Applied and Environmental Microbiology, Nov. 1996, p. 4019–4025 Vol. 62, No. 11
0099-2240/96/$04.00+0
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# Characterization of the Gene Encoding Nitrite Reductase and the Physiological Consequences of Its Expression in the Nondenitrifying *Rhizobium* "*hedysari*" Strain HCNT1

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Received 3 April 1996/Accepted 6 September 1996

***Rhizobium* "*hedysari*" HCNT1 is an unclassified rhizobium which contains a nitric oxide-producing nitrite reductase but is apparently incapable of coupling the reduction of nitrite to energy conservation. The gene encoding the nitrite reductase, *nirK*, has been cloned and sequenced and was found to encode a protein closely related to the copper-containing family of nitrite reductases. Unlike other members of this family, *nirK* expression in HCNT1 is not dependent on the presence of nitrogen oxides, being dependent only on oxygen concentration. Oxygen respiration of microaerobically grown Nir-deficient cells is not affected by concentrations of nitrite that completely inhibit oxygen respiration in wild-type cells. This loss of sensitivity suggests that the product of nitrite reductase, nitric oxide, is responsible for inhibition of oxygen respiration. By using a newly developed chemically modified electrode to detect nitric oxide, it was found that nitrite reduction by HCNT1 produces significantly higher nitric oxide concentrations than are observed in true denitrifiers. This indicates that nitrite reductase is the only nitrogen oxide reductase active in HCNT1. The capacity to generate such large concentrations of freely diffusible nitric oxide as a consequence of nitrite respiration makes HCNT1 unique among bacteria.**

Dissimilatory nitrite reductases occur in a wide variety of bacteria. These enzymes catalyze either the six-electron reduction of nitrite to ammonia or the one-electron reduction of nitrite to the gas nitric oxide (NO) (6). Nitrite reductases that reduce nitrite to NO are members of a series of enzymes required for the dissimilatory reduction of nitrate to nitrogen gas (21). Nitrate reduction to dinitrogen, termed denitrification, is an alternative form of respiration that occurs when oxygen concentrations are low. Reduction of nitrogen oxides is coupled to energy conservation and permits cell growth under anaerobic conditions.

As nitrate is reduced to nitrogen, the intermediates that occur are, in order, nitrite, NO, and nitrous oxide (27). While denitrification typically refers to reduction of nitrate to nitrogen gas, many bacteria have been described that carry out incomplete denitrification (33). The most common end product in incomplete denitrification is nitrous oxide. Several bacteria have been described that lack nitrous oxide reductase, including *Chromobacterium violaceum*, *Pseudomonas aureofaciens*, and several rhizobia (33). There have also been bacteria described that catalyze only the latter steps in denitrification. For example, *Wolinella succinogenes*, which reduces nitrite to ammonia, appears to be capable of NO and nitrous oxide reduction (28). Some strains of *Campylobacter fetus* subsp. *fetus* cannot reduce NO but can reduce nitrous oxide (28). Both *Wolinella* and *Campylobacter* cells can grow with nitrous oxide as a terminal electron acceptor, indicating nitrous oxide reduction is coupled to energy conservation.

*Rhizobium* "*hedysari*" HCNT1 is an unclassified rhizobium that is symbiotic with French honeysuckle (*Hedysarum coronarium*) (11). This diazotroph is unique among denitrifiers in that it can reduce nitrite but is apparently incapable of coupling this reduction to energy conservation (13). Cell growth is not observed under strict anaerobic conditions with nitrite as a terminal electron acceptor. Another unusual feature of HCNT1 is that cells grown with low levels of oxygen are extremely sensitive to nitrite. Micromolar nitrite concentrations significantly reduce cell yields and inhibit oxygen respiration in free-living cells and bacteroids (12). Nitrogenase activity in HCNT1 also shows an extreme sensitivity to nitrite (12). Given the sensitivity of respiration and nitrogenase to nitrite, it has been suggested that nitrite reductase has a detoxifying role in HCNT1 (13). It is unclear why the reduction of nitrite is not coupled to cell growth. It is possible that HCNT1 contains a nitrite reductase that is dissimilar from other known NO-evolving nitrite reductases. However, there has been no molecular or biochemical characterization of the nitrite reductase from HCNT1.

In this paper, we report on the isolation and molecular characterization of the gene encoding the nitrite reductase from *R.* "*hedysari*" HCNT1. The protein encoded by the gene is closely related to copper-containing nitrite reductases from other true denitrifiers. Regulatory studies indicate the enzyme is induced under microaerobic conditions but does not require the presence of nitrite for maximal expression. Inactivation of the gene encoding nitrite reductase, *nirK*, eliminates sensitivity of oxygen respiration to nitrite. By using a chemically modified electrode to detect the evolution of NO, it was shown that HCNT1 accumulates significant concentrations of NO as a consequence of nitrite reduction. Therefore, the accumulation of NO, not nitrite, is responsible for the sensitivity of HCNT1 to nitrite.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *R.* "*hedysari*" HCNT1 (ATCC 43676) and CC1335 were grown aerobically as described by Casella et al.

