

Insertion of a MalE β -Galactosidase Fusion Protein into the Envelope of *Escherichia coli* Disrupts Biogenesis of Outer Membrane Proteins and Processing of Inner Membrane Proteins

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The synthesis of a membrane-bound MalE β -galactosidase hybrid protein, when induced by growth of *Escherichia coli* on maltose, leads to inhibition of cell division and eventually a reduced rate of mass increase. In addition, the relative rate of synthesis of outer membrane proteins, but not that of inner membrane proteins, was reduced by about 50%. Kinetic experiments demonstrated that this reduction coincided with the period of maximum synthesis of the hybrid protein (and another maltose-inducible protein, LamB). The accumulation of this abnormal protein in the envelope therefore appeared specifically to inhibit the synthesis, the assembly of outer membrane proteins, or both, indicating that the hybrid protein blocks some export site or causes the sequestration of some limiting factor(s) involved in the export process. Since the MalE protein is normally located in the periplasm, the results also suggest that the synthesis of periplasmic and outer membrane proteins may involve some steps in common. The reduced rate of synthesis of outer membrane proteins was also accompanied by the accumulation in the envelope of at least one outer membrane protein and at least two inner membrane proteins as higher-molecular-weight forms, indicating that processing (removal of the N-terminal signal sequence) was also disrupted by the presence of the hybrid protein. These results may indicate that the assembly of these membrane proteins is blocked at a relatively late step rather than at the level of primary recognition of some site by the signal sequence. In addition, the results suggest that some step common to the biogenesis of quite different kinds of envelope protein is blocked by the presence of the hybrid protein.

Several periplasmic and outer membrane proteins in *Escherichia coli* are synthesized as pre-proteins which are processed by removal of the N-terminal signal sequence to form the mature polypeptide (5, 14, 17, 27). The signal peptidase(s) involved in processing some or all of these proteins has not been specifically identified, but some evidence suggests that it is located in the inner membrane (8, 21, 25). Unprocessed intermediates if detected in vivo are normally short lived (10, 19). Furthermore, kinetic studies have indicated that the synthesis and assembly of many outer membrane proteins are tightly coupled (11), and in particular we have demonstrated that growing chains of the 36,500-molecular-weight matrix protein (26) in *E. coli* B/r are inserted directly into the envelope (9). The studies are consistent with the loop model used by Halegoua and Inouye (14) for protein secretion in which, for example, certain

proteins are extruded directly into the outer membrane, accompanied by processing within the inner membrane.

The total polypeptide content per unit surface area of the *E. coli* and *Salmonella typhimurium* outer membrane is strictly regulated (1, 6), and different polypeptides appear to compete with each other for correct processing and assembly into the outer membrane (6). Smit and Nikaido (29) have presented electron microscope evidence for the emergence of protein molecules at about 200 discrete locations on the surface of *S. typhimurium*. These could represent nascent polypeptides being assembled at any one particular time and need not necessarily indicate the existence of specific structural sites for protein export.

Strong evidence for the role of the N-terminal signal sequences in the localization of membrane proteins in *E. coli* has been obtained by Bassford

et al. (3, 4), Emr et al. (12), and Silhavy et al. (28) by the construction of hybrid genes coding for proteins containing the N-terminal region of membrane or periplasmic proteins fused to an active β -galactosidase molecule. However, fusion proteins involving the N-terminus of the periplasmic, MalE protein, do not apparently cause β -galactosidase to be transported to the periplasm but result in its association with the cytoplasmic membrane when synthesis of the hybrid is induced by the addition of maltose (3). Bassford and Beckwith (3) also observed that strain PB72-47, producing the MalE β -galactosidase hybrid protein, grew as filaments in liquid culture and failed to form colonies after the addition of maltose. From these results, it was concluded that the large-molecular-weight fusion protein blocked the export sites to the periplasm, causing disruption of normal growth. In addition, while this paper was in preparation, Ito et al. (18) reported that precursors of both periplasmic and outer membrane proteins accumulated in strain PB72-47 after the addition of maltose, indicating that processing but not synthesis of at least some exported proteins was also blocked.

Previous studies in this laboratory (6, 9, 16) indicate that a high level of outer membrane protein synthesis is an essential requirement for division. We therefore decided to investigate in detail the rate of division and the synthesis of outer membrane proteins during the development of maltose sensitivity in a strain carrying a MalE β -galactosidase hybrid gene. Surprisingly, in the course of these studies, we also observed that processing not only of outer but also of inner membrane proteins was inhibited.

