The *Bradyrhizobium japonicum* napEDABC genes encoding the periplasmic nitrate reductase are essential for nitrate respiration

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The napEDABC gene cluster that encodes the periplasmic nitrate reductase from *Bradyrhizobium japonicum* USDA110 has been isolated and characterized. napA encodes the catalytic subunit, and the napB and napC gene products are predicted to be a soluble dihaem c and a membrane-anchored tetrahaem c-type cytochrome, respectively. napE encodes a transmembrane protein of unknown function, and the napD gene product is a soluble protein which is assumed to play a role in the maturation of NapA. Western blots of the periplasmic fraction from wild-type cells grown anaerobically with nitrate revealed the presence of a protein band with a molecular size of about 90 kDa corresponding to NapA. A *B. japonicum* mutant carrying an insertion in the napA gene was unable to grow under nitrate-respiring conditions, lacked nitrate reductase activity, and did not show the 90 kDa protein band. Complementation of the mutant with a plasmid bearing the napEDABC genes restored both nitrate-dependent anaerobic growth of the cells and nitrate reductase activity. A membrane-bound and a periplasmic c-type cytochrome, with molecular masses of 25 kDa and 15 kDa, respectively, were not detected in the napA mutant strain incubated anaerobically with nitrate, which identifies those proteins as the NapC and the NapB components of the *B. japonicum* periplasmic nitrate reductase enzyme. These results suggest that the periplasmic nitrate reductase is the enzyme responsible for anaerobic growth of *B. japonicum* under nitrate-respiring conditions. The promoter region of the napEDABC genes has been characterized by primer extension. A major transcript initiates 66·5 bp downstream of the centre of a putative FNR-like binding site.

INTRODUCTION

Nitrate (NO₃⁻) is reduced through nitrite (NO₂⁻), nitric oxide (NO) and nitrous oxide (N₂O) to dinitrogen (N₂) by denitrifying bacteria. Nitrate reductase catalyses the first step of the denitrification pathway, the two-electron reduction of NO₃⁻ to NO₂⁻. Two types of dissimilatory nitrate reductases have been found in denitrifying bacteria. One is known as the respiratory membrane-bound nitrate reductase (Nar enzyme), such as NADH dehydrogenase I (Berks et al., 1995; Moreno-Vivián & Ferguson, 1998; Richardson & Watmough, 1999; Richardson et al., 2001; Potter et al., 2001). Membrane-bound nitrate reductase (Nar enzyme) employs a redox loop to couple quinol oxidation with proton translocation and energy conservation, which permits cell growth under oxygen-limited conditions. Periplasmic nitrate reductase (Nap enzyme) also oxidizes quinol, but this enzyme is thought to participate indirectly in nitrate respiration by functioning in an electron-transport chain with a proton-translocating enzyme, such as NADH dehydrogenase I (Berks et al., 1995; Moreno-Vivián & Ferguson, 1998; Richardson, 2000). Nap enzymes are heterodimers consisting of a large (90 kDa) catalytic subunit, NapA, containing the molybdopterin guanine-dinucleotide cofactor (MGD) and a [4Fe–4S] cluster, and a small (13–19 kDa) electron-transfer subunit, dihaem cytochrome c, NapB. NapC is a 25 kDa membrane-bound c-type tetrahaem cytochrome which participates in electron transfer between the quinol/quinone pool and the periplasmic NapAB complex, and NapD seems to be necessary for maturation of NapA. In addition to the napDABC genes in *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) (Siddiqui et al., 1993), other nap genes present...
Bradyrhizobium japonicum is a facultatively anaerobic soil bacterium with the capability to reduce NO\textsubscript{3} and NO\textsubscript{2} (Sears et al., 2000; Ellington et al., 2002; Gavira et al., 2002), catalyses the first step of aerobic denitrification in P. denitrificans (Richardson et al., 1998), and might also have a role in adaptation to anaerobic metabolism in Ralstonia eutropha (Warnecke-Eberz & Friedrich, 1993; Siddiqui et al., 1993). In E. coli, Nap is necessary for anaerobic respiration at extremely low nitrate concentrations (Wang et al., 1999; Stewart et al., 2002). Although it has been considered that the first step of anaerobic nitrate reduction is catalysed by the classical membrane-bound nitrate reductase, the demonstration that Nap was competent in anaerobic denitrification came from studies with Pseudomonas sp. G-179 (Bedzyk et al., 1999), Rhodobacter sphaeroides f. sp. denitrificans (Liu et al., 1999), and Azospirillum brasiliense Sp245 (Steenhoudt et al., 2001).