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TABLE 1. List of plasmids and strains used in this study

| Strain or plasmid | Genotype or phenotype[a] | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* K-12 | | |
| DH5αF′ | F′/*endA1 hsdR supE44 thi-1 recA1 gyrA relA1* Δ(*lacIZYA-argF*)U169 *deoR* ϕ80dlacΔ(*lacZ*)M15 | Laboratory collection |
| S-17-1 | C600:RP4-2 (Tc::Mu) (Km::Tn*7*) *thi pro hsdR recA* $Tra^+$ | Simon et al. (28b) |
| JM109λ*pir* | F′ *traD36 lacI*$^q$ *proA*$^+$*B*$^+$/*rpsL* e14$^-$(McrA$^-$) Δ(*lac-proAB*) *thi gyrA96 endA1 hsdR17* ($r_k^- m_k^-$) *relA1 supE44* λ*pir* | Laboratory collection |
| S-17-1λ*pir* | S-17-1 λ*pir* | Simon et al. (28b) |
| *R.* "*hedysari*" | | |
| HCNT1 | Wild type | Casella et al. (13) |
| HCAT2 | $Kn^r$; *nirK* interrupted by a single crossover of pAT3 | This work |
| HCAT3 | HCAT2 (pRKAT7) | This work |
| HCAT4 | HCNT1 (pAT707) | This work |
| HCAT5 | HCAT2 (pAT707) | This work |
| CC1335 | Wild type | Casella et al. (12) |
| CCAT1 | CC1335 (pAT707) | This work |
| *R. sphaeroides* 2.4.3 (ATCC 17025) | Wild type | American Type Culture Collection |
| Plasmids | | |
| pT7/T3α-18 and -19 | $Ap^r$ | GIBCO BRL |
| pRK415 | $Tc^r$ | Keen et al. (21a) |
| pJP5603 | $Km^r$ | Penfold and Pemberton (28a) |
| pKOK6 | $Tc^r$ | Kokotek and Lotz (23) |
| pAT2 | $Ap^r$; ∼1.5-kb *Pst*I fragment containing 3′ end of *nirK* in pT7/T3-18U | This work |
| pAT3 | $Km^r$; ∼0.6-kb internal *nirK* fragment generated by PCR in pJP5603 | This work |
| pAT4 | $Ap^r$; ∼1.4-kb *Pst*I-*Bam*HI fragment containing 5′ end of *nirK* in pT7/T3-18U | This work |
| pAT5 | $Ap^r$; ∼1.5-kb *Pst*I-*Kpn*I fragment containing 3′ end of *nirK* in pT7/T3-18U | This work |
| pAT7 | $Ap^r$ *nirK*; ∼2.9-kb *Bam*HI-*Pst*I fragment in pT7/T3-18U | This work |
| pRKAT7 | $Tc^r$ *nirK*; ∼2.9-kb *Bam*HI-*Pst*I fragment in pRK415 | This work |
| pAT8 | $Tc^r$; ∼1.4-kb *Pst*I-*Bam*HI fragment containing 5′ end of *nirK* in pRK415 | This work |
| pAT707 | $Tc^r$ $Km^r$; *nirK-lacZ* in pRK415 | This work |

[a] $Ap^r$, ampicillin resistant; $Kn^r$, kanamycin resistant; $Tc^r$, tetracycline resistant.

(13), except that TY medium (tryptone at 5 g liter$^{-1}$, yeast extract at 3 g liter$^{-1}$, and $CaCl_2$ at 0.8 g liter$^{-1}$) was used. To culture cells microaerobically, 125 ml of TY medium in a 250-ml Erlenmeyer flask was inoculated with 750 μl of an aerobically grown overnight culture. The flask was then sealed with a rubber stopper and grown with agitation. Samples for the enzymatic assay were removed with a syringe to permit sampling without unsealing the flask. When necessary, 500 μM nitrite was added to cultures. Antibiotics were added to HCNT1 cultures at the following concentrations: tetracycline, 5 μg ml$^{-1}$; kanamycin, 100 μg ml$^{-1}$ in solid medium and 15 μg ml$^{-1}$ in liquid medium. Antibiotics were added to *Escherichia coli* cultures at the following concentrations: ampicillin, 100 μg ml$^{-1}$; tetracycline, 15 μg ml$^{-1}$; kanamycin, 25 μg ml$^{-1}$. *Rhodobacter sphaeroides* 2.4.3 cultures were grown as described previously (31). Plasmids and strains used in this work are shown in Table 1.

**DNA manipulation.** Chromosomal DNA was isolated from bacterial cells with the Puregene system (Gentra Systems). Plasmid isolations were done by the alkaline lysis method (4). Standard methods were used for restriction digests, agarose gel electrophoresis, fragment isolation, and ligations (25). Transformation was done by the method of Chung et al. (14a). Plasmids were moved into HCNT1 by conjugation. S-17-1 was the *E. coli* donor strain in biparental matings. Exconjugants were isolated on minimal medium to prevent growth of *E. coli.*

For Southern hybridizations, DNA fragments were transferred to nylon membranes (GeneScreen; NEN Research Products). Labeling of the PCR-derived fragment with digoxigenin was carried out as described by the manufacturer (Boehringer Mannheim). Hybridization was done overnight at 42°C in a solution containing 50% formamide. Membranes were washed twice at 65°C in 0.5× standard saline citrate–0.1% sodium dodecyl sulfate for 30 min before autoradiography. Detection of hybridizing fragments by chemiluminescence was done as described by the manufacturer.

The DNA sequence was obtained from single-stranded DNA by the chain termination method with materials and protocols from the Sequenase version 2.0 kit (U.S. Biochemicals) and $^{35}$S-dATP (Amersham). Both strands of the region encoding *nirK* were sequenced by making subclones and by using the M13 reverse sequencing primer. Database searches of sequences were performed with the BLAST programs (1).

