

Ralstonia eutropha TF93 Is Blocked in Tat-Mediated Protein Export

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***Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) TF93 is pleiotropically affected in the translocation of redox enzymes synthesized with an N-terminal signal peptide bearing a twin arginine (S/T-R-R-X-F-L-K) motif. Immunoblot analyses showed that the catalytic subunits of the membrane-bound [NiFe] hydrogenase (MBH) and the molybdenum cofactor-binding periplasmic nitrate reductase (Nap) are mislocalized to the cytoplasm and to the inner membrane, respectively. Moreover, physiological studies showed that the copper-containing nitrous oxide reductase (NosZ) was also not translocated to the periplasm in strain TF93. The cellular localization of enzymes exported by the general secretion system was unaffected. The translocation-arrested MBH and Nap proteins were enzymatically active, suggesting that twin-arginine signal peptide-dependent redox enzymes may have their cofactors inserted prior to transmembrane export. The periplasmic destination of MBH, Nap, and NosZ was restored by heterologous expression of *Azotobacter chroococcum tata* mobilized into TF93. *tata* encodes a bacterial Hcf106-like protein, a component of a novel protein transport system that has been characterized in thylakoids and shown to translocate folded proteins across the membrane.**

Periplasmic enzymes binding redox cofactors play a central role in alternative energy metabolism of gram-negative bacteria. In contrast to cytochrome *c*-type proteins, various periplasmic enzymes binding constituents, such as the molybdenum cofactor, a [NiFe] site, copper centers, or iron-sulfur clusters, contain a conserved, positively charged -S/T-RRXFLK- (twin-arginine) element within their N-terminal signal peptides, pointing to a special translocation pathway (2). Very recently, a system responsible for the membrane targeting and translocation of [NiFe] hydrogenases, as well as the molybdenum enzymes dimethylsulfoxide reductase, trimethylamine *N*-oxide reductase, and periplasmic nitrate reductase, has been characterized for *Escherichia coli* (32, 43). Mutant analyses suggested that the translocation of the twin-arginine signal peptide-bearing enzymes proceeds independently of the general secretion machinery of the cell (Sec), presumably via intermediates which have their cofactors inserted (31).

Ralstonia eutropha (formerly *Alcaligenes eutrophus* [7]) is the host of at least three periplasmic cofactor-containing enzymes synthesized with an N-terminal twin-arginine signal peptide: the membrane-bound hydrogenase (MBH) (involved in energy generation from H₂), the periplasmic nitrate reductase (Nap) (reduces nitrate to nitrite), and the nitrous oxide reductase (NosZ) (a component of the denitrification pathway). All three enzymes are encoded in *R. eutropha* H16 by megaplasmid-borne genes.

The MBH of *R. eutropha* is a member of the [NiFe] hydrogenases (15) composed of a heterodimer (HoxKG) attached to the periplasmic surface of the inner membrane by a cytochrome *b*-type anchor protein (HoxZ) (4, 13). Hydrogen is activated at the [NiFe] site of HoxG (62 kDa), and the electrons are transferred via three iron-sulfur clusters within HoxK (35 kDa) to the physiological electron acceptor HoxZ (4). The small subunit HoxK contains a twin-arginine signal peptide. A deletion in this region blocks the membrane targeting of the

MBH dimer and leads to the accumulation of inactive HoxG protein in the cytoplasm (3). The N-terminal amino acid sequence of the mature HoxG protein is colinear with the sequence predicted from the nucleotide sequence (17), indicating the absence of an export-triggering signal peptide at the N terminus. In contrast, HoxG contains a peptide extension of 15 amino acids (aa) at the C terminus, which is removed by a specific protease during [NiFe] cofactor assembly and plays a role in metal insertion (3). From these results, it was concluded that the two MBH subunits are cotranslocated in a tandem fashion and that this process is directed by the twin-arginine signal peptide-bearing small subunit. This conclusion gained support by a recent report on hydrogenase-2 of *E. coli* (27).

The periplasmic nitrate reductase, Nap, belongs to a large family of respiratory nitrate reductases, which appears to participate in denitrification, at least in some organisms (1, 28). Nap has been isolated from *R. eutropha* as a heterodimeric enzyme consisting of a 90-kDa subunit (NapA) and a 17-kDa subunit (NapB). NapA carries the catalytic site and exhibits sequence similarity with molybdopterin guanine dinucleotide (MGD) binding polypeptides of bacterial assimilatory nitrate reductases and formate dehydrogenases, both of which bind an iron-sulfur cluster at the N terminus (35). In fact, crystal structure analyses of NapA from *Desulfovibrio desulfuricans* show two MGD moieties per polypeptide and a [4Fe-4S] cluster (10). Comparison of the N-terminal amino acid sequence of the mature *R. eutropha* NapA subunit, as determined by Edman degradation, with the predicted primary structure identified a 29-aa twin-arginine signal peptide in NapA. NapB, which contains two binding sites for heme *c*, is synthesized with an N-terminal signal peptide resembling those required for translocation by Sec (35).

