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MINIREVIEW

Transferable Denitrification Capability of *Thermus thermophilus*

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Laboratory-adapted strains of *Thermus* spp. have been shown to require oxygen for growth, including the model strains *T. thermophilus* HB27 and HB8. In contrast, many isolates of this species that have not been intensively grown under laboratory conditions keep the capability to grow anaerobically with one or more electron acceptors. The use of nitrogen oxides, especially nitrate, as electron acceptors is one of the most widespread capabilities among these facultative strains. In this process, nitrate is reduced to nitrite by a reductase (Nar) that also functions as electron transporter toward nitrite and nitric oxide reductases when nitrate is scarce, effectively replacing respiratory complex III. In many *T. thermophilus* denitrificant strains, most electrons for Nar are provided by a new class of NADH dehydrogenase (Nrc). The ability to reduce nitrite to NO and subsequently to N_2O by the corresponding Nir and Nor reductases is also strain specific. The genes encoding the capabilities for nitrate (*nar*) and nitrite (*nir* and *nor*) respiration are easily transferred between *T. thermophilus* strains by natural competence or by a conjugation-like process and may be easily lost upon continuous growth under aerobic conditions. The reason for this instability is apparently related to the fact that these metabolic capabilities are encoded in gene cluster islands, which are delimited by insertion sequences and integrated within highly variable regions of easily transferable extrachromosomal elements. Together with the chromosomal genes, these plasmid-associated genetic islands constitute the extended pangenome of *T. thermophilus* that provides this species with an enhanced capability to adapt to changing environments.

Denitrification is a fundamental process in the nitrogen cycle (Fig. 1) by which many prokaryotes and a few fungi are able to use nitrogen oxides instead of oxygen as terminal electron acceptors (1). The full process involves four consecutive reduction steps, starting with nitrate and ending with dinitrogen that is released to the atmosphere (2–4). However, the four steps of the denitrification process are not always carried out by a single microorganism. Some bacteria can catalyze only the reduction of nitrate to nitrite, which is secreted into the medium as a final product. Other bacteria reduce nitrite to ammonium instead of nitric oxide. However, as the presence of the nitrous oxide reductase in denitrificant microorganisms is not widespread, N_2O consequently becomes the final product of the process, leading to a relevant increase in the atmospheric concentrations of this powerful greenhouse gas (5, 6–8).

Most of our present knowledge about denitrification derives from studies on mesophilic model bacteria, especially in the *Proteobacteria Pseudomonas* spp. and *Paracoccus denitrificans* (9–12), although several works have also concentrated on species of the *Firmicutes*, especially of the genus *Bacillus*, among which the denitrification process is widespread and likely underestimated (13).

In the mesophilic models studied so far, denitrification starts with a membrane-bound (NarGHI) and/or a periplasmic (NapABC) nitrate reductase. In both molibdoenzymes, the electron donor is a quinol from the membrane, although the details of the energy conservation process by asymmetric reduction for the periplasmic enzyme are not clear (1, 14, 15). In contrast, assimilatory nitrate reduction is carried out by a NADH-dependent cytoplasmic nitrate reductase (NasA) that is likely in complex with assimilatory nitrite reductase (NasC) (16). Nitrite produced in the cytoplasm by NarGHI has to be secreted to the periplasm through a membrane transporter from the NarK family of the major facilitator superfamily (MFS). In the periplasm, nitrite is reduced to ammonium by a multiheme periplasmic enzyme (NrfA) or to nitric oxide either by heme (NirS)- or by copper (NirK)-containing nitrite reductases. Although the natural electron donors for these enzymes are not well characterized, periplasmic cytochromes are likely involved, and energy conservation is associated with the oxidation of membrane quinols by a specific subunit of NrfA or by respiratory complex III in the cases of NirS and NirK (1, 17–19). As the NO produced by these enzymes is highly toxic, it has to be reduced to N_2O immediately by a membrane-bound polytopic enzyme (Nor). Depending on the type of Nor enzyme used, electrons for this reaction can be supplied by a periplasmic cytochrome *c* (cNor) or by quinones (qNor). The recent availability of crystal structures of these enzymes from *Pseudomonas aeruginosa* (cNor) (20) and *Geobacillus stearothermophilus* (qNor) (21) supports the idea that these are not proton pumping enzymes despite their sequence and structural similarity to cytochrome *c* and quinol oxidases (1). In several denitrificant microorganisms, N_2O is finally reduced to N_2 by a periplasmic reductase (NosZ), for which a cytochrome *c* or a type I copper protein, e.g., pseudoazurin or azurin, has to act as an electron donor (22). However, N_2 is also produced by organisms that do not encode NosZ homologues, supporting the idea that a different type of N_2O reductase might exist (23, 24).

The presence of the different denitrification genes is in many cases species specific or even strain specific. The *nir* and *nor* operons are usually clustered in the genome of denitrificant strains and expressed in a coordinated form to avoid the accumulation of NO, whereas *nar*, *nap*, and *nos* genes can appear in separate loci in the genome (12, 25). This uneven distribution of denitrification genes does not fit with the phylogeny based on the sequence of the 16S

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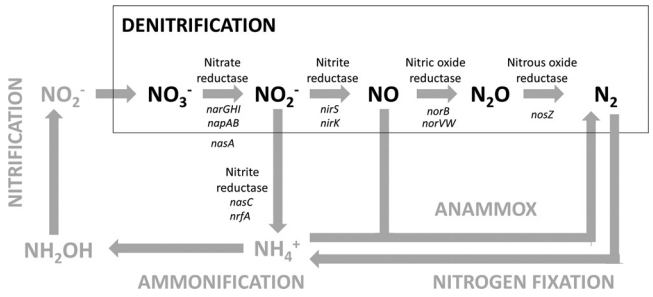

FIG 1 The nitrogen cycle. Denitrification consists of the sequential reduction of nitrogen to dinitrogen. The enzymes involved in the denitrification pathway are listed next to the reaction they catalyze. Nitrite can be alternatively reduced through the ammonification process. Nitrification proceeds from ammonia to nitrite via hydroxylamine (NH_2OH). Ammonia can also be oxidized to dinitrogen during the anammox process (anaerobic ammonium oxidation). Finally, nitrogen fixation is responsible for incorporating the gaseous dinitrogen into the cycle as assimilable compounds.

rRNA, supporting the idea of both an ancient origin for this pathway and its frequent spreading through lateral gene transfer (LGT) (25–27). In fact, the genomes of several phylogenetically ancestral extreme thermophiles and hyperthermophiles include genes encoding enzymes for denitrification, which is one of the few steps of the nitrogen cycle that remain active above 65°C (Fig. 1). The probable ancestral nature of thermophiles and of the denitrification process itself is an important argument in favor of the study of denitrification in thermophilic models, such as *Thermus* spp.

The genus *Thermus* is ubiquitously spread in natural and man-made thermophilic environments all over the world. It includes hundreds of strains that constitute an important source for enzymes of biotechnological interest (28). In addition, most strains can grow to high cell densities under laboratory conditions and at least some of them can be genetically manipulated due to the presence of a highly efficient natural competence system (29). These properties and the greater ability of protein complexes from thermophiles to crystallize compared to their counterparts from mesophiles (30) have led to the use of isolates of this species as a major model for structural biology programs. Examples such as the high-resolution structures of the 70S ribosome (31), the bacterial RNA polymerase (32), and respiratory complex I (33) are based on the analysis of these complexes from *Thermus* spp.

Among the great panoply of *Thermus* isolates, the strains *T. thermophilus* HB27 and HB8 have been by far the most studied under laboratory conditions. Natural competence, particularly in the HB27 strain, is highly efficient and apparently constitutive, allowing incorporation rates of 40 kbp/cell/s without much difference regarding its origin (34). This promiscuity has made this strain the ideal thermophilic laboratory model, allowing the development of a complete genetic toolbox to analyze the function of any given protein *in vivo* (28).

The aerobic components of the respiratory electron transport chain from *T. thermophilus* have been the subject of many biochemical and structural studies. A great variety of carbon substrates can be catabolized, including most amino acids, different sugars, and fatty acids. Most NADH is generated through a complete tricarboxylic acid cycle, and it is oxidized essentially by respiratory complex I, the proton-translocating type I NADH dehydrogenase (NqoA-N). The three-dimensional (3D) structures of both the soluble domain and all of complex I of *T. thermophilus* have been recently obtained by X-ray crystallography (33, 35). A putative type II NADH oxidase (36) and the tricarboxylic acid cycle component succinate dehydrogenase (complex II) provide electrons to the respiratory chain as well (37). Electrons are transferred to menaquinone-8, the major quinone in the membrane of this organism (38). The corresponding quinol is oxidized by heterotetrameric complex III (39, 40), which donates the electrons to the terminal oxidases (complex IV) through soluble periplasmic cytochrome c_{552} (41, 42). There are two terminal cytochrome oxidases in this bacterium: caa_3, expressed under conditions of fully aerobic growth, and ba_3, induced under low oxygen pressures (43–47). ATP is synthesized by complex V, an A/V-type ATP synthase that could have been obtained from archaea by LGT (28, 48).

