

# Control of the respiratory metabolism of *Thermus thermophilus* by the nitrate respiration conjugative element NCE

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## Summary

The strains of *Thermus thermophilus* that contain the nitrate respiration conjugative element (NCE) replace their aerobic respiratory chain by an anaerobic counterpart made of the Nrc-NADH dehydrogenase and the Nar-nitrate reductase in response to nitrate and oxygen depletion. This replacement depends on DnrS and DnrT, two homologues to sensory transcription factors encoded in a bicistronic operon by the NCE. DnrS is an oxygen-sensitive protein required *in vivo* to activate transcription on its own *dnr* promoter and on that of the *nar* operon, but not required for the expression of the *nrc* operon. In contrast, DnrT is required for the transcription of these three operons and also for the repression of *nqo*, the operon that encodes the major respiratory NADH dehydrogenase expressed during aerobic growth. Thermophilic *in vitro* assays revealed that low DnrT concentrations allows the recruitment of the *T. thermophilus* RNA polymerase  $\sigma^A$  holoenzyme to the *nrc* promoter and its transcription, whereas higher DnrT concentrations are required to repress transcription on the *nqo* promoter. In conclusion, our data show a complex auto-inducible mechanism by which DnrT functions as the transcriptional switch that allows the NCE to take the control of the respiratory metabolism of its host during adaptation to anaerobic growth.

## Introduction

In many prokaryotes, nitrogen oxides substitute for oxygen during denitrification, a series of four consecutive steps ( $\text{NO}_3^-$ :  $\text{NO}_2^-$ :  $\text{NO}$ :  $\text{N}_2\text{O}$ :  $\text{N}_2$ ) catalysed by the respective reductases (Zumft, 1997; Richardson *et al.*, 2001). For a few of them, a hierarchical regulatory mechanism has been shown that co-ordinates the expression of each of these reductases in response to two specific environmental signals: (i) oxygen depletion and (ii) presence of the appropriate nitrogen oxide. In most denitrifiers, oxygen depletion is detected through a [4Fe–4S] iron–sulphur redox centre located at the N-terminal domain of a CRP (cAMP receptor protein)-like transcription factor, similar to the *Escherichia coli* FNR (fumarate and nitrate reductase regulatory protein) (Korner *et al.*, 2003). In the presence of oxygen, the iron–sulphur centre is oxidized, causing the factor to monomerize and become inactive (Crack *et al.*, 2004). In the absence of oxygen, the reduction of the [4Fe–4S] centre leads to protein dimerization through a central  $\alpha$ -helix, allowing the protein to bind to a palindromic sequence known as anaerobox through its C-terminal helix–turn–helix (HTH) motif (Korner *et al.*, 2003). Also, there are examples of FNR and other CRP-like factors acting as transcriptional repressors at specific promoters (Busby and Ebright, 1999; Korner *et al.*, 2003).

Additionally, the presence of an appropriate nitrogen oxide must be detected by specific sensory proteins. Nitrate is usually detected by a membrane sensor (NarX) that phosphorylates a response regulator (NarL), which then binds to specific sequences upstream from the transcription start site of the corresponding nitrate reductase operons (Stewart, 1993; 2003). However, in organisms like *Paracoccus pantotrophus*, which lacks NarL homologues, a member of the CRP family of regulators named NarR detects nitrate through a yet unknown mechanism (Wood *et al.*, 2001). Interestingly, the other main signal in denitrification, nitric oxide (NO), is also detected through a subgroup of the CRP family known as Dnr (Vollack *et al.*, 1999; Korner *et al.*, 2003).

The genus *Thermus* sp. belongs to one of the oldest lineages of the bacterial phylogenetic tree (Hartmann *et al.*, 1989), and most of its isolates show an overall aerobic nature. The extreme thermophilic *Thermus*