MATERIALS AND METHODS

Bacterial strains. Strain PB72-47 ($\Delta lac-169$ *araD139 rpsL relA thiA* λ p72-47) carrying the *malE-lacZ* fusion and its wild-type parent MC4100 and MC4100 (λ pRI-1), containing a mutation in the fusion gene rendering the hybrid protein cytoplasmic, have all been described previously (3). For UV- λ infection experiments, MC4100 and PB72-47 were transformed with pGY101 carrying λ *imm*⁴³⁴ and Kan^r. λ BS10 carrying *dacA* (penicillin-binding protein 5 [PBP5]) and pGY101 (20) were obtained from B. G. Spratt and N. Stoker. Bacteria were grown in 0.4% (wt/vol) glycerol-M9 minimal medium at 30°C supplemented with 0.4% (wt/vol) maltose where indicated.

Cell number. Bacterial cell numbers were measured in a model B Coulter counter as previously described (6).

Isolation of membranes. Bacterial envelopes were isolated from sonicated cells and separated into inner and outer membranes by treatment with Sarkosyl NL97 as previously described (6). Unless stated otherwise, inner membranes are defined as the Sarkosyl-soluble fraction and outer membranes are defined as the Sarkosyl-insoluble fraction. In some experiments,

envelopes were also isolated from spheroplasts after osmotic lysis and inner and outer membranes separated by centrifugation to equilibrium on sucrose density gradients as described by Osborn et al. (23).

Measurement of rates of synthesis of membrane proteins. Samples (1 ml) of cultures were pulse-labeled with [³⁵S]methionine (15 μ Ci; 50 μ Ci/ μ g) for 5 min; incorporation was terminated by the addition of excess methionine and chloramphenicol (250 μ g/ml) and incorporation into separated inner and outer membranes determined as described previously (6). To minimize errors in measurements of incorporation into different cell fractions and into individual proteins separated by polyacrylamide gel electrophoresis, a standard sample of cells previously grown for several generations in the presence of [³H]leucine (50 Ci/mmol) was added to [³⁵S]methionine-labeled cells as an internal standard before isolation of envelopes (6). The rates of synthesis of membrane fractions and individual proteins are therefore expressed as the ratio of ³⁵S to ³H. In addition, synthesis is expressed as a relative rate, i.e., the rate of synthesis of membrane proteins relative to total protein synthesis measured in the same pulse.

SDS-PAGE. Envelope samples or total cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 11% gels as previously described (6). The radioactivity in individual gel bands was measured as described by Ames (2).

Infection of heavily irradiated cells with λ . Infection of heavily UV-irradiated bacterial hosts PB72-47 (pGY101) or MC4100 (pGY101) with the transducing phage λ pBS10 to label the *dacA* gene product with [³⁵S]methionine was carried out as described previously (24). To identify the PBP5 precursor, cultures of PB72-47 were grown in the presence of 0.4% maltose before irradiation and infection.

Penicillin-binding protein assay. Cell envelopes were incubated with [¹⁴C]benzylpenicillin to specifically label penicillin-binding proteins in the inner membrane as described by Spratt (30).

Isotopes. Radioactive isotopes were obtained from the Radiochemical Centre, Amersham, U.K.

Molecular weight standards. Proteins used as molecular weight standards were phosphorylase A (94,000), transferrin (74,000), bovine serum albumin (68,000), lactic dehydrogenase (36,000), chymotrypsinogen (25,000), and lysozyme (14,300).

RESULTS

Effect of maltose on the growth and division of a strain synthesizing the fusion protein. Strain PB72-47, carrying the *malE-lacZ* fusion gene, and its wild-type parent were grown to exponential phase in minimal glycerol medium, and then maltose was added. The appearance of the fusion protein was readily detected in total cell lysates as described by Bassford and Beckwith (3). The rate of mass increase in the mutant compared with the wild type is reduced particularly between 2 and 3 h after maltose addition (Fig. 1). The rate of division was also reduced in the mutant, with the mass per cell increasing almost threefold over 4 h as the cells filamented.

