Molybdate-dependent expression of the periplasmic nitrate reductase in *Bradyrhizobium japonicum*

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Abstract

The napEDABC genes of *Bradyrhizobium japonicum* encode the periplasmic nitrate reductase, an Mo-containing enzyme which catalyses the reduction of nitrate to nitrite when oxygen concentrations are limiting. In this bacterium, another set of genes, modABC, code for a high affinity ABC-type Mo transport system. A *B. japonicum* modΔ mutant has been obtained that is not capable of growing anaerobically with nitrate and lacks nitrate reductase activity. Under nitrate respiring conditions, when Mo concentrations are limiting, the *B. japonicum* modΔ mutant lacked both the 90 kDa protein corresponding to the NapA component of the periplasmic nitrate reductase, and the membrane-bound 25 kDa c-type cytochrome NapC.

Regulatory studies using a napE-lacZ fusion indicated that napE expression was highly reduced in the modΔ mutant background when the cells were incubated anaerobically with nitrate under Mo-deficient conditions.

Introduction

Species of the order *Rhizobiales* are best characterized for their ability to establish N₂-fixing symbioses with leguminous plants. *Bradyrhizobium japonicum*, the microsymbiont of soybeans, is also able to respire nitrate under oxygen-limiting conditions. During denitrification, the Mo-containing Nap (periplasmic nitrate reductase) catalyses the first step of nitrate reduction leading to the formation of N₂ via intermediates such as nitric oxide (NO) and nitrous oxide (N₂O) [1]. In *B. japonicum*, the modABC genes code for a high-affinity ABC-type Mo transport system. A *B. japonicum* modΔ mutant has been obtained that is not capable of growing anaerobically with nitrate and lacks nitrate reductase activity. Under nitrate respiring conditions, when Mo concentrations are limiting, the *B. japonicum* modΔ mutant lacked both the 90 kDa protein corresponding to the NapA component of the periplasmic nitrate reductase, and the membrane-bound 25 kDa c-type cytochrome NapC.

Regulatory studies using a napE-lacZ fusion indicated that napE expression was highly reduced in the modΔ mutant background when the cells were incubated anaerobically with nitrate under Mo-deficient conditions.

Materials and methods

Bacterial strains and growth conditions

*B. japonicum* USDA110 (U.S. Department of Agriculture, Beltsville, MD, U.S.A.) and the modΔ 0512 mutant derivative (A. Tresierra-Ayala, C. Talbi, E.J. Bedmar and M.J. Delgado, unpublished work) have been used in this study. YEM (yeast extract-mannitol) medium [4] was used for routine aerobic cultures of *B. japonicum*. Mo-limiting conditions were obtained by preparing the YEM medium with MulliQ water and high-quality chemical products. Anaerobic cultures were kept at 28 °C in Mo-deficient YEM medium supplemented with 10 mM KNO₃ in completely filled, rubber-stoppered serum bottles as described previously [1]. When required, 0.5 μM Na₂MoO₄·2H₂O was added. *E. coli* strains were cultured in Luria–Bertani medium [5]. *E. coli* DH5α (Stratagene) was used as host in standard cloning procedures, and *E. coli* S17.1 [6] served as the donor in conjugal plasmid transfer. Appropriate antibiotics were added to bacterial cultures.

Analysis of NapA and haem-c proteins

Preparation of periplasmic proteins was carried out as described previously [1]. NapA was detected by Western blotting with specific rabbit antisera generated against the purified NapA subunit of *Paracoccus pantotrophus* [7]. Membrane preparations and detection of NapC by the chemiluminescence haem-dependent peroxidase method was performed as described earlier [1].

Construction of a napE-lacZ fusion

To construct a napE-lacZ fusion, the EcoRI–BamHI 1.5 kb fragment from plasmid pPM200P9134 [1] containing the

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**Key words:** anaerobiosis, gene regulation, molybdate transport, nap genes, nitrate respiration.

**Abbreviations used:** YEM medium, yeast extract-mannitol medium; Nap, periplasmic nitrate reductase.

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Figure 1 | Western-blot analysis (a) and haem-stained proteins (b) in periplasmic and membrane fractions, respectively, from cells of B. japonicum strains USDA110 (lane 1) and modA mutant derivative 0512 (lane 2)

Periplasmic and membrane fractions were prepared from cells incubated anaerobically in Mo-deficient YEM medium with 10 mM KNO₃. Proteins were separated by SDS/PAGE, transferred to nitrocellulose and immunostained (a) or stained for covalently bound haem proteins (b). Each lane contained about 30 µg protein. Haem-stained c-type cytochromes identified previously [1] are specified in the right margin. Apparent molecular mass of the proteins is shown on the left.

napE promoter region was subcloned into the EcoRI–BamHI sites of pMP220 [8] yielding plasmid pBG0612. To monitor napE expression, pBG0612 was used to transform E. coli S17-1, and then transferred by conjugation into B. japonicum strains USDA110 and 0512 to obtain strains 110-0612 and 0512-0612 respectively.

Analytical methods

β-Galactosidase activity was determined as previously described [5]. Protein concentration was estimated by using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with BSA as the standard.

Results and discussion

In contrast with the wild-type strain B. japonicum USDA110, the modA derivative mutant strain 0512 was not capable of growing anaerobically with nitrate in Mo-deficient YEM medium (results not shown). It is possible that traces of Mo in the medium could support growth of the parental strain, whereas the mutation in modA prevented the entry of Mo within the mutant cells. The phenotype of the parental strain was recovered in the modA mutant when the medium was supplemented with 0.5 µM sodium molybdate (results not shown). This suggests that, in addition to the high affinity ABC-type Mo transporter, B. japonicum possesses an alternative, low-affinity Mo-uptake system.

Since cells of B. japonicum 0512 did not grow anaerobically with nitrate, to determine the presence of NapA, cells of the parental and of the mutant strains were grown first aerobically in YEM medium and then incubated anaerobically in Mo-deficient YEM medium supplemented with nitrate. The periplasmic fraction from cells of B. japonicum 0512 lacked the 90 kDa protein band corresponding to NapA [1], which is present in the parental strain USDA110 (Figure 1a, lanes 1 and 2 respectively). Similarly, the protein band of about 25 kDa found in wild-type cells, which corresponds to NapC [1], was also absent in membranes from the modA mutant (Figure 1b, lanes 1 and 2 respectively).

Rates of β-galactosidase activity in cells of B. japonicum 0512-0612 containing the napE–lacZ fusion, and incubated anaerobically with nitrate in a Mo-deficient medium, were almost 6-fold lower than those detected in cells of strain 110-0612 with the napE–lacZ fusion (Table 1). These results indicate that, in addition to anaerobiosis and nitrate [1], Mo is also required for expression of the nap genes. Inclusion of 0.5 µM sodium molybdate in the incubation medium restored the level of β-galactosidase activity in the modA mutant (Table 1). While repressing expression of the modABCD operon, ModE acts as the transcriptional activator of the byc [9], narGHJI [9], dmsABC [10] and napFDAGHBC [11] operons encoding the Mo-containing enzymes hydrogenase, membrane-bound nitrate reductase, dimethyl sulphoxide reductase, and Nap respectively. MopB, a ModE homologue, has been characterized as the molybdate-responsive transcriptional repressor of modABCD gene expression in Rhodobacter capsulatus [12]. Similarly, MopB positively regulates expression of the dorCDA genes involved in dimethyl sulphoxide respiration, but is not required for the expression of the Nap or xanthine dehydrogenase in R. capsulatus [12]. The results in this paper show that

Table 1 | Expression of β-galactosidase from a napE–lacZ fusion in B. japonicum parental and modA mutant backgrounds

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Mo-deficient YEM medium</th>
<th>Mo-deficient YEM medium + 0.5 µM sodium molybdate</th>
</tr>
</thead>
<tbody>
<tr>
<td>110-0612</td>
<td>napE–lacZ wild-type</td>
<td>194 ± 10</td>
<td>184 ± 12</td>
</tr>
<tr>
<td>0512-0612</td>
<td>napE–lacZ modA</td>
<td>34 ± 3</td>
<td>162 ± 8</td>
</tr>
</tbody>
</table>

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molybdate has an effect on transcription of the B. japonicum napEDABC operon, but ModE/MopB-like proteins have not been found in the complete genome sequence of B. japonicum USDA110 ([13], see also http://www.kazusa.or.jp/rhizobase).

We thank D. Richardson for the gift of the P. pantotrophus NapA polyclonal antibodies. This work was supported by grants BMC2002-04126-C03-02 and FIT-050000-2001-30 from Dirección General de Investigación to E.J.Br. The support of the Junta de Andalucía (PAI/CVI-275) is also acknowledged.

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

Received 24 September 2004