The periplasmic, copper-dependent nitrous oxide reductase, NosZ, is a key enzyme of denitrification which converts nitrous oxide to molecular dinitrogen. The primary structures of NosZ from various bacteria, including *R. eutropha*, are highly conserved and are all characterized by an unusually long N-terminal twin-arginine signal peptide of 45 to 56 aa (11, 47).

In this study, we reexamined the H₂-oxidizing *R. eutropha* TF93 (ATCC 17697) strain, which had been reported in early

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
<i>R. eutropha</i>		
H16	Wild type, pHG1	ATCC 17699
HF345	Δ <i>hoxM</i> ; isogenic mutant of H16	3
HF359	Δ <i>hoxG</i> ; isogenic mutant of H16	3
HF405	Δ <i>hoxZ</i> ; isogenic mutant of H16	3
TF93	Wild type, pHG2	ATCC 17697
TF100	Megaplasmid-free derivative of TF93; Nap negative	14
TF140	Derivative of TF93 harboring pHG1 instead of pHG2	B. Friedrich (Humboldt-University Berlin)
<i>E. coli</i>		
S17-1	<i>Tra</i> ⁺ ; <i>recA pro thi hsdR chr:RP4-2</i>	36
NovaBlue(DE3)	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proA⁺B⁺ lac1^qZΔM15::Tn10]</i>	Novagen
Plasmids		
pBluescript II SK(+)	<i>Ap</i> ^r , <i>lacZ'</i> , T7 ϕ 10 promoter, <i>f1 ori</i>	Stratagene
pEDY309	<i>Tc</i> ^r , pRK290 replicon with multiple cloning site	23
pGE144	Derivative of pVK101 harboring the <i>nap</i> gene region	35
pGE151	Derivative of pRK404, containing <i>Plac</i>	17
pGY12	2.7-kb <i>HindIII/BamHI</i> fragment harboring <i>tatABC'</i> of <i>A. chroococcum</i>	45
pGE600	2.7-kb <i>HindIII/BamHI</i> fragment of pGY12 in pGE151	This work
pGE601	366-bp <i>HindIII/BamHI</i> fragment with <i>tatA</i> from pGY12 in pGE151	This work
pGE602	468-bp <i>HindIII/BamHI</i> fragment with <i>tatB</i> from pGY12 in pGE151	This work
pGE603	1.4-kb <i>NdeI/XbaI</i> fragment with <i>E. coli phoA</i> in pEDY309	This work
pCH800	366-bp <i>HindIII/BamHI</i> fragment with <i>tatA</i> from pGY12 in pBluescript SK(+)	This work
pCH801	468-bp <i>HindIII/BamHI</i> fragment with <i>tatB</i> from pGY12 in pBluescript SK(+)	This work
pCH802	1.6-kb <i>SphI</i> fragment of pGY12 in pUC18	This work

studies to form a membrane-type of hydrogenase which occurred in the soluble fraction of the cell (14). We show that due to a missing or nonfunctional chromosomal factor, the strain is affected not only in membrane targeting of the twin-arginine signal peptide-bearing [NiFe] hydrogenase but also in the translocation of the periplasmic nitrate reductase and the nitrous oxide reductase. The mislocalized MBH and Nap proteins were active with artificial electron acceptors and donors, supporting the interpretation that these metalloenzymes have their cofactors inserted prior to translocation.

MATERIALS AND METHODS

Strains and plasmids. Bacterial strains and plasmids are listed in Table 1. Plasmid pGE600 is a derivative of the mobilizable vector pGE151, carrying the 2.7-kb *BamHI/HindIII* fragment of pGY12. DNA fragments extending from nucleotides 1268 to 1620 and from 1555 to 2006 of the *Azotobacter chroococcum* *tat* region (GenBank accession no. ACU48408) were synthesized by PCR, using plasmid pGY12 as a template. Amplification of *tatA* with primer pair 5'-ACCC AAGCTTGGCGAAAAGGCCGACGGCC-3' and 5'-CGGGATCCAGCAG GAGTTCGCTGAAGCCG-3' produced a 366-bp fragment containing an additional *HindIII* and *BamHI* site. All the PCR amplifications were carried out with *Pfu* DNA polymerase (Stratagene). The resulting fragment was cut with *HindIII* and *BamHI* and ligated into pBluescript SK(+) (pCH800). In the same way, the primers 5'-ACCCAAGCTTGAAGGTCGAGGAACCGGCCAGG-3' and 5'-CGGGATCCCTGCGCAGCAGGCGCGAGCGC-3' were used for amplification of a 468-bp fragment containing *tatB* and ligated into pBluescript SK(+) (pCH801). The identity of the subcloned PCR fragments was verified by nucleotide sequence determination. The *HindIII/BamHI* fragments containing either *tatA* or *tatB* were excised from the pBluescript derivatives and cloned into plasmid pGE151, yielding plasmids pGE601 and pGE602, respectively. For nucleotide sequence determination of the fusion in the *tat* region (see Fig. 6A), the native 1,638-bp *SphI* fragment of pGY12 was cloned into pUC18, yielding pCH802. *E. coli phoA* was PCR amplified on a 1.4-kb fragment with the primers 5'-TACATATGAAACAAAGCACTATTGCACTGG-3' and 5'-TATCTAGAT TATTTCAGCCCGAGCGGC-3', with total DNA as the template. The PCR product was cut by *NdeI* and *XbaI* and cloned downstream of the strong *R. eutropha* promoter of the soluble hydrogenase into the broad-host-range plasmid pEDY309 as previously described (23), yielding pGE603.

