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A Third Subunit in Ancestral Cytochrome *c*-Dependent Nitric Oxide Reductases

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Reduction of NO to N_2O by denitrifiying bacteria is catalyzed either by a monomeric quinol-nitric oxide reductase (qNor) or by a heterodimeric cytochrome *c*-dependent nitric oxide reductase (cNor). In ancient thermophilic bacteria belonging to the *Thermales* and *Aquificales* phylogenetic groups, the cluster encoding the cNor includes a small third gene (*norH*), in addition to those encoding homologues to the subunits of a typical cNor (*norC* and *norB*). We show in *Thermus thermophilus* that the three genes are cotranscribed in a single mRNA from an inducible promoter. The isolation of individual *nor* mutants and the production *in vivo* of His-tagged NorH protein followed by immobilized-metal affinity chromatography (IMAC) allowed us to conclude that NorH constitutes a third subunit of the cNor from *T. thermophilus*, which is involved in denitrification *in vivo*, likely allowing more efficient electron transport to cNor.

The nitrogen cycle is one of the most important biogeochemical cycles of the biosphere. The biochemistry of this cycle depends on several redox reactions divided into assimilative or respiratory reactions carried out mainly by microorganisms. One of these processes is the denitrification pathway, where nitrogen oxides are used as electron acceptors for anaerobic respiration in four reduction steps ($NO_3^- > NO_2^- > NO > N_2O > N_2$), each one catalyzed by the corresponding reductase (1, 2).

The genes encoding these reductases are distributed along the whole phylogenetic trees from both *Bacteria* and *Archaea*, being in many cases species and even strain specific. This fact and phylogenetic comparisons with related enzymes support both an ancestral nature for the process (1) and the existence during evolution of frequent events of loss or acquisition by lateral gene transfer (3, 4).

NO is a strongly cytotoxic gas that efficiently inhibits metalloproteins involved in respiration. Thus, most organisms in the three domains contain enzymes that are able to cope with the toxicity of this gas (5). In fact, in most sequenced denitrifying bacteria, the genes encoding the nitrite reductase responsible for NO production are clustered with the genes encoding nitric oxide reductases (NOR) that reduce NO to N_2O, a less soluble and less toxic gas. In contrast, the nitrous oxide reductase is not present in many denitrifying organisms, so the denitrification process frequently ends in N_2O.

The main enzymes involved in the reduction of NO during the denitrification process (NOR) are integral to the membrane. These Nor enzymes belong to the same heme-copper oxidase superfamily as the O_2-reducing cytochrome oxidases. However, Nor enzymes seem not to conserve energy as they are devoid of the proton pathways from the cytoplasm that allow the cytochrome oxidases to pump protons outwards (6–8).

Three types of nitric oxide reductases have been described so far: the cytochrome *c* type NOR (cNor), the quinol-dependent NOR (qNor), and the copper-dependent qNor (CuqNor) (9). The cNor is the most thoroughly studied nitric oxide reductase. It is built by two subunits, a cytochrome *c* subunit (NorC) and a catalytic subunit (NorB) (2).

The genus *Thermus* belongs to one of the oldest phylogenetic groups of the *Bacteria* lineage (10). Despite the extreme thermophily of many of its members, all strains of the genus prefer oxygen as the electron acceptor in respiration, and many of them are described as strict aerobes (11). However, many natural strains of *Thermus* spp. that have not been subjected to routine aerobic growth under laboratory conditions can grow anaerobically with nitrate, producing nitrite or N_2O (12), the final products depending on the strain (13). Actually, a high rate of N_2O emissions has been detected *in situ* in thermal springs where *Thermus thermophilus* constitutes a major population (14). The instability of the denitrification capability in this genus is related to the localization of the two gene clusters that encode the denitrification pathway (12) within an insertion sequence-rich region of a megaplasmid. In *T. thermophilus*, nitrate respiration is encoded by the nitrate respiration conjugative element (NCE) (15), a cluster of genes that can be transferred laterally to aerobic strains (16, 17). The NCE cluster encodes a heterotetrameric nitrate reductase (Nar) (18), one or two nitrate/nitrite transporters (13, 19), and a sensory system that allows switching from aerobic to anaerobic respiration (15). In addition, many but not all of the NCEs encode a dedicated NADH dehydrogenase that provides electrons to the Nar (20, 21). On the other hand respiration of nitrite is encoded by the nitrite respiration cluster (*nic*) located downstream of NCE and is also transferable (17). In *T. thermophilus* PRQ25, the *nic* cluster includes genes encoding a cd_1-type nitrite reductase (NirS), a protein involved in its maturation (NirJ), and a periplasmic cytochrome *c* (NirM) (22). Also within the *nic* cluster, a putative operon exists that encodes NorC and NorB, the two subunits of a cNor (17). Recently, we have shown that NorC and NorB form a functional nitric oxide reductase when expressed recombinantly

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TABLE 1 Strains used in this work

