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The general protein-export pathway is directly required for extracellular pullulanase secretion in *Escherichia coli* K12

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Summary

Pullulanase is an extracellular, cell surface-anchored lipoprotein produced by Gram-negative bacteria belonging to the genus *Klebsiella*. Its correct localization in recombinant *Escherichia coli* requires the products of 14 genes that are linked to the enzyme structural gene in the *Klebsiella* chromosome. In addition, we show here that six *sec* genes (*secA*, *secB*, *secD*, *secE*, *secF* and *secY*) are all required for processing of the prepullulanase signal peptide to occur. This implies that pullulanase crosses the cytoplasmic membrane via the general export pathway of which the *sec* gene products are essential components. Removal or drastic alteration of the prepullulanase signal peptide cause the enzyme to remain cytoplasmic. We propose that pullulanase secretion occurs in two steps, the first of which is common to all signal peptide-bearing precursors of exported and secreted proteins, whereas the second is specifically involved in translocating pullulanase to the cell surface.

Introduction

All periplasmic and outer-membrane proteins of *Escherichia coli* K12, as well as a few cytoplasmic membrane proteins, are made as signal peptide-bearing precursors that are transported out of the cytoplasm, across the cytoplasmic membrane, by the general export pathway (GEP) (reviewed in Pugsley, 1989). This pathway comprises (i) several cytosolic chaperones including SecB protein (Kumamoto and Beckwith, 1985; Weiss *et al.*, 1988; Lecker *et al.*, 1989), GroEL protein (Kusukawa *et al.*, 1989; Lecker *et al.*, 1989) and possibly also DnaK protein (Phillips and Silhavy, 1990) that maintain nascent polypeptides in an export-competent state (Pugsley, 1989), (ii) the SecA protein that binds to proteins with signal peptides (Akita *et al.*, 1990) and functions as a translocation ATPase (Cunningham and Wickner, 1989), (iii) at least four integral cytoplasmic membrane proteins (SecD, SecE(PrlG), SecF and SecY(PrlA)) that probably form the signal peptide receptor and/or translocation channel (Bieker and Silhavy 1989; 1990; Brundage *et al.*, 1990; Gardel *et al.*, 1990), and (iv) two signal peptidases, one of which (lipoprotein signal peptidase) is specific for a subclass of secretory proteins in which glycerylcysteine forms part of the cleavage site and is inhibited by the antibiotic globomycin (Pugsley, 1989). Many of the components of this pathway were identified through the analysis of mutations that diminished the export of several different proteins or that increased the export of proteins with debilitated signal peptides (reviewed by Schatz and Beckwith, 1990).

With few exceptions (e.g. Felmlee *et al.*, 1985; Delepelaire and Wandersman, 1989), extracellular proteins secreted by Gram-negative bacteria also possess typical *N*-terminal signal peptides, implying that they too might cross the cytoplasmic membrane via GEP (Pugsley, 1988). In many cases, the extracellular secretion of these proteins is blocked by mutations that are without effect on the export of periplasmic or outer-membrane proteins, indicating that if GEP is involved, it is not sufficient for extracellular secretion (Pugsley *et al.*, 1990b). In the case of pullulanase (PulA), a cell surface/extracellular lipoprotein produced by *Klebsiella oxytoca* and *Klebsiella plantolytica* (*Klebsiella pneumoniae*/*Klebsiella aerogenes*), we have shown that all but one of these genes are in an operon that is coregulated with the structural gene, *pulA*. One of these genes codes for a cytoplasmic protein while several others code for cytoplasmic membrane proteins (Pugsley *et al.*, 1990b). This led us to suggest that some of these pullulanase-specific secretion factors might partially or completely replace GEP for pullulanase translocation across the cytoplasmic membrane (Pugsley *et al.*, 1990b). In the present paper, we make use of the fact that the entire pullulanase secretion pathway has been reconstituted in *E. coli* K12 (d'Enfert *et al.*,

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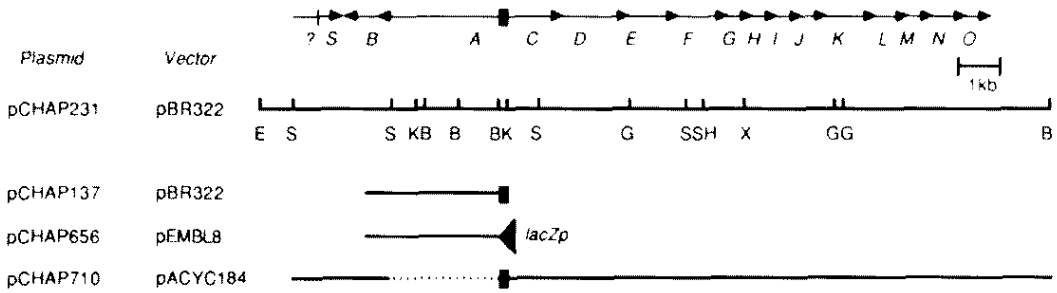

