Hierarchical Control of Nitrite Respiration by Transcription Factors Encoded within Mobile Gene Clusters of Thermus thermophilus

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Abstract: Denitrification in Thermus thermophilus is encoded by the nitrate respiration conjugative element (NCE) and nitrite and nitric oxide respiration (nic) gene clusters. A tight coordination of each cluster’s expression is required to maximize anaerobic growth, and to avoid toxicity by intermediates, especially nitric oxides (NO). Here, we study the control of the nitrite reductases (Nir) and NO reductases (Nor) upon horizontal acquisition of the NCE and nic clusters by a formerly aerobic host. Expression of the nic promoters PnirS, PnirJ, and PnorC, depends on the oxygen sensor DnrS and on the DnrT protein, both NCE-encoded. NsrR, a nic-encoded transcription factor with an iron–sulfur cluster, is also involved in Nir and Nor control. Deletion of nsrR decreased PnorC and PnirJ transcription, and activated PnirS under denitrification conditions, exhibiting a dual regulatory role never described before for members of the NsrR family. On the basis of these results, a regulatory hierarchy is proposed, in which under anoxia, there is a pre-activation of the nic promoters by DnrS and on the DnrT, and then NsrR leads to Nor induction and Nir repression, likely as a second stage of regulation that would require NO detection, thus avoiding accumulation of toxic levels of NO. The whole system appears to work in remarkable coordination to function only when the relevant nitrogen species are present inside the cell.

Keywords: denitrification; nitrite respiration; regulation; thermophiles; Thermus thermophilus

1. Introduction

Many bacteria, archaea, and a few fungi species are able to utilize nitrogen oxides (NOX) as electron acceptors at low oxygen concentrations [1]. In this process, known as denitrification, water-soluble nitrate and nitrite are eliminated from the local environment through conversion into gaseous nitrous oxide (N2O) or dinitrogen (N2) that escape to the atmosphere [2–4]. The conversion of nitrate to dinitrogen is carried out in four steps (nitrate > nitrite > nitric oxide > nitrous oxide > dinitrogen) by the corresponding membrane-bound or periplasmic reductases (Nar/Nap, Nir, Nor, and Nos). All four reductases are found in complete denitrifying microorganisms, such as Pseudomonas spp. and Paracoccus denitrificans, employed as models of the whole denitrification process [1,5–9].

In well-characterized denitrifying bacteria, the genes encoding the denitrification reductases are transcribed in response to two signals: low oxygen levels, and presence of the appropriate nitrogen...
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Oxide species [7]. Low oxygen is most frequently signaled in denitrification by homologues of fumarate and nitrate reduction regulatory protein (FNR) a transcription factor of the catabolite repressor protein (CRP) family that contains an oxygen-sensitive [4Fe-4S] iron–sulfur cluster, or by heme-containing proteins of the FixL family, whereas nitrate and nitrite are detected through two-component systems of the NarX/NarL type. Downstream enzymes of the denitrification pathway are expressed upon detection of NO, driven by a specialized and diverse set of sensory transcription factors [10]. Proteins of the CRP family, designated as NNR and DNR, sense NO through an N-terminal domain containing a heme group, and are the main NO sensors in Pseudomonas spp. and related bacteria [11,12]. Proteins of the NorR family, present in diverse bacteria, sense NO likely through nitrosylation of a mono-nuclear non-heme iron present in a N-terminal cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA (GAF) domain. Finally, members of the NsrR family regulate the expression of NO detoxifying enzymes or the expression of denitrification genes in different Gram-positive and Gram-negative bacteria, by an N-terminal [4Fe–4S] cluster that is oxidized to a 2Fe–2S cluster upon exposure to O2, and likely to NO, abolishing its DNA binding capability [13].

The genus Thermus includes hundreds of aerobic or facultatively anaerobic isolates, of which the Thermus thermophilus HB8 and HB27 strains have been extensively used as laboratory models, due to their fast growth and highly efficient natural competence system [14], facilitating the development of a complete genetic toolbox [15]. These two strains are obligate aerobes, and their respiratory complexes are well known, even at the structural level [16–21]. However, many environmental Thermus isolates grow anaerobically by reducing nitrate to nitrite, or by carrying out almost complete denitrification, N2O being the final product [22–24]. Transformation efficiencies of these environmental denitrifying strains is quite low, and therefore, in order to ease the study of the denitrification process, the gene clusters encoding denitrification enzymes were transferred to the T. thermophilus HB27 strain, both by transformation and by “transjugation”, a cell-to-cell transfer mechanism unrelated to common conjugation [23,25,26]. In contrast with the natural denitrifying strains, the derivative T. thermophilus HB27d shows high transformation efficiency, allowing for the generation of directed insertion mutants needed for the analysis of the denitrification pathway in extreme thermophiles.

As observed in the natural donor strains, the genes encoding denitrification in T. thermophilus HB27d localize to a highly variable region of the pTT27 megaplasmid. Nitrate respiration is encoded within a 30-kbp genetic island named the nitrate respiration conjugative element (NCE) [23,27], and includes operons for a nitrate reductase and nitrate/nitrite transporters (narCGHJIKT), signaling, and regulatory proteins (dnrST, drpAB), and a dedicated type II NADH dehydrogenase (nrcDEFN). Nitrite respiration is encoded by the nitrite and nitric oxide respiration (nic) cluster, a 7-kb region separated from the NCE and surrounded by insertion sequences [24]. The nic cluster encodes NirS, a cd1-type nitrite reductase, a protein likely involved in its maturation (NirI), an electron transporter (NirM) [28], a heterotrimeric NO reductase (NorCBH) [29] and putative regulatory genes (nsrRST).