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*thermophilus* shows the same overall aerobic nature of the genus, but there are isolates of this species that carry out the first step of the denitrification pathway, growing anaerobically with nitrate and excreting nitrite as the end-product (Ramirez-Arcos *et al.*, 1998a), and even others that fulfil a complete denitrification, reducing nitrite to N<sub>2</sub> as final product (Rainey and da Costa, 2001). This ability is encoded within a > 30 kb DNA fragment that can be transferred by conjugation to aerobic strains of the same species through an Hfr-like mechanism, allowing the new hosts to grow as facultative anaerobes (Ramirez-Arcos *et al.*, 1998b). This nitrate respiration conjugative element (NCE) encodes two main operons, named *nar* and *nrc*. The *nar* operon codes for a thermophilic membrane nitrate reductase (Nar), which contains a periplasmic cytochrome *c* as a fourth subunit in addition to homologues to the NarG, NarH and NarI enzyme subunits found in other bacteria (Zafra *et al.*, 2005). The *nrc* operon codes for a new class of respiratory NADH dehydrogenase (NDH) made of four subunits, in contrast to the 14- to 16-meric multimer that constitutes the type I NDH, or to the monomeric type II NDH found in most bacteria (Cava *et al.*, 2004). Phylogenetic comparisons of the amino acid sequences of Nar and Nrc subunits are in agreement with the 16S RNA-based phylogeny of the genus, and also suggest an ancient origin for both enzymes (Philippot, 2002; Cava *et al.*, 2004). When a NCE-carrying *T. thermophilus* strain grows in a nitrate-rich medium under laboratory conditions, depletion of oxygen either due to consumption by the increasing population or experimentally induced leads to the apparently simultaneous induction of the *nar* and *nrc* operons (Ramirez-Arcos *et al.*, 1998a; Cava *et al.*, 2004). Interestingly, transcription of the *nqo* operon, which encodes the aerobic type I NDH of the host, is concomitantly repressed (Cava *et al.*, 2004). Therefore, the *T. thermophilus* strains carrying NCE replace not just the final electron acceptor enzyme, but the whole respiratory chain, during transition to anaerobic growth with nitrate. In contrast, menaquinone-8 is common to both respiratory processes (Cava *et al.*, 2004).

In a previous work we observed that the nitrate- and anoxia-dependent transcription of the *nar* operon promoter required the presence of the NCE (Moreno *et al.*, 2003), and therefore we concluded that the regulatory factors required for the respiratory chain replacement in response to these signals were encoded within this transferable element. Here we identify a bicistronic operon (*dnr*) in the NCE that encodes DnrS and DnrT (RegA and RegB in Cava and Berenguer, 2006), two homologues to bacterial transcription and sensory factors. The development of new promoter-probe and expression vectors allow us to show here that both proteins are required for the expression of the operons of the NCE when the cells

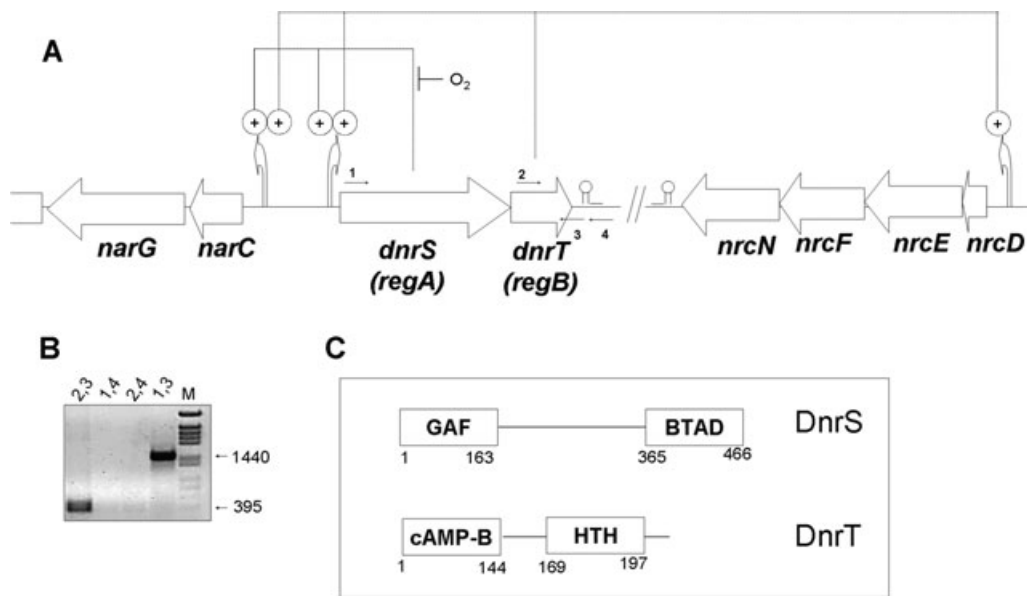
grow in a medium with nitrate under low oxygen concentrations. We find that DnrS is an oxygen-sensitive protein required for the expression of the *nar* and *dnr* operons under anaerobic conditions, whereas DnrT is the central regulatory factor required not only to activate the transcription from all the NCE operons but also to repress the transcription from the *nqo* operon. To our knowledge, this is the first time in which a single thermophilic transcription factor is shown to activate and to repress *in vitro* transcription by the RNA polymerase of *T. thermophilus* on two different promoters. Its dual role as transcription activator of the NCE operons and as repressor of the chromosomally encoded Nqo points to DnrT as the central switching mechanism by which the NCE controls the respiratory metabolism of its host during adaptation to anaerobic growth.

