The EMBO Journal vol.12 no.9 pp.3373–3383, 1993

Bradyrhizobium japonicum TlpA, a novel membrane-anchored thioredoxin-like protein involved in the biogenesis of cytochrome aa_3 and development of symbiosis

Hannes Loferer, Michael Bott and Hauke Hennecke[1]

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, ETH-Zentrum, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland

[1]Corresponding author

Communicated by A.Holmgren

We report the discovery of a bacterial gene, *tlpA*, that codes for a hitherto unknown type of thioredoxin-like protein. The gene was found in the course of studying a Tn*5* insertion mutant of the soybean root nodule symbiont *Bradyrhizobium japonicum*. The TlpA protein shared up to 31% amino acid sequence identity with various eukaryotic and prokaryotic thioredoxins and protein disulfide isomerases, and possessed a characteristic active-site sequence, Trp-Cys-Val-Pro-Cys. In contrast to all members of the thioredoxin family known to date, TlpA was shown to be anchored to the cytoplasmic membrane by means of an N-terminal transmembrane domain, while the active site-containing part of the protein faced the periplasm. The *tlpA* mutant had a pleiotropic phenotype in that it was defective in the development of a nitrogen fixing endosymbiosis and exhibited a strongly decreased oxidase activity, as compared with the wild-type. Holocytochrome aa_3 was spectroscopically undetectable in the mutant, whereas the apoprotein of subunit one (CoxA) of this oxidase was still synthesized and incorporated into the cytoplasmic membrane. Since cytochrome aa_3 is not a prerequisite for the development of symbiosis, the results suggest that TlpA is involved in at least two independent cellular processes, one of which is an essential periplasmic step in the maturation of cytochrome aa_3.

Key words: cytochrome aa_3/cytochrome biogenesis/ disulfide bond/membrane anchor/thioredoxin-like protein

Introduction

Thioredoxins are known as soluble redox proteins of low molecular mass (~12 kDa), which contain the unique active site sequence Trp-Cys-Gly-Pro-Cys (Gleason and Holmgren, 1988). Thioredoxins from different prokaryotic and eukaryotic sources share ~50% sequence identity at the amino acid level. They have been discovered in Archea (Guagliardi *et al.*, 1992), Eubacteria (Gleason and Holmgren, 1988), yeast (Gan, 1991), plants and mammals (Holmgren, 1985), which suggests that thioredoxins are ubiquitous in living cells. The three-dimensional structure of the oxidized form of *Escherichia coli* thioredoxin (Trx-S_2) has been determined to 1.68 Å resolution by X-ray crystallography (Katti *et al.*, 1990). Special features of this protein are the high degree of secondary structure with ~75% of the amino acid residues in α-helices, β-sheets or reverse turns, and the exposure of the active site on the surface of the protein molecule. Various biochemical functions of thioredoxins have been described. The reduced forms [Trx$(SH)_2$] are powerful protein disulfide reductases (Holmgren, 1985, 1989). The *E.coli* thioredoxin has been characterized (i) as a hydrogen donor *in vitro* for ribonucleotide reductase (Thelander and Reichard, 1979) as well as for sulfur-metabolism enzymes (Tsang and Schiff, 1976), (ii) as an essential subunit of phage T7 DNA polymerase (Mark and Richardson, 1976) and (iii) as a component required for the assembly of the filamentous phages f1, fd and M13 (Russel and Model, 1985; Holmgren, 1989). There is evidence from experiments with *Rhodobacter sphaeroides* that thioredoxin activates 5-amino levulinic acid synthase, the first enzyme in porphyrin biosynthesis in this organism (Clement-Metral, 1979). Two types of thioredoxins exist in spinach chloroplasts: thioredoxin *f*, which activates fructose-1,6-bisphosphatase, and thioredoxin *m*, which activates NADP-dependent malate dehydrogenase (Schürmann *et al.*, 1981; Buchanan, 1986).

Protein disulfide isomerase (PDI), a protein located in the rough endoplasmatic reticulum of eukaryotic cells, consists of four domains, two of which show considerable homology to bacterial thioredoxins (Edman *et al.*, 1985). PDI is supposed to catalyze native disulfide bond formation in secreted proteins. This protein also serves as the β-subunit of prolyl-4-hydroxylase, an enzyme involved in collagen biosynthesis. The glycosylation site binding protein involved in cotranslational N-glycosylation of secreted proteins in eukaryotes shares 90% amino acid sequence identity with rat PDI. Finally, the isoenzyme I of rat phosphoinositide-specific phospholipase C contains two domains that are homologous to thioredoxins in the same relative positions as they occur in PDI (for reviews see Freedman, 1989; Noiva and Lennarz, 1992). In conclusion, there are members of a so-called thioredoxin family which seem to participate in various cellular processes and whose precise functions still remain obscure in many cases.

Recently a 21 kDa periplasmic protein (DsbA) of *E.coli* was identified which catalyzes the formation of disulfide bonds in several exported proteins (Bardwell *et al.*, 1991; Kamitani *et al.*, 1992). Biochemical studies showed that DsbA has a PDI-like activity *in vitro* (Akiyama *et al.*, 1992). Subsequent work led to the detection of similar proteins in *Vibrio cholerae* and *Haemophilus influenzae*, with ~40% amino acid sequence identity to DsbA (Peek and Taylor, 1992; Tomb, 1992; Yu *et al.*, 1992). All of these proteins share a conserved active-site sequence, Cys-Pro-His-Cys, comparable to that found in thioredoxins and protein disulfide isomerases, yet no overall amino acid sequence homology can be detected. This suggests that the DsbA-like proteins form a new family of enzymes involved in the formation of disulfide bonds within proteins located in the bacterial periplasm.

Previous genetic work done in our laboratory on

© Oxford University Press

Bradyrhizobium japonicum, a Gram-negative nitrogen-fixing soil bacterium capable of establishing a root nodule symbiosis with soybean, resulted in the identification of several components involved in respiration (Thöny-Meyer *et al.*, 1989; Bott *et al.*, 1990, 1991; Preisig *et al.*, 1993). When grown aerobically, *B.japonicum* expresses a mitochondria-type respiratory chain with cytochrome aa_3 as the terminal oxidase (Appleby, 1969; Bott *et al.*, 1990). Despite an increasing body of genetic and biochemical information about this oxidase type in various bacterial systems (Saraste, 1990), the questions of how these membrane protein complexes are correctly assembled and how their cofactors (Cu and heme) are incorporated are largely unanswered.

In this paper we report on the identification of a *B.japonicum* gene (named *tlpA*) encoding a novel thioredoxin-like protein. The data suggest that TlpA is membrane anchored at its N-terminus, whereas its active site is exposed to the periplasm. A mutation in the *tlpA* gene leads to defects in cytochrome aa_3 maturation and the development of a nitrogen fixing symbiosis. To our knowledge, TlpA is the first thioredoxin-like protein described as being membrane anchored and essential for the biogenesis of a cytochrome oxidase.

Results

Phenotypic characterization of B.japonicum mutant COX64

A random Tn*5*-induced mutagenesis of *B.japonicum* was performed previously, and the insertion mutants were screened for a cytochrome *c* oxidase-negative phenotype using *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as the artificial electron donor (Bott *et al.*, 1990). Mutant COX64 was one of 16 TMPD$^-$ mutants obtained. Southern blot hybridizations proved that only one copy of Tn*5* was present in the chromosome of strain COX64 (data not shown).

A TMPD$^-$ mutant is generally expected to have one or several defective respiratory components. To identify such a defect, the mutant's cytochrome composition was investigated by *in vivo* difference spectroscopy and compared with that of the wild-type (Figure 1) using aerobically grown cells. In the wild-type spectrum, peaks characteristic for *c*-type cytochromes at 522 and 552 nm, as well as shoulders at 529 and 560 nm characteristic for *b*-type cytochromes, were detected. The presence of cytochrome aa_3 in the wild-type was indicated by the absorpion peak at 603 nm. By contrast, the COX64 spectrum clearly showed an absence of cytochrome aa_3, while *b*- and *c*-type cytochromes were still present (Figure 1). The undisturbed presence of all *c*-type cytochromes in COX64 was further verified by SDS–PAGE of proteins present in the soluble and membrane fractions, as detected with a heme-staining procedure (Francis and Becker, 1984). There was no difference in the heme-staining pattern between wild-type and COX64 (data not shown). Southern blot hybridization experiments verified that the Tn*5* insertion occurred neither in *coxA*, the structural gene of subunit I of the cytochrome aa_3 complex (Bott *et al.*, 1990), nor in the DNA region that is supposed to encode the other subunits of *B.japonicum* cytochrome aa_3 (using specific *Paracoccus denitrificans* DNA probes; all data not shown). These results indicate that COX64 is unable to synthesize a functional cytochrome aa_3, even though the cytochrome aa_3 structural genes are not affected by the Tn*5* insertion.