**METHODS**

**Bacterial strains and growth conditions.** *B. japonicum* USDA110 (US Department of Agriculture, Beltsville, MD, USA) was used in this study. Peptone-salts-yeast extract (PSY) medium (Regensburger & Hennecke, 1983) and yeast extract-mannitol (YEM) medium (Vincent, 1974) were used for routine aerobic cultures of *B. japonicum*. Anaerobic cultures were kept at 28 °C in YEM medium supplemented with 10 mM KNO\textsubscript{3} in completely filled, rubber-stoppered serum bottles. Antibiotics were added to *B. japonicum* cultures at the following concentrations (µg ml\textsuperscript{-1}): gentamicin, 100; spectinomycin 200; streptomycin, 200; and kanamycin, 200. *E. coli* strains were cultured in Luria–Bertani (LB) medium (Miller, 1972) at 37 °C. *E. coli* DH5α (Stratagene) was used as host in standard cloning procedures and for the production of double-stranded plasmid DNA sequencing templates, and *E. coli* S17-1 (Simon et al., 1983) served as the donor in conjugative plasmid transfer. The antibiotics used were (µg ml\textsuperscript{-1}): ampicillin, 75–200; gentamicin, 10; streptomycin, 20; spectinomycin, 20; and kanamycin, 25.

**Routine DNA work and sequence analysis.** Chromosomal and plasmid isolations, restriction enzyme digestions, agarose gel electrophoresis, ligations, and *E. coli* transformations, were performed according to standard protocols (Sambrook et al., 1989). Hybridizations were performed with digoxigenin-dUTP-labelled probes (Roche), and the chemiluminescence method. Alternatively the direct staining method, using nitro blue tetrazolium chloride (NBT) and X-phosphate as the chromogenic substrates, was applied to detect hybridization bands. In a shotgun cloning experiment *Pst* fragments of *B. japonicum*, preselected for their size within the range of 6–15 kb, were ligated to the mobilizable vector pQ200 (Quandt & Hynes, 1993). Plasmid pPM200P9134 was thus isolated containing a 6·3 kb *Pst* fragment of cloned *B. japonicum* DNA (data not shown). After restriction analysis and partial DNA sequencing of pPM200P9134, a 1·96 kb *Apal–Pst* internal fragment of pPM200P9134 was subcloned into pQ200, yielding plasmid pPM0610 (Fig. 1). Genomic DNA of strain GRPA1 (see below)
DNA sequencer, model 4000 (MWG Biotech). The results of the separation were obtained and processed on a LI-COR.

Homology searches were performed by using the National Center Gene Base (Applied Maths, 1.0) and vector suite NTI (Informax).

Gradually cooling to 52°C for 1 h at a specific temperature regime: 30 s RNA denaturation at 78°C, cooling quickly to 62°C, and more slowly to 57-3°C with a plateau (30 s) at the optimal primer annealing temperature, then gradually cooling to 52-3°C. Primer extension was carried out withavian myeloblastosis virus reverse transcriptase (Roche) for 1 h at 42°C. Segueal Complete and Segueal XR solutions (Biozym) were used for the electrophoretic separation of cDNA products. The results of the separation were obtained and processed on a LI-COR DNA sequencer, model 4000 (MWG Biotech).