## Results

### *NCE encodes two homologues of bacterial transcription factors*

Sequence analysis of the NCE revealed the presence of two genes, preliminarily named *regA* and *regB* (Cava and Berenguer, 2006), encoded in the DNA region that separates the *nar* and the *nrc* operons (EMBL Accession No. AM161043) (Fig. 1A). The proximity of the coding sequences of both genes (4 bp) and the presence of a Rho-independent transcription terminator downstream of the translation stop codon of *regB* suggested that they form a bicistronic operon. This was confirmed by reverse transcription polymerase chain reaction (RT-PCR) assays on RNA isolated from cells incubated for 4 h under anoxic conditions with nitrate (*Experimental procedures*), in which the expected 1440 bp amplification fragment was obtained with forward primer 1, specific for *regA*, and reverse primer 3, specific for *regB* (Fig. 1B). In contrast, the use of reverse primer 4, which hybridizes downstream the putative transcription terminator, was unsuccessful.

The amino acid sequence motifs and main similarities to proteins from the databank of the hypothetical proteins encoded by *regA* and *regB* are also shown in Fig. 1. The *regA* gene encodes a 467-amino-acid-long protein, with a theoretical size of 52.6 kDa. A search for sequence motifs at the NCBI server revealed the presence of an N-terminal (positions 1–120) GAF (cGMP-specific and -stimulated phosphodiesterases, *Anabaena* adenylate cyclases and *E. coli* FhlA) domain usually involved in signal detection in several proteins (Aravind and Ponting, 1997; Galperin, 2004). There is an additional BTAD domain (bacterial transcriptional activator domain) shared by all the members of the DNRI/REDD/AFSR family of transcription regulators involved in secondary metabolism in *Streptomyces* sp. and related Actinobacteria (Yeats *et al.*,



**Fig. 1.** The *dnr* operon.

A. Scheme of the *dnr* operon, and the relative position of the *nrc* and part of the *nar* operons that surrounds it. Dotted lines on the top indicate the roles of DnrS and DnrT as transcription activators of the indicated NCE promoters shown in this article.

B. RT-PCR assay with the indicated primers pairs on total RNA isolated from cells grown for 4 h under nitrate/anoxia conditions. Primers used were regANde1 (1), o27-77 (2), dnrTstop (3) and o27-81 (4), and their approximate annealing positions are indicated in (A).

C. Scheme of the positions of the domains identified in DnrS and DnrT.

Details are described in the text.

