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Biotinylation In Vivo as a Sensitive Indicator of Protein Secretion and Membrane Protein Insertion

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***Escherichia coli* biotin ligase is a cytoplasmic protein which specifically biotinylates the biotin-accepting domains from a variety of organisms. This in vivo biotinylation can be used as a sensitive signal to study protein secretion and membrane protein insertion. When the biotin-accepting domain from the 1.3S subunit of *Propionibacterium shermanii* transcarboxylase (PSBT) is translationally fused to the periplasmic proteins alkaline phosphatase and maltose-binding protein, there is little or no biotinylation of PSBT in wild-type *E. coli*. Inhibition of SecA with sodium azide and mutations in SecB, SecD, and SecF, all of which slow down protein secretion, result in biotinylation of PSBT. When PSBT is fused to the *E. coli* inner membrane protein MalF, it acts as a topological marker: fusions to cytoplasmic domains of MalF are biotinylated, and fusions to periplasmic domains are generally not biotinylated. If SecA is inhibited by sodium azide or if the SecE in the cell is depleted, then the insertion of the MalF second periplasmic domain is slowed down enough that PSBT fusions in this part of the protein become biotinylated. Compared with other protein fusions that have been used to study protein translocation, PSBT fusions have the advantage that they can be used to study the rate of the insertion process.**

The outer membrane, periplasmic, and inner membrane proteins of *Escherichia coli* all have hydrophilic parts which must be translocated across the hydrophobic environment of the inner membrane. A variety of genetic and biochemical methods have been used to identify the cellular machinery responsible for this translocation (39). So far, the known components of the secretion apparatus comprise SecA, SecB, SecD, SecE, SecF, SecG, and SecY (5, 21, 25, 28, 30, 32, 46). Of these, only SecA, SecE, and SecY are absolutely essential in an in vitro system (20, 46), and the remaining proteins appear to have an accessory role in enhancing protein translocation across the inner membrane.

While most outer membrane and periplasmic proteins clearly make use of the Sec machinery, the results concerning inner membrane proteins are somewhat equivocal. Some inner membrane proteins, notably M13 coat protein (47), insert efficiently even when there are severe secretion defects. On the other hand, the insertion of leader peptidase (45, 48) is clearly Sec dependent, and certain results suggest that the chemotaxis receptor Tsr (17) is also Sec dependent. MalF insertion was initially found to be unaffected by SecD or SecA mutations (26). More recently, however, it has been reported that SecA is involved in the insertion of the second periplasmic loop of MalF and that this Sec dependence is very much a function of sequence context (37). It has also been suggested that large periplasmic domains, such as the second periplasmic loop of MalF, may generally be translocated in a Sec-dependent manner, while smaller ones (<60 residues) are translocated in a Sec-independent manner (2).

Genetic fusions to inner membrane proteins have frequently been used for studying membrane topology and insertion (9). Such fusion systems have included alkaline phosphatase (AP) (24), β-galactosidase (16), β-lactamase (34), and the biotin-targeting domain from *Klebsiella pneumoniae* oxaloacetate decarboxylase (KPBT) (50). These fusions share a useful feature in that the ligand has properties that differ depending on its location relative to the membrane; e.g., AP is active only in the periplasm and β-galactosidase is active only in the cytoplasm. *E. coli* biotin ligase, which efficiently biotinylates a lysine residue in KPBT, is a strictly cytoplasmic enzyme. Thus, if protein translocation proceeds more rapidly than biotinylation, one might expect that a KPBT membrane protein fusion would be biotinylated only if it is attached to a cytoplasmic part of the protein.

Biotinylated protein sequences, such as KPBT, are conserved throughout biology. Strong conservation, along with the resistance of the peptides to proteolysis, indicated that these sequences were probably stably folded domains. This prediction was recently confirmed by both the two-dimensional nuclear magnetic resonance structure (36) and the crystal structure (3) of the C-terminal segment of *E. coli* biotin carboxyl carrier protein (BCCP). In the case of the nuclear magnetic resonance study, both the biotinylated and nonbiotinylated forms of this 86-residue C-terminal peptide were overproduced (12), and their structures were solved (36). The two structures are essentially identical and consist of two four-stranded antiparallel β-sheets folded into a barrel structure. The hydrogen-bonded N and C termini are at one end of the barrel. At the opposite end, biotin is attached to a lysine at the tip of a β-hairpin loop. As originally predicted (10), this structure is strikingly similar to that of domains modified by the attachment of lipoic acid. The biotinylated domains swing on a proline/alanine-rich hinge immediately upstream of the folded barrel. These and other findings indicate that biotin ligase recognizes domain structures rather than linear sequences of amino acid residues.

When KPBT was genetically fused to the C termini of two secreted proteins in *E. coli*, AP and β-lactamase, biotinylated products were detected in the periplasm of the cells (35). In the

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TABLE 1. Strains, plasmids, and phage used in this study

Strain, plasmid, or phage	Relevant characteristics	Reference or source
Strain		
BM1161	MC4100 Φ(*bioA-lacZ*)*301 argE3* Rif^r	4
CM263	*phoA* Δ*Pvu*II *pcnB80 zad*::Tn*10*Tet^s Str^r *secE*Δ19-111 *recA*::Cm^r pCM110	Chris Murphy
DHB3	Δ(*ara-leu*)*7697 araD139* Δ*lacX74 galE galK phoA* ΔpvuII *phoR malF*	8
DHB4	DHB3 F′(*lacI*^{q} *lacZ*$^+$)	8
GJ55	DHB3 Φ(*bioA-lacZ*)*301* pMS421	This study
GJ64	GJ55 *malB* Δ*101* pMS421	This study
GJ117	CM263 F′(*lacI*^{q} *lacZ*$^+$), pGJ110 replaces pCM110	This study
HS3018	MC4100 Δ*malE444 malT*(Con)-1	41
KJ173	KJ180 *secD29 zaj*::Tn*10*	33
KJ180	*phoR araD139* Δ(*ara-leu*)*7679 galE galK* Δ*lacX*7 *rpsL150 thi*	33
KJ184	KJ180 *secF62 zaj*::Tn*10*	33
MC4100	*araD139* Δ*lacU169 rpsH* Str^r *thi mal*	11
MM24	MC4100 *malB* Δ *101*	Beckwith collection
MM152	MC4100 *malT*(Con) *malT*::Tn*10 secB*::Tn*5*	22
Plasmid		
pBAD30	*araBAD* promoter, Amp^r, pACYC184 origin	19
pBoneIV	*phoA* with N- and C-terminal polylinkers, Amp^r	Michael Ehrmann
pCM110	*secE* under *araBAD* promoter, Amp^r, pBR322 origin	Chris Murphy
pCY66	PSBT Amp^r, Kan^r	14
pCY100	*birA*, Cm^r	14
pDHB60	*tac* promoter with polylinker, Amp^r	Dana Boyd
pGJ44	*malE*-PSBT, Amp^r	This study
pGJ47	*phoA* under *tac* promoter with C-terminal polylinker, Amp^r	This study
pGJ48	*phoA*-PSBT, Amp^r	This study
pGJ110	*secE* under *ara* promoter, Amp^r, pACYC184 origin	This study
pGJ61	PSBT, Kan^r, *phoA′*	This study
pGJ78 series	*malF*-PSBT fusions at positions H, I, J, K, L, M, N, O, P, Q, and R, Kan^r	This study
pMS421	*lacI*^{q} Spc^r	18
pPL2	Kan^r *phoA*	Ping Li
pPR683	*malE* with C-terminal polylinker, Amp^r	Paul Riggs
pPR890	*malE*, Amp^r	Paul Riggs
Phages		
λGJ38	λGT11 Amp^r *phoA′*, Kan′	This study
λGJ61	λGJ38 with PSBT from pGJ61, Kan^r	This study
λGJ78 series	λGJ38 *malF*-PSBT fusions at positions H, J, K, L, and M, Kan^r *phoA′ I*21 *S*$^+$	This study
λGT11	*lac5* Δ*sh*ndIIIλ2-3 *sr*Iλ3° *cIts*857 *sr*Iλ4° *nin5 sr*Iλ5° *S*am100	49
λI21H80	*I*21 *H*80	Beckwith collection

