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# A signal sequence is not required for protein export in *prlA* mutants of *Escherichia coli*

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**The *prlA/secY* gene, which codes for an integral membrane protein component of the *Escherichia coli* protein export machinery, is the locus of the strongest suppressors of signal sequence mutations. We demonstrate that two exported proteins of *E.coli*, maltose-binding protein and alkaline phosphatase, each lacking its entire signal sequence, are exported to the periplasm in several *prlA* mutants. The export efficiency can be substantial; in a strain carrying the *prlA4* allele, 30% of signal-sequenceless alkaline phosphatase is exported to the periplasm. Other components of the *E.coli* export machinery, including SecA, are required for this export. SecB is required for the export of signal-sequenceless alkaline phosphatase even though the normal export of alkaline phosphatase does not require this chaperonin. Our findings indicate that signal sequences confer speed and efficiency upon the export process, but that they are not always essential for export. Entry into the export pathway may involve components that so overlap in function that the absence of a signal sequence can be compensated for, or there may exist one or more means of entry that do not require signal sequences at all.**

*Key words:* alkaline phosphatase/maltose-binding protein/*prlA* mutants/protein export/signal sequence

## Introduction

Proteins to be exported from the cytoplasm are synthesized with an amino-terminal extension known as the leader or signal sequence. The signal hypothesis, which states that this sequence is responsible for directing the protein into the export pathway, has directed thinking about protein export for two decades (Milstein *et al.*, 1972; Blobel and Dobberstein, 1975; Emr and Silhavy, 1982). Observations from both *in vivo* and *in vitro* studies involving a variety of organisms are consistent with a central role for the signal sequence in protein export. In *Escherichia coli*, for example, signal sequence mutations impede export of the protein and result in the accumulation of its precursor in the cytoplasm (Emr *et al.*, 1978, 1980; Emr and Silhavy, 1980; Bassford and Beckwith, 1979; Bedouelle *et al.*, 1980).

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The functions of signal sequences have been investigated largely in *E.coli*, where techniques ranging from classical genetics to biophysics have been brought to bear (Gennity *et al.*, 1990). Signal sequences have been proposed to facilitate entry into the export pathway by retarding the folding of the protein in the cytoplasm into a conformation that is incompatible with export and by interacting directly with the cytoplasmic components of the export machinery. The folding kinetics of the purified precursors of maltose-binding protein, ribose-binding protein and β-lactamase support a role for the signal sequence in the retardation of the folding of the mature portion of the protein (Park *et al.*, 1988; Laminet and Plückthun, 1989). Recognition of signal sequences by SecA has been inferred from the observation that both the OmpA signal peptide and the mature portion of the protein are required, in conjunction, to substitute for the intact OmpA precursor in its capacity to activate the ATPase activity of SecA (Lill *et al.*, 1990). An OmpF-Lpp fusion protein could be crosslinked to SecA and the efficiency of crosslinking depended upon the positive charge at the amino-terminus of the signal sequence (Akita *et al.*, 1990). Although the interaction of SecB with the mature portion of the protein to be exported is central to its function (Hardy and Randall, 1991), it has been proposed that SecB interacts directly with the signal sequence as well (Watanabe and Blobel, 1989; Altman *et al.*, 1990).

Signal sequences have also been proposed to initiate the process of translocation across the cytoplasmic membrane. Biophysical studies have demonstrated that signal peptides can insert spontaneously into membrane-mimetic environments (Jones *et al.*, 1990). Signal sequences defective for promoting translocation *in vivo* were unable to insert *in vitro* as peptides (Hoyt and Gierasch, 1991a,b). It has been hypothesized that insertion of the signal sequence into the cytoplasmic membrane facilitates subsequent interactions of the precursor protein with the export machinery by restricting its diffusion and insuring its proper orientation (Jones *et al.*, 1990). It was recently demonstrated that large aqueous channels could be opened by the addition of LamB signal peptide to *E.coli* cytoplasmic membrane preparations. This observation has prompted an alternative model for the initiation of translocation, wherein the signal sequence binds within a proteinaceous channel that mediates protein translocation (Simon and Blobel, 1992).

Direct interaction of the signal sequence with an integral membrane protein component of the export machinery was originally proposed a decade ago on the basis of genetic studies. The *prlA* gene was identified as a locus to which suppressors of signal sequence mutations map (Emr *et al.*, 1981). Several mutations in *prlA* enhance the export of proteins with defective or mutant signal sequences, which are exported poorly in a wild-type cell (Schatz and Beckwith, 1990; Bieker *et al.*, 1990). Conditional lethal mutations (*secY*) were obtained in the *prlA* gene that result in the accumulation of precursors of exported proteins (Ito *et al.*,

1983). PrlA/SecY is an integral membrane protein that spans the cytoplasmic membrane 10 times (Akiyama and Ito, 1987), prompting it to be regarded as either the machinery or part of the machinery that physically transfers proteins across the membrane, the so-called 'translocase' (Brundage *et al.*, 1992). Most *in vitro* studies have shown complete dependence upon PrlA/SecY for translocation of precursor proteins into vesicles (Brundage *et al.*, 1990; Fandl and Tai, 1990; Nishiyama *et al.*, 1991).

On the basis of the signal sequence suppression phenotype, it was hypothesized originally that the product of the *prlA/secY* gene is involved in signal sequence recognition (Emr *et al.*, 1981). However, *prlA* mutations lack allele specificity and suppress a variety of signal sequence mutations in a variety of genes. Suppressors are appropriately categorized as 'strong' or 'weak' because their behavior in one system generally carries over to the next (Randall *et al.*, 1987). It is often assumed, nonetheless, that *prlA* suppression involves recognition of a mutant signal sequence by a mutant PrlA/SecY protein. This seems possible for those signal sequence mutations that leave the signal sequence largely intact. But for those mutations that are more disruptive, notably the large deletion in the hydrophobic core of the *lamB* signal sequence that gave rise to the earliest and some of the most potent *prlA* suppressor mutations (Emr *et al.*, 1981), this recognition seems less likely. To determine whether signal sequence recognition is required for *prlA* suppression and to address the larger question of whether the signal sequence is truly essential for the many functions that have been ascribed to it, we determined whether *prlA* mutations would permit the export of periplasmic proteins lacking any signal sequence whatsoever.

## Results

### *Periplasmic proteins deprived of their signal sequences are exported in prlA mutants*

The DNA that codes for the signal sequences of two well characterized periplasmic proteins of *E.coli*, maltose-binding protein (MBP) and alkaline phosphatase (AP), was deleted, to yield *malEΔ2−26* and *phoAΔ2−22*. The initiator methionine codon is followed by the first codon of the mature sequence in *malEΔ2−26* (Duplay *et al.*, 1984; Weiss *et al.*, 1989) and by the second codon of the mature sequence in *phoAΔ2−22* (Chang *et al.*, 1986). Uptake of maltose requires that MBP be present in the periplasm (Shuman, 1982). The expression of *malEΔ2−26* was unable to support growth on maltose minimal medium (Puziss *et al.*, 1992; Figure 1). AP is inactive if not exported from the cytoplasm (Boyd *et al.*, 1987). The expression of *phoAΔ2−22* resulted in the appearance of only 1% of the AP protein as active enzyme (Table I). The phenotypes associated with these two signal sequence deletions resemble those of the strongest signal sequence mutations that have been isolated in the *malE* and *phoA* genes (Bassford and Beckwith, 1979; Michaelis *et al.*, 1986).