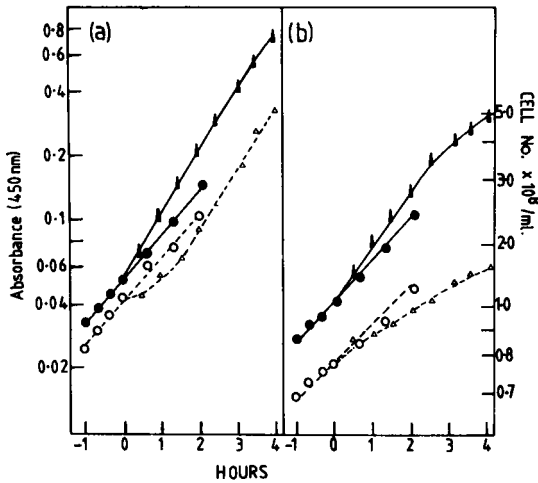


FIG. 1. Effect of addition of maltose on mass increase (absorbance at 450 nm) and cell number (Coulter counts) in strain MC4100 (a) and PB72-47 (b). Cultures were grown in M9-glycerol medium at 30°C, and maltose was added at time zero. Solid curve, mass; broken curve, cell number. Symbols: ▲ and △, maltose added; ● and ○, no maltose added.

The mass per cell in the wild type also increased to nearly double after the addition of maltose (Fig. 1), but this quickly adjusted to a new steady-state level. MC4100(λ pRI-1), a maltose-resistant revertant of PB72-47, in which a mutation affecting the MalE signal sequence causes the fusion protein to accumulate in the cytoplasm (3), was also analyzed and was found to behave similarly to the wild-type parental strain (data not shown). Although clearly cells do filament after maltose induction of PB72-47 as observed by Bassford and Beckwith (3), division

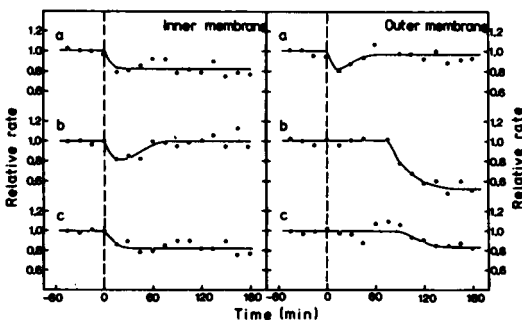


FIG. 2. Effect of maltose addition on the relative rate of synthesis of bulk inner membrane and outer membrane protein in MC4100 (a), PB72-47 (b), and MC4100 (λ pRI) (c). Maltose was added at time zero to cultures growing exponentially in M9-glycerol medium at 30°C, and samples were pulse-labeled at intervals with [³⁵S]methionine as in Materials and Methods.

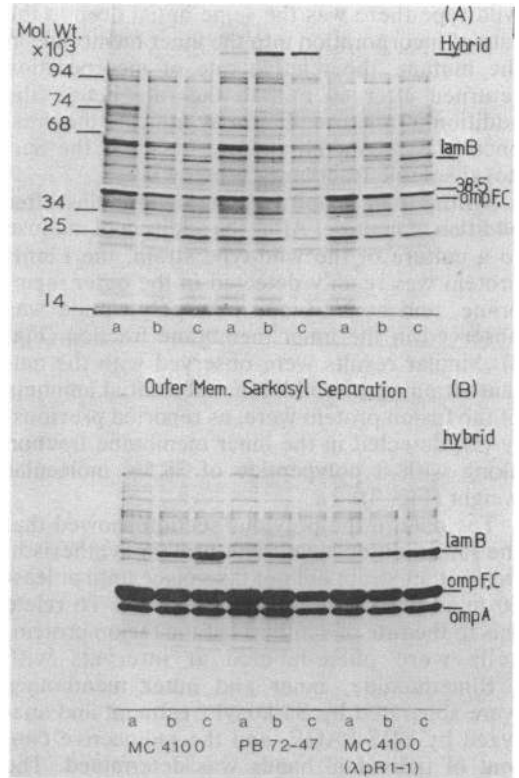


FIG. 3. Coomassie blue-stained gel profiles after SDS-PAGE analysis of inner membrane (A) and outer membrane (B) from cultures growing in M9-glycerol medium at 30°C. Samples were taken at time zero (a), 90 min (b), and 180 min (c) after the addition of maltose.