Nitrate respiration in T. thermophilus HB27d also responds to low oxygen and nitrate, but scarcely to nitrite [24]. Although no homologues of FNR or NarX/NarL exist in its genome [24], the sensory-signaling system for nitrate and anoxia in Thermus spp. is genetically linked to the NCE [27,30]. Two proteins (DnrS and DnrT) encoded in a bicistronic operon upstream of the nitrate reductase were identified as essential for the transcription of the nitrate reductase. DnrS is a large cytoplasmic protein with an N-terminal GAF and a C-terminal bacterial transcriptional activator domain (BTAD) domains, which is required for transcriptional activation of the nar and dnr operons, but not for expression of nrc [30]. As oxygen induces conformational changes in DnrS that make it highly susceptible to proteases [30], we hypothesized that it functions as the systemic oxygen sensor, through a yet unknown mechanism related to its GAF domain. However, every attempt to obtain biochemical data on its function, in vitro, has failed, due to difficulties expressing an active form in Escherichia coli. On the other hand, DnrT belongs to the DNR subgroup of the CRP family of transcription activators [31], and contains a C-terminal helix-turn-helix (HTH) DNA binding motif, and a putative N-terminal cyclic nucleotide binding motif, but lacks the cysteine motif required
for incorporation of an iron-sulfur cluster as in FNR. DnrT is insensitive to oxygen, and functions as a transcriptional activator required for the expression of its own operon, and for that of the nar and nrc operons [30]. Though electrophoretic mobility shift assays (EMSA) with DnrT have been unsuccessful, in vitro transcription assays with the nrc operon promoter have revealed that DnrT recruits the *T. thermophilus* RNA polymerase, allowing the identification of its binding site [30].

DnrS and DnrT are not just local regulators acting at the level of the NCE; DnrT also functions as a transcriptional repressor of the chromosomal *nqo* and *fbc* operons, encoding the respiratory complexes I and III, respectively [22,30], consequently controlling the transition from aerobic respiration to denitrification [30]. Moreover, derivative mutants of complete denitrifying strains lacking either DnrS or DnrT are unable to grow with nitrite as electron acceptor, supporting that they are also required for the expression of the nitrite and/or NO reductases [22].

In this work, we study the regulation of the promoters in the *nic* nitrite respiration cluster. Our results demonstrate that DnrS and DnrT, the denitrification master regulators, and a *nic*-encoded local regulator of the NsrR family (named NsrR in *Thermus* as well), act in concert to control the expression of the Nir and Nor reductases in *T. thermophilus*. These data support that genetic regulation of denitrification in the ancient genus *Thermus* spp. significantly differs from that found in modern mesophilic bacteria.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Bacterial strains used in this work are described in Table 1. Aerobic growth of *T. thermophilus* strains was carried out at 70 °C with rotational shaking (Thermos Scientific, MAXQ 420 HP, 150 rpm) in flasks filled up to 1/5 of capacity with *Thermus* broth (TB) [32]. Anaerobic growth was achieved in screw-capped tubes containing 10 mL of TB supplemented with potassium nitrate (20 mM), sodium nitrite (5 mM), or sodium nitroprusside (SNP) (100 μM), and overlaid by mineral oil. *T. thermophilus* colonies were grown aerobically on *Thermus* agar (1.5% w/v agar) plates. For liquid or solid selection, kanamycin (30 mg L⁻¹), hygromycin B (100 mg L⁻¹), and/or bleomycin (15 mg L⁻¹) were added when required.

*E. coli* strain DH5α was used for construction of plasmids, whereas *E. coli* strain BL21(DE3) was used for overexpression and purification of recombinant proteins. *E. coli* was grown at 37 °C in liquid or solid lysogeny broth (LB) media, with kanamycin (30 mg L⁻¹), ampicillin (100 mg L⁻¹), hygromycin B (100 mg L⁻¹), or bleomycin (3 mg L⁻¹) added when required.

| Name              | Genotype/Phenotype | Reference   |
|-------------------|--------------------|-------------|
| DH5α              |                    | [33]        |
|                   | *F-*endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR napG*Φ80 |            |
|                   | lacZΔM15 Δ(lacZYA-argF)U169, hsdR17 (rK- mK+), λ-           | [34]       |
| BL21 (DE3)        | *F-*ompT gal dcm lon hsdSB (rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 |            |
|                   | ind1 sam7 nin5]) |            |
| *T. thermophilus* HB27 | Wild type        | Dr. Koyama |
| *T. thermophilus* HB27d | Denitrifying strain | [23]       |
| HB27d ΔnsrS::kat  | *dnrS* mutant     | This work  |
| HB27d ΔdnrT::kat  | *dnrT* mutant     | This work  |
| HB27d ΔnsrRST::kat| *nsrRST* mutant   | This work  |

2.2. Nucleic Acid Manipulation and Transformation

Primers and plasmids used in this work are listed in Tables 2 and 3, respectively. Total DNA from *T. thermophilus* was purified using a DNeasy Blood and Tissue kit (Qiagen), following a bacterial culture-adapted protocol. Plasmid construction, purification, restriction analysis, and DNA sequencing were performed by standard methods [35]. DNA was amplified by polymerase chain reaction
(PCR) using 1 U mL\(^{-1}\) of DNA polymerase from *Pyrococcus furiosus* (Roche Molecular Biochemicals, Basel, Switzerland) in its recommended buffer with 3 mM MgCl\(_2\), 0.5 mM dNTP mixture, and 50 pmol of each primer (Sigma-Aldrich, St. Louis, MO, USA).