Despite the fact that *T. thermophilus* strains HB27 and HB8 are obligate aerobes, there are different isolates of the species and of other *Thermus* spp. that have been shown to grow anaerobically with nitrogen oxides, sulfur, or even metals as electron acceptors. In fact, some genes usually associated with the anaerobic metabolism were revealed when the sequence of the HB27 strain was published (49). Among *T. thermophilus*, different isolates grow anaerobically by reducing nitrate to nitrite whereas others are true denitrificants as they can also use nitrite for growth with the concomitant production of gas (50) (Table 1). However, specific studies on the denitrificant strain *T. thermophilus* PRQ25 revealed N_2O as the accumulated gas from nitrate, with no traces of N_2. These results have been supported by the detection of high rates of N_2O emission produced in the main pool and high-temperature shallow sediments from the natural hot spring of Great Boiling Spring in the U.S. Great Basin, where *T. thermophilus* and *T. oshimai* constituted the most abundant bacterial strains. In this environment, the N_2O fluxes measured were comparable to the N_2O rates obtained from soils and sediments impacted with agricultural fertilizers, such as coastal marine environments, freshwater streams, or riparian zone environments (51). These results suggest that the absence of an efficient N_2O reductase (Nos) is a common trait among species of the genus *Thermus*.

NITRATE RESPIRATION

A gene encoding a putative respiratory nitrate reductase (*narG*) was incidentally identified during the analysis of a gene library from a strain of *T. thermophilus* originally labeled HB8 (52) and further renamed NAR1 once the sequence of the HB8 strain in Japan was published and significant differences from this strain were found. Involvement of *narG* in nitrate respiration was further confirmed (53), and its connection with a genetic element of approximately 30 kbp that had the capability to be transferred by conjugation to the HB27 aerobic strain was subsequently demonstrated (54). This genetic element was named NCE (for "nitrate respiration conjugative element"), and its sequence revealed the presence of four operons encoding the nitrate reductase and nitrate/nitrite transporters (*narCGHJIKT*), a new type of NADH dehydrogenase (*nrcDEFN*), and signaling and regulatory proteins (*dnrST* and *drpAB*) (Fig. 2). A whole-genome sequence approach for investigation of this strain revealed that the NCE is actually located in a variable region of a megaplasmid (55).

THE UNUSUAL NITRATE REDUCTASE OF *T. THERMOPHILUS*

Four genes of the *nar* operon (*narGHJI*) encode homologues to the three classic subunits of the heterotrimeric nitrate reductases

TABLE 1 Capability for anaerobic growth with nitrate and nitrite, gas production from nitrate, and presence of *nrcE* and *norB* in different *Thermus* strains[a]

Strain[b]	Nitrate	Nitrite	Gas[c]	*nrcE*[d]	*norB*[d]
T. thermophilus					
HB27	−	−	−	−	−
HB8	−	−	−	−	−
NAR1	+	−	−	+	−
B	+	−[e]	+	+	−
Fiji3A1	+	−	−	+	−
PRQ14	+	+	+	+	+
PRQ15	+	+	+	+	+
PRQ16	+	+	+	−	+
PRQ17	+	+	+	+	+
PRQ21	+	+	+	−	+
PRQ23	+	+	+	−	+
PRQ24	+	+	+	+	+
PRQ25	+	+	+	−	+
PRQ26	+	+	+	+	+
PRQ27	+	+	+	−	+
PRQ28	+	+	+	+	+
PRQ30	+	+	+	+	+
PRQ31	+	+	+	+	+
VG-7	+	+	+	−	+
SG0.5	+	+	+	−	+
T. scotoductus					
SA01	+	+	+	−	+

[a] Data are from reference 70. +, present; −, absent.
[b] *Thermus thermophilus* strains were provided by Milton da Costa except for HB27, NAR1, and SG0.5 and *T. scotoductus* SA01.
[c] Accumulated into inverted Durham tubes after 48 h of anaerobic growth with 40 mM nitrate.
[d] Data were determined by PCR with primers for *nrcE* (ACGCGTCGACGATGGCCTT GCCCTCGGGCCT and ATTCGATATCGCTTCTCCACCAGAAACCGGT) and for *norB* (AAAATCTAGAATGATCCAGGCTTTACCGCAG and AAAAGAATTCCTAGTC ATGGGCCCCCAC) or deduced directly from genome complete or draft sequences (*T. thermophilus* Fiji3A1, VG7, and SG0.5 and *T. scotoductus* SA01).
[e] Low concentrations of nitrate are required to start anaerobic growth with nitrite.

from mesophiles (NarG, NarH, and NarI) and the corresponding chaperone required for its maturation (NarJ) (53). The additional genes in the operon code for a cytochrome *c* (NarC) (56) and two membrane transporters of the MFS family (NarKT, formerly NarK1K2) (57). Except for the transporters, the inactivation of any of the *nar* genes in the operon, including the membrane NarI cytochrome *b*, and the NarC protein, leads to a complete absence of activity. In contrast, absence of NarI in the *Escherichia coli* enzyme produces soluble but active enzyme, supporting the idea of a model for enzyme maturation different from that of mesophilic Nar (58).

NarG and NarH subunits of the nitrate reductase of *T. thermophilus* show 50% sequence identity with that of the enzyme from *E. coli*, allowing the identification of the main residues involved in electron transfer centers in the structure of the mesophilic enzyme. NarH contains the corresponding residues required for the coordination of four iron-sulfur clusters, three of the [4Fe-4S] type (FS1 to FS3) and one of the [3Fe-4S] type (FS4). Also, in NarG the residues involved in the coordination of its [4Fe-4S] center (FS0) and the several domains and residues implicated in the binding of the bis-MGD (bis-molibdopterin guanil dinucleotide) cofactor are conserved. The conservation of all these groups clearly supports the idea of an electron transfer pathway within the enzyme similar to that suggested by the structure of the *E. coli* enzyme: FS4 → FS3 → FS2 → FS1 of NarH to the FS0 cluster and bis-MGD of NarG (59).

In contrast to NarG and NarH, NarI shows lower (28%) sequence identity to its homologue in *E. coli*. However, this similarity is high enough to establish some relevant structural comparisons. Similarly to its mesophilic counterpart, NarI is predicted to contain 5 transmembrane α-helices (TM), two of which (TM2 and TM5) contain the histidine residues that coordinate the Fe atoms in two heme *b* groups, one of which is situated closer to the cytoplasmic side and is likely involved in electron transfer to the FS4 of NarH.

Despite these sequence similarities, genetic analyses support the idea of the existence of relevant differences between the *E. coli* and the *T. thermophilus* enzymes. The most relevant difference is the presence in the thermophilic enzyme of NarC, a cytochrome *c* with two heme *c* binding motifs predicted to have a periplasmic location, while being anchored to the membrane through a C-terminal domain. The relevance of this protein was revealed by the inability of *narC* null mutants to synthesize an active nitrate reductase and to attach it to the membrane (56, 58). These results, along with interaction data provided by bacterial two-hybrid assays, support the idea that NarC constitutes a fourth subunit in the thermophilic enzyme that is absent from the nitrate reductase of *E. coli* and most other mesophilic bacteria (58).

Unlike its relevance for the synthesis and activity of the nitrate reductase of *T. thermophilus*, the actual role of NarC in the electron pathway through the enzyme is controversial. There are no accurate data on the redox potentials of the two heme *c* groups, although they are probably higher than those of the heme *b* groups of NarI. Consequently, electrons would flow from NarI to NarC and toward unidentified periplasmic acceptors. In fact, *narC* mutants defective in heme *c* binding site 1 (proximal) or 2 (distal) are still able to complement the anaerobic growth of *narC*::*kat* mutants despite their hindered NarG maturation (50), supporting the idea that electron transport through NarC is not a requirement for nitrate reduction. In fact, mutants in NarC are defective in nitrite respiration, supporting the idea that nitrite and NO receive electrons through NarC (50). This is also in agreement with the induction of nitrate reductase by nitrite and NO, as described elsewhere in this review. Therefore, the most probable role for NarC is to allow the transfer of electrons from menaquinol to extracellular acceptors when nitrate becomes scarce inside the cells.

In this context, it is relevant to note that similar *narCGHJI* clusters are carried not only by bacteria of the *Thermus-Deinococcus* group (e.g., *Meiothermus silvanus*) but, intriguingly, also by mesophilic nitrifying bacteria of the genus *Nitrobacter*. In this organism, the *nxr* cluster is annotated as nitrite oxidoreductase (*nxrABIC*) and has been involved in oxidation of nitrite to nitrate, with electrons being transferred through the membrane cytochrome *b* (NxrI) and the periplasmic cytochrome *c* (NxrC) to the cytochrome oxidase. However, as the purified enzyme requires ferricyanide and has more activity as a nitrate reductase and as bacteria are also capable of growing anaerobically with nitrate, the actual role of Nxr is under discussion (1, 60).

