JOURNAL OF BACTERIOLOGY, Jan. 2001, p. 604–610 Vol. 183, No. 2
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.2.604–610.2001

Specificity of Signal Peptide Recognition in Tat-Dependent Bacterial Protein Translocation

NATASCHA BLAUDECK,[1] GEORG A. SPRENGER,[1] ROLAND FREUDL,[1] AND THOMAS WIEGERT[2]*

Institut für Biotechnologie 1, Forschungszentrum Jülich GmbH, D-52425 Jülich,[1] and Lehrstuhl für Genetik, Universität Bayreuth, D-95440 Bayreuth,[2] Germany

Received 24 August 2000/Accepted 25 October 2000

The bacterial twin arginine translocation (Tat) pathway translocates across the cytoplasmic membrane folded proteins which, in most cases, contain a tightly bound cofactor. Specific amino-terminal signal peptides that exhibit a conserved amino acid consensus motif, S/T-R-R-X-F-L-K, direct these proteins to the Tat translocon. The glucose-fructose oxidoreductase (GFOR) of *Zymomonas mobilis* is a periplasmic enzyme with tightly bound NADP as a cofactor. It is synthesized as a cytoplasmic precursor with an amino-terminal signal peptide that shows all of the characteristics of a typical twin arginine signal peptide. However, GFOR is not exported to the periplasm when expressed in the heterologous host *Escherichia coli*, and enzymatically active pre-GFOR is found in the cytoplasm. A precise replacement of the pre-GFOR signal peptide by an authentic *E. coli* Tat signal peptide, which is derived from pre-trimethylamine *N*-oxide (TMAO) reductase (TorA), allowed export of GFOR, together with its bound cofactor, to the *E. coli* periplasm. This export was inhibited by carbonyl cyanide *m*-chlorophenylhydrazone, but not by sodium azide, and was blocked in *E. coli tatC* and *tatAE* mutant strains, showing that membrane translocation of the TorA-GFOR fusion protein occurred via the Tat pathway and not via the Sec pathway. Furthermore, tight cofactor binding (and therefore correct folding) was found to be a prerequisite for proper translocation of the fusion protein. These results strongly suggest that Tat signal peptides are not universally recognized by different Tat translocases, implying that the signal peptides of Tat-dependent precursor proteins are optimally adapted only to their cognate export apparatus. Such a situation is in marked contrast to the situation that is known to exist for Sec-dependent protein translocation.

Besides the well-characterized Sec system, which is used for the translocation of the majority of exported proteins across the cytoplasmic membrane (8, 10, 28), another export pathway is existent in bacteria, the so-called twin-arginine translocation (Tat) pathway (for a recent review, see reference 2). There is strong evidence that, in contrast to the Sec pathway, the twin-arginine translocase exclusively exports across the cytoplasmic membrane folded proteins which, in most cases, contain a bound cofactor (17, 29–31, 41, 46). Precursor proteins that are exported via the Tat pathway possess amino-terminal signal peptides which are substantially longer than typical Sec signal peptides and contain an S/T-R-R-X-F-L-K consensus motif in their amino-terminal region (1, 2). The two arginine residues of the conserved motif are of crucial importance, and mutagenesis of one or both of these residues severely affects membrane translocation of the corresponding mutant precursor proteins (7, 9, 13, 36). Furthermore, the central hydrophobic core (h region) of Tat signal peptides is less hydrophobic than the h region of Sec signal peptides (7). In the more polar carboxy-terminal region that precedes the processing site, basic amino acid residues are frequently observed in Tat signal peptides, whereas signal peptides of the Sec pathway show a strong bias against such residues near the signal peptidase cleavage site (2, 3, 38).

Four integral cytoplasmic membrane proteins, encoded by *tatA*, *tatB*, *tatC*, and *tatE*, have been shown to be involved in the Tat translocation process in *Escherichia coli*, although very little is known about their function (2). The bacterial Tat pathway is closely related to the ΔpH-dependent protein import pathway of the plant chloroplast thylakoid membrane (4, 34, 35). Their common phylogenetic origin is stressed by the fact that bacterial Tat substrates can be translocated by the ΔpH pathway and that their signal peptides are interchangeable (14, 24, 42).

The glucose-fructose oxidoreductase (GFOR) of the gram-negative bacterium *Z. mobilis* exhibits the typical characteristics of a Tat substrate. The homotetrameric protein contains four tightly bound NADP molecules as a cofactor and is found in the periplasm in a soluble form (20, 21). GFOR is synthesized as a cytoplasmic precursor (pre-GFOR) with an extraordinary long signal sequence of 52 amino acid residues containing the typical twin-arginine consensus motif (43). The replacement of one or both of the arginine residues by lysine prevents export of the corresponding pre-GFOR proteins (15). Furthermore, the export kinetics of mutant forms of pre-GFOR which have substantially decreased affinities for the NADP cofactor is significantly slower than that of the wild-type enzyme, suggesting that cytoplasmic cofactor insertion and tight folding are prerequisites for Tat-dependent membrane translocation of GFOR (15). Moreover, it has been shown that pre-GFOR can be translocated in vitro into isolated plant thylakoids in a ΔpH-dependent manner (14).

In previous experiments we have observed that pre-GFOR is not exported to the periplasm of the heterologous host *E. coli*, although cofactor insertion and formation of correctly folded and enzymatically active pre-GFOR take place in the cytosol (44). These results suggest that the foreign GFOR precursor

* Corresponding author. Mailing address: Lehrstuhl für Genetik, Universität Bayreuth, Universitätsstrasse 30, D-95440 Bayreuth, Germany. Phone: 49-921-552724. Fax: 49-921-552710. E-mail: thomas.wiegert@uni-bayreuth.de.

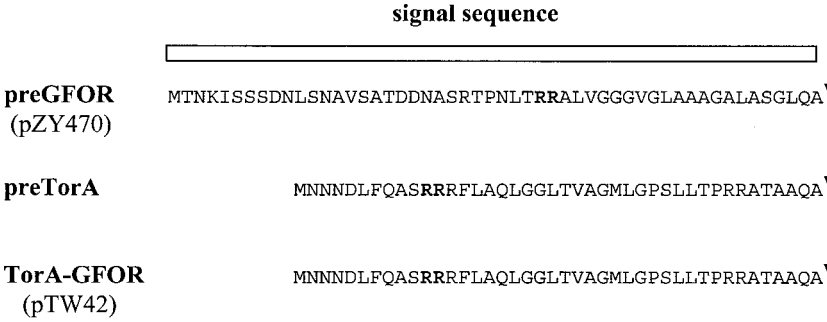

FIG. 1. Signal peptide and early mature region of pre-GFOR, pre-TorA, and the TorA-GFOR fusion protein. Processing sites are indicated by inverted triangles; twin arginine residues are in boldface.

protein is not recognized by the *E. coli* Tat machinery. Replacement of the genuine GFOR signal sequence by the OmpA signal peptide, which is a typical Sec signal peptide, results in efficient Sec-dependent export of the corresponding hybrid precursor without its cofactor and in the subsequent degradation of the translocated mature part in the periplasm by proteases (44).