case of the AP-KPBT fusion, the biotinylation was enhanced by a *secB*::Tn*5* mutation, which presumably slowed down protein export. Insertions of KPBT, together with a factor Xa protease site, into two normally periplasmic loops of lactose permease resulted in fusions that were biotinylated, and the biotinylated product was not exported into the periplasm (50). In addition, two of four insertions into cytoplasmic loops of lactose permease (LacY) were not biotinylated, prompting the authors to conclude that biotinylation cannot be used to study the topology of complex membrane proteins.

In this study, we fused the biotinylatable domain from the 1.3S subunit of *Propionibacterium shermanii* transcarboxylase (PSBT) (14) to the C termini of two periplasmic proteins, AP and maltose-binding protein (MBP), and at several positions in the inner membrane protein MalF. We used PSBT instead of KPBT in our experiments because this domain was available to us as a convenient plasmid-encoded cassette. Like KPBT, the 76-amino-acid PSBT fragment is recognized in the cytoplasm by *E. coli* biotin ligase, which biotinylates a lysine residue 35 amino acids from the end of the protein. With PSBT fusions to periplasmic proteins, we show a general correlation between defects in secretion (SecA, SecB, SecD, and SecF) and increases in the level of biotinylation of PSBT. Furthermore, we show that 10 of 11 MalF-PSBT fusion proteins are biotinylated or not biotinylated, in agreement with the known topology of MalF, and that inhibition of SecA or depletion of SecE slows down the translocation of PSBT fusions to the second periplasmic domain of MalF enough that they can become biotinylated.

MATERIALS AND METHODS

Strains, plasmids, and phages. Strains, plasmids, and phages used are described in Table 1. Strain GJ55 was constructed from strain DHB3 by P1 transducing Φ(*bioA-lacZ*) from strain BM1161, selecting for growth on lactose under biotin-limiting conditions. Strain GJ64 was derived from strain GJ55 by P1 transducing Δ*malB* from strain MM24, selecting for λ resistance. Strain GJ117 was derived from strain CM263 by replacing pCM110 with pGJ110 and mating in F′(*lacI*^{q} *lacZ*$^+$) from strain DHB4. pGJ38 was constructed by inserting an *Eco*RI fragment encoding Amp^r into pPL2, replacing parts of both the Kan^r gene and *phoA*. pGJ44 was made by cloning an *Xba*I-*Hin*dIII fragment containing the *P. shermanii* biotin targeting sequence from pCY66 into the polylinker of pPR683. pGJ47 was made by cloning a *Bam*HI fragment with *phoA* from pBoneIV into the *Bam*HI site of pDHB60. pGJ48 was derived from pGJ47 by cloning the biotin domain from pCY66 into the polylinker C terminal to *phoA*, using *Xba*I and *Hin*dIII. pGJ61 was made from pPL2 by moving in the PSBT from pCY66 with *Eco*RI and *Hin*dIII. The MalF-biotin domain fusions of the pGJ78 plasmids were made by using PCR (one primer with *tac* promoter sequence and one primer complementary to *phoA*) to amplify the MalF fragments of plasmids with MalF-AP fusions described previously (8). These fragments were then cloned into pGJ61, resulting in C-terminal biotin fusions at the positions where the AP fusions previously were. λGJ38 was made by cloning *phoA′*, Amp^r, and Kan′ from pGJ38 into λGT11. λGJ78 constructs were made by recombining λGJ38 with pGJ78 plasmids, selecting for Kan^r phage lysogens, and screening these for Amp^s and expression of the MalF-biotin fusion.

Media and reagents. NZ medium containing 0.5% yeast extract (Difco), 1.0% NZamine (Difco), 1.5% agar, and 0.8% NaCl was used as rich medium. M63, with carbon sources added at 0.2%, was used as minimal medium (27). Amino acids were added as described previously (23). Restriction enzymes, DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs. [^{3}H]biotin, [^{35}S]methionine, and [^{14}C]maltose were purchased from Amersham. Trypsin, lysozyme, and biotin were purchased from Sigma. Antibiotics were used at the following concentrations: ampicillin, 200 μg/ml; kanamycin, 60 μg/ml; chloramphenicol, 10 μg/ml; and spectinomycin, 100 μg/ml. Anti-MBP antibodies were prepared by Kathy Strauch, anti-MalF antibodies were prepared by Beth Traxler, and anti-AP antibodies were prepared by Claudette Gardel.

Tritium labeling and detection of secreted proteins. Overnight cultures of strains in M63-glucose supplemented with 20 amino acids and the appropriate antibiotics were diluted 1:50 in the same medium and were allowed to grow at 37°C until the cells had reached an optical density at 600 nm of approximately 0.3. At this time, 5 μCi of [^{3}H]biotin per ml, 41 nM unlabeled biotin (to prevent endogenous biotin synthesis), and 0.5 mM isopropylthiogalactopyranoside (IPTG) were added. For SecA inhibition, sodium azide was added at 2 mM 2 h before harvesting of the cells. After 2 h of additional growth at 37°C, cells were harvested by centrifugation. When needed, separation into periplasmic and cytoplasmic-plus-membrane fractions was done by chloroform shock (1). Cell extracts were electrophoresed on 10% polyacrylamide gels. Gels were treated with Amplify (Amersham) as instructed by the manufacturer, and ^{3}H-labeled proteins were detected by fluorography.

Measurement of maltose uptake. Overnight cultures in M63-glycerol medium with 20 amino acids and appropriate antibiotics were diluted 1:50 in the same medium and were grown to an optical density at 600 nm of approximately 0.3. IPTG (1 mM) was added, and cells were grown for a further 1.5 h at 37°C. [^{14}C] maltose (2 μCi/ml) and unlabeled maltose (7.8 nM) were added, and 100-μl samples were collected at 10, 20, 30, 45, and 60 s by aspiration onto Millipore filter disks. Samples were washed with 10 to 20 ml of M63 salts immediately after collection to prevent further uptake of maltose. Total ^{14}C uptake of the samples was measured with an LKB 1212 liquid scintillation counter.