*E. coli* competence was induced following Inoue’s method [36], and transformation was carried out following the method described by Hanahan [33]. Transformation of *T. thermophilus* with linear or circular DNA was achieved by natural competence as described [37].

Table 2. Oligonucleotides used in this work. Underlined sequences correspond to restriction sites included for cloning purposes.

| Name            | Sequence                        | Amplicon/Purpose        |
|-----------------|---------------------------------|-------------------------|
| PnirRXbaIdir    | AAAATCTAGCCAGTCTCACGTCAGGTT     | PnirR/complementation plasmid |
| PnirRNdeIdir    | AAAATCTAGCCAGTCTCACGTCAGGTT     | PnirR/complementation plasmid |
| PnirSbgaA       | AAAATCTAGCCAGTCTCACGTCAGGTT     | PnirR/complementation plasmid |
| PnorSbgaA       | AAAATCTAGCCAGTCTCACGTCAGGTT     | PnirR/complementation plasmid |

Table 3. Plasmids used in this work.

| Plasmid          | Use                     | Reference |
|------------------|-------------------------|-----------|
| pMHbgaA          | Empty promoter probe vector. HygR. | [30]      |
| pMHPnsrRbgaA     | PnsrR promoter probe vector. HygR. | This work |
| pMHPnsrSbgaA     | PnsrS promoter probe vector. HygR. | This work |
| pUC19            | Cloning vector. AmpR     | [38]      |
| pUC19 ΔdnrT::kat | dnrT mutant construction. AmpR, KanR. | This work |
| pUC19 ΔdnrS::kat | dnrS mutant construction. AmpR, KanR. | This work |
| pUC19 nsrRST::kat | nsrRST mutant construction. AmpR. KanR. | This work |
| pMHPnirSbgaA     | PnirS promoter probe vector. HygR. | [28]      |
| pMHPnirJbgaA     | PnirJ promoter probe vector. HygR. | [28]      |
Table 3. Cont.

| Plasmid                | Use                                                                 | Reference                  |
|------------------------|----------------------------------------------------------------------|----------------------------|
| pMHPnorCbgA            | *PnorC* promoter probe vector. HygR.                                 | [39]                       |
| pWUR12/77-1            | Expression vector in *T. thermophilus*. BleoR.                       | [40]                       |
| pWURnsrR               | Complementation of NsrR<sup>Th</sup>. BleoR.                       | This work                  |
| pWURnsrRST             | Complementation of NsrR<sup>Th</sup>ST. BleoR.                      | This work                  |
| pET22b(+)              | Expression vector in *E. coli*. AmpR.                               | Novagen, Merck KGaA, Darmstadt, Germany |
| pET28b(+)              | Expression vector in *E. coli*. KanR.                               | Novagen, Merck KGaA, Darmstadt, Germany |
| pET28 dnrT             | Overexpression of His-DnrT. KanR.                                   | This work                  |
| pET28 nsrR             | Overexpression of NsrR<sup>Th</sup>-His. AmpR.                     | This work                  |
| pET28 nsrS             | Overexpression of His-NsrS. KanR.                                   | This work                  |
| pET28 nsrT             | Overexpression of His-NsrT. KanR.                                   | This work                  |
| pET22 nsrRC93A         | Overexpression of NsrR<sup>C93A</sup>-His. AmpR.                   | This work                  |

2.3. Construction of Deletion Mutants

Plasmid pUC19 [38] was used for the construction Δdnr<sup>T</sup>, Δdnr<sup>S</sup>, and Δnsr<sup>RST</sup> mutants in *T. thermophilus* through double recombination. For this, 500 bp regions upstream and downstream of each target gene were amplified by PCR with the appropriate primers (Table 2), and cloned into pUC19 using the restriction sites included in the primers. A gene cassette devoid of transcription terminator encoding thermostable resistance to kanamycin (*kat*) [41] was inserted between the cloned fragments using the *XbaI* restriction site incorporated in each construct. Downstream orientation of the resistance cassette was selected, in all cases, to allow the expression of downstream genes. Insertional mutants were subsequently obtained by transformation of *T. thermophilus* with linearized derivatives of each constructs, followed by selection on TB plates with kanamycin (30 mg L<sup>−1</sup>); mutations were confirmed by PCR and, when possible, by western blot.

Complementation assays were carried out with plasmid pWUR, in which cloned genes are expressed under the control of the constitutive promoter *PslpA* (*S*-layer promoter) and with thermostable resistance to bleomycin (15 mg L<sup>−1</sup>).

2.4. Promoter Activity Assays

The putative promoter regions that include the 300–500 bp preceding the start codon of the *nsrR* and *nsrS* genes were cloned in the promoter probe plasmid pMHbgaA [30]. Expression from these promoters was assayed on transformed *T. thermophilus* strains in different growth conditions by measuring thermostable β-galactosidase activity. For anaerobic expression, cells were grown aerobically up to exponential phase and then subjected to anaerobic conditions for 16 h in the absence or presence of 20 mM nitrate, 5 mM nitrite, or 100 µM SNP.