In the present work, we addressed the question of why *Z. mobilis* pre-GFOR is not exported by the *E. coli* Tat pathway, despite the fact that it is an efficient Tat substrate in its original host. There are several possible explanations for the failure of pre-GFOR to be exported in *E. coli*: (i) *E. coli* may lack certain accessory protein factors that are necessary for GFOR export and that are present in pea thylakoids and *Z. mobilis*, (ii) the folded structure of GFOR may not be compatible with the *E. coli* Tat machinery, or (iii) the GFOR signal peptide may not be recognized by the *E. coli* Tat apparatus. Here, we show that a precise replacement of the GFOR signal peptide by an authentic *E. coli* Tat signal peptide is sufficient to promote the Tat-dependent export of GFOR in *E. coli*. These results strongly suggest that there exists a recognition event between Tat signal peptides and one or more components of the Tat export apparatus that goes beyond the recognition of the conserved general features found in all Tat signal peptides and that this recognition event seems to have a species-specific component. Our findings imply that the signal peptides of Tat-dependent precursor proteins are optimally adapted only to their cognate export apparatus, which is in marked contrast to the situation that is known for Sec-dependent protein translocation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* K-12 strain JM109 (47) was used for standard cloning procedures. *E. coli* strains MC4100Δ*tatAE* (JARV15) and MC4100Δ*tatC* (B1LK0) are derivatives of MC4100 (6) with deletions in the respective *tat* genes (4, 30). *E. coli* cells were grown aerobically in Luria-Bertani medium (23) or in mineral salts medium (37) with 0.4% glycerol as a carbon source and ampicillin at a concentration of 100 mg/liter, as required.

A PCR megaprimer method was used to replace the genuine GFOR signal sequence coding region in plasmid pZY470 by introducing unique *Bgl*II and *Eco*47-III restriction sites essentially as described earlier (43). A first round of PCR, with primer 5′-GCTGGCACCAGCAGGCGTCGCAGCGCTCATAG ATCTTGTTTCTTTCTTAACTAACCAACA-3′ and the pUC/M13 reverse-sequencing primer together with a 471-bp *Pvu*II fragment of pZY470 (43), yielded a 258-bp megaprimer. The use of this megaprimer, the M13/pUC universal and reverse-sequencing primers, and a 1.5-kb *Pvu*II fragment of pZY470 in a second round of PCR gave a 1.5-kb fragment that was digested with *Eco*RI and *Pst*I. A 222-bp *Eco*RI-*Pst*I fragment was ligated to the 3.8-kb *Eco*RI-*Pst*I fragment of pZY470. The correctness of the resulting plasmid pTW40 was verified by DNA sequencing.

The coding region of the TorA signal peptide was cloned by PCR with chromosomal DNA of *E. coli* MC4100 as the template and oligonucleotides torA-5′ (5′-GGCCATAGATCTATGAACAATAACGATCTCTTTCAGGCA-3′) and torA-3′ (5′-GGCCATCAGCTGCGCCGCAGTCGCACGTCGCGGCGT-3′) as primers. The 152-bp *torA* PCR fragment was restricted with *Bgl*II and *Pvu*II and ligated to the *Bgl*II–*Eco*47-III fragment of pTW40, resulting in plasmid pTW42 (encoding the TorA signal sequence fused to GFOR) (Fig. 1). The correctness of the fusion was verified by DNA sequencing.

Plasmid pTW43, with point mutations S116D, K121A, K123Q, and I124K in the NADP binding site, was constructed by replacing a 289-bp *Pst*I-*Sph*I fragment of pTW42 with the respective fragment of plasmid pZY470/S116D/K121A/K123Q/I124K (45).

Pulse-chase experiments, preparation of spheroplasts, and tryptic digestion. Pulse-chase experiments were performed as described earlier (44). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was dissolved in ethanol at a concentration of 10 mM and added to samples where indicated to give a final concentration of 0.1 mM. In a control experiment using the same amount of ethanol without CCCP, the processing kinetics were the same as in the absence of ethanol (data not shown).

For spheroplast formation, cells were grown in mineral salts medium to an optical density at 578 nm of 1. A 1.5-ml aliquot of the culture was withdrawn and incubated in a 37°C water bath for 5 min. Isopropyl-1-thio-ß-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM in order to induce the expression of the gene encoding pre-GFOR or TorA-GFOR which were cloned under the regulatory control of the *lac* promoter-operator system. After 1 min, the cells were labeled with [^{35}S]methionine (500 μCi), and after 1 min of labeling time, chase solution was added (1 mg of nonradioactive methionine/ml, 2 mg of chloramphenicol/ml [final concentrations]). After a 5- to 60-min chase, cells were pelleted by centrifugation at 4°C. The cells were resuspended in 1.8 ml of ice-cold 30 mM Tris-HCl–20% sucrose (pH 8.0). EDTA and lysozyme were added to final concentrations of 1 mM and 100 μg/ml, respectively. After incubation on ice for 1 h, the sample was divided into three aliquots. The first aliquot was left untreated, while the second and third received trypsin (1 mg/ml final concentration). In addition, the third aliquot was sonicated in an ice bath for cell disruption (Branson Sonifier B15; 50% duty cycle; output control, 3.5, three 10-pulse sonications with 30-s interruptions). After incubation on ice for 1 h, a trypsin inhibitor (5 mg/ml final concentration) was added to all three aliquots; this was followed by a 10-min incubation on ice. Finally, the first aliquot received trypsin (1 mg/ml final concentration), and after 5 min of further incubation, trichloroacetic acid was added to each aliquot (10% final concentration).

Proteins were precipitated on ice for 1 h and prepared for immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorography as described earlier (44). For immunoprecipitation, each aliquot was divided into three parts and antibodies against GFOR (21), OmpA (22), and DnaK (5) were added, respectively.