Media and growth conditions. Lithotrophic cultures of *R. eutropha* strains were grown in mineral salts medium under an atmosphere of hydrogen, carbon dioxide, and oxygen (8:1:1 [vol/vol/vol]) supplemented with 0.8 μ M NiCl₂ in

place of the standard trace element mixture SL6 (12). Synthetic media for aerobic heterotrophic growth, in order to optimally express Nap, contained 0.4% (wt/vol) gluconate or 0.4% (wt/vol) fructose and SL6 as described previously (42). Anaerobic heterotrophic growth, at the expense of 0.2% (vol/vol) nitrate (denitrification), was performed in medium containing fructose as previously described (30). Strains of *E. coli* were grown in Luria-Bertani medium (20). Solid media contained 1.5% (wt/vol) agar. Antibiotics were added as appropriate for *R. eutropha* (tetracycline, 12.5 μ g/ml) and for *E. coli* (tetracycline, 12.5 μ g/ml; ampicillin, 80 μ g/ml).

Conjugative plasmid transfer. Mobilizable plasmids were transferred from *E. coli* S17-1 to *R. eutropha* by a spot mating technique (36). Transconjugants were selected on mineral medium plates containing the appropriate antibiotic under lithotrophic growth conditions.

DNA techniques. Standard techniques were used in this study (29). Plasmid DNA isolation was carried out by the alkaline lysis procedure and ion-exchange chromatography according to the manufacturer's instructions (QIAGEN Inc.). DNA and PCR fragments used in plasmid constructions were isolated from agarose gels by QiaEx (QIAGEN Inc.). Nucleotide sequence determination of pCH802 was done by the dideoxy chain termination method and by cycle sequencing with sequence-derived fluorescence-labelled primers and the thermostable sequenase kit (Amersham Pharmacia Biotech) in an automatic sequencing device, as recommended by the manufacturer (LICOR).

In vivo expression of *tatAB* gene products. Expression of *tatA* and *tatB* from plasmids pCH800 and pCH801 was under control of the phage T7 ϕ 10 promoter. The plasmids were transformed into strain NovaBlue(DE3), which carries a chromosomally encoded T7 polymerase. Synthesis of the *tat*-encoded gene products was induced by IPTG (isopropyl- β -D-thiogalactopyranoside) and labelling with [³⁵S]methionine followed the procedure previously described (38).

Isolation of subcellular fractions. Subcellular fractions were prepared according to a method described by Bernhard et al. (4), with modifications. *R. eutropha* cells (100 ml) were grown in the presence of oxygen either under lithotrophic conditions for 36 h or heterotrophically on gluconate for 24 to 48 h and then harvested by centrifugation (4,000 \times g, 4°C). Cells were washed with 10 ml of 10 mM Tris-HCl, pH 7.5 (4,000 \times g, 4°C), and resuspended in 5 ml of 10 mM Tris-HCl, pH 7.8, containing 0.5 M sucrose. After a 10-min incubation at 30°C in the presence of 1 mM EDTA, the incubation was continued at room temperature for 30 min with lysozyme (10 mg/g [wet weight] of cells). The suspension was centrifuged (4,000 \times g; 20 min; 4°C), and the supernatant contained the periplasmic fraction. The spheroplasts were washed and lysed by osmotic shock with 5 ml of 10 mM Tris-HCl, pH 7.8. Cell debris was removed (5,000 \times g, 15 min, 4°C), and the membrane and cytoplasmic fractions were obtained by ultracentrifugation (88,000 \times g, 45 min, 4°C). Membranes used for the detection of the MBH were washed three times and suspended in 50 mM KPO₄, pH 7.0. Membranes used for the detection of NapA were resuspended in 250 mM NaKPO₄, pH 6.5.

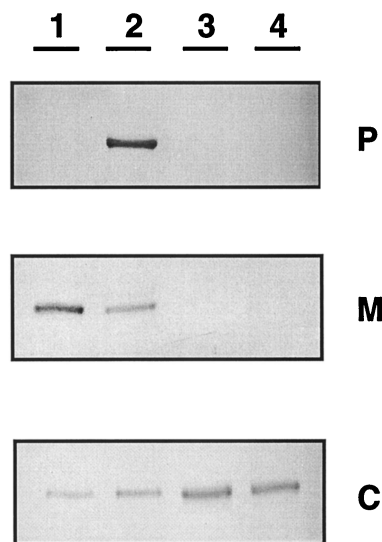


FIG. 1. Cellular localization of the [NiFe] center-bearing subunit (HoxG) of MBH in strains of *R. eutropha*. Equal amounts of protein (25 μ g) were loaded onto the gel except for the periplasmic fractions (100 μ l each), because they contained high concentrations of lysozyme protein. The subcellular fractions are indicated: P, periplasm; M, membrane; C, cytoplasm. The strains tested are as follows: lane 1, H16 (harboring pHG1); lane 2, HF405, carrying Δ hoxZ in pHG1; lane 3, TF93 (harboring pHG2); lane 4, TF140 (harboring pHG1).