Fig. 1. Organization of plasmids used in this study. pCHAP231 was described by d'Enfert *et al.* (1987) and pCHAP656 and pCHAP710 by Kornacker and Pugsley (1990). The construction of pCHAP137 is described in the *Experimental procedures*. The sites for the restriction enzymes *Eco*RI (E), *Sma*I(S), *Kpn*I (K), *Bam*HI (B), *Bgl*II (G), *Hin*dIII (H) and *Xho*I (X) are shown on the map of pCHAP231, with which all other maps are aligned. Vector sequences are omitted for clarity. The line above the map of pCHAP231 shows the positions of the *pul* genes (see Pugsley *et al.*, 1990b). The black box between *pulA* and *pulC* represents the divergent, MalT regulated promoters of these genes. The arrowhead on the map of pCHAP656 shows the orientation of the *lacZ* promoter. This plasmid lacks the *pulA–pulC* promoters (Kornacker and Pugsley, 1990). The broken line on the representation of pCHAP710 indicates *pulA* sequences that are missing from this plasmid.

1987), making it possible to analyse the effects of mutations or conditions that reduce the efficiency of protein export via GEP on pullulanase processing by lipoprotein signal peptidase and on its secretion.

Results

The integrity of the prepullulanase signal peptide is required for exit from the cytoplasm

In order to test whether the pullulanase signal peptide is required for pullulanase secretion, we constructed three different *pulA* mutations in derivatives of pCHAP656 (Fig. 1; Kornacker and Pugsley, 1990) which were designed to render the signal peptide inactive (Pugsley, 1989) or to remove it completely (Fig. 2 and *Experimental procedures*).

The wild-type and mutant plasmids were introduced into strain PAP105 carrying pCHAP710, a compatible pACYC184-derived plasmid carrying all pullulanase-specific secretion genes (Fig. 1; Kornacker and Pugsley, 1990). The resulting strains were maintained in early exponential phase in minimal medium to which 1 mM isopropyl-β-D-thiogalactoside (IPTG) and 0.4% maltose were added to induce expression of *pulA* and the *pulC–pulO* operon of secretion genes, respectively (Fig. 1). As reported previously, pullulanase produced under these conditions by strains carrying wild-type *pulA* was almost entirely located on the cell surface, as determined by its accessibility to the non-penetrating substrate pullulan (Kornacker and Pugsley, 1990; Pugsley *et al.*, 1990b). Cells carrying pCHAP656 derivatives bearing the *pulA107*, *pulA201*, or *pulA301* alleles produced approximately the same amount of pullulanase as those carrying wild-type *pulA* but little of this activity was detectable in whole cells, indicating that the enzyme had not reached the cell surface (Table 1). Fractionation studies indicated that the activity encoded by these mutant plasmids was mainly cytoplasmic (Table 1), whereas wild-type pullulanase was apparently present in the envelope, cytoplasmic and periplasmic fractions as well as the cell debris, irrespective of the presence of pCHAP710 (Table 1). The aberrant behaviour of wild-type pullulanase during cell fractionation by procedures such as those used here is due to its tendency to form protein micelles upon release from the outer or cytoplasmic membranes (e.g. Pugsley *et al.*, 1990a). Furthermore, sphaeroplasts produced from cells in which *pulA* is highly expressed are rather fragile and release some cytoplasmic proteins.

Only wild-type pullulanase could be labelled with [^{3}H]-palmitate (not shown), indicating the absence of fatty acylation of the mutant polypeptides (Pugsley *et al.*, 1986). This result was expected in the case of the *pulA301*-encoded protein, which lacks the Cys residue that is normally fatty-acylated (Fig. 2). In the remaining two cases, however, the data agree with the fact that the mutant enzymes remain in the cytoplasm and thus cannot

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                                    ▼
pulA wt          MLRYTRNALVLGSLVLLSGCDNGSSS---
pulA107           MLRYTRNLLSGCDNGSSS---
pulA201  MLRYTRNALVLGSRSRSLVLLSGCDNGSSS---
pulA301           MTMITNSRGSRSSS---
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Fig. 2. Amino acid sequences (single-letter code) of the *N*-termini of pullulanases encoded by wild type *pulA* and its *107*, *201*, and *301* alleles. The arrow indicates the processing site for lipoprotein signal peptidase in wild-type pullulanase.

Table 1. Activity and location of pullulanase encoded by wild-type *pulA* and by *pulA* derivatives mutated to affect the pullulanse signal peptide.

pulA allele	pCHAP710	Total exposed activity	(%)	Percent Recovery peri.	envel.	cyto.	debris
wt	-	1587	12	6	39	29	26
	+	676	86	37	27	17	19
107	-	2215	7	17	5	67	10
	+	2418	4	14	6	66	13
201	-	926	9	20	5	65	9
	+	686	16	13	8	70	9
301	-	2243	3	23	4	65	8
	+	2776	6	15	8	69	8

Cells were grown in minimal maltose medium and induced for 2 h with 1 mM IPTG. Total (lysed cells) and exposed (whole cells) pullulanase activities were then determined (units are nmoles of reducing sugar liberated min^{-1} mg^{-1} cell protein). The cells were then converted into sphaeroplasts to release *periplasmic proteins and subsequently lysed. The periplasmic, cytoplasmic, envelope and cell debris* fractions were then assayed for pullulanase, amylomaltase (cytoplasmic marker) and β-lactamase (periplasmic marker) activities. The percent activities of the latter two enzymes in the various fractions were respectively 11–33% and 72–90% in the periplasm, < 2–24% and <2% in the envelope, 48–84% and 7–22% in the cytoplasm, and <2–10% and 1–5% in the debris.

be modified by fatty-acyl transferases located in the cytoplasmic membrane. We conclude that the prePulA signal peptide is essential for pullulanase secretion.

