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Nitrosomonas europaea Expresses a Nitric Oxide Reductase during Nitrification

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In this paper, we report the identification of a *norCBQD* gene cluster that encodes a functional nitric oxide reductase (Nor) in *Nitrosomonas europaea*. Disruption of the *norB* gene resulted in a strongly diminished nitric oxide (NO) consumption by cells and membrane protein fractions, which was restored by the introduction of an intact *norCBQD* gene cluster in *trans*. NorB-deficient cells produced amounts of nitrous oxide (N_2O) equal to that of wild-type cells. NorCB-dependent activity was present during aerobic growth and was not affected by the inactivation of the putative *fnr* gene. The findings demonstrate the presence of an alternative site of N_2O production in *N. europaea*.

The production of NO and N_2O by the lithoautotrophic ammonia (NH_3)-oxidizing bacterium *Nitrosomonas europaea*, as well as by other NH_3-oxidizing bacteria, represents a long-standing and unresolved question in the biology of nitrifying bacteria (2, 17). These gaseous nitrogen oxides are produced by a mechanism that is reminiscent of the production of NO and N_2O by organisms from the group of heterotrophic denitrifying bacteria (15, 16). Denitrification is an anaerobic mode of respiration that involves the enzymes nitrate reductase, nitrite reductase (Nir), nitric oxide reductase, and nitrous oxide reductase, which catalyze the stepwise reduction of nitrate (NO_3^-), via the intermediates nitrite (NO_2^-), NO, and N_2O, to dinitrogen (24). Accordingly, full expression of the denitrifying pathway in heterotrophic denitrifying bacteria occurs in response to a combination of oxygen (O_2) limitation and the presence of one, or more, of the denitrification substrates NO_3^-, NO_2^-, and NO (24). Recently, we reported the identification of a gene that encodes a copper-type nitrite reductase (NirK) in *N. europaea* (2). In addition, genes with homology to c-Nor-type *nor* genes are present in the genome of this bacterium (4).

N. europaea acquires all its free energy from the oxidation of NH_3 to NO_2^- via the intermediate hydroxylamine (NH_2OH), which is catalyzed by the enzymes ammonia monooxygenase and hydroxylamine oxidoreductase (HAO) (23). While this nitrification pathway is relatively well characterized, the structure, functioning, and physiological relevance of its putative denitrification pathway(s) still remain largely unknown (1, 2, 15, 19, 20). It has been suggested that the putative denitrification pathway of *N. europaea* may allow the use of NO_2^- as an alternative terminal electron acceptor under O_2-limiting conditions, facilitating the use of all available O_2 for the monooxygenation of NH_3 (1, 19). Alternatively, the finding that NirK-deficient cells of *N. europaea* had a lower tolerance to NO_2^- suggests that this denitrification enzyme may be recruited to protect the cell against the NO_2^- produced during NH_3 oxidation (2, 20). In the heterotrophic denitrifying bacteria, the toxic NO produced by Nir is maintained at a low concentration by Nor (24). It may be hypothesized that the maintenance of NO homeostasis in *N. europaea*, which produces NO during nitrification, also involves Nor (16).

In this work, we show that the *norCBQD* homologues of *N. europaea* encode a functional Nor that is expressed under fully aerobic conditions. We address the role of this denitrification enzyme in (i) the production of N_2O, (ii) the defense against NO and NO_2^-, and (iii) respiration under O_2-limiting conditions on the bases of physiological characterizations of a Nor-deficient strain of *N. europaea*.

The *nor* homologues of *N. europaea*. A cluster of genes with high homology to the *norCBQD* loci of heterotrophic denitrifying bacteria is present in the genome of *N. europaea* (4) (Fig. 1). In these bacteria, *norC* encodes a membrane-anchored *c*-type cytochrome that forms a complex with the major membrane-bound catalytic subunit, which is encoded by *norB* (10). In *Paracoccus denitrificans*, *norQ* and *norD* encode accessory proteins that are essential for the activation of NorCB (5). The *nor* gene cluster of *N. europaea* is flanked by uncharacterized open reading frames and is separated from the *nirK* gene cluster by 1.15 Mb on the 2.81-Mb chromosome.