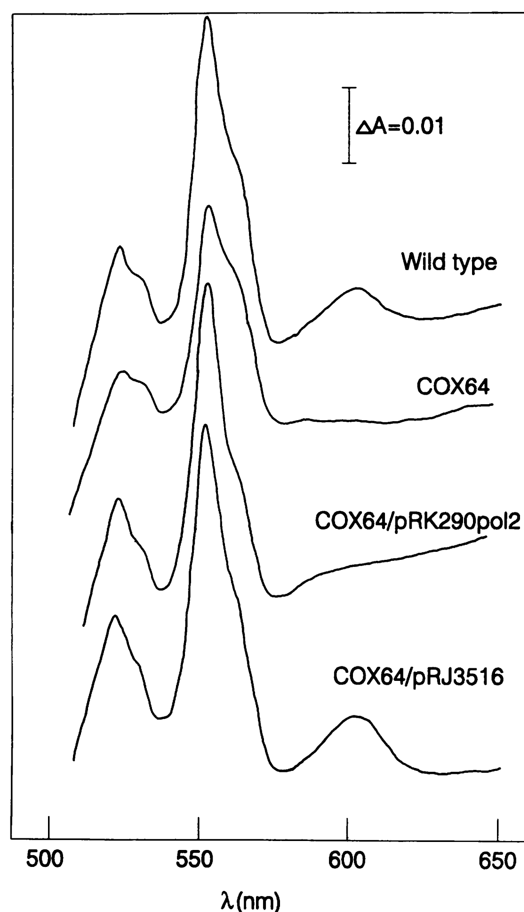

Fig. 1. *In vivo* difference spectra (dithionite-reduced minus air-oxidized) of aerobically grown cells (3.5 mg protein/ml) of *B.japonicum* wild-type, mutant COX64 without or with vector (pRK290pol2) and mutant COX64 complemented with plasmid pRJ3516.

Table I. Whole cell TMPD-oxidase activity of *B.japonicum* wild-type and mutant strains

Strains	Specific activity (nmol O_2/min/mg)	% of wild-type activity
Wild-type	25.2 ± 7.3	100
COX64 (*tlpA*$^-$)	5.3 ± 4.1	21
COX132 (*coxA*$^-$)	5.3 ± 2.5	21

Respiration rates were then determined using a Clark-type oxygen electrode and TMPD/ascorbate as the artificial electron-donating system. Aerobically grown cells of the *B.japonicum* wild-type, of mutant COX64 and of mutant COX132 (a *coxA*::Tn*5* mutant included as control) were harvested in the late exponential phase and analyzed for specific TMPD-oxidase activity (see Materials and methods). The respiration rates of the mutant strains COX64 and COX132 were found to be drastically affected, both showing only 21% of residual activity as compared with wild-type cells (Table I). This residual activity is probably brought about by the expression of alternative oxidases in *B.japonicum*, as this bacterium has an obligatory respiration-

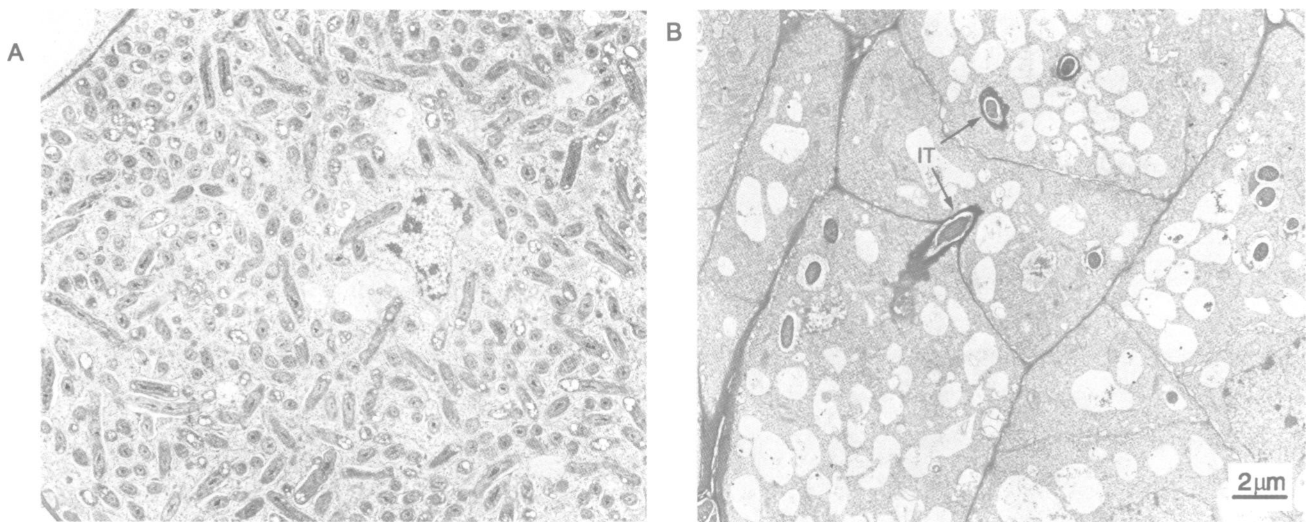

Fig. 2. Electron microscopic examination of soybean root nodule cells. (**A**) Nodules induced by *B.japonicum* wild-type. (**B**) Nodules induced by mutant COX64. IT = infection thread. Bar in the lower right corner applies to both parts of the figure. Photograph was taken by Dr René Hermann.

dependent mode of energy conservation (Bott *et al.*, 1992; Preisig *et al.*, 1993). When grown aerobically in PSY medium, strain COX64 showed only a marginally longer generation time (9 h) than the wild-type (7 h). COX64 also grew well in minimal media, showing that the Tn*5* insertion did not result in auxotrophy.

While the COX64 mutant was able to induce nodule formation on soybean (*Glycine max* L. Merr.), no symbiotic nitrogen-fixation activity could be detected (Fix^- phenotype). The nodules were green inside, indicating that functional leghemoglobin was not synthesized. Electron microscopic analysis of nodules harvested 10 days after inoculation revealed that there were no bacteroids in nodules induced by strain COX64 (Figure 2B), whereas bacteroids had already been formed in wild-type nodules (Figure 2A). The few bacteria seen in nodules of COX64 still reside within the infection threads. Obviously, the mutant bacteria fail to establish themselves as bacteroids inside the plant cell. An inspection of nodules harvested 21 days after inoculation led to similar results (not shown).

Cloning and sequencing

The Tn*5*-containing 9.5 kb *Eco*RI DNA fragment of strain COX64 was cloned out of total DNA in the Bluescript vector pKS+, yielding plasmid pRJ3506. The cloned DNA fragment was re-introduced into the *B.japonicum* wild-type by double homologous recombination, which resulted in a mutant strain that had phenotypic properties indistinguishable from those of COX64. This proved that the insertion itself rather than any secondary mutation was the cause of the observed phenotypes. A 1.75 kb *Eco*RI–*Bam*HI DNA fragment isolated from pRJ3506, which consists of *B. japonicum* DNA flanking Tn*5*, was then used as a hybridization probe to screen a genomic library of *B.japonicum* 110*spc*4 DNA constructed in a λ EMBL4 vector. Two hybridizing recombinants were detected, one of which was chosen for detailed analysis. A 1.6 kb *Xho*I DNA fragment was subcloned into Bluescript vector pSK+ (giving pRJ3519) and sequenced on both strands. Figure 3 shows a restriction map of this DNA fragment together with the sequencing strategy.

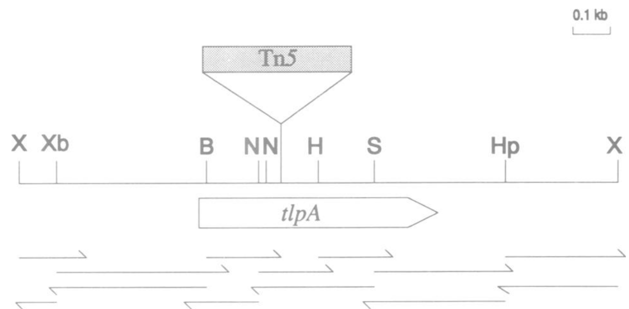

Fig. 3. Physical map of the cloned and sequenced 1.6 kb *Xho*I fragment carrying the *tlpA* gene. The position of the Tn*5* insertion in the mutant strain COX64 is indicated. The sequencing strategy is shown at the bottom. Restriction sites: B, *Bam*HI; H, *Hin*dIII; Hp, *Hpa*I; N, *Nco*I; S, *Sal*I; X, *Xho*I; Xb, *Xba*I.

The nucleotide sequence is presented in Figure 4. With the aid of the UWGCG computer program 'CodonPreference' in combination with the codon usage table of *B.japonicum* 'group III genes' (Ramseier and Göttfert, 1991), only one ORF of 666 nucleotides was found, which translated into 221 amino acids. The ORF started with an ATG at position 355 and ended with a TAA codon at position 1020. No obvious transcription terminator structure followed the stop codon. The ATG at position 343 was not considered as a start codon, because there was no putative ribosome binding site in the preceding sequence. The precise position of the Tn*5* insertion in COX64 was determined by sequencing clones containing the junction between Tn*5* and *B.japonicum* DNA (Figure 4). A target site duplication of nine nucleotides (5'-CCCCTGAAG-3', positions 568–576), which is characteristic after Tn*5* transposition events (Berg and Berg, 1983), was detected at both ends of the transposon (Figure 4).