β-Galactosidase activity was assayed twice in triplicate experiments on the chromogenic substrate ortho-nitrophenyl-galactopyranoside (ONPG), (Sigma) at 60 °C. Briefly, cells were permeabilized with 0.2% (w/v) SDS for 15 min at 37 °C, followed by addition of the reaction buffer (phosphate 80 mM pH 7.5, containing 0.2% (w/v) ONPG). The reaction was incubated for 20 min at 60 °C in a plate reader, and the absorbance variation was measured at 410 nm. β-Galactosidase activity was expressed as nanomoles of o-nitrophenol produced per min and mg of protein.

2.5. Directed Mutagenesis

QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) was used for the construction of the mutant NsrR<sup>C93A</sup> following manufacturer’s instructions. pET22 nsrR plasmid was used as template for the construction of the mutant. Primers used are described in Table 2. The sequence of the mutant was confirmed.
2.6. Recombinant Protein Overexpression and Purification

The genes encoding transcriptional regulators \textit{dnrT}, \textit{nsrR}, \textit{nsrS}, and \textit{nsrT} were cloned into pET22b or pET28b vectors (Novagen, Merck KGaA, Darmstadt, Germany) for expression in \textit{E. coli} BL21(DE3) cells, with a His-tag fused to the N- or C-terminus. Expression was induced at OD$_{600}$ = 0.4 with 1 mM isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) for 3 h, after which cell pellets were collected and disrupted by French press. His-tagged proteins were purified by affinity chromatography from the soluble cell fraction on TALON CellThru Resin columns following the manufacturer’s instructions (Clontech Laboratories, Inc., Mountain View, CA, USA). Purified proteins were eluted in elution buffer (50 mM phosphate buffer pH 7.0, 300 mM NaCl, 150 mM imidazol) and further dialyzed and concentrated in 50 mM phosphate buffer (pH 7.0) using Amicon Ultra concentrator tubes (3 or 10 kDa cutoff) (Millipore, Burlington, MA, USA). Proteins were visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) following the manufacturer’s instructions. Spectrophotometric analysis of the samples was carried out at room temperature in 0.2 mL cuvettes in a V-730 spectrophotometer (Jasco, Oklahoma City, OK, USA).

2.7. Western Blot

\textit{NarG} (\(\alpha\)-subunit of the Nar), \textit{NirS} (cd$_1$ type Nir), and \textit{NorC} (c subunit of the Nor) were visualized in total cell extracts from nitrate- or nitrite-induced cultures by western blot with specific rabbit antisera [23,32] and horseradish peroxidase-labeled anti-rabbit antibodies; detection was carried out through a electroluminescense (ECL), (Amersham International, Little Chalfont, UK).

2.8. Electrophoretic Mobility Shift Assays

Labeled promoter probes were obtained by PCR amplification of the promoter sequences. A 5′-DY782-labeled reverse oligonucleotide (Eurofins MWG Operon, Louisville, KY, USA) was used in the experiments of Figure 5, Figure S5 and S6. PCR products were separated in agarose gels and bands were purified and concentrated using commercial kits (Qiagen, Hilden, Germany).

Interaction reactions were performed in interaction buffer (20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) pH 6.9, 50 mM NaCl 50 mM, BSA 0.1 mg mL$^{-1}$, 5 mM \(\beta\)-mercaptoethanol) using 25–50 nM labeled DNA and 1:5–1:10 molar ratio of purified transcriptional regulators; 0.5 \(\mu\)g poly(dI–dC) was added as a competitor to reduce non-specific interactions. Reactions were performed for 10 min at 60°C, after which 2 \(\mu\)L glycerol 85% (v/v) was added for gel loading. Six percent EMSA polyacrylamide gels (composition for 10 mL:1 mL 10× EMSA buffer (Tris-Acetate-EDTA (TAE) 50×, 0.5 M EDTA, 0.27% (v/v) acetic acid, pH 7.5), 1.5 mL acrylamide/bisacrylamide 40% (37.5:1), 250 \(\mu\)L 1% (w/v) APS, 20 \(\mu\)L 100% (v/v) tetramethylethlenediamine (TEMED) and water up to 10 mL) were prepared using 1.5 mM glass separators.

Prior to sample loading, gels were pre-run for 20 min at 5 mA in 1× EMSA buffer (see composition above). Electrophoresis was performed at room temperature, protected from the light at 5 mA per gel for approximately 30 min for smaller fragments (100 bp), and up to 3 h for larger fragments (400 bp). Gels were scanned using a LI-COR Odyssey Scanner (LI-COR Biosciences, Lincoln, NE, USA) (excitation 782 nm and emission 800 nm), and analyzed with the Odyssey Infrared Imaging System v.3.0.21 software (LI-COR Biosciences, Lincoln, NE, USA) for those DNA labeled with the DY782 fluorochrome. For unlabeled DNA fragments, staining with SYBR Gold gel staining reagent (Invitrogen, Carlsbad, CA, USA) was used, and the DNA detected at 300 nm.