Transcript analysis. Transcripts of nap genes were analysed by primer extension. RNA was isolated from B. japonicum USDA110 grown aerobically and anaerobically in YEM medium supplemented or not with 10 mM KNO₃. In each case, 100 ml of cells were collected in prechilled tubes, cooled with liquid nitrogen, pelleted and subjected to RNA isolation as previously described (Nienaber et al., 2000). Primer extension was performed with the primer NEX-5′ (5′-GGAAACAGGAAGGGGAAAATC-3′), which is complementary to positions 51–75 downstream of the ATG start codon of napE. Five picomoles of IRD-800-labelled primer was added to anneal to 20 μg total RNA using a Biometra gradient cycler for the application of a specific temperature regime: 30 s RNA denaturation at 78°C, cooling quickly to 62-3°C, and more slowly to 57-3°C with a plateau (30 s) at the optimal primer annealing temperature, then gradually cooling to 52-3°C. Primer extension was carried out with avian myeloblastosis virus reverse transcriptase (Roche) for 1 h at 42°C. Segueal Complete and Segueal XR solutions (Biozym) were used for the electrophoretic separation of cDNA products. The results of the separation were obtained and processed on a LI-COR DNA sequencer, model 4000 (MWG Biotech).

Construction of a napA mutant. The napA gene was mutated by performing gene-directed mutagenesis by marker exchange. A 1-2 kb BamHI–PstI fragment from pPM2009P134 was subcloned into pK18mobsac (Schäfer et al., 1994) to obtain plasmid pBG602 (data not shown). Finally, the 2 kb Smal fragment (Ω SpaI/SmaI interposon) of pH45Δ (Penn et al. & Krisch, 1984) was inserted to replace a central 24 bp NrdA fragment within pBG602 (Fig. 1). The resulting plasmid pBG602Ω was transferred via conjugation into B. japonicum USDA110 using E. coli S17-1 as donor. Double recombination events were favoured by growth on agar plates containing sucrose. Mutant strains resistant to spectinomycin/streptomycin but sensitive to kanamycin were checked by Southern hybridization experiments (data not shown) for correct replacement of the wild-type fragment by the Ω interposon. The mutant derivative GRPA1, used in this study, was obtained.

Genetic complementation of the napA mutant. To obtain a recombinant plasmid containing the complete napEDABC region, the PstI–BamHI deletion derivative of pPM606-1 was cloned into pQE200 to construct plasmid pPM606-1ΔAB. pPM606-1ΔAB was then linearized by double digestion with Apal and PstI and ligated with the 1-96 kb fragment of pPM60610, which was excised by the identical enzymes (Fig. 1). The resulting recombinant plasmid (5-1 kb Apal–BamHI–BamHI fragment carrying napEDABC cloned in pQE200) was designated pPM60611 (Fig. 1). Plasmid pPM60611 was integrated by homologous recombination into the chromosome of B. japonicum GRPA1 using E. coli S17-1 as a donor, resulting in strain GRPA1C. Recombinant strains were selected for gentamicin resistance and their correct genomic structure was confirmed by Southern blot analysis of genomic DNA preparations.

Determination of nitrate reductase activity. Cells of B. japonicum grown aerobically in YEM medium were harvested by centrifugation (8000 g for 10 min at 4°C), washed twice with YEM, resuspended in 150 ml of the same medium supplemented with 10 mM KNO₃, and finally incubated under anaerobic conditions for 96 h. After incubation, the cells were washed with 50 mM Tris/HCl buffer (pH 7.5) until no nitrite was detected, and then resuspended in 1 ml of the same buffer. The reaction mixture contained 50 mM Tris/HCl buffer (pH 7-5), 10 mM KNO₃, 200 μM methyl viologen (MV⁺) or benzyl viologen (BV⁺), and 50 μl cell suspension (0-3–0-5 mg protein). The reaction was started by addition of 50 μl freshly prepared sodium dithionite solution (8 mg ml⁻¹) in Tris/HCl buffer. After incubation for 10 min at 30°C, the reaction was stopped by vigorous shaking until the samples had lost their blue colour. Nitrite was estimated after diazotation by adding the sulfanilamide/naphthylethylen diamine dihydrochloride reagent (Nicholas & Nason, 1957).