Introduction of the *prlA4* mutation, a potent suppressor of signal sequence mutations (Emr and Bassford, 1982), restored the ability of cells expressing *malEΔ2−26* to grow on maltose (Puziss *et al.*, 1992; Figure 1). Colonies grew more slowly than a true Mal$^+$ strain, but looked virtually identical to such a strain after 48 h on minimal maltose agar. This observation indicated that export of signal-sequenceless

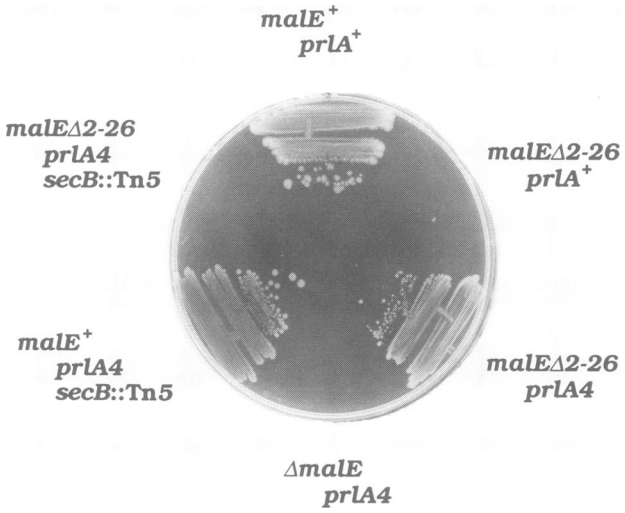


**Fig. 1.** Growth on maltose minimal agar of strains expressing MBPΔ2−26. Strains are isogenic derivatives of strain BAR1091. MBPΔ2−26 is expressed from phagemid pUZ226. The plate was incubated at 37°C for 48 h.

**Table I.** Export of APΔ2−22 in *prlA* mutants

| *prlA* allele | Alkaline phosphatase activity | % Total protein exported |
|---|---|---|
| *prlA*$^+$ | 14 ± 1.6 (9) | 1 |
| *prlA8910* | 24 (1) | 2 |
| *prlA8911* | 275 ± 25.3 (7) | 20 |
| *prlA8912* | 21 (1) | 1 |
| *prlA8913* | 167 ± 14.4 (6) | 12 |
| *prlA8914* | 210 ± 19.9 (8) | 15 |
| *prlA4* | 411 ± 24.9 (6) | 29 |

Cultures of strains expressing APΔ2−22 from phagemid pAID135 in the appropriate *prlA* backgrounds were grown, washed and assayed as described in Materials and methods. Activities reported are the mean and standard error of the mean for the number of trials indicated in parentheses. The percentages in the last column were calculated by taking the activity measured in the *prlA*$^+$ strain to represent an export level of 1%. The derivation of this number is described in Materials and methods.

MBP (MBPΔ2−26) was not as efficient as that of wild-type MBP. The amount of MBPΔ2−26 that was exported could not be inferred, however, because only a small amount of MBP is required in the periplasm for growth on maltose (Bassford and Beckwith, 1979).

The *prlA4* mutation had a similar effect upon *phoAΔ2−22*. The resulting strain formed blue colonies on plates containing the chromogenic indicator 5-bromo-4-chloro-3-indolyl phosphate (XP) in contrast to the white to pale blue colonies of the parent strain. AP activity was increased ~30-fold, to nearly 30% of wild-type levels (Table I).

Other *prlA* mutations were also found to suppress *malEΔ2−26* and *phoAΔ2−22*. The recently described suppressor *prlA666* was even more effective than *prlA4* in suppression of *malEΔ2−26* (Puziss *et al.*, 1992), but the suppressor *prlA402* was ineffective (Bankaitis and Bassford, 1985). Several but not all members of the *prlA8900* series suppressed *phoAΔ2−22*, but none as effectively as did *prlA4* (Table I).

The observed Mal$^+$ and Pho$^+$ phenotypes are very reliable indicators of export. For confirmation, subcellular

**Table II.** Subcellular fractionation of AP activity of APΔ2−22 in *prlA* mutants

| Enzyme | Fraction | *prlA*$^+$ | *prlA* allele | | |
|---|---|---|---|---|---|
| | | | *prlA8911* | *prlA4* | *prlA*$^+$ (*phoA*$^+$) |
| Alkaline phosphatase | lysed cells | 44 | 447 | 722 | 1476 |
| | spheroplasts | 26 | 26 | 26 | 31 |
| | supernatants | 41 | 520 | 785 | 1633 |
| β-lactamase | lysed cells | 1505 | 1706 | 1952 | 19 |
| | spheroplasts | 46 | 76 | 56 | 3 |
| | supernatant | 1732 | 2130 | 2184 | 24 |
| β-galactosidase | lysed cells | 158 | 172 | 150 | 140 |
| | spheroplasts | 180 | 141 | 104 | 188 |
| | supernatant | 32 | 30 | 35 | 32 |

Fractionations were carried out as described in Materials and methods. The experiment was carried out twice with slightly different induction times and essentially the same results were obtained. The data presented are from one trial. The *prlA*$^+$ *phoA*$^+$ strain was AD158.

fractionation was carried out. When spheroplasts were prepared from the *prlA4* and *prlA8911* mutants in which *phoAΔ2−22* was expressed, the AP activity was released to the supernatants as it was from a *phoA*$^+$ strain (Table II). The presence of the β-lactamase activity and the absence of the β-galactosidase activity in these supernatants confirmed that they were indeed periplasmic fractions. Pulse-labelling experiments confirmed that the amount of signal-sequenceless AP (APΔ2−22) synthesized was the same in all of the suppressor *prlA* backgrounds, within a factor of two, as in the wild-type strain (data not shown). The increase in AP activity was therefore not due to enhanced synthesis of APΔ2−22.

Further evidence for export of APΔ2−22 comes from the ability to distinguish cytoplasmic from periplasmic AP on the basis of its protease sensitivity. Whereas the native periplasmic AP is highly resistant to cellular proteases, the cytoplasmic form is extremely sensitive. Export of a substantial fraction of the proteolytically unstable APΔ2−22 protein, and consequent folding into the protease-resistant conformation of the active enzyme would be expected to extend the apparent half-life of the protein (Michaelis *et al.*, 1986; Boyd *et al.*, 1987). This stabilization was observed in pulse−chase experiments. The magnitude of the slope of the decay curve for APΔ2−22 was reduced substantially in *prlA4* cells (Figure 2). All of the *prlA* mutations that suppressed *phoAΔ2−22* stabilized APΔ2−22 (data not shown).