Identification of a pullulanase precursor

Signal peptide processing is usually indicative of translocation across the cytoplasmic membrane via GEP. In order to investigate the GEP-dependence of pullulanase secretion, we developed a gel system capable of separating mature PulA from prePulA accumulated in globomycin-treated cells carrying *pulA* from *K. oxytoca* strain UNF5023. Because of their large size (>116 kDa), these forms of PulA could only be separated in 30-cm-long gels of 8% acrylamide containing 0.1% bisacrylamide, which is half the normal concentration of cross-linker. PrePulA produced by globomycin-treated *E. coli* expressing *pulA* from pCHAP137 or *pulA* and all pullulanase-specific secretion genes (*pulS* and *pulC-O*) from pCHAP231 migrated slightly more slowly than mature PulA (Fig. 3, lanes C, D and E). This is the reverse of the situation previously observed with pullulanase produced by *K. plantolytica* K21 (Kornacker *et al.*, 1989a).

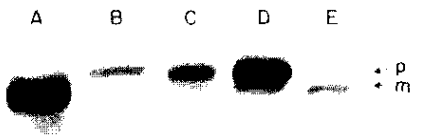

Fig. 3. Separation of processed from unprocessed pullulanase by SDS-polyacrylamide gel electrophoresis. The gel contained 8% *polyacrylamide and 0.1% bisacrylamide, and the cells were labelled with* [^{35}S]-methionine for 1 min.
A, MM7(pCHAP137) cells producing exclusively mature PulA (see text). B, MM18(pCHAP137) producing almost exclusively precursor PulA (see text). C, PAP105(pCHAP231) pretreated before labelling with 200μg ml^{-1} globomycin for 10 min, producing exclusively precursor PulA; D, as C, but with PAP105(pCHAP137); E, as C, but without globomycin treatment. m, mature pullulanase; p, precursor pullulanase polypeptides.

Effect of the MalE–LacZ hybrid on pullulanase secretion

We reported previously that expression of the *malE–lacZ72-47* gene fusion (strain MM18) whose product (a maltose-inducible, maltose binding protein–β-galactosidase hybrid) blocks protein export (and hence precursor processing) (Ito *et al.*, 1981) prevented prepullulanase processing in *E. coli* expressing *pulA* and *pulS* from *K. plantolytica* ATCC15050 (Pugsley *et al.*, 1986). *This is also the case for E. coli* MM18(pCHAP231) *in* which *pulA* and all pullulanase-specific secretion genes are expressed (Fig. 4), and in a strain with *pulA* alone (MM18(pCHAP137); Fig. 3, lane B and data not shown). The effects of MalE–LacZ were abolished by a mutation (*malE19-1*) that inactivated the MalE signal peptide of the hybrid protein and prevented its entry into GEP (strain MM7 in Figs 3 and 4; see Bedouelle *et al.*, 1979, for details).

The effects of the *malE–lacZ* gene fusion on preMalE processing were almost identical to those on prePulA processing (Fig. 4 and data not shown). Both prePulA and preMalE were stable and could not be chased to the *mature forms even after 30 min. The pullulanase pre*cursor that accumulated in maltose-induced MM18-(pCHAP137) or MM18(pCHAP231) cells migrated slightly more slowly in SDS-polyacrylamide gels than the precursor that accumulated in globomycin-treated cells (Fig. 3, lanes B and C). It was expected that the two precursors would behave differently because the latter probably has

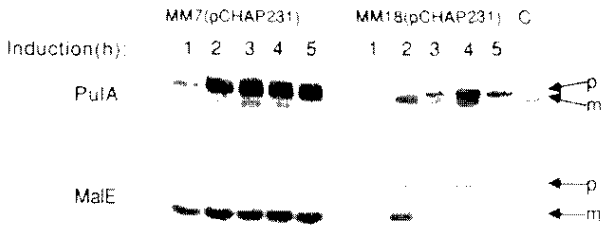

Fig. 4. Appearance of prePulA in cells of MM18 (*malE–lacZ*) (pCHAP231) after addition of 0.4% maltose to the cultures at 1 h. Samples were removed from the cultures and labelled for 1 min with [^{35}S]-methionine. PulA and MalE were immunoprecipitated with appropriate antisera and separated by SDS-polyacrylamide gel electrophoresis. m, mature protein; p, precursor protein. PrePulA and PreMalE accumulate only in the MM18 culture. Identical results were obtained when the cells carried pCHAP137 instead of pCHAP231. C, control containing mature PulA and mature MalE immunoprecipitated from a wild-type *E. coli* strain.

a diacylglyceride-modified Cys^{+1} residue whereas the former does not (Watanabe *et al.* 1988).