Analysis of NapA and haem-c proteins. Cells of B. japonicum grown aerobically in YEM medium (500 ml) were harvested by centrifugation as above, washed twice with YEM, resuspended in 1 l of the same medium supplemented with 10 mM KNO₃, and finally incubated under anaerobic conditions for 96 h. Preparation of periplasmic proteins was carried out as described previously (McEwan et al., 1984). Essentially, after incubation, cells were resuspended in 10 ml 100 mM Tris/HCl (pH 8-0) containing 0-5 M sucrose, 3-0 mM EDTA and 250 μg lysosome ml⁻¹, and incubated for 2 h at 30°C. The cell suspension was then centrifuged at 12 000 g for 15 min at 4°C, and the resulting supernatant, containing periplasmic proteins, was concentrated to about 250 μl by using Amicon Centriprep 3 and Centricon 3 filters. The concentrated periplasmic fractions were stored at −20°C until use. Membrane preparations were performed as described earlier (Fischer et al., 2001). For this purpose, anaerobically incubated cells were washed with 50 mM sodium phosphate buffer (pH 7-0) containing 1 mM MgCl₂, 0-1 mM CaCl₂ and 0-9 % NaCl, and resuspended in 3 ml of the same buffer containing 1 mM amidoaminophenylmethanesulfonyl fluoride (APMSF), 20 μg DNase 1 ml⁻¹ and 20 μg RNase A ml⁻¹ (fractionation buffer). Cells were disrupted by three passages through an ice-cold French pressure cell (SLM-Aminco) at a pressure of about 120 MPa. Unbroken cells were removed by centrifugation (10 000 g for 10 min at 4°C). Membranes were prepared by further centrifugation of the supernatant at 140 000 g for 2 h at 4°C. The membrane pellet was washed once with fractionation buffer, resuspended in 200 μl of the same buffer and stored at −20°C.

Periplasmic and membrane protein aliquots (30 μg) were diluted in sample buffer (124 mM Tris/HCl pH 7-0, 20% v/v glycerol, 4-6% SDS and 50 mM 2-mercaptoethanol), and incubated at room temperature for 10 min for haem-c staining, or at 100°C for 3 min for immunostaining. Membrane proteins were separated at 4°C in SDS-12% polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose filter and stained for haem-dependent peroxidase activity by chemiluminescence as described previously (Vargas et al., 1993). Periplasmic proteins were electrophoresed on a SDS 12% polyacrylamide gel for immunostaining assays or on an SDS 15–20% gel gradient for haem-c staining assays. NapA was detected by Western blotting with specific rabbit antiserum generated against the purified...
NapA subunit of Paracoccus pantotrophus (Richardson et al., 1998). Protein concentration was estimated by using the Bio-Rad assay with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Isolation and sequencing of the napEDABC genes