RESULTS

The signal peptide of the *E. coli* TMAO reductase (TorA) precursor, but not the authentic GFOR signal peptide, allows translocation of the *Z. mobilis* GFOR protein across the *E. coli* plasma membrane. Previously, we have observed that the pre-

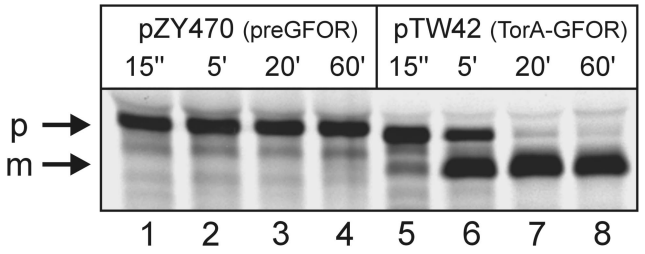

FIG. 2. Processing of pre-GFOR and TorA-GFOR in *E. coli* JM109. *E. coli* JM109 carrying either plasmid pZY470, encoding wild-type pre-GFOR (lanes 1 to 4), or plasmid pTW42, encoding the TorA-GFOR fusion protein (lanes 5 to 8), was grown in mineral salts medium to early logarithmic phase and labeled for 1 min with [^{35}S]methionine, after which nonradioactive methionine was added. Samples were withdrawn at chase times of 15 s and 5, 20, and 60 min and subjected to immunoprecipitation with antiserum against GFOR, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. p, precursor form; m, mature protein.

GFOR of *Z. mobilis* is not translocated across the *E. coli* plasma membrane despite the fact that the GFOR signal peptide mediates efficient translocation of the enzyme, together with its cofactor, into the periplasm of *Z. mobilis* (43, 44). To test whether the nature of the GFOR signal peptide is responsible for the lack of export of this protein in *E. coli*, we constructed a hybrid precursor protein containing the mature GFOR protein fused to an *E. coli* Tat signal peptide. To do so, the GFOR signal peptide was precisely replaced by the signal prepeptide of the *E. coli* pre-trimethylamine *N*-oxide (TMAO) reductase (pre-TorA), an enzyme which is known to be efficiently translocated by the *E. coli* Tat translocase (29) (Fig. 1). Expression of the gene encoding the TorA-GFOR hybrid protein in *E. coli* JM109 resulted in GFOR enzyme activities that were similar to the activities which were found when the wild-type *gfo* gene was expressed in *E. coli* (data not shown). Hence, correct folding of the GFOR protein and correct insertion of the NADP cofactor do occur in both cases. To analyze whether the signal peptide replacement has an effect on the export behavior of GFOR, pulse-chase experiments were performed. As shown in Fig. 2, lanes 1 to 4, and as described earlier (44), no processing of the wild-type pre-GFOR was observed even after a 60-min chase, confirming that the pre-GFOR was not translocated by the *E. coli* Tat machinery. In contrast, the TorA-GFOR fusion protein was completely processed to mature GFOR during a 20-min chase (Fig. 2, lanes 5 to 8), and this processing occurred with relatively slow kinetics, similar to the pre-GFOR export kinetics in *Z. mobilis* (43).

Since cleavage of the signal peptide is an indication of membrane translocation but does not necessarily prove that export of the protein across the plasma membrane has occurred, we examined whether the processing of the TorA-GFOR fusion protein is accompanied by export of mature GFOR protein into the *E. coli* periplasm. Cells expressing the genes for wild-type GFOR or the TorA-GFOR hybrid protein were labeled with [^{35}S]methionine, converted to spheroplasts, and treated with trypsin. Samples were taken and immunoprecipitated with anti-GFOR and, as a control, anti-OmpA and anti-DnaK antibodies as outlined in Materials and Methods. As shown in Fig. 3, the processed form of the TorA-GFOR fusion protein was clearly susceptible to tryptic digestion in spheroplasts and was degraded to a smaller tryptic GFOR fragment, whereas the unprocessed form remained protease resistant (Fig. 3A; compare lanes 1 and 2). When the spheroplasts were broken up by ultrasonication, the unprocessed TorA-GFOR protein also became trypsin sensitive (Fig. 3A, lane 3). In contrast, the wild-type pre-GFOR protein, with its genuine signal sequence, and some smaller GFOR-derived protein bands, which most