Proteolysis of MalF-PSBT. Overnight cultures grown in NZ medium were diluted 1:100 into M63-glucose with appropriate antibiotics and 1.6 nM biotin. If proteins were to be detected by autoradiography, then the cultures also received 3.2 μCi of [^{3}H]biotin per ml. After 4 h of shaking at 37°C, cultures were induced for 0.5 h with 1 mM IPTG, and 1.5 ml of cells was spun out and resuspended in 1.5 ml of 18% sucrose–100 mM Tris-HCl (pH 8). After addition of lysozyme (5 μg/ml) and EDTA (2.5 mM), cells were left on ice for 20 min. Each spheroplast sample was divided into three parts: 0.5 ml was not treated further, 0.5 ml received 10 μg of trypsin per ml, and 0.5 ml was lysed with 1% Triton X-100 prior to addition of 10 μg of trypsin per ml. After 20 min on ice, 20 μl of phenylmethylsulfonyl fluoride (35 mg/ml) was added to all samples, which were left on ice for an additional 5 min. The spheroplasts and trypsin-treated spheroplasts were spun out at 4°C. Pellets were taken up in 50 μl of sodium dodecyl sulfate (SDS) sample buffer (6). Supernatants and Triton-lysed spheroplasts were trichloroacetic acid precipitated (6) and then taken up in SDS sample buffer. Samples were separated on SDS-acrylamide gels, and biotinylated proteins were detected either by fluorography after treatment with Amplify (Amersham) or with streptavidin-horseradish peroxidase and the ECL (enhance chemiluminescence) detection system (Amersham).

Steady-state labeling of MalF-PSBT. Overnight cultures grown in NZ medium were diluted 1:100 into M63-glucose medium with 19 amino acids and 1.6 nM biotin and grown for 2.5 h. After addition of 10 μM methionine, 6.6 μCi of [^{35}S] methionine per ml, and 0.5 mM IPTG, cells were grown for an additional 1.5 h. Samples were lysed and immunoprecipitated with anti-MalF antibodies as described previously (33). Proteins were separated on 10% gels and detected by autoradiography.

Chemiluminescence detection of biotinylated proteins. Proteins separated by electrophoresis were transferred to nitrocellulose. Membranes were blocked for 1 h with 20 mM Tris-HCl–137 mM NaCl–0.1% Tween 20–3% bovine serum albumin (BSA) (pH 7.6). After blocking, gels were exposed to streptavidin-horseradish peroxidase (Amersham) at 1:1,500 dilution in the same buffer containing 1% BSA for 1 h. Gels were washed four times with 20 mM Tris-HCl–137 mM NaCl–0.1% Tween 20 prior to detection with an ECL kit (Amersham) as instructed by the manufacturer.

SecE depletion. Cells with *secE* under control of the arabinose promoter were grown overnight in NZ medium with 0.2% arabinose and were diluted 1:50 into M63 minimal medium with either 0.2% arabinose or 0.2% glucose and all amino acids except methionine. After 3 h of growth, 1 mM IPTG was added, and cells were grown for an additional hour before samples were collected for pulse-labeling and MalF-PSBT detection. Growth curves for a representative strain were compiled by removing 1-ml samples for readings of optical density at 600 nm at 1-h intervals.

Pulse-labeling. Cells collected at the end of the SecE depletion experiment described above were pulse-chase labeled with [^{35}S]methionine (33). Briefly, log-phase cells were added to prewarmed, shaking tubes containing 30 μCi of [^{35}S]methionine per ml, and after 30 s, pulse samples were removed and added to chilled tubes containing 0.1 volume of 1% methionine. The remaining cells received 0.1 volume of 1% methionine, and chase samples were moved to prechilled tubes after 1 and 5 min. Proteins were immunoprecipitated with anti-MBP antibodies as described previously (33), separated on 12% acrylamide gels, and detected by autoradiography.

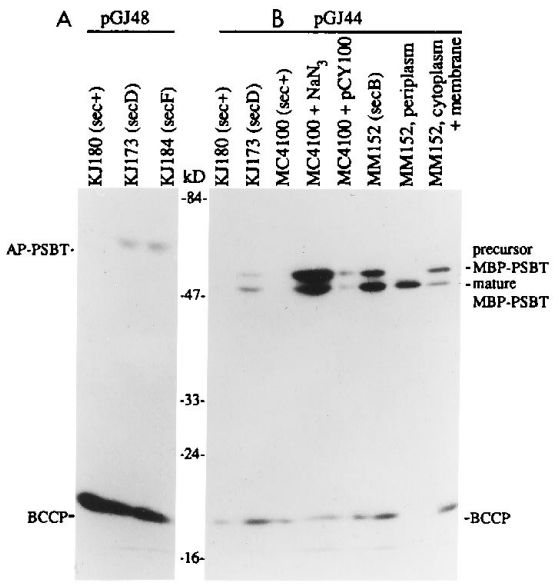

FIG. 1. (A) Biotinylation of AP-PSBT produced from pGJ48 in a wild-type strain (KJ180), in a *secD* strain (KJ173), and in a *secF* strain (KJ184); (B) biotinylation of MBP-PSBT produced from pGJ44 in wild-type strains (KJ180 and MC4100), in a *secD* strain (KJ173), after treatment with sodium azide (MC4100 + NaN_3), with overexpressed biotin ligase (MC4100 + pCY100), in a *secB* strain (MM152), and in MM152 subjected to cell fractionation by chloroform shock.

Sodium azide inhibition of membrane insertion. Overnight cultures grown in NZ medium were diluted 1:100 in the same medium. After 2 h of growth, production of MalF-PSBT fusions was initiated with 1 mM IPTG. Fifteen minutes after induction, the samples were split into two parts, one of which received 2 mM sodium azide for 10 min.

RESULTS

PSBT fusions to periplasmic proteins are biotinylated when there is a secretion defect. Mutations of *sec* genes generally slow down the secretion of periplasmic proteins. Thus, if PSBT is fused to a secreted protein such as MBP or AP, it should be more easily biotinylated by the cytoplasmic enzyme biotin ligase in *sec* mutant backgrounds. We used in vitro techniques to make pGJ44, which encodes an MBP-PSBT fusion protein in which PSBT is fused to the last amino acid of MBP. The presence of the desired fusion joint was verified by DNA sequencing, and the production of a fusion protein of approximately the correct size was detected by Western blotting (immunoblotting). Maltose uptake experiments comparing MBP-PSBT and wild-type MBP produced from pPR890 showed that addition of the biotin domain had only a small effect on maltose transport activity (data not shown). Thus, MBP-PSBT was presumably properly located in the periplasm of the cell.