Sequence analysis of the DNA cloned in plasmids pPM0610, pPM060-1 and pPM060-3 showed that it contained five ORFs with a G+C content of 65 mol%. The napE, napD, napA, napB and napC ORFs are 186, 333, 2517, 471 and 642 bases in length, respectively, and encode proteins of 61 (6-6 kDa), 110 (11-8 kDa), 838 (94-5 kDa), 156 (16-9 kDa) and 213 (23-9 kDa) aa residues, respectively. The deduced primary sequences of NapA, NapB and NapC have between 46 % and 76 % identity with the translated sequences of the napA, napB and napC genes from Ralstonia eutropha H16 (Siddiqui et al., 1993), Thiosphaera pantotropha (proposed for reclassification as a strain of Paracoccus denitrificans) (Berks et al., 1995), Rhodobacter sphaeroides DMS158 (Reyes et al., 1996, 1998), R. sphaeroides f. sp. denitrificans (Liu et al., 1999), Pseudomonas sp. G-179 (Bedzyk et al., 1999), Pseudomonas aeruginosa PAO1 (Stover et al., 2000), Azospirillum brasilense (Steenhoudt et al., 2001) and Sinorhizobium meliloti (Galibert et al., 2001). In the N-terminal region of NapA, the sequence 7DRRQLMK14 corresponds closely to the twin arginine motif identified in a large number of periplasmic metalloproteins that are exported via the Sec-independent Tat translocase (Berks, 2000). A conserved [4Fe–4S] binding motif is also present at the N-terminal end of NapA. In contrast, the amino acid sequence 8RFGIALLAVAIAAG17 at the N-terminal end of NapB indicates that the protein is translocated by the general secretory pathway (Pugsley, 1993). In the NapB sequence two CXXCH motifs are identified (82CLSCH86 and 122CTECH126), representing di- haem cytochrome c binding sites, whereas in NapC there are four such motifs (69CTGCH73, 99CPDCH103, 139CRNCH163 and 159CRNCH163). Sequence analysis predicts NapE to be a membrane protein of no currently known function. The postulated role for NapD is that of a private chaperone involved in maturation of NapA prior to export to the periplasm (Berks et al., 1995; Potter & Cole, 1999). Additional nap genes such as napF, napG, napH or napK have not been detected in the B. japonicum nap genetic region. The complete genome sequence of B. japonicum USDA10 (Kaneko et al., 2002) confirms the absence of the nap genes in this micro-organism as described, and the absence of other related nap genes. Overlapping coding regions between napA and napB, as well as between the napB and napC stop and start codons, suggest translational couplings between napA, napB and napC, which has also been found in many other bacterial genomes (Richardson et al., 2001). However, unlike the translational coupling between napD and napA which was found in all cases analysed so far (Richardson et al., 2001), in B. japonicum there is a short intergenic region of 16 nt between these two genes.

Analysis of the promoter region and Nap activity

Inspection of the DNA sequence (Fig. 2c) revealed a purine-rich Shine–Dalgarno-like sequence (AGAGAGA) 13 bases upstream of the putative translational start codon of napE. Located 101 bp upstream of the putative initiation codon of

Fig. 2. (a) Mapping of the transcription start site of B. japonicum napE by primer extension. The sequence ladder shown was generated with pPM0610 DNA and the same napE primer as used for the transcript mapping. The transcriptional start site is marked with an arrow. (b) MV+-dependent nitrate reductase activity in B. japonicum USDA110. Data, in nmol NO2 produced (mg protein)−1 min−1, are shown as means, with the standard error of the mean in parentheses, for at least three cultures which were assayed in duplicate. For RNA isolation (a) and nitrate reductase assays (b), cells were cultured aerobically (lanes 1 and 2) and anaerobically (lanes 3 and 4) in YEM medium supplemented (lanes 2 and 4) or not (lanes 1 and 3) with 10 mM KNO3. (c) The napE promoter sequence. The nucleotide at which transcription initiates is shown in bold and marked +1 above. The putative FNR-binding site is indicated by two arrows. A potential Shine–Dalgarno (RBS) sequence is underlined. The putative translation start codon is shown in bold. The N-terminal sequence of the NapE protein is presented in the one-letter code.
There is the sequence $5'$-TTGAT-N$_2$-ATCAA-3'$, which has 10 out of 10 matches with the FNR consensus sequence $5'$-TTGAT-N$_2$-ATCAA-3'$ (Spiro, 1994), and 8 out of 10 matches with the FixK consensus sequence $5'$-TTGAT-N$_2$-GTCAA-3'$ (Fischer, 1994; Zumft, 1997) (Fig. 2c). Among bacteria containing periplasmic nitrate reductases, FNR boxes have been only identified in the promoter regions of *Pseudomonas* sp. G-179 *napE* (Bedzyk et al., 1999) and *E. coli napF* (Darwin et al., 1998). Computer searches revealed no other totally conserved recognition motifs in the *nap* promoter region.