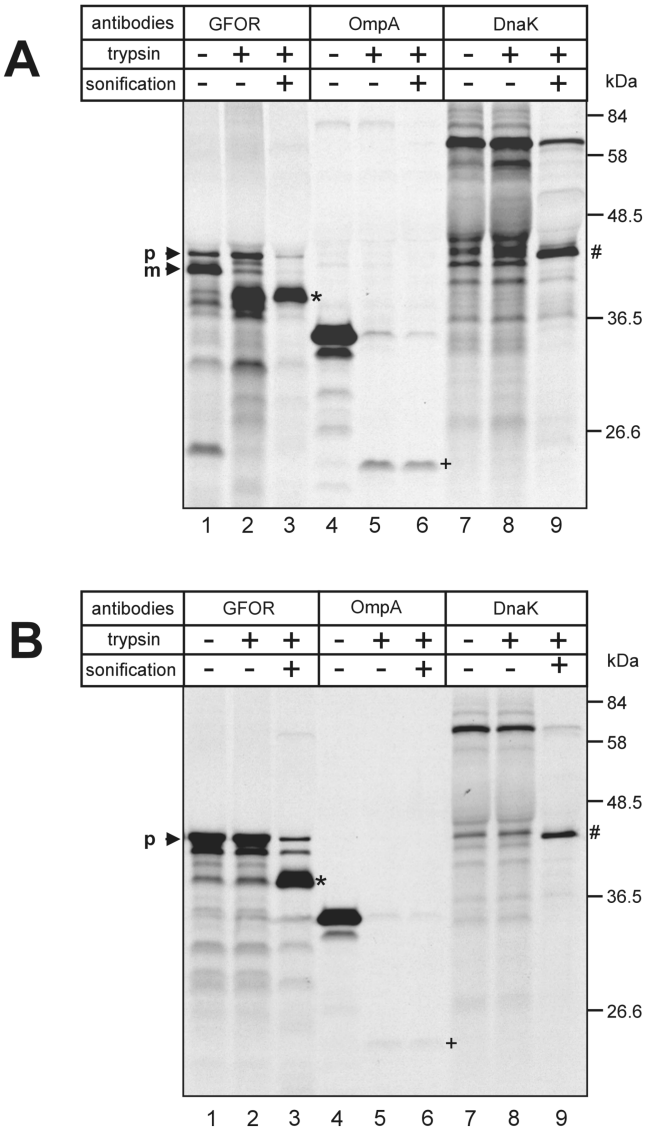

FIG. 3. Trypsin treatment of spheroplasts. *E. coli* JM109 carrying either plasmid pTW42, encoding the TorA-GFOR fusion protein (A), or plasmid pZY470, encoding the wild-type pre-GFOR (B), was labeled with [^{35}S]methionine for 1 min. After a 5-min chase, cells were converted to spheroplasts and divided into three aliquots. Trypsin was added where indicated (+) to digest periplasmic proteins. As a control, cells in one aliquot were disrupted by ultrasonication after trypsin addition. After trypsin treatment, each aliquot was divided into three parts and subjected to immunoprecipitation with antisera against GFOR (lanes 1 to 3), OmpA (lanes 4 to 6), and DnaK (lanes 7 to 9). p, precursor form of TorA-GFOR (A) or pre-GFOR (B); m, mature GFOR. Positions of tryptic fragments are indicated for GFOR (*), OmpA (+), and DnaK (#). The numbers at the right margin are positions of molecular mass markers.

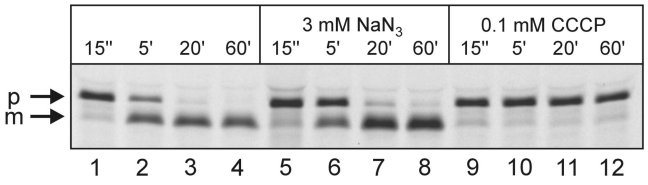

FIG. 4. Effect of CCCP and sodium azide (NaN_3) on processing of TorA-GFOR. Pulse-chase experiments were performed with *E. coli* JM109 carrying plasmid pTW42, encoding the TorA-GFOR fusion protein, as described in the legend to Fig. 2. Sodium azide (3 mM final concentration) (lanes 5 to 8) or CCCP (0.1 mM final concentration) (lanes 9 to 12) was added to the cultures prior to the chase. Samples were taken at the indicated chase times and submitted to immunoprecipitation with GFOR-specific antibodies. p, precursor form; m, mature protein.

likely represented cytoplasmic degradation products, were totally resistant to tryptic digestion in *E. coli* spheroplasts (Fig. 3B; compare lanes 1 and 2). The reliability of the method was verified by using the outer membrane protein OmpA and the cytoplasmic protein DnaK as internal controls. In spheroplasts, the periplasmic part of OmpA is degraded by trypsin, resulting in a protease-resistant fragment of 24 kDa located in the outer membrane (11, 48). DnaK is digested by trypsin to a resistant fragment of about 46 kDa (5). Because it is located in the cytoplasm, DnaK should not be attacked in spheroplasts.

As expected, the periplasmic part of the OmpA protein was degraded by trypsin, yielding a fragment of 24 kDa that corresponds to the membrane part of OmpA (Fig. 3; compare lanes 4 and 5), while the cytoplasmic DnaK was not converted to its tryptic fragment (Fig. 3; compare lanes 7 and 8) unless the spheroplasts were broken by ultrasonication (Fig. 3, lanes 9). These results clearly show that GFOR is exported to the *E. coli* periplasm when the GFOR signal peptide is replaced by the TorA signal sequence.

The tryptic GFOR fragment of about 38 kDa is formed only when the cofactor NADP is bound to the GFOR apoprotein. Without the cofactor, GFOR is completely degraded by trypsin (44). Since a fragment of the same size was observed upon trypsin treatment of spheroplasts expressing the TorA-GFOR fusion protein, and since obviously no NADP is present in the *E. coli* periplasm (44), we conclude that with the aid of the TorA signal peptide, GFOR is exported in a correctly folded state with its bound NADP cofactor.

Export of the TorA-GFOR fusion protein can be blocked by CCCP but not by the SecA inhibitor sodium azide. The protonophore CCCP inhibits both the Tat- and the Sec-dependent translocation pathways, showing that both processes require an intact membrane potential (7, 29, 33). In contrast, sodium azide, which severely inhibits Sec-dependent protein export by interfering with the translocation-ATPase activity of the SecA protein (27), only slightly affects Tat translocation (29). To analyze whether the observed export of the TorA-GFOR hybrid protein occurs via the Tat or the Sec pathway, sodium azide (3 mM) or CCCP (0.1 mM) was added in the pulse-chase experiments prior to the chase. As shown in Fig. 4, the processing of the TorA-GFOR fusion protein was not inhibited by sodium azide. In contrast, the processing was completely blocked by the addition of 0.1 mM CCCP. In a parallel control experiment, processing of chromosomally encoded OmpA, which is exported in a Sec-dependent manner, was inhibited both by sodium azide and by CCCP (data not shown). The insensitivity of the processing of the TorA-GFOR fusion protein to azide is typical for a Tat-dependent precursor protein, indicating that export of TorA-GFOR is mediated by the Tat translocase.