NITRATE AND NITRITE TRANSPORT

The last two genes of the *nar* operon from *T. thermophilus* NAR1 encode the NarK and NarT transporters (Fig. 2), both belonging

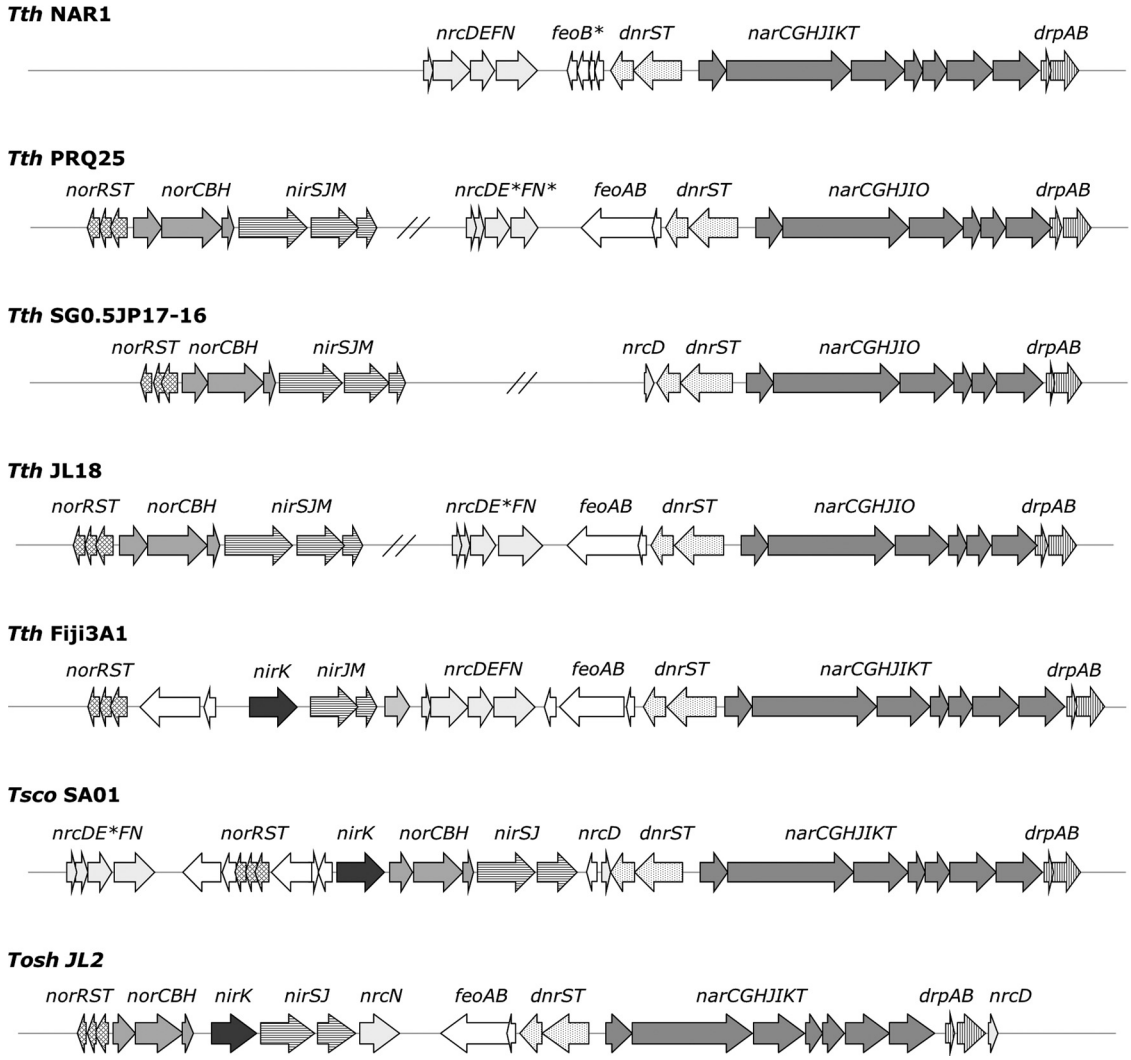

FIG 2 Genetic comparisons of the denitrification clusters among different *Thermus* spp. Gene clusters for nitrate, nitrite and NO respiration are schematized for *T. thermophilus* NAR1 (Y10124.3) (82), *T. thermophilus* PRQ25 (FN666415.2) (55), *T. thermophilus* SG0.5JP17-16 (CP002777.1 and CP002778.1), *T. thermophilus* JL18 (CP003252.1, CP003253.1, and CP003254.1) (83), *T. thermophilus* Fiji3A1 (unpublished), *T. scotoductus* SA01 (CP001962.1 [67] and CP001963.1), and *T. oshimai* JL2 (CP003249.1, CP003250.1, and CP003251.1) (84). Genes encoding homologues are labeled with the same pattern. Gene names labeled with an asterisk (*) represent genes that contain inactivating mutations. Discontinuities in the sequence are indicated as double bars. Gene names are as described in the text.

to the nitrate/nitrite porter (NNP) family within the major facilitator superfamily (MFS) of transporters (57). Genes encoding proteins from this family share two conserved glycine-rich nitrate signature motifs, are commonly clustered close to the nitrate reductase, and have been implicated in nitrite extrusion and/or nitrate import (11, 14), forming a distinct subfamily known as NarK. These proteins are predicted to contain 12 transmembrane helices in two 6-helix domains connected by a longer loop and can be divided into two distinct subfamilies. NarK1-like transporters, such as NarK from *T. thermophilus* NAR1, are proposed to be nitrate/proton symporters, whereas NarK2 transporters, like NarT transporters, are proposed to be nitrate/nitrite antiporters (61, 62).

Recently, the structures of the two NNP transporters of *E. coli* that belong to the NarK2 subfamily, NarU and NarK, have been solved (63, 64). Based on the structure of NarU, a model was proposed in which nitrate transport is catalyzed by subtle conformational changes in a group of internal membrane helices facilitating transition from an "outward-open" state that allows the entrance of the anion from the periplasm into its binding site in the center of the protein and into the "inward-open" state that allows the exit of the anion to the cytoplasm. On the other hand, the model based on the NarK structure predicts a massive rocker switch mechanism in which the protein can adopt 6 different conformational states, allowing the exchange of nitrate with nitrite apparently without the involvement of proton transfer. Both mechanisms could explain the nitrate/nitrite antiporter activity but not that of the nitrate/proton symporter also described for these proteins.

In addition to *Thermus* spp., the presence of two clustered NarK homologues belonging to the NarK1 and NarK2 subfamilies has been reported for different bacteria, such as *Pseudomonas aeruginosa*, where the transporters are expressed as the first genes of the *narK1K2GHJI* operon (65). There are even examples in

which transporters of the NarK1 and NarK2 subfamilies are fused into a single protein with 24 transmembrane helices. In such cases, the N-terminal half of the protein has been hypothesized to function as a nitrate/proton symporter whereas the C-terminal half would function as nitrite/nitrate antiporter (66).

In the nitrate-respiring *T. thermophilus* NAR1 strain, single *narK* or *narT* mutants are still able to grow anaerobically with nitrate, although at lower rates than the wild type. Since the *narKT* double mutant is unable to grow anaerobically, not only is the absence of alternative transporters evident, but it can also be concluded that both proteins are able to act as nitrate/nitrite antiporters. However, owing to the differential behavior patterns of the two mutants and to *narT* mutants being apparently more affected, the possibility exists that one of them could function preferentially as a nitrate/proton symporter and the other as a nitrite/nitrate antiporter as previously suggested (57).

Interestingly, the presence of two NarK homologues is not a conserved trait among the *nar* operons of *Thermus* spp., and a single gene for a MFS transporter is encoded at the end of the *nar* operon in certain *Thermus* denitrificant strains (Fig. 2). For example, whereas in *T. scotoductus* SA01 (67), *T. thermophilus* Fiji3A1 (unpublished results), and *T. oshimai* JL-2 (accession CP003250.1), homologues to NarK and NarT with identities between 88% and 92% with those of NAR1 are found, in *T. thermophilus* SG0.5JP17-16 (accession numbers CP002777 and CP002778), *T. thermophilus* JL18 (accession CP003253.1), and *T. thermophilus* PRQ25 (FN666415.2), a single member of the family is present (NarO) which intriguingly shows identical amino acid sequences in the three strains. This hyperconserved NarO protein has low similarity to both NarK (32% identity) and NarT (33% identity) proteins of the NAR1 strain. Actually, NarO presents higher similarity to NarK proteins from other genera such as *Oceanithermus profundus* DSM 14977 (YP_004058054.1, 73% identity) or *Marinithermus hydrothermalis* DSM 14884 (YP_004368685.1, 62% identity).