In MC4100/pGJ44, a wild-type strain with MBP-PSBT under *tac* promoter control, we failed to detect any labeling of MBP-PSBT when cells were fed [^{3}H]biotin (Fig. 1). On the other hand, when the same plasmid was expressed in MM152 (*secB*::Tn*5*), we detected biotin labeling of both the precursor and the mature forms of MBP-PSBT. We used chloroform shock (1) to separate labeled MM152/pGJ44 into periplasmic and cytoplasmic-plus-fractions. Proper secretion and processing of some biotin-labeled MBP-PSBT was indicated by the fact that the periplasmic fraction contains only the mature form of the protein. The cytoplasmic-plus-membrane fraction, which is probably contaminated with some unlysed cells, contains both

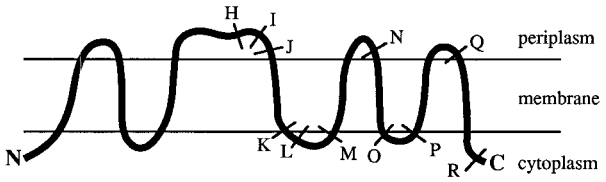

FIG. 2. Topology of MalF in the inner membrane of *E. coli*. The approximate locations of 11 MalF-PSBT fusions are indicated.

precursor and mature forms of the protein. BCCP, the only normally biotinylated protein in *E. coli*, is, as expected, found entirely in the cytoplasmic fraction of the cell. Addition of 2 mM sodium azide, which specifically inhibits SecA (31), also had a strong effect on the level of biotinylation of MBP-PSBT. KJ173/pGJ44, which is secretion compromised at 37°C by a *secD29* mutation, showed a higher level of MBP-PSBT biotinylation than the isogenic background strain KJ180/pGJ44.

Since conditions that slowed down protein export allowed biotinylation of MBP-PSBT, we reasoned that increasing the amount of biotin ligase in the cytoplasm might show a similar effect by increasing the rate of biotinylation. This was indeed the case when pGJ44 and pCY100, which overexpresses biotin ligase from the *birA* gene, were both moved into MC4100 (Fig. 1). Again, both the precursor and the mature forms of the protein are visible on the autoradiograph.

We examined a second periplasmic protein, AP, in a similar manner. PSBT was fused to AP by using in vitro techniques, and the presence of the desired construct was verified by DNA sequencing and Western blotting. Using DHB4 as a background strain, we found that the AP activity of AP-PSBT produced from pGJ48 was comparable to that of wild-type AP produced from pGJ47 (data not shown). AP activity assays are a direct measure of the export of this protein, since it is active only in the periplasm. As with the MBP fusion, we were not able to detect any [^{3}H]biotin-labeled AP-PSBT in the cells that were not secretion compromised (Fig. 1). When AP-PSBT was expressed in *secD* or *secF* cells, on the other hand, we detected some labeled fusion protein. The amount of biotin that was attached was even less than that which was detected when MBP-PSBT was expressed in a *secD* strain. There is only one band visible for labeled AP-PSBT, which, from size comparisons with Western-blotted proteins, we believe is the mature form of the protein. We have not carried out cell fractionation experiments with these constructs.

Cytoplasmic MalF-PSBT fusions are biotinylated. AP fusions have been used to study the topology of the cytoplasmic membrane protein, MalF. We wished to see whether the pattern of biotinylation of MalF-PSBT fusion proteins would indicate the same topology, that is, whether fusions to cytoplasmic domains would be biotinylated whereas fusions to periplasmic domains would remain nonbiotinylated. These studies were of particular interest, since certain of the AP fusions to cytoplasmic domains of MalF had given anomalous results (7, 8).

We constructed MalF-PSBT fusions at sites that are identical to those used by Boyd et al. (8) for MalF-AP fusions (Fig. 2). In each fusion protein, the MalF residues C terminal to the fusion joint are deleted. We made fusions only in the latter half of MalF because the available anti-MalF antibodies recognize the second periplasmic loop of the protein. As verified by DNA sequencing, the nucleotides of *malF* at the fusion joints are at the following positions: H, 682; I, 712; J, 826; K, 907; L, 920; M, 958; N, 1051; O, 1177; P, 1249; Q, 1417; and R, 1543.

The production of MalF-PSBT by each of the fusions was ascertained with [^{35}S]methionine steady-state labelings of GJ55/pGJ78 strains (Fig. 3A). All of the fusion proteins except the Q fusion protein were produced in comparable amounts. Overproduction of MalF-PSBT fusion proteins slowed down the growth rate of the cells. The greater growth defect of longer fusions was compensated for by loading equal amounts of cell material in each lane of the gel. Thus, it is possible either that strain GJ55/pGJ78.Q made less MalF-PSBT or that this strain did not take up [^{35}S]methionine as well as the other strains. The same cultures were also used to visualize MalF-PSBT with streptavidin-horseradish peroxidase and an ECL detection kit (Fig. 3B). As expected, all of the cytoplasmic fusions (K, L, M, O, P, and R) were biotinylated. Three periplasmic fusions (I, J, and Q) were not biotinylated. The N fusion, which should be periplasmic, was biotinylated, but at a slightly lower level than the cytoplasmic fusions. Strain GJ55/pGJ78.H, expressing the MalF-PSBT H fusion, was not included in this experiment, but Fig. 5 shows that this fusion is not biotinylated. We have detected the H fusion protein in other experiments by Western blotting (data not shown).

Proteolysis of MalF-PSBT K, L, and M fusions. The AP part of MalF-AP fusions in which the fusion joint is at the beginning of a cytoplasmic loop tends to be exported slowly into the periplasm of the cell (43). The mislocalization of AP in such fusions is due to the absence of the basic amino acids that normally stabilize the cytoplasmic localization of these domains. While MalF-AP fusions at the K and L positions have AP activities that are almost as high as those of periplasmic fusions, the MalF-AP M fusion has a level of activity that is almost 20-fold lower than that of periplasmic fusions (8). Therefore, it was conceivable that the K and L MalF-PSBT fusions, although biotinylated, had actually exported the biotin-containing domain to the periplasm of the cells.

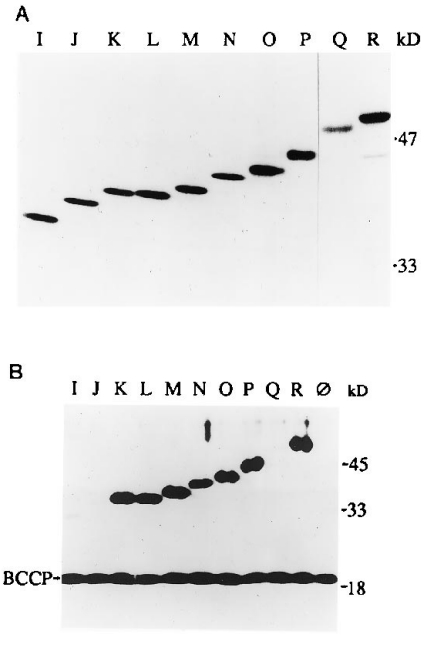

FIG. 3. (A) MalF-PSBT I, J, K, L, M, N, O, P, Q, and R fusions expressed from pGJ78 plasmids in strain GJ55, steady-state labeled with [^{35}S]methionine, immunoprecipitated, and detected by autoradiography; (B) MalF-PSBT I, J, K, L, M, N, O, P, Q, and R fusions expressed from pGJ78 plasmids in strain GJ55 and visualized with streptavidin-horseradish peroxidase and chemiluminescence detection.