Strain	Genotype	Reference or source
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	32
T. thermophilus		
HB27	Wild type	Y. Koyama[a]
PRQ14	Wild type	33
PRQ15	Wild type	33
PRQ16	Wild type	33
PRQ17	Wild type	33
PRQ21	Wild type	33
PRQ23	Wild type	33
PRQ24	Wild type	33
PRQ25	Wild type	33
PRQ26	Wild type	33
PRQ27	Wild type	33
PRQ28	Wild type	33
PRQ30	Wild type	33
PRQ31	Wild type	33
RQ-1	Wild type	34
B	Wild type	33
HB27d	HB27 transformed with DNA from strain PRQ25; denitrifying strain	17
Δ*norC* mutant	HB27d *norC* deletion *mutant*	This work
Δ*norB*::*kat* mutant	HB27d Δ*norB*::*kat* Kan^r	This work
Δ*norH* mutant	HB27d *norH* deletion mutant	This work
Δ*norC*(pMKcNOR) mutant	HB27d Δ*norC*(pMKcNOR) Kan^r	This work
HB27d *norH*-His mutant	HB27d pK18norH-His Kan^r	This work

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in *Escherichia coli* (8). Intriguingly, the *norC* and *norB* genes of *Thermus* spp. are always followed by a third gene (*norH*) encoding a small membrane protein, which is also conserved in the *nor* clusters of thermophilic *Aquificales*, supporting a relevant role at high temperatures. In this work, we analyze the physiological role of NorH and find that it is associated *in vivo* with the cNor, thus constituting a third subunit required for efficient denitrification.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this work are described in Table 1. *T. thermophilus* strains were routinely grown aerobically in *Thermus* broth (TB) (23) at 70°C with mild shaking (150 rpm). Anaerobic growth was achieved in Hungate tubes containing 10 ml of TB supplemented with potassium nitrate (10 mM) or sodium nitrite (5 mM), leaving a headspace for the collection of gas samples. After the tubes were sealed with thermoresistant butyl rubber stoppers and metal caps, the tubes were bubbled with N_2 to eliminate oxygen before sterilization. Once inoculated with the study strain, residual oxygen was rapidly consumed by the cells. The aerobic strain *T. thermophilus* HB27 was always used as negative control for anaerobic growth in all experiments. *T. thermophilus* colonies were isolated on TB agar plates. Kanamycin (Kan [30 mg/liter]) and hygromycin B (Hyg [50 mg/liter]) were added for selection when required.

Escherichia coli DH5α was used for the construction of plasmids. *E. coli* strains were grown at 37°C on liquid or solid LB (24). Kan (30 mg/liter), ampicillin (Amp [100 mg/liter]), and Hyg (50 mg/liter) were used when needed.

Plasmids and isolation of mutants. The plasmids used in this work are described in Table 2. For the isolation of clean deletion mutants, we followed a pop-in/pop-out strategy with different suicide plasmids. In essence, 500-bp regions upstream and downstream of the target gene were amplified by PCR with the primers indicated in Table 3 and cloned into pK18, a plasmid that replicates in *E. coli* but not in *Thermus* spp., and the constructs were further used to transform the parental strain. Kanamycin-resistant (Kan^r) colonies obtained by single recombination (pop-in) were grown in TB without antibiotics for 10 to 25 generations to allow the loss of the plasmid by back recombination (pop-out). Clones sensitive to kanamycin were analyzed by PCR to identify those in which the target gene was not present.

For larger genes like *norB*, a replacement strategy with a selectable *kat* gene cassette through double recombination was followed. For this, a pUC119 derivative was constructed that carried upstream and downstream regions of *norB* separated by the *kat* gene in the forward orientation. The construct (pUC19Δ*norB*::*kat*) was linearized and used to transform *T. thermophilus*. The presence of the NorC protein in the mutants was assayed by Western blotting with specific rabbit antisera (17) and anti-rabbit antibodies labeled with horseradish peroxidase. Detection was carried out through a bioluminescence assay (ECL; Amersham International).

Promoter induction assays. Quantitative measurement of the transcription of the *nor* operon was tested in cultures of *T. thermophilus* strains transformed with pMHPnorβgal, a promoter probe plasmid in which the $norC_p$ promoter was cloned after amplification with primers PnorXbaIdir and PnorNdeIrev (Table 3), controlling the expression of a thermostable β-galactosidase. The activity assays were carried out in 96-well plates with 25 μl of cell suspensions at optical density at 550 nm (OD_{550}) of 1 in 50 mM phosphate buffer (pH 7.5). Cells were first permeabilized by incubation at 37°C for 15 min with 0.1% (wt/vol) of sodium dodecyl sulfate (SDS). The samples were then incubated at 70°C with 3 mM orthonitrophenyl-β-D-galactopyranoside (ONPG). The β-galactosidase activity is presented as the average of two independent experiments with three rep-