Complementation of B.japonicum COX64

In order to prove that the observed phenotypes of COX64 were due not to polar effects of the Tn*5* insertion on downstream genes but to disruption of the identified ORF, a complementation experiment was performed. For this

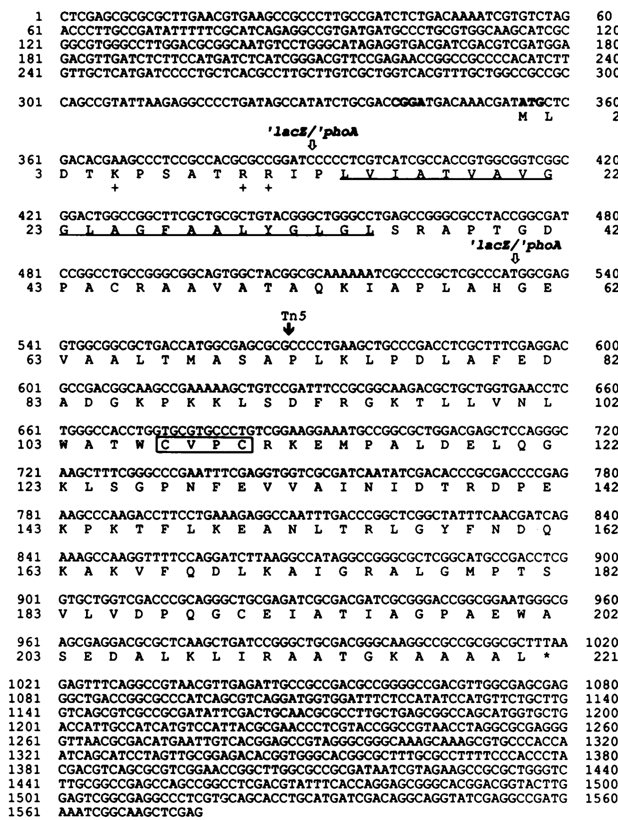

Fig. 4. Nucleotide sequence of the 1.6 kb *Xho*I fragment and the deduced amino acid sequence of the TlpA protein. A putative ribosome binding site and the start codon are shown in bold-face letters. The active-site amino acid sequence is boxed. The putative transmembrane helix is underlined and positively charged amino acids at the N-terminus are marked by the symbol +. The translational fusion sites to *'lacZ* and *'phoA* as well as the site of Tn*5* insertion in COX64 are indicated by vertical arrows. These sequence data will appear in the EMBL/Genbank Nucleotide Sequence Data Libraries under the accession number Z23140.

purpose, the 1.6 kb DNA fragment from plasmid pRJ3519 (Figure 3) was cloned into the broad host range vector pRK290pol2, resulting in plasmid pRJ3516. This plasmid was then mobilized into the *B.japonicum* wild-type (strain 110*spc*4) and into the mutant COX64. Both recipient strains (containing only the vector pRK290pol2) served as controls. Aerobically grown cells were tested for their ability to oxidize TMPD (Bott *et al.*, 1990). COX64 carrying pRJ3516 showed an immediate blue colour reaction in this test (<5 s) just like the wild-type control, whereas COX64 carrying the vector pRK290pol2 had a strong delay in colour formation like the original COX64 mutant (>30 s). Reduced-minus-oxidized difference spectroscopy was also carried out to examine the formation of cytochrome aa_3 (Figure 1). *B.japonicum* COX64 containing pRJ3516 produced cytochrome aa_3, whereas the control strain carrying only the vector was still deficient in the formation of that cytochrome. Finally, plasmid pRJ3516 was tested for its ability to complement the symbiotic phenotype of COX64. As much as 60% of wild-type Fix activity could be measured in plants infected with COX64 containing pRJ3516 (20 days after inoculation). The reddish colour of nodules, indicative of functional leghemoglobin, also reappeared. The fact that only partial complementation occurred can be explained by plasmid loss, which is usual in this type of experiment. Consistent with this explanation was that only ~55% of the bacteroids re-isolated from the nodules still carried the tetracycline resistance. All of these results showed that the 1.6 kb *Xho*I DNA fragment was able to complement the *B.japonicum* mutant COX64. We conclude, therefore, that the disruption of the sequenced ORF by the Tn*5* insertion is solely responsible for the observed phenotypes of COX64.

The tlpA gene product is a novel member of the thioredoxin family

The ORF coded for a polypeptide of 221 amino acids with a calculated molecular weight of 23 260 and a theoretical isoelectric point of 8.97. The gene product, TlpA (mnemonic for thioredoxin-like protein), showed several interesting features. After screening the NBRF protein sequence database for homologous proteins using the FASTA program (UWGCG), we found that TlpA had a striking homology to proteins of the thioredoxin family. There is 43–67% amino acid sequence identity between the bacterial thioredoxins, whereas the eukaryotic thioredoxins contain only 26–30% amino acids at identical positions to the *E.coli* protein. *B.japonicum* TlpA is 31% identical with *E.coli* TrxA. An alignment of TlpA with several members of this protein family is shown in Figure 5. Many of the amino acid residues that are of structural importance in thioredoxins are also conserved in TlpA. The region around the active site sequence (Trp-Cys-Val-Pro-Cys) is especially highly conserved. Nevertheless, there are also major differences between TlpA and other members of the thioredoxin protein family. All thioredoxins are ~100 amino acid residues long. *B.japonicum* TlpA is considerably larger (221 amino acids), primarily owing to an N-terminal extension of 72 amino acid residues. A stretch of 22 mainly hydrophobic amino acids (positions 14–35; Figures 4 and 5) is present in this part of the protein. Computer-assisted analysis using several different algorithms predicted that this part of the sequence was a transmembrane helix. Both the length of the hydrophobic stretch (22 residues) and its distance of 15 residues from a first possible leader peptidase cleavage site (Ala-Val-Ala, at positions 48–50) argued against the presence of a signal sequence (von Heijne, 1985). Furthermore, TlpA has three positively charged amino acid residues near the N-terminus, which precede the putative transmembrane helix. It is known from studies of various bacterial membrane proteins that positively charged residues flanking transmembrane helices are the main determinants of the topology of these proteins in the cytoplasmic membrane (Boyd *et al.*, 1987; McGovern *et al.*, 1991). Taking all of these features together, we had good reason to suggest that *B.japonicum* TlpA represents a novel type of a thioredoxin-like protein that is membrane anchored at its N-terminus, while its active site is exposed to the periplasmic space.

Analysis of TlpA's membrane topology

We made use of a genetic approach, which is based on the fact that translational fusions to reporter proteins such as alkaline phosphatase (PhoA) and β-galactosidase (LacZ) of *E.coli* exhibit different enzymatic activities dependent on the location of the fusion sites with respect to the cytoplasmic membrane (Manoil and Beckwith, 1986). Alkaline phosphatase is only active in the periplasm (Derman and Beckwith, 1991), whereas β-galactosidase shows activity

```
                    +    ++
BjTlpA      MLDTKPSATRRIPLVIATVAVGGLAGFAALYGLGLSRAPTGDPACRAAVATAQKIAPLAH   60

BjTlpA      GEVAALTMASAPLKLPDLAFEDADGKPKKLSDFRGKTLLVNLWATWCVPCRKEMPALDEL  120
EcTrxA                     SDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEI   45
RsTrxA                       STVPVTDATFDTEVRKSDVPVVVDFWAEWCGPCRQIGPALEEL   43
RrTrxA                        MKQVSDASFEEDVLKADGPVxVDFWAEWCGPCRQxAPALEEL   42
CnTrx-c2                   SATIVNTTDENFQADVLDAETPVLVDFWAGWCAPCKAIAPVLEEL   45
BjORF132                     MQADNVQVPGLDPAAFKGKVSLVNVWASWCVPCHDEAPLLTEL   43
SpTrxm                        VQDVNDSGWKEFVLQSSEPSMVDFWAPWCGPCKLIAPVIDEL   42
HsTrx                      MVKQIESKTAFQEALDAAGDKLVVVDFSATWCGPCKMINPFFHSL   45
BovPDI-A1     H2N-19aa-GAPDEEDHVLVLHKGNFDEALAAHKYLLVEFYAPWCGHCKALAPEYAKA   68
BovPDI-A2         -QELPDDWDKQPVKVLVGKNFEEVAFDEKKNVFVEFYAPWCGHCKQLAPIWDKL  412
                                   D               V FWA WCGPCK  AP L EL

BjTlpA      QGKLSGPNFEVVAINIDTRDPEKPKTFLKEANLTRLGYFNDQKAKVFQDLKAIGRALGMP  180
EcTrxA      ADEY-QGKLTVAKLNIDQNPGTAPKYGIR----------------------------GIP   76
RsTrxA      SKEY-AGKVKIVKVNVDENPxSPAMLGVR----------------------------GIP   74
RrTrxA      ATAL-GDKVTVAKINIDENPQTPSKYGVR----------------------------GIP   73
CnTrx-c2    SNEY-AGKVKIVKVDVTSCEDTAVKYNIR----------------------------NIP   76
BjORF132    GKDKRFQLVGINYKDAADNARRFLGRYGNPFGRVGVDANGRASIEWGVY--------GVP   95
SpTrxm      AKEY-SGKIAVTKLNTDEAPGIATQYNIR----------------------------SIP   73
HsTrx       SEKY-SNVIFLEVDVDDCQDVASECEVK-----------------------------CTP   75
BovPDI-A1   AGKLKAEGSEIRLAKVDATEESDLAQQYGVR--------------------------GYP  102
BovPDI-A2   GETYKDHEN-IVIAKMDSTANEVEAVKVH----------------------------SFP  443
               Y            D                                        G P

BjTlpA      TSVLVDPQGCEIATIAGPAEWASEDALKLIRAATGKAAAAL                     221
EcTrxA      TL-LLFKNGEVAATKVGALSKGQLKEFLDANLA                             108
RsTrxA      AL-FLFKNGQVVSNKVGAAPKAALATWIASAL                              105
RrTrxA      TL-MIFKDGQVAATKIGALPKTKLFEWVEASV                              104
CnTrx-c2    AL-LMFKDGEVVAQQVGAAPRSKLAAFIDQNI                              107
BjORF132    GVPETFVVGREGTIVYKLVGPITPDNLRSVLLPQMEKALK                      135
SpTrxm      TV-LFFKNGERKESIIGDVSKYQL                                       96
HsTrx       TF-QFFKKGQKVGEFSGANKEKLEATINELV                               105
BovPDI-A1   TI-KFFKNGDTASPKEYTA-240aa-PDI-A2-                             120
BovPDI-A2   TL-KFFPASADRTVIDYN-50aa-COOH                                  460
            T    F  G       G
```