The periplasmic, cytoplasmic, and membrane fractions were used directly or stored at -20°C until used for enzyme assays, activity staining, and immunoblotting analysis. The quality of the subcellular fractions of the lithotrophically grown cells was monitored by examining the distribution of the cytoplasmic, NAD-reducing hydrogenase activity of the soluble hydrogenase (SH) of *R. eutropha* and immunoblotting with SH-specific antibodies, as previously described (4). The purity of subcellular fractions of heterotrophically grown cells was controlled by immunoblotting with antiserum raised against the cytoplasmic marker flavohemoglobin of *R. eutropha* (9).

Immunoblot analysis. Proteins were resolved by electrophoresis in sodium dodecyl sulfate (SDS)-10% or 12% (wt/vol) polyacrylamide gels and were transferred to nitrocellulose membranes, and the immunoblot analysis was done according to a standard protocol (41). Specific proteins were detected with polyclonal rabbit antisera and an alkaline phosphatase-labelled goat anti-rabbit immunoglobulin G (Jackson Immuno Research Laboratories). The proteins were applied at the following dilutions: anti-flavohemoglobin (1:5,000), anti-HoxH (1:10,000), anti-HoxG (1:2,000), anti-PhoA (1:1,000; 5 Prime \rightarrow 3 Prime, Inc.), anti-nitrite reductase (1:10,000) (30), and anti-NapA (1:1,000).

Analytical procedures. SH (hydrogen:NAD⁺ oxidoreductase [EC 1.12.1.2]) activity was assayed by spectrophotometric determination of H₂-dependent NAD reduction (14). MBH (ferredoxin:H⁺ oxidoreductase [EC 1.18.99.1]) activity was determined according to a previously described method (37), with modifications. Hydrogenase activities of membranes were determined in N₂-saturated 50 mM KPO₄, pH 7.0. Soluble extracts at pH 5.5 were measured with 0.5 mM methylene blue and 86 μ M H₂. One unit of hydrogenase activity was the amount of enzyme which catalyzed the consumption of 1 μ mol of substrate per min. Nap reductase activity was determined in subcellular fractions obtained from aerobically grown cells (42) exploiting the formate-dependent nitrate reduction (35). One unit of Nap activity was the amount of enzyme which catalyzed the formation of 1 μ mol of nitrite per min. Nitrite concentration was determined colorometrically at 546 nm (18).

In-gel chromogenic detection of hydrogenase activity after native polyacrylamide gel electrophoresis was done according to a previously described method (4).

c-Type cytochromes were detected after SDS-polyacrylamide gel electrophoresis of subcellular fractions and specific staining for covalently attached heme, as previously described (39).

Protein determination of cells and subcellular fractions was done by the method of Lowry et al. (19).

Determination of nitrous oxide and dinitrogen by gas chromatography was done as previously described (9).

RESULTS AND DISCUSSION

The membrane-bound hydrogenase of *R. eutropha* TF93 is mislocalized to the cytoplasm. The majority of *R. eutropha*

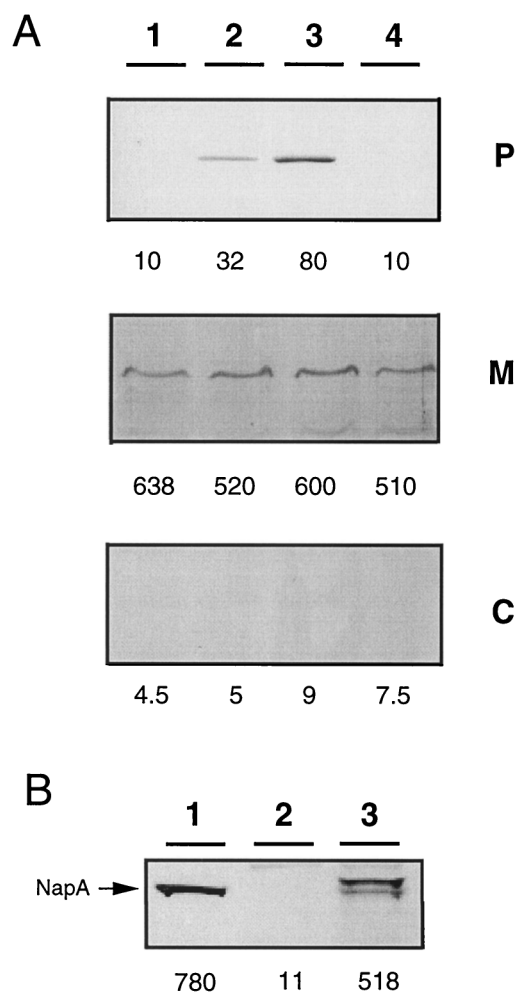


FIG. 2. Mislocalization of NapA in TF140 and restoration of NapA export by *tat* genes (A) and specificity of the NapA detection system (B). The subcellular fractions are indicated: P, periplasm; M, membrane; C, cytoplasm. Nap activity (milliunits per milligram of protein) is given below the immunoblot. Note, the Nap activity in the periplasm is given as milliunits per gram (wet weight) of cells. (A) Lane 1, TF140; lane 2, TF140 harboring *tatAB* on pGE600; lane 3, TF140 harboring *tatA* on pGE601; lane 4, TF140 harboring *tatB* on pGE602. (B) Lane 1, partially purified NapA (arrow); lane 2, membrane of TF100 cells; lane 3, membrane of TF100 cells harboring the complete *nap* genes on pGE144 (35).