The arrest of protein export by MalE–LacZ is lethal to cells, which are consequently maltose-sensitive (Ito *et al.*, 1981) even when the cells carried pCHAP231. This implies that secretion factors encoded by pCHAP231 do not provide a bypass pathway by which pullulanase (or essential envelope proteins) can be exported when GEP is blocked by MalE–LacZ.

Pullulanase activity in cultures of strain MM18 carrying pCHAP231 or pCHAP137 increased as incubation in maltose-containing medium continued. In MM18(pCHAP37), the enzyme remained cryptic (<2% exposed to the substrate pullulan) until after prolonged induction of *malE–lacZ*, which resulted in lysis. In contrast, pullulanase produced by MM18(pCHAP231) was initially entirely cell-surface exposed, but prolonged induction of *malE–lacZ* gradually reduced the proportion of total enzyme activity that could be detected in whole cells to 50–60% after 5 h incubation. These results suggest that inhibition of prepullulanase processing caused by a MalE–LacZ-induced block in GEP prevents the enzyme from reaching the cell surface, probably because pullulanase uses GEP to cross the cytoplasmic membrane (see below).

Effects of mutations affecting GEP on pullulanase processing and secretion

In the next series of experiments, we tested the effects on pullulanase processing and secretion of mutations that are known to affect protein export via GEP. The mutants belong to three phenotypic classes: heat (42 C)-sensitive (*secA*, *secY* and *groEL*), cold (24 C)-sensitive (*secD*, *secE* and *secF*) and non-conditional (*secB*). The permissive temperatures for the temperature-conditional mutants were 30 C and 34 C, respectively. Figures 5 and 6 and Table 2 show representative results of experiments designed to study the effects of the three classes of mutations on the processing of prePulA and four other precursor proteins, namely preMalE, preBlaM (β-lactamase encoded by the *pulA*-bearing plasmids), prePhoA (alkaline phosphatase) and preOmpA (outer-membrane protein A). As above, the experiments were performed with isogenic strains carrying pCHAP137 or pCHAP231 (Fig. 1) to assess the effects of pullulanase secretion factors.

The *secA*^{ts} and *secY*^{ts} mutations both caused prePulA to accumulate at 42 C (Fig. 5). The effect was first detected in the *secA* mutant after 2 h at 42 C and was maximal (>80% inhibition) shortly thereafter. In the *secY* mutant, reduced prePulA processing was detected as early as one hour after the shift to 42 C and was again maximal (>70%) shortly thereafter. Both mutations caused similar defects in preBlaM, preMalE and preOmpA processing, except that preOmpA was not detected in the *secA*^{ts} mutant until after 3 h at 42 C (not shown).

The *groEL*^{ts} mutation was totally without effect on preOmpA (not shown) and prePulA (Fig. 5) processing 15 min to 4 h after the culture was shifted to 42 C, but small amounts of preBlaM were detected after 20-second pulse labelling in the *groEL*^{ts} mutant after one hour or more at 42 C. This precursor was not detectable after a further 40 s chase. The result is in line with previous observations on the requirement for GroEL protein for rapid preBlaM processing (Kusukawa *et al.*, 1989).

The *secD*^{cs}, *secE*^{cs} and *secF*^{cs} mutations all caused defects in processing of preOmpA and prePulA (Table 2) and the *secD* and *secF* mutations were also found to block prePhoA processing (not shown).

PreOmpA, preMalE and prePulA were all detected in the *secB* mutant (Fig. 6 and not shown), but the mutation

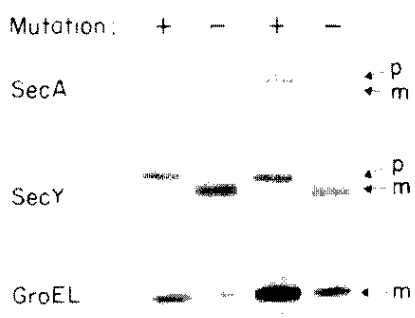

Fig. 5. [^{35}S]-methionine-labelled prePulA (p) and mature PulA (m) immunoprecipitated from strains with (+) or without (−) *secA*^{ts}, *secY*^{ts} or *groEL*^{ts} mutations and bearing pCHAP137 or pCHAP231 (see Table 3 for details of strains). The cultures were pregrown at 30 C and switched to 42 C for 3 h before labelling for 1 min.

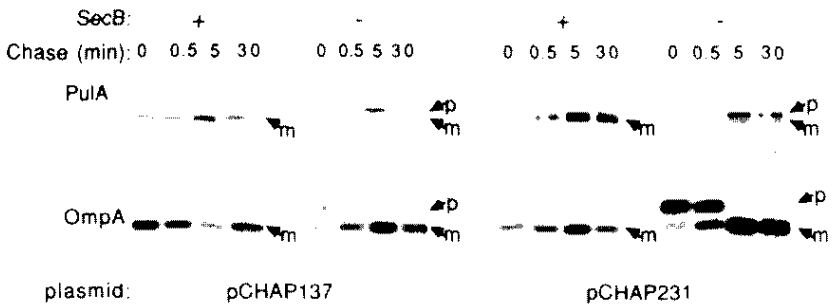

Fig. 6. SecB requirement for processing of prePulA and preOmpA. Strains MC4100 (SecB$^+$) or CK1959 (*secB*::Tn*5*, SecB$^-$) carrying pCHAP137 or pCHAP231 were labelled for 1 min with [^{35}S]-methionine and then chased for the indicated time before the proteins were immunoprecipitated with appropriate antisera and run on SDS-polyacrylamide gels. p, precursor proteins; m, mature proteins.

was apparently without effect on preBlaM processing (not shown), as reported previously (Kusakawa *et al.*, 1989). PreOmpA detected in the *secB* mutant was slowly chased to the mature form (Fig. 6), whereas both prePulA (Fig. 6) and preMalE (not shown) were apparently stable.