Fig. 5. Alignment of TlpA with thioredoxins (Trx) and thioredoxin-homologous domains of protein disulfide isomerases (PDIs). Thioredoxin sequences are from *E.coli* (Ec), *Rhodobacter sphaeroides* (Rs), *Rhodospirillum rubrum* (Rr), *Corynebacterium nephredii* (Cn), spinach (Sp) and human (Hs). BovPDI-A1/A2 are the two thioredoxin-homologous domains of bovine PDI. All of these sequences were taken from the NBRF protein data base. BjORF132 is a Trx-like protein with 26% amino acid sequence identity to *E.coli* TrxA, whose gene is located in a cluster of *B.japonicum* genes important for the biogenesis of *c*-type cytochromes (Ramseier *et al.*, 1991). Conserved amino acid residues are printed in bold, with an overall consensus shown in the bottom line. The N-terminal transmembrane domain of TlpA is underlined, and the positively charged amino acids at its N terminus are indicated by + symbols.

Table II. Reporter enzyme activities derived from translational fusions of *tlpA* to *E.coli 'phoA* and *'lacZ*

Plasmid[a]	Fusion construct	Enzyme activity (Miller units)[b]	
		β-Galactosidase	Alkaline phosphatase
pRK290pol2		0.69 ± 0.29	3.27 ± 0.59
pRJ3525	H_2N–'PhoA	n.d.	3.82 ± 0.57
pRJ3527	H_2N–'LacZ	13.70 ± 1.53	n.d.
pRJ3526	H_2N–■–'PhoA	n.d.	27.41 ± 0.97
pRJ3528	H_2N–■–'LacZ	0.90 ± 0.01	n.d.

[a]The fusion constructs were present in the *B.japonicum* wild-type (110*spc*4) on the broad host range vector pRK290pol2. The black box indicates the putative membrane anchor of TlpA.
[b]Values are the mean of two independent experiments with five samples of each construct tested in parallel. n.d. not determined.

only when retained in the cytoplasm (Bieker and Silhavy, 1989). We created two translational fusions of *tlpA* to each reporter gene (*phoA* and *lacZ*) at sites that either preceded or followed the putative transmembrane domain in the translated protein. The fusion sites are marked in Figure 4. The DNA fragments encoding the four fusion proteins were cloned into the broad host range vector pRK290 and mobilized into *B.japonicum* strain 110*spc*4, and then the enzyme activities were determined (Table II). High alkaline phosphatase activity could be measured only when the fusion protein contained the putative transmembrane helix of TlpA, whereas the fusion protein lacking this domain was inactive. By contrast, β-galactosidase activity was high when the fusion protein lacked the putative transmembrane helix, whereas the form containing this domain gave only background activity (Table II). These data show that the thioredoxin-like domain of TlpA, which contains the presumptive active site, is exposed to the periplasmic space.

To prove further that the N-terminal transmembrane helix of TlpA indeed functions as a membrane anchor and is not cleaved off, cell fractionation experiments were performed. A *B.japonicum* strain, which expressed the active TlpA–PhoA fusion protein containing the putative membrane anchor, was fractionated by ultracentrifugation

Table III. Cellular location of the TlpA–PhoA hybrid protein that contains the putative transmembrane helix

Cellular fraction of 110*spc*4/pRJ3526	PhoA activity (A_{420} × 1000/min)	Cyt *c* oxidase activity (nmol cyt *c* oxidized/min)	Malate dehydrogenase activity (μmol NADH oxidized/min)
Crude extract	887.46 ± 4.2	60.0 ± 8.0	77.30 ± 3.5
Soluble fraction	32.45 ± 2.1	< 2.0	76.12 ± 4.3
Membrane fraction	813.83 ± 76.9	64.0 ± 4.0	1.85 ± 0.01

for the separation of membrane and soluble fractions. Alkaline phosphatase activities in crude extracts, soluble fractions and membranes were determined along with marker enzymes. Table III shows clearly that the PhoA activity co-fractionated with the membrane-bound cytochrome *c* oxidase activity, and separated from the soluble malate dehydrogenase activity. When the same cell fractions were subjected to Western blotting and probed with a polyclonal antiserum against alkaline phosphatase, a band migrating at the expected molecular weight (~60 kDa) could be detected in the crude extract and in the membrane fraction, but not in the soluble fraction (data not shown). These results confirmed that TlpA is anchored to the cytoplasmic membrane by means of its N-terminal hydrophobic domain.

Influence of TlpA on maturation of cytochrome aa_3

As mentioned, the mutation in the *tlpA* gene resulted in the absence of the characteristic cytochrome aa_3 absorption peak at 603 nm in the reduced-minus-oxidized difference spectrum, suggesting an absence of functional cytochrome aa_3 in the mutant. The bacterial cytochromes aa_3 are homologous to the cytochrome *c* oxidase present in mitochondria except that their subunit composition is much simpler than that of the eukaryotic homologue. Bacterial cytochrome aa_3 usually consists of only three subunits (Saraste, 1990), whereas the yeast enzyme, for example, has nine subunits (Nobrega *et al.*, 1990). Subunit I (COI), which has 12 transmembrane helices, contains the six-coordinated low-spin heme *a*, the five-coordinated high-spin heme a_3 and copper B (Cu_B), whereby the latter two form the binuclear center responsible for oxygen binding. Subunit II (COII) is membrane anchored by two helices and contains the copper A (Cu_A) and the putative binding site for cytochrome *c*. Subunit three (COIII) has seven transmembrane helices and does not contain cofactors. While COIII is not necessary for catalytic activity, it is needed for proper assembly of the enzyme complex (Haltia *et al.*, 1989). Keeping this structural information in mind, one can predict that the maturation of cytochrome aa_3 must be a complex multistep process.

Far less is known about cytochrome aa_3 from *B.japonicum*. The only information available is the DNA sequence of the *coxA* gene that encodes subunit I (COI) of the oxidase complex (Bott *et al.*, 1990). We raised anti-CoxA antibodies in rabbits as a tool to test whether or not the apoprotein of COI of *B.japonicum* cytochrome aa_3 was still expressed in the COX64 (*tlpA*$^-$) mutant. Peptide epitopes on CoxA were chosen according to an antigenicity index calculated with the aid of the computer program PEPTIDESTRUCTURE (UWGCG), and by taking into account the periplasmic or cytoplasmic loops according to the current topological models of *P.denitrificans* COI (Saraste, 1990). Two amino acid stretches were chosen for peptide synthesis (cf. Figure 4 in Bott *et al.*, 1990): H_2N-