3. Results

3.1. Genes and Putative Promoters of the Nic Cluster

In previous studies, we have shown that the genes for nitrite and NO respiration in \textit{T. thermophilus} PRQ25 are expressed from the \textit{PnirS}, \textit{PnirJ}, and \textit{PnorC} promoters [28,29] (Figure 1). In addition, the \textit{nic}
The putative promoter responsible for the expression of nsrR is likely located within the 129 bp region that separates its coding sequence from that of the divergent norC gene (Figure 1). The 47 bp that separate the nsrR stop codon from the start codon of nsnS is much longer than the usual distance between co-transcribed genes in the compact genome of T. thermophilus, suggesting the existence of a specific promoter that controls the expression of the NsrS–NsrT proteins. In contrast, the stop codon of nsnS overlaps the start codon of nsnT, strongly supporting nsnS–nslT co-transcription. However, transcripts spanning nsnR, nsnS, and nsnT were undetectable by real-time reverse transcriptase polymerase chain reaction (RT-PCR) (data not shown), suggesting that the three genes are transcribed at very low levels, even under denitrification conditions. To check this, we tested the expression of a thermostable β-galactosidase reporter from the PnsrR and PnsrS putative promoters in a multicopy promoter probe plasmid, both in the aerobic strain HB27 and its denitrifying derivative HB27d. Only the putative PnsrS promoter showed a two-fold increase in expression above the background in the HB27d strain under anoxia plus nitrate growth conditions (Supplementary Figure S2). Low expression levels from the putative PnsrR promoter were confirmed in these assays, which demonstrated the presence of an independent promoter that controls the expression of a nsnST transcript.

3.2. Regulation of the Nitrite and NO Reductases Gene Promoters

The low expression levels from the PnsrR and PnsrS promoters precluded their further analysis; in contrast, the PnorC, PnirS, and PnirJ promoters showed high expression levels using the same promoter probe vectors (Figure 2). Expression from PnirS was almost constitutive in the aerobic strain HB27, and its expression was increased two-fold by nitrate under anoxic conditions in HB27d, consistent with previous observations [28]. Deletion mutants were used to analyze the roles of
the NsrR^{Th}, NsrS, and NsrT proteins, and the DnrS and DnrT regulators in Nir transcription. Nitrate-mediated anaerobic induction was not detected in the absence of DnrS, whereas absence of DnrT had almost no effect on \( P_{\text{nirS}} \) expression (Figure 2). On the other hand, deletion of the \( \text{nsrRST} \) cluster produced around two-fold upregulation of \( P_{\text{nirS}} \), especially under anoxic conditions, which was confirmed at the protein level by Western blot tests against NirS (Figure 3). In this figure, the holo (larger) and apo (smaller) forms of NirS were clearly detected in the \( \Delta \text{nsrRST} \) mutant grown in the absence of nitrate (Figure 3, lane 7), in contrast to the negligible detection in the parental strain under the same conditions (Figure 3, lane 1). Further, the absence of induction in \( P_{\text{nirS}} \) transcription of the \( \Delta \text{dnrS} \) mutant upon growth with nitrate observed in Figure 2 was confirmed by Western blot (Figure 3, lane 4 vs. lane 2), as was the lack of effect of \( \Delta \text{dnrT} \) mutation (Figure 3, lane 6 vs. lane 2). Parallel detection of the nitrate reductase (NarG) encoded within the NCE confirmed its requirement for DnrS but not for DnrT [30], and also independence of NsrR^{Th} (Figure 3, lanes 2, 4, 6, and 8).

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**Figure 2.** Transcriptional activity from the \( \text{nic} \) promoters. (a) Scheme of the promoters assayed; (b) \( \beta \)-galactosidase activity of the strains HB27, HB27d, HB27d \( \text{dnrS}:\text{kat} \) (\( \Delta \text{dnrS} \)), HB27d \( \text{dnrT}:\text{kat} \) (\( \Delta \text{dnrT} \)), and HB27d \( \text{nsrRST}:\text{kat} \) (\( \Delta \text{nsrRST} \)) carrying the promoter probe plasmids, pMHP\( \text{nirS} \text{bgaA} \) (\( P_{\text{nirS}} \)), pMHP\( \text{nirJ} \text{bga} \) (\( P_{\text{nirJ}} \)) or pMHP\( \text{norC} \text{bgaA} \) (\( P_{\text{norC}} \)). Transcriptional activity was measured in aerobic cultures (1) or after induction for 16 h under anaerobic conditions in the absence (2) or presence of 20 mM nitrate (3); 5 mM nitrite (4); or 100 \( \mu \)M SNP (5). \( \beta \)-Galactosidase activity is expressed as nanomoles of o-nitrophenol produced per min and mg of protein. Data represent mean values from triplicate samples in at least two independent experiments; bars indicate standard error.
Figure 3. Expression of denitrification reductases in the regulator mutants. Immunodetection of NarG (α-subunit of the Nar, 136 kDa), NirS (cd1 type Nir, 57 and 48 kDa), and NorC (c subunit of the Nor, 25 kDa) after 16 h under anaerobic conditions in the absence (−) or presence of 20 mM nitrate (+) as electron acceptor. Strains: HB27d, HB27dΔdnrS::kat (ΔdnrS), HB27dΔdnrT::kat (ΔdnrT), and HB27dΔnsrRST::kat (ΔnsrRST).