A SPECIFIC NADH DEHYDROGENASE FOR NITRATE RESPIRATION

The NCE of *T. thermophilus* NAR1 includes the *nrcDEFN* operon that encodes a NADH dehydrogenase complex (Nrc), which is expressed under the same conditions as Nar (68). NrcD is a small ferredoxin with two iron-sulfur clusters ([3Fe-4S]-[4Fe-4S]) similar in sequence (85% identity) to the aerobic FdTt ferredoxin from *T. thermophilus* (69) but containing a 10-amino-acid C-terminal extension absent in FdTt. NrcF is homologous to the iron-sulfur (B subunit) from respiratory complex II (succinate:quinone oxydoreductase) and the quinol:fumarate reductase. As with its homologues, NrcF contains the conserved cysteine and the aspartate residues that constitute the sequence motifs for the coordination of three iron-sulfur clusters ([2Fe-2S], [4Fe-4S], and [3Fe-4S]). In addition, the operon encodes a homologue to NADH dehydrogenases of type II (NrcN) that is able to oxidize NADH in the presence of quinones *in vitro* (36). NrcE is an integral membrane protein that contains a cytoplasmic domain in which a sequence motif for the binding of an iron-sulfur cluster is present. Furthermore, a heme *b* group has been detected upon expression of NrcE in *E. coli* (36). *Thermus thermophilus* NAR1 strains with mutations in *nrcF*, *nrcE*, or *nrcN* show a decreased rate of anaerobic growth with nitrate and a lower capability to reduce nitrate from NADH *in vitro* (68). These data, together with those from two-hybrid interaction assays, support the idea that the *nrc* operon encodes a heterotetrameric NADH dehydrogenase (Nrc) specifically dedicated to nitrate respiration, likely forming a Nrc-Nar respiratory supercomplex. However, attempts to detect the complex by heterologous expression in *E. coli* have failed (36).

An active Nrc complex is not always found in the denitrificant *Thermus* strains so far sequenced. In particular, *nrcE* is deleted from different strains (Fig. 2). For example, the *nrcE* gene is not present in *T. scotoductus* (67) and the *nrcD* ferredoxin gene is duplicated. Also, among a series of *T. thermophilus* strains isolated in the Azores islands (PRQ series), 40% lacked the *nrcE* gene (Table 1) (70). Moreover, there are strains such as *T. thermophilus* SG0.5JP17-16 in which only the *nrcD* gene remains, with no traces of *nrcEFN*. In contrast, other strains such as *T. thermophilus* Fiji3A1 encode the whole *nrc* operon described for NAR1 (unpublished results). Therefore, major electron donors for the nitrate reductase through the quinone pool can differ among the denitrificant strains, and evolution of the NCE in specific strains has led to the loss of the Nrc in many of them, likely due to the presence of alternative NADH dehydrogenases.

In some of the strains analyzed, another set of genes, *feoAB*, is present between the *dnr* and *nrc* operons. These genes code for a putative iron transporter apparently not required for nitrate reduction, since inactivating mutations and deletions are detected in these genes in several strains (*T. thermophilus* strains NAR1 and SG0.5JP17-16 and *T. scotoductus* strain SA01) (Fig. 2).

NITRITE RESPIRATION

As commented above, different isolates of *T. thermophilus* can use nitrite for anaerobic growth, leading to the production of N_2O without any traces of N_2, at least in *T. thermophilus* PRQ25 (70). According to this capability, the sequence of the megaplasmid from *T. thermophilus* PRQ25 reveals the presence of a cluster of genes encoding putative Nir and Nor (55). The 6.9-kbp DNA *nor-nir* cluster encodes a homologue of cytochrome cd_1 nitrite reductase (*nirS*), followed by genes (*nirJ* and *nirM*) coding for homologues found in *nir* gene clusters from other bacteria. Similarly to its homologues from *Proteobacteria*, the NirS protein is predicted to have a periplasmic localization and to contain heme *c* and heme d_1 groups (71). The *nirJ* gene encodes a member of the radical SAM (*S*-adenosylmethionine) family of proteins that, as seen with its homologues, could be implicated in an as-yet-unknown step of heme d_1 synthesis or incorporation into NirS (72), whereas NirM seems to be a soluble single heme *c* periplasmic protein homologue to cytochrome c_{552}.

NirS homologues are not present in all the denitrificant strains of *T. thermophilus* (Fig. 2). In fact, strains such as Fiji3A1 and VG7 carry homologues to genes encoding the copper nitrite reductase (NirK) that are usually clustered with genes encoding cNor or even with those encoding NirJ (unpublished results). Moreover, there are strains such as *T. scotoductus* SA01 with genes that encode homologues to both NirS and NirK (67), a very unusual fact that could be related to the metal-rich environment in which this deep-mine strain was isolated. However, there is no evidence at present on the simultaneous functionality of both classes of nitrite reductases in this organism.

Upstream of the *nirS* gene, *T. thermophilus* PRQ25 and many other strains have a putative *nor* operon that encodes homologues of the subunits of the cytochrome-dependent cNor (NorC and NorB) and a third gene (*norH*) coding for a small integral membrane protein. The heterodimer NorC-NorB has been recombi-

nantly expressed in *E. coli* and characterized biochemically as a bona fide cNor, likely having more than one pathway for proton delivery from the periplasm (36), as evidenced by bioinformatic analysis and site-directed mutagenesis. However, the presence of *norH* as the third gene in the operon (*norCBH*) is intriguing, especially because it is conserved in all the denitrificant *Thermus* spp. so far analyzed that encode a cNor and also among the *nor* operon of many *Aquificae*. Preliminary data from our group support the idea that this third gene has a role in nitric oxide reduction *in vivo* but not *in vitro* (70).

CONTROL OF THE DENITRIFICATION APPARATUS

As in other denitrificant bacteria, the enzymes for denitrification are expressed when a suitable nitrogen oxide is present and the oxygen concentration in the medium decreases below an as-yet-undefined threshold level. However, none of the genomes of *Thermus* spp. analyzed so far contain homologues either to FNR, the main oxygen sensor involved in denitrification in most bacteria, or to the nitrate/nitrite two-component sensor system NarX/NarL (28). In addition, it seems clear from previous work that the sensor signaling system, at least for nitrate respiration, is associated with the NCE and acquired upon transfer of this element (54, 73).

A detailed analysis of the NCE sequence from the nitrate-respiring strain NAR1 revealed the presence of two genes (*dnrST*) encoding homologues to transcription factors and regulatory proteins between the *nrc* and the *nar* operons. The high degree of conservation of these two genes in the same position relative to *nar* in all the nitrate-respiring strains of *T. thermophilus* supports the idea of their relevance for the process (Fig. 2). DnrS is a large cytoplasmic protein with a N-terminal GAF (c**G**MP-specific phosphodiesterases, **a**denylyl cyclases, and **F**hlA) domain, which is usually involved in signal detection in several proteins (74), and a C-terminal BTAD (**b**acterial **t**ranscriptional **a**ctivator **d**omain), first described as involved in secondary metabolism in *Streptomyces* spp. and other *Actinobacteria* (73, 75). Transcriptional analysis with promoter probe vectors in *T. thermophilus* mutants showed that DnrS is required for the transcriptional activation of the *nar* and *dnr* operons but not for the expression of *nrc* (73). However, its activating function depends on the absence of oxygen, since its presence induces conformational changes in the protein, making it highly susceptible to proteases. Based on these data, we hypothesize that DnrS is the oxygen sensor of the system, operating through an as-yet-unknown mechanism related to its GAF domain. However, all the attempts to obtain biochemical data on its function *in vitro* have failed due to the hypersensitivity of this protein to oxygen and the difficulties associated with its heterologous expression in *E. coli*.

The other protein encoded by the *dnr* operon is DnrT, a protein that belongs to the DNR subgroup of the CRP family of transcription activators (76). As with all the CRP-like proteins, DnrT contains a C-terminal HTH (helix-turn-helix) DNA binding motif and a putative N-terminal cyclic nucleotide binding motif but not the cysteine motif required for the incorporation of an iron-sulfur cluster as in FNR. DnrT is required for the expression of its own operon and for that of *nar* and *nrc* (73). In contrast to DnrS, DnrT seems insensitive to the presence of oxygen or nitrate and, in fact, the recombinant His-tagged DnrT is able to recruit the RNA polymerase to the *nrc* promoter and activate its transcription *in vitro*. However, *in vitro* transcription of *nar* and *dnr* promoters failed, likely due to the aforementioned requirement for active DnrS or another as-yet-unknown factor(s). On the other hand, DnrT represses the transcription of the Nqo and Fbc operons, which encode respiratory complex I and complex III, respectively (73, 77).

With these data, a regulatory model was proposed (Fig. 3) for nitrate respiration in which the DnrS protein is expressed aerobically at low levels and the absence of oxygen stabilizes it, provided enough nitrate is present in the medium. Consequently, its own promoter is stimulated (with the participation of DnrT) to achieve enough production of DnrS and DnrT to activate the transcription of the *nar* and *nrc* promoters. In this scenario, the increase in DnrT expression concomitantly represses the transcription of operons for respiratory complexes I and III, thus playing a switching role in the transition from aerobic growth to anaerobic nitrate respiration (73). In this model, the signal for nitrate presence has to be transmitted by an independent system not yet characterized. The best candidates for this role are a group of highly conserved genes (*drpAB*) carried downstream from the *nar* operon in all the *T. thermophilus* strains so far analyzed. DrpA is a small periplasmic protein, whereas DrpB seem to have repressor activity on the Pdnr promoter (71).