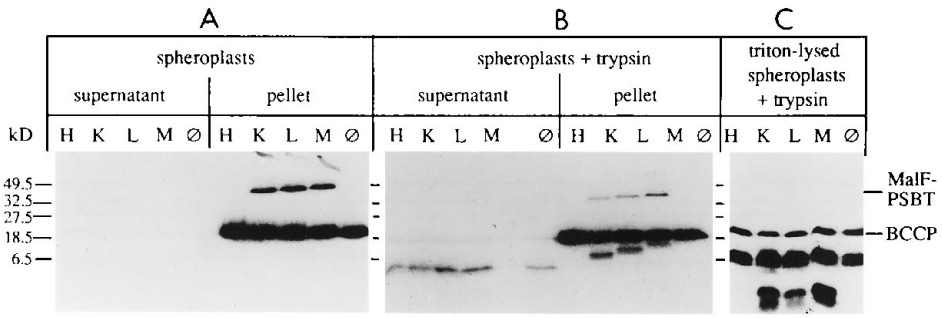

FIG. 4. (A) Strains GJ64/pGJ78.H, -K, -L, and -M and strain GJ64/pGJ61 (control; lane ø) labeled with [^{3}H]biotin, made into spheroplasts with lysozyme-EDTA treatment, centrifuged into supernatant and pellet fractions, separated on a 22.5% acrylamide gel, and detected by fluorography. Neither MalF-PSBT nor BCCP is released in significant amounts by the spheroplast treatment. (B) Same samples as in panel A but treated with trypsin (10 μg/ml) for 20 min after spheroplasting. Degradation products of the MalF-PSBT K, L, and M fusions are visible in the pellet fraction as a ladder of three bands between 6.5 and 18.5 kDa. Bands at 6.5 kDa in all lanes of the supernatant fraction are due to leaky spheroplasts and degradation of BCCP. (C) Same samples as in panel B but with lysis with 1% Triton prior to trypsin treatment and no separation by centrifugation. Breakdown products of the K, L, and M fusions are all the same size and are smaller than the 6.5-kDa breakdown products of BCCP.

The locations of the biotinylated domains of the K, L, and M fusions were determined by proteolysis of spheroplasts labeled with [^{3}H]biotin. These experiments were done with GJ64 (Δ*malB*) as a background strain, since the presence of MalG and MalK in a strain can reduce the protease sensitivity of MalF (42). Figure 4A shows the supernatant and pellet fractions of the spheroplasts. The K, L, and M fusions are labeled in approximately equal amounts. In control strains with pGJ78.H and pGJ61, only BCCP is labeled by [^{3}H]biotin. The small amount of BCCP, a cytoplasmic protein, in the supernatant fraction indicates that the spheroplasts are mostly intact. Figure 4B shows the spheroplasts after treatment with trypsin. The K, L, and M fusions are now partially degraded as a result of the action of the protease from the outside of the membrane. The three fragments are of progressively larger size, in the order K, L, M, as a result of the increasing amount of MalF present between the cleavage site at the membrane surface and the PSBT fragment. There was some leakage of the spheroplasts during proteolysis, as evidenced by the fact that there is a breakdown product of BCCP in all lanes of the supernatant fraction. If the spheroplasts were lysed prior to addition of the protease (Fig. 4C), then the breakdown products of the MalF-PSBT fusions were all the same size, probably because of cutting at one of the trypsin-sensitive sites within the PSBT fragment. BCCP was degraded to fragments that are approximately the same size as those in the supernatant fraction in Fig. 4B. We interpret these results as indicating that the MalF-PSBT K, L, and M fusions all have their biotinylated domains located on the cytoplasmic side of the inner membrane.

Proteolysis of the MalF-PSBT N fusion. The MalF-PSBT N fusion is of particular interest because this fusion is to a periplasmic domain of MalF but nevertheless is biotinylated. There are two possible explanations for this observation: (i) the protein is not inserted and PSBT remains in the cytoplasm; and (ii) the protein inserts more slowly than other periplasmic fusions, giving biotin ligase a chance to biotinylate PSBT before it is translocated through the membrane. To determine the localization of PSBT in this construct, we performed a trypsin proteolysis experiment with strain GJ64/pGJ78.N (Fig. 5). When spheroplasts of this strain are treated with trypsin, most of the biotinylated breakdown product is released into the medium. Thus, at least some of the MalF-PSBT N fusion is inserted properly into the membrane, supporting the hypothesis that biotinylation in this situation is the result of a slowdown of membrane protein insertion.

SecA inhibition allows biotinylation of MalF-PSBT. Sääf et al. (37) used proteolysis to show that inhibition of SecA with sodium azide can affect the insertion of the second periplasmic loop of MalF under some conditions. We find that an effect of inhibition of SecA on membrane protein insertion can be more easily visualized as a change in the biotinylation of MalF-PSBT fusions in the second periplasmic loop. MalF-PSBT fusions H, J, K, L, and M were crossed onto λ phage, and the production of fusion proteins of about the right size was verified by Western blotting with anti-MalF antibodies (data not shown). MC4100/pMS421 with λGJ61 (control) or λGJ78.H, -J, -K, or -M was subjected to sodium azide treatment as described in Materials and Methods, and biotinylated proteins were detected by fluorography. The K and M fusions were biotinylated approximately equally in the treatment and control cells (Fig. 6). The H and J fusions, on the other hand, were biotinylated only when membrane insertion was inhibited by interference with SecA function. It is not possible to make a direct comparison of the intensities of the bands in this experiment because the cytoplasmic MalF-PSBT fusions were biotinylated

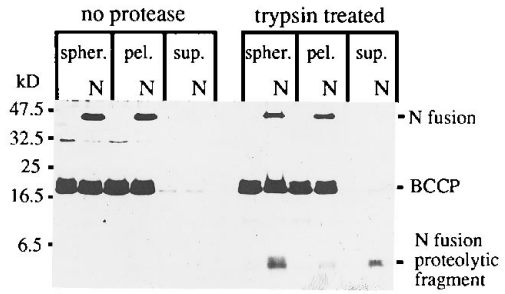

FIG. 5. Proteolysis of the MalF-PSBT N fusion. Spheroplasts with or without trypsin treatment (lanes labeled sphero.) were separated by centrifugation into periplasmic (sup.) and cytoplasmic-plus-membrane fractions (pel.). Lanes N contain samples produced from strain GJ64/pGJ78.N. Adjacent unlabeled lanes are samples prepared from strain GJ64/pGJ61. Samples were separated on a 15% acrylamide gel, and biotinylated proteins were detected with streptavidin-horseradish peroxidase and the ECL detection system. The MalF-PSBT N fusion, a membrane protein, and BCCP, a cytoplasmic protein, are located primarily in the pellet fractions, an indication of the integrity of the spheroplasts. After trypsin treatment, a biotinylated breakdown product that is specific to the N fusion is located primarily in the periplasmic fraction, an indication that at least some of the PSBT domain has been translocated. The nonspecific band running near the 32.5-kDa standard appears in some samples when the ECL detection system is used.