NO consumption by *N. europaea*. *N. europaea* strain ATCC 19718 (wild type) (18) and the Nor-deficient mutants BLnt (ATCC 19718 derivative; *norB*::pNORBsu, Km^r [this study]) and QLnt (ATCC 19718 derivative; *norQ*::pNORQsu, Km^r [this study]) were cultured in batch cultures at 30°C, 175 rpm, in the dark, as described by Hyman and Arp (1.5 liters in 2-liter Erlenmeyer flasks for the growth of cells for the NO consumption assays, and 150 ml in 500-ml flasks for determination of growth curves) (13). Cells that had been harvested in the early stationary growth phase were assayed for NO consumption in an anaerobic reaction vial that contained phosphate buffer (9.2 mM KH_2PO_4 and 10.7 mM K_2HPO_4, pH 7) in the presence of 100 μM NO, 10 mM ascorbate (electron donor), and 100 μM

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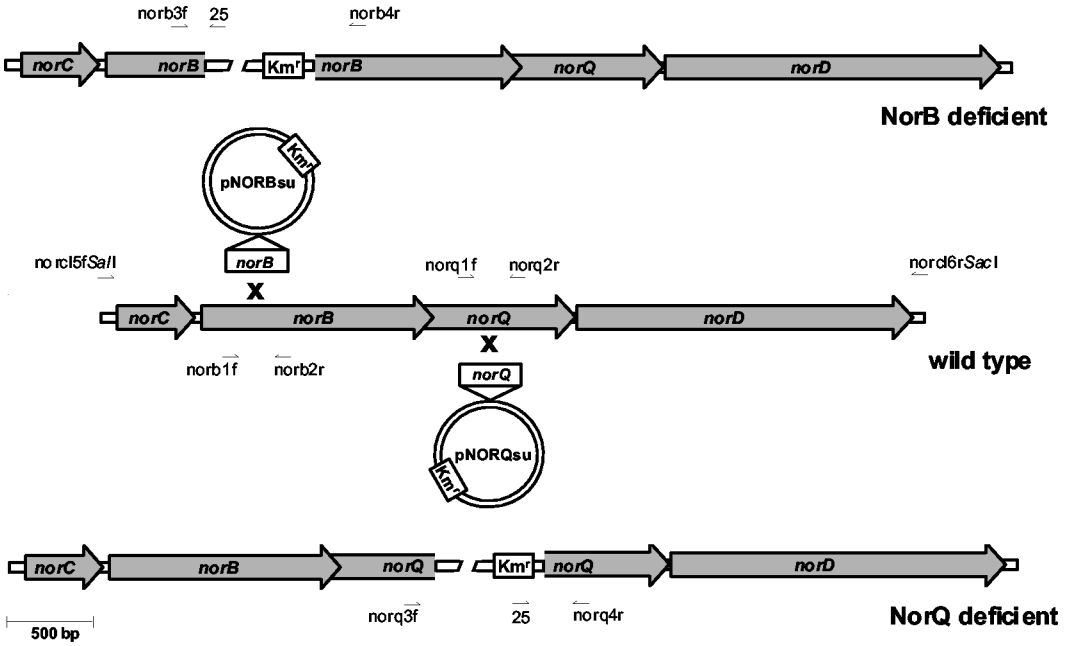

FIG. 1. Schematic representation of the *nor* gene cluster in wild-type *N. europaea* and in the Nor-deficient strains. *norC* starts at genomic position 2163869, and *norD* ends at genomic position 2166533 (4). Nor-deficient strains were engineered by insertion of the suicide vectors pNORBsu and pNORQsu as indicated. Arrows indicate primers used for construction of the suicide vectors, verification of correct integration, and construction of the complementation vector.