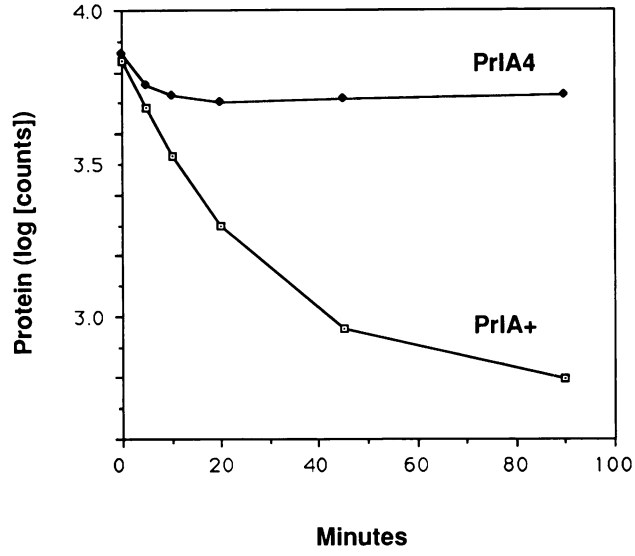


**Fig. 2.** Stabilization of APΔ2−22 in a *prlA4* strain. Growth of cultures, radiolabelling, immunoprecipitation and gel quantification were carried out as described in Materials and methods (Calculation of the export efficiency of APΔ2−22 in wild-type cells) except that cultures of strains expressing APΔ2−22 in either a *prlA*$^+$ or *prlA4* background were induced with 5 mM IPTG for 25 min, pulse-labelled with [$^{35}$S]methionine for 1 min and chased for 90 min. Cells were removed and added to chilled TCA at the end of the pulse and at 5, 10, 20, 45 and 90 min into the chase. After induction, the *prlA*$^+$ strain had 12 units of AP activity and the *prlA4* strain had 382 units. The half-life of APΔ2−22 was 11 min in the *prlA*$^+$ strain. The apparent half-life of APΔ2−22 in the *prlA4* strain was not calculated.

### *MBPΔ2−26 and APΔ2−22 fractionate anomalously in wild-type strains*

Cold osmotic shock fractionation was carried out to determine the cellular location of MBPΔ2−26 in the *prlA4* strain. Although ~50% of the radiolabelled MBPΔ2−26 appeared in the osmotic shock fluid of the *prlA4* strain, we were surprised to find that a similar amount of MBPΔ2−26 appeared in the osmotic shock fluid of the control *prlA*$^+$ strain (Figure 3A). The fractionation profiles of MBPΔ2−26 in the *prlA4* and *prlA*$^+$ strains appeared to be identical. Cold osmotic shock gave otherwise predictable results, releasing the majority of wild-type MBP but little MBP19-1, which carries a point mutation in the MBP signal sequence (Figure 3B; Bassford and Beckwith, 1979; Bedouelle *et al.*, 1980). Similarly, when spheroplasts were prepared, nearly 50% of the MBPΔ2−26 was released to the spheroplast supernatant of either a *prlA4* strain or a *prlA*$^+$ strain (data not shown).

Anomalous fractionation of APΔ2−22 was sometimes observed as well. In some experiments, ~50% of the APΔ2−22 was released from a *prlA*$^+$ strain, and the fractionation profile resembled that which would be expected from a *prlA4* strain. However, if fractionations were carried out in the presence of iodoacetamide so as to allow the redox state of the cysteines of AP to be monitored (Bardwell *et al.*, 1991; Derman and Beckwith, 1991), the profiles of APΔ2−22 in *prlA* and *prlA4* were shown to differ. Oxidized APΔ2−22 was present in the supernatant of the *prlA4* mutant (Figure 4A) and the *prl8911* mutant (data not shown), but

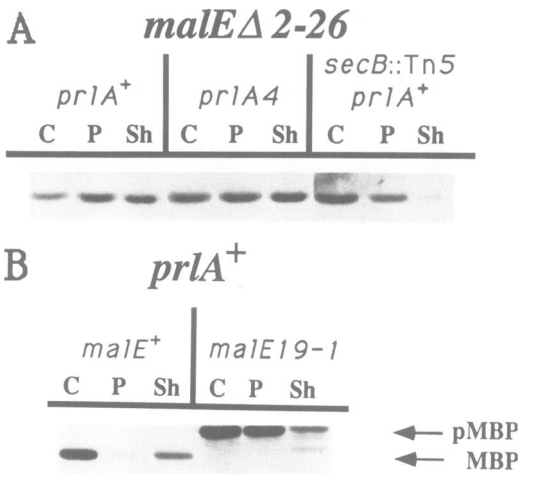


**Fig. 3.** Fractionation of MBPΔ2−26 by osmotic shock. Growth of cultures, radiolabelling, osmotic shock and immunoprecipitation were carried out as described in Materials and methods. Cultures were radiolabelled with [$^{35}$S]methionine for 10 min. The MBP or MBP variant was immunoprecipitated from solubilized whole cells (C), from shock fluid (Sh) and from the pellet that remained following osmotic shock (P). pMBP, MBP precursor.

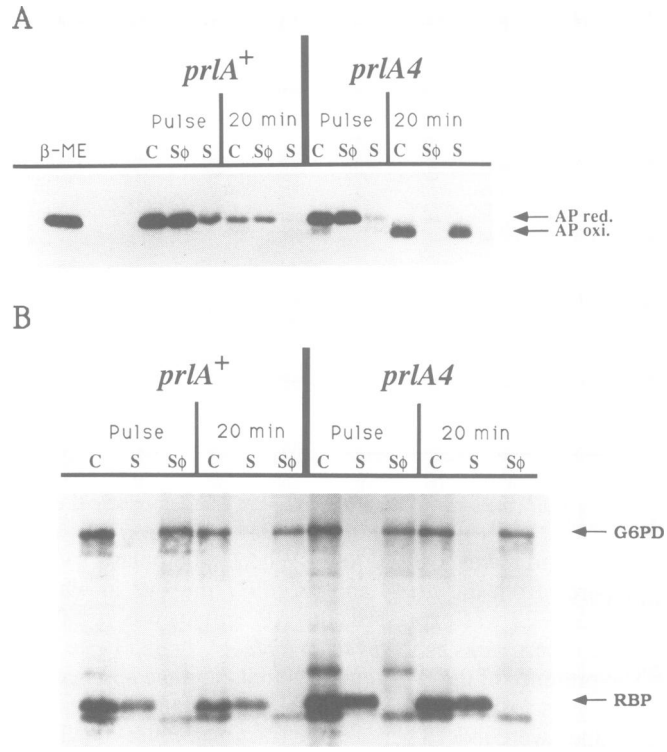


**Fig. 4.** Fractionation of APΔ2−22 by spheroplast preparation. Growth of cultures, radiolabelling, spheroplast preparation and immunoprecipitation were carried out as described in Materials and methods. Cultures were induced with 5 mM IPTG for 10 min, radiolabelled with [$^{35}$S]methionine for 1 min, and chased for 20 min. At the end of the pulse and at 20 min, cells were removed and added to chilled iodoacetamide to give a final concentration of 50 mM. After 10 min, the cells were pelleted and spheroplasts prepared. Two sets of immunoprecipitations were performed on the solubilized whole cells (C), the spheroplasts (Sφ), and the spheroplast supernatants (S). (**A**) APΔ2−22 was immunoprecipitated. (**B**) The cytoplasmic marker glucose-6-phosphate dehydrogenase (G6PD) and the periplasmic marker ribose-binding protein (RBP) were immunoprecipitated. β-mercaptoethanol was present only in the lane designated β-ME. This lane provides a marker for the migration of the reduced conformation of APΔ2−22.

not in supernatant of the *prlA*$^+$ strain, indicating that export and disulfide bond formation occurred only if a suppressor were present. In both *prlA*$^+$ and *prlA4* strains, the APΔ2−22 that remained associated with the spheroplasts was in the fully reduced conformation, as would be expected for cytoplasmic AP. In the spheroplast supernatants from both strains, however, some reduced APΔ2−22 was inexplicably present. The same profile was observed if osmotic shock was performed (data not shown).