On the other hand, both the PnirJ and the PnorC promoters behaved in a similar way to each other. As expected, these promoters were not active in the strictly aerobic HB27 strain (Figure 2). However, both were induced by anoxia and nitrate in the denitrifying HB27d strain, consistent with the requirement for specific transcriptional activators encoded within the denitrification clusters. Other nitrogen oxides, such as nitrite or NO (produced from SNP), also led to an increased transcriptional activity, but with lower efficiency than nitrate. For the PnirJ and the PnorC promoters, absence of the DnrS, DnrT, or nsrRST cluster led to complete loss of transcription under all the conditions assayed (Figure 2). Detection of NorC by Western blot confirmed the absence of NorC expression in ΔdnrT mutants (Figure 3, lane 6), and very low expression in ΔdnrS and ΔnsrRST (Figure 3, lanes 4 and 8) mutants, compared to the wild type parental strain. Expression from PnirJ could not be confirmed by Western blot, due to the lack of an available NirJ-specific antibody.

In conclusion, while induction of NirS by nitrate depended only partially on DnrS and was repressed by protein(s) expressed from the nsrRST cluster, expression of NorC and NirJ required both DnrS and DnrT, and at least one of the proteins encoded by the nsrRST cluster, likely NsrRTh.

3.3. NsrRTh is a Transcription Factor

To elucidate the role of NsrRTh in the expression of the nic promoters ΔnsrRST complementation studies were carried out. For this purpose, the nsrR gene and the whole nsrRST cluster were expressed from plasmids pWURNsrR and pWURnsrRST, derived from pWUR112/77-1 [40], a bifunctional E. coli–Thermus vector conferring thermostable bleomycin resistance that permits medium-to-high levels of constitutive expression of the cloned genes. Each plasmid was co-transformed with the promoter probe vectors into the ΔnsrRST mutant, and thermostable β-galactosidase activity was measured under aerobic and anaerobic conditions. As shown in Figure 4, the plasmid expressing NsrRTh produced strong activation of PnirJ and partial activation on PnorC, whereas PnirS was partially repressed. The collective ectopic expression of all three genes (nsrR, nsrS and nsrT) produced a similar effect as NsrRTh alone, although reaching lower activity levels.
Figure 4. Complementation of promoter expression by NsrR<sup>th</sup>. Transcriptional activity of promoters PnirS, PnirJ, and PnorC (positions indicated in (a)) in HB27, HB27d and HB27d ΔnsrRST::kat (ΔRST) strains complemented with plasmids overexpressing NsrR<sup>th</sup> (ΔRST + NsrR), or all three proteins (ΔRST + NsrRST) (b). Transcriptional activity was measured in aerobic cultures (white) or after induction for 16 h under anaerobic conditions with 20 mM nitrate (grey). β-Galactosidase activity is expressed as nanomoles of o-nitrophenol produced per min and mg of protein. Data represent mean values from triplicate samples in at least two independent experiments; bars indicate standard error.

3.4. NsrR<sup>th</sup> Binds to the Main Nic Promoters

The above results support the notion that NsrR<sup>th</sup> can function as a transcriptional activator for the PnirJ and PnorC promoters, and as repressor of PnirS promoter. To determine the ability of NsrR<sup>th</sup> to bind DNA, His-tagged NsrR<sup>th</sup>, NsrS, NsrT, and DnrT proteins were expressed and purified by affinity chromatography; products of expected size were obtained, though NsrT seemed to produce SDS-resistant dimers (Supplementary Figure S3). With these proteins, EMSA assays were carried out using DNA fragments of 417, 344, and 353 base pairs (bp) containing the PnirS, PnirJ, and PnorC putative promoters, respectively. Significant DNA–protein complexes were detected for NsrR<sup>th</sup> in all three promoters, whereas none of the other proteins, including DnrT, had any detectable effect on DNA mobility (Figure 5).
Figure 5. NsrR\textsuperscript{Th} binding to the nic promoters. Promoters PnirS, PnirJ, and PnorC labeled with DY782 were used as probes in electrophoretic mobility shift assays (EMSA) with the indicated purified proteins. Arrows indicate the mobility of specific DNA–protein complexes. Promoter probes (50 nM) were incubated in interaction buffer with each regulator (500 nM) at a 1:10 ratio for 10 min at 60 °C. Lane (−) corresponds to control without protein.

As NsrS and NsrT are always encoded in tandem with NsrR\textsuperscript{Th} in diverse Thermus spp., we searched for the effects of NsrS and NsrT on NsrR\textsuperscript{Th}'s DNA binding. Addition of each protein alone, or in combination, did not affect the binding capacity of NsrR\textsuperscript{Th} to the PnorC promoter under the conditions assayed (Supplementary Figure S5).

3.5. Identification of the Putative Binding Sites of NsrR\textsuperscript{Th}

A sequence analysis revealed the presence of a highly conserved palindromic sequence “CTTGACNGGTCATGG” in those promoters regulated by NsrR\textsuperscript{Th} (Figure 6). This sequence partially overlaps with the binding fingerprint generated by DnrT on the Pnrc promoter [30] (framed in Figure 6a). This sequence localizes upstream of the translation start (−50 for the PnirJ promoter, −73 for the PnorC promoter, −61 for the Pnrc promoter), except for the PnirS promoter, in which it is localized immediately before the identified Shine–Dalgarno sequence (−30). The proximity to the translation start of NirS, and the lower sequence conservation, points out to a repressive effect of NsrR\textsuperscript{Th} on the nirS expression compared to the activation that takes place on the nirJM and the norCBH operons, in agreement with our previous results. Both the PnsrR and the PnsrS promoter regions contain this conserved sequence “CTTGACNGGTCATGG” at a similar distance from the ATG start codon (−72 and −52, respectively) found in the PnirJ promoter, supporting that the nsrRST genes are also regulated by NsrR\textsuperscript{Th}.