TABLE 2 Plasmids used in this work

Name	Characteristics	Reference or source
pH118	Hyg^r; modified pUC118 with Amp^r cassette replaced by Hyg^r one	22
pK18	Kan^r; modified pUC118 with Amp^r cassette replaced by Kan^r one	35
pMH Pnorß-gal	Hyg^r; pMH184 derivative where $norC_p$ has been cloned controlling a thermostable β-galactosidase	This work
pMH184	Hyg^r pMK184 derivative; Hyg^r cassette (*hph5*) replaces *kat* (NdeI-BglII)	15
pMK18	Kan^r PlacZ′; *slpA* promoter(PslpA); bifunctional plasmid (*Thermus-E. coli*)	36
pMK184	Kan^r pMK18 derivative.	15
pUC18/19	Amp^r PlacZ′; cloning vector	37
pK18*norH*-His	Kan^r pK18 derivative; *norH* cloned with His tag in its C terminus.	This work
pK18Δ*norC*	Kan^r pK18 derivative; upstream (800 bp) and downstream (800 bp) regions of *norC* cloned for clean deletion mutant construction	This work
pK18Δ*norH*	Kan^r pK18 derivative; upstream (800 bp) and downstream regions (800 bp) regions of *norH* cloned for clean deletion mutant construction	This work
pUC19Δ*norB-kat*	Kan^r pUC19 derivative; upstream (500 bp) and downstream (500 bp) regions of *norB* cloned for insertional mutant construction	This work
pMHPnorβgal	Promoter probe plasmid containing $norC_p$ promoter between XbaI and HindIII sites	This work

TABLE 3 Oligonucleotides used in this work as primers

Name	Sequence (5′→3′)	Purpose or amplified sequence
1b	AAAATCTAGACTCCGGCTCATAAACTCCGAC	*norC* upstream region
1b-dir	AAAAGTCGACGCGGGCCTTTTCCAAGAGGT	*norC* upstream region
1c	AAAATCTAGAGGAGGTGAAGCATGACCCAG	*norC* downstream region
1c-rev	AAAAGAATTCCGATCCAGAAGTAGTGGTGGC	*norC* downstream region
2b	AAAATCTAGACTACTCCGCCGCCGCAAA	*norB* upstream region
2b-dir	AAAAAAGCTTGCCATGGCCCCATGCTAAC	*norB* upstream region
2c	AAAATCTAGAGGAGGTGGGGGCCCATGA	*norB* downstream region
2c-rev	AAAAGAATTCCCTTCCAGGTCTTGCGGATCT	*norB* downstream region
3a	AAAAGAATTCCCATCGCCCAGGCCTTCG	*norH* upstream region
3b	AAAATCTAGACTAGTCATGGGCCCCCAC	*norH* upstream region
3b-dir	AAAAGAATTCGAGGCCACTTGGGAGGTC	*norH* His tag
3c	AAAATCTAGATCCCGGTCCGGACCCC	*norH* downstream region
3c-rev	AAAAGAATTCCTTGAACTTGCTGGACTCGATG	*norH* downstream region
3d	AAAAGTCGACGGCACGTAGACCTTCCAGGT	*norH* downstream region
5b	AAAATCTAGAGTGGGGGCGCGTTTCCAT	*nirS*
7a	AAAAGAATTCCTTCCCCGGCCTTGGCC	Region upstream of *norC*
badM-Fw	AAAACATATGGCCCTTCGGAGCCTTC	Region upstream of *norC*
kat1	CCTTTTTCCCCGCATCC	Detection of *kat* gene
kat2	GAAACTTCTGGAATCGC	Detection of *kat* gene
kat3	GGAACGAATATTGGATA	Detection of *kat* gene
kat4	AGAAATTCTCTAGCGAT	Detection of *kat* gene
norB_Fw	AAAATCTAGAATGACCCAGGCTTTACCGCAG	*norB*
norB_Rv	AAAAGAATTCCTAGTCATGGGCCCCCAC	*norB*
norC_Fw	AAAATCTAGAATGGAGATCGGCTGGATAGAAAC	*norC*
norC_Rv	AAAAGAATTCCTACTCCGCCGCCGCAAA	*norC*
norH-histag Rev	AAAAAGCTTCAGTGGTGGTGGTGGTGGTGCCCGGATCCGTGAGCGTGCTC	*norH* His tag
orf85_Rv	AAAAGAATTCTCAGTGAGCGTGCTCCTTCT	*norH*
PnorXbaIdir	AAAATCTAGACTTGGGCCACACCCCTC	$norC_p$ promoter
PnorNdeIrev	AAAACATATGCTTACCCTCCTTTCACCTCCG	$norC_p$ promoter

licates and is expressed as the variation in OD_{420} as a function of time (ΔOD_{420}) normalized to the amount of cells.

RT-PCR analysis. Total RNA from exponential cultures of *T. thermophilus* was extracted with the RNeasy minikit (Qiagen). The extraction was performed from aerobic cultures and from anaerobic cultures grown for 4 h with 20 mM potassium nitrate. The RNA was retrotranscribed to cDNA using Superscript III reverse transcriptase (Invitrogen), following the manufacturer's instructions, and the product was analyzed by PCR.

Detection of nitrogen oxides. Nitrite was detected by colorimetric assays (25). Nitrous oxide was measured from gas samples taken from the headspace of cultures grown for 24 h at 70°C in Hungate tubes. Gas samples were injected into a Hewlett Packard 5890 series II gas chromatograph with a Porapak 80-100 column installed, and the electron capture data were registered by a Spectra Physics SP 4290 integrator. The injector temperature was maintained at 300°C, the column at 40°C, and the electron capture detector at 300°C. The concentration of N_2O (peak with retention time of 4.6 ± 0.14 min), was calculated using 0.4-, 100-, and 1,000-ppm standards (Scientific and Technical Gases, Ltd., Newcastle under Lyme, United Kingdom).