phenazine ethosulfate (PES) (electron mediator) by using a Clark-type electrode, as described by Girsch and de Vries (8). This revealed that wild-type cells of *N. europaea* consumed NO at a specific rate of approximately 0.04 μmol of NO min^{-1} mg of $protein^{-1}$ (Fig. 2a). The kinetics of NO consumption varied between experiments in that NO was sometimes consumed at a constant rate and sometimes at a rate that changed in time. In all cases, NO was consumed to a concentration below the detection level of approximately 2 μM. In the absence of PES-ascorbate, a transient consumption of NO occurred that was also observed with heat-inactivated cells (Fig. 2b and c). This may partially be the result of the reaction of NO with O_2 but also appears to involve other reactions of sample components with NO, based on the disappearance of more NO than is predicted by the 2:1 reaction of NO with O_2. To determine whether the PES–ascorbate-dependent activity was membrane associated, soluble and membrane protein fractions were assayed for NO-consuming activity. The assay conditions were the same as for cells, with the exception of the additional presence of horse heart cytochrome *c* (0.25 g $liter^{-1}$). NO was not consumed by the soluble protein fraction. In contrast, membrane protein fractions consumed NO to a concentration below the detection level at a specific rate of 0.19 μmol of NO min^{-1} mg of $protein^{-1}$ (Fig. 2f).

Disruption of the *nor* genes diminishes NO consumption. To determine if the PES–ascorbate-dependent, membrane-associated NO-consuming activity was encoded by the *nor* homologues, *norB* and *norQ* were independently inactivated. This was achieved by the insertion of suicide vectors, harboring an internal fragment of *norB* or *norQ*, into the genome via homologous recombination (Fig. 1). For the construction of the suicide vectors, internal fragments of *norB* and *norQ* were obtained by PCR and cloned into the vector pRVS3 (22). The resulting suicide vectors were transferred from cells of *Escherichia coli* to wild-type cells of *N. europaea* via conjugation. Integration of these vectors into the targeted loci, involving a single crossover, resulted in the disruption of the genes, yielding the NorB- and NorQ-deficient strains (Fig. 1). Correct integration was confirmed by PCR (data not shown). The structure of the *nor* gene cluster of *N. europaea* suggests that it is transcribed as an operon, in which case it is likely that the insertion of the suicide vectors also silenced genes downstream of the targeted gene due to polar effects. NorB-deficient cells exhibited NO consumption kinetics that differed markedly from those of wild-type cells (Fig. 2d). NO was consumed by cells of this mutant at a rate that continuously decreased in time and ceased before all NO was consumed. The addition of wild-type cells at this point resulted in the complete consumption of the remaining NO (Fig. 2e). Likewise, the consumption of NO by membrane protein fractions of NorB- and NorQ-deficient cells was also strongly diminished and decreased in time (Fig. 2g and h). In this study, we did not specifically address the residual NO-consuming activity that was observed; at present, it remains unresolved whether this disappearance of NO is enzymatic or chemical. The insertion of a complementation vector, which harbored an intact copy of the *norCBQD* gene cluster under the control of its native promoter, in the strain in which *norB* was disrupted resulted in the restoration of the NO-consuming activity in membrane protein fractions to wild-type levels (Fig. 2i).

NorCB-dependent NO-consuming activity is present at a constant level throughout growth in aerobic batch cultures. Membrane protein fractions were prepared from cells that had been harvested at various cell densities in order to monitor the

