–342.
Takeshita,S., Sato,M., Toba,M., Masahashi,W. and Hashimoto-Goto,T. (1987) *Gene*, **61**, 63–74.

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