To confirm the requirement of this sequence for the binding of NsrR\textsuperscript{Th} to the promoter sequences, we performed EMSA assays with PnorC fragments of different length, with or without the putative NsrR\textsuperscript{Th} binding site (Supplementary Figure S6a). Our results show that addition of NsrR\textsuperscript{Th} did not cause a shift when PnorC fragments lacking the palindromic sequence were used (Supplementary Figure S6b, fragments −56 and −51), thus confirming that NsrR\textsuperscript{Th} binds to this conserved sequence.
4. Discussion

In this article, we show that the expression of the nitrite reductase and the nitric oxide reductases of the denitrification pathway of *T. thermophilus* depends on the oxygen sensitive global regulator DnrS and on the local regulator NsrR\textsubscript{Th}, which in contrast to homologues in other bacteria, functions both as repressor and as activator of specific denitrification promoters.

4.1. DnrS and DnrT as Master Regulators of Denitrification.

In nitrate-respiring isolates of *T. thermophilus*, the master regulators DnrS and DnrT are required for the expression of the nitrate reductase, and consequently, deletion mutants in either of these proteins prevented anaerobic growth with nitrate [30]. In denitrifying isolates of the same species, the deletion of these regulators also prevents anaerobic growth with nitrite and NO [22], supporting that both proteins are involved in the control of the expression of the genes encoding the nitrite and the nitric oxide reductases in their natural host. Here, we analyze this hierarchy of control in a formerly aerobic surrogate host that has received the adaptive NCE and nic clusters by consecutive transformation events [23]. In this host, de novo adapted to an anaerobic lifestyle, we demonstrate the role of these NCE-encoded transcription factors as master regulators of the promoters in the nic cluster, thus showing the hierarchical dominance of the NCE over the nic cluster. Ultimately, we show that the transcription factor NsrR\textsubscript{Th} regulates the nic cluster subordinated to the NCE master regulators.

Under aerobic conditions, the nitrite reductase gene is transcribed from the *PnirS* promoter at basal levels, whereas anaerobic incubation in nitrate-containing media produces a DnrS-dependent two-fold induction (Figure 2). In contrast, DnrT seems to play no role in NirS expression (Figures 2 and 3). In addition, our data support the involvement of a transcriptional repressor encoded within the *nsrRST* cluster in the control of *PnirS*, as mutants lacking this cluster show upregulation of *PnirS* transcription and NirS expression under anoxia, independently of the presence or absence of nitrogen oxides (Figures 2 and 3).

In contrast, transcription from the *PnirJ* and *PnorC* promoters is not induced at all by anoxia, except when nitrogen oxides are present (nitrate > nitrite > NO). Such anoxia plus nitrogen oxide activation requires both DnrS and DnrT, and at least one of the proteins encoded by the *nsrRST* cluster (Figure 2). Their similar behavior suggests that both *PnirJ* and *PnorC* promoters are co-regulated in an analogous way, with at least three transcription activators working on them. However, a more
simplistic alternative could involve a dependence effect between these putative activators affecting their own expression. Actually, DnsS mutants (ΔdnrS::kat) express DnrT in a constitutive form, and therefore, absence of transcription from PnorC and PnirJ promoters implies a direct effect of DnrS. In contrast, DnrT mutants (ΔdnrT::kat) do not express DnrS at all [30], making it impossible to discriminate if the detected effects on PnorC and PnirJ are due to DnrT or to DnrS absence. In any case, both promoters contain sequences that partially overlap with the binding fingerprint generated by DnrT on the Pnrc promoter [30] (framed in Figure 6), supporting the existence of a direct binding of DnrT also on PnorC and PnirJ.

4.2. The Role of NsrRTh

The conserved nsrcst cluster is proximal to the Nir and Nor reductase genes in different Thermus spp., such as T. thermophilus SG0.5P17-16 and T. scotoductus SA01. Mutants of the HB27d strain lacking the nsrcst cluster used in this work were unable to grow anaerobically with nitrite (data not shown), consistent with the lack of transcription from the PnorC and PnirJ promoters. Of the three hypothetical proteins encoded in the cluster, only NsrRTh shows a canonical DNA binding motif and in vitro binding capability to the PnirS, PnirJ, and PnorC promoters in EMSA assays (Figure 5). In addition to this DNA binding motif, NsrRTh contains three cysteines conserved in NsrR homologs from other bacteria, where they coordinate a [4Fe–4S] iron–sulfur cluster that functions as NO sensor [13], with a conserved fourth ligand absent in NsrRTh. The presence of an iron–sulfur cluster in NsrRTh has been demonstrated (Figure S4), suggesting that another glutamatic residue in the vicinity (i.e., E82) could constitute the fourth ligand for its iron–sulfur cluster.

Our promoter assays suggest that, under denitrification conditions, NsrRTh represses PnirS, whereas it induces PnirJ and PnorC (Figure 4). Our EMSA assays actually show binding capability of NsrRTh to all the nic promoters. It remains to be analyzed whether the dual activator/repressor role of NsrRTh can be modulated by the oxidation/reduction state of its iron–sulfur cluster. Since EMSA assays were carried out under aerobic conditions, it is likely that the NsrRTh recombinant protein used was actually a partially oxidized form of the protein, already able to bind DNA.