***nirK* isolation and plasmid construction.** A fragment of *nirK* was isolated by PCR with *nir*-specific primers. The forward primer was 5′-GCGCGGTACCT(C G)CACAACATCGACTTCCAC-3′, and the reverse primer was 5′-GCGCGAATTC(GC)AGGTTGTGGTT(GC)AC(GA)TA(GC)GC-3′. Residues in parentheses indicate degeneracies which were based on the high GC content of rhizobia. To facilitate cloning of the PCR product, *Kpn*I and *Eco*RI restriction sites and GC clamps were added to the primers (underlined regions in primers). The isolated fragment was cloned and verified by sequencing to encode a copper-type Nir. The fragment was labeled and used to probe HCNT1 chromosomal DNA digested with *Eco*RI, *Bam*HI, *Hin*dIII, and *Pst*I singly and in combinations. Southern hybridization analysis revealed that a 1.5-kb *Pst*I fragment, which was uncut by the other enzymes, hybridized to the probe. HCNT1 chromosomal DNA was then digested with all four enzymes and separated on an agarose gel. Fragments with a size of 1.5 kb were isolated and cloned into pT7/T3-18 digested with *Pst*I. Southern hybridizations with the PCR-generated probe were used to screen plasmids containing 1.5-kb inserts. A total of 50 plasmids were screened, and 1 hybridized to the probe. This was subcloned and partially sequenced with the M13 reverse sequencing primer. The sequence indicated the fragment contained about 60% of the *nirK* open reading frame. To isolate the remaining fragment, chromosomal DNA from strain HCAT2 was digested with *Bam*HI and religated. Restriction with *Bam*HI allowed most of pJP5603 along with an insert to be recircularized and to be stably replicated in JM109λ*pir*. This construct was subcloned and shown to contain an additional 1.4 kb, including the remaining fraction of the *nirK* open reading frame, upstream of the previously isolated fragment. To join the two fragments containing the *nirK* open reading frame, a 150-bp *Pst*I-*Kpn*I fragment from pAT3 was ligated to the *Pst*I-*Bam*HI insert from pAT4 to give a 1.5-kb *Bam*HI-*Kpn*I fragment in pT7/T3-18 (pAT5) (Table 1). The 1.35-kb *Kpn*I-*Pst*I fragment from pAT3 was ligated to the 1.5-kb *Bam*HI-*Kpn*I fragment from pAT5 to make pAT7, which contains the entire open reading frame (Fig. 1). This 2.9-kb *Pst*I-*Bam*HI fragment was cloned into pRK415, yielding pRKAT7, for complementation experiments.

To construct the *nirK-lacZ* fusion, a 1.4-kb *Bam*HI-*Pst*I fragment from pAT7 was cloned into pRK415, yielding pAT8 (Fig. 1). The *lacZ*-Kan$^r$ cassette from pKOK6, digested with *Pst*I, was cloned into pAT8 linearized with *Pst*I, yielding pAT707. The correct orientation of the *lacZ* cassette was confirmed with *Eco*RI digests.

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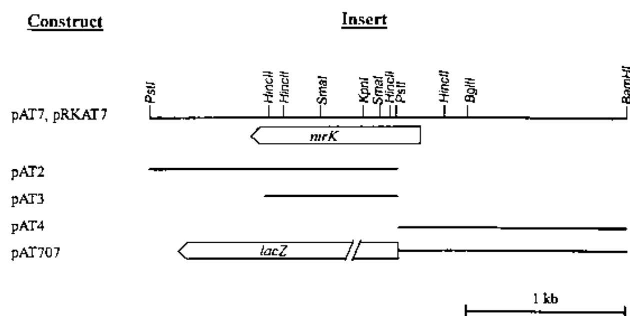


FIG. 1. Schematic representation of the region of the chromosome of *R. "hedysari"* HCNT1 containing *nirK*. The boxed arrow indicates the location and orientation of the deduced open reading frame. The DNA fragments used to generate important plasmid constructs are shown beneath the complete region.

**Enzyme assays.** β-Galactosidase activities were determined with permeabilized cells from at least two independently grown cultures by standard methods (25). Cells removed from stoppered flasks were not kept anaerobic but were used immediately for assays. Nitrite reductase was assayed by monitoring the disappearance of nitrite in the presence of the electron donor methyl viologen (30). If necessary, the cells were washed to remove nitrite present in the growth medium. The presence of nitrite in the growth medium was determined by adding small aliquots of medium, typically 10 μl, to 900 μl of phosphate buffer (pH 7.5) and, without adding electron donor, assaying for nitrite. Typically, assays for both β-galactosidase and nitrite reductase activities were taken from samples withdrawn from flasks at various times during the growth cycle. The reported activities are averages of the maximal values.

**Oxygen uptake assays.** Aerobically or microaerobically grown cells were harvested by centrifugation when cell density, as measured by $A_{600}$, reached 1.0 or 0.6, respectively. Cells were resuspended in a 100-fold-smaller volume of 50 mM phosphate buffer (pH 7.1) and kept on ice until use. Oxygen consumption was measured polarographically essentially as described by Casella et al. (13). The volume of buffer and cells in the reaction chamber was kept at 2.0 ml by addition of cells and resuspension buffer. Typically, 200 μl of cells was added to the reaction vessel. To minimize dilution, nitrite was added in small volumes of at most 25 μl.

**NO evolution assays.** Cells for NO evolution assays were harvested as described for the oxygen uptake assays. NO detection was carried out with an NO-sensitive chemically modified electrode. The nature of this electrode and its application to monitoring NO evolution in denitrifiers have been described elsewhere (26). To determine the specific activities of NO evolution protein, concentrations of the whole cells were determined by the bicinchoninic acid protein assay (Pierce).