Export of the TorA-GFOR fusion protein in *E. coli* is Tat dependent. For direct proof that membrane translocation of the TorA-GFOR fusion protein is in fact mediated by the Tat translocase, pulse-chase experiments were performed with mutant derivatives of *E. coli* MC4100 harboring a deletion of *tatAE* or *tatC* (4, 30) and, as a control, the isogenic wild-type strain. First of all, we noticed that processing of the TorA-GFOR fusion protein in the wild-type strain MC4100 was significantly slower than in *E. coli* JM109 (compare Fig. 5A, lanes 1 to 4, with Fig. 2, lanes 5 to 8); the reason for this is unknown. Nevertheless, we found that processing of the TorA-GFOR fusion protein was clearly affected in the *tatAE* (Fig. 5A, lanes 5 to 8) and *tatC* (Fig. 5A, lanes 9 to 12) mutant strains. However, a mature-sized form also appeared in the *tat* mutants after a 20-min chase. Quantification of the pulse-chase experiments showed that, after a 60-min chase, about 30% (*tatC* mutant) or 50% (*tatAE* mutant) of the immunoprecipitated GFOR protein was in a mature-sized form (Fig. 5B). Since we expected processing of Tat substrates to be completely inhibited in the *tatAE* and *tatC* mutants (4, 30), we thought that it might be possible that the mature-sized GFOR protein, which accumulates in the *tat* mutant strains, is a result of the degradation of the TorA-GFOR precursor by proteases in the cytosol, rather than being caused by export and subsequent processing of the signal peptide. If so, the respective mature-sized GFOR protein should be localized in the cytosol and therefore should be resistant to protease digestion in spheroplasts. To determine the localization of the mature-sized forms of the TorA-GFOR fusion protein in wild-type *E. coli* MC4100 and in the *tatAE* and *tatC* mutant strains, the corresponding cells were converted to spheroplasts after a 60-min chase and treated with trypsin as described above (Fig. 5C). In all experiments, the quality of the spheroplasts and the effectiveness of the tryptic digestions were verified again with OmpA and DnaK as controls (data not shown). Like the situation in *E. coli* JM109 (Fig. 3A, lanes 1 and 2), the mature-sized GFOR protein was also clearly sensitive to trypsin in spheroplasts derived from the MC4100 wild-type strain (Fig. 5C, upper panel), showing that this mature protein is localized in the periplasm and therefore is a result of membrane translocation and signal peptide processing. In contrast, the mature-sized forms which accumulate in the *tatAE* (Fig. 5C, middle panel) and the *tatC* (Fig. 5C, lower panel) mutant strains were completely resistant to tryptic digestion after spheroplast conversion of the corresponding cells, showing that these forms are localized in the cytosol. Cytosolic degradation of a TorA fusion protein in the case of blocked Tat-dependent export was also described in an earlier report; there, a TorA-LepA fusion protein was completely degraded in *tat* mutant strains or when the RR amino acid residues of the twin-arginine motif were mutated to KK (7). We assume that the TorA signal peptide of the hybrid TorA-GFOR protein is accessible to cytoplasmic proteolysis when interaction with Tat components is disturbed. Taken together, our results clearly show that membrane trans-

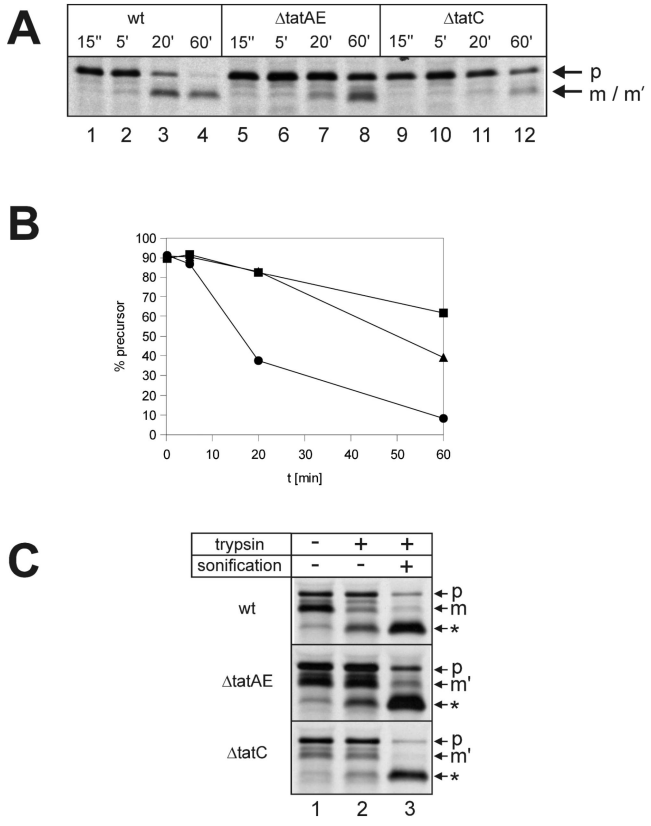

FIG. 5. Processing of TorA-GFOR in *E. coli tat* mutants and localization of GFOR gene products by trypsin treatment of spheroplasts. (A) Pulse-chase experiments with *E. coli* strains MC4100, MC4100 Δ*tatC*, and MC4100 Δ*tatAE*, containing plasmid pTW42, encoding the TorA-GFOR fusion protein, were performed as described in the legend to Fig. 2. (B) The bands of the gel in panel A were quantified using a PhosphorImager and the FragmeNT Analysis (version 1.1) software (Molecular Dynamics). The percentages of precursor present at the indicated chase times were calculated $[p/(p + m) \times 100$, where p is the amount of precursor and m is the amount of mature form]. Circles, MC4100 wild type; triangles, MC4100 *tatAE* mutant; squares, MC4100 *tatC* mutant. (C) To examine the subcellular localization of the different GFOR forms in *E. coli* MC4100, MC4100 Δ*tatAE*, and MC4100 Δ*tatC*, labeled cells were converted to spheroplasts and submitted to tryptic digestion as described in the legend to Fig. 3, with the exception that, due to the slower processing of TorA-GFOR in *E. coli* MC4100, cells were converted to spheroplasts after a 60-min chase. wt, wild type; p, precursor; m, mature GFOR; m′, mature-sized cytosolic GFOR fragment; ∗, tryptic fragment of GFOR.

location of the TorA-GFOR hybrid protein is mediated by the Tat export apparatus.

Tight binding of the NADP cofactor is essential for membrane translocation of the TorA-GFOR fusion protein. Previously, we have described various mutant derivatives of the GFOR protein which contain an alteration in one or more amino acid residues located in the NADP-binding Rossman fold and which are impaired in tight binding of the NADP cofactor (45). In addition, we demonstrated that these GFOR mutant proteins with reduced affinity for NADP lost the ability to oxidize glucose and reduce fructose but instead were able to exchange reduced cofactor (NADPH) for the oxidized form (NADP), therefore acting as glucose dehydrogenases (45). Since these mutant forms of GFOR still exhibit enzymatic activity, the overall three-dimensional structure should be intact. Interestingly, these mutant forms were not or were only very slowly processed when examined in pulse-chase experiments with *Z. mobilis* (15). This led us to the hypothesis that the *Z. mobilis* Tat pathway is able to recognize proper cofactor binding and folding prior to protein export. To test whether the Tat-dependent export of the TorA-GFOR fusion protein in *E. coli* also requires tight NADP binding, the point mutations S116D, K121A, K123Q, and I124K were introduced into the TorA-GFOR hybrid protein (plasmid pTW43). In pulse-chase experiments with *E. coli* MC4100, the TorA-GFOR mutant protein was nearly completely degraded during a 60-min chase (Fig. 6). Furthermore, no processed mature form accumulated during the chase, which is in sharp contrast to what was observed with the TorA-GFOR wild-type protein (compare Fig. 6, lanes 1 to 4, with Fig. 5A, lanes 1 to 4). Likewise, in the faster-processing *E. coli* strain JM109 as well, no processing of the TorA-GFOR cofactor-binding mutant protein to the mature form was detected in pulse-chase experiments and, also in this case, the mutant protein was degraded (data not shown). An identical behavior was found when the TorA-GFOR mutant protein was expressed in the *tatC* deletion strain (Fig. 6, lanes 5 to 8), showing that the observed degradation most probably had occurred in the cytosol and that point mutations S116D, K121A, K123Q, and I124K in the NADP-binding Rossman fold of GFOR, also in *E. coli*, severely impairs the Tat-dependent translocation. These results support the current view that correct folding and cofactor insertion are generally an important prerequisite for export of cofactor-containing proteins via the Tat pathway.