Whenever subcellular fractionation of strains expressing APΔ2−22 was performed, some reduced APΔ2−22 was found in the periplasmic fractions. The amount ranged from a small fraction to nearly 50% of the whole cell total (compare Figure 4A with Figure 5A), but was fairly uniform among all strains within a single experiment. In all experiments, the cytoplasmic marker glucose-6-phosphate dehydrogenase fractionated properly, as did the periplasmic markers β-lactamase and ribose-binding proteins (Figures 4B and 5B). It appeared, then, that some release of APΔ2−22 from the cytoplasm occurred during fractionation regardless of whether the *prlA* allele present in the strain was wild-type or mutant. By contrast, genuine export of APΔ2−22 to the periplasm occurred only in *prlA* mutants.

### *Export of APΔ2−22 is slow and post-translational*

Export as mediated through *prlA* suppression has been characterized as slow and post-translational (Ryan and Bassford, 1985). This was the case for APΔ2−22. Whereas the spheroplast supernatants of pulse-labelled *prlA4* cells contained only a small amount of oxidized APΔ2−22, substantially more oxidized APΔ2−22 was present after a 20 min chase (Figure 4A).

### *Export of MBPΔ2−26 and APΔ2−22 requires other components of the export machinery*

To understand how export was being effected through suppressor PrlA/SecY in the absence of a signal sequence, we assessed the contribution of other components of the export machinery. SecA, a cytoplasmic protein that is also peripherally associated with the cytoplasmic membrane, is an ATPase, the activity of which can be inhibited by sublethal concentrations of azide (Cunningham *et al.*, 1989; Lill *et al.*, 1989; Oliver *et al.*, 1990). AP activity was measured after brief induction in the presence of 0.5 mM sodium azide of the *phoAΔ2−22* gene in three *prlA* mutants. Azide treatment led in all cases to a reduction in activity similar to that observed for wild-type AP (Table III). A mutant allele of *secA* that allows normal function of the protein in the presence of azide was introduced in order to test whether this reduction represented a direct effect upon export resulting from the action of azide on SecA. This was the case since the reduction was considerably dampened by this allele (Table III). Thus, functional SecA is required for suppressor PrlA/SecY-mediated export of APΔ2−22.

SecB is a cytoplasmic protein that interacts with a subset of exported proteins and maintains their competence for export (Kumamoto, 1990). Export of MBP is greatly dependent upon SecB. To test the involvement of SecB in suppressor PrlA/SecY-mediated export of MBPΔ2−26, a disrupted *secB* gene was introduced into a strain in which MBPΔ2−26 is expressed in the presence of the suppressor *prlA4*. This resulted in loss of the ability to grow on maltose, indicating

**Table III.** SecA dependence of PrlA-mediated export of APΔ2−22

| *prlA* allele | Azide (mM) | AP activity | |
|---|---|---|---|
| | | *secA*$^+$ | *secA azi*-4 |
| | 0 | 78 | 69 |
| *prlA*$^+$ (*phoA*$^+$) | 0.5 | 54 (70%) | 63 (90%) |
| | 2.5 | 8 (10%) | 46 (70%) |
| | 0 | 8 | 42 |
| *prlA8911* | 0.5 | 3 (40%) | 32 (80%) |
| | 2.5 | <1 | 16 (40%) |
| | 0 | 10 | 12 |
| *prlA8913* | 0.5 | 1 (10%) | 9 (75%) |
| | 2.5 | <1 | 5 (40%) |
| | 0 | 7 | 7 |
| *prlA8914* | 0.5 | 3 (40%) | 6 (90%) |
| | 2.5 | 1 (10%) | 6 (90%) |

For each strain, an exponential phase culture, grown as described in Materials and methods, was distributed into fresh culture tubes containing (a) IPTG to yield a final concentration of 5 mM; (b) IPTG to 5 mM and $NaN_3$ to 0.5 mM; (c) IPTG to 5 mM and $NaN_3$ to 2.5 mM; (d) neither IPTG nor $NaN_3$. After 5 min of aeration, the four samples were treated with iodoacetamide, washed and assayed. The value obtained from (d) represented the activity accumulated in the culture prior to induction and was subtracted from the values obtained from the other three samples to generate the data in the table. The experiment was carried out several times with slight variations in procedure; the data presented are derived from a single typical experiment. The fraction of activity measured in the untreated induced sample, (a), that remained after treatment with $NaN_3$ is given as a percentage.

**Table IV.** SecB dependence of PrlA-mediated export of APΔ2−22

| *prlA* allele | AP activity | |
|---|---|---|
| | (*secB*$^+$) | (*secB*::Tn*5*) |
| *prlA8911* | 479 | 55 (11%) |
| *prlA8913* | 322 | 43 (13%) |
| *prlA8914* | 156 | 49 (31%) |
| *prlA4* | 654 | 25 (4%) |
| *prlA*$^+$ (*phoA*$^+$) | 1339 | 1127 (85%) |

Cultures of strains expressing APΔ2−22 from pAID135 in either a *secB*$^+$ or *secB*::Tn*5* background were grown, washed and assayed as described in Materials and methods, except that cultures were induced for 60 min. The experiment was carried out twice and essentially the same results were obtained. The data presented are from one trial. The fraction of activity measured in the *secB*$^+$ background that was present in the *secB*::Tn*5* background is given as a percentage.

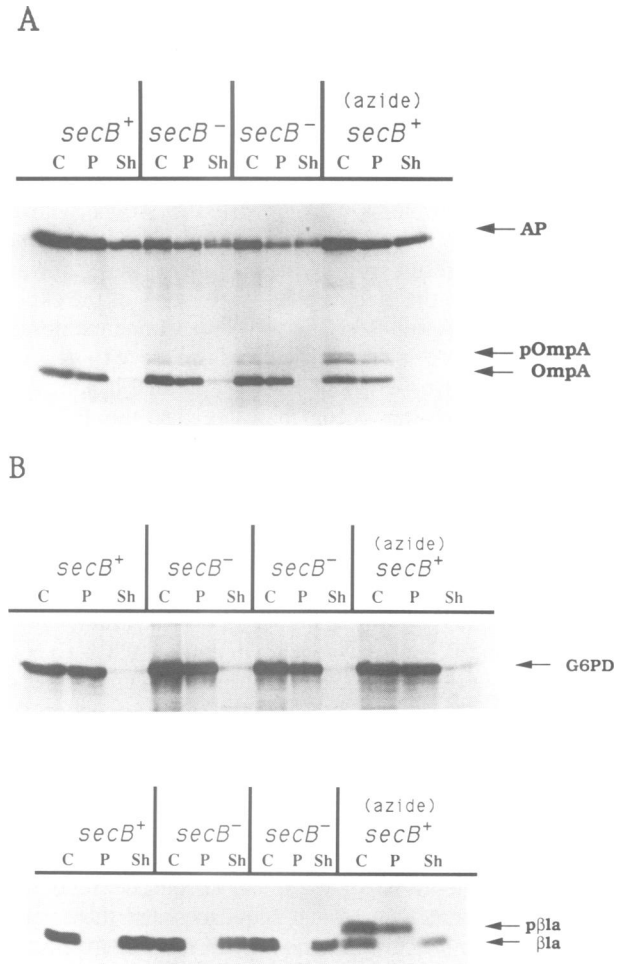


**Fig. 5.** Fractionation of APΔ2−22 in a *secB*::Tn*5* strain. Growth of cultures, radiolabelling, osmotic shock and immunoprecipitation were carried out as described in Materials and methods. Cultures were induced with 5 mM IPTG for 10 min, radiolabelled with [$^{35}$S]methionine for 30 s and chased for 2 min, whereupon osmotic shock was carried out. Two isolates of a *secB*::Tn*5* were radiolabelled. The *secB*$^+$ strain was radiolabelled both in the absence and in the presence of 2 mM $NaN_3$. Two sets of immunoprecipitations were performed on the solubilized whole cells (C), the shock fluid (Sh) and the pellet that remained following osmotic shock (P). (**A**) APΔ2−22 and OmpA were immunoprecipitated. The SecB$^-$ phenotype of the two *secB*::Tn*5* strains is confirmed by the presence of OmpA precursor (pOmpA) in cells and pellet. (**B**) The cytoplasmic marker glucose-6-phosphate dehydrogenase (G6PD) and the periplasmic marker β-lactamase (βla) were immunoprecipitated. The two panels are derived from different exposures of the same gel. The presence of pOmpA in (A) and β-lactamase precursor (pβla) in (B) confirm inhibition of protein export by $NaN_3$.

that export of MBPΔ2−26 was either greatly diminished or eliminated entirely (Figure 1). AP, unlike MBP, does not require SecB for export. To our surprise, however, in the absence of SecB, suppressor PrlA/SecY-mediated export of APΔ2−22 was also greatly diminished (Table IV).