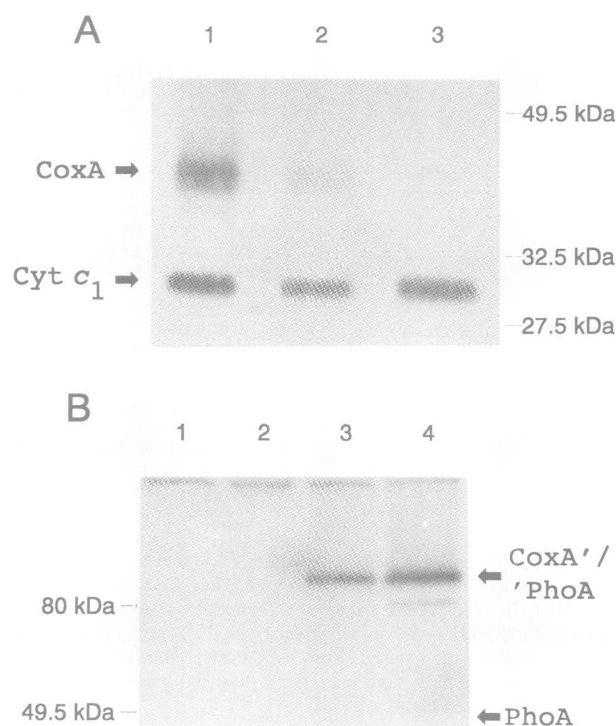

Fig. 6. Membrane protein analyses. (**A**) Western blot of membrane proteins of the *B.japonicum* wild-type (lane 1) and mutants COX64 (*tlpA*$^-$, lane 2) and COX132 (*coxA*$^-$, lane 3). Fifty micrograms of membrane proteins of each strain were separated by SDS–PAGE (12%), blotted on to a nitrocellulose membrane (see Materials and methods), and probed with anti-CoxA polyclonal serum (directed against peptide PEP2). Polyclonal antiserum against cytochrome c_1 served as an internal standard. (**B**) Western blot analysis of membrane proteins from the *B.japonicum* wild-type (lane 1), mutant COX64 (*tlpA*$^-$, lane 2) and the same two strains carrying a chromosomally integrated *coxA'*–*'phoA* translational fusion (lanes 3 and 4). Separated and blotted proteins (50 μg each) were probed with a polyclonal antiserum against *E.coli* alkaline phosphatase (kindly provided by Lars Hederstedt, University of Lund, Sweden). Molecular weight markers, the CoxA'–'PhoA fusion protein, and the position of the *E.coli* PhoA protein are indicated in the margins.

GHDEHAHPTGWRRYV-COOH, positions 16–30 in the CoxA sequence (PEP1), and H_2N-LTDRNFGTTFFAPD-GGGDPV-COOH, positions 238–257 in the CoxA sequence (PEP2). PEP1 probably corresponds to a cytoplasmic domain that precedes the first transmembrane helix of CoxA. PEP2 probably corresponds to a periplasmic loop between helices five and six (Bott *et al.*, 1990; Saraste, 1990). The peptides served as antigens in the immunization of rabbits (see Materials and methods). *B.japonicum* strains 110*spc*4 (wild-type), COX64 (*tlpA*::Tn*5*) and COX132 (*coxA*::Tn*5*, a suitable negative control) were grown aerobically to mid-exponential phase, then the membrane fractions were prepared, subjected to Western blotting and probed with antisera against PEP2 of CoxA and against

Table IV. Alkaline phosphatase activity derived from the translational *coxA'*−*'phoA* fusion integrated into the chromosome of *B.japonicum* wild-type and mutant strain COX64

B.japonicum strain	Specific PhoA activity (A_{420} × 1000/min/mg)[a]	
	Crude extract	Membrane fraction
Wild-type	0.98 ± 0.01	0.50 ± 0.06
COX64 (*tlpA*::Tn*5*)	0.80 ± 0.05	0.45 ± 0.08
W3544 (*tlpA*$^+$, *coxA'*−*'phoA*)	2.98 ± 0.06	8.37 ± 0.22
63544 (*tlpA*::Tn*5*, *coxA'*−*'phoA*)	3.24 ± 0.58	8.34 ± 0.18

[a]The values are the mean of two independent experiments with five samples tested in parallel.

cytochrome c_1 (Thöny-Meyer *et al.*, 1991), the latter serving as an internal standard (Figure 6A). In wild-type membranes, a band migrating at ~45 kDa was detected (lane 1). This band was absent in membranes of strain COX132 (lane 3) and is therefore specific for CoxA. Its different migration in SDS−PAGE as opposed to the predicted molecular weight of 59 247 is not unusual for integral membrane proteins (Lübben *et al.*, 1992). The same band could also be detected in experiments with antisera against PEP1; however, the cross-reaction was much weaker (not shown). In membranes of the *tlpA* mutant, COX64, CoxA can be detected clearly, although it is apparently present in much smaller amounts than in the wild-type (Figure 6A, lane 2). This result can be interpreted in at least two ways: (i) CoxA is expressed in strain COX64, but is more unstable than the wild-type protein, perhaps because of an improper assembly of the enzyme complex; (ii) the mutation in *tlpA* affects *coxA* expression at the transcriptional or translational level.

To obtain additional information on how TlpA may affect CoxA, we measured expression of the *coxA* gene with the aid of translational fusions. A *Bst*BI restriction site at position 1674 in the *coxA* DNA sequence (Bott *et al.*, 1990) was chosen for the construction of translational fusions to *phoA* and *lacZ*. The DNA fragments encoding the fusion proteins were cloned into the suicide vector pSUP202 and co-integrated at their homologous sites in *B.japonicum* strain 110*spc*4 (wild-type) and strain COX64 (*tlpA*$^-$). In these constructs the fusion site is located on the C-terminal side of the last transmembrane helix of CoxA, which is therefore devoid only of its last seven amino acid residues. The current topological models of COI proteins predict that this last hydrophobic domain is located on the cytoplasmic side of the membrane. For this reason we expected the translational fusion to *lacZ* to be active. However, this was not the case: no activity was detected. By contrast, the translational fusion to *phoA* showed activity. Possibly one of the last two proposed transmembrane helices near the C-terminus of CoxA is only a membrane-associated domain, thereby directing the fused PhoA to the periplasm. Table IV shows the results of an experiment, in which alkaline phosphatase activity was measured in crude extracts and in membranes. It is obvious from these data that the CoxA protein is synthesized in equal amounts in the *B.japonicum* wild-type as well as in strain COX64. Moreover, both in the wild-type and the *tlpA* mutant carrying the co-integrated fusions, a protein of ~85 kDa was found to cross-react with polyclonal antibodies specific to PhoA in a Western blot (Figure 6B, lanes 3 and 4). This protein corresponds well with the expected molecular mass of the CoxA−PhoA fusion protein. In membranes of those strains that did not carry the co-integrates, no cross-reacting protein was detected (Figure 6B, lanes 1 and 2). All of these data suggest that the CoxA protein can be synthesized and incorporated into the cytoplasmic membrane of the mutant COX64. The observed instability of the CoxA protein in strain COX64 as compared with the wild-type (Figure 6A) is therefore indicative of an improper assembly of the cytochrome aa_3 complex rather than of an effect on *coxA* expression.

Discussion

This paper deals with the discovery of a hitherto unreported bacterial thioredoxin-like protein that has all of the characteristics potentially enabling it to function as a novel periplasmic protein disulfide oxidoreductase. On the basis of the three-dimensional structure of the *E.coli* thioredoxin (Katti *et al.*, 1990), structural models of thioredoxins from different species were constructed with the aid of computer graphics (Eklund *et al.*, 1991). The models predict very similar structures for all thioredoxins and assign possible functions to conserved amino acid residues. The active-site cysteines 32 and 35 (the numbering relates to the *E.coli* thioredoxin sequence; see Figure 5) can switch between an oxidized disulfide form and a reduced dithiol form. A basic amino acid at position 36 is supposed to stabilize the thiolate anion in the active site. Tryptophan 31 may be required for protein−protein interactions. Proline at position 34 is characteristic for all thioredoxins, whereas protein disulfide isomerases have a histidine at this position. Proline 40 creates a kink in α-helix 2 of the *E.coli* thioredoxin and thereby correctly positions the active site on the surface of the protein (Eklund *et al.*, 1991). All of these amino acid residues are conserved in the *B.japonicum* TlpA protein (Figure 5). Furthermore, the two domains preceding and following the active-site cysteines, which form β-sheet 2 and α-helix 2 in the thioredoxins, share strong homology with corresponding domains in TlpA (Figure 5). According to the recently published tertiary structure predictions for thioredoxin-like proteins (Ellis *et al.*, 1992), these are the main structural characteristics of the region around the active site of thioredoxins. Taken together, a close relationship between TlpA and the thioredoxin family seems compelling.