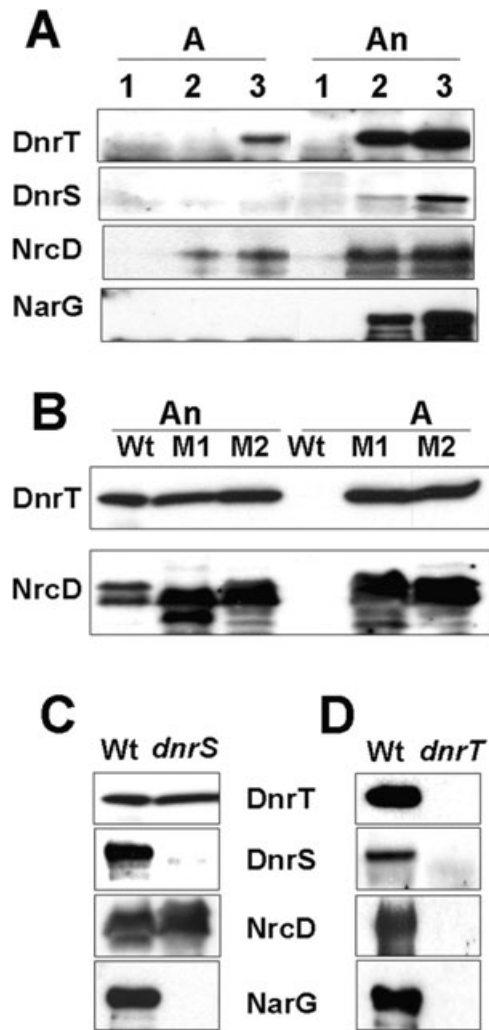
2003). This domain structure suggests a putative role as a signal-sensitive transcription factor for the hypothetical RegA protein. This protein will be named DnrS thereafter to avoid confusion with proteins from other microorganisms.

On the other hand, *regB* encodes a 217-amino-acid-long protein with a theoretical size of 24.8 kDa. Its coding sequence starts with a GTG codon. Sequence comparisons revealed that RegB belongs to the CRP family of transcription activators, sharing the C-terminal HTH DNA-binding motif common to all the family members, and also showing an N-terminal nucleotide-binding motif, like the CRP protein. More precisely, the sequence alignment with members of this family (Fig. S1) revealed the highest percentages of identity with members of the Dnr subgroup of this CRP family, most of which are implicated in signal transduction of NO (DnrE, DnrD) or nitrate (NarR) presence in denitrifying Proteobacteria (Korner *et al.*, 2003). Due to these similarities and to the role in nitrate respiration that we describe in this article, we renamed *regB* as *dnrT*. As all its closest homologues, DnrT lacks the four cysteine residues that in members of the CRP family, like the FNR protein, are responsible for the detection of oxygen through a 4Fe-4S cluster. Interestingly, the sequence of the HTH motif of DnrT keeps the highly conserved residues E182, S185 and R186, which interact directly with the DNA sequence target in most proteins of the CRP family (Korner *et al.*, 2003) (Fig. S1).

#### *DnrS* and *DnrT* are induced in *T. thermophilus* during anaerobic respiration

To be sure that hypothetical DnrS and DnrT proteins were actually synthesized in *T. thermophilus*, their respective coding genes were cloned, overexpressed in *E. coli*, and specific polyclonal rabbit antisera were prepared for each of them (*Experimental procedures*). These antisera showed similar sensitivities for their respective proteins in parallel Western blot assays (Fig. S2A), thus making it possible to compare DnrS and DnrT amounts by their respective signals.

As shown in Fig. 2A, specific signals for proteins of the sizes expected for DnrS and DnrT were immunodetected in the soluble fraction of the facultative wild-type strain NAR1 (lane 3) treated for 4 h under anoxic conditions with nitrate (An conditions). Parallel immunodetection of NarG (Ramirez-Arcos *et al.*, 1998a) and NrcD (Cava *et al.*, 2004) were carried out on the corresponding membrane fractions to confirm the expression of the Nar and Nrc respiratory enzymes under these conditions. As expected, none of these proteins (DnrT, DnrS, NrcD and NarG) were detected in the aerobic strain *T. thermophilus* HB27 (lane 1) subjected to the same treatment, but they did in its HB27c derivative (lane 2) that carries the NCE element (Ramirez-Arcos *et al.*, 1998b). Interestingly, when the facultative wild-type strain was grown aerobically (A), a low amount of DnrT was detected by Western blot (lane 3),



**Fig. 2.** Requirement of the *dnr* operon genes for the expression of the Nar and Nrc respiratory complexes.

A. Total proteins from the *T. thermophilus* strains HB27 (1), HB27c (2) and NAR1 (3), grown either aerobically (A) or after 4 h under nitrate/anoxia conditions (An), were subjected to parallel Western blots to detect DnrT, DnrS, NrcD and NarG.

B. Western blots showing DnrT and NrcD were carried out on cultures of the NAR1 wild-type strain (Wt) and in two independent *dnrS::kat* null mutants (M1 and M2) grown either aerobically (A) or treated for 4 h under nitrate/anoxia-inducing conditions (An). Note the constitutive expression of DnrT and NrcD under aerobic conditions in the mutants.