In addition to its role in the control of the expression of the nitrate respiration, DnrT and DnrS are also involved in nitrite respiration. Actually, mutants of the denitrificant PRQ16 strain in DnrS or DnrT are unable to grow with nitrite or NO, thus supporting the idea that the transcription of the genes in the *nor-nir* cluster also requires these proteins (77). Furthermore, in these denitrificant strains, nitrogen oxides other than nitrate (e.g., nitrite or NO) can activate the expression of the *nar* operon, although at lower levels, supporting the idea that the *Pnar* promoter may be subjected to control by additional factors, likely NO. The reason for such an apparently wasteful induction process is related to a role of the nitrate reductase in further steps of the denitrification pathway. Actually, the absence of the nitrate reductase or mutation of the membrane-distal heme *c* group of its NarC subunit has been shown to limit the rate of anaerobic growth and the oxidation of NADH with nitrite and NO, supporting the idea that the nitrate reductase plays a role as an electron transporter for the Nir and Nor reductases (50).

Regarding NO signaling, all the nitrite-respiring *Thermus* spp. encode three small proteins (NorR, NorS, and NorT) upstream and in the complementary DNA strand with respect to the structural *nor* operon. NorR shows similarities to transcriptional regulators of the BadM/Rrf2 family, some of whose members are involved in the metabolism of nitrite, iron, and nitrogen. NorR is a homologue of NsrR, a member of the Rrf2 family of transcription repressors specifically dedicated to sensing nitric oxide (NO) in a variety of pathogenic and nonpathogenic bacteria. The NsrR protein from *Streptomyces coelicolor* has been characterized in detail and contains a C-terminal [2Fe-2S] cluster that is insensitive to oxygen but that can be nitrosylated by NO, leading to the loss of DNA binding activity by its N-terminal HTH motif (78). The sequence of NorR also contains a conserved DNA binding domain and a three-cystein motif likely involved in coordination of an iron-sulfur cluster, thus supporting the idea of a role in NO detection and in transcription control. The other two proteins show low similarities to any other protein of known function. NorS shows low similarity (36% identity) to the N terminus of DnrN, a NO-dependent regulator from *Geobacillus thermodenitrificans* HG80-2, and presents a ScdA domain identified in genes impli-

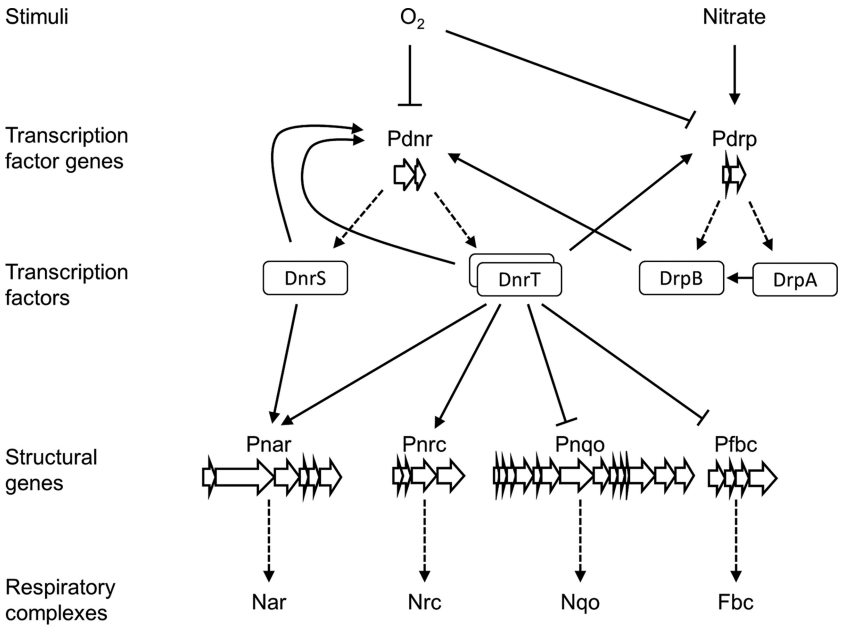

FIG 3 General regulation of the facultative metabolism of *T. thermophilus* NAR1. Low oxygen and nitrate levels are the signals that initiate the switching to anaerobic respiration. DnrS is stabilized at low O_2 levels, acting on its own promoter and allowing an increase in its own concentration and in that of DnrT. For this to happen, repression on Pdnr has to be alleviated by the presence of nitrate, likely sensed through DrpAB. Higher concentrations of DnrS and DnrT activate transcription of the promoters for nitrate respiration. DnrT is also responsible for repression of the promoters for complex I (Nqo) and complex III (Fbc). Arrows indicate activation; bars indicate repression. The expression of the nitrite and nitric oxide reductases is also under the control of the *dnrST* operon (77), but specific details are not yet known.

cated in protection against NO (79). Finally, NorT has a Cupin-2 domain found widespread among thousands of hypothetical proteins with rather different functions. However, data from sequence comparisons and gene location are not completely conclusive and further studies are required to confirm a role of this *norRST* cluster in regulation.

LATERAL GENE TRANSFER OF THE DENITRIFICATION APPARATUS

In addition to the LGT of the NCE by conjugation from the nitrate-respiring strain NAR1 to the aerobe HB27, the whole denitrification pathway can also be transferred by this same process from the denitrificant strain PRQ25 to the aerobe HB27 (55). The conjugative mechanism of this LGT process seems to be quite unconventional (80), as neither the donor nor the receptor strains encode homologues to proteins involved in conjugative transfer in other bacteria, generally known as type IV secretion systems (T4SS). However, and regardless of the mechanism, it is evident from conjugation assays that genes located within the megaplasmid are transferred by conjugation at frequencies at least 10-fold higher than those seen with the genes located in the chromosome (80). In addition to conjugation, the whole denitrification pathway can also be acquired through the highly efficient natural competence system of the receptor strain HB27 (29). Nevertheless, under laboratory conditions, this requires at least two strictly ordered transformation steps (55): first, transfer of the NCE for nitrate respiration; second, acquisition of the *nor-nir* cluster. This obligate transfer sequence would be in agreement with the above-commented dependence on the NCE for nitrite respiration (77).

The denitrification clusters of *T. thermophilus* strains, including those most studied (e.g., NAR1 and PRQ25), are located in a highly variable region of the pTT27-like megaplasmids, supporting the idea of an easier spread of these genes than of any other chromosomal gene. In fact, a comparison between the available genomes of *T. thermophilus* and of the phylogenetically related *Deinococcus radiodurans* suggested the presence of genes in the pTT27 megaplasmid of *T. thermophilus*, likely acquired from thermophilic archaea, which could have a putative chief biological role in the adaptation to the thermophilic lifestyle, including reverse gyrase and DNA repair systems (81). Interestingly, the denitrification clusters are also relatively easily lost under laboratory aerobic growth conditions (unpublished data). In fact, the pTT27 megaplasmids of the HB8 and HB27 aerobic strains contain genetic "scars" that indicate the former presence of the denitrification NCE and *nor-nir* cluster in ancestors of these "aerobic" strains. As shown in Fig. 4, a small fragment of *nrcN* is present in HB8's pTT27 megaplasmid as part of the misannotated TTHB137 open reading frame. Similar events occurred in the HB27 strain, where open reading frame TTP0089 includes part of the *nirM* gene.

CONCLUDING REMARKS AND PERSPECTIVES

The analysis of the denitrification pathway in *Thermus thermophilus* has revealed the existence of two sets of genes, the NCE, encoding the nitrate respiration, and the *nor-nir* cluster, encoding the ability to respire nitrite and NO, whereas neither a nitrous oxide reductase activity nor the corresponding coding genes have been detected so far. The two denitrification clusters can be transferred by conjugation either as separate pieces or together, as a consequence of their proximity in the megaplasmid of the donor strain. However, separate transfer of the *nor-nir* cluster to aerobic strains