Apart from the similarity to thioredoxins, the TlpA protein also has some special, unprecedented structural features. Neither its molecular mass (23 kDa, which is twice as large as that of the known bacterial thioredoxins) nor its N-terminally located transmembrane anchor have been encountered in other thioredoxins. Using translational fusions to *lacZ* and *phoA* and by investigating the cellular location of one fusion protein, we have shown that TlpA is anchored to the cytoplasmic membrane while the bulk of the protein is exposed to the periplasm. A topological model of TlpA that considers all of the aforementioned characteristics is shown in Figure 7. The model implies that TlpA interacts with one or several target molecules in the periplasmic compartment. In that sense there is functional analogy between TlpA and the recently discovered family of periplasmic DsbA-homologous proteins (Bardwell *et al.*, 1991; Akiyama *et al.*, 1992; Kamitani *et al.*, 1992; Peek

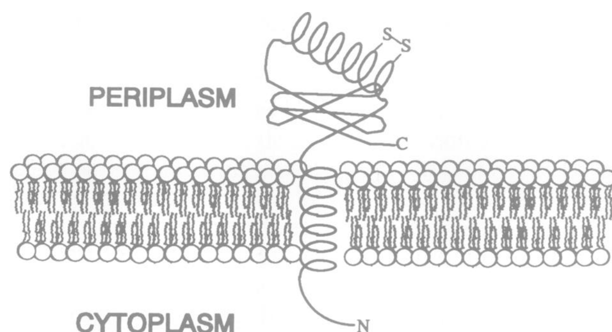

Fig. 7. Topological model of the *B.japonicum* TlpA protein in the cytoplasmic membrane. See text for details.

and Taylor, 1992; Tomb, 1992; Yu *et al.*, 1992), even though TlpA and DsbA do not share amino acid sequence similarity except for the two active-site cysteines. In contrast to TlpA, the DsbA-like proteins are soluble. It remains to be seen in future work whether or not the solubility of DsbA and the membrane location of TlpA are requirements that reflect the substrate specificity and selectivity of these two proteins. In this context it is of interest to note that *E.coli* alkaline phosphatase, a recognized natural substrate for DsbA (Bardwell *et al.*, 1991), is enzymatically active as a fusion protein in the *B.japonicum* COX64 (*tlpA*$^-$) background. Either this speaks for the presence of a DsbA-like protein also in *B.japonicum*, or else it argues against a more general role of TlpA in disulfide bond formation within periplasmic enzymes such as alkaline phosphatase. Recently it was shown that a thioredoxin is part of an enzyme complex that catalyzes a key step in β-lactam antibiotic synthesis in *Streptomyces clavuligerus* (Aharonowitz *et al.*, 1993). This raises the possibility that TlpA acts as a subunit of a larger enzyme complex.

At present, the only clues as to the biochemical function of TlpA in *B.japonicum* can be derived from the peculiar phenotypes of the *tlpA* mutant. While it may be difficult, if not impossible, to uncover the cause of the defective development of a nitrogen fixing endosymbiosis, the absence of cytochrome aa_3 in aerobically grown cells may help direct us towards the elucidation of a possible target for TlpA. As shown previously, lack of cytochrome aa_3 *per se* is clearly not detrimental to the development of symbiosis (Bott *et al.*, 1990). Hence, the defects of the *tlpA* mutant COX64 in both symbiosis and cytochrome aa_3 formation probably reflect an involvement of TlpA in at least two, if not several, independent cellular processes.

TlpA's role in cytochrome aa_3 formation could be narrowed down to some extent. We showed that *coxA* gene expression was independent of TlpA. Moreover, the membrane insertion of the apoprotein of subunit I (CoxA protein) of cytochrome aa_3 did not appear to be seriously affected in the *tlpA* mutant, although we observed a decreased steady state level of the CoxA protein, but not of the CoxA–PhoA fusion protein, in the mutant's membrane. This may be due to an increased turnover of improperly assembled cytochrome oxidase, a process that appears to be prevented by the PhoA moiety. It has been shown that mutations in yeast affecting the assembly of cytochrome oxidase also led to a destabilization of the enzyme (Nobrega *et al.*, 1990). In conclusion, we interpret our data to mean that TlpA is concerned with the maturation of cytochrome aa_3 after incorporation of the apoprotein into the membrane. This leaves three possible maturation steps in which TlpA might act: (i) synthesis and incorporation of heme *a*; (ii) uptake and incorporation of the copper cations; and (iii) correct folding of protein factors that facilitate cytochrome oxidase assembly. With regard to the first possibility, there is evidence from experiments with *Saccharomyces cerevisiae* and *Neurospora crassa* that heme is necessary for the synthesis and assembly of cytochrome aa_3 in mitochondria (Saltzgaber-Müller and Schatz, 1978; Kumar and Padmanaban, 1980). With regard to the third possibility, there is evidence for the existence of cytochrome aa_3-specific assembly factors in bacteria and yeast (Raitio *et al.*, 1987; Haltia *et al.*., 1989; Nobrega *et al.*, 1990; Saraste, 1990; Tzagoloff *et al.*, 1990; Steinrücke *et al.*, 1991; Svennson and Hederstedt, 1992). Similar proteins have not yet been found in *B.japonicum*. Finally, it seems unlikely that TlpA acts directly on the *B.japonicum* CoxA protein, because this protein contains only one cysteine residue (Bott *et al.*, 1990). It will be a major challenge in our future work to identify a target for TlpA action.

Materials and methods

Media and growth of cells

B.japonicum strains were grown aerobically at 28°C in PSY medium (Regensburger and Hennecke, 1983). Antibiotics were added at the following concentrations (μg/ml): kanamycin, 100; spectinomycin, 100; streptomycin, 100; tetracycline, 60; chloramphenicol, 10. *E.coli* was routinely grown in LB medium (Sambrook *et al.*, 1989) at 37°C. Strain JM101 was cultured as described in the Amersham protocol (1984). Antibiotics were added at the following concentrations (μg/ml): ampicillin, 100; kanamycin, 100; tetracycline, 10. Bacterial strains used or constructed in this work are listed in Table V.

Tn5 mutagenesis

Tn*5* mutagenesis of *B.japonicum* 110*spc*4 and screening for oxidase-negative mutants have been described previously (Bott *et al.*, 1990).

Cytochrome spectra

In vivo difference spectroscopy of aerobically grown *B.japonicum* cells was done at room temperature in 10 mm light-path cuvettes as described by Bott *et al.* (1990), using the double-beam mode of a Shimadzu UV-3000 spectrophotometer (600 nm/min; 2 nm slit).

DNA manipulations

Standard procedures were used for cloning, restriction mapping, Southern blotting and hybridization (Sambrook *et al.*, 1989). Chromosomal DNA of *B.japonicum* was isolated as described by Hahn and Hennecke (1984). Plasmids used or constructed in this work are listed in Table V.

DNA sequence analysis

The 1.6 kb *Xho*I DNA fragment was sequenced on both strands using overlapping M13 subclones. For overlapped sequencing of the *Bam*HI site (position 387) and the *Xho*I site (position 1572), two 15mer oligonucleotides were synthesized in a DNA synthesizer (model 380A; Applied Biosystems, Foster City, CA) and used as primers. The precise location of the Tn*5* insertion in strain COX64 was determined by sequencing the regions flanking the transposon, using a Tn*5*-specific oligonucleotide primer (Thöny *et al.*, 1985). DNA sequencing was carried out with the chain-termination method (Sanger *et al.*, 1977) and the equipment for automated DNA sequencing (Sequencer model 370 and M13-specific fluorescent dye primers from Applied Biosystems, Foster City, CA). Computer-assisted sequence analyses and comparisons were done using programs of the Genetics Computer Group of the University of Wisconsin (Madison, WI).

Construction of translational fusions

The *tlpA'*–*'phoA* fusions were constructed as follows. Plasmid pRJ3519 was digested with *Bam*HI or *Nco*I, and the ends were made blunt and ligated to a *Pst*I linker (Pharmacia, Uppsala, Sweden), yielding pRJ3520 and