is by no means completely blocked, continuing at about 50% of the normal rate. Figure 2 shows the effect of the addition of maltose on the rate of synthesis and assembly of inner and outer membrane proteins, expressed relative to the total rate of protein synthesis, in strain PB72-47, its wild-type parent, and the maltose-resistant revertant MC4100(λ pRI-1). Inner and outer membrane fractions were obtained by Sarkosyl treatment of envelopes from sonicated cells. In the wild-type strain, the relative rate of outer membrane protein synthesis was essentially unaffected by maltose. In contrast, after about 75 min, the rate of outer membrane protein synthesis in strain PB72-47 fell rapidly to about 50% of the initial rate, indicating that synthesis of the hybrid protein was blocking the sites of synthesis, assembly, or both, of outer membrane proteins. There was also a slight reduction of outer membrane protein synthesis in the revertant strain consistent with the presence of small amounts of fusion protein in the membrane of this strain (Fig. 3). Finally, although as in the

wild type there was the same initial drop in the rate of incorporation into the inner membrane in the mutant, the relative rate of incorporation returned after 60 min to the rate before the addition of maltose. This may indicate the presence of outer membrane precursors in the Sarkosyl-soluble fraction (see below).

Synthesis of LamB and fusion proteins after addition of maltose. After the addition of maltose to a culture of the wild-type strain, the LamB protein was readily detected in the outer membrane, and at least one new polypeptide was observed in the inner membrane fraction (Fig. 3). Similar results were observed with the mutant strain, but in addition, substantial amounts of the fusion protein were, as reported previously (3), detected in the inner membrane fraction along with a polypeptide of 38.5K molecular weight (Fig. 3).

The data in the previous section showed that the fall in outer membrane protein synthesis in the mutant strain did not take place until at least 60 min after the addition of maltose. To relate this to the rate of synthesis of the fusion protein, cells were pulse-labeled at intervals with [³⁵S]methionine, inner and outer membranes were separated by Sarkosyl treatment and analyzed by SDS-PAGE, and the radioactive content of individual bands was determined. The results obtained (expressed relative to the rate of total protein synthesis determined in each pulse) are shown in Fig. 4b. Two major features should be noted; the addition of maltose was followed by a lag of 30 to 45 min before synthesis of the hybrid protein and the LamB protein was detected (in the inner and outer membrane fractions, respectively), and the maximal rates of synthesis at 60 to 75 min coincided exactly with the overall fall in outer membrane protein synthesis shown in Fig. 2. These results clearly suggest that as maximal rates of synthesis of the fusion protein are reached, a substantial portion of sites for the synthesis and assembly of outer membrane proteins are blocked by the fusion protein. These results can be seen in better perspective when compared with the kinetics of LamB synthesis in the wild-type strain shown in Fig. 4a. First, the initial kinetics are essentially the same, demonstrating that the early lag in synthesis is not due to the presence of the hybrid protein. Second, the maximal rate of synthesis again peaked after about 75 min and then gradually declined to a new steady-state level approximately 40% of the maximum rate. This presumably reflects the gradual adjustment, involving several factors, to a new steady-state level of transcription from the maltose operons. The gradual decline in the synthesis of the fusion protein can be attributed to similar regulation of the *malE* promoter. This in turn should result in the appearance of some

inner membrane export sites free of fusion protein, thus allowing outer membrane protein synthesis eventually to continue at the reduced rate shown in Fig. 2

Accumulation of unprocessed forms of envelope protein precursors in PB72-47 after addition of maltose. As indicated above, at least one new protein, in addition to the hybrid protein, was detected in the Sarkosyl-soluble fraction of isolated envelopes after addition of maltose to cultures of PB72-47 (Fig. 3). Kinetic studies indicated (Fig. 4) that the appearance of this polypeptide also coincided with the reduction in rate of outer membrane protein synthesis. The molecular weight and partial peptide analysis (data not shown) of this protein indicated its close relatedness to the OmpF protein. In fact, while this manuscript was in preparation, Ito et al. (18) reported that several outer membrane proteins, including OmpF and periplasmic protein precursor forms, accumulated in PB72-47 after addition of maltose. These results indicated that