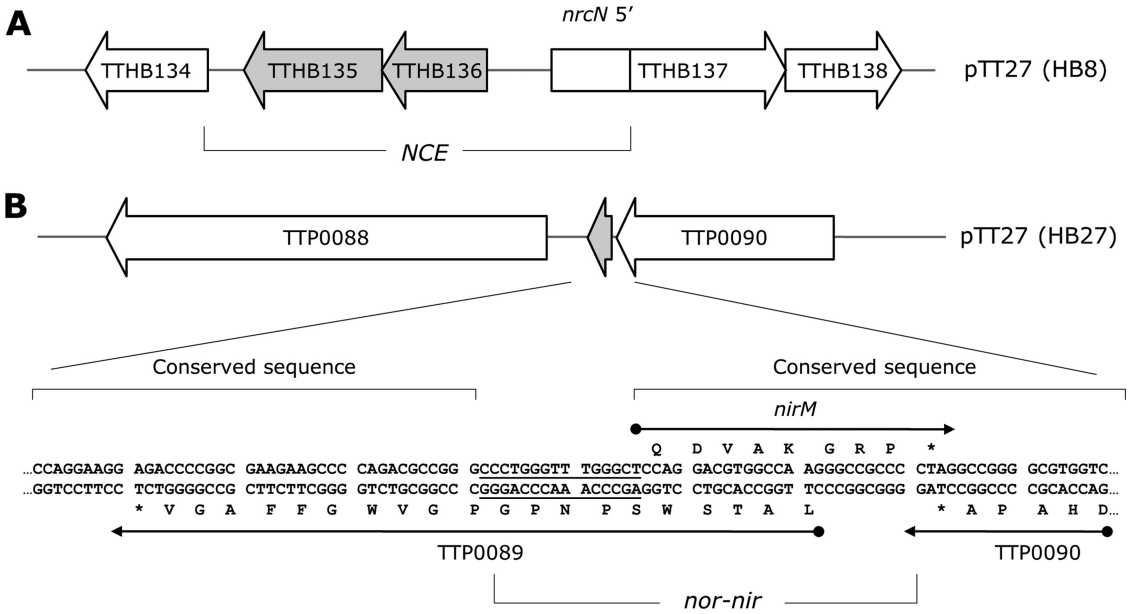

FIG 4 Genetic "scars" of the denitrification island in *T. thermophilus* aerobic strains. (A) NCE location within the pTT27 megaplasmid of strain HB8. TTHB137 is a chimeric gene derived from the fusion of *nrcN* and a pre-existing sequence in the megaplasmid after deletion of the NCE as deduced by a DNA comparison (BLASTn) between the sequence of the HB27 aerobic megaplasmid and the sequence of the NCE from the NAR1 strain (Y10124.3). Sequences that are not present in the denitrificant strains are indicated in gray. (B) *nor-nir* cluster location within the pTT27 megaplasmid of strain HB27. TTP0089 is actually a chimeric gene that comprehends the 3′ end of *nirM* from the *nor-nir* cluster of the PRQ25 strain (FN666415.2) and 32 bp of the conserved sequence of the megaplasmid. A 15-bp sequence is not present in the denitrificant strains (underlined). Data are from reference 71.

is not functional due to the lack of transcription factors encoded within the NCE, thus leading to an ordered process of LGT.

As previously reported for other systems, the expression of the denitrification enzymes is regulated by the absence of oxygen and the presence of the appropriate nitrogen oxide. However, the sensory signal-transduction systems required for this sensing are apparently distant from those studied in other denitrificant bacteria, since no homologues to FNR or NarX/NarL were found to be involved in the process. Induction of the nitrate reductase by nitrogen oxides other than nitrate and the results of biochemical assays support the idea that the presence of an extra cytochrome *c* subunit in the nitrate reductase has an additional role as an electron transporter toward the Nor and Nir reductases in substitution for complex III. Therefore, and despite its codification in two separate loci, the whole denitrification pathway seems to function as a single, transcriptionally coordinated respiratory unit, whose presence in a variable region of a megaplasmid, abundant with insertion sequences, allows a higher frequency of lateral transfer. In this context, the aerobic strains *T. thermophilus* HB8 and HB27 present evidence in their genomes of the loss of both denitrification islands.

Several questions remain to be answered regarding the denitrification process in these bacteria. The enzymatic characterization of purified Nar and Nir with identification of natural electron donors could clarify their probable interdependence. The unconventional sensory systems for nitrate, nitrite, and NO also require further investigation. Finally, the area of cluster mobilization and spreading in a population is an attractive field that merits a deeper investigation.

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REFERENCES

1. **Simon J, Klotz MG.** 2013. Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. Biochim. Biophys. Acta **1827:**114–135. http://dx.doi.org/10.1016/j.bbabio.2012.07.005.
2. **Betlach MR.** 1982. Evolution of bacterial denitrification and denitrifier diversity. Antonie Van Leeuwenhoek **48:**585–607.
3. **Knowles R.** 1982. Denitrification. Microbiol. Rev. **46:**43–70.
4. **Tiedje JM.** 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium, p 179–244. *In* Biology of anaerobic microorganisms. John Wiley & Sons, New York, NY.
5. **Richardson D, Felgate H, Watmough N, Thomson A, Baggs E.** 2009. Mitigating release of the potent greenhouse gas N_2O from the nitrogen cycle—could enzymic regulation hold the key? Trends Biotechnol. **27:**388–397. http://dx.doi.org/10.1016/j.tibtech.2009.03.009.
6. **Chapuis-Lardy L, Wrage N, Metay A, Chotte JL, Bernoux M.** 2007. Soils, a sink for N_2O? A review. Global Change Biol. **13:**1–17. http://dx.doi.org/10.1111/j.1365-2486.2006.01280.x.
7. **Philippot L, Andert J, Jones CM, Bru D, Hallin S.** 2011. Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N_2O emissions from soil. Global Change Biol. **17:**1497–1504. http://dx.doi.org/10.1111/j.1365-2486.2010.02334.x.
8. **Houlton BZ, Bai E.** 2009. Imprint of denitrifying bacteria on the global terrestrial biosphere. Proc. Natl. Acad. Sci. U. S. A. **106:**21713–21716. http://dx.doi.org/10.1073/pnas.0912111106.
9. **Philippot L, Mirleau P, Mazurier S, Siblot S, Hartmann A, Lemanceau P, Germon JC.** 2001. Characterization and transcriptional analysis of *Pseudomonas fluorescens* denitrifying clusters containing the *nar*, *nir*, *nor* and *nos* genes. Biochim. Biophys. Acta **1517:**436–440. http://dx.doi.org/10.1016/S0167-4781(00)00286-4.
10. **Vollack KU, Xie J, Hartig E, Romling U, Zumft WG.** 1998. Localization of denitrification genes on the chromosomal map of *Pseudomonas aeruginosa*. Microbiology **144**(Pt 2):441–448. http://dx.doi.org/10.1099/00221287-144-2-441.