### *The fractionation profile of MBPΔ2−26 but not of APΔ2−22 was altered in the absence of SecB*

When osmotic shock was performed on a strain carrying the *secB* disruption, very little MBPΔ2−26 was released (Figure 3A). This finding suggested that the unexpected release of MBPΔ2−26 from the wild-type strain was not an artifact of the fractionation process but a reflection of the physiological state of the protein. These was no significant difference, however, in the amount of APΔ2−22 released by osmotic shock from a *secB*$^+$ or *secB*$^-$ strain (Figure 5A). Osmotic shock was also performed on cells that had been treated with 0.5 mM azide in order to inhibit the activity of SecA. The accumulation of the precursors of OmpA (Figure 5A) and of β-lactamase (Figure 5B) indicated that inhibition of SecA had been effected. Nevertheless, azide treatment had no effect upon the amount of APΔ2−22 that was released by osmotic shock. In both systems the same results were obtained if fractionation was carried out by spheroplast preparation (data not shown).

## Discussion

### *Proteins lacking their signal sequences can be exported*

The importance of signal sequences for protein export is firmly established. Complete deletion of the signal sequence of two *E.coli* proteins curtailed drastically their export to the periplasm, as would be expected. Unexpectedly, however, these proteins were exported in strains with an export machinery altered by mutations in the *prlA/secY* gene. Export did not result from bypass of the normal pathway. On the contrary, the cytoplasmic components of the export machinery, SecA and SecB, as well at least one membrane component, PrlA/SecY, participated in the export. The finding that relatively efficient export of proteins lacking their signal sequences can occur indicates that the functions proposed for signal sequences must be reconsidered.

### *The proposed functions for signal sequences reconsidered*

It has been proposed that signal sequences maintain the export-competence of proteins, either by interfering directly with the folding of proteins or by recruiting factors that do so (Park *et al.*, 1988; Laminet and Plückthun, 1989; Watanabe and Blobel, 1989; Altman *et al.*, 1990). Export-competence of both MBP and AP was retained in the absence of their signal sequences, however, so signal sequences are not essential for this function.

Signal sequences are not required for the interaction of SecB with proteins to be exported. MBP required and could make use of SecB for its export when deprived of its signal sequence. AP actually acquired a dependence upon SecB for export when deprived of its signal sequence. SecB may ordinarily interact with signal sequences and these interactions may enhance interactions with the mature portion of the protein. Our findings, however, support the conclusion that the SecB interactions that are crucial for export are those with the mature portion of the protein (Randall *et al.*, 1990; Hardy and Randall, 1991).

Dependence upon SecA persisted for the export of MBP and AP in the absence of their signal sequences. Moreover, the basic amino acids at the amino-terminus of the signal sequence that have been proposed to mediate interaction with SecA are not present at the amino-terminus of either MBPΔ2−26 or APΔ2−22 (Akita *et al.*, 1990). Recognition of the signal sequence by SecA is therefore not essential for protein export.

It has been proposed that insertion of the signal sequence into the cytoplasmic membrane is a prerequisite for translocation. Of course, insertion of the signal sequence cannot be a step in the export of the two signal-sequenceless proteins, so we conclude that signal sequence insertion cannot be essential for export.

The existence of *prlA* mutations has been adduced as evidence for direct interaction between PrlA/SecY and signal sequences (Emr *et al.*, 1981). It is clear, however, that no signal sequence is required for export in these *prlA* strains. It could be argued that *prlA* mutations so relax the specificity of recognition that sequences in the mature protein are recognized as signal sequences. We have inspected the sequences of both MBPΔ2−26 and APΔ2−22 for potential surrogate signal sequences. The earliest stretch in AP with a high hydrophobic index extends from amino acid 46 to 50. The stretch, Ile-Ile-Leu-Leu-Ile, is situated in an otherwise hydrophilic region (Chang *et al.*, 1986). A similar stretch is found early on in MBP [amino acids 7−11 (Duplay *et al.*, 1984)]. It could be that these short sequences pass for signal sequences in *prlA* mutants. If this is so, however, *prlA* mutants must be able to recognize such signals within the protein and to promote export of portions of the protein that are situated both amino-terminal and carboxy-terminal to these signals. We favor the alternative and simpler explanation that the export requires no particular recognition sequence at all. In other words, a recognition function that is present in $prlA^+$ may be relaxed to the point of being suspended completely in *prlA* mutants, or no such recognition function is present in $prlA^+$ to begin with. Our findings weaken the hypothesis that one of the functions of signal sequences is to be recognized by PrlA/SecY.

In sum, signal sequences are not needed for maintaining the export competence of proteins to be exported or for recruitment and binding of SecB. Mediating interaction with SecA and insertion into the cytoplasmic membrane, if these are functions of the signal sequence, can be circumvented. And the case for direct interaction with PrlA/SecY is weak. What then do signal sequences do?

### *Signal sequences confer speed and efficiency upon the export process*

Signal sequences are clearly important for export in wild-type cells, for export of a protein is diminished greatly by mutations that disrupt its signal sequence or alter its features. When the signal sequence of AP was deleted, it was actually surprising that about one in 100 molecules was still exported. It would seem that while signal sequences are necessary to promote the level of export that is physiologically necessary, their elimination does not abolish export completely even in wild-type cells.

We propose that the basic function of signal sequences is to promote protein export that is rapid and efficient. The stronger suppressor *prlA* alleles can be relatively efficient, but they mediate export that is always slow and post-translational. Export of APΔ2−22 in the wild-type strain proceeded with slow kinetics as well (Figure 4a). The ability of signal sequences to ensure rapid export is most likely associated with their position at the amino-terminus of the protein. As the first part of the protein to emerge from the ribosome, a signal sequence can promote entry into the export pathway with synthesis of the protein still ongoing. It is very likely that signal sequences do help to perform many of the functions proposed for them and in so doing they contribute to the speed and efficiency of export. For example, signal sequences may interact with SecA and with the cytoplasmic membrane as proposed, particularly if SecB is not involved in the export of protein. Our findings are also consistent with and, in fact, support a function for signal sequences in the retardation of folding of the mature portion of the protein. The rapid export of the wild-type AP precursor does not require SecB but the slow export of APΔ2−22 does. SecB, which can retard the folding of proteins to be exported, may substitute for the absent signal sequence in this capacity (Collier *et al.*, 1988).