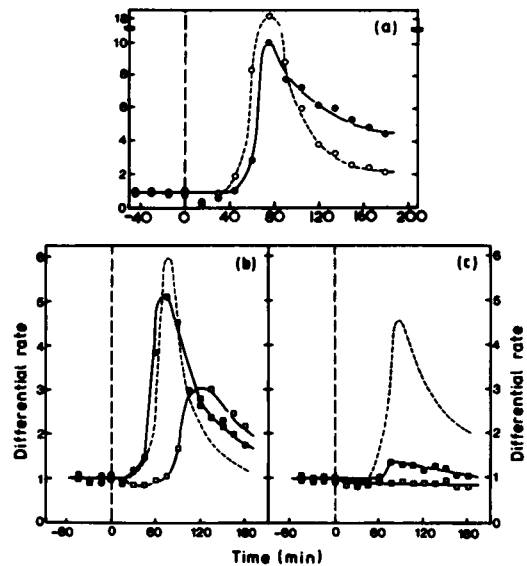


FIG. 4. (a) The relative rate of synthesis of the *lamB* protein in MC4100 (wild type) and in PB72-47 are compared (solid and broken lines, respectively). The relative rate of synthesis of *lamB* (broken line), the fusion protein (■) and the putative OmpF and C 38.5K precursor (□) are shown for PB72-47 (b) and MC4100 (ΔpRI) (c). The OmpF and C porins were not resolved in the particular gel conditions used here. Similarly the putative precursor band may consist of both porins. Maltose was added at time zero, and the [³⁵S]methionine/[³H]leucine values (see Materials and Methods) are normalized to a value 1 for glycerol grown cells. For the analysis of *lamB*, bands were cut out from gel samples of outer membranes, whereas bands were cut out from gels of inner membrane samples for fusion protein and the porin precursor.

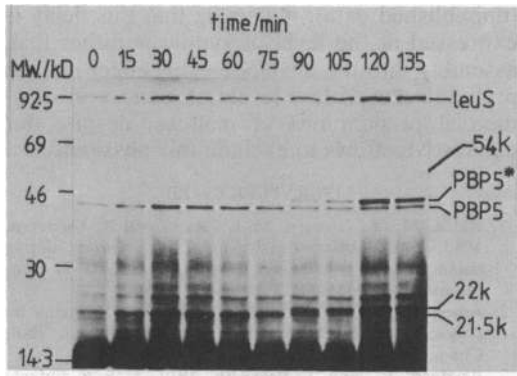


FIG. 5. Kinetics of inhibition of PBP5 processing on maltose induction of PB72-47(pGY101). Maltose was added at time zero to an exponential culture of glycerol grown PB72-47(pGY101). At intervals, samples were removed to examine the proteins synthesized after λ pBS10 infection of the UV-irradiated cells, using a [35 S]methionine label. The products of the labeled samples were then separated by SDS-PAGE (11% gel). The position of molecular weight standards is indicated on the left, and the polypeptides coded by the transducing phage are indicated on the right. Other details are described in the text.

the fusion protein not only blocked sites of export for outer membrane and periplasmic proteins but also blocked access to the signal peptidase(s). To determine whether this effect was specific to outer membrane and periplasmic proteins, we also investigated the effect of induction of the fusion protein on the processing of inner membrane proteins. We have recently shown (25) that two inner membrane polypeptides, PBP5 and PBP6, are synthesized as larger-molecular-weight precursors which can be processed *in vitro* by the addition of inner membrane vesicles. Labeling of cells with [14 C]benzylpenicillin after growth of PB72-47 in maltose indeed demonstrated the presence of new proteins (data not shown) identical in molecular weight to the PBP5 and PBP6 precursors synthesized *in vitro*. Finally, this same higher-molecular-weight form of PBP5 labeled with [35 S]methionine can be detected when maltose grown cells (after heavy UV irradiation) are infected with the λ -transducing phage pBS10 (31) carrying *dacA* (PBP5) (Fig. 5). The appearance of the precursor could be detected as early as 75 min after addition of maltose, again coincident with the maximal rate of synthesis of the hybrid protein. Further inspection of the gel in Fig. 5 indicates the presence of an additional protein, apparently accumulating as a higher-molecular-weight precursor in the presence of maltose. This protein, approximate molecular weight 22,000, is identical to a protein coded by phage λ pBS10 previously iden-

tified by Spratt et al. (31; N. Stoker, personal communication) and predominantly localized in the inner membrane.