NO reductase activity assay. The following chemical reaction was used to produce the substrate NO used for these experiments: $2\ NaNO_2 + 2\ FeSO_4 + 3\ H_2SO_4 \rightarrow 2\ NO + Fe_2(SO_4)_3 + 2\ NaHSO_4 + 2\ H_2O$.

The reaction was carried out in 20-ml Hungate tubes after first adding 0.25 g of $NaNO_2$. Oxygen was removed in from inside the bottle by vacuum aspiration. Afterwards 3 ml of saturated ferrous sulfate acid solution were injected, and the gas produced was collected in 10-ml syringes and used to prepare a saturated NO solution.

The NO reductase activity was measured from membrane extracts. A 100-μl sample of NO-saturated solution was injected into 1.8 ml of 100 mM Tris-HCl (pH 7.7) with 10 mM ascorbate and 0.5 mM TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine) in 5-ml Vacutainer tubes containing 10 μl of membrane extracts (25 μg/ml of proteins) from anaerobically grown cultures of the different strains of *T. thermophilus*. The reaction mixture was incubated at 60°C for 16 h, and the N_2O produced was measured by gas chromatography as described above.

Expression and purification of wild-type and NorH-deficient cNOR proteins. Protein expression and purification were done as previously described (8). In essence, we expressed and purified the wild-type and NorH-deficient enzymes from an *E. coli* strain that coexpresses the genes required for the assembly of cytochromes *c*, pEC86 (26), and the pRARE plasmid carrying tRNA genes needed for reading rare codons (pRARE). The enzymes were purified by affinity to NorB or NorH His-tagged subunits as described previously (8).

Steady-state activity of the isolated enzymes. NO consumption rates were measured in an ISO-NO electrode equipped with ISO-NO Mark II (World Precision Instruments) in a 2-ml anaerobic reaction chamber at 42°C. The data were recorded on a Duo 18 device (World Precision Instruments). The assay solution contained 50 mM Na citrate (pH 6.0), 0.05% dodecyl-β-D-maltoside (DDM), 5 mM ascorbate, 0.5 or 2.5 mM TMPD, or 10 μM phenazine methosulfate (PMS), as indicated. After 20 min of argon flux, NO-saturated solution was added to the reaction mixture at a final concentration of approximately 25 μM. Once the background consumption of NO was stable, NO reduction (consumption) was initiated by the addition of cNOR (final concentration between 0.1 and 0.5 μM). The enzyme turnover (mol electrons/min mol enzyme) was calculated from the slope of the nitric oxide consumption traces.

RESULTS

The *nor* operon. In *T. thermophilus* PRQ25, a third gene exists (*norH*) downstream of *norB* that encodes a 98-amino-acid-long membrane protein (NorH [11.2 kDa]). The *norH* gene is con-

TABLE 4 Sequence homology of Nor protein from *T. thermophilus* PRQ25

Organism	% of identity to[a]: NorC (221 aa)	NorB (476 aa)	NorH (98 aa)
T. thermophilus			
NAR1	−	−	−
SG0.5JP17-16	99	99	98
JL-18	99	99	98
T. scotoductus	92	95	90
Meiothermus silvanus	−	−	−
Hydrogenobacter thermophilus	62	68	55
Hydrogenivirga sp.	56	67	34
Pseudomonas stutzeri	38	40	−
Paracoccus denitrificans	35	40	−

[a] Shown are the percentages of identity of NorC, NorB, and NorH to homologues from different organisms. Lengths (amino acids [aa]) are given in parentheses. −, no homologues present.

TABLE 5 Distribution of *nor* genes in *T. thermophilus*[a]

Strain	Growth with: NO_3^-	Growth with: NO_2^-	Gas production	Presence of gene: *norC*	Presence of gene: *norB*	Presence of gene: *norH*
HB27	−	−	−	−	−	−
NAR1	+	−	−	−	−	−
RQ1	+	−	+	−	−	−
B	+	−	+	−	−	−
PRQ14	+	+	+	+	+	+
PRQ15	+	+	+	+	+	+
PRQ16	+	+	+	+	+	+
PRQ17	+	+	+	+	+	+
PRQ21	+	+	+	+	+	+
PRQ23	+	+	+	+	+	+
PRQ24	+	+	+	+	+	+
PRQ25	+	+	+	+	+	+
PRQ26	+	+	+	+	+	+
PRQ27	+	+	+	+	+	+
PRQ28	+	+	+	+	+	+
PRQ30	+	+	+	+	+	+
PRQ31	+	+	+	+	+	+

[a] Shown is a characterization of the ability to grow anaerobically with nitrate or nitrite and to produce gas anaerobically from nitrate (40 mM) in different isolates of *T. thermophilus*. The presence of the *nor* genes was checked by PCR amplification with the primers indicated in Table 3. +, positive; −, negative.

served in the same position in *nor* clusters from all of the denitrifying strains of *Thermus* spp. so far sequenced: *T. thermophilus* strains SG0.5JP17-16 (SG0.5) and JL-18 (27), *Thermus scotoductus* SA1 (28), and *Thermus oshimai* JL2 (29) (Table 4). Moreover, PCR-based assays on genomic DNA from several strains of *T. thermophilus* showed the presence of the *norH* gene in all strains that grow anaerobically with nitrite but not in the aerobic strain HB27 or in those that only reduce nitrate to nitrite, like NAR1 (Table 5). In addition, homologues to NorH are also encoded downstream from *norB* in several bacteria belonging to the *Aquificae* phylum, such as *Hydrogenobacter thermophilus* TK-6 or *Hydrogenivirga* sp. (Table 4).