In summary, mutations in *secA*, *secB*, *secD*, *secE*, *secF*, and *secY* all block prePulA processing under conditions where processing of other precursors was also blocked to a similar extent. The extent of prePulA processing (in pulse-labelling experiments) and the incubation time required at the non-permissive temperature for prePulA to be first detected were both similar to those observed for other precursors tested. More importantly, the kinetics of appearance of prePulA and the extent to which processing occurred were identical in strains carrying pCHAP137 or pCHAP231 (Figs 5 and 6 and Table 2). *This means that the pullulanase secretion factors do not* influence prePulA processing.

The effects of the MalE–LacZ hybrid suggested that PrePulA processing was required for cell-surface exposition in cells carrying pCHAP231. We predicted that a similar defect would be observed in cells carrying *sec* mutations. Experiments designed to test this were complicated, however, by the fact that very little pullulanase was produced after shifting cultures to 24 °C, and that pullulanase activity is unstable at 42 °C. Nevertheless, the proportion of the total amount of pullulanase exposed at the cell surface in pre-induced *secA*^{ts} or *secY*^{ts} strains carrying pCHAP231 declined from 100% at 30 °C to 60–80% after 4 h at 42 °C. This defect in cell-surface exposition was even more obvious (40–60%) in cells simultaneously induced with maltose and shifted to 42 °C. No change in per cent exposition was observed in wild-type strains or in the *groEL*^{ts} mutant at 42 °C.

The percent pullulanase exposition in CK1953 (pCHAP231) (*secB*) was consistently 20–40% compared with 100% in MC4100(pCHAP231). At least part of the pullulanase exposition in the *secB* mutant may be due to lysis because 15–30% of the pullulanase activity in CK1953(pCHAP137) was detected in whole cells (compared to 2–5% in MC4100(pCHAP137)).

Effect of pullulanase overproduction

PrePulA was sometimes detected in large quantities in wild-type cells carrying pCHAP656, a pEMBL8 (pUC8) *derivative in which pulA is under exclusive lacZp control* (Fig. 1; Dente *et al.*, 1983; Kornacker and Pugsley, 1990). PrePulA accumulated in fully-induced strains carrying pCHAP656 was not chased to the mature form (Fig. 7).

Table 2. Accumulation of PrePulA and preOmpA in strains carrying *secE*^{ts}, *secD*^{ts} or *secF*^{ts} mutations at 24 °C

			Percent of Protein as Precursor					
			15 min		60 min		120 min	
Strain	Allele	Plasmid	OmpA	PulA	OmpA	PulA	OmpA	PulA
PS264	*secE*$^+$	pCHAP137	<2	<2	<2	<2	<2	<2
		pCHAP231	<2	<2	<2	<2	<2	<2
PS266	*secE*^{ts}	pCHAP137	25	15	60	60	85	90
		pCHAP231	40	40	70	65	95	95
G62	*secDF*$^+$	pCHAP137	<2	<2	<2	<2	<2	<2
		pCHAP231	<2	<2	<2	<2	<2	<2
KJ175	*secD*^{ts}	pCHAP137	5	5	30	15	60	60
		pCHAP231	10	<2	40	25	60	60
KJ184	*secF*^{ts}	pCHAP137	50	30	70	45	90	75
		pCHAP231	60	45	80	60	90	90

Samples were removed at indicated times after the shift to 24 °C and labelled for 1 min with [^{35}S]-methionine. Values of <2% were not above background radioactivity.

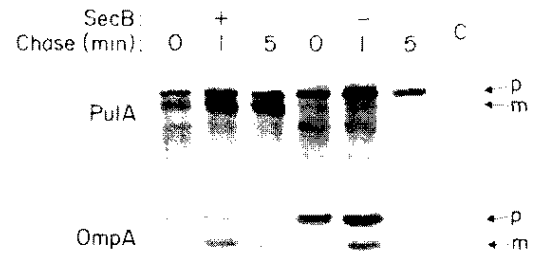

Fig. 7 Effect of the absence of SecB protein and high level *pulA* expression on prePulA and preOmpA processing. Strains MC4100 (*secB*$^+$) or CK1953 (*secB*$^-$ Tn*5* SecB$^-$) carrying F' Tn*10* from PAP105 and pCHAP656 were grown under conditions (subculture from saturated precultures, prolonged induction with 1 mM IPTG) known to favour accumulation of prePulA in these strains. Cells were labelled for 1 min with [^{35}S]-methionine and chased as indicated. Other details are as for Fig. 7. C is a control sample containing immunoprecipitated mature PulA and mature OmpA.