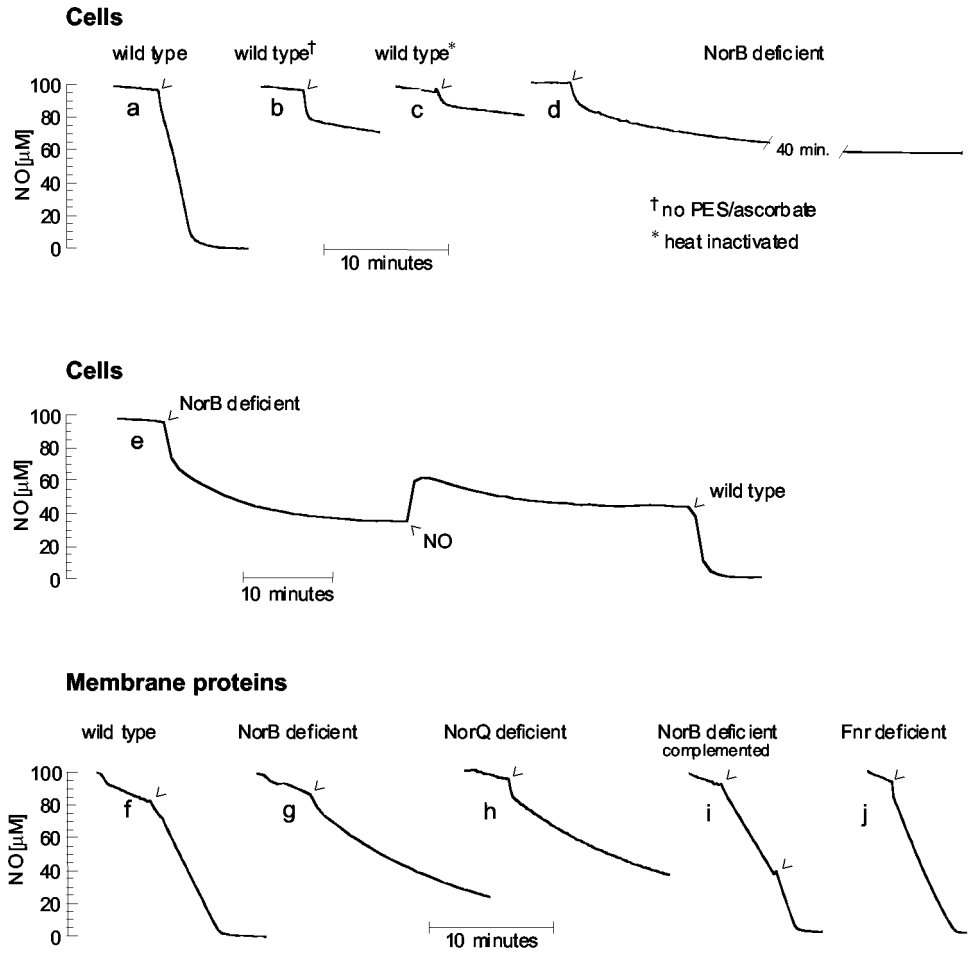

FIG. 2. Nitric oxide consumption by cells and membrane protein fractions measured under anoxic conditions with a Clark-type electrode with phenazine ethosulfate (PES) as a mediator of ascorbate-derived electrons. Additionally, horse heart cytochrome *c* was present when assaying membranes. Cells were harvested in the early stationary growth phase and stored at 0°C overnight. The disappearance of NO before the addition of cells or protein represents the background rate of NO consumption via chemical conversion. Arrowheads in a to d mark the addition of cells to a final OD_{600} of 0.9 (50 μl). The arrowheads in e mark the addition of NorB-deficient cells, NO from a saturated solution, and wild-type cells, respectively. Equal amounts of cells of both strains were added to a combined OD_{600} of 2.7 (two times, 50 μl). Arrowheads in f to j mark the addition of membrane proteins: wild type (0.17 mg in 20 μl), NorB deficient (0.59 mg in 50 μl), NorQ deficient (0.28 mg, 75 μl), NorB deficient complemented (0.20 mg, 20 μl) (twice), and Fnr deficient (0.28 mg in 75 μl).

level of NorCB activity during exponential growth and in the stationary phase. Aerobic conditions were inferred from the occurrence of exponential growth and confirmed in the early exponential growth phase with a Clark-type electrode (data not shown). The specific NO consumption rates of these membrane preparations, as estimated by linear approximation of the initial rate (3-min interval after the PES–ascorbate-independent NO consumption), did not vary significantly (i.e., between 0.18 ± 0.02 and 0.24 ± 0.02 μmol of NO min^{-1} mg of $protein^{-1}$ [95% confidence interval of activity measurement, $n = 3$]). Membrane proteins isolated from cells that had been harvested from O_2-limited cultures in the linear growth phase (optical density at 600 nm [OD_{600}] of 0.04) consumed NO at a rate of 0.30 ± 0.01 μmol of NO min^{-1} mg of $protein^{-1}$ (95% confidence interval of activity measurement, $n = 3$). O_2-limited growth was achieved by shaking at 70 rpm instead of 175 rpm. Under these conditions, the O_2 concentration, as measured with a Clark-type electrode during linear growth, was below the detection level of approximately 1 μM. Membrane protein fractions prepared from NorB-deficient cells that had been harvested at various cell densities all exhibited the described impaired NO consumption kinetics (data not shown).