 Primer extension experiments were performed to analyse *nap* transripts in cells of *B. japonicum* grown under different conditions (Fig. 2a). No transcript was detected when RNA from aerobically grown cells was used, regardless of the presence or the absence of nitrate in the medium (Fig. 2a, lanes 1 and 2). In contrast, reverse transcription with RNA isolated from anaerobically grown *B. japonicum* USDA110 revealed the presence of a transcriptional start site that initiates at an A, 26 nt upstream of the putative translational start codon (Fig. 2a, c). Levels of cDNA obtained after extension with the primer PEX-nap were higher in nitrate-respiring cells than in those that were incubated anaerobically in the absence of nitrate (Fig. 2a, lanes 3 and 4, respectively). Only basal rates of periplasmic MV$^+$-dependent nitrate reductase activity were found in cells of *B. japonicum* USDA110 grown aerobically with or without nitrate (Fig. 2b, lanes 1 and 2). Activity values increased when the cells were incubated anaerobically in the absence of nitrate, and maximal rates were obtained after growth under anaerobic conditions with nitrate (Fig. 2b, lanes 3 and 4, respectively). These results agree with those observed in the transcript mapping experiments. Anaerobic induction of the *nap* genes is in line with the presence of a putative FNR box upstream of the transcriptional start of *napE*. The region homologous to the FNR-binding domain is centred 66-5 bases upstream of the transcriptional start site (Fig. 2c), which suggests that *B. japonicum* *napE* promoter could be considered as a class I FNR-dependent promoter (Wing et al., 1995). The *E. coli* FNR-dependent *napF* promoter is also a type I promoter centred 64-5 bases upstream of the transcriptional start site (Darwin et al., 1998). The Fnr homologue responsible for anaerobic control of *B. japonicum* *nap* genes expression is not known. Anaerobic expression of the *B. japonicum nirK* (Velasco et al., 2001) and *norCBQD* (Mesa et al., 2002) denitrification genes is regulated by FixK$_2$, a member of the FNR- (fumarate and nitrate reductase regulator) and CRP- (cyclic AMP receptor) like transcriptional regulators from bacteria. FixK$_2$ has also been shown to activate genes involved in anaerobic and microaerobic metabolism (Fischer, 1994; Nellen-Anthamatten et al., 1998). Thus the possibility exists that FixK$_2$ also regulates *nap* transcription in response to low-oxygen conditions. As indicated above, besides the absence of oxygen, expression of the *B. japonicum* *nap* genes was further induced by the presence of nitrate. Induction of *nap* transcription by nitrate under anaerobic conditions has also been demonstrated in *E. coli* (Darwin et al., 1998), but has not been observed in other bacteria such as *Paracoccus pantotrophus* (Sears et al., 2000) and *Rhodobacter sphaeroides* DSM158 (Gavira et al., 2002). These findings highlight differences in the regulation of functionally different Nap systems. Besides FixK$_2$, another protein, NnrR, has been shown to be required for N-oxide regulation of the *B. japonicum nirK* and *norCBQD* genes (Mesa et al., 2003). Whether or not this is the case for the *nap* genes is not known.

**Mutation of napA and complementation analysis**

To confirm the function of the *napA* gene product in nitrate metabolism, the gene was mutated by marker-exchange mutagenesis (Fig. 1). In contrast to *B. japonicum* USDA110, cells of the mutant strain GRPA1 were unable to grow anaerobically with nitrate as the final electron acceptor (Fig. 3). Both strains, however, grew well when the YEM medium was amended with 0.5 mM nitrite (data not shown). Complementation of strain GRPA1 with the chromosomally integrated plasmid pPM0611 containing the wild-type *napEDABC* genes restored the ability of the cells to grow on nitrate as the alternative electron acceptor (Fig. 3). Because antibiotics were used to keep the selective pressure on strain GRPA1C, growth of the complemented mutant was most likely delayed as compared with that observed for the parental strain.