## RESULTS

**Isolation and sequencing of *nirK*.** It has been shown previously that the nitrite reductase activity in HCNT1 is inhibited by the addition of the chelator diethyldithiocarbamate (13). Only copper-type nitrite reductases are sensitive to this compound. PCR of HCNT1 genomic DNA, with oligonucleotide primers whose design was based on two areas of conserved sequence in copper-type Nir, produced a single DNA fragment of the expected size. Analysis of the sequence of this fragment revealed it encoded a portion of a copper-type Nir. With this fragment, a 1.5-kb *Pst*I fragment containing part of the *nirK* open reading frame was isolated. By digesting genomic DNA from strain HCAT2 with the appropriate enzymes, the remaining portion of *nirK* was isolated. A restriction map of the region encoding *nirK* is shown in Fig. 1.

**Analysis of *nirK*.** The *nirK* open reading frame (GenBank accession no. U65658) is 1,134 bp long and has a G+C content of 59 mol%. The G+C content is in the range expected for rhizobia. The deduced amino acid sequence encoded by *nirK* is 377 amino acid residues long and has a molecular mass of 40,724 Da. The deduced sequence has significant homology with sequences of other copper-type nitrite reductases. In pairwise alignments of Nir from HCNT1 with other Nir proteins, excluding the signal sequences, the percentage of identity ranged from 65 to 80% (Fig. 2). The high degree of amino acid identity among the Nir sequences is striking.

FIG. 2. Alignment of amino acid sequences of copper-containing nitrite reductases. The sequences are from (in order from top to bottom) *R. "hedysari"* HCNT1 (Rh), *P. aureofaciens* (Pf), *A. cycloclastes* (Ac), and *Pseudomonas aeruginosa* G-179 (Pa). Asterisks indicate residues that are ligands to the copper centers. Numbers coincide with the HCNT1 Nir sequence.

All of the amino acid residues that structural studies have identified as copper ligands are present in the deduced Nir sequence from HCNT1 (18) (Fig. 2). Copper-containing nitrite reductases contain two coppers per monomer. One of these is designated type 1 and is required for electron transfer. The other center is designated type 2 and is the site of nitrite binding and reduction. On the basis of an alignment with Nir from *Achromobacter cycloclastes*, His-132, Cys-173, His-182, and Met-187 are ligands to the type 1 center. His-137, His-172, and His-343 are ligands to the type 2 center.

There are two consecutive ATG initiation codons at the probable start of the open reading frame (Fig. 3). Immediately upstream of the putative translation start codons is a purine-rich Shine-Dalgarno-like sequence (AGGAGAA). To permit a reasonable distance between the Shine-Dalgarno site and the translation start site, the second ATG was chosen as the most probable initiation codon. Starting at position −91 in the promoter region, there is a sequence, -TTGCT(N4)ATCAA-, homologous to the recognition motif for the transcriptional activator Fnr (Fig. 3). The sequence has 9 out of 10 matches with the Fnr consensus sequence, -TTGAT(N4)ATCAA- (29). The presence of this sequence is consistent with *nirK* transcription being oxygen regulated (2, 17).

**Phenotypic effects of *nirK* inactivation.** Inactivation of *nirK* was accomplished by crossing pAT3 into HCNT1, generating strain HCAT2. Since the insert in pAT3 is from within the

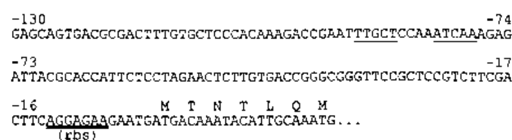


FIG. 3. DNA sequence of the promoter region of *nirK*. The recognition motif of members of the Fnr/Crp family of transcriptional activators is underlined. A potential ribosome binding site (rbs) is doubly underlined. The second of the two consecutive ATG codons was chosen as the translation start to allow sufficient spacing between it and the ribosome binding site. The A in the putative translation initiation codon is residue +1.

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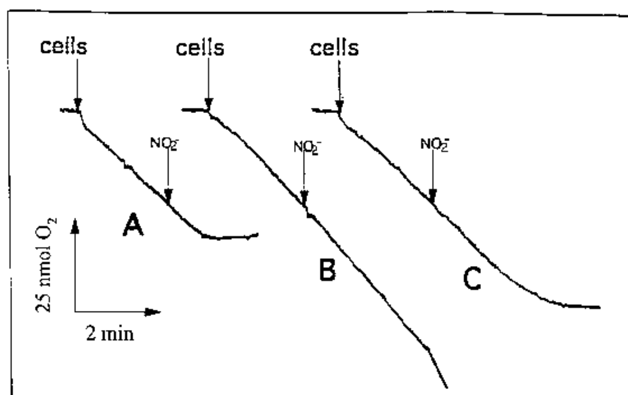


FIG. 4. Influence of nitrite on oxygen uptake of cells of HCNT1 (A), HCAT2 (B), and HCAT3 (C). A downward deflection in the curve indicates oxygen consumption, which was measured polarographically. Equal concentrations of cells were used in all experiments. Cells were added after the baseline had stabilized, as evidenced by the flat region at the top of the curve. Nitrite was added in all experiments to a final concentration of 100 μM.

open reading frame, single crossovers into the chromosome insertionally inactivate *nirK*. Strain HCAT2 has no detectable nitrite reductase activity. This result is consistent with the Southern analysis indicating only a single gene encoding a copper-type Nir. Conjugation of pRKAT7, containing the entire *nirK* open reading frame, into HCAT2 restored nitrite reductase activity to wild-type levels (data not shown).