Fnr is not essential for expression of NorCB-dependent NO-consuming activity. The putative *fnr* gene of *N. europaea* appears to encode an Fnr protein that contains four conserved cysteine residues, which are involved in the ligation of a [4Fe-4S] cluster that is specific for the O_2-responsive Fnr proteins (14). The *fnr* gene of *N. europaea* is not localized in the vicinity of the *nir* or *nor* gene clusters on the chromosome (separated by 0.31 and 0.85 Mb, respectively). Membrane preparations of cells in which the putative *fnr* gene had been disrupted by insertion of a suicide vector displayed wild-type NO consumption kinetics (Fig. 2j).

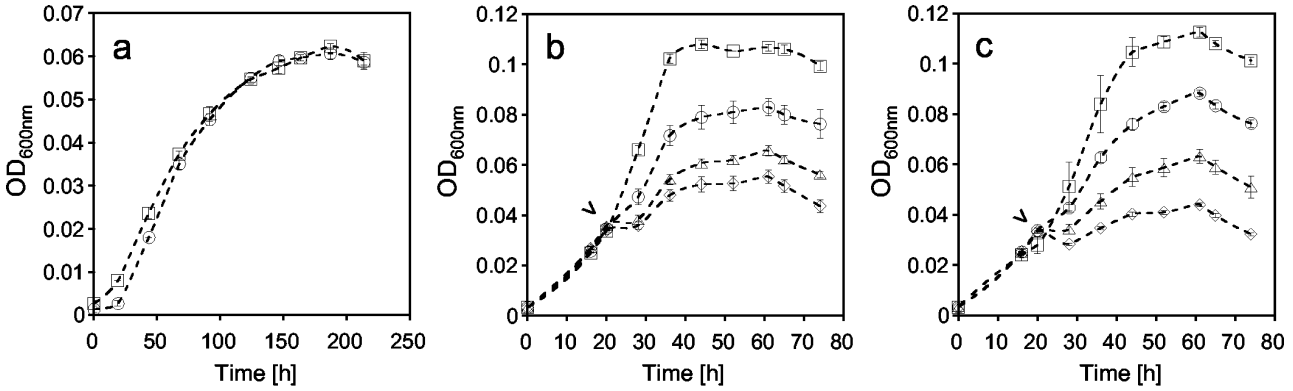

FIG. 3. (a) Growth curves of cells of wild-type and NorB-deficient strains of *N. europaea* during cultivation in O_2-limited batch cultures. Squares, wild-type cells; circles, NorB-deficient cells. Error bars indicate the 95% confidence interval of replicate cultures ($n = 3$). (b and c) Growth curves of wild-type (b) and NorB-deficient (c) cells of *N. europaea* in aerobic batch cultures to which 0 (squares), 50 (circles), 100 (triangles), and 200 (diamonds) μM SNP was added at a *t* of 20 h. Arrowheads indicate the addition of SNP. Error bars indicate the 95% confidence interval of replicate cultures ($n = 3$).

NorB-deficient cells still produce N_2O. To address the role of Nor in the production of N_2O by *N. europaea*, the concentration of this gas was determined in the headspace of sealed 150-ml batch cultures in 500-ml bottles after 3 days of incubation. The NorB-deficient strain produced amounts of N_2O similar to that for wild-type cells (i.e., 31 ± 5 μM and 40 ± 10 μM [95% confidence interval of replicate cultures, $n = 3$], respectively).

Wild-type and NorB-deficient cells have similar growth characteristics under O_2 limitation. In aerobic batch cultures, NorB-deficient cells had wild-type growth characteristics (Fig. 3b and c, upper curves). To assess whether NorCB is involved in the optimization of the O_2 requirements of *N. europaea* during O_2 limitation, the growth characteristics of wild-type and NorB-deficient cells were determined under O_2-limiting growth conditions. Both wild-type and NorB-deficient cells displayed identical, nonexponential, transiently linear growth and reached similar maximal cell densities under these conditions (Fig. 3a).