### *How does prlA suppression work?*

If *prlA* mutations do not restore protein export by allowing for the recognition of altered or surrogate signal sequences, it could be argued that *prlA* mutations so compromise the selectivity of the export machinery that cytoplasmic proteins are freely exported. MBPΔ2−26 and APΔ2−22 thus would

be representative cytoplasmic proteins. This interpretation is probably incorrect. If the AP data are taken as a gauge of the extent of export, PrlA4 would be mediating export of 30% of cytoplasmic proteins. Mislocalization of so much protein would be expected to be lethal. The tremendous load on the export machinery should adversely affect the export of proteins that are supposed to be exported. Instead, *prlA* mutants are healthy and do not accumulate precursors of exported proteins. Spheroplast fractionation experiments performed on *prlA* mutants yielded a profile of cytoplasmic and periplasmic proteins that is indistinguishable from that of a *prlA*$^+$ strain (data not shown).

It is likely, therefore, that MBPΔ2−26 and APΔ2−22 retain features that distinguish them from other cytoplasmic proteins and that enable them to be exported. In particular, MBPΔ2−26 and APΔ2−22 must fold slowly in the cytoplasm and be targeted efficiently to the export machinery in the cytoplasmic membrane. Slow folding is likely to be the critical feature that distinguishes exported from cytoplasmic proteins, since SecB recognizes and binds to proteins that are unfolded (Hardy and Randall, 1991) and very likely targets these proteins as well (Kumamoto, 1990; De Cock and Tommassen, 1992). Thus, any protein that folds slowly enough should be insured delivery to the export machinery in the cytoplasmic membrane in both *prlA*$^+$ and *prlA* mutants. Only in the *prlA* mutants, however, does the translocation of proteins such as MBPΔ2−26 and APΔ2−22 proceed. It is likely that the signal sequence is important for allowing the wild-type PrlA/SecY protein to discriminate at this stage. It is unclear how this discrimination is accomplished, but it need not involve direct interaction of the PrlA/SecY protein with the signal sequence. The signal sequence may direct either the protein to be exported or its chaperone, i.e. SecB, to assume the proper conformation for recognition by PrlA/SecY. In *prlA* mutants, these conformational requirements would be relaxed or suspended altogether. So, for example, if the PrlA/SecY protein is part of a protein conducting channel, *prlA* mutants would allow the channel to initiate translocation in the absence of a signal sequence, but the presence of a chaperone such as SecB would still be required for this to occur.

In short, our model proposes that a salient feature of exported proteins is the tendency to fold slowly in the cytoplasm even in the absence of their signal sequences and that this feature is sufficient for export in *prlA* mutants. The model predicts that a cytoplasmic protein carrying a mutation that retards its folding would be exported to the periplasm of *prlA* mutants.

### *Targeting of MBPΔ2−26 and APΔ2−22 to the cytoplasmic membrane may be responsible for their anomalous fractionation*

In wild-type cells, both MBPΔ2−26 and APΔ2−22 were released by osmotic shock or were present in the supernatant when cells were converted to spheroplasts. These fractions are commonly considered to represent the contents of the periplasm. Nonetheless the physiological data indicate that in wild-type cells, in the absence of a *prlA* mutation, there is very little functional MBP or AP in the periplasm. Moreover, the presence of reduced sulfhydryls in the apparently periplasmic APΔ2−22 was not consistent with true localization to the periplasm. This apparently periplasmic APΔ2−22 is therefore more likely to be derived from the cytoplasm. Although the same analysis cannot be performed with MBPΔ2−26, we assume that the same explanation obtains for the anomalous release of that protein.

The fractionation of the periplasmic protein β-lactamase, if expressed with no signal sequence, resembles that of MBPΔ2−26 and APΔ2−22 (Bowden *et al.*, 1992). Anomalous fractionation of certain cytoplasmic proteins has been observed before (Beacham, 1979). The translational elongation factor Tu is released from cells by osmotic shock, as is thioredoxin (Jacobson *et al.*, 1976; Lunn and Pigiet, 1986). In part for this reason, and also on the basis of electron microscopy, thioredoxin has been argued to reside in an osmotically sensitive compartment of the cytoplasm (Nygren *et al.*, 1981; Bayer *et al.*, 1987). If the same is true for MBPΔ2−26 and APΔ2−22, their anomalous fractionation may result from the targeting of these proteins to the export machinery at the cytoplasmic membrane. SecB appears to be be responsible for the targeting of MBPΔ2−26 since in its absence, MBPΔ2−26 failed to be released to the supernatant of spheroplasts or by osmotic shock. In contrast, about the same amount of APΔ2−22 was released to the supernatant of spheroplasts or by osomotic shock whether or not SecB was present in the cells. This difference may indicate that targeting of APΔ2−22 is effected by factors other than SecB.

We are aware of the risks entailed in drawing conclusions about physiology from anomalous fractionation, particularly of proteins which have been engineered to be mislocalized to begin with (Tommassen, 1986). It is possible that release is an artifact associated, for example, with sudden exposure of cells to cold, and that release does not reflect the location of these proteins in growing cells. However, the SecB requirement for release of MBPΔ2−26 is consistent with a physiological basis for this phenomenon.

### *Entry into the export pathway*

Our findings have revealed that protein export as mediated by certain mutant PrlA proteins does not require signal sequences and that signal sequences are therefore not indispensable for protein export in *E.coli*. We suspect that some of the export that has been observed in prior studies with *prlA* mutants, particularly in conjunction with severely defective signal sequences, was inaccurately attributed to a residual activity in these signal sequences. In truth, the functions that would ordinarily be carried out by signal sequences can be carried out by other factors such as SecB. Signal sequences are the principal and the most efficient access to the protein export pathway, but they are not the only access. There may exist a distinct route of entry into the export pathway, access to which does not require a signal sequence. Alternatively, there may exist but one route of entry that consists of components that so overlap in function that they can supplement and even substitute for one another fairly effectively.

## Materials and methods

### *Materials*

Media used for growth and maintenance of *E.coli* strains have been described by Miller (1972). NZ-amine-A (Sheffield Products, Norwich, NY) was substituted for bacto-tryptone in media used for strains expressing APΔ2−22. The chromogenic indicator for AP, 5-bromo-4-chloro-3-indolyl phosphate (XP) (Bachem), was present in agar plates at a concentration of 40 μg/ml. Isopropyl-thio-β-D-galactosidase (IPTG, Bachem) was added to agar plates (1 mM) or to liquid medium (5 mM) to induce expression of MBPΔ2−26

from the *lacUV5* promoter; IPTG was added to all media to a concentration of 5 mM to induce expression of APΔ2−22 from the *tac* promoter. Reagents for recombinant DNA work were obtained from New England Biolabs unless otherwise noted. L-[$^{35}$S]Methionine was obtained either from New England Nuclear (1000 Ci/mmol), Wilmington, DE or from Amersham (1000 Ci/mmol). The rabbit anti-MBP serum and the rabbit anti-RBP serum used for immunoprecipitation experiments involving MBPΔ2−26 have been described previously (Emr and Bassford, 1982; Collier *et al.*, 1990). Rabbit anti-AP serum was prepared by C.Gardel and the rabbit anti-RBP serum used in immunoprecipitation experiments involving APΔ2−22 was prepared by J.Garwin. β-lactamase was immunoprecipitated with anti-ampicillinase antibody obtained from 5 Prime→3 Prime, Incorporated. Rabbit anti-G6PD and rabbit anti-OmpA serum were, respectively, gifts of the laboratories of D.Fraenkel and C.Kumamoto. All other biochemicals were obtained from Sigma Chemical Co. unless otherwise indicated.