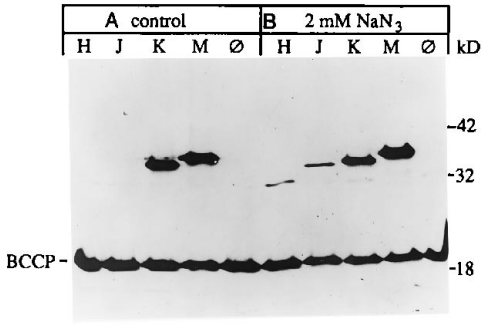

FIG. 6. MalF-PSBT from strain MC4100/λGJ61 (lanes ø) and strains MC4100/λGJ78.H, -J, -K, and -M visualized with streptavidin-horseradish peroxidase and the ECL detection system. (A) Control experiment; (B) inhibition of SecA by treatment with 2 mM sodium azide for 10 min. MalF-PSBT H and J fusions are biotinylated only after sodium azide treatment.

for the full 25-min induction period, whereas the periplasmic fusions were biotinylated only during the last 10 min after sodium azide addition.

The biotinylated domains of periplasmic MalF-PSBT fusions that are seen after inhibition with azide could be in either the cytoplasmic or the periplasmic compartment of the cell. We performed a trypsin proteolysis experiment with spheroplasts of strain GJ64/λGJ78.J and found that just as in the case of the N fusion, a biotinylated breakdown product was released into the periplasm (data not shown). Therefore, at least some of this protein was inserted properly after azide treatment, and the observed biotinylation is probably the result of a slowdown in membrane translocation.

SecE depletion causes a membrane insertion defect. Of all conditions that have been tested, depletion of SecE expressed from an arabinose promoter results in the strongest secretion defect (44), but it was not known whether SecE depletion also causes a defect in membrane protein insertion. We constructed GJ117 as a strain that is suitable for detecting an effect of SecE on the insertion of MalF-PSBT fusions. This strain carries a chromosomal *secE* deletion and SecE expressed on a plasmid from the tightly controlled *araBAD* promoter. This promoter is induced by arabinose and repressed by glucose (19).

Strain GJ117/λGJ78.J was used in initial experiments to show that there is a secretion defect in this strain background. An overnight culture was diluted 1:50 into M63-glucose and M63-arabinose. The growth rate in glucose was much slower than that in arabinose, and after 8 h (about 2.5 doublings), the cells in M63-glucose stopped growing completely (Fig. 7). Addition of 1 mM IPTG after 3 h had no discernible effect on the growth rate. No revertants appeared in depleted cultures grown overnight. To minimize the chance of seeing secondary effects resulting from long-term depletion of SecE, we examined effects of SecE depletion at the 4-h time point, when the growth rates of the glucose- and arabinose-grown cultures were relatively similar.

Four hours after the start of SecE depletion, the secretion defect in strain GJ117/λGJ78.J was visualized by pulse-labeling and immunoprecipitating MBP (Fig. 8). When cells were grown in M63-arabinose, all of the MBP was in the mature, secreted form at the end of the pulse-labeling. In M63-glucose, on the other hand, most of the MBP was in the precursor form at the end of the pulse-labeling, and much of it chased into the mature form over a period of 5 min. Thus, at this time point, there is a partial secretion block due to SecE depletion.

GJ117/λGJ61 (control strain) and GJ117/λGJ78.H, -J, -K, -L, and -M were used to show the effect of SecE depletion on membrane insertion. Cultures were diluted as described above, and production of MalF-PSBT was induced with 1 mM IPTG after 3 h. After 1 h of induction, cells were harvested and biotinylated proteins were identified with the ECL detection system (Fig. 9). While the K, L, and M fusions were biotinylated approximately equally in arabinose and in glucose medium, the H and J fusions were biotinylated only when there was a depletion of SecE in M63-glucose medium. The H fusion, which runs very close to a background band in this gel, was biotinylated to a somewhat lesser extent than the J fusion.

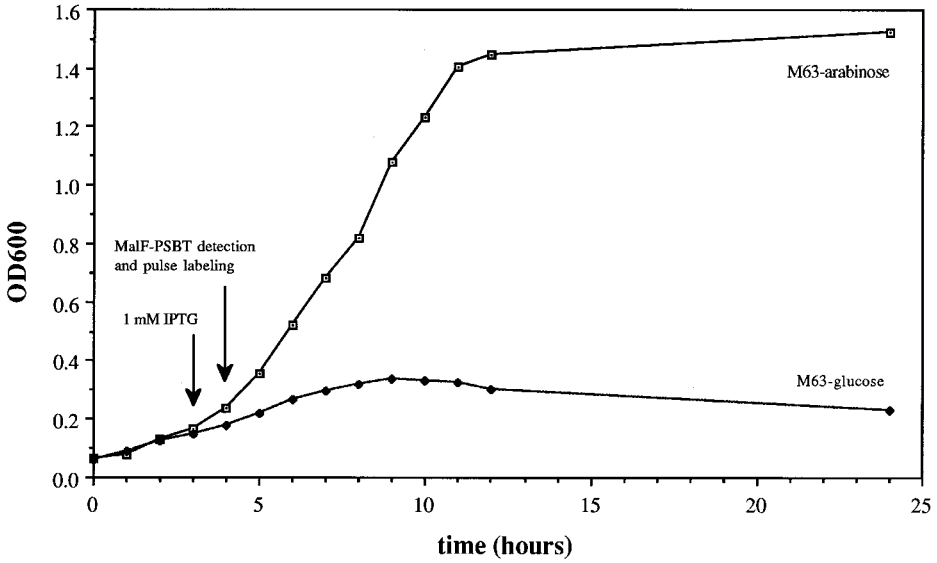

FIG. 7. Growth curves of strain GJ117/λGJ78.J with (M63-glucose) and without (M63-arabinose) SecE depletion. Cells grown overnight in NZ medium with 0.2% glucose were diluted 1:100 into M63 minimal medium with 0.2% glucose or 0.2% arabinose at time zero. Addition of IPTG for MalF-PSBT induction and the collection of samples for subsequent experiments are indicated. OD600, optical density at 600 nm.

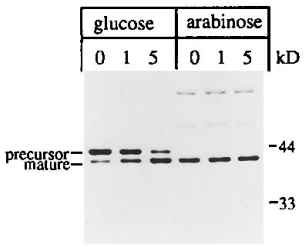

FIG. 8. Production of MBP from strain GJ117/λGJ78.J with (M63 medium with 0.2% glucose) and without (M63 medium with 0.2% arabinose) depletion of SecE for 4 h. Samples were subjected to [^{35}S]methionine labeling with pulse (lane 0) and 1- and 5-min chase (lanes 1 and 5) time points. Precursor and mature forms of MBP are indicated.