strains form two enzymes for energy conservation from molecular hydrogen: a cytoplasmic NAD-dependent SH and an MBH. Both proteins have been shown to be also present in the natural isolate *R. eutropha* TF93; however, the MBH was identified in the soluble fraction instead of in the membrane. Mislocalization of the enzyme could not be restored by a megaplasmid exchange using a donor which synthesized the MBH properly attached to the membrane (14).

To determine the precise cellular localization of the MBH dimer, we examined periplasmic, membrane, and cytoplasmic fractions of autotrophically grown TF93 cells by immunoblot analysis using antiserum raised against the large MBH subunit (HoxG) of *R. eutropha* H16. HoxG of TF93 was exclusively found in the cytoplasm (Fig. 1, lane 3), unlike the MBH of strain H16, which occurred predominantly in the membrane fraction and only in trace amounts in the cytoplasm (Fig. 1, lane 1). Export of the MBH to the periplasm of TF93 can be excluded on the basis of the release of the MBH into the

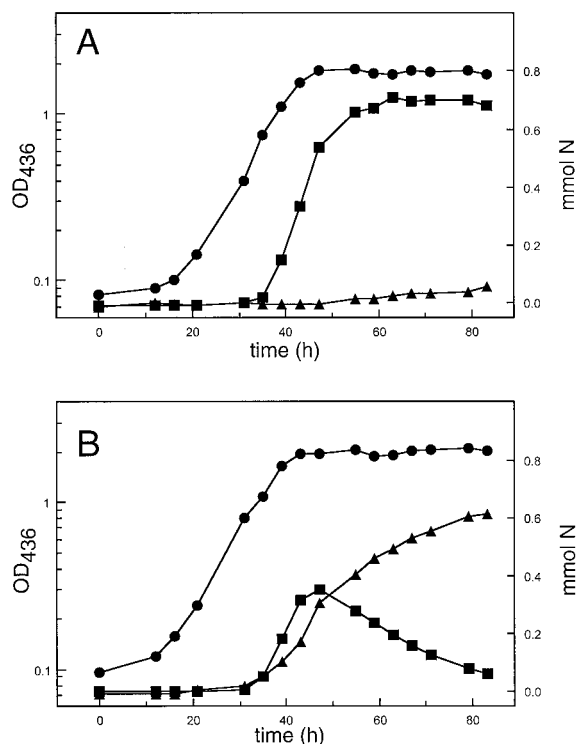


FIG. 3. Blocked nitrous oxide reduction in *R. eutropha* TF140 (A), which was restored by the expression of *A. chroococcum* *tatA* on pGE601 (B). Growth was performed anaerobically on fructose containing 10 mM nitrate, and the gaseous denitrification products were monitored by gas chromatography. ●, growth of *R. eutropha* culture at 436 nm; ■, nitrous oxide; ▲, dinitrogen. OD, optical density.

periplasm by a mutant of H16 impaired in the membrane anchor HoxZ (Fig. 1, lane 2) (4). These results unambiguously show that the MBH of TF93 is restricted to the cytoplasm, which was also the case in a transconjugant, TF140, harboring the megaplasmid pHG1 of *R. eutropha* H16 (Fig. 1, lane 4). This confirmed that no mutations in megaplasmid genes are responsible for the mislocalization of the MBH in TF93, but pointed to a defective or missing chromosomally encoded factor which is required for the proper targeting of the MBH to the membrane. Since pHG1 of strain H16 is better characterized than the native plasmid pHG2 of TF93, subsequent experiments were done with TF140 (Table 1).

***R. eutropha* TF93 is pleiotropically deficient in translocation of redox enzymes.** To examine whether the deficiency of TF93 plays a more general role in the export of cofactor-containing enzymes, we investigated the localization of Nap and of NosZ in comparison to proteins which are translocated via the general secretion system. Subcellular fractions from TF140 cells grown heterotrophically in synthetic medium in presence of oxygen were analyzed for Nap. Immunoblots with polyclonal NapA antiserum showed that the catalytic subunit NapA was trapped in the membrane, whereas both the periplasm and the cytoplasm were almost free of any cross-reacting material (Fig. 2, lane 1). This clearly pointed to a pleiotropic nature of the export deficiency in TF140. Identical results were obtained with the parental TF93 strain (data not shown).