11. **Zumft WG.** 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61:**533–616.
12. **Zumft WG, Korner H.** 1997. Enzyme diversity and mosaic gene organization in denitrification. Antonie Van Leeuwenhoek **71:**43–58. http://dx.doi.org/10.1023/A:1000112008026.
13. **Verbaendert I, De Vos P, Boon N, Heylen K.** 2011. Denitrification in Gram-positive bacteria: an underexplored trait. Biochem. Soc. Trans. **39:**254–258. http://dx.doi.org/10.1042/BST0390254.
14. **Berks BC, Ferguson SJ, Moir JW, Richardson DJ.** 1995. Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. Biochim. Biophys. Acta **1232:**97–173. http://dx.doi.org/10.1016/0005-2728(95)00092-5.
15. **Richardson DJ.** 2000. Bacterial respiration: a flexible process for a changing environment. Microbiology **146**(Pt 3)**:**551–571.
16. **Luque-Almagro VM, Gates AJ, Moreno-Vivian C, Ferguson SJ, Richardson DJ, Roldan MD.** 2011. Bacterial nitrate assimilation: gene distribution and regulation. Biochem. Soc. Trans. **39:**1838–1843. http://dx.doi.org/10.1042/BST20110688.
17. **Rinaldo S, Cutruzzola F.** 2007. Nitrite reductases in denitrification, p 37–55. In Biology of the nitrogen cycle. Elsevier, Amsterdam, The Netherlands.
18. **Nordling M, Young S, Karlsson BG, Lundberg LG.** 1990. The structural gene for cytochrome c551 from *Pseudomonas aeruginosa*. The nucleotide sequence shows a location downstream of the nitrite reductase gene. FEBS Lett. **259:**230–232.
19. **Pearson IV, Page MD, Van Spanning RJ, Ferguson SJ.** 2003. A mutant of *Paracoccus denitrificans* with disrupted genes coding for cytochrome c550 and pseudoazurin establishes these two proteins as the in vivo electron donors to cytochrome cd1 nitrite reductase. J. Bacteriol. **185:**6308–6315. http://dx.doi.org/10.1128/JB.185.21.6308-6315.2003.
20. **Hino T, Matsumoto Y, Nagano S, Sugimoto H, Fukumori Y, Murata T, Iwata S, Shiro Y.** 2010. Structural basis of biological N_2O generation by bacterial nitric oxide reductase. Science **330:**1666–1670. http://dx.doi.org/10.1126/science.1195591.
21. **Matsumoto Y, Tosha T, Pisliakov AV, Hino T, Sugimoto H, Nagano S, Sugita Y, Shiro Y.** 2012. Crystal structure of quinol-dependent nitric oxide reductase from *Geobacillus stearothermophilus*. Nat. Struct. Mol. Biol. **19:**238–245. http://dx.doi.org/10.1038/nsmb.2213.
22. **Dell'acqua S, Moura I, Moura JJ, Pauleta SR.** 2011. The electron transfer complex between nitrous oxide reductase and its electron donors. J. Biol. Inorg. Chem. **16:**1241–1254. http://dx.doi.org/10.1007/s00775-011-0812-9.
23. **Zumft WG, Kroneck PM.** 2007. Respiratory transformation of nitrous oxide (N2O) to dinitrogen by Bacteria and Archaea. Adv. Microb. Physiol. **52:**107–227.
24. **Sanford RA, Wagner DD, Wu Q, Chee-Sanford JC, Thomas SH, Cruz-García C, Rodríguez G, Massol-Deyá A, Krishnani KK, Ritalahti KM.** 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proc. Natl. Acad. Sci. U. S. A. **109:**19709–19714. http://dx.doi.org/10.1073/pnas.1211238109.
25. **Philippot L.** 2002. Denitrifying genes in bacterial and archaeal genomes. Biochim. Biophys. Acta **1577:**355–376. http://dx.doi.org/10.1016/S0167-4781(02)00420-7.
26. **Heylen K, Vanparys B, Gevers D, Wittebolle L, Boon N, De Vos P.** 2007. Nitric oxide reductase (*norB*) gene sequence analysis reveals discrepancies with nitrite reductase (*nir*) gene phylogeny in cultivated denitrifiers. Environ. Microbiol. **9:**1072–1077. http://dx.doi.org/10.1111/j.1462-2920.2006.01194.x.
27. **Jones CM, Stres B, Rosenquist M, Hallin S.** 2008. Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. Mol. Biol. Evol. **25:**1955–1966. http://dx.doi.org/10.1093/molbev/msn146.
28. **Cava F, Hidalgo A, Berenguer J.** 2009. *Thermus thermophilus* as biological model. Extremophiles **13:**213–231. http://dx.doi.org/10.1007/s00792-009-0226-6.
29. **Averhoff B.** 2009. Shuffling genes around in hot environments: the unique DNA transporter of *Thermus thermophilus*. FEMS Microbiol. Rev. **33:**611–626. http://dx.doi.org/10.1111/j.1574-6976.2008.00160.x.
30. **Jenney FE, Jr, Adams MW.** 2008. The impact of extremophiles on structural genomics (and vice versa). Extremophiles **12:**39–50. http://dx.doi.org/10.1007/s00792-007-0087-9.
31. **Yusupov MM, Yusupova GZ, Baucom A, Lieberman K, Earnest TN, Cate JH, Noller HF.** 2001. Crystal structure of the ribosome at 5.5 A resolution. Science **292:**883–896. http://dx.doi.org/10.1126/science.1060089.
32. **Severinov K.** 2000. RNA polymerase structure-function: insights into points of transcriptional regulation. Curr. Opin. Microbiol. **3:**118–125. http://dx.doi.org/10.1016/S1369-5274(00)00062-X.
33. **Sazanov LA, Hinchliffe P.** 2006. Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*. Science **311:**1430–1436. http://dx.doi.org/10.1126/science.1123809.
34. **Schwarzenlander C, Averhoff B.** 2006. Characterization of DNA transport in the thermophilic bacterium *Thermus thermophilus* HB27. FEBS J. **273:**4210–4218. http://dx.doi.org/10.1111/j.1742-4658.2006.05416.x.
35. **Efremov RG, Baradaran R, Sazanov LA.** 2010. The architecture of respiratory complex I. Nature **465:**441–445. http://dx.doi.org/10.1038/nature09066.
36. **Schurig-Briccio LA, Venkatakrishnan P, Hemp J, Bricio C, Berenguer J, Gennis RB.** 30 July 2013. Characterization of the nitric oxide reductase from *Thermus thermophilus*. Proc. Natl. Acad. Sci. U. S. A. [Epub ahead of print.] http://dx.doi.org/10.1073/pnas.1301731110.
37. **Kolaj-Robin O, O'kane SR, Nitschke W, Leger C, Baymann F, Soulimane T.** 2011. Biochemical and biophysical characterization of succinate: quinone reductase from *Thermus thermophilus*. Biochim. Biophys. Acta **1807:**68–79. http://dx.doi.org/10.1016/j.bbabio.2010.10.009.
38. **Fee JA, Kuila D, Mather MW, Yoshida T.** 1986. Respiratory proteins from extremely thermophilic, aerobic bacteria. Biochim. Biophys. Acta **853:**153–185. http://dx.doi.org/10.1016/0304-4173(86)90009-1.
39. **Mooser D, Maneg O, Corvey C, Steiner T, Malatesta F, Karas M, Soulimane T, Ludwig B.** 2005. A four-subunit cytochrome bc(1) complex complements the respiratory chain of *Thermus thermophilus*. Biochim. Biophys. Acta **1708:**262–274. http://dx.doi.org/10.1016/j.bbabio.2005.03.008.
40. **Mooser D, Maneg O, Macmillan F, Malatesta F, Soulimane T, Ludwig B.** 2006. The menaquinol-oxidizing cytochrome bc complex from *Thermus thermophilus:* protein domains and subunits. Biochim. Biophys. Acta **1757:**1084–1095. http://dx.doi.org/10.1016/j.bbabio.2006.05.033.
41. **Hon-Nami K, Oshima T.** 1977. Purification and some properties of cytochrome c-552 from an extreme thermophile, Thermus thermophilus HB8. J. Biochem. **82:**769–776.
42. **Soulimane T, Von Walter M, Hof P, Than ME, Huber R, Buse G.** 1997. Cytochrome-c_{552} from *Thermus thermophilus*: a functional and crystallographic investigation. Biochem. Biophys. Res. Commun. **237:**572–576. http://dx.doi.org/10.1006/bbrc.1997.7041.
43. **Fee JA, Choc MG, Findling KL, Lorence R, Yoshida T.** 1980. Properties of a copper-containing cytochrome c_1aa_3 complex: a terminal oxidase of the extreme thermophile *Thermus thermophilus* HB8. Proc. Natl. Acad. Sci. U. S. A. **77:**147–151. http://dx.doi.org/10.1073/pnas.77.1.147.
44. **Keightley JA, Zimmermann BH, Mather MW, Springer P, Pastuszyn A, Lawrence DM, Fee JA.** 1995. Molecular genetic and protein chemical characterization of the cytochrome ba3 from *Thermus thermophilus* HB8. J. Biol. Chem. **270:**20345–20358. http://dx.doi.org/10.1074/jbc.270.35.20345.
45. **Mather MW, Springer P, Hensel S, Buse G, Fee JA.** 1993. Cytochrome oxidase genes from *Thermus thermophilus*. Nucleotide sequence of the fused gene and analysis of the deduced primary structures for subunits I and III of cytochrome caa3. J. Biol. Chem. **268:**5395–5408.
46. **Soulimane T, Buse G, Bourenkov GP, Bartunik HD, Huber R, Than ME.** 2000. Structure and mechanism of the aberrant ba(3)-cytochrome c oxidase from *Thermus thermophilus*. EMBO J. **19:**1766–1776. http://dx.doi.org/10.1093/emboj/19.8.1766.
47. **Zimmermann BH, Nitsche CI, Fee JA, Rusnak F, Munck E.** 1988. Properties of a copper-containing cytochrome ba_3: a second terminal oxidase from the extreme thermophile *Thermus thermophilus*. Proc. Natl. Acad. Sci. U. S. A. **85:**5779–5783. http://dx.doi.org/10.1073/pnas.85.16.5779.
48. **Müller V, Grüber G.** 2003. ATP synthases: structure, function and evolution of unique energy converters. CMLS **60:**474–494. http://dx.doi.org/10.1007/s000180300040.
49. **Henne A, Bruggemann H, Raasch C, Wiezer A, Hartsch T, Liesegang H, Johann A, Lienard T, Gohl O, Martinez-Arias R, Jacobi C, Starkuviene V, Schlenczeck S, Dencker S, Huber R, Klenk HP, Kramer W, Merkl R, Gottschalk G, Fritz HJ.** 2004. The genome sequence of the extreme thermophile *Thermus thermophilus*. Nat. Biotechnol. **22:**547–553. http://dx.doi.org/10.1038/nbt956.
50. **Cava F, Zafra O, Berenguer J.** 2008. A cytochrome c containing nitrate reductase plays a role in electron transport for denitrification in *Thermus*