No differences in nitrate reductase activity were found in cells of USDA110 when either MV$^+$ or BV$^+$ was used as electron donor (Table 1). Because the cell membrane is permeable to BV$^+$ (Bell et al., 1990), the presence of membrane-bound nitrate reductase would result in a higher activity when BV$^+$ is used as an electron donor as compared to that obtained with MV$^+$. Whereas no BV$^+$- or MV$^+$-dependent activity was detected in the *napA* mutant, activities were found in cells of USDA110 when either MV$^+$ or BV$^+$ was used as electron donor (Table 1). Because the cell membrane is permeable to BV$^+$ (Bell et al., 1990), the presence of membrane-bound nitrate reductase would result in a higher activity when BV$^+$ is used as an electron donor as compared to that obtained with MV$^+$.

**Fig. 3.** Nitrate-dependent anaerobic growth of wild-type *B. japonicum* USDA110 (■), *napA* mutant derivative GRPA1 (●), and *napA* mutant complemented with plasmid pPM0611 (▲). Cells were grown anaerobically in YEM medium supplemented with 10 mM KNO$_3$. Growth of the cells under anaerobic conditions was measured by monitoring the OD$_{550}$.
levels of nitrate reductase activity in cells of strain GRPA1C containing plasmid pPM0611 were similar to those detected in the parental strain (Table 1). Taken together, these results clearly indicate that the periplasmic nitrate reductase encoded by the \( \text{napEDABC} \) genes is the primary enzyme carrying out nitrate reduction in \( \text{B. japonicum} \) under oxygen-limiting conditions. In many of the best-characterized denitrification systems, it was long considered that the membrane-bound nitrate reductase catalyses the first step of anaerobic denitrification (Zumft, 1997) and that the Nap system was more important for aerobic denitrification (Richardson \textit{et al}., 1998). However, this work clearly gives another example among the order \textit{Rhizobiales}, where, as has been shown in \textit{Pseudomonas} sp. G-179 (Bedzyk \textit{et al}., 1999), the Nap enzyme can support anaerobic growth by reducing nitrate to nitrite which can be further reduced by the reactions of denitrification. DNA sequences showing homology with those published encoding the Nar system (\( \text{narGHI} \) genes) have not been found in the complete genomic sequence of \( \text{B. japonicum} \) USDA110 (Kaneko \textit{et al}., 2002). Other members of the \textit{Rhizobiales} such as \textit{Sinorhizobium meliloti} (Galibert \textit{et al}., 2001), \textit{Agrobacterium tumefaciens} (accession no. AE008245) and \textit{Pseudomonas} sp. G-179 (Bedzyk \textit{et al}., 1999) lack a membrane-bound-like nitrate reductase, but contain a periplasmic nitrate reductase.

### Immunoblot and haem-c analysis

Proteins from the periplasmic fraction of \( \text{B. japonicum} \) cells were isolated and Western blots were developed with \( \text{P. pantotrophus} \) NapA polyclonal antibodies. Whereas a protein band with a molecular size of about 90 kDa was detected in cells of USDA110, a similar band was not observed in cells of the mutant strain GRPA1 (Fig. 4a, lanes 1 and 2, respectively). These results demonstrate that the \( \text{B. japonicum} \) NapA protein is located in the periplasmic fraction, consistent with its sequence-deduced properties. When proteins from the periplasmic fraction were stained for covalently bound haem proteins, two stained bands, of 15 and 12 kDa, respectively, were detected in the periplasmic fraction of \( \text{B. japonicum} \) USDA110 (Fig. 4b, lane 1).