Processing of preOmpA was also delayed but not abolished (Fig. 7). The combined effects of full induction of *pulA* on pCHAP656 and the presence of the *secB* mutation completely blocked prePulA processing (Fig. 7).

Are other genes required for pullulanase secretion?

Earlier experiments in which the *K. plantolytica* strain K21 chromosome was mutated with Tn*10* suggested that mutations outside the region containing *pulS* and *pulC–O* could prevent pullulanase secretion (Kornacker *et al.*, 1989b). Unlike *secB* mutations, these mutations were not lethal in rich medium and did not appear to affect the export of other proteins. We reasoned that the corresponding genes should also exist in *E. coli* K12 and therefore mutagenized strain W3110(pCHAP231) with Tn*5* and screened the mutants for defects in pullulanase production on red pullulan plates (d'Enfert *et al.*, 1988). Of the 8868 clones tested, 24 produced smaller hydrolysis zones. Of these, 3 carried inserts in the *pulD-E* region of pCHAP231, 15 had reduced pCHAP231 copy number, 4 were Mal$^-$ and 2 had reduced levels of pullulanase and of amylomaltase, indicating a partial defect in the maltose regulon, possibly at the level of maltose accumulation. Thus, none of the mutants was specifically affected in terms of pullulanase secretion.

We also tested a number of mutations that are known to affect outer-membrane function, including *tolA* and *tolB* that have pleiotropic effects on outer membrane composition (Levengood and Webster, 1989), *tolC* that prevents α-haemolysin secretion via a signal peptide-independent pathway in *E. coli* (Wandersman and Delepelaire, 1990) and *tonB* and *exbB* that prevent energy coupling to certain outer-membrane transport systems (Postle, 1990) (see Table 3 for strains). None of these mutations had any effect on pullulanase exposition in maltose-induced transformants carrying pCHAP231, and only the *tolA* and *tolB* mutations reduced the level of pullulanase activity (to approximately 10% of that in control cells).

Discussion

The results presented here suggest that pullulanase secretion in *E. coli* requires not only its cognate secretion factors (Pugsley *et al.*, 1990b) but also the products of six *sec* genes that were originally characterized as required for the export (processing) of cell envelope proteins. This is the first time that *sec* gene dependence has been demonstrated for secretion of an extracellular protein. Since only *sec* mutations prevent processing of the pre-PulA signal peptide, it seems probable that pullulanase is translocated across the cytoplasmic membrane via GEP and that signal peptide processing and fatty acylation occur as for other lipoproteins (Watanabe *et al.*, 1988). This process may constitute a discrete step in the pullulanase secretion pathway that is distinct from the second step in which pullulanase-specific secretion factors participate only after signal peptide processing and possibly complete translocation across the cytoplasmic membrane (Pugsley *et al.*, 1990a,b) have occurred.

It is not yet known whether cytoplasmic membrane-anchored pullulanase that accumulates in the absence of *pul* gene-encoded secretion factors (Pugsley *et al.*, 1990a,b) adopts a fully folded, active conformation or indeed whether it is a bona fide intermediate in the normal secretion pathway. Thus, although GEP alone is sufficient for signal peptide processing, pullulanase secretion functions may be required to complete translocation across the cytoplasmic membrane, or may interact with the polypeptide during translocation. This could explain the requirement for pullulanase-specific secretion factors in the cytoplasm and in the cytoplasmic membrane.

In considering the effects of the *sec* mutations on protein export and secretion, it is important to be clear about what the accumulation of a precursor protein actually means. The fact that a particular precursor accumulates when a given *sec* mutant is grown under non-permissive conditions need not mean that the product of that *sec* gene is required for the export of that protein. Consider, for example, the effects of a mutation in a hypothetical *sec* gene that is required only at a late (e.g. post-processing) stage in the export of a small subset of envelope proteins via GEP. The block imposed by this mutation might cause proteins to pile up at earlier stages in the pathway until it becomes entirely jammed up, preventing the export

of all proteins via GEP. The presently available data on the functions of GEP components suggest that SecA and SecB act at an early stage, prior to contact between precursor proteins and the translocation machinery in the cytoplasmic membrane. The fact that *secA* and *secB* mutations both prevent pullulanase processing and secretion (cell-surface exposition) must therefore indicate that both proteins are required for these events. Among the identified or putative components of the translocation machinery, SecE may act prior to SecY (Bieker and Silhavy, 1990) while SecD and SecF have relatively large periplasmic domains which might indicate that they act at a still later stage (Gardell *et al.*, 1990). Thus, the evidence that *sec* genes coding for components of the translocation machinery are required for pullulanase secretion is strongest for the *secE* gene.

Another possible source of confusion in the interpretation of the effects of *sec* mutations is that they will almost certainly affect the export of PulA-specific secretion factors to the cell envelope. For technical reasons, we cannot rule out such effects as one of the causes of the reduced efficiency of cell-surface exposition of pullulanase when GEP is blocked by *sec* mutations or by the MalE–LacZ hybrid protein. We can, however, discount such effects as a cause of the reduced processing of prepullulanase, which is entirely independent of the *pul* gene products.