DISCUSSION

The results described above indicate that although the MalE protein is normally localized in the periplasm, attempted export of this protein fused to β -galactosidase blocked at least some of the sites of synthesis and assembly of outer membrane proteins. In addition, these results indicate that the syntheses of both periplasmic proteins, e.g., the MalE protein, and outer membrane proteins are also normally subject to the availability of the same limiting factor(s). However, our study does not necessarily provide evidence for specific structural sites concerned with outer membrane biosynthesis. We have argued previously (6) that the capacity of *E. coli* cells to synthesize outer membrane proteins is normally saturated, and therefore the presence of the fusion protein in the inner membrane may simply reduce the availability of some limiting factor essential for translocation, synthesis, or both, of outer membrane proteins.

Although outer membrane proteins in *E. coli* are synthesized at a constant rate which doubles at a discrete time in the cell cycle (6, 9; A. Boyd, Ph.D. thesis, University of Leicester, U.K., 1979), the limiting factor might be due either to an abrupt doubling in the number of export sites or equally to a doubling in the rate of expansion for the underlying peptidoglycan (6). If such a bilinear mode of growth of the cell surface is an essential element in division control (15), differential inhibition of outer membrane protein synthesis might be expected to be correlated with inhibition of cell division. We have recently reported such a correlation (16) under conditions in which polynucleotide gyrase is inactivated. In some contrast, in this study, whereas the synthesis and assembly of outer membrane proteins were markedly reduced, the rate of cell division, although also reduced, still continued at about 50% of the rate before the addition of maltose. In this case, either we underestimated the amount of protein assembled into the outer membrane or high levels were not required for the division process. Although the membrane fractionation procedure may have been perturbed when high levels of the hybrid protein were present (see below), the slight inflation of the inner membrane fraction in the strain PB72-47 compared with the wild type (Fig. 2) was not sufficient to account for the apparent deficit of outer membrane protein. An alternative explanation is that the rate of peptidoglycan synthesis might be the primary determinant in bilinear surface growth, in which case the rate of outer membrane pro-

tein assembly might not be a major limiting factor as observed here.

Under conditions in which outer membrane protein synthesis was reduced, evidence was obtained for the accumulation of a precursor form of at least one of the major porins (22) in the envelope of strain PB72-47. While this manuscript was in preparation, Ito et al. (18), using a quite different approach, identified several outer membrane and periplasmic proteins in precursor form in strain PB72-47 after the addition of maltose. These results indicate that the presence of the fusion protein blocks some translocation system in the envelope or access to the signal peptidase, presumably located in the inner membrane. The processing of at least two inner membrane polypeptides, PBP5 and PBP6, is also inhibited, suggesting that this inhibition is due to a general disruption of the organization of inner membrane polypeptides or the sequestering of many signal peptidase molecules into abortive processing complexes, rather than the blockage of a specific export system common to outer membrane and periplasmic proteins. We have not ruled out the possibility that some envelope proteins accumulate in the cytoplasm and are therefore not processed, but clearly unprocessed polypeptides can be recovered with the envelope fraction. For PBP5 and PBP6, we could not detect any proteins capable of binding [¹⁴C]benzylpenicillin in the cytoplasmic fraction after growth on maltose.

In an attempt to localize the fusion polypeptide in the envelope, we observed that the protein fractionated either with the outer or the inner membrane, depending upon the method (sucrose gradient or Sarkosyl) of membrane separation used (18; unpublished data). This apparent ambiguity may reflect the association of unprocessed periplasmic and other membrane proteins *in vivo* with both membranes, as found previously by Halegoua and Inouye (13). Nevertheless, until the precise localization of the fusion protein and in particular any unprocessed outer membrane proteins is established, it is perhaps premature to conclude whether processing is being blocked at an early or a late step in the normal translocation and final maturation of envelope proteins.

Finally, the kinetics of induction of the LamB and MalE proteins in both wild-type and mutant strains were quite unexpected. Although the cultures responded immediately in terms of the increased growth rate upon the addition of maltose, indicating that maltose was being metabolized, the synthesis of these proteins was not detected for at least 30 min. Identical kinetics were obtained in two other wild-type strains. Moreover, the appearance of the hybrid protein in total cell lysates followed the same kinetics

(unpublished data), indicating that this delay is expressed at the level of synthesis rather than assembly into the envelope. This effect is most probably due to low levels of glucose in commercial preparations of maltose, despite our exhaustive efforts to exclude this possibility.

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