DISCUSSION

In the present work, we have shown that a precise replacement of the authentic signal peptide of the *Z. mobilis* GFOR precursor protein with a signal peptide derived from the *E. coli* Tat substrate TorA is sufficient to promote export of GFOR to the *E. coli* periplasm and that this export is mediated by the Tat translocase. These results clearly demonstrate that the mature part of GFOR is compatible with translocation by the *E. coli* Tat export apparatus and that the previously described export defect of wild-type pre-GFOR in *E. coli* is due to an incompatibility of the GFOR signal peptide with Tat-dependent protein translocation in *E. coli*.

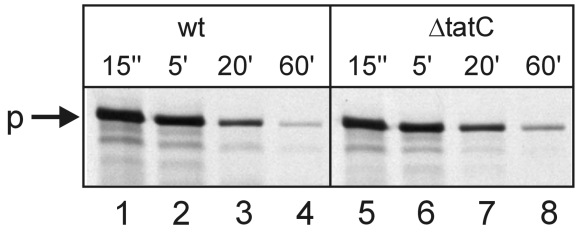

FIG. 6. Processing of a cofactor-binding-defective TorA-GFOR fusion protein. Pulse-chase experiments were performed, as described in the legend to Fig. 2, with *E. coli* strains MC4100 (lanes 1 to 4) and MC4100Δ*tatC* (lanes 5 to 8) carrying plasmid pTW43, encoding a mutant form of TorA-GFOR with point mutations in the NADP binding site that result in decreased cofactor binding affinity. wt, wild type; p, precursor form.

What is the nature of such an incompatibility? For cofactor-containing Tat substrates, it was proposed that their signal peptides might have distinct structural features, allowing specific protein-protein interactions with the mature protein and/or assembly factors (2), which ensure cofactor binding prior to export. In such a model, the signal peptide is sheltered either by the mature part of the apoprotein or by a special accessory protein until cofactor binding takes place (29). Because enzymatically active pre-GFOR is formed in the *E. coli* cytoplasm, the possibility that the GFOR signal peptide is not accessible to the Tat translocon due to improper folding of the mature part of GFOR or problems with cofactor incorporation can be excluded.

The GFOR signal peptide shows characteristics of a typical twin-arginine signal peptide. Although the twin-arginine motif T-R-R-A-L-V-G does not completely match the motif with the highest consensus of Tat signal peptides (S/T-R-R-X-F-L-K) (1), alignments of Tat signal sequences show that the F-L-K residues are more variable than the invariant R-R residues and that L-V-G residues at respective positions can be found in other Tat signal sequences (1, 7). Moreover, it was most recently shown that the F residue of the consensus (which is L in the GFOR signal peptide) can be functionally replaced by an L and that the K residue in the consensus (which is a G in the GFOR signal peptide) even retards Tat transport (36). In addition, the h region of the GFOR signal peptide, which contains several glycine residues, is less hydrophobic than the corresponding region of typical Sec signal peptides, which is another critical determinant for efficient Tat-dependent protein translocation (7). Thus, the export defect of pre-GFOR in *E. coli* cannot be explained by differences in the Tat signal peptide consensus features alone, since exactly the same signal peptide mediates efficient Tat-dependent export in the original host, *Z. mobilis* (43).

One possible explanation for the lack of export of pre-GFOR in *E. coli* is that there exist specific recognition events between Tat signal peptides and one or more components of the Tat translocase (or some as-yet-unrecognized factors) that involve more than just the recognition of the generally conserved features in the Tat signal peptides. This would mean that Tat signal peptides optimally interact only with the Tat translocase of the same organism, whereas interactions of a certain Tat precursor with the Tat translocase of a heterologous host organism might not occur or might be nonproductive. In light of this, it is most intriguing that an efficient in vitro translocation of pre-GFOR into isolated plant thylakoids via the ΔpH pathway is possible (14); this may be due to a lesser stringency of the ΔpH translocon for foreign signal peptides.

In contrast to the situation observed with the pre-GFOR protein, it is known that signal peptides are, in general, interchangeable in the Sec protein translocation pathway (18, 39). Signal sequences of gram-negative bacteria are recognized by Sec translocons of gram-positive bacteria and vice versa (22, 25, 32), and even eukaryotic signal peptides of the Sec-related pathway for protein import into the endoplasmatic reticulum and bacterial Sec signal peptides are interchangeable (12, 40). The observed species specificity of pre-GFOR export via the Tat pathway is therefore in marked contrast to the general Sec pathway.

A similar specificity of signal peptide recognition was observed when expression of the thylakoidal Tat substrate pre-23K in *E. coli* was examined. Whereas the pre-23K protein containing its authentic signal peptide was not exported in *E. coli* (16), replacement of that signal peptide with the signal peptide of the *E. coli* TorA protein resulted in Tat-dependent membrane translocation (30). However, it should be mentioned that there are also known cases in which a Tat signal peptide is functional in a heterologous host. Fusion of mature ß-lactamase to the signal peptide of the Tat substrate protein [NiFe]-hydrogenase of *Desulfovibrio vulgaris* Hildenborough allowed significant translocation of the ß-lactamase into the *E. coli* periplasm (26).

Two alternative scenarios can be imagined to be responsible for the lack of export of pre-GFOR in *E. coli*. First, specific recognition of the signal peptide or keeping the GFOR precursor in an export-competent state might require an as-yet-unidentified factor which is present in *Z. mobilis* but lacking in *E. coli*. Second, it cannot be excluded that an unknown *E. coli* protein or nonproteinaceous component unspecifically interacts with the GFOR signal peptide and, despite correct folding of the mature protein and successful cofactor binding, prevents the pre-GFOR from entering the Tat secretion pathway.