The second twin-arginine signal peptide-bearing redox enzyme, NosZ, was investigated on the physiological level. Cells of TF140 were grown anaerobically under denitrifying conditions with nitrate as the electron acceptor. Nitrous oxide production and its conversion to molecular dinitrogen were monitored by gas chromatography. Figure 3A shows that TF140 accumulated nitrous oxide as the final product, indicating that NosZ was incapable of converting nitrous oxide. Physiologically active periplasmic NosZ of *R. eutropha*, however, readily converts nitrous oxide, which only transiently accumulates in the gas phase (30). The result is compatible with the conclusion

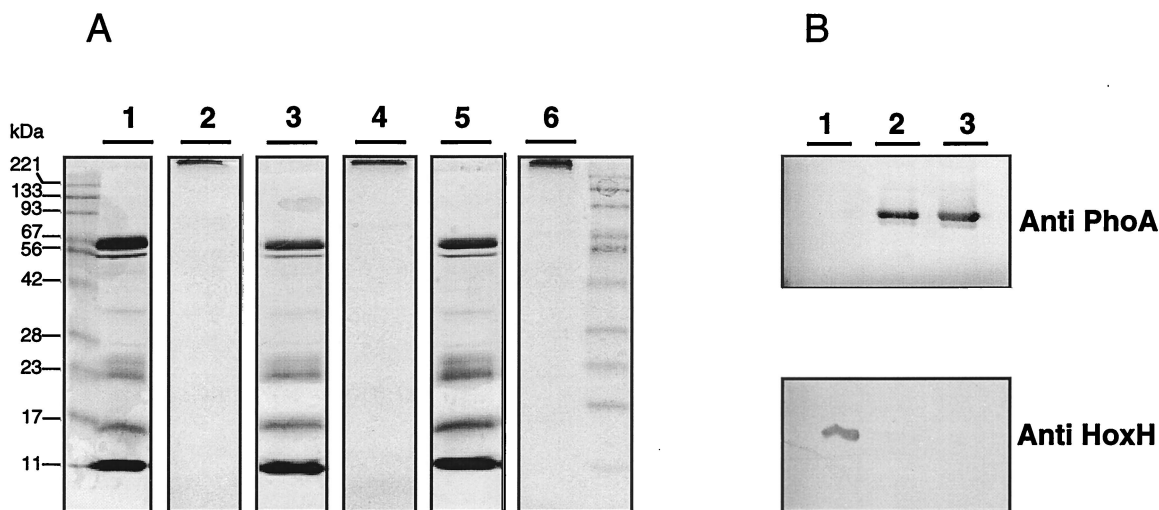


FIG. 4. Periplasmic *c*-type cytochromes (A) and export of heterologously expressed *E. coli* PhoA to the periplasm (B) in *R. eutropha* strains. (A) Cytochrome *c* was visualized by heme staining of periplasmic (lanes 1, 3, and 5) and cytoplasmic (lanes 2, 4, and 6) fractions of TF140 cells grown anaerobically on nitrate. The proteins were separated on SDS-15% (vol/vol) polyacrylamide gels. Lanes 1 and 2, TF140; lanes 3 and 4, TF140 harboring *tatAB* on pGE600; lanes 5 and 6, TF140 harboring *tatA* on pGE601. Prestained protein markers are on the left and right of the stain. The nature of the staining at the border of the stacking and the running gel in the cytoplasmic fractions (lanes 2, 4, and 6) is unclear. (B) Immunoblot analysis of the periplasmic fractions of autotrophically grown *R. eutropha* strains with antiserum directed against *E. coli* PhoA (upper panel) and a purity control with antiserum raised against the SH protein (anti-HoxH) as a cytoplasmic marker (4) (lower panel). Lane 1, 1 μ g of purified SH; lane 2, periplasm of TF140 expressing a copy of *E. coli* *phoA* on pGE603; lane 3, periplasm of H16 derivative (HF405) expressing a copy of *E. coli* *phoA* on pGE603.

that the mislocalization of NosZ in TF140 impairs its physiological function. Indeed, mutational analysis of the NosZ system from *Pseudomonas stutzeri* has shown that the export to the periplasm is a prerequisite of the protein to be physiologically active (11). Due to the lack of an appropriate NosZ antibody, it was not possible to investigate the cellular localization of the enzyme. Attempts to use heterologous antiserum raised against NosZ by *P. stutzeri* (47) were not successful. Nevertheless, the results provide an excellent explanation for the failure of *R. eutropha* TF93 to produce dinitrogen gas during anaerobic nitrate respiration (44). Furthermore, the data demonstrate that the export deficiency in TF93 has an enormous physiological impact on the cells since it blocks the function of at least three independent redox systems.

To definitely show that the translocation of twin-arginine signal peptide-dependent redox enzymes is specifically blocked and that the translocation of proteins across the cytoplasmic membrane is not generally impaired in *R. eutropha* TF93, the distribution of cytochrome *c*-type proteins of this strain was tested. Periplasmic and cytoplasmic fractions obtained from cells of TF140 grown aerobically and anaerobically were subjected to heme *c* staining (Fig. 4). Heme *c*-type proteins were identified exclusively in the periplasm; they were absent in the cytoplasmic fraction (Fig. 4A, lanes 1 and 2). Furthermore, immunoblot analyses confirmed the periplasmic localization of the heme *cd*₁-containing nitrite reductase (data not shown) and also of the alkaline phosphatase (PhoA), which is a well-known substrate of the general secretory pathway (Sec) in *E. coli* (25). When heterologously expressed from plasmid pGE603, *E. coli* PhoA is exported to the periplasmic space of TF140 in the same manner as in an H16 derivative, as expected (Fig. 4B, lanes 2 and 3, top panel). Purity controls eliminated the possibility of a contamination by cytoplasmic proteins (Fig. 4B, bottom panel).