***norH* is cotranscribed with *norCB*.** The *norC* and *norB* genes are separated by short DNA sequences, and *norB* and *norH* overlap (Fig. 1A). A putative Rho-independent transcriptional terminator is located immediately downstream of *norH*, suggesting that the three genes are cotranscribed into a single mRNA. This hypothesis was confirmed by RT-PCR assays on total RNA isolated from cultures of *T. thermophilus* PRQ25 grown aerobically or anaerobically with nitrate. As shown in Fig. 1B, RT-PCR products were detected extending the gene pairs *norC-norB* and *norB-norH* in RNA from anaerobic cultures but not in RNA isolated from aerobic ones, whereas the control *groES* genes were detected in both samples. Therefore, *norH* is cotranscribed with *norC* and *norB* from a putative promoter located upstream from *norC* ($norC_p$).

In order to identify the promoter, a 340-bp region upstream of *norC* was cloned into a promoter probe vector (pMHPnorβgal), and its expression under different conditions was assayed. As shown in Fig. 1C, this promoter was expressed at a very low level under aerobic conditions (condition 1) in the denitrifying strain HB27d but was strongly induced by nitrate under anoxia (condition 3). Nitrite had a minor effect, whereas sodium nitroprusside (SNP), a generator of NO, produced a 2-fold increase compared to the aerobic culture. It is noteworthy that this promoter was basically inactive in the aerobic strain HB27, thus supporting that the promoter depends on transcription activators encoded by the denitrification island transferred to the HB27d strain (17).

NorH is required for efficient respiration of NO. To analyze the role of the *nor* genes in denitrification and particularly that of *norH*, we isolated null mutants for each of the three *nor* genes. Δ*norC*, Δ*norH*, and Δ*norB*::*kat* deletion mutants were obtained in which the corresponding coding sequences were deleted or replaced by the *kat* gene (Fig. 2A) as further confirmed by PCR (not shown). Western blot assays revealed that the *norC* and *norB* mutants did not contain any detectable NorC protein, whereas the *norH* mutant expressed similar levels of NorC to the parental HB27d strain (Fig. 2B). As expected, the NorC protein was not present in the aerobic HB27 strain.

Under anaerobic growth conditions with nitrate as the electron acceptor, the three mutants showed similar growth rates in the early exponential phase but a decrease in growth rate above an OD_{550} of 0.3. After 12 h, the three mutants reached a lower cell density than the wild type. However, after 24 h, the differences from the wild-type strain in cell mass were in the range of 20% lower (Fig. 3A). Parallel analysis revealed that the amounts of N_2O accumulated by the *norC* and *norB* mutants in these growth experiments were almost negligible, whereas the *norH* mutant produced roughly one-third of the amount produced by the wild-type strain.

Effects of the absence of NorH on *in vitro* NO reductase activity. The data presented above confirmed the expected requirements for NorC and NorB for Nor activity but also suggested a role for NorH in N_2O production. To check if this effect was due to a role of NorH in proper folding or maturation of the NorCB enzyme, we assayed the NO reductase activity of membrane fractions isolated from the *norH* mutant and its parent, as well as with the purified recombinant enzymes with or without NorH. As shown in Fig. 4, membranes of the *norH* mutant show a capability to produce N_2O from NO similar to that of the wild type after 16 h of incubation using ascorbate-TMPD as the reductant. In contrast, the membrane extracts from the *norC* and *norB* mutants produced significantly smaller amounts of N_2O from NO in these *in vitro* assays. Hence, the *in vitro* assays with purified membranes do not reproduce the differences in N_2O production between the