DISCUSSION

Gene fusion systems have been used to study problems in protein secretion and in membrane protein topology and assembly. In these studies, the usual approach is to determine whether, under steady-state conditions, the fusion tag protein has been exported across the cytoplasmic membrane. These approaches can miss more subtle effects on protein translocation, in which the polypeptide chain does cross the membrane but, under certain conditions, more slowly than in the wild-type situation. Our results suggest that such subtle effects can be detected by using a biotinylatable domain as a fusion tag. When the export of the fusion protein is slowed down, there is increased opportunity for biotinylation before the protein crosses the inner membrane. The studies with the two periplasmic proteins, MBP and AP, are consistent with previous findings on the effects of *sec* mutants on their export and the slow posttranslational export that takes place in *sec* mutant backgrounds.

The sensitivity of the biotinylation domain fusion approach has allowed us to detect effects on the membrane insertion of the MalF protein that have not been previously observed. While in a previous study there was no decrease in export of AP in periplasmic MalF-AP fusions as a result of *sec* mutants, we detect biotinylation of analogous PSBT fusion proteins under conditions of SecE depletion or inhibition of SecA. These results, along with those of Traxler and Murphy (44), indicate that the insertion of MalF into the cytoplasmic membrane does utilize the *sec* machinery. The failure to observe these effects previously (26) may have been due to both the lesser sensitivity of the assay system and the strength of the MalF export signals. That is, stronger *sec* mutant alleles, such as the SecE-depleted strain used here, may be necessary to observe effects on insertion of membrane proteins with highly hydrophobic export signals. These proteins may have higher affinity for the Sec machinery than periplasmic proteins. Thus, most or all membrane proteins may utilize the Sec machinery for their insertion.

Protein secretion. Our MBP-PSBT and AP-PSBT fusions are biotinylated only when their secretion is slowed down (Fig. 2). Inhibiting the function of SecB, a cytoplasmic protein, SecA, a peripheral membrane protein, and SecD and SecF, integral membrane proteins results in increased biotinylation of PSBT. This rate competition is not unique to the *P. shermanii* biotinylation domain. In an earlier study, Reed and Cronan (35) showed that a mutation in one secretion gene, *secB*, can increase the biotinylation of AP-KPBT. An alternative explanation for these results, that the rate of secretion is unimportant and the PSBT domain is somehow sequestered in a wild-type secretion complex and more accessible to biotin ligase in a *sec* mutant, seems unlikely. We tested three classes of Sec proteins, which would affect different parts of the secretion pathway, and found similar phenotypes in all cases. The observation that overproduction of biotin ligase results in the biotinylation of MBP-PSBT further supports the notion that there is a competition between biotinylation and export of these fusion proteins.

Membrane protein topology. A rate competition between biotinylation and translocation also occurs when PSBT is fused to the inner membrane protein MalF. Six PSBT fusion proteins with fusion joints that lie in cytoplasmic domains of MalF are biotinylated. Four of five fusions with fusion joints in periplasmic domains were not biotinylated. The Q fusion, although it may be produced at a lower level (Fig. 3A), is clearly not biotinylated (Fig. 3B). Thus, we presume that with these fusions, the PSBT domain is exported rapidly enough that it cannot be biotinylated. The only exception to this biotinylation pattern was the N fusion PSBT domain, which was biotinylated and also exported to the periplasm. One explanation for this latter result is that the third periplasmic loop of MalF inserts more slowly than the second and fourth periplasmic loops. This explanation is at odds with the results of Traxler et al. (43), who showed by proteolysis of MalF-AP fusions at positions J, N, and Q that the fourth periplasmic loop inserts more slowly than the second and the third loops. Perhaps these differences in results indicate that an important MalF topogenic signal has been truncated in the N and Q fusions. Then, the rate of insertion might be partly determined by the protein sequence, either PSBT or AP, that replaces the truncated domain.

Our results are markedly different from those of Zen et al. (50), who found that only two of six LacY-KPBT fusions were biotinylated in a manner consistent with the known topology of LacY. The two different biotinylation domains used in these experiments are unlikely to be the cause of the discrepancy. PSBT and KPBT have very similar sequences (38), are recognized by the same highly specific *E. coli* enzyme, and behave similarly when fused C terminally to *E. coli* periplasmic proteins. Instead, the difference may result from the fact that the LacY-KPBT fusions are not C-terminal deletions of LacY but instead have KPBT and a factor Xa protease site inserted into the middle of the protein. *K. pneumoniae* oxaloacetate decarboxylase, like *P. shermanii* transcarboxylase and most other biotin-containing proteins, is biotinylated on a highly conserved C-terminal domain (14). Biotinylation of *P. shermanii* transcarboxylase, when the protein is expressed in *E. coli*, is sensitive to small C-terminal changes (29). Thus, the LacY-

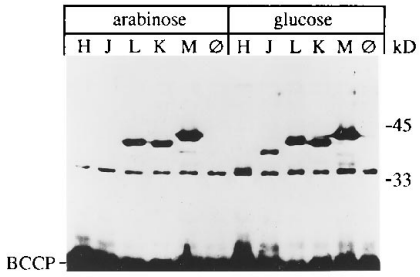

FIG. 9. Strain GJ117/λGJ61 (control; lane ø) and strains GJ117/λGJ78.H, -J, -K, -L, and -M with (0.2% glucose) and without (0.2% arabinose) depletion of SecE for 4 h. MalF-PSBT was visualized with streptavidin-horseradish peroxidase and chemiluminescence detection. The MalF-PSBT H and J fusions are biotinylated only after SecE depletion. The H fusion protein is visible just barely above a background band, which is present in all lanes. This band appears in some strains when biotinylated proteins are detected by chemiluminescence but is not visible with [^{3}H]biotin labeling of these cells.

KPBT fusions, although they contain the entire target site, may not all be properly biotinylated because KPBT is C-terminally constrained.

The AP part of the MalF-AP K and L fusions, even though fused to the cytoplasmic end of a MalF membrane-spanning segment, is slowly exported into the periplasm (7, 43). As a result, these two fusions have a much higher phosphatase activity than the M fusion, which is in the same cytoplasmic loop (8). In contrast, the K, L, and M MalF-PSBT fusions all behave the same; they are all biotinylated, and the biotinylated domain remains in the cytoplasm of the cell (Fig. 4). These results are analogous to those found with MalF–β-lactamase fusions. In those cases, fusions at positions K, L, and M all showed a low level of β-lactamase activity, consistent with the β-lactamase being on the cytoplasmic side of the membrane (34). Both β-lactamase, a normally periplasmic protein, and biotinylated PSBT (reference 35 and results presented here) are capable of being translocated across the cytoplasmic membrane if given a sufficiently strong secretion signal. Thus, the inconsistent localization of the MalF-AP K and L fusions is probably a function of the AP moiety. Perhaps there are internal secretion signals in AP, or the protein folds slowly enough in the cytoplasm that the unusually long transmembrane domain preceding the K and L fusions can act as a weak signal sequence. AP simply requires a stronger signal for cytoplasmic localization than either PSBT or β-lactamase. In the case of MalF-AP fusions, this signal consists of the positively charged amino acids that are present in the M fusion but not in the K and L fusions (7).