NO_2^- tolerance is not compromised in NorB-deficient cells. To test whether Nor is involved in the protection of the cell against NO_2^-, growth characteristics of wild-type and NorB-deficient cells were determined in cultures to which increasing amounts of NO_2^- had been added at the start of culturing. In this assay, the growth rate and maximal cell density of a NirK-deficient strain were much more strongly affected by the addition of NO_2^- than those of wild-type cells (2). In contrast, the growth rate and maximal cell density of NorB-deficient cells were comparable to those of wild-type cells in a similar experiment (data not shown).

Relatively high NO tolerance does not depend on NorCB. With the aim to address a possible role of Nor in the defense against NO, the effects of externally added NO on respiration of wild-type and NorB-deficient cells, harvested in the mid-exponential growth phase, were determined in an oxygraph. The addition of 15 μl of NO-saturated buffer (40 mM KH_2PO_4, 3.5 mM NaH_2PO_4, adjusted with NaOH to pH 8.0), resulting in a final concentration of 30 μM NO, had no significant effects on the NH_3-dependent O_2 consumption of either strain (data not shown). In order to provide a point of reference for this particular experimental setup, the experiment was also performed with wild-type and NorB-deficient cells of the heterotrophic denitrifying bacterium *P. denitrificans* (5, 6). The concentration of *P. denitrificans* cells that was used was approximately four times higher than that of *N. europaea*, OD_{600} of 0.24 and 0.06, respectively. *P. denitrificans* was cultured under O_2-limiting conditions in the presence of NO_3^-, to ensure the expression of both Nor and terminal oxidase (21). In contrast to *N. europaea*, the addition of 15 μl of NO-saturated buffer resulted in a transient inhibition of the succinate-dependent O_2 uptake by both wild-type and NorB-deficient cells of *P. denitrificans* (data not shown). The duration of inhibition of the NorB-deficient cells was approximately twofold longer than was observed for wild-type cells.

A possible role of Nor in the protection of growing cells of *N. europaea* against NO was studied in cultures of wild-type and NorB-deficient cells to which increasing amounts of the NO-releasing agent sodium nitroprusside (SNP) were added in the early exponential growth phase (Fig. 3b and c). SNP had negative effects on the growth rate and the maximal cell density of both wild-type and NorB-deficient cells. NorB-deficient cells were only affected to a larger extent than wild-type cells at the highest concentration (200 μM), as judged by a significantly lower growth rate of the NorB-deficient cells after the addition of SNP and the larger negative effect on the maximal cell density reached.

Conclusions. Based on the findings presented, we conclude that cells of *N. europaea* express a membrane-bound NorCB during fully aerobic nitrification. The specific NorCB activity in membrane preparations was comparable to those reported for the heterotrophic denitrifying bacterium *P. denitrificans* during denitrifying growth (7). The role of NorCB in *N. europaea* that was revealed in this study differed from that expected on the basis of extrapolation of the roles of its homologues in the heterotrophic denitrifying bacteria. NorCB was not the only N_2O-producing mechanism present in *N. europaea*. The relative contributions of NorCB and the alternative N_2O-producing pathway(s) cannot be deduced from the observations because of possible pleiotropic effects of the mutation of *norB*. NorCB did not play a vital role in the tolerance of *N. europaea*

to NO_2^- or NO produced during growth on NH_3. The relatively high NO tolerance was only compromised by the inactivation of *norB* in the presence of high concentrations of SNP, suggesting that an alternative NO-consuming mechanism might be present. NorCB did not appear to play a crucial role during oxygen-limited growth. Taken together, the findings reveal an inorganic nitrogen metabolism of *N. europaea* that is complex in terms of sources and sinks of gaseous nitrogen oxides. Several lines of biochemical evidence put forward HAO as an important candidate for a role in the production of N_2O by *N. europaea*. HAO was demonstrated to produce NO and N_2O during the oxidation of NH_2OH in vitro (11, 12). More recently, HAO has been described to catalyze the reduction and oxidation of NO in vitro (3, 9).

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