### Bacterial strains

Experiments involving MBPΔ2−26 were carried out in *E.coli* strain BAR1091, a derivative of MC4100. The strain carries the *malE Δ312* mutation, an in-frame, non-polar deletion that removes the DNA sequence that codes for residue 15 of the MBP signal peptide to residue 159 of the mature moiety (Rasmussen *et al.*, 1985). Experiments involving APΔ2−22 were carried out in *E.coli* strain DHB4, a derivative of strain MC1000 (Boyd *et al.*, 1987). This strain carries the *phoA ΔPvu*II deletion (Inouye *et al.*, 1981). Present also in the strain was one of two derivatives of plasmid pACYC184 that carry an $I^Q$ fragment (Calos, 1978), either the previously described pACYC*lacI*$^Q$ (Boyd *et al.*, 1987), which confers resistance to tetracycline or, pACYC*lacI*$^Q$-CAM, which confers resistance to chloramphenicol (see below).

### Plasmid/phagemid constructions

Standard recombinant DNA techniques were employed unless otherwise noted. Materials from Qiagen, Inc. were used for large-scale plasmid isolation.

Plasmid pACYC*lacI*$^Q$-CAM was constructed by restricting plasmid pACYC*lacI*$^Q$ with *Eco*RI to release the $I^Q$ fragment, treating the gel-purified fragment with the large (Klenow) fragment of *E.coli* DNA polymerase, and then ligating into the *Eco*RV site of pACYC184 (T4 DNA ligase, FPLC pure, Pharmacia LKB Biotechnology). Recombinant plasmids were selected for the ability to confer resistance to chloramphenicol and screened for the inability to confer resistance to tetracycline. The construction was confirmed by restriction endonuclease digestion of plasmid DNA isolated by the method of He *et al.* (1990). The promoter orientation of the *lac* repressor in pACYC*lacI*$^Q$-CAM corresponds to that of the tetracycline resistance gene.

Strains carrying pACYC*lacI*$^Q$ were grown and maintained in the presence of tetracycline at 20 μg/ml (rich medium) or 10 μg/ml (minimal medium). Strains carrying pACYC*lacI*$^Q$-CAM were grown and maintained in the presence of chloramphenicol at 30 μg/ml (rich medium) or 10 μg/ml (minimal medium).

The construction of phagemid pUZ226, from which MBPΔ2−26 is expressed under control of the *lacUV5* promoter, has been described previously (Weiss *et al.*, 1989). In a similar fashion, oligonucleotide-directed site-specific mutagenesis was used to delete the 63 nucleotides of the *phoA* gene that code for the signal sequence and the first codon of mature AP (Chang *et al.*, 1986; Boyd *et al.*, 1987). Phagemid pDHB5059 carries the intact *phoA* gene, having been constructed by the replacement of a 1742 bp *Bst*XI−*Mlu*I fragment of the phagemid pSX102 which expresses the *malF−phoA* fusion J under *tac* promoter control (Boyd *et al.*, 1987) with an 882 bp *Rsa−Bst*XI fragment of plasmid pHI-1, which expresses the *phoA* gene (Inouye *et al.*, 1981). Cells carrying pDHB5059 form dark blue colonies on agar plates supplemented with XP. Cells of strain CJ236 (*dut*$^-$ *ung*$^-$; a gift from Bio-Rad) were transformed with pACYC*lacI*$^Q$ and with this phagemid, then infected with helper phage M13K07 (Vieira and Messing, 1987; kindly provided by Yichen Lu). Mutagenesis was carried out according to the procedure provided by Bio-Rad using as template the single-stranded DNA prepared from the progeny phage and as mutagenic oligonucleotide the 41mer 5′-CCAGAACAGGCATTTCTGGTGTCACTTTATTTTCT-CCATGT-3′ (synthesized on an Applied Biosystems DNA Synthesizer by Steve Lynn at Harvard Medical School). Strain DHB85 (Boyd and Beckwith, 1989) was transformed and phagemid DNA was isolated from the pale blue colonies that arose on agar plates supplemented with XP. The appropriate regions of DNA were sequenced in order to confirm the constructions. The resulting phagemid, expressing APΔ2−22 under *tac* promoter control, was designated pAID135.

Strains carrying pUZ226 were grown and maintained in the presence of ampicillin at 50 μg/ml (rich medium) or 25 μg/ml (minimal medium). Strains carrying pAID135 were grown and maintained in the presence of either ampicillin at 100 μg/ml or carbenicillin at 150 μg/ml (rich or minimal medium).

### Strain constructions

Standard techniques of bacterial genetics were used for strain construction (Miller, 1972). Alleles of *prlA/secY* were introduced into strains carrying pUZ226 by cotransduction with closely linked chromosomal markers conferring resistance to spectinomycin or streptomycin; alleles of *prlA/secY* were introduced into strains carrying pAID135 by cotransduction with a closely linked transposon. Antibiotic-resistant colonies were screened for a Mal$^+$ phenotype or for blue color on agar plates supplemented with XP. Appearance of these phenotypes was taken as an indication of successful cotransduction. For some constructions, pUZ226 was introduced into appropriate *prlA* mutants by transformation. The *azi-4* allele of *secA* was derived from strain DO309 (Oliver *et al.*, 1990); recipient strains, which carried the chromosomal deletion *Δaraleu769*, which is closely linked to *secA*, were transduced to Leu$^+$. Resistance to azide was scored on agar plates that were spread with $NaN_3$ to a final concentration of 0.5 or 1 mM. The *secB*::Tn*5* allele was introduced by transduction of recipient strains to kanamycin resistance (Kumamoto and Beckwith, 1985). Colonies were then screened for inhibited growth on rich medium, a phenotype of SecB$^-$ strains.

Strain AD158 is a derivative of DHB4 with a single chromosomal copy of wild-type *phoA* under *tac* promoter control, constructed by integration of a derivative of phagemid pDHB5059 at the *phoA* locus (*phoAΔPvu*II deletion). Phagemid pDHB5059 was restricted with *Ava*I and *Bam*HI, treated with the large (Klenow) fragment of *E.coli* DNA polymerase, then diluted and religated. The resulting construct lacks the ColE1 origin of replication and depends upon the M13 origin of replication to be maintained episomally. Replication requires the M13 geneII protein, present on the transducing phage λ CH616 (Geider *et al.*, 1985). The episome was introduced into a λ CH616 lysogen of DHB85, the resulting strain was then infected with helpher phage R408 (Russel *et al.*, 1986), kindly provided by Marjorie Russel), transducing particles were used to infect DHB85 itself, and integrants were selected as colonies resistant to 50 μg/ml ampicillin. Although the integrated episome was not stabilized by the introduction of a mutation in *recA*, maintenance of the strain in the absence of ampicillin was found to be sufficient to insure a stable single copy.

### Isolation of prlA8900 mutants

The *phoA2AB* allele codes for an AP that has a wild-type signal sequence but the amino-terminus of the mature portion of the protein contains positive charge in excess of that present in wild-type AP. This AP is exported with 5% of the efficiency of wild-type AP (Li *et al.*, 1988). The 8900 series *prlA* suppressor alleles were isolated by UV mutagenesis of strain Mph113a, which carries the *phoA2AB* mutation at the chromosomal *phoA* locus. Colonies were screened on agar plates containing XP, those that were darker blue than those of Mph113a were purified, and their AP activity was determined by the standard *p*-nitrophenylphosphate assay. Where an increase was observed, P1 transduction was used to determine whether the mutation responsible was linked to the *phoA* or to the *prlA/secY* locus. More than half of the mutations were linked to the *prl/secY* locus. It was concluded that these were indeed *prlA* mutations because they were capable of suppressing the well-characterized *phoA61* signal sequence point mutation (Michaelis *et al.*, 1986) and did not confer a Pho$^+$ phenotype upon a strain that carries a deletion of the *phoA* gene.