thermophilus without involvement of the bc respiratory complex. Mol. Microbiol. **70:**507–518. http://dx.doi.org/10.1111/j.1365-2958.2008.06429.x.
51. **Hedlund BP, Mcdonald AI, Lam J, Dodsworth JA, Brown JR, Hungate BA.** 2011. Potential role of *Thermus thermophilus* and *T. oshimai* in high rates of nitrous oxide (N_2O) production in approximately 80 degrees C hot springs in the US Great Basin. Geobiology **9:**471–480. http://dx.doi.org/10.1111/j.1472-4669.2011.00295.x.
52. **Fernández-Herrero LA.** 1995. Caracterización de genes implicados en la regulación y la síntesis de la envoltura celular de *T. thermophilus HB8*. Universidad Autónoma, Madrid, Spain.
53. **Ramírez-Arcos S, Fernandez-Herrero LA, Berenguer J.** 1998. A thermophilic nitrate reductase is responsible for the strain specific anaerobic growth of *Thermus thermophilus* HB8. Biochim. Biophys. Acta **1396:**215–227. http://dx.doi.org/10.1016/S0167-4781(97)00183-8.
54. **Ramírez-Arcos S, Fernandez-Herrero LA, Marin I, Berenguer J.** 1998. Anaerobic growth, a property horizontally transferred by an Hfr-like mechanism among extreme thermophiles. J. Bacteriol. **180:**3137–3143.
55. **Alvarez L, Bricio C, Gomez MJ, Berenguer J.** 17 December 2010. Lateral transfer of the denitrification pathway among *Thermus thermophilus* strains. Appl. Environ. Microbiol. http://dx.doi.org/10.1128/AEM.02048-10.
56. **Zafra O, Ramirez S, Castan P, Moreno R, Cava F, Valles C, Caro E, Berenguer J.** 2002. A cytochrome c encoded by the nar operon is required for the synthesis of active respiratory nitrate reductase in *Thermus thermophilus*. FEBS Lett. **523:**99–102. http://dx.doi.org/10.1016/S0014-5793(02)02953-8.
57. **Ramírez-Arcos S, Moreno R, Zafra O, Castan P, Valles C, Berenguer J.** 2000. Two nitrate/nitrite transporters are encoded within the mobilizable plasmid for nitrate respiration of *Thermus thermophilus* HB8. J. Bacteriol. **182:**2179–2183. http://dx.doi.org/10.1128/JB.182.8.2179-2183.2000.
58. **Zafra O, Cava F, Blasco F, Magalon A, Berenguer J.** 2005. Membrane-associated maturation of the heterotetrameric nitrate reductase of *Thermus thermophilus*. J. Bacteriol. **187:**3990–3996. http://dx.doi.org/10.1128/JB.187.12.3990-3996.2005.
59. **Bertero MG, Rothery RA, Palak M, Hou C, Lim D, Blasco F, Weiner JH, Strynadka NC.** 2003. Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A. Nat. Struct. Biol. **10:**681–687. http://dx.doi.org/10.1038/nsb969.
60. **Sundermeyer-Klinger H, Meyer W, Warninghoff B, Bock E.** 1984. Membrane-bound nitrite oxidoreductase of *Nitrobacter*:evidence for a nitrate reductase system. Arch. Microbiol. **140:**153–158. http://dx.doi.org/10.1007/BF00454918.
61. **Moir JW, Wood NJ.** 2001. Nitrate and nitrite transport in bacteria. CMLS **58:**215–224. http://dx.doi.org/10.1007/PL00000849.
62. **Wood NJ, Alizadeh T, Richardson DJ, Ferguson SJ, Moir JW.** 2002. Two domains of a dual-function NarK protein are required for nitrate uptake, the first step of denitrification in *Paracoccus pantotrophus*. Mol. Microbiol. **44:**157–170. http://dx.doi.org/10.1046/j.1365-2958.2002.02859.x.
63. **Yan H, Huang W, Yan C, Gong X, Jiang S, Zhao Y, Wang J, Shi Y.** 2013. Structure and mechanism of a nitrate transporter. Cell Rep. **3:**716–723. http://dx.doi.org/10.1016/j.celrep.2013.03.007.
64. **Zheng H, Wisedchaisri G, Gonen T.** 2013. Crystal structure of a nitrate/nitrite exchanger. Nature **497:**647–651. http://dx.doi.org/10.1038/nature12139.
65. **Schreiber K, Krieger R, Benkert B, Eschbach M, Arai H, Schobert M, Jahn D.** 2007. The anaerobic regulatory network required for Pseudomonas aeruginosa nitrate respiration. J. Bacteriol. **189:**4310–4314. http://dx.doi.org/10.1128/JB.00240-07.
66. **Goddard AD, Moir JW, Richardson DJ, Ferguson SJ.** 2008. Interdependence of two NarK domains in a fused nitrate/nitrite transporter. Mol. Microbiol. **70:**667–681. http://dx.doi.org/10.1111/j.1365-2958.2008.06436.x.
67. **Gounder K, Brzuszkiewicz E, Liesegang H, Wollherr A, Daniel R, Gottschalk G, Reva O, Kumwenda B, Srivastava M, Bricio C, Berenguer J, Van Heerden E, Litthauer D.** 2011. Sequence of the hyperplastic genome of the naturally competent *Thermus scotoductus* SA-01. BMC Genomics **12:**577. http://dx.doi.org/10.1186/1471-2164-12-577.
68. **Cava F, Zafra O, Magalon A, Blasco F, Berenguer J.** 2004. A new type of NADH dehydrogenase specific for nitrate respiration in the extreme thermophile *Thermus thermophilus*. J. Biol. Chem. **279:**45369–45378. http://dx.doi.org/10.1074/jbc.M404785200.
69. **Macedo-Ribeiro S, Martins BM, Pereira PJ, Buse G, Huber R, Soulimane T.** 2001. New insights into the thermostability of bacterial ferredoxins: high-resolution crystal structure of the seven-iron ferredoxin from *Thermus thermophilus*. J. Biol. Inorg. Chem. **6:**663–674. http://dx.doi.org/10.1007/s007750100243.
70. **Bricio C.** 2012. Producción de óxidos de nitrógeno gaseosos en *Thermus thermophilus*. Universidad Autónoma, Madrid, Spain.
71. **Alvarez L.** 2012. Análisis de la respiración de nitrito en *Thermus thermophilus*. Universidad Autónoma de Madrid, Madrid, Spain.
72. **Brindley AA, Zajicek R, Warren MJ, Ferguson SJ, Rigby SE.** 2010. NirJ, a radical SAM family member of the d1 heme biogenesis cluster. FEBS Lett. **584:**2461–2466. http://dx.doi.org/10.1016/j.febslet.2010.04.053.
73. **Cava F, Laptenko O, Borukhov S, Chahlafi Z, Blas-Galindo E, Gomez-Puertas P, Berenguer J.** 2007. Control of the respiratory metabolism of Thermus thermophilus by the nitrate respiration conjugative element NCE. Mol. Microbiol. **64:**630–646. http://dx.doi.org/10.1111/j.1365-2958.2007.05687.x.
74. **Aravind L, Ponting CP.** 1997. The GAF domain: an evolutionary link between diverse phototransducing proteins. Trends Biochem. Sci. **22:**458–459. http://dx.doi.org/10.1016/S0968-0004(97)01148-1.
75. **Yeats C, Bentley S, Bateman A.** 2003. New knowledge from old: in silico discovery of novel protein domains in Streptomyces coelicolor. BMC Microbiol. **3:**3. http://dx.doi.org/10.1186/1471-2180-3-3.
76. **Körner H, Sofia HJ, Zumft WG.** 2003. Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. FEMS Microbiol. Rev. **27:**559–592. http://dx.doi.org/10.1016/S0168-6445(03)00066-4.
77. **Cava F, Zafra O, Da Costa MS, Berenguer J.** 2008. The role of the nitrate respiration element of *Thermus thermophilus* in the control and activity of the denitrification apparatus. Environ. Microbiol. **10:**522–533. http://dx.doi.org/10.1111/j.1462-2920.2007.01472.x.
78. **Tucker NP, Hicks MG, Clarke TA, Crack JC, Chandra G, Le Brun NE, Dixon R, Hutchings MI.** 2008. The transcriptional repressor protein NsrR senses nitric oxide directly via a [2Fe-2S] cluster. PLoS One **3:**e3623. http://dx.doi.org/10.1371/journal.pone.0003623.
79. **Pohlmann A, Cramm R, Schmelz K, Friedrich B.** 2000. A novel NO-responding regulator controls the reduction of nitric oxide in *Ralstonia eutropha*. Mol. Microbiol. **38:**626–638. http://dx.doi.org/10.1046/j.1365-2958.2000.02157.x.
80. **César CE, Álvarez L, Bricio C, van Heerden E, Littauer D, Berenguer J.** 2011. Unconventional lateral gene transfer in extreme thermophilic bacteria. Int. Microbiol. **14:**187–199. http://dx.doi.org/10.2436/20.1501.01.148.
81. **Bruggemann H, Chen C.** 2006. Comparative genomics of *Thermus thermophilus*: Plasticity of the megaplasmid and its contribution to a thermophilic lifestyle. J. Biotechnol. **124:**654–661. http://dx.doi.org/10.1016/j.jbiotec.2006.03.043.
82. **Cava F, Berenguer J.** 2006. Biochemical and regulatory properties of a respiratory island encoded by a conjugative plasmid in the extreme thermophile *Thermus thermophilus*. Biochem. Soc. Trans. **34:**97–100. http://dx.doi.org/10.1042/BST0340097.
83. **Murugapiran SK, Huntemann M, Wei CL, Han J, Detter JC, Han CS, Erkkila TH, Teshima H, Chen A, Kyrpides N, Mavrommatis K, Markowitz V, Szeto E, Ivanova N, Pagani I, Lam J, Mcdonald AI, Dodsworth JA, Pati A, Goodwin L, Peters L, Pitluck S, Woyke T, Hedlund BP.** 24 January 2013. Whole genome sequencing of *Thermus oshimai* JL-2 and *Thermus thermophilus* JL-18, incomplete denitrifiers from the United States Great Basin. Genome Announc. [Epub ahead of print.] http://dx.doi.org/10.1128/genomeA.00106-12.
84. **Murugapiran SK, Huntemann M, Wei CL, Han J, Detter JC, Han C, Erkkila TH, Teshima H, Chen A, Kyrpides N, Mavrommatis K, Markowitz V, Szeto E, Ivanova N, Pagani I, Pati A, Goodwin L, Peters L, Pitluck S, Lam J, Mcdonald AI, Dodsworth JA, Woyke T, Hedlund BP.** 2013. *Thermus oshimai* JL-2 and *T. thermophilus* JL-18 genome analysis illuminates pathways for carbon, nitrogen, and sulfur cycling. Stand. Genomic Sci. **7:**449–468. http://dx.doi.org/10.4056/sigs.3667269.