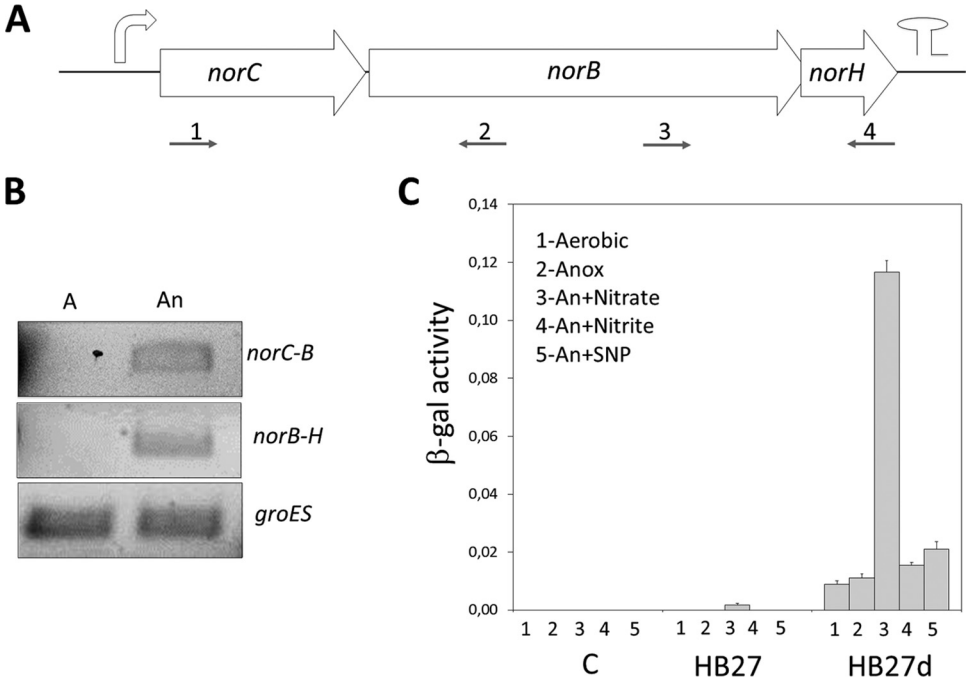

FIG 1 The *norCBH* operon. (A) Scheme of the *nor* operon. The loop indicates a Rho-independent transcriptional terminator located downstream of *norH*. The bent arrow indicates the $norC_p$ promoter. Gray arrows indicate the approximate hybridization positions of the primers (Table 3) used for the RT-PCR assays shown in panel B: norC_Fw (arrow 1), 1c_Rev (arrow 2), 3b_dir (arrow 3), and orf85_Rv (arrow 4). (B) Cultures of *T. thermophilus* PRQ25 grown aerobically (lane A) or incubated for 4 h with 20 mM nitrate under anaerobic conditions (lane An) were used as the source of RNA for RT-PCR experiments with the primers shown in panel A. The *groES* housekeeping gene was used as a control. (C) β-Galactosidase (β-gal) activity produced by cells transformed with pMHPnor-βgal under the following conditions: Aerobic (no. 1), anaerobic without electron acceptors (no. 2), or anaerobic with nitrate (no. 3), nitrite (no. 4), or SNP (no. 5). The HB27 strain was used as an aerobic control. The β-galactosidase activity was expressed as the change in OD_{420} as a function of time (ΔOD_{420}) normalized to the OD_{550} of the cultures.

wild type and the *norH* mutants observed with entire cells *in vivo* (Fig. 3B).

Further information was obtained by assaying purified enzymes with or without the NorH protein by using different electron donors (Table 6). These assays can be completed within a few minutes, in contrast to 16 h, as done for membrane extracts. TMPD, which donates electrons to the heme *c* component of the enzyme, showed about a 3-fold increase in activity in the NorH-

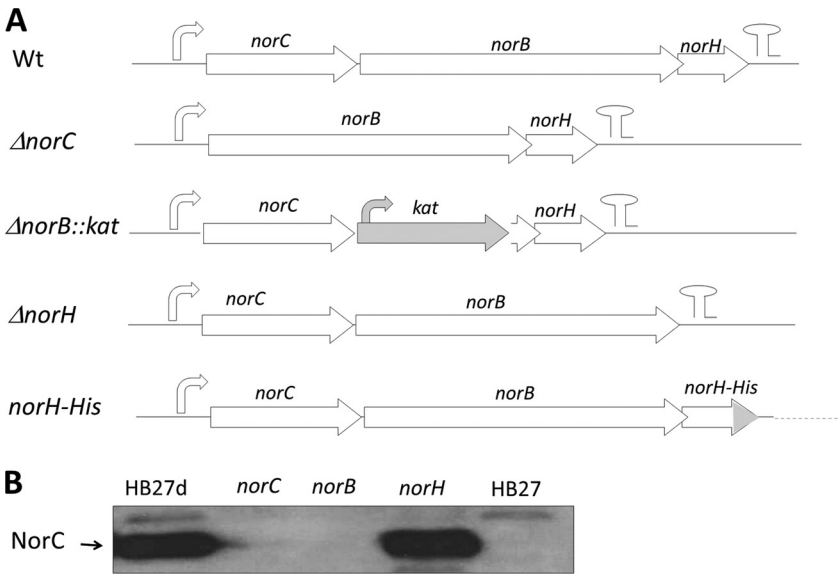

FIG 2 Isolation of *nor* mutants. (A) Scheme showing the genetic structure of the *nor* operon in the *nor* mutants. Bent arrows indicate the $norC_p$ (white) and $slpA_p$ (gray) promoters that control the *nor* operon and the *kat* gene, respectively. Note that absence of transcription terminator after the *kat* gene allows the expression of the *norH* gene. Wt, wild type. (B) Western blot to detect the NorC protein in the *nor* mutants. The aerobic HB27 strain was used as a negative control.