Table V. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source
E.coli strains		
DH5α	*supE*44 Δ*lacU*169 (φ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *relA*1	Hanahan (1983)
HB101	*hsdR hsdM recA*13 Str[r]	Davis *et al.* (1980)
S17-1	*hsdR* /RP4-2 *kan*::Tn*5* *tet*::Mu, integrated in the chromosome	Simon *et al.* (1983)
JM101	Δ*lac-proAB thi supE*, F'(*traD*36 *pro AB*$^+$ *lacI*^{q} *Z* ΔM15)	Messing (1983)
NM539	*lacY*, *metB*, *hsdR*, *supF*, P2 *cox3*	Frischauf *et al.* (1983)
B.japonicum strains		
110*spc*4	Spc[r] (wild-type)	Regensburger and Hennecke (1983)
COX64	*tlpA*::Tn*5*, Kan[r]	This work
COX132	*coxA*::Tn*5*, Kan[r]	Bott *et al.* (1990)
W3544	110*spc*4, chromosomally integrated *coxA'*−*'phoA*	This work
63544	COX64, chromosomally integrated *coxA'*−*'phoA*	This work
Plasmids		
pKS+/−	Amp[r], f1 (±) origin	Stratagene, La Jolla, CA
M13mp18/mp19	Vectors for sequencing	Norrander *et al.* (1983)
pNM481/pNM482	Amp[r], *'lacZ*	Minton (1984)
pCH2/pCH39	Tet[r], *'phoA*	Hoffman and Wright (1985)
pSUP202	Amp[r], Tet[r], Cam[r], *oriT* from RP4	Simon *et al.* (1983)
pSUP202pol5	Tet[r], *oriT* from RP4; contains a *Bal*I site	L.Schaffner, unpublished
pRK2013	Kan[r], *tra*$^+$	Figurski and Helinski (1979)
pRK290pol2	Tet[r], pKS+ polylinker in *EcoR*I site of pRK290	P.Grob, unpublished
pRK290polSca2	*Sma*I site of pRK290pol2 replaced by *Sca*I	D.Holzhäuser, unpublished
pRJ3506	9 kb Tn*5*-containing *Eco*RI fragment from *B.japonicum* COX64	This work
pRJ3516	1.6 kb *tlpA*-containing *Xho*I fragment in pRK290pol2	This work
pRJ3517	Amp[r], *tlpA'*−*'lacZ* fused at *Bam*HI	This work
pRJ3518	Amp[r], *tlpA'*−*'lacZ* fused at *Nco*I	This work
pRJ3519	Amp[r], 1.6 kb *Xho*I fragment containing *tlpA* in pKS+	This work
pRJ3520	Amp[r], *Pst*I linker at *Bam*HI site of pRJ3519	This work
pRJ3521	Amp[r], *Pst*I linker at *Nco*I site of pRJ3519	This work
pRJ3522	Amp[r], *tlpA'*−*'phoA* fused at *Bam*HI (pKS+)	This work
pRJ3523	Amp[r], *tlpA'*−*'phoA* fused at *Nco*I (pKS+)	This work
pRJ3525	Tet[r], 2.9 kb *Xho*I fragment from pRJ3522 in pRK290pol2	This work
pRJ3526	Tet[r], 3.05 kb *Xho*I fragment from pRJ3523 in pRK290pol2	This work
pRJ3527	Tet[r], 5.5 kb *Sma*I−*Stu*I fragment from pRJ3517 in pRK290polSca2	This work
pRJ3528	Tet[r], 5.65 kb *Sma*I−*Stu*I fragment from pRJ3518 in pRK290polSca2	This work
pRJ3529	1.6 kb *tlpA*-containing *Xho*I fragment in pKS+	This work
pRJ3533	6 kb *coxA*-containing *Bam*HI fragment in pKS+	This work
pRJ3537	*Pst*I linker at *Bst*BI site of pRJ3533	This work
pRJ3539	Amp[r], *coxA'*−*'phoA* fusion at *Bst*BI site	This work
pRJ3544	*Bam*HI−*Apa*I fragment from pRJ3539 in pSUP202pol5	This work

pRJ3521. A 2.9 kb *Pst*I fragment from pCH2 or pCH39 was cloned into the *Pst*I site of pRJ3520 and pRJ3521, resulting in pRJ3522 and pRJ3523. The *Xho*I fragments containing the translational fusions (2.9 kb fragment from pRJ3522, 3.05 kb fragment from pRJ3523) were cloned into pRK290pol2, which resulted in pRJ3525 and pRJ3526, respectively.

The *tlpA'*−*'lacZ* fusions were generated as follows. A 500 bp *Xho*I−*Bam*HI fragment and a 650 bp *Xho*I−*Nco*I fragment of pRJ3519 were cloned into pNM482 and pNM481, respectively; the vectors had been digested with *Hin*dIII and the ends were made blunt and digested with *Sal*I prior to cloning. This resulted in plasmids pRJ3517 and pRJ3518. DNA containing the translational fusions was excised from these plasmids by *Sma*I−*Stu*I digestion and cloned into pRK290pol2, resulting in pRJ3527 and pRJ3528.

The *coxA'*−*'phoA* fusion was made as follows. Plasmid pRJ3533 was digested with *Bst*BI, and the ends were made blunt and ligated to a *Pst*I linker (Pharmacia, Uppsala, Sweden), yielding plasmid pRJ3537. After cloning the *phoA*-containing *Pst*I fragment from pCH39 into pRJ3537, the resulting plasmid, pRJ3539, was digested with *Bam*HI and *Apa*I, and the isolated fragment was cloned into pSUPpol5, which resulted in pRJ3544.

The fusion sites in all of the plasmid constructs were confirmed by DNA sequencing. The pRK290 derivatives were mobilized into *B.japonicum* using the *E.coli* strain HB101 containing plasmid pRK2013 as a helper strain. Integration of the *coxA'*−*'phoA* fusion into the chromosome was achieved by mobilizing the pSUP202-derivative pRJ3544 from *E.coli* S17-1 into the *B.japonicum* wild-type and strain COX64 and selecting for tetracycline-resistant clones. Co-integration at the homologous site was confirmed by appropriate Southern blot analyses.

Enzyme assays

β-Galactosidase activity was measured with 100 μl samples of five independent cultures as described by Miller (1972). Alkaline phosphatase activity of strains harbouring the *tlpA'*−*'phoA* fusions was measured as follows. Cells from 1 ml of a late exponential-phase culture were resuspended in an equal volume of 1 M Tris−HCl (pH 8.0) and lysed with 50 μl of a 0.1% solution of SDS plus 50 μl in chloroform. After equilibration of the samples at 28°C, the reaction was started by the addition of 100 μl *p*-nitrophenyl-phosphate (Sigma, St Louis, MO; 0.4% in 1 M Tris−HCl, pH 8.0). The reaction was stopped by the addition of 100 μl 1 M KH_2PO_4 and then the absorption at 420 nm was measured. Specific activity was calculated by analogy with 'Miller units' (Miller, 1972). Alkaline phosphatase activities in crude extracts and in membranes of *B.japonicum* strains W3544 and 63544 was determined in the same way, but without addition of SDS and chloroform. The specific activity was determined as A_{420} × 1000/mg protein/min. In all of the experiments it was ensured that the increase in A_{420} was linear within the investigated time range.

TMPD-oxidase activity was measured polarographically with a Clark-

type oxygen electrode (RANK Brothers, Cambridge, UK). *B.japonicum* cells were harvested in the late exponential phase, washed in 100 mM potassium phosphate buffer (pH 7.0) and injected into the electrode chamber that had been filled with the same buffer and kept at a temperature of 28°C. The final protein concentration during the measurements was ~100 μg/ml. Subsequently, 10 mM ascorbate and 0.2 mM TMPD (final concentrations) were added, and the consumption of oxygen was recorded. The activity could be inhibited completely by addition of 100 μM KCN. Net TMPD oxidase activity was determined by subtracting the endogenous and the ascorbate-induced respiration rates.

Cytochrome *c* oxidase activity was used as a marker for the membrane fraction and measured essentially as described by Gerhus *et al.* (1990). Malate dehydrogenase activity was used as a cytoplasmic marker enzyme and measured by following the oxidation of NADH at 340 nm (Kitto, 1969). Samples containing 25–100 μg protein were used.

Cell fractionation

Cells were grown to the late exponential phase, washed and resuspended in 3 ml of 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM phenylmethylsulfonylfluoride. After addition of RNase A (Sigma, St Louis, MO) and DNase I (Boehringer, Mannheim, Germany) to final concentrations of 20 μg/ml, cells were disrupted by passing them twice through a French pressure cell (18 000 p.s.i.; SLM Aminco; Lightning Instruments, Lausanne, Switzerland). After removal of the cell debris by centrifugation at 15 000 r.p.m. for 20 min (Sorvall SS34 rotor), membranes were collected via ultracentrifugation at 150 000 *g* for 90 min. The supernatant (soluble fraction) was concentrated using a Centricon-3000 ultrafiltration device (Amicon, Beverly, MA). The pellet (membrane fraction) was washed and resuspended in the same buffer described above.

Western blotting

Proteins were separated by SDS–PAGE (Laemmli, 1970) and electroblotted on to Hybond-C nitrocellulose filters (Amersham, Buckinghamshire, UK). Polyclonal rabbit antibodies raised against *E.coli* alkaline phosphatase were kindly provided by Lars Hederstedt (University of Lund, Sweden). Polyclonal antibodies specific for *E.coli* β-galactosidase were from Sigma (St Louis, MO). Anti-CoxA-peptide antibodies were custom-synthesized by Cambridge Research Biochemicals (Cambridge, UK: antibodies against PEP1) or by Neosystem (Strasbourg, France: antibodies against PEP2). Immunoglobulins bound to cross-reacting *B.japonicum* proteins were probed with goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA), and bands were finally visualized by colour development using nitrotetrazolium blue and 5-bromo-4-chloro-3-indolyl phosphate. In the course of experiments with the anti-peptide antibodies we realized that the way in which the samples were prepared prior to SDS–PAGE was crucial for the detection of the CoxA protein. When the samples were routinely boiled for 2 min before electrophoresis, no CoxA-specific band was detected. This problem was overcome simply by equilibrating the samples in SDS sample buffer at room temperature for 15 min. One possible interpretation of this phenomenon is that the highly hydrophobic CoxA protein forms aggregates during boiling which do not migrate into the gel.

Protein determination

Protein concentrations were measured using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) with bovine γ-globulin as the standard.