Another important factor concerning the regulation of these promoters is the location of the putative NsrRTh binding site, because most probably it can determine the outcome of the regulation in each specific promoter context, that being induction of the PnirJ and PnorC promoters, or repression of the PnirS promoter (Figure 6). The localization of this sequence, close to the translation start in the PnirS promoter and further upstream in the PnirJ and PnorC promoters, agrees with the role of NsrRTh as repressor of PnirS and activator of PnirJ and PnorC, deduced from our in vivo assays.

4.3. The Role of NsrS and NsrT

The PnrsS promoter region contains the conserved sequence “CTTGACCNGGTCATGG” at a similar distance from the ATG start codon found in the PnirJ and PnorC promoters, supporting that the NsrS and NsrT proteins are also regulated by NsrRTh. However, the role of these proteins remains to be elucidated. There is no doubt about their involvement in denitrification both from their sequence conservation and from their clustering downstream of nsrc homologues in denitrifying Thermus spp. A search for homologues in GenBank revealed a ScdA domain (PF04405) within NsrS, taking its name from the protein ScdA of Staphylococcus aureus, also present in proteins YfE of E. coli and DnrN of Neisseria gonorrhoeae, which are both described as di-iron proteins involved in the repair of iron–sulfur clusters or repair of iron centers (RIC) [47]. Mutations in these RIC proteins in model organisms produce pleiotropic effects when subjected to NO and other oxidative stresses. Interestingly, RIC proteins are upregulated by NO [50], and their coding genes are usually located immediately upstream or downstream of regulatory proteins of the NsrR family in denitrifying bacteria, such as Ralstonia eutropha, Ralstonia solanacerarum, Hahella chejuensis, and Anaeromyxobacter dehalogenans, supporting a similar role for all of them. In fact, a detailed analysis of NsrS showed sequence homology with the N-terminal region of RIC proteins, including a highly conserved DfCCgG motif of unknown
function [50]. However, most RIC proteins are much larger (around 220 aa) than NsrS (65 aa), and their C-terminal domains are involved in binding of a non-heme bi-nuclear iron center. We hypothesize that the cupin-2 domain of the NsrT protein, which also contains two cysteine residues, could play a role in iron binding, similar to that of the C-terminal domain of the RIC proteins. Overall, these data suggest a joint role for the NsrS and NsrT proteins, likely co-translated in 1:1 stoichiometry, in the repair of the iron–sulfur center of NsrR<sub>Th</sub>, in a similar way to the role played by the RIC proteins of other bacteria. In any case, further investigations are required to determine the specific role of NsrS and NsrT in denitrification.

4.4. A Regulatory Model for Nitrite Respiration

A complex regulatory network of the nic cluster can be depicted in <i>T. thermophilus</i> (Figure 7). In natural denitrifying strains, the denitrification reductases initiate a cascade of reactions that lead to the subsequent reduction of nitrate, nitrite, and NO, leading to the final production of N<sub>2</sub>O. In this process, reduction of nitrite to NO has to be immediately followed by further reduction to N<sub>2</sub>O, given the toxic effects of NO accumulation. Therefore, a tight regulation is required. As a first control level, the nic cluster cannot be expressed in the absence of the NCE, because DnrS and DnrT are master regulators required to pre-activate the nitrite respiration promoters upon oxygen depletion. However, transcription of nirS is semi-constitutive, except for a two-fold enhancement that depends on DnrS, but not on DnrT. This fact could lead to the production of NO up to toxic levels, even in strains that only have the nic cluster by lateral gene transfer, so preventive repressor systems should be in place. According to our model, NsrR<sub>Th</sub> could constitute such a security mechanism in which reaction with NO excess allows binding to the PnirS promoter with subsequent reduction in NO production by Nir. Concomitantly, NsrR<sub>Th</sub> would also activate the production of Nor through binding to its gene promoter and decreasing the NO levels in the cell. Finally, the other two proteins encoded along with NsrR<sub>Th</sub>, NsrS and NsrT, could play a key role in restoring the activity of the NsrR<sub>Th</sub> iron–sulfur cluster, returning the system to its basal level.

![Figure 7](image_url)

**Figure 7.** The regulatory network of the nic cluster. DnrS and DnrT encoded by the nitrate respiration cluster play a major role in the control of the denitrification pathway, being required for transcription of nitric oxide (NO) reductase and also of the nir<sub>J</sub> gene. The transcription of nirS is semi-constitutive except for a two-fold enhancement that depends on DnrS but not on DnrT. Activation (+) and repression (−) are indicated. Dashed arrows indicate a putative indirect effect of DnrT and DnrS. NsrR<sub>Th</sub> is a transcription factor required for the expression of the nic operons. NsrR<sub>Th</sub> binds to the regulated promoters, activating NO reduction by Nor and limiting its production by NirS. The putative activity of NsrS and NsrT in the repair of the iron–sulfur cluster of NsrR<sub>Th</sub> remains to be elucidated.
Supplementary Materials: The following are available online at www.mdpi.com/2073-4425/8/12/361/s1. Figure S1: Sequence alignment of NsrR<sup>Th</sup> with NsrR family members; Figure S2: Transcriptional activity from the putative promoters of the nsrK, nsrS and nsrT genes; Figure S3: Production of recombinant His-tagged proteins; Figure S4: Production and spectroscopic analysis of NsrR<sup>Th</sup> and its C93A mutant; Figure S5: Effects of NsrT and NsrS on the binding of NsrR<sup>Th</sup> to the PnorC promoter; Figure S6: NsrR<sup>Th</sup> binds to a conserved palindromic sequence.

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