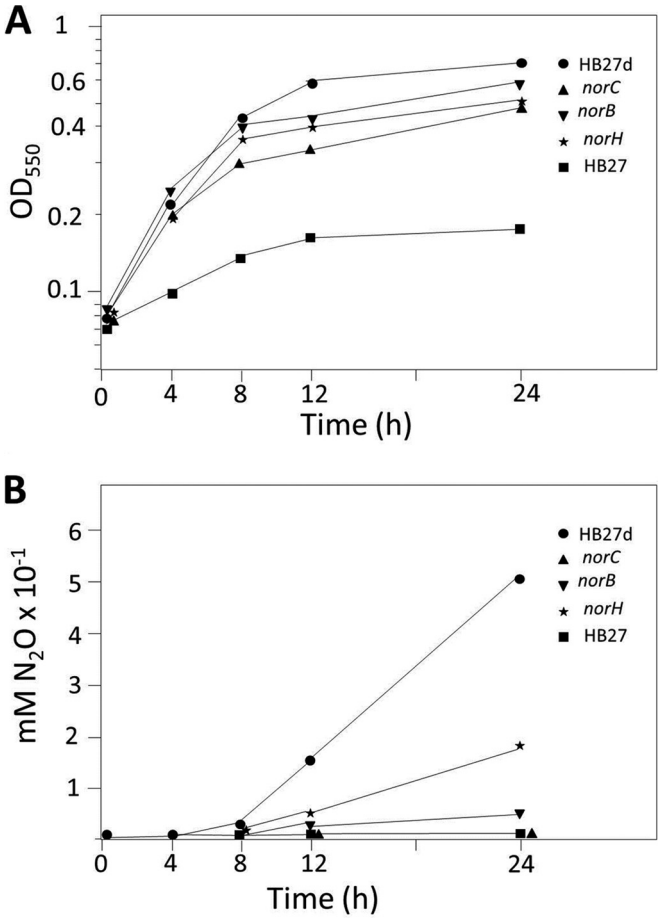

FIG 3 Anaerobic growth and N_2O accumulation in the *nor* mutants. (A) Anaerobic growth of the wild type (HB27d) and the indicated *nor* mutant derivatives at 70°C in the presence of 10 mM nitrate. (B) Production of nitrous oxide from the cultures shown in panel A. The aerobic strain HB27 strain was used as a negative control.

deficient protein compared to the wild type. However, PMS, which presumably donates electrons directly to the active site in the NorB subunit without involving the heme *c* in the NorC subunit (6), did not show any change in activity with respect to three enzyme subunits (Table 6). The data implicate the NorH subunit as possibly regulating the kinetics of electron transfer to the heme c component of the cNOR.

NorH interacts with the nitric oxide reductase. The results obtained with purified recombinant enzymes isolated by His tag affinity show the existence of direct interactions between NorH and the NorCB complex. To test if this was also true in the native enzyme, an insertion mutant of the *T. thermophilus* denitrifying strain HB27d was generated by recombination with a suicide plasmid that produced a NorH protein with a C-terminal His tag (Materials and Methods). A pulldown experiment using a Ni-agarose affinity column with proteins solubilized from membranes of this mutant allowed the detection of the NorC protein by Western blotting in fractions of protein specifically bound to the column (lanes 2 and 3). In contrast, NorC was not detected when equivalent cell extracts from the wild-type non-His-tagged strain were used (not shown). Therefore, the NorH-His protein was able to pull down NorC, supporting that NorH is associated with the enzyme.

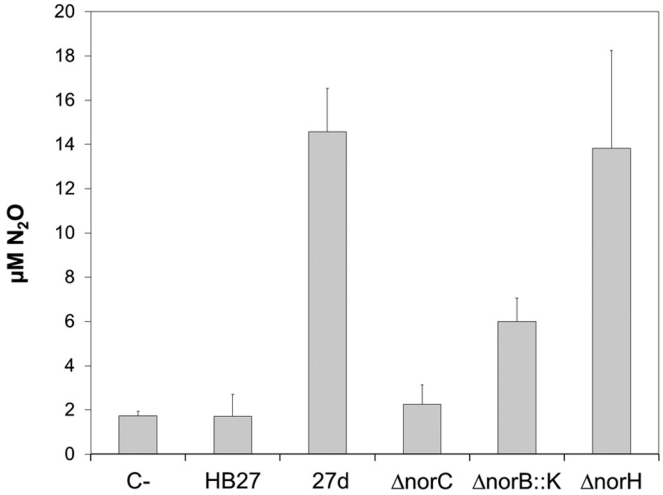

FIG 4 Nitric oxide reductase activity in membrane fractions of *nor* mutants. The figure shows the amount of N_2O produced from NO (100 μM) at 70°C after 24 h of incubation with membrane fractions isolated from cultures of the indicated strains grown anaerobically with nitrate. Ascorbate (5 mM) and TMPD (5 mM) were used as electron donors.

DISCUSSION

Recently it has been shown that NorC and NorB form a heterodimeric complex active in NO reduction when expressed recombinantly in *E. coli* (8), and the three-dimensional (3D) structure of this complex was modeled based on the crystal of cNor from *Pseudomonas aeruginosa*. Therefore, the results showing that *norC* and *norB* deletion mutants are unable to produce significant amounts of N_2O *in vivo* were expected (Fig. 3B). Significantly, it is shown here that a third protein (NorH), encoded at the end of the *nor* operon of the ancient clades *Thermales* and *Aquificales*, is relevant for efficient NO respiration *in vivo* and influences the kinetics of cNOR activity with the isolated enzyme when assayed with TMPD.

The *norH* gene is cotranscribed with *norC* and *norB* (Fig. 1B) from a promoter located immediately upstream of *norC*, which is induced under denitrifying conditions (Fig. 1C). Although we do not have specific antibodies to monitor the expression of NorH, the presence of a ribosome binding site supports that the protein is translated as well. This translation of *norH* is also suggested by the fact that Δ*norH* mutants show defective production of N_2O (Fig. 3B).