Plant infection test

Plant infection tests with soybean (*Glycine max* L. Merr. cv Williams) were performed as described previously (Hahn and Hennecke, 1984). With each *B.japonicum* strain, 8–10 soybean seedlings were inoculated. For electron microscopical studies (Studer *et al.*, 1987) nodules were harvested from 10- and 21-day old plants. Whole root nodule nitrogen fixation activity was determined by the acetylene reduction assay (Turner and Gibson, 1980). Additionally, the number, distribution, colour and dry weight of the nodules were recorded.

Acknowledgements

We thank our colleagues Gonzalo Acuña for providing the lambda library, René Hermann for the electron microscopic analysis, Christoph Kündig for synthesizing the oligonucleotides, and Linda Thöny-Meyer and Daniel Ritz for stimulating discussions throughout this work. We gratefully acknowledge Lars Hederstedt (University of Lund, Sweden) for generously giving anti-*E.coli* alkaline phosphatase immunoglobulins. This work was supported by grants from the Federal Institute of Technology, Zürich, and the Swiss National Foundation for Scientific Research.

References

Aharonowitz,Y., Av-Gay,Y., Schreiber,R. and Cohen,G. (1993) *J. Bacteriol.*, **175**, 623–629.

Akiyama,Y., Kamitani,S., Kusukawa,N. and Ito,K. (1992) *J. Biol. Chem.*, **267**, 22440–22445.

Amersham International plc (1984) *M13 Cloning and Sequencing Handbook*. Amersham, Buckinghamshire.

Appleby,C.A. (1969) *Biochim. Biophys. Acta*, **172**, 88–105.

Bardwell,J.C.A., McGovern,K. and Beckwith,J. (1991) *Cell*, **67**, 581–589.

Berg,D.E. and Berg,C.M. (1983) *Biotechnology*, **1**, 417–435.

Bieker,K.L. and Silhavy,T.J. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 968–972.

Bott,M., Bolliger,M. and Hennecke,H. (1990) *Mol. Microbiol.*, **4**, 2147–2157.

Bott,M., Ritz,D. and Hennecke,H. (1991) *J. Bacteriol.*, **173**, 6766–6772.

Bott,M., Preisig,O. and Hennecke,H. (1992) *Arch. Microbiol.*, **158**, 335–343.

Boyd,D., Manoil,C. and Beckwith,J. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 8525–8529.

Buchanan,B.B. (1986) In Holmgren,A., Brändén,C.-I., Jörnvall,H. and Sjöberg,B.-M. (eds) *Thioredoxin and Glutaredoxin Systems*. Raven Press, New York, pp. 233–242.

Clement-Metral,J.D. (1979) *FEBS Lett.*, **101**, 116–120.

Davis,R.W., Botstein,D. and Roth,J.R. (1980) *Advanced Bacterial Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. NY.

Derman,A.I. and Beckwith,J. (1991) *J. Bacteriol.*, **173**, 7719–7722.

Edman,C.C., Ellis,L., Blacher,R.W., Roth,R.A. and Rutter,W.J. (1985) *Nature*, **317**, 267–270.

Eklund,H., Gleason,F.K. and Holmgren,A. (1991) *Proteins*, **11**, 13–28.

Ellis,L.B.M., Saurugger,P. and Woodward,C. (1992) *Biochemistry*, **31**, 4882–4891.

Figurski,D.H. and Helinski,D.R. (1979) *Proc. Natl Acad. Sci. USA*, **76**, 1648–1652.

Francis,R.T. and Becker,R.R. (1984) *Anal. Biochem.*, **136**, 509–514.

Freedman,R.B. (1989) *Cell*, **57**, 1069–1072.

Frischauf,A.-M., Lehrach,H., Poustka,A. and Murray,N. (1983) *J. Mol. Biol.*, **170**, 827–842.

Gan,Z.-R. (1991) *J. Biol. Chem.*, **266**, 1692–1696.

Gerhus,E., Steinrücke,P. and Ludwig,B. (1990) *J. Biol. Chem.*, **172**, 2392–2400.

Gleason,F.K. and Holmgren,A. (1988) *FEMS Microbiol. Rev.*, **54**, 271–298.

Guagliardi,A., Cerchia,L., De Rosa,M., Rossi,M. and Bartolucci,S. (1992) *FEBS Lett.*, **303**, 27–30.

Hahn,M. and Hennecke,H. (1984) *Mol. Gen. Genet.*, **193**, 46–52.

Haltia,T., Finel,M., Harms,N., Nakari,T., Raitio,M., Wikström,M. and Saraste,M. (1989) *EMBO J.*, **8**, 3571–3579.

Hanahan,D. (1983) *J. Mol. Biol.*, **166**, 557–563.

Hoffman,C.S. and Wright,A. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 5107–5111.

Holmgren,A. (1985) *Annu. Rev. Biochem.*, **54**, 237–271.

Holmgren,A. (1989) *J. Biol. Chem.*, **264**, 13963–13966.

Kamitani,S., Akiyama,Y. and Ito,K. (1992) *EMBO J.*, **11**, 57–62.

Katti,S.K., LeMaster,D.M. and Eklund,H. (1990) *J. Mol. Biol.*, **212**, 167–184.

Kitto,G.B. (1969) *Methods Enzymol.*, **13**, 106–107.

Kumar,C.C. and Padmanaban,G. (1980) *J. Biol. Chem.*, **255**, 11130–11134.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lübben,M., Kolmerer,B. and Saraste,M. (1992) *EMBO J.*, **11**, 805–812.

McGovern,K., Ehrmann,M. and Beckwith,J.(1991) *EMBO J.*, **10**, 2773–2782.

Manoil,C. and Beckwith,J. (1986) *Science*, **233**, 1403–1408.

Mark,D. and Richardson,C.C. (1976) *Proc. Natl Acad. Sci. USA*, **73**, 780–784.

Messing,J. (1983) *Methods Enzymol.*, **101**, 20–78.

Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

Minton,N.P. (1984) *Gene*, **31**, 269–273.

Nobrega,M.P., Nobrega,F.G. and Tzagoloff,A. (1990) *J. Biol. Chem.*, **265**, 14220–14226.

Noiva,R. and Lennarz,W.J. (1992) *J. Biol. Chem.*, **267**, 3553–3556.

Norrander,J., Kempe,T. and Messing,J. (1983) *Gene*, **26**, 101–106.

Peek,J.A. and Taylor,R.K. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 6210–6214.

Preisig,O., Anthamatten,D. and Hennecke,H. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3309–3313.
Raitio,M., Jalli,T. and Saraste,M. (1987) *EMBO J.*, **6**, 2825–2833.
Ramseier,T.M. and Göttfert,M. (1991) *Arch. Microbiol.*, **156**, 270–276.
Ramseier,T.M., Winteler,H.V. and Hennecke,H. (1991) *J. Biol. Chem.*, **266**, 7793–7803.
Regensburger,B. and Hennecke,H. (1983) *Arch. Microbiol.*, **135**, 103–109.
Russel,M. and Model,P. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 29–33.
Saltzgaber-Müller,J. and Schatz,G. (1978) *J. Biol. Chem.*, **253**, 305–310.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
Saraste,M. (1990) *Q. Rev. Biophys.*, **23**, 331–336.
Schürmann,P., Maeda,K. and Tsugita,A. (1981) *Eur. J. Biochem.*, **116**, 37–45.
Simon,R., Priefer,U. and Pühler,A. (1983) In Pühler,A. (ed.), *Molecular Genetics of the Bacteria–Plant Interaction.* Springer Verlag, Heidelberg. pp. 98–106.
Steinrücke,P., Gerhus,E. and Ludwig,B. (1991) *J. Biol. Chem.*, **266**, 7676–7681.
Studer,D., Gloudemans,T., Franssen,H.J., Fischer,H.-M., Bisseling,T. and Hennecke,H. (1987) *Eur. J. Cell Biol.*, **45**, 177–184.
Svennson,B. and Hederstedt,L. (1992) *EBEC Short Reports*, **7**, 36.
Thelander,L. and Reichard,P. (1979) *Annu. Rev. Biochem.*, **48**, 133–158.
Thöny,B., Kaluza,K. and Hennecke,H. (1985) *Mol. Gen. Genet.*, **198**, 441–448.
Thöny-Meyer,L., Stax,D. and Hennecke,H. (1989) *Cell*, **57**, 683–697.
Thöny-Meyer,L., James,P. and Hennecke,H. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 5001–5005.
Tomb,J.-F. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 10252–10256.
Tsang,M.L.-S. and Schiff,J.A. (1976) *J. Bacteriol.*, **125**, 923–933.
Turner,G.L. and Gibson,A.H. (1980) In Bergersen,F.J. (ed.), *Methods for Evaluating Biological Nitrogen Fixation.* Wiley and Sons, Chichester, pp. 111–138.
Tzagoloff,A., Capitanio,N., Nobrega,M.P. and Gatti,D. (1990) *EMBO J.*, **9**, 2759–2764.
von Heijne,G. (1985) *J. Mol. Biol.*, **184**, 99–105.
Yu,J., Webb,H. and Hirst,T.R. (1992) *Mol. Microbiol.*, **6**, 1949–1958.

Received on May 11, 1993