### Enzymatic assays of AP, β-galactosidase, and β-lactamase in whole cells

Cultures were grown in minimal M63 medium containing 0.2% each ribose and glycerol, 50 μg/ml each 18 amino acids (no cysteine or methionine) and appropriate antibiotics. Cultures were induced with 5 mM IPTG for 25 min. Final optical density at 600 nm was ~0.3. Following induction, cells were added to prechilled microfuge tubes containing iodoacetamide to yield a final concentration of 1 mM. Iodoacetamide prevents the spontaneous activation of APΔ2−22 that occurs in non-growing cells (A.I.Derman and J.Beckwith, unpublished data). Cells were washed twice with a buffer consisting of 50 mM NaCl, 10 mM $NH_4Cl$, 10 mM $MgCl_2$, 1 mM iodoacetamide, 40 mM 3-[*N*-morpholino]propanesulfonate (MOPS, $K^+$ salt), pH 7.3, and then resuspended in this buffer. Washed cells were assayed at 28°C for AP or β-galactosidase activity as described by Brickman and Beckwith (1975) and Miller (1972). For β-lactamase assays, the washed cells were concentrated 10-fold and lysed as described in the following section. An aliquot of the lysate was added to a buffer consisting of 600 μg/ml penicillin G (Squibb), 100 mM $K^+/Na^+$ $PO_4$, pH 7.3, and the decrease in $A_{240}$ due to hydrolysis of the penicillin G was monitored (Ross and

O'Callaghan, 1975). Units of β-lactamase were calculated by the following formula:

$$\frac{1000 \cdot (\Delta_{240}/\text{min})}{OD_{600} \cdot (\text{volume of culture assayed})}$$

All enzyme assays were carried out in duplicate.

***Subcellular fractionation of AP activity***

Cultures were grown as described in the previous section and induced with 5 mM IPTG for 1 h. The final optical density at 600 nm was ~0.3. Cells were washed once with the MOPS buffer described in the previous section except that $MgCl_2$ was omitted, and then resuspended in 0.5 vol 40% sucrose, 33 mM Tris−HCl, pH 8.0. An aliquot was removed for optical density measurement. Half of the remaining cell suspension was treated with 8.3 mM EDTA and 83 μg/ml chicken egg white lysozyme for 15 min on ice, and then pelleted in a microfuge for 5 min. The supernatants were then removed. The spheroplasts were resuspended in 0.1 vol 10 mM EDTA, 10 mM Tris−HCl, pH 8.0 and then lysed by three cycles of freezing in a dry ice/ethanol bath and thawing in a 28°C water bath. The other half of the cell suspension was pelleted, concentrated 10-fold in 10 mM EDTA, 10 mM Tris−HCl, pH 8.0, treated with 2 mg/ml lysozyme for 30 min on ice, and lysed. $MgCl_2$ and $ZnCl_2$ were then added to the spheroplast supernatants, the lysed spheroplasts and the lysed whole cells so as to offset the EDTA. Spheroplast and lysis buffers were supplemented with 1 mM iodoacetamide and with the protease inhibitors phenylmethylsulfonyl fluoride (150 μg/ml), leupeptin (1 μg/ml, Boehringer Mannheim), pepstatin (1 μg/ml, Boehringer Mannheim) and soybean trypsin inhibitor (10 μg/ml, Boehringer Mannheim). Fractions and lysed whole cells were assayed for AP, β-galactosidase and β-lactamase activity.

***Radiolabelling and immunoprecipitation***

The procedures employed for the radiolabelling of cells and the immunoprecipitation of their contents have been described previously. Growth and radiolabelling was carried out at 30°C for strains expressing MBPΔ2−26 and at 37°C for strains expressing APΔ2−22. The procedure used for MBPΔ2−26 (Puziss *et al.*, 1992) differed slightly from that used for APΔ2−22 (Derman and Beckwith, 1991). The protease inhibitors listed in the previous section were included in the immunoprecipitation reactions where the latter procedure was employed.

Subcellular fractionation of radiolabelled cells expressing MBPΔ2−26 was carried out by cold osmotic shock (Ryan and Bassford, 1985) or by spheroplast preparation (Bankaitis and Bassford, 1984). Subcellular fractionation of radiolabelled cells expressing APΔ2−22 was carried out by cold osmotic shock (Strauch and Beckwith, 1988) or by spheroplast preparation as described above; protease inhibitors were present throughout the fractionation procedure. In experiments where preservation of the redox state of the AP sulfhydryls was necessary, labelled cells were added to prechilled tubes containing iodoacetamide to yield a final concentration of 50 mM. This procedure was found to be sufficient to trap quantitatively the APΔ2−22 in its fully reduced form [see Figure 4A, *prlA*$^+$, pulse-labelled whole cells (C)], suggesting that the cytoplasmic membrane is permeable to iodoacetamide, at least under these conditions. Consequently iodoacetamide was routinely omitted both from the spheroplast buffer and from the buffer in which the cells were boiled and lysed.

SDS−PAGE and autoradiography were carried out as described previously (Ryan and Bassford, 1985; Derman and Beckwith, 1991). In experiments where iodoacetamide was used, sample preparation buffer contained no β-mercaptoethanol.

***Calculation of the export efficiency of APΔ2−22 in wild-type cells***

Strain AD135, which expresses APΔ2−22 from pAID135, was grown in minimal M63 medium containing 0.2% each ribose and glycerol, 50 μg/ml each 18 amino acids (no cysteine or methionine), and 5 mM IPTG to an optical density at 600 nm of ~0.3. Part of the culture was pulse-labelled with [$^{35}$S]methionine 1000 Ci/mmol at 50 μCi/ml for 1 min and then 650 μl were transferred to an equal volume of 10% trichloracetic acid prechilled in ice-water. Trichloroacetic acid precipitation of the labelled cells was allowed to proceed on ice for at least 1 h. The pelleted precipitation was then washed, dried, boiled and subjected to immunoprecipitation with rabbit anti-AP serum and rabbit anti-RBP serum. The same procedure was carried out with strain AD158, and with strain Mph42, which expresses wild-type AP constitutively from the native promoter. During the labelling, an additional 1 ml was withdrawn from the culture and added to prechilled tubes containing iodoacetamide to yield a final concentration of 1 mM (see above). These cells were washed and assayed for AP activity. For each strain, four independent cultures were radiolabelled and assayed in this manner.

The immunoprecipitates were fractionated on SDS−PAGE and band areas were quantified by applying ImageQuant Software v3.0. FastScan (area integration) to a Molecular Dynamics 400 Series PhosporImage scan. For each culture, a quotient representing the percentage of AP protein that was enzymatically active, i.e. exported, was calculated by dividing the units of AP activity by the area of the AP band (normalized to the area of the corresponding RBP band). The average quotient for AD135 (three cultures) was then divided by the average quotient for AD158 and Mph42 (three and four cultures respectively). This ratio, 1% represents the export efficiency of APΔ2−22 relative to that of wild-type AP (San Millan *et al.*, 1989).

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