Binding of NADP is necessary to stabilize the GFOR quaternary structure in which the NADP binding pocket of one subunit is covered by the amino-terminal arm of another subunit (19). When directed to the Sec pathway by replacement of the GFOR signal peptide with the OmpA signal sequence, the OmpA-GFOR fusion protein is exported, processed to mature GFOR, and then very quickly degraded by periplasmic proteases (44). In contrast, we have now shown that GFOR, which is translocated by the Tat pathway with its bound cofactor, is completely stable during a 60-min chase period. Therefore, the previously observed proteolytic instability of GFOR exported by the Sec pathway is due to the lack of NADP cofactor, which is not present in the *E. coli* periplasm and which cannot be cotranslocated with the apoprotein, which, in the case of translocation by the Sec machinery, is threaded through the membrane in a more or less unfolded form.

In contrast to the cofactor-binding-proficient TorA-GFOR fusion protein, the TorA-GFOR S116D/K121A/K123Q/I124K fusion protein with mutated amino acid residues in the NADP binding pocket was not processed to a mature form but instead was almost completely degraded during a 60-min chase period in a wild-type strain as well as in a *tatC* mutant. These results further strengthen the view that correct folding, which in the case of cofactor-containing proteins requires cofactor insertion or, in other words, the absence of unfolded structures, seems to be an absolute requirement for Tat-dependent protein translocation.

ACKNOWLEDGMENTS

We are very grateful to Tracy Palmer for kindly providing strains B1KL0 (MC4100 Δ*tatC*) and JARV15 (MC4100 Δ*tatAE*), to Karl-Ludwig Schimz for OmpA and GFOR antibodies, to Bernd Bukau for DnaK antibodies, and to Hermann Sahm for continuous support. Thomas Wiegert is indebted to Wolfgang Schumann for constant support.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Sp503/1-4 und Sp503/2-1).