MBH and Nap are enzymatically active in the translocation-arrested state. In the process of cytochrome *c* maturation, the polypeptide and the prosthetic group heme *c* are translocated separately (reference 40 and references therein). Thus, neither preapocytochromes nor holo-cytochrome *c* containing the cofactor essential for the catalytic activity is detectable when export is blocked. Immunochemical studies showed that cofactor-free apofoms are unstable (21, 22).

The detection of export-blocked MBH and Nap prompted us to examine whether both are also enzymatically active. MBH activity was visualized by in-gel H₂-dependent phenazine methosulfate reduction and showed a strong activity staining in the cytoplasmic compartment of TF140 (Fig. 5A, lane 3). For H16, only a faint stain corresponding to traces of anti-MBH material detected in the cytoplasm was observed (Fig. 1 and 5A, lane 1). No staining appeared in an H16 derivative which is devoid of the [NiFe]-containing subunit (HF359; Δ *hoxG*) (Fig. 5A, lane 2). This demonstrated that translocation-arrested MBH accumulates in its catalytically active form in the cytoplasm of TF140. Further immunoblot analyses showed that the MBH is processed in TF140, compared to a mutant in which the protease gene had been deleted (HF345; Δ *hoxM*) (Fig. 5B, compare lanes 1 and 2). This confirms that for metal center assembly, the mislocalized MBH undergoes the same proteolytic processing at the C terminus of the [NiFe]-containing subunit in TF140 as that documented for the H16 enzyme (4). These results are in agreement with the previous finding of the partially purified active MBH from the soluble fraction of *R. eutropha* TF93 (24). Nitrate reductase assays showed that the NapA protein trapped in the membrane of TF140 is also catalytically active (Fig. 2, lane 1). Only basal nitrate-reducing activities were found in the periplasmic and cytoplasmic frac-

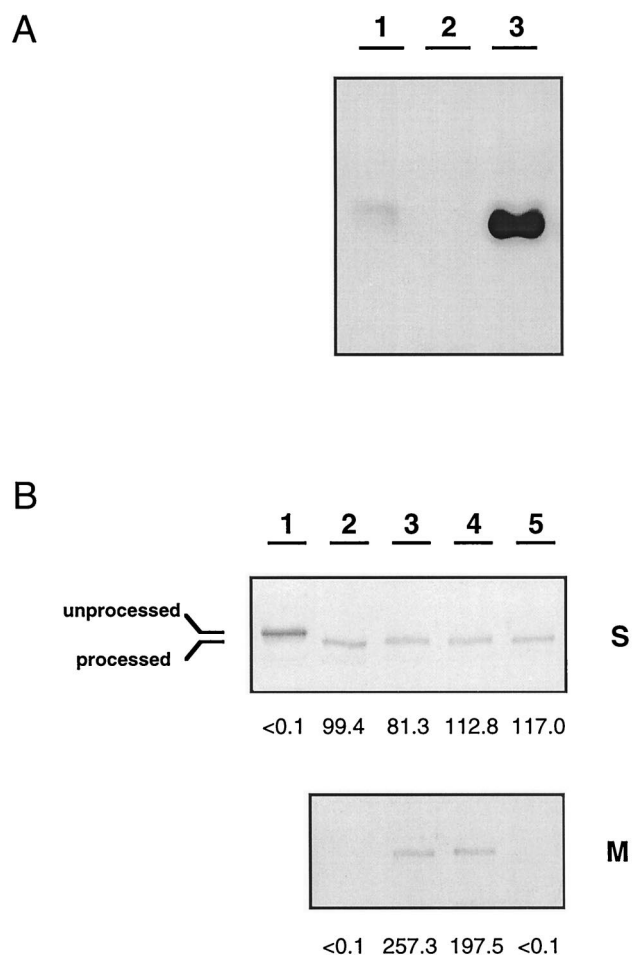


FIG. 5. In-gel detection of MBH-dependent hydrogenase activity in the cytoplasmic fraction of TF140 (A) and the restoration of MBH targeting to the membrane by *tat* genes (B). (A) Lane 1, H16; lane 2, HF359, defective in the [NiFe]-containing subunit of MBH (Δ *hoxG* in pHG1); lane 3, TF140. (B) Analysis of restoration of MBH translocation was carried out by immunoblot analysis. S, the soluble extract consisting of the periplasmic and cytoplasmic fractions; M, the membrane fraction. MBH activity (milliunits per milligram of protein) is given below the blot. Lane 1, HF345, defective in the MBH-specific protease (Δ *hoxM* in pHG1); lane 2, TF140; lane 3, TF140 with *tatAB* (pGE600); lane 4, TF140 with *tatA* (pGE601); lane 5, TF140 with *tatB* (pGE602).

tions (Fig. 2, lane 1). The nitrate reductase activity measured is exclusively due to Nap and not due to the respiratory membrane-bound nitrate reductase, which is known to be expressed anaerobically only when nitrate serves as the alternative electron acceptor (42). Figure 2B shows that a Nap-negative variant of TF93 formed no anti-NapA reacting material, and in consequence, no Nap activity was trapped in the membrane of the aerobically grown cells. However, NapA-specific protein and the corresponding enzyme activity were detectable again upon complementation of the mutant *nap* strain by the *nap* genes residing on pGE144 (Fig. 2B, compare lanes 2 and 3). In summary, the results demonstrate that MBH and NapA have their respective cofactors incorporated in an identical manner appropriate for the physiological situation despite their translocation being blocked.