In addition, when a gene coding for a His-tagged NorH was used to replace the wild-type *norH* gene in the genome of the strain, we were able to pull down the NorC protein by immobilized-metal affinity chromatography (IMAC) both in the recom-

TABLE 6 NOR activity of the purified *T. thermophilus* cNOR wild-type and *norH*-deficient enzymes using different electron donors

	Turnover no. (min^{-1})[a]	
Electron donor	cNOR	Δ*norH* mutant
TMPD	5.5 ± 0.5	15.6 ± 1.2
PMS	9 ± 0.7	10 ± 0.8

[a] Turnover number is expressed as mol of electrons/min per mol of enzyme. Turnover refers to the mol NO/min per mol of enzyme that can be obtained by dividing by 2 the data shown. The data are expressed as the average ± standard deviation from 4 independent experiments.

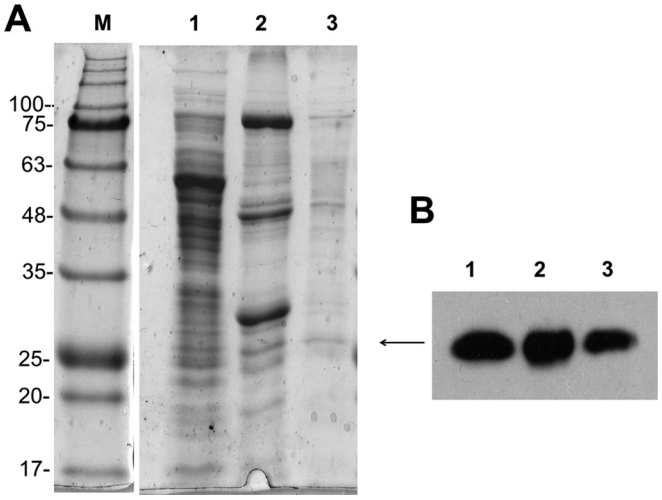

FIG 5 NorH interacts with NorC. (A) Membrane proteins from the *norH*-His mutant grown anaerobically with 20 mM nitrate for 16 h at 70°C were solubilized with DDM and subjected to IMAC. The following fractions were analyzed by SDS-PAGE and Coomassie blue staining: lane 1, DDM-solubilized proteins; lane 2, concentrate of proteins eluted with 250 mM imidazole; lane 3, concentrate of proteins eluted with 500 mM imidazol; lane M, molecular markers of the indicated sizes (kDa). (B) Western blot to detect NorC.

binant enzyme produced in *E. coli* (Table 6) and that produced in *T. thermophilus* (Fig. 5), showing first that NorH is actually expressed and second that it interacts at least with NorC. In the pulldown experiments with *T. thermophilus* the amount of NorC detected was small, as expected for a terminal reductase expressed from a single-copy gene, and immunoblots were required for detection. Despite this low abundance, most of the NorC protein from the solubilized extracts of NorH-His-expressing cells was bound to the IMAC column, supporting a high binding affinity of NorH for NorC. As NorC forms a complex with NorB to render an active enzyme (8) a direct interaction between NorH and NorB is also possible. In either case, our data with *in vivo* His-tagged NorH and with recombinant Nor-tagged protein support that NorH is bound to NorBC.

NorH is predicted to be a small highly hydrophobic integral membrane protein with three transmembrane helices. Therefore, its interaction with NorCB is likely to take place within the membrane bilayer. The possibility that NorH could function as a chaperone required for folding or maturation seems unlikely because it is possible to form NorC-NorB heterodimers in *E. coli* that are quite active in NO reduction without NorH (Table 6) (8). On the other hand, a role for NorH in the stabilization of the complex at high temperatures (>60°C) also seems unlikely, as membrane extracts from Δ*norH* mutants of *T. thermophilus* showed no defects in NO reduction to N_2O when artificial electron donors were used (Fig. 4). Moreover, it seems that the stability of NorC depends on NorB and not on NorH, as Δ*norB*::*kat* mutants do not express any detectable amount of NorC (Fig. 2B). Thus, a role for NorH in respiration itself is more likely than in enzyme stabilization.

The activity of the recombinant wild-type and NorH-deficient enzymes using different substrates may provide a clue as to the function of NorH (Table 6). NOR activity using PMS, which donates electrons directly to the enzyme active site (6), is not influenced by the absence of NorH. In contrast, NOR activity using TMPD as the electron donor is increased by 3-fold in the NorH-deficient enzyme compared to the wild type. The results with intact cells show that the production of N_2O is lower in the absence of NorH, but it must be recalled that the physiological electron donor is not TMPD but a *c*-type cytochrome. Putative candidates are the nitrate reductase, whose periplasmic cytochrome *c* has been speculated to be required as an electron donor to the terminal reductases (30) or the cytochrome c_{552} shown to be the electron donor for the cytochrome ba_3 oxidase (31). At this point, the data are merely suggestive that the role of NorH may involve modulating the interaction between the physiological electron donor and the cytochrome *c* component of cNOR. Further biochemical analysis and a structural analysis of the whole NorCBH complex are needed to clarify the role of the NorH subunit of these thermophilic cNORs.

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