C. Western blots to detect DnrT, DnrS, NrcD and NarG on nitrate/anoxia induced cultures of the wild-type NAR1 strain (Wt) or of a *dnrS::kat* null mutant (*dnrS*).

D. Western blots to detect DnrT, DnrS, NrcD and NarG were carried out as in (C) on the wild-type strain and on the *dnrT* null mutant.

and longer exposure times allowed its detection also in the HB27c strain. Detection of NrcD followed the same pattern. In contrast, detection of DnrS under aerobic conditions was not evident unless larger protein amounts and longer exposure times were used. The final conclusion from these experiments was that DnrT and DnrS are not

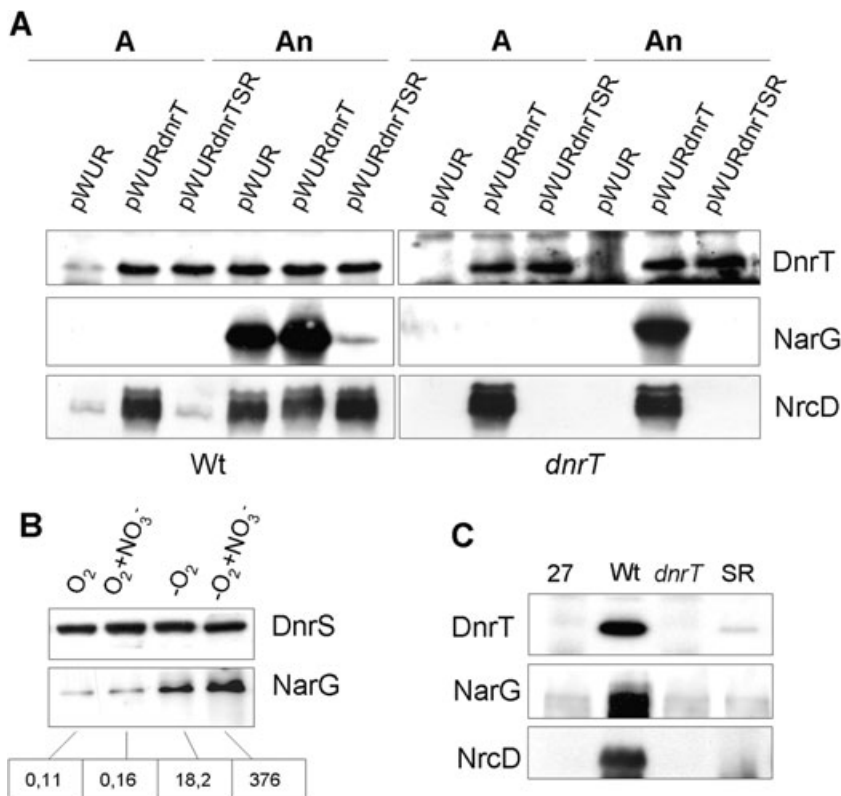
hypothetical but actual proteins, which are expressed in *T. thermophilus* at low levels during aerobic growth but induced during anaerobic growth with nitrate. Having in mind that the respective antisera show similar sensitivities for immunodetection of both proteins by Western blot (Fig. S2A), and that the two proteins are translated from a single mRNA (Fig. 1B), these data suggest for DnrS either lower translation efficiency or a less stable character than for its co-expressed DnrT counterpart.

#### *DnrS* and *DnrT* are required for anaerobic growth

Null mutants of *dnrT* and *dnrS* were isolated (*Experimental procedures*). Due to their operon nature and to the absence of a transcription terminator at the end of the *kat* gene, the DnrT protein was constitutively expressed in two independent *dnrS::kat* mutants (Fig. 2B, lanes M1 and M2), in such a way that similar levels of DnrT were detected in cultures grown aerobically (A) as in those treated for 4 h with nitrate under anoxic conditions (An). By comparison, the DnrT protein was expressed by the wild-type strain under aerobic conditions at such a low level that it was not detected under the exposure time shown in this figure. On the other hand, no polar effect of the *dnrT* null mutation on the expression of the DnