Characterization of the norB Gene, Encoding Nitric Oxide Reductase, in the Nondenitrifying Cyanobacterium Synechocystis sp. Strain PCC6803

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A norB gene encoding a putative nitric oxide reductase is present in the genome of the nondenitrifying cyanobacterium Synechocystis sp. strain PCC6803. The gene product belongs to the quinol-oxidizing single-subunit class of nitric oxide reductases, discovered recently in the denitrifier Ralstonia eutropha. Heterologous complementation of a nitric oxide reductase-negative mutant of R. eutropha with norB from Synechocystis restored nitric oxide reductase activity. With reduced menadione as the electron donor, an enzymatic activity of 101 nmol of NO per min per mg of protein was obtained with membrane fractions of Synechocystis wild-type cells. Virtually no nitric oxide reductase activity was present in a norB-negative mutant of Synechocystis. Growing cells of this mutant are more sensitive toward NO than wild-type cells, indicating that the presence of a nitric oxide reductase is beneficial for Synechocystis when the cells are exposed to NO. Transcriptional fusions with the chloramphenicol acetyltransferase reporter gene were constructed to monitor norB expression in Synechocystis. Transcription of norB was not enhanced by the addition of the NO-generating agent sodium nitroprusside.

Nitric oxide is an important molecule in cell signaling and host defense mechanisms of eukaryotes. In prokaryotes, NO is a true intermediate of denitrification and is produced from nitrite by the dissimilatory nitrite reductase. In the course of denitrification, NO is further reduced to nitrous oxide by nitric oxide reductase. Bacterial nitric oxide reductases are integral membrane proteins which are connected to an electron transport chain. Nitric oxide reductases purified from denitrifying bacteria are classified as cytochrome cb heterodimers, consisting of a catalytic subunit, NorB, and a small subunit, NorC (NorCB enzyme) (12, 36, 42).

The prototype of a novel class of nitric oxide reductases was discovered in the β-proteobacterium Ralstonia eutropha (5). The carboxy terminus of this monomeric NorB protein corresponds to the catalytic subunit of NorCB enzymes. The amino terminus of NorB is extended and contains a large, probably periplasmic domain flanked by two additional transmembrane segments (3). The purified protein accepts electrons from quinols but fails to oxidize cytochrome c (3). To distinguish between the two types of nitric oxide reductases, the NorCB enzymes are designated cNor, and the single-component enzymes are referred to as qNor (12). Database analyses of unfinished genome sequences suggest that several bacteria harbor qNor enzymes. The majority of these hosts are classified as nondenitrifying pathogens.

A norB gene homologue was discovered in the genome sequence of the unicellular cyanobacterium Synechocystis sp. strain PCC6803 (16). Recently, the question was raised as to whether the putative norB gene product is a member of the class of qNor proteins, since its sequence predicts an N-terminal extension similar to that of qNor from R. eutropha and a norC-like gene is absent in the genome of Synechocystis (5). In this study, we show that the product of norB from Synechocystis is indeed physiologically active with reduced menadione. We demonstrate that an active nitric oxide reductase confers on its host an elevated tolerance to toxic NO.

MATERIALS AND METHODS

Bacterial strains and culturing. R. eutropha H16 (ATCC 17699) is a wild-type strain harboring endogenous megaplasmid pHG1. HF420 is a nitric oxide reductase-negative mutant of strain H16 (5). Escherichia coli S17-1 (29) served as the donor in conjugative transfers. E. coli XL1-blue (Stratagene) was used as a host in standard cloning procedures. Synechocystis sp. strains M320 and M321 are norB-negative mutants of wild-type Synechocystis sp. strain PCC6803.

E. coli strains were grown in Luria-Bertani broth at 37°C. R. eutropha strains were cultivated at 30°C in mineral salts medium (27) with 0.4% (wt/vol) fructose as the carbon source (FN medium). Under denitrifying conditions, the cells were cultivated in 150-ml glass flasks sealed with a rubber septum and containing 100 ml of FN medium supplemented with 0.1% (wt/vol) sodium nitrate. The gas phase consisted of helium. Synechocystis sp. strain PCC6803 (wild type) and mutant strains were grown under continuous illumination at 30°C in BG-11 medium (30) supplemented with either 18 mM nitrate or 5 mM ammonia. Media for phototrophic cultivation of Synechocystis strains were amended with 5 mM glucose.

Solid media contained 1.5% (wt/vol) agar. Antibiotics were added as follows: for R. eutropha, kanamycin (360 μg/ml) and tetracycline (15 μg/ml); for E. coli, kanamycin (50 μg/ml), tetracycline (20 μg/ml), and ampicillin (50 μg/ml); and for Synechocystis, kanamycin (40 μg/ml).

Nucleic acid manipulations. Isolation of plasmids, transformation, and cloning were carried out by standard methods (26). Southern hybridization was performed by using a digoxigenin-11-dUTP kit (Roche, Mannheim, Germany) for primer labeling and detection. Genomic DNA of Synechocystis was isolated as described by Franke and Damerval (10). A cosm id clone (cos0502) containing a 39-kb genomic DNA fragment of Synechocystis sp. strain PCC6803 was obtained from the Kazusa Research Institute (Kazusa, Japan). Plasmids pCH670, pCH681, and pGE409 (Fig. 1) were constructed by cloning a 4-kb HindIII fragment of cos0502 into pBlueScript KS(+) (Stratagene), pBlueScript SK(+) (Stratagene), and pVK101 (17), respectively. Transcriptional fusions were con-
FIG. 2. Nitrous oxide production by R. eutropha strains. Symbols: ○, wild-type H16; ▲, nitric oxide reductase-negative mutant HF420; ●, HF420 complemented with the norB gene region from Synechocystis (pGE409); ◇, HF420 complemented with Synechocystis norB under the control of the norAB promoter of R. eutropha (pGE453).

RESULTS

Complementation of a norB-negative mutant of R. eutropha.

To test whether the norB gene product of Synechocystis is physiologically active, complementation studies were conducted with norB-negative mutant HF420 of R. eutropha as a recipient. Due to the high toxicity of NO, the absence of the nitric oxide reductase is lethal to denitrifying mutant cells of R. eutropha (5). Complementation with a physiologically active norB gene should therefore rescue the cells, and transconjugants derived from such experiments should produce nitrous oxide under anaerobic conditions with nitrate as the electron acceptor. A gene region including norB and dnr of Synechocystis was cloned into broad-host-range vector pVK101, yielding plasmid pGE409. In a parallel approach, the norB gene of Synechocystis was cloned under the control of the norAB promoter of R. eutropha, yielding plasmid pGE453. Plasmid pGE409 restored nitrous oxide production, although to a moderate degree, presumably due to the low expression of norB. An increase in nitrous oxide production was obtained with HF420(pGE453), reaching almost the R. eutropha wild-type level (Fig. 2). In contrast to the wild-type cells, however, the transconjugant cells did not consume nitrous oxide. A similar phenotype is occasionally observed for several R. eutropha mutants which have an altered nitric oxide reductase expression pattern (unpublished observations).

Nitric oxide reductase activity in Synechocystis membranes.

Complementation analysis indicated that the norB gene from Synechocystis encodes a functional nitric oxide reductase. To investigate whether the norB gene is also active in its natural host, a knockout mutation in Synechocystis was constructed. A kanamycin resistance cartridge was inserted into norB in both orientations with the aid of plasmids pCH836 and pCH837 (Fig. 1). The corresponding mutants, M320 and M321, were assayed for nitric oxide reductase activity by monitoring NO consumption with membrane fractions and a Clark electrode. With ascorbate and phenazine methosulfate as the electron-donating agents, 50 nmol of NO per min per mg of protein was consumed by wild-type membranes of Synechocystis, corre-

FIG. 1. Synechocystis norB gene region. A physical map and relevant restriction sites are shown. DNA fragments of subclones are indicated by black bars. The orientation of a kanamycin resistance cartridge (Km) is indicated by arrows. The norB promoter of R. eutropha is depicted by a white box with an arrow.

structured by using vector pSB2A (22), which contains a promoterless chloramphenicol acetyltransferase (cat) reporter gene. The integenic region between dnr and norB of Synechocystis was amplified from plasmid pCH861 by PCR with primers 445 (5′-TCTTTGCTATATGCCAATTCACCTTTGATCC-3′) and 446 (5′-TGCTGCATACACAAAGAATGAAAAA-3′), which generate FspI sites at both ends of the PCR product. The resulting 426-bp fragment was digested with FspI and inserted into the HpaI site of vector pSB2A in both orientations, yielding plasmids pCH834 (Pcat-catr) and pCH835 (Pcat-catr) (Fig. 1).

An Ndel site was introduced 5′ of the start codon of norB by PCR with primers 451 (5′-TCTTTGCTATATGCCAATTCACCTTTGATCC-3′) and 452 (5′-CATATGGTTTCTCCGTCTGAAGTTACGC-3′) and plasmid pCH670 as a template. The 327-bp PCR product was cloned as an Ndel-EagI fragment into pET22b(+) (Novagen), yielding plasmid pCH838. The complete norB gene was restored in plasmid pCH839 by inserting a 2.46-kb EagI-XhoI fragment from pCH670 into pCH838. Ndel and BglII sites were introduced in the norAB promoter region of R. eutropha by PCR with primers 453 (5′-CCCAAGATCTCGTGACACCGCCGGCAGGTG-3′) and 454 (5′-CCAGGGTCATATGGTTTCTCCGTCTGAAGTTACGC-3′) and plasmid pCH510 (5) as a template. The resulting 321-bp Ndel-BglII fragment containing PnorAB was cloned into pCH839. The resulting plasmid, pCH840, contains a fusion of PnorAB of R. eutropha with the complete norB gene of Synechocystis. For complementation of R. eutropha strains, a 3.1-kb BglII-HindIII fragment of pCH840 was cloned into HmI-dhII-linearized broad-host-range vector pVDZ’2 (8), yielding pGE453 (Fig. 1). For inactivation of the norB gene of Synechocystis, a 1.5-kb Psel kanamycin resistance cartridge from pRM1 (W. Messer, Berlin, Germany) was cloned in both orientations into the NsiI site of plasmid pCH670, yielding pCH836 and pCH837, respectively (Fig. 1).

Analytical procedures. Protein was determined as described by Lowry et al. (20). N2O and N2 were analyzed by gas chromatography from the headspace of cultures bottles as described previously (4). Cat protein formed by cells was assayed for nitric oxide reductase activity by monitoring NO production at 30°C (using a Clark electrode). The reaction mixture (2 ml) contained 50 mM sodium phosphate buffer (pH 7.0), 20 mM d-glucose, 10 U of glucose oxidase, and 250 U of catalase. NO reduction was measured by using 10 μmol of ascorbate plus 0.25 μmol of phenazine methosulfate. NADH-dependent NO reduction was measured by using 0.25 μmol of NADH and 0.35 μmol of 2-methyl-1,4-naphthoquinone (menadione). Quinols were formed from the corresponding quinones in the presence of 40 U of diaphorase from Clostridium klyveri. After an incubation period of 5 min, NO-saturated buffer (100 mM of NO) was added. The reaction was started by the addition of membrane extracts.

Inactivation of Synechocystis norB. Synechocystis cells were transformed with plasmids pCH836 and pCH837 by following the protocol of Ermakova and coworkers (9). Cells were grown for 6 h on nonselective agar plates and subsequently overlaid with a kanamycin solution (final concentration, 10 μg/ml). Homozygous strains were obtained after four serial streak purifications of single colonies on plates containing increasing concentrations of kanamycin to a final concentration of 40 μg/ml (37) and were confirmed by Southern analysis.
sponding to 10% of the level obtained with membrane fractions of *R. eutrophpha*. The addition of NADH-reduced 2-methyl-1,4-naphthoquinone (menadione) to membrane extracts of *Synechocystis* enhanced NO-reducing activity twofold (101 nmol of NO per min per mg of protein). Virtually no NO-reducing activity was detected with mutants M320 and M321. This result shows that NO reduction in *Synechocystis* is mediated by the *norB* gene product. Furthermore, the enzymatic activity seen with menadione supports the notion that the enzyme belongs to the group of qNor proteins.

**Promoter analysis of** **P** *norB* and **P** _dnr_, **norB** of *Synechocystis* is preceded by a _dnr_ gene, which is transcribed in the opposite direction. The putative _dnr_ gene product belongs to a subfamily of Fnr-like transcriptional activators which presumably respond to effectors other than oxygen. To assess a possible effect of NO on the expression of _norB_ in *Synechocystis*, the intergenic region between _dnr_ and _norB_ was cloned in both orientations into vector pSB2A. This construct should allow the identification of promoter activities exerted from _P* _norB_, as well as _P* _dnr_, if fused to an appropriate reporter gene. In this instance, transcriptional fusions were constructed with the chloramphenicol acetyltransferase-encoding gene on plasmid pSB2A. Plasmids pCH834 (P _dnr-cat_ ) and pCH835 (P _norB-cat_ ) and control vector pSB2A were transferred by conjugation into wild-type cells of *Synechocystis*. Transconjugant cells were grown photoautotrophically by using either ammonia or nitrate as the nitrogen source. The rationale for using nitrate, which is converted to nitrite by the assimilatory nitrate reductase, was to provide to the cells nitrogen oxide compounds, which may have an inducing effect on _norB_ transcription.

The _P* _dnr-cat_ fusion showed weak promoter activity that was not affected by the nitrogen source. An approximately threefold-higher level of promoter activity was obtained with the _P* _norB-cat_ fusion (Fig. 3). Cells grown with nitrate showed a slightly higher level of promoter activity than did cell cultures amended with ammonia. Therefore, we cannot exclude the possibility that nitrate and/or nitrite modulates the expression of _norB_ in *Synechocystis*.

The Dnr/Nnr regulators from *Paracoccus denitrificans* (34) and *Rhodobacter sphaeroides* (19) and the sigma N-dependent regulator NorR from *R. eutrophpha* (24) have been shown to respond to the NO-generating (1) and nitrosylating (25) agent sodium nitroprusside (SNP). To investigate possible induction of *Synechocystis* _norB_ by exogenous NO, photoautotrophically grown cultures of *Synechocystis* were amended with 5 mM SNP in the presence of ammonia as the nitrogen source. As shown in Fig. 3, the transcription of neither _dnr nor norB_ was enhanced by the addition of SNP. Similar results were obtained with lower SNP concentrations, in the range of 0.5 to 4 mM (data not shown). Cells cultivated under oxygen limitation or grown phototrophically were also not susceptible to induction by SNP-released NO (data not shown).

**Sensitivity of** **Synechocystis** **to** **NO.** So far the data indicate that *Synechocystis* forms a catalytically active nitric oxide reductase even in the absence of its substrate, NO. This behavior enables the cells to respond immediately to the toxic compound if encountered in the environment. To examine the hypothesis of a protective role of NorB, the effects of exogenously added SNP and NO were investigated with growing cells of *Synechocystis*.

In a first attempt, paper disks soaked with SNP were placed on agar plates spread with a layer of *Synechocystis* cells. After 36 h of incubation, a clearance zone was visible around the paper disks containing SNP, indicating inhibition of growth by the NO-donating agent (Fig. 4A). The diameter of the area of inhibition was clearly enlarged when cells of NorB-deficient mutant M321 were used, indicating an increased sensitivity to the toxic compound.

To test if gaseous NO affects the growth of *Synechocystis*, wild-type and _norB_ mutant cells were mixed with BG-11 soft agar in gastight tubes and subsequently incubated with or without NO in the headspace. A similar method was used by Cross and coworkers (6) to show increased NO sensitivity of *Rhodobacter capsulatus* cytochrome c’ mutants. After 36 h of incubation at 30°C in light, the mutant cells showed an inhibition of growth in the upper layer of the agar directly exposed to NO (Fig. 4B). NO growth inhibition occurred in tubes with wild-type cells or in tubes without NO in the headspace. On the basis of these experiments, we concluded that the _norB_ gene product is beneficial for *Synechocystis*, since it protects the cells to a certain degree from the detrimental effect of NO.

**DISCUSSION**

Several bacteria have been described to contain incomplete denitrification pathways. The lack of nitrous oxide reductase, which mediates the production of dinitrogen, is most common (41). *Wolinella succinogenes* appears to be devoid of an NO-producing nitrite reductase but, on the other hand, is able to convert NO and nitrous oxide to the final product, dinitrogen (40). Some strains of *Campylobacter fetus* have the capacity to reduce nitrous oxide despite the lack of a nitrous oxide-producing activity (23). The nondenitrifying strain *R. sphaeroides*
the enzymatic pattern seen in \textit{R. sphaeroides} 2.4.1. The regulation of these two systems, however, seems to be different. The expression of the nitric oxide reductase in \textit{R. sphaeroides} 2.4.1 is induced by exogenously or endogenously provided NO (18), whereas the qNor of \textit{Synechocystis} is expressed independently of NO. A largely nonregulated phenotype was unexpected, since norB is neighbored by a \textit{dnr} gene whose product likely is a regulator of the Dnr/Nnr subgroup of Fnr-like proteins that lack the N-terminal cysteines critical for oxygen sensing. Dnr/Nnr proteins have been shown to control the NO-sensitive transcription of \textit{nor} genes in \textit{R. sphaeroides} (18, 19, 32), \textit{Pseudomonas stutzeri} (35), and \textit{Paracoccus denitrificans} (33, 34). Interestingly, a change of the conserved residue Tyr93 to Phe in Nnr of \textit{P. denitrificans} resulted in severe downregulation, explained by less tight contact of the mutant protein with RNA polymerase (14). In Dnr of \textit{Synechocystis}, the corresponding residue in the native sequence is Phe. Therefore, the lack of induction by SNP in \textit{Synechocystis} indicates that Dnr, if involved in regulation at all, responds to a different signal or is nonfunctional.

Most, if not all, organisms that contain a cNor protein also contain sets of accessory genes for the NorD, NorE, NorF, and NorQ/NirQ proteins, which affect the activity, assembly, or stability of the NorC\textit{B} heterodimer in \textit{P. denitrificans} (7). None of these genes is present in the genome of \textit{Synechocystis}, suggesting that the function of qNor enzymes does not rely on these accessory genes. This observation is surprising, since qNor and the NorB subunit of cNor share similar overall structures and equipment with prosthetic groups. The broad phylogenetic distribution of qNor-encoding genes among proteobacteria, cyanobacteria, green algae (21), and even archaea (28) may reflect a selective advantage of qNor whose function relies only on a single gene transfer.

We have demonstrated the function of the NorB product of \textit{Synechocystis} by complementation of a nitric oxide reductase-negative mutant of \textit{R. eutropha}. Wild-type cells of \textit{Synechocystis} tolerate high concentrations of approximately 10 mM NO in the headspace. Steady-state levels of NO in the environment of denitrifying organisms are supposed to be in the nanomolar range (11, 15, 41). Therefore, Kwiatkowski and coworkers (18) have proposed that the nitric oxide reductase of \textit{R. sphaeroides} 2.4.1 uses NO from the environment to gain energy rather than to protect cells from the toxic compound. The same function may account for the nitric oxide reductase of \textit{Synechocystis}. Moreover, the recent discovery of NO formation by nitrifying bacteria (2), cyanobacteria, green algae (21), and even archaea (28, 39) suggests that the function of qNor enzymes does not rely on endogenously produced NO.

The presence of a nitric oxide reductase and the absence of an NO-producing nitrite reductase in \textit{Synechocystis} resemble

![Image](http://aem.asm.org/)
REFERENCES