The pretranslocational fashion of cofactor insertion accompanied with folding of the preproteins precludes translocation by the Sec system (25). In fact, it has been shown recently that the twin-arginine signal peptide-dependent translocation of

transconjugants were tested for the localization of MBH and of NapA. Figure 5B shows that the *tatAB*- and *tatA*-harboring derivatives targeted the MBH correctly to the membrane and restored hydrogenase activity in this fraction. Likewise, NapA was correctly exported in the *tatAB*- and *tatA*-harboring cells, accompanied by the occurrence of Nap activity in the periplasm (Fig. 2). Moreover, expression of *A. chroococcum* *tatA* (on pGE600 and pGE601) restored nitrous oxide reduction in TF140; the resulting transconjugants accumulated dinitrogen in the gas phase, as illustrated representatively for TF140 harboring a copy of *tatA* (Fig. 3B). Transfer of *A. chroococcum* *tatB* alone (on pGE602) did not restore any deficiency observed in TF140 (Fig. 2, 3, and 5). In contrast, the expression of the *A. chroococcum* *tat* genes had no effect at all on the export of cytochrome *c*-type proteins (Fig. 4A). These results strongly point to a lesion in a TatA-like protein in *R. eutropha* TF93 which appears to be required for the translocation of folded twin-arginine signal peptide-bearing redox enzymes.

Although we cannot exclude the possibility that the folding constraints for MBH and Nap in export-blocked TF93 are different from the export-competent cells, the results presented could be interpreted in favor of a cytoplasmic assembly rather than a periplasmic assembly pathway for twin-arginine signal peptide-bearing redox enzymes, as previously proposed (2). This interpretation also gains support by studies indicating that metal insertion has to take place before the molybdenum-dependent dimethylsulfoxide reductase from *Rhodobacter sphaeroides* f. sp. *denitrificans* (46) and hydrogenase-2 from *E. coli* (26) are translocated. Both enzymes are examples of bacterial redox proteins carrying the unusual signal peptide (2).

Hcf106-like proteins in targeting and translocation of bacterial enzymes binding different redox cofactors. Very recently it was shown that *E. coli* contains a Sec-independent transport apparatus required for the membrane targeting and translocation of twin-arginine signal peptide-bearing enzymes (31, 32, 43). The novel system, Tat (twin-arginine translocator, formerly called Mtt [membrane targeting and translocation]), is encoded by the *tatABCD* operon and the unlinked *tatE* gene (32). Although controversial, it is now apparent that the *E. coli* Tat system comprises at least three gene products regarded as Hcf106-like proteins, designated TatA (11 kDa), TatB (18 kDa), and TatE (13 kDa) (Fig. 6) (32, 33; A. Chanal, C. Santini, and L. F. Wu, Letter, *Mol. Microbiol.* **30**:674–676, 1998). Like the corresponding gene products identified in *A. chroococcum*, which are 8 (TatA) and 12 (TatB) kDa in size, they are predicted to have a very similar N-terminal membrane-spanning domain, followed by an amphipathic helix (Fig. 6B). The fourth component identified in *E. coli* is predicted to constitute a membrane-integral protein, TatC (29 kDa), and forms the essential core component of the translocation system (6). Although we have not yet identified any of the corresponding genes in *R. eutropha*, successful transcomplementation with a copy of *tatA* suggests that a homolog or analog to TatC could be functional in this organism. We report that *A. chroococcum* TatA promoted the translocation of three basically different twin-arginine signal peptide-proteins carrying the cofactors [NiFe], MGD, and polynuclear copper sites. Our observation that TatA recognizes a broad range of proteins is consistent with the very first analysis of bacterial Hcf106-like proteins in *E. coli*. Mutational analysis has suggested that they can compensate for each other in the translocation of molybdoenzymes and [NiFe] hydrogenases to a certain extent, depending on the enzyme studied (32, 43). One may speculate that the Hcf106 analogs function in concert as a membrane-bound receptor complex and thus respond to variations in the tertiary and

oligomeric structures of the different Tat substrates. The question remains whether the Hcf106 analogs are sufficient to select, proofread, and guide the various metalloproteins through a core component. From this point of view, it is noteworthy that the formation of physiologically active Tat-dependent enzymes requires individual sets of auxiliary proteins. We have shown that eight accessory genes are involved in the energy generation by the MBH in *R. eutropha*. However, the functions of six of these gene products are still unknown (3). Even much less is known about accessory genes of the *nap* cluster and the *nos* locus of *R. eutropha*. Work is in progress to elucidate whether those accessory gene products assist the coordination of cofactor insertion and Tat-mediated translocation.

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