### Table 1. Nitrate reductase activity in \( \text{B. japonicum} \) parental and mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Electron donor</th>
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<tr>
<td></td>
<td></td>
<td>MV(^+)</td>
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<tr>
<td>USDA110</td>
<td>Wild-type</td>
<td>50±3.0</td>
</tr>
<tr>
<td>GRPA1</td>
<td>( \text{napA} )</td>
<td>0.6±0.3</td>
</tr>
<tr>
<td>GRPA1C</td>
<td>( \text{napA} ) with chromosomally integrated pPM0611</td>
<td>48±3.5</td>
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Fig. 4. Western blot analysis (a) and haem-stained proteins (b) in periplasmic fractions from cells of \( \text{B. japonicum} \) strains USDA110 (lane 1) and GRPA1 (lane 2). (c) Haem-stained proteins in membranes from cells of \( \text{B. japonicum} \) strains USDA110 (lane 1) and GRPA1 (lane 2). Periplasmic and membrane fractions were prepared from cells incubated anaerobically in YEM medium supplemented with 10 mM KNO\(_3\). Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunostained (a) or stained for covalently bound haem proteins (b, c). Each lane contained about 30 \( \mu \)g protein. Haem-stained c-type cytochromes identified previously (Preisig \textit{et al}., 1993) are indicated on the right (c). Apparent molecular masses of the proteins are shown on the left.
Whereas the 12 kDa protein was clearly visible in the napA mutant, the concentration of the 15 kDa protein was lower than that in the parental strain (Fig. 4b, lane 2). The 12 kDa c-type cytochrome has been identified previously as the cytochrome c$_{550}$ encoded by cycA and required for nitrate respiration (Bott et al., 1995). It is possible that the weak 15 kDa protein band still present in the periplasmic fraction of the napA mutant might correspond to another soluble c-type cytochrome co-migrating with NapB, which is absent in the mutant. In fact, the presence of a soluble cytochrome c$_{550}$ of about 15 kDa has been demonstrated in soluble crude extracts from B. japonicum (Bott et al., 1995), which might also be present in anaerobically incubated cells.

To establish the presence of the NapC cytochrome, proteins from the membrane fractions of the wild-type and mutant strain were separated by SDS-PAGE and stained for covalently bound haem proteins. Six stained bands, of 32, 28, 25, 24, 20 and 16 kDa, were detected (Fig. 4c, lane 1). The proteins of 28, 20 and 16 kDa have been identified previously as the B. japonicum cytochrome c$_{1}$ (Thöny-Meyer et al., 1988), CycM (Bott et al., 1991) and the NorC subunit of the nitric oxide reductase enzyme (Mesa et al., 2002), respectively. As described by Preisig et al. (1993), there is even a seventh protein of 28 kDa co-migrating with cytochrome c$_{1}$. This 28 kDa protein and the 32 kDa c-type cytochrome have been identified as the B. japonicum FixP and FixO proteins, respectively, of the cbb$_{3}$-type, high-affinity cytochrome oxidase encoded by the fixNOQP operon (Preisig et al., 1993, 1996). A haem-stainable band of approximately 25 kDa, which is the predicted size for NapC, is very prominent in anaerobic wild-type membranes and absent in the napA mutant GRPA1 (Fig. 4c, lanes 1 and 2), which identifies this protein as the NapC component of the B. japonicum USDA110 periplasmic nitrate reductase. The concentration of FixP and FixO/c$_{1}$ c-type cytophores in the napA mutant was lower than in the parental strain (Fig. 4c, lanes 1 and 2). FixP and FixO are not synthesized under aerobic conditions and, because of the inability of the mutant strain to grow anaerobically with nitrate, the expression of those proteins during the anaerobic incubation period might be reduced.

Membranes from strain GRPA1 also lacked the 16 kDa NorC protein (Fig. 4c, lane 2), which suggests that nitrate reduction by NapA is required for NorC expression.

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