We reported previously that pullulanase accumulated in the cytoplasm of cells in which *pulA* was fully expressed for long periods from pCHAP656, a high copy-number plasmid (Pugsley *et al.*, 1990a). We have shown here that prePulA accumulates under the conditions described in the previous report, and that there is an absolute correlation between the accumulation of this precursor and reduced efficiency of cell-surface exposition of the enzyme. It is important to note that the non-secreted, cytoplasmic enzyme is active, at least when the cells are lysed, which is contrary to previous predictions (Kornacker and Pugsley, 1990; Pugsley *et al.*, 1990a).

Given that most extracellular proteins produced by Gram-negative bacteria are, like pullulanase, made as signal peptide-bearing precursors, it would not be surprising if they too required factors corresponding to the *E. coli sec* gene products for transport across the cytoplasmic membrane. Complex secretion pathways with components structurally similar to those encoded by the *pul* genes are now known to exist in several other species of Gram-negative bacteria (A. Lazdunski, G. Salmond and J. Tommassen, personal communications). It is not yet possible to test whether these pathways include a GEP component because *sec*-type mutations are not yet available in the bacteria concerned and because the complete pathways have not yet been reconstituted in *E. coli*.

Experimental procedures

Bacterial strains, plasmids and media

E. coli K12 strains are listed in Table 3. The plasmids used (Fig. 1) are all derived from pCHAP231 (d'Enfert *et al.*, 1987). pCHAP656 and pCHAP710 are described by Kornacker and Pugsley (1990). pCHAP137 comprises an *Eco*RI–*Hin*dIII fragment including *pulA* subcloned from pCHAP601 (Kornacker and Pugsley, 1990) in place of the *Eco*RI–*Hin*dIII fragment of pBR322. These plasmids are introduced into strains listed in Table 3 by transformation. Hosts for pCHAP656 also carried the F' Tn*10* plasmid from PAP105 (Table 3) in order to repress the *lacZ* promoter. Strain CK1953 (*secB*) was refractory to transformation by standard procedures. The protocol was therefore modified slightly by growing cells in minimal medium (see below), washing them once in ice-cold distilled water and then resuspending them in ice-cold 100 mM $CaCl_2$. Plasmid pCHAP909 carrying the *pulA107* mutation was created by site-directed mutagenesis of DNA in an m13mp10 subclone carrying the *Eco*RI–*Apa*I DNA fragment from pCHAP765 (Kornacker and Pugsley, 1990). The oligonucleotide 5'-GCAGCCGCTAAGTAAA TTACGGGTATATCTGAGCCCTGGTTCTGGGATCGCTGGTT-3' was used to delete the sequence 5'GCCCTGGTTCTGGG-ATCGCTGGTT-3' that is normally located after the 15th base of the sequence corresponding to the oligonucleotide, using an Amersham mutagenesis kit. The mutated DNA fragment was used to replace the corresponding fragment in pCHAP765 and its sequence was verified as previously (Kornacker and Pugsley, 1990). pCHAP1052, carrying the *pulA201* mutation, was created by inserting the 12 bp linker 5'-pGATCTACATCTA-3' into the *Sau* 3AI site (5'-GGATCG-3') in the signal peptide-coding portion of *pulA* in pCHAP656 (see Kornacker and Pugsley, 1990). The same 12 bp sequence was also introduced into the first *Bam*HI site of *pulA* in pCHAP656 (see Kornacker and Pugsley, 1990) to create a unique *Bgl* II site. The *Bgl* II–*Hin*dIII fragment of this plasmid (the entire *pulA* sequence 3' of codon 24) was subcloned into pEMBL8 cleaved with *Bam*HI and *Hin*dIII so that the 5' end of *lacZ* of the vector was fused in-frame to *pulA*. The resulting plasmid (pCHAP1051) carries the *pulA301* allele. The sequences of the PulA derivatives encoded by these mutated genes are shown in Fig. 2.

Cells were grown in L-broth, or M63 minimal medium containing 0.4% glycerol, 0.4% maltose and 0.4% casamino acids (except for labelling experiments; see below). CK1953 could only be grown in minimal medium. Antibiotics used were ampicillin (Ap, 200 μg ml^{-1}, for CHAP231, pCHAP137, and pCHAP656 and its derivatives), chloramphenicol (Cm, 25 μg ml^{-1}, pCHAP710) and tetracycline (Tet, 15 μg ml^{-1}), for F' Tn*10*. Media were solidified with 1.5% agar as appropriate for use in Petri dishes. Cultures were normally incubated at 30°C except for strains carrying temperature-conditional mutations (see Table 3). These were grown at the permissive temperatures, and the absence of suppressor mutations was confirmed by plating out at the permissive and non-permissive temperatures. In all cases, <0.1% of cells formed colonies at the non-permissive temperature.

Assays

Pullulanase was assayed as previously (Michaelis *et al.*, 1985).