It has been suggested that a component of the electron transport chain, present only in microaerobically grown cells, is sensitive to nitrite (13). The presence of a nitrite-sensitive component was inferred from the observation that nitrite inhibits oxygen consumption of microaerobically cultured cells. An alternative explanation is that a product of nitrite reduction inhibits oxygen uptake. This would explain the positive correlation between respiratory inhibition and the presence of nitrite reductase. To determine if nitrite reductase activity is required for inhibition of oxygen consumption, the sensitivity of microaerobically grown wild-type and HCAT2 cells to nitrite was determined. Oxygen uptake in wild-type cells was completely inhibited, with no recovery within 10 min, by addition of 100 μM nitrite (Fig. 4A). This is similar to previously reported concentrations and demonstrates the extreme sensitivity of wild-type HCNT1 to nitrite (13). Addition of nitrite to microaerobically grown HCAT2 cells had no effect on oxygen consumption. It can be seen in Fig. 4B that the rate of oxygen uptake of microaerobically grown cells was unaffected by the addition of 100 μM nitrite. Nitrite concentrations as high as 10 mM had no effect on oxygen consumption (not shown). Aerobically grown HCNT1 exhibits a similar insensitivity to nitrite. Oxygen consumption by microaerobically grown *R. sphaeroides* 2.4.3, a true denitrifier, was also not affected by 10 mM nitrite (data not shown). HCAT2 cells containing *nirK* in *trans* (strain HCAT3) regained nitrite sensitivity (Fig. 4C). The nitrite insensitivity of HCAT2 is not consistent with the presence of a nitrite-sensitive component of the respiratory chain. Instead, it is the activity of nitrite reductase that appears to be obligatory for inhibition of oxygen consumption.

**Regulation of *nirK*.** To study in more detail the regulation of *nirK* expression in HCNT1, a *nirK-lacZ* transcriptional fusion was constructed. Plasmid pAT707 contains a 1.4-kb *Bam*HI-*Pst*I fragment of *nirK*, which includes 1 kb of upstream DNA, cloned to the *lacZ*-Kan$^r$ cassette of pKOK6 (Fig. 1) (23). It should be noted that wild-type HCNT1 has no detectable β-galactosidase activity under the conditions used in this study. Cells containing the *nirK-lacZ* fusion had maximal levels of β-galactosidase activity under microaerobic conditions (Fig. 5). Expression increased nearly 45-fold as cells shifted from aerobic to microaerobic conditions. Consistent with the results of Casella et al. (13), the presence of a nitrogen oxide was not required for expression. In fact, the inclusion of 500 μM nitrite in the medium slightly decreased expression (Fig. 5).

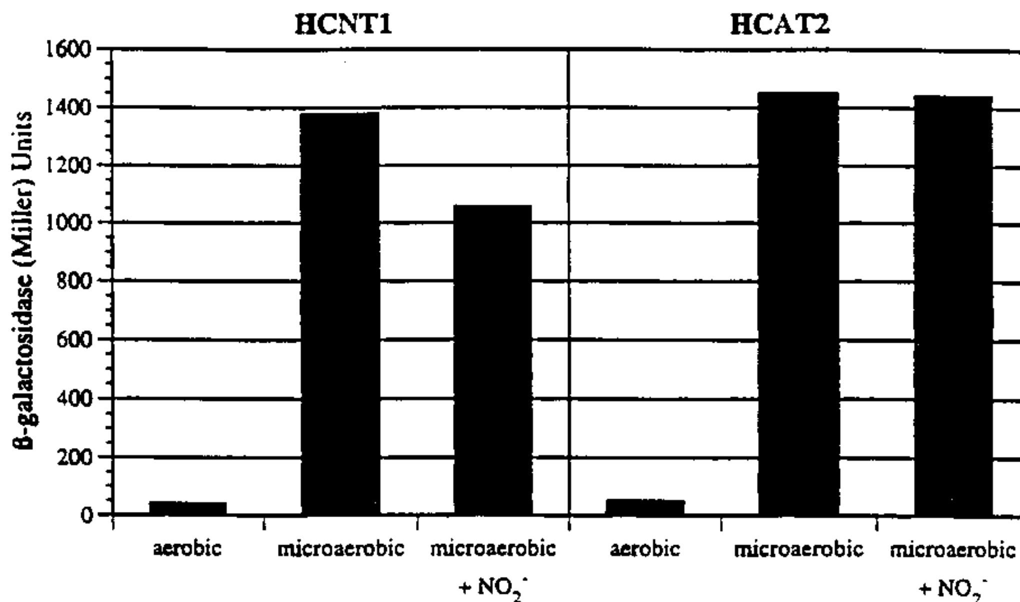


FIG. 5. Expression of *nirK-lacZ* in HCNT1 and HCAT2 under various growth conditions. Aerobic and microaerobic cells were grown identically, except that after inoculation of the microaerobic culture, a rubber stopper was placed on the flask to prevent oxygen exchange. Nitrite was added to a final concentration of 500 μM. The standard deviations were never larger than 10% for any of the measurements.

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To test if the decrease in expression in the presence of nitrite is an indirect result of growth inhibition or is directly due to nitrite, expression of *nirK-lacZ* was monitored in HCAT2. In medium lacking nitrogen oxides, microaerobically grown cells of HCAT2 and HCNT1 had identical levels of *nirK-lacZ* expression. Addition of 500 μM nitrite had no effect on expression of the fusion in HCAT2 (Fig. 5). This indicates that nitrite does not directly affect expression but probably stops cell growth before maximal levels of expression are reached. These results also demonstrate that *nirK* expression HCNT1 is only responsive to oxygen concentration; nitrogen oxides play no role in activating expression.