REFERENCES

1. **Berks, B. C.** 1996. A common export pathway for proteins binding complex redox cofactors? Mol. Microbiol. **22:**393–404.
2. **Berks, B. C., F. Sargent, and T. Palmer.** 2000. The Tat protein export pathway. Mol. Microbiol. **35:**260–274.
3. **Bogsch, E., S. Brink, and C. Robinson.** 1997. Pathway specificity for a ΔpH-dependent precursor thylakoid lumen protein is governed by a 'Sec-avoidance' motif in the transfer peptide and a 'Sec-incompatible' mature protein. EMBO J. **16:**3851–3859.
4. **Bogsch, E. G., F. Sargent, N. R. Stanley, B. C. Berks, C. Robinson, and T. Palmer.** 1998. An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. J. Biol. Chem. **273:** 18003–18006.
5. **Buchberger, A., H. Theyssen, H. Schröder, J. S. McCaety, G. Virgallita, P. Milkereit, J. Reinstein, and B. Bukau.** 1995. Nucleotide-induced conformational changes in the ATPase and substrate binding domains of the DnaK chaperone provide evidence for interdomain communication. J. Biol. Chem. **270:**16903–16910.
6. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-lac bacteriophage: *in vivo* probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA **76:**4530–4533.
7. **Cristobal, S., J. W. de Gier, H. Nielsen, and G. von Heijne.** 1999. Competition between Sec- and Tat-dependent protein translocation in *Escherichia coli*. EMBO J. **18:**2982–2990.
8. **Danese, P. N., and T. J. Silhavy.** 1998. Targeting and assembly of periplasmic and outer-membrane proteins in *Escherichia coli*. Annu. Rev. Genet. **32:** 59–94.
9. **Dreusch, A., D. M. Bürgisser, C. W. Heizmann, and W. G. Zumft.** 1997. Lack of copper insertion into unprocessed cytoplasmic nitrous oxide reductase generated by an R20D substitution in the arginine consensus motif of the signal peptide. Biochim. Biophys. Acta. **1319:**311–318.
10. **Fekkes, P., and A. J. M. Driessen.** 1999. Protein targeting to the bacterial cytoplasmic membrane. Microbiol. Mol. Biol. Rev. **63:**161–173.
11. **Freudl, R., H. Schwarz, M. Degen, and U. Henning.** 1987. The signal sequence suffices to direct export of outer membrane protein OmpA of *Escherichia coli* K-12. J. Bacteriol. **169:**66–71.
12. **Gray, G. L., J. S. Baldridge, K. S. McKeown, H. L. Heyneker, and C. N. Chang.** 1985. Periplasmic production of correctly processed human growth hormone in *Escherichia coli*: natural and bacterial signal sequences are interchangeable. Gene **39:**247–254.
13. **Gross, R., J. Simon, and A. Kröger.** 1999. The role of the twin arginine motif in the signal peptide encoded by the *hydA* gene of the hydrogenase from *Wolinella succinogenes.* Arch. Microbiol. **172:**227–232.
14. **Halbig, D., B. Hou, R. Freudl, G. A. Sprenger, and R. B. Klösgen.** 1999. Bacterial proteins carrying twin-R signal peptides are specifically targeted by the ΔpH-dependent transport machinery of the thylakoid membrane system. FEBS Lett. **447:**95–98.
15. **Halbig, D., T. Wiegert, N. Blaudeck, R. Freudl, and G. A. Sprenger.** 1999. The efficient export of NADP-containing glucose-fructose oxidoreductase to the periplasm of *Zymomonas mobilis* depends both on an intact twin-arginine motif in the signal peptide and on the generation of a structural export signal induced by cofactor binding. Eur. J. Biochem. **263:**543–551.
16. **Henry, R., M. Carrigan, M. McCaffrey, X. Ma, and K. Cline.** 1997. Targeting determinants and proposed evolutionary basis for the Sec and Delta pH protein transport systems in chloroplast thylakoid membranes. J. Cell Biol. **136:**823–832.
17. **Hynds, P. J., D. Robinson, and C. Robinson.** 1998. The Sec-independent twin arginine translocation system can transport both tightly folded and malfolded proteins across the thylakoid membrane. J. Biol. Chem. **273:**34868–34874.
18. **Izard, J. W., and D. A. Kendall.** 1994. Signal peptides: exquisitely designed transport promoters. Mol. Microbiol. **13:**765–773.
19. **Kingston, R. L., R. K. Scopes, and E. N. Baker.** 1996. The structure of glucose-fructose oxidoreductase from *Zymomonas mobilis*: an osmoprotective periplasmic enzyme containing non-dissociable NADP. Structure **4:** 1413–1428.
20. **Loos, H., H. Sahm, and G. A. Sprenger.** 1993. Glucose-fructose oxidoreductase, a periplasmic enzyme of *Zymomonas mobilis*, is active in its precursor form. FEMS Microbiol. Lett. **107:**293–298.
21. **Loos, H., M. Völler, B. Rehr, Y.-D. Stierhof, H. Sahm, and G. A. Sprenger.** 1991. Localisation of the glucose-fructose oxidoreductase in wild type and overproducing strains of *Zymomonas mobilis*. FEMS Microbiol. Lett. **84:** 211–216.
22. **Meens, J., E. Frings, M. Klose, and R. Freudl.** 1993. An outer membrane protein (OmpA) of *Escherichia coli* can be translocated across the cytoplasmic membrane of *Bacillus subtilis*. Mol. Microbiol. **9:**847–855.
23. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Mori, H., and K. Cline.** 1998. A signal peptide that directs non-Sec transport in bacteria also directs efficient and exclusive transport on the thylakoid delta pH pathway. J. Biol. Chem. **273:**11405–11408.
25. **Nakamura, K., Y. Fujita, Y. Itoh, and K. Yamane.** 1989. Modification of length, hydrophobic properties and electric charge of *Bacillus subtilis* α-amylase signal peptide and their different effects on the production of secretory proteins in *B. subtilis* and *Escherichia coli* cells. Mol. Gen. Genet. **216:**1–9.
26. **Nivière, V., S.-L. Wong, and G. Voordouw.** 1992. Site-directed mutagenesis of the hydrogenase signal peptide consensus box prevents export of a β-lactamase fusion protein. J. Gen. Microbiol. **138:**2173–2183.
27. **Oliver, D. B., R. J. Cabelli, K. M. Dolan, and G. P. Jarosik.** 1990. Azide-resistant mutants of *Escherichia coli* alter the SecA protein, an azide-sensitive component of the protein export machinery. Proc. Natl. Acad. Sci. USA **87:**8227–8231.
28. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57:**50–108.
29. **Santini, C.-L., B. Ize, A. Chanal, M. Müller, G. Giordano, and L.-F. Wu.** 1998. A novel Sec-independent periplasmic protein translocation pathway in *Escherichia coli*. EMBO J. **17:**101–112.
30. **Sargent, F., E. G. Bogsch, N. R. Stanley, M. Wexler, C. Robinson, B. C. Berks, and T. Palmer.** 1998. Overlapping functions of components of a bacterial Sec-independent protein export pathway. EMBO J. **17:**3640–3650.
31. **Sargent, F., N. R. Stanley, B. C. Berks, and T. Palmer.** 1999. Sec-independent protein translocation in *Escherichia coli*. A distinct and pivotal role for the TatB protein. J. Biol. Chem. **274:**36073–36082.
32. **Sarvas, M.** 1986. Protein secretion in bacilli. Curr. Top. Microbiol. Immunol. **125:**103–125.
33. **Schiebel, E., A. J. M. Driessen, F.-U. Hartl, and W. Wickner.** 1991. $\Delta\mu H^+$ and ATP function at different steps of the catalytic cycle of preprotein translocase. Cell **64:**927–939.
34. **Settles, A. M., and R. Martienssen.** 1998. Old and new pathways of protein export in chloroplasts and bacteria. Trends Cell Biol. **8:**494–501.
35. **Settles, A. M., A. Yonetani, A. Baron, D. R. Bush, K. Cline, and R. Martienssen.** 1997. Sec-independent protein translocation by the maize Hcf106 protein. Science **278:**1467–1470.
36. **Stanley, N. R., T. Palmer, and B. C. Berks.** 2000. The twin arginine motif of Tat signal peptides is involved in Sec-independent protein targeting in *Escherichia coli*. J. Biol. Chem. **275:**11591–11596.
37. **Tanaka, H., S. A. Lerner, and E. C. C. Lin.** 1967. Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. J. Bacteriol. **93:**642–648.
38. **von Heijne, G.** 1985. Signal sequences. The limits of variation. J. Mol. Biol. **184:**99–105.
39. **von Heijne, G.** 1990. The signal peptide. J. Membr. Biol. **115:**195–201.
40. **Wanker, E., E. Klingsbichel, and H. Schwab.** 1995. Efficient secretion of *Bacillus subtilis* levanase by *Saccharomyces cerevisiae*. Gene **161:**45–49.
41. **Weiner, J. H., P. T. Bilous, G. M. Shaw, S. P. Lubitz, L. Frost, G. H. Thomas, J. A. Cole, and R. J. Turner.** 1998. A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. Cell **93:** 93–101.
42. **Wexler, M., E. G. Bogsch, R. B. Klösgen, T. Palmer, C. Robinson, and B. C. Berks.** 1998. Targeting signals for a bacterial Sec-independent export system direct plant thylakoid import by the ΔpH pathway. FEBS Lett. **431:**339–342.
43. **Wiegert, T., H. Sahm, and G. A. Sprenger.** 1996. Export of the periplasmic NADP-containing glucose-fructose oxidoreductase of *Zymomonas mobilis.* Arch. Microbiol. **166:**32–41.
44. **Wiegert, T., H. Sahm, and G. A. Sprenger.** 1997. Expression of the *Zymomonas mobilis gfo* gene for NADP-containing glucose-fructose oxidoreductase (GFOR) in *Escherichia coli*. Formation of enzymatically active preGFOR but lack of processing into a stable periplasmic protein. Eur. J. Biochem. **244:** 107–112.
45. **Wiegert, T., H. Sahm, and G. A. Sprenger.** 1997. The substitution of a single amino acid residue (Ser-116→Asp) alters NADP-containing glucose-fructose oxidoreductase of *Zymomonas mobilis* into a glucose dehydrogenase with dual coenzyme specificity. J. Biol. Chem. **272:**13126–13133.
46. **Wu, L.-F., A. Chanal, and A. Rodrigue.** 2000. Membrane targeting and translocation of bacterial hydrogenases. Arch. Microbiol. **173:**319–324.
47. **Yanish-Perron, C., J. Vieira, and J. Messing.** 1985. Improved phage M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
48. **Zimmermann, R., and W. Wickner.** 1983. Energetics and intermediates of the assembly of protein OmpA into the outer membrane of *Escherichia coli*. J. Biol. Chem. **258:**3920–3925.