To ensure that the biotinylated forms of MalF-PSBT are not merely an insignificant fraction of the total MalF-PSBT produced by the cells, we performed calculations to estimate the percentage of protein that is being biotinylated. The periplasmic MalF-AP fusions of Boyd et al. (8), which are produced from the *tac* promoter in a chromosomally inserted construct, produce an average of 27 Miller units of AP activity. From this AP activity and the known activity of purified *E. coli* AP (purchased from Sigma), we calculate that there are approximately 650 molecules of MalF-AP produced in these strains under steady-state conditions. The MalF-PSBT K and M fusions seen in Fig. 6 are in a similar strain under the *tac* promoter on the chromosome. The amount of biotinylated MalF-PSBT is somewhat greater than the amount of BCCP. Since there are roughly 100 to 1,000 molecules of BCCP per cell, this is an indication that a significant portion of the cytoplasmic MalF-PSBT fusions are being biotinylated. These results are in agreement with an earlier observation that about 25% the 1.3S subunit of *P. shermanii* transcarboxylase, from which PSBT is derived, is biotinylated when the protein is expressed from a plasmid in *E. coli* (40). The actual level of biotinylation of any construct expressed in *E. coli* will depend on a number of factors, including the growth rate of the cells and the amount of biotin domain protein that is being produced.

The use of PSBT fusions adds a new approach to analyzing membrane protein topology. While each of the fusion systems used before, AP, β-lactamase, and β-galactosidase, allows the proposal of detailed models of topology, occasional anomalies have been observed with all of them (34). The added feature of PSBT fusions is that one may be able to detect the end result (biotinylation) of a reduction in the rate of protein translocation, as with the K and L fusions. The disadvantage of this sensitivity is that weaker export signals (such as those in the N fusion), which in the context of the intact membrane protein are perfectly satisfactory, may slow down translocation enough to allow some biotinylation. One possible solution to this problem would be to put the *birA* gene, which encodes biotin ligase, under an inducible promoter. It would then be possible to produce just enough biotin ligase that the cytoplasmic MalF-PSBT fusions are biotinylated but the periplasmic MalF-PSBT N fusion is not biotinylated.

Dependence of membrane protein insertion on the Sec machinery. We used MalF-PSBT fusions to detect inhibition of membrane insertion of the MalF protein under conditions such that the cell's secretion machinery is impaired. Biotinylation of the MalF-PSBT H and J fusions is clearly caused by the inhibition of SecA with sodium azide and the depletion of SecE. Sodium azide had the same rapid effect on membrane insertion that it is known to have on protein secretion (31), and at least some of the biotinylated domains were exported into the periplasm. The SecE result is probably not a secondary effect due to long-term depletion of SecE, since the cells used in this experiment were only beginning to be depleted of SecE, were still growing at close to wild-type rates, and showed only a partial MBP secretion defect.

After dilution of strain GJ117 into glucose medium, there is a progressive depletion of SecE and a progressive increase in the protein translocation defect. If the induction of MalF-PSBT fusions was started an hour earlier after the onset of SecE depletion and samples were collected at the 3-h time point, the level of biotinylation of the H and J fusions (data not shown) was lower than that seen in Fig. 9. At the 3-h time point, the cells did not take up the [^{35}S]methionine well enough to give a clear picture of the defect in MBP secretion. If SecE depletion is continued for several hours, there eventually is a complete block of MBP secretion, as measured by signal sequence processing (44). Thus, the most likely explanation for the fact that the H and J bands in Fig. 9 are less intense than the K, L, and M bands is that there is still a partially functional secretion apparatus and thus only a partial membrane insertion defect. This type of comparison cannot be made with the SecA inhibition experiment (Fig. 6), since the cytoplasmic and periplasmic MalF-PSBT fusions were not being biotinylated for the same amount of time.

It is likely that the other known Sec proteins are also involved in membrane protein insertion. Preliminary results show a slight insertion defect due to depletion of SecD and SecF. Strain JP352, which has *secD* and *secF* under control of the *araBAD* promoter (15), was used to express MalF-PSBT fusions from lysogenic λ phage. After 1:100 dilution into NZ medium supplemented with either 0.2% glucose or 0.2% arabinose and 8 h of growth at 30°C, the MalF-PSBT J fusion was biotinylated only under the SecD- and SecF-depleting conditions (20a). The level of biotinylation was lower than in the SecA and SecE experiments, and no biotinylation of the MalF-PSBT H fusion was detected. The biotinylation of periplasmic MalF-PSBT seen under these conditions correlates well with the effects seen on the secretion of periplasmic proteins in other assay systems. SecE depletion has a stronger effect on secretion than azide inhibition, which, in turn, results in a greater secretion defect than SecD and SecF depletion.

An alternate explanation for our results, that the insertion of the second periplasmic domain of MalF is Sec independent and that what we are observing is merely Sec dependence of the transfer of PSBT, seems unlikely. Both our results and those presented previously (35) show that PSBT per se did not inhibit secretion when it was fused to a periplasmic protein. In addition, results of two other groups (38, 44) have also shown a Sec dependence for the insertion of the second periplasmic loop of MalF. Therefore, we think that biotinylation of the PSBT domain is a valid indicator of impaired membrane protein insertion.

Although it is clear that the *E. coli* secretion apparatus plays

a role in the insertion of MalF, we cannot rule out the possibility that additional proteins needed for membrane insertion have not yet been discovered. While several different genetic selections have been used to detect protein secretion mutations (39), there is not yet a good screen or selection for mutations in the membrane protein insertion machinery. A screen based on the biotinylation of MalF-PSBT is another approach for detecting membrane insertion defects. The *bioA-lacZ* fusion in strain GJ55 is derepressed when there is depletion of the biotin in the cell (4, 13). Such a depletion occurs when a cytoplasmic MalF-PSBT fusion becomes biotinylated. On M63-glucose plates with 41 nM biotin, 1 mM IPTG, and 20 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), strains GJ55/pGJ78.H, -I, and -J make white colonies and strains GJ55/pGJ78.K, -L, and -M make blue colonies (20a). Mutations of *E. coli* that slow down the insertion rate of the H, I, and J fusions should result in a blue colony color or perhaps better growth on lactose minimal medium for these strains. Initial screens of this kind with X-Gal indicator plates resulted in a high level of background mutations, but it may be possible to surmount this problem by using a less sensitive detection medium such as MacConkey-lactose agar.

Concluding remarks. Biotin-targeting domain fusions will be a useful tool for studying protein secretion and membrane protein insertion. These fusions have several advantages over genetic fusions that have been used previously. (i) The PSBT fragment is fairly small (76 amino acids) and therefore less likely to affect the protein to which it is attached. (ii) The biotinylated domain is easily and specifically detected with [^{3}H] biotin labeling or with streptavidin-horseradish peroxidase and a chemiluminescence detection system. (iii) Commercially available avidin beads and columns can be used to purify biotinylated proteins with high specificity. (iv) Unlike assays possible with other types of fusion proteins, biotinylation can provide an easy assay for the detection of altered rates of membrane translocation of a particular protein domain.

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