Maximal expression of *nirK* in the denitrifier *R. sphaeroides* 2.4.3 has been shown to be limited to only denitrifying strains of *R. sphaeroides* (31). *R. "hedysari"* strains lacking Nir have been described. *R. "hedysari"* CC1335 is a variant that has no detectable nitrite reductase activity when grown microaerobically (12). The absence of *nirK* in CC1335 was confirmed by chromosomal Southern blots with *nirK* from HCNT1 as a probe (data not shown). Plasmid pAT707, containing the HCNT1 *nirK-lacZ* fusion, was conjugated into CC1335, and its expression was monitored under microaerobic conditions. The maximum levels of LacZ activity measured were about 2,100 U, which is slightly higher than those in HCNT1 cells grown under identical conditions. These results suggest that *nirK* expression in HCNT1 does not rely on some regulatory pathway unique to nitrogen oxide reduction but instead is controlled solely by oxygen-sensitive regulatory proteins. The *nirK-lacZ* fusion was not expressed in *R. sphaeroides* 2.4.3, however, suggesting that expression is limited to certain rhizobia (data not shown).

**NO production by HCNT1.** Work in this study and elsewhere has shown that the nitrite inhibition of oxygen respiration in HCNT1 requires microaerobic conditions and the presence of nitrite reductase (12). The product of nitrite reductase is NO, a well-known inhibitor of many metalloenzymes, including cytochrome oxidase (8). An attractive explanation for nitrite sensitivity in HCNT1 is that nitrite reductase activity produces inhibitory levels of NO. This could occur because either NO reductase is absent or is kinetically incompetent to keep pace with Nir activity. In true denitrifiers, steady-state concentrations of NO are kept in the nanomolar range by NO reductase activity (19). To directly test if nitrite reduction by HCNT1 leads to NO accumulation, a chemically modified electrode sensitive to NO was used to monitor the products of nitrite reduction in HCNT1 and HCAT2. This electrode has a limit of detection of about 100 nM (26).

Cyclic voltammograms, showing the time course of NO evolution in HCNT1 and HCAT2 cells after addition of nitrite, are shown in Fig. 6. NO evolution is seen as an increase in peak current in the −0.90-V region of the voltammogram. In experiments with HCNT1 cells, significant concentrations of NO are detectable after the addition of nitrite (Fig. 6A). NO concentrations typically reached 15 μM 5 min after addition of 100 μM nitrite. Assuming linearity of NO production over the 5-min period, the specific activity of NO evolution in HCNT1 was ~4 nmol $min^{-1}$ mg of $protein^{-1}$ upon addition of 100 μM nitrite. As expected, there was no detectable NO evolution from HCAT2 (Fig. 6B).

The production of NO by HCNT1 is very similar to that observed with a mutant of *R. sphaeroides* 2.4.3 in which the NO reductase is insertionally inactivated (26). In both strains, 10 to 20 μM NO accumulates within 5 min after nitrite addition. The maximum steady-state level of NO produced by both strains is about 20 to 30 μM. With the identical electrode, only 200 nM NO was detected after nitrite addition to wild-type *R. sphaeroides* 2.4.3 (26). The accumulation of NO by HCNT1 suggests NO reductase is absent or only marginally active.

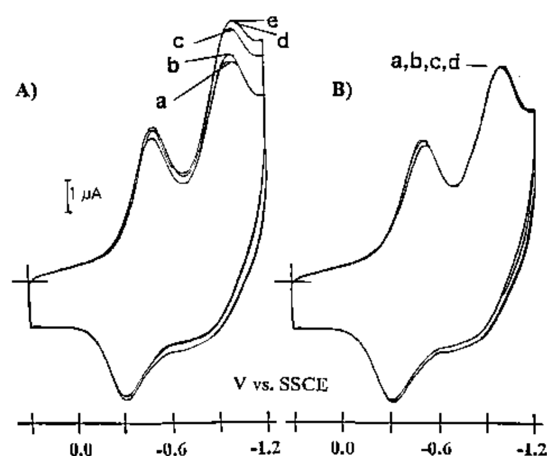


FIG. 6. Cyclic voltammetry measurements with a glassy-carbon electrode coated with an electropolymerized film of [$Cr^{3+}$ (v-tpy)$_2$] and a film of Nafion in 0.05 M Tris buffer (pH 7.0) containing 0.05 M $NaClO_4$ and 100 μM $NO_2^-$ and either HCNT1 (A) or HCAT2 (B) cells after zero (a), 1 (b), 5 (c), 10 (d), or 15 (e) min. The increase of the peak current in panel A is due to the electrocatalytic reduction of NO, produced in the cell at the modified electrode. All potentials are referenced to a sodium chloride-saturated calomel electrode (SSCE).

## DISCUSSION

We have shown that *R. "hedysari"* HCNT1 encodes a copper-containing nitrite reductase closely related to nitrite reductases found in true denitrifiers. What makes HCNT1 unusual is that none of the other terminal reductases associated with denitrification appear to be present. Previous work has shown that HCNT1 cannot reduce or grow anaerobically with nitrate or nitrous oxide (13). Moreover, nitrate reductase and nitrous oxide reductase activities have not been detected, indicating both proteins are absent. Our results suggest there is no NO reductase (Fig. 6A). It should be noted, however, that experiments monitoring the products of nitrogen oxide reduction by gas chromatography have shown that microaerobically grown cells of HCNT1 produce nitrous oxide when nitrite or NO is present (14). Other nondenitrifiers have also been shown to be capable of generating nitrous oxide from nitrite or NO by an unknown pathway that does not involve NO reductase (7). The nitrous oxide production observed in HCNT1 may be due to a similar mechanism. Irrespective of the exact mechanism of nitrous oxide production, it is obvious that the rate of NO reduction is not sufficient to prevent NO accumulation.

The accumulation of NO as a consequence of nitrite reductase activity is responsible for all of the unique, nitrite-dependent phenotypic characteristics of HCNT1. In many ways, HCNT1 is phenotypically equivalent to a true denitrifier that is Nor deficient. For example, both HCNT1 and denitrifiers lacking NO reductase are incapable of anaerobic growth with nitrate or nitrite (5). This is a direct result of NO accumulation to toxic levels. The sensitivity of oxygen respiration in HCNT1 to nitrite is also a result of NO accumulation. NO binds with high affinity to most proteins containing transition metals. In particular, proteins containing high-spin hemes, such as cytochrome *c* oxidase, can bind NO present at nanomolar concentrations (9). Once formed, nitrosyl-heme complexes are very stable, making NO a potent inhibitor of oxidase activity. The NO concentration produced in a typical experiment (Fig. 6) is

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well above the concentrations shown to inhibit oxidase activity (8).

Another important metalloenzyme in HCNT1 is also inhibited by the activity of nitrite reductase. Casella et al. (12) have shown that nitrogen fixation in bacteroids is inhibited by exposure to small concentrations of nitrite. Like other metalloenzymes, nitrogenase is inhibited by NO at low concentrations (10). It seems likely that bacteroids of HCNT1, like microaerobically grown free-living cells, express nitrite reductase, and since NO reductase activity is lacking, NO accumulation inhibits its nitrogen fixation.

One significant difference between *nirK* in HCNT1 and the genes encoding nitrite reductases in true denitrifiers is that its expression does not require the presence of nitrite. Studies with either enzyme activity assays or specific antibodies to monitor expression of the terminal reductases in true denitrifiers have shown that there is little expression unless an intermediate in denitrification is present (15, 24). In a more recent study with gene fusions, the expression of *nirK* in *R. sphaeroides* 2.4.3 has been examined in detail (31). In this denitrifier, not only is a nitrogen oxide required for expression, but the activity of nitrite reductase is required as well. In contrast, expression of Nir in HCNT1 appears to be solely dependent on oxygen concentration. The results shown in Fig. 5 show that inclusion of nitrite in the medium does not enhance Nir expression. An active nitrite reductase is also not required, since *nirK-lacZ* expression in HCAT2 and CC1335, both lacking nitrite reductase, was as high or higher than that observed in HCNT1.

In this work, we have shown that *R.* "*hedysari*" HCNT1 contains a gene encoding a copper-type Nir whose product, NO, is lethal to the cell. However, this seems counterintuitive. Why does HCNT1 carry a gene that encodes a protein whose reaction product is lethal, particularly under those conditions when the gene is most highly expressed? The most obvious explanation is that in the environment where these bacteria are found, nitrite concentrations are so low that even if anaerobic conditions occur, NO cannot accumulate to toxic levels. Nitrate concentrations in the soil are very low, typically in the low micromolar range (3). Nitrite concentrations are typically 100-fold lower than nitrate concentrations (3). Nitrite concentrations less than 25 μM are not lethal to HCNT1, so it is unlikely that toxic NO concentrations would occur in the environment. In this context, it is important to note that HCNT1 does not possess nitrate reductase activity. Since soil nitrate concentrations are significantly higher than nitrite concentrations, the loss of nitrate reductase may prevent reduction of nitrate to lethal levels of nitrite.

If nitrite reductase expression is not disadvantageous to HCNT1, is it advantageous? This is unclear. Inactivation of *nirK* resulted in no obvious phenotypic changes in HCNT1, other than those linked to the loss of NO production. Strains of *R.* "*hedysari*" that lack nitrite reductase have been isolated (CC1335, for example), and they nodulate *Hedysarum* spp. as effectively as HCNT1, indicating nitrite reductase is not required for infection and nodulation (11). Nitrite reduction in denitrifiers is coupled to energy conservation, and it is possible that reduction of small amounts of nitrite could be used for energy conservation when oxygen concentrations are low (22). If Nir is used for energy conservation, it might be expected that the genes encoding NO reductase and nitrous oxide reductase would be found in HCNT1 to maximize the energy available from nitrite. Why are they absent? If the genes for denitrification can be transferred horizontally among bacteria, perhaps HCNT1 received, and had the genetic background to stably maintain, a subset of the entire denitrification complement. Other facultative aerobes that express only a single nitrogen oxide reductase have been described (33). What makes HCNT1 unique is that it is the only member of this group of bacteria whose sole terminal nitrogen oxide reductase is nitrite reductase.

## ACKNOWLEDGMENTS

We thank Jiarong Shi for assistance in sequencing *nirK*.

A.T. was supported by a grant from the CNR. This work was supported by the Office of Naval Research, National Science Foundation, Italy/USA Bilateral Projects (grant number 94.00072.CT06 [CNR]), and the Department of Energy (95ER20206).

## REFERENCES


1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Anthamatten, D., and H. Hennecke.** 1991. The regulatory status of the *fixL*- and *fixJ*-like genes in *Bradyrhizobium japonicum* may be different from that in *Rhizobium meliloti*. Mol. Gen. Genet. **225:**38–48.
3. **Binnerup, S. J., and J. Sørensen.** 1992. Nitrate and nitrite microgradients in barley rhizosphere as detected by a highly sensitive denitrification bioassay. Appl. Environ. Microbiol. **58:**2375–2380.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Braun, C., and W. G. Zumft.** 1991. Marker exchange of the structural genes for nitric oxide reductase blocks the denitrification pathway of *Pseudomonas stutzeri* at nitric oxide. J. Biol. Chem. **266:**22785–22788.
6. **Brittain, T., R. Blackmore, C. Greenwood, and A. J. Thomson.** 1992. Bacterial nitrite-reducing enzymes. Eur. J. Biochem. **209:**793–802.
7. **Brons, H. J., W. R. Hagen, and A. J. B. Zehnder.** 1991. Ferrous iron dependent nitric oxide production in nitrate reducing cultures of *Escherichia coli*. Arch. Microbiol. **155:**341–347.
8. **Brown, G. C.** 1995. Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. FEBS Lett. **369:**136–139.
9. **Brown, G. C., and C. E. Cooper.** 1994. Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Lett. **356:**295–298.
10. **Caballero, F. J., M. I. Igeno, R. Quiles, and F. Castillo.** 1992. In-vivo inhibition of nitrogenase by hydroxylamine in *Rhodospirillaceae*: role of nitric oxide. Arch. Microbiol. **158:**14–18.
11. **Casella, S., R. R. Gault, K. C. Reynolds, J. E. Dyson, and J. Brockwell.** 1984. Nodulation studies on legumes exotic to Australia: *Hedysarum coronarium*. FEMS Microbiol. Lett. **22:**37–45.
12. **Casella, S., J. P. Shapleigh, F. Lupi, and W. J. Payne.** 1988. Nitrite reduction in bacteroids of *Rhizobium* "*hedysari*" strain HCNT1. Arch. Microbiol. **149:**384–388.
13. **Casella, S., J. P. Shapleigh, and W. J. Payne.** 1986. Nitrite reduction in *Rhizobium* "*hedysari*" strain HCNT1. Arch. Microbiol. **146:**233–238.
14. **Casella, S., A. Toffanin, S. Ciompi, N. Rossi, and W. J. Payne.** 1994. Metabolism of nitrogen oxides and hydroxylamine in cells of true denitrifiers and *Rhizobium* "*hedysari*" HCNT1. Can. J. Microbiol. **40:**1–5.

14a. **Chung, C. T., S. L. Niemela, and R. H. Miller.** 1989. One-step transformation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86:**2172–2175.

15. **Coyne, M. S., and J. M. Tiedje.** 1990. Induction of denitrifying enzymes in oxygen-limited *Achromobacter cycloclastes* continuous culture. FEMS Microbiol. Ecol. **73:**263–270.
16. **Fenderson, F. F., S. Kumar, E. T. Adman, M.-Y. Liu, W. J. Payne, and J. Legall.** 1991. Amino acid sequence of nitrite reductase: a copper protein from *Achromobacter cycloclastes*. Biochemistry **30:**7180–7187.
17. **Fischer, H.-M.** 1994. Genetic control of nitrogen fixation in rhizobia. Microbiol. Rev. **58:**352–386.
18. **Godden, J. W., S. Turley, D. C. Teller, E. T. Adman, M. Y. Liu, W. J. Payne, and J. Legall.** 1991. The 2.3 angstrom X-ray structure of nitrite reductase from *Achromobacter cycloclastes*. Science **253:**438–442.
19. **Goretski, J., and T. C. Hollocher.** 1988. Trapping of nitric oxide produced during denitrification by extracellular hemoglobin. J. Biol. Chem. **263:**2316–2323.
20. **Goretski, J., O. C. Zafiriou, and T. C. Hollocher.** 1990. Steady-state nitric oxide concentrations during denitrification. J. Biol. Chem. **265:**11535–11538.
21. **Hochstein, L. I., and G. A. Tomlinson.** 1988. The enzymes associated with denitrification. Annu. Rev. Microbiol. **42:**231–261.

21a. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.

22. **Koike, I., and A. Hattori.** 1975. Energy yield of denitrification: an estimate from growth yield in continuous cultures of *Pseudomonas aeruginosa* under nitrate, nitrite and nitrous oxide limited conditions. J. Gen. Microbiol. **88:**11–19.
23. **Kokotek, W., and W. Lotz.** 1989. Construction of a *lacZ*-kanamycin-resis-


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tance cassette, useful for site-directed mutagenesis and as a promoter probe. Gene **84:**467–471.
24. **Korner, H., and W. G. Zumft.** 1989. Expression of denitrification enzymes in response to the dissolved oxygen and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. Appl. Environ. Microbiol. **55:**1670–1676.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
26. **Pariente, F., M. Maskus, Q. Wu, A. Toffanin, J. P. Shapleigh, and H. D. Abruna.** 1996. Electrocatalytic reduction of nitric oxide at electrodes modified with electropolymerized films of [Cr(v-tpy)2]+3 and their applications to cellular NO determinations. Anal. Chem. **68:**3128–3134.
27. **Payne, W. J.** 1981. Denitrification. John Wiley & Sons, New York.
28. **Payne, W. J., M. A. Grant, J. Shapleigh, and P. Hoffman.** 1982. Nitrogen oxide reduction in *Wolinella succinogenes* and *Campylobacter* species. J. Bacteriol. **152:**915–918.
28a. **Penfold, R. J., and J. M. Pemberton.** 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene **118:**145–146.
28b. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.
29. **Spiro, S.** 1994. The FNR family of transcriptional regulators. Antonie Leeuwenhoek **66:**23–36.
30. **Stewart, V., and J. Parales, Jr.** 1988. Identification and expression of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. J. Bacteriol. **170:**1589–1597.
31. **Tosques, I. E., A. V. Kwiatkowski, J. Shi, and J. P. Shapleigh.** Characterization and regulation of the gene encoding nitrite reductase in *Rhodobacter sphaeroides* 2.4.3. Submitted for publication.
32. **Ye, R. W., M. R. Fries, S. G. Bezborodnikov, B. A. Averill, and J. M. Tiedje.** 1993. Characterization of the structural gene encoding a copper-containing nitrite reductase and homology of this gene to DNA of other denitrifiers. Appl. Environ. Microbiol. **59:**250–254.
33. **Zumft, W. G.** 1992. The denitrifying prokaryotes, p. 554–582. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes. Springer-Verlag, Berlin.

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