Table 3. Strains of *E. coli* K12

Strain no	Genotype	Source
PAP105	*Δ(lac-pro) F′(lacZM15, pro⁺, Tn10)*	Laboratory strain
MC4100	*F⁻ araD139, ΔargF–lac)169, rpsL, relA flbB5301, deoC1, ptsF25, rbsR*	Laboratory strain
CK1953	MC4100 *secB*::Tn*5*	C. Kumamoto
IQ85	MC4100 Tn*10*	K. Ito
IQ86	As IQ85 but *secY24*[ts]	K. Ito (Shiba *et al.* 1984)
MM18	MC4100 (*O(malE-lacZ)72 4*[cs])	K. Ito
MM7	As MM18 but (*O(malE19-1-lacZ)*)	K. Ito
MM52	MC4100 *secA51*[ts]	J. Beckwith
G62	*F⁻ araD139, Δ(araABC leu)7679, galU galK, ΔlacX174, rpsL, thi, phoR*	J. Beckwith
KJ173	G62 *secD29*[cs]*, zag*::Tn*10*	J. Beckwith (Gardel *et al.* (1990))
KJ184	G62 *secF62*[cs]*, zag*::Tn*10*	J. Beckwith (Gardel *et al.* (1990))
PS264	MC4100 *ziiRK498*::Tn*5*	J. Beckwith
PS266	PS264 *secE15*[cs]	J. Beckwith
W3110	F⁻	Laboratory strain
CG2241	W3110 *galE*	C. Georgopoulos
CG2245	CG2241 *groEL44*[ts]	C. Georgopoulos
1292	*supE, hsdS, met, gal, lacY, thuA*	N. Lefebvre
JC3411	1292 *tolA207*	N. Lefebvre
JC3417	1292 *tolB236*	N. Lefebvre
AB2847Mal⁻	*aroB, thi, tsx*	V. Braun
BR158Mal⁻	AB2847Mal⁻ *tonB*	V. Braun
W3110-6	W3110 *exbB*	V. Braun
C600	*thr, leu, thuA, supE44, lacY*	Laboratory strain
C600TolC	C600 *tolC*::Tn5	C. Wandersman

cs and ts indicate cold- and heat-sensitivity, respectively. Δ indicates deletion and O indicates gene fusion.

Cells were lysed (to release cryptic enzyme) with 0.5% octylpolyoxyethylene. β-lactamase was assayed using nitrocefin (O'Callaghan *et al.*, 1972) using cells lysed by sonication. Amylomaltase was assayed according to Pugsley and Dubreuil (1988).

Cell fractionation

Cells from 20 ml of culture grown to mid-exponential phase (OD_{600} = 1.0) were harvested and resuspended in 2 ml of 10 mM TrisCl (pH 7.4) containing 0.6 M sucrose. Lysozyme (final concentration 100 μg ml^{-1}) was then added, and this was followed by 2 ml of ice-cold 10 mM TrisCl (pH 7.4) containing 1 mM ethylenediamine tetraacetic acid (EDTA). Cells were gently shaken on ice for up to 30 min, by which time >98% of the cells had been converted to sphaeroplasts, as judged by phase-contrast microscopy. The cell suspension was then centrifuged and the supernatant fraction retained as the periplasm. The pellets were resuspended in 10 mM TrisCl (pH 7.4) containing 1 mM $MgSO_4$, and lysis was assisted by brief sonication at 4°C. Debris was removed from the resulting mixture by low-speed centrifugation, and the membranes pelleted in an ultracentrifuge at 163000 × *g* for 2 h.

Labelling and immunoprecipitation

Cells were labelled for the times indicated at the temperature at which they had previously been grown. [^{35}S]-methionine and [^{3}H]-palmitate were used at 20–100 μCi and 100 μCi ml^{-1}, respectively. [^{35}S]-methionine was chased by the addition of unlabelled methionine to 0.05%. Labelling was stopped by the addition of SDS to 0.8% and heating immediately to 100°C. Control experiments showed that this procedure gave the same results as precipitation of cells with 20% trichloroacetic acid.

For immunoprecipitation, the SDS-lysed cells were diluted 1/20 in TOPS buffer (TrisCl pH 8.0, 150 mM NaCl, 1%-octylpolyoxyethylene) containing 1 μl of specific antiserum and a 50-fold excess of heated, SDS-lysed cells of a strain lacking the protein(s) under analysis. The mixture was incubated at 37°C for 20 min, and then supplemented with protein A-Sepharose (Pharmacia) in TOPS. This mixture was shaken gently at 37°C for 10 min, centrifuged, and the supernatant fraction discarded. The Sepharose beads were washed twice with TOPS and resuspended in SDS sample buffer (100 mM TrisCl, pH 8.0, 5% SDS, 13% glycerol, 1% 2-mercaptoethanol) and heated to 100°C for 5 min. Palmitate labelling was performed under similar conditions except that labelling was continued for 30 min.

Samples were separated by SDS-polyacrylamide gel electrophoresis on Tris/glycine-buffered gels (Pugsley and Schnaitman, 1978). Gels used to detect pullulanase contained 8% and 0.1% bisacrylamide. Other gels contained 10% acrylamide and 0.2% bisacrylamide.

Dried gels were exposed to Kodak XA-R film for autoradiography. Pretreatment with Amplify (Amersham) was used for gels loaded with [^{3}H]-palmitate-labelled samples. Quantification was performed by cutting out the radioactive bands identified by autoradiography, dissolving them in Protosol (New England Nuclear) and counting in a scintillation counter.

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