Partial and complete denitrification in *Thermus thermophilus*: lessons from genome drafts

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Abstract

We have obtained draft genomic sequences of PD (partial denitrificant) and CD (complete denitrificant) strains of *Thermus thermophilus*. Their genomes are similar in size to that of the aerobic strains sequenced to date and probably contain a similar megaplasmid. In the CD strain, the genes encoding a putative cytochrome cd_1 Nir (nitrite reductase) and ancillary proteins were clustered with a cytochrome *c*-dependent Nor (nitric oxide reductase), and with genes that are probably implicated in their regulation. The Nar (nitrate reductase) and associated genes were also clustered and located 7 kb downstream of the genes coding for the Nir. The whole *nar–nir–nor* denitrification supercluster was identified as part of a variable region of a megaplasmid. No homologues of NosZ were found despite nitrogen balance supports the idea that such activity actually exists.

The genus *Thermus*

The genus *Thermus* is widespread in natural and man-made thermophilic environments all over the world. It includes hundreds of strains that constitute an important source of enzymes of biotechnological interest [1]. In addition, most strains can grow up to high cell densities under laboratory conditions and at least some of them can be genetically manipulated because of the presence of a highly efficient natural competence system [2]. These properties and the greater ability of protein complexes from thermophiles to crystallize compared with their counterparts from mesophiles [3] have led to the use of isolates of this species as the main model in structural biology. Examples such as the high-resolution structures of the 70S ribosome [4], the bacterial RNA polymerase [5] and the respiratory Complex I [6] are based on the analysis of these complexes from *Thermus* spp.

At the ultrastructural level, the envelope of *Thermus* spp. shows characteristics typical of Gram-negative bacteria, with an external membrane that defines a perisplasmic space. However, at the biochemical level, the peptidoglycan composition and the presence of an S-layer homology domain attached to secondary cell wall polymers is more common among Gram-positive bacteria [1]. In any case, it is relevant to state that *Thermus* forms, along with *Deinococcus*, a distinct bacterial phylum [7], for which an ancient origin has been proposed and discussed [8].

Among the great panoply of *Thermus* spp. isolates in existence, *T. thermophilus* strains HB27 and HB8 have been by far the most studied under laboratory conditions. Both strains are strict aerobes, despite the presence of some genes usually associated with anaerobic metabolism, which was revealed when their sequences were published [9]. Natural competence, in particular in the HB27 strain, is highly efficient and apparently constitutive, allowing incorporation rates of 40 kb per cell and secondly without much difference regarding its origin [10]. This promiscuity has made this strain an ideal laboratory model, allowing the development of a complete genetic toolbox, through which it is possible to analyse the actual function of a given protein *in vivo* [1].

Energy metabolism in *T. thermophilus*

Energy is obtained in *T. thermophilus* preferentially through aerobic respiration. Its respiratory enzymes have been the subject of many biochemical as well as structural studies, making it one of the best known respiratory systems. A great variety of carbon substrates can be catabolized, including most amino acids, different sugars and fatty acids. NADH from a complete tricarboxylic acid cycle is oxidized by Complex I, the proton-translocating type I NADH dehydrogenase (NqoA-N). The 3D (three-dimensional) structure of the soluble domain of this complex enzyme has been solved at high resolution, and that of the whole enzyme, including the membrane domain, has been also obtained recently [11]. Complex II (succinate dehydrogenase) also provides electrons to the respiratory chain. A putative type II NADH oxidase (TTC1829) could also provide electrons to the respiratory chain. Menaquinone-8, the major quinone in membranes of this organism, is oxidized by Complex III, encoded by the operon *fbcCXFB*. Electrons are then transported to the terminal oxidases through a soluble periplasmic cytochrome c_{552}, or directly through Complex III. There are two terminal cytochrome oxidases: a caa_3-type expressed under fully aerobic growth and a ba_3-type that

Key words: denitrification, genomics, nitrate, nitrite, reductase, thermophile, *Thermus thermophilus*.

Abbreviations used: CD, complete dentitrificant; Nar, nitrate reductase; NCE, nitrate respiration conjugative element; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase; ORF, open reading frame; PD, partial denitrificant; 3D, three-dimensional.

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is induced under low oxygen pressures. The 3D structure of the latter enzyme is also available. ATP is synthesized by Complex V, a V/A-type ATP-synthase that could have been the consequence of a lateral gene transfer from Archaea (reviewed in [1]).

Despite the sequenced HB27 and HB8 strains not being able to grow in the absence of oxygen, there are other isolates of *Thermus* spp. that are able to grow anaerobically by using nitrogen oxides, sulfur or even metals as electron acceptors. Actually, different isolates identified as *T. thermophilus* have been shown to grow anaerobically by reducing nitrate to nitrite [PD (partial denitrificant)] or by reducing nitrite to a gas form [CD (complete denitrificant)] [12].

The ability to grow by nitrate respiration is encoded by a cluster of genes that could be transferred from the NAR1 PD strain to the aerobic HB27 strain. The group of genes encoding this capability was named NCE (nitrate respiration conjugative element) and included a cryptic replicative origin [13]. The sequence of NCE from NAR1 includes a seven-gene operon that encodes a heterotetrameric Nar (nitrate reductase) (NarCGHI), its dedicated chaperone (NarJ) and two nitrate/nitrite transporters (NarK and NarT). A second operon was found to encode a four-subunit NADH dehydrogenase (NrcDEFN). In addition, an operon (*dnrST*) was found between *nrc* and *nar* that encoded putative transcription factors. DnrT is a CRP (cAMP receptor protein) family member required as an activator of the Pnar, Pnrc and Pdnr promoters, whereas DnrS is required for Pnar and Pdnr expression [14]. Our findings support the hypothesis that the oxygen sensitivity of DnrS is the factor that triggers the activation of the nitrate respiration by low oxygen pressure as there is no FNR (fumarate-nitrate reduction regulator)-like factors in the genome [15]. Probably, its N-terminal GAF domain constitutes the oxygen sensor. Another operon exists downstream of *nar* that encodes two small proteins (provisional names DrpA and DrpB). Their actual function is not known, but there is evidence suggesting a possible role in nitrate sensing (Z. Chahlafi, F. Cava, L. Alvarez and J. Berenguer, unpublished work).

In contrast with nitrate respiration, the enzymes implicated in further reduction steps of the denitrification pathway in CD strains are not known. Actually, it is not known whether or not the denitrification proceeds up to N_2. To understand the whole process, we have sequenced in parallel the CD strain PRQ25 and the PD strain NAR1 of *T. thermophilus.* In the following sections, we provide some clues to the denitrification process derived from physiological and genomic data.

Completeness of the denitrification pathway in *T. thermophilus* PRQ25

The PRQ25 strain grows anaerobically with nitrate and nitrite, with the concomitant production of gas (Figure 1A), suggesting that nitrogen was reduced at least to the level of N_2O (nitrous oxide) (L. Alvarez, C. Bricio, M.J. Gómez and J. Berenguer, unpublished work). This was confirmed by gas chromatography on cultures grown anaerobically for 48 h at 70°C in the presence of different concentrations of nitrate. From these assays, we observed that the production of N_2O reached a maximum with 10 mM nitrate and decreased to almost undetectable levels above 20 mM (Figure 1B), in agreement with the decrease in the size of the bubble accumulated inside inverted tubes (results not shown). It is noteworthy that nitrite accumulated in cultures at high nitrate concentrations and that residual nitrate remained at 40 mM. Therefore it seems that an excess of nitrate inhibits the

Figure 1 | Denitrification in *T. thermophilus*

(**A**) Growth curve of PRQ25 in TB (*Thermus* broth) medium with nitrate (40 mM) or nitrite (5 mM) under anaerobic conditions and the corresponding production of gas. The aerobic HB27 strain was incubated anaerobically with nitrate (40 mM) as a control. (**B**) Concentration of N_2O produced from the indicated amount of nitrate after 2 days of incubation at 70°C under anaerobic conditions (Hungate tubes). The presence (+) or absence (−) of nitrite after the incubation period is indicated. The aerobic HB27 strain was used as control. O.D., optical density (attenuance).

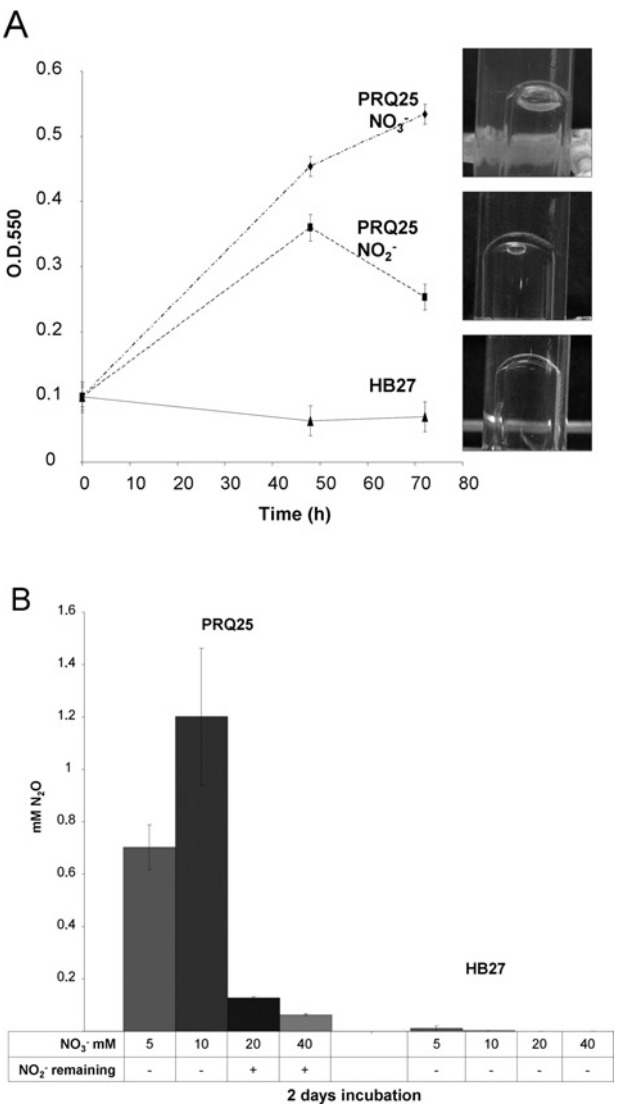


Table 1 | **Main genome data from the NAR1 and PRQ25 strains**

Parameter	HB27 (chromosome)	HB27 (pTT27)	NAR1 (total)	PRQ25 (total)
Size (bp)	1 894 877	232 605	2 168 704	2 185 944
G+C (%)	69.4	69.2	67.8	68.9
Coding sequence (%)	95	89	91.3	91.5
Total genes (*n*)	1988	230	2564	2351
tRNAs (*n*)	47	–	64	47
Transposases (*n*)	12	9	11	11

production of N_2O either directly or through an indirect way. Actually, the excess of nitrate does not result in an increase in cell growth, which is lower with 20 or 40 mM nitrate than with 10 mM, supporting a toxic effect probably associated with the accumulation of nitrite/NO. This inhibition was not detected in the PD strain NAR1, despite being able to quantitatively reduce nitrate to nitrite.

These findings support the hypothesis that PRQ25 has active Nars, Nirs (nitrite reductases) and Nors (nitric oxide reductases). The presence of Nos (nitrous oxide reductase) activity is also likely, because the amount of N_2O produced with 10 mM nitrate is much smaller (1.4 mM) than that expected if all of the nitrate used was reduced to this gas. Actually, neither nitrate nor nitrite remains in the medium, and NO accumulation is too toxic to be a possible means of nitrogen losses.

Comparative genomics of the PD and CD strains

Draft sequences of the PRQ25 and NAR1 strains were obtained through pyrosequencing (454 technology; Roche). An average of ten reads/sequence was obtained. The raw reads obtained were assembled into contigs using the 454 *de novo* Newbler Assembly software, and the resulting contigs were annotated through an automatic pipeline developed by the Sequencing and Bioinformatic Unit of the Centro de Astrobiología (CSIC-INTA). Manual inspection of the automatic annotations was focused on genes with putative interest in the denitrification pathway.

The comparison of the sequences obtained with the genome of the HB27 strain shown in Table 1 indicates that PRQ25 and NAR1 have a C+G genome content similar to that of HB27. If we assume that a genome size for a typical *T. thermophilus* strain is 2.15–2.2 Mb, our findings indicate that almost all of the genome from both strains has been sequenced. The higher number of ORFs (open reading frames) predicted in NAR1 and PRQ25 in comparison with the HB27 genome is because the system computes as different ORF fragments of a single gene found at the extreme of two different contigs, and we probably have many of these cases. It is also relevant that approx. 40% of the sequences of the pTT27 megaplasmid from the HB27 strain are identified in both NAR1 and PRQ25, supporting the idea that a similar megaplasmid exists in both strains.

Nitrate respiration genes

A search revealed the presence of NCE in NAR1 and PRQ25. The corresponding NCEs, however, were not identical in both strains. For example, the *nar* operon of PRQ25 encodes a single nitrate/nitrite transporter (NarO) that shows relatively low identity with either NarK or NarT from NAR1. The other genes of the *nar* operon are well conserved between both strains, as well as the regulatory operon *dnrST* and the *drpAB* operon. The *nrc* operon of this strain seems to lack the membrane subunit of the complex (NrcE), so it is not clear how the complex can provide electrons to the Nar. The NCE of PRQ25, however, encodes a putative ferrous iron transporter that is mutated (pseudogene) in NAR1.

The analysis of the regions flanking the NCE, i.e. a search for sequences upstream and downstream of the NCE that show high identity with sequences of the aerobic strains HB27 and/or HB8, indicated that the NCE of both strains was inserted between sequences almost identically with those encoding TTHB134 (5′) and TTHB137 (3′) of the pTT27 megaplasmid from HB8. These genes are part of one of the variable regions of this megaplasmid [16] (Figure 2). Therefore our data support the hypothesis that the NCE of NAR1 and PRQ25 is located in a variable region of a megaplasmid equivalent to pTT27.

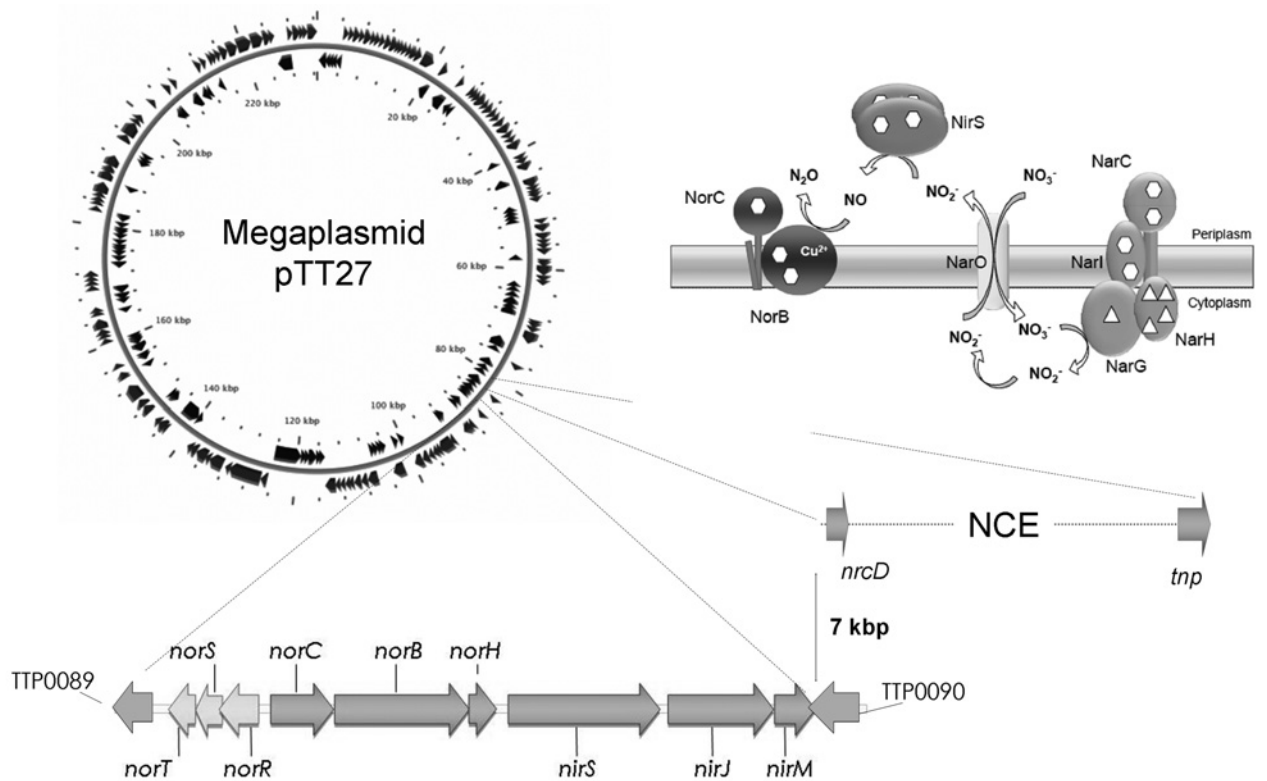
Nitrite respiration genes

In our search among the PRQ25 sequences, we found genes encoding putative Nirs and Nors that were absent from the NAR1 and from the aerobic HB27 and HB8 strains. Actually the *nor* and *nir* genes were clustered in a 6.9 kb DNA fragment that is probably responsible for the reduction of nitrite into N_2O in PRQ25 (Figure 2). The cluster encodes homologues of the subunits of the cytochrome-dependent Nor (NorC and NorB) within an operon that includes a third gene (*norH*) encoding a small membrane protein. Downstream of *norH*, a homologue of *cytochrome* cd_1 Nir (*nirS*) is encoded apparently within a monocistronic operon. This is followed by a two-gene operon (*nirJM*) encoding homologues of proteins found in the Nir clusters of other bacteria. Upstream of *norC* there are three genes encoding small proteins, the first of which (norR) shows similarities to NO-sensory transcription factors of the MarR family.

Interestingly, genes around this *nor–nir* cluster are almost identical with genes TTP89 (5′) and TTP90 (3′) from the

Figure 2 | The denitrification supercluster of *T. thermophilus* PRQ25

Left-hand panel: structure of the *nor–nir* cluster and its approximate location on the map of the pTT27 megaplasmid of the aerobic HB27 strain. The NCE was found 7 kb downstream of this cluster and surrounded by genes belonging to the same region of the pTT27 plasmid in the HB8 strain. The names of the genes are explained in the text, except for *tnp*, which encodes a putative transposase. Right-hand panel: putative localization of the proteins implicated in denitrification of PRQ25 is shown. Triangles indicate Fe–S clusters, and hexagons represent haem groups.



pTT27 plasmid of the HB27 strain. These genes also belong to the same variable region in which the NCE gene was found, supporting that they are clustered. This was finally confirmed by joining the corresponding contigs through PCR, leading us to the conclusion that the *nor–nir* cluster is 7 kb upstream of the first NCE gene (*nrcD*). Therefore three of the enzymes in the denitrification pathway (Nar, Nir and Nor) are clustered in a variable region of a megaplasmid from PRQ25.

Despite our efforts, no homologues of typical Nos were found within the proteins encoded by the PRQ25 and NAR1 genomes, suggesting that either this process is not carried out in PRQ25 or that another enzyme plays this role.

Concluding remarks

Our draft genomic data from the CD strain PRQ25 shows the existence of a supercluster of genes encoding homologues of Nar, Nir and Nor from other organisms. This denitrification supercluster is surrounded by genes belonging to a variable region of the pTT27 megaplasmid found in the aerobic strains HB8 and HB27. In the NAR1 strain, only the NCE was present, but, unexpectedly, within an equivalent position to that of PRQ25, supporting that it was integrated in such a position in an ancestral *T. thermophilus* strain from which both NAR1 and PRQ25 derive. It is interesting to note that the last gene in the NCE insertion in both organisms encodes a putative transposase (Figure 2) that could be implicated in its integration. It is also relevant to note that, despite this likely common origin, differences such as a single nitrate–nitrite transporter in PRQ25 (NarO) instead of the two found in NAR1 (NarK and NarT) were found. Such differences could be related with the ability of the former to eliminate the nitrite through reduction to N_2O, whereas the latter has to synthesize a more efficient nitrite secretion system to tolerate its toxicity. Other differences such as the lack of a full-size *nrcE* gene in PRQ25 and the presence of a complete iron transporter gene in this strain instead of the pseudogene found in NAR1 are not so straightforward to explain.

The denitrification supercluster also encodes homologues of putative regulatory proteins (NorR) and of proteins

probably implicated in electron transport (NirM) or in the synthesis of the *haem* d_1 (NirJ). It is interesting to note that genes hypothetically essential for NirS maturation are not part of the cluster. An example is NirE (uroporphyrinogen-III methyltransferase), usually encoded in *nir* operons of other bacteria. As a homologue of this enzyme is encoded within the chromosome of PRQ25 and HB27 (PRQ25_1676, 95% identical with TTC0308), no need for the clustering with *nirS* of additional homologues of this enzyme exists in *T. thermophilus*.

Regarding the completeness or not of the denitrification pathway carried out by PRQ25, our results suggest that, despite no homologues of NosZ appearing in the genome draft, part of the N_2O could be reduced to N_2 by another enzyme, as the nitrogen balance reveals a loss of nitrogen in the process. An alternative explanation could be that part of the nitrite was transformed into ammonia by an enzyme (PRQ25_1681) 98% identical with TTC0313 from HB27, a protein annotated as a ferredoxin-nitrite reductase. However, this enzyme more likely represents a sulfite reductase, as it clusters with enzymes such as phosphoadenonine phosphosulfate reductase (PRQ25_1678), which has been implicated in the assimilation of sulfate. Future genetic work will allow us to definitively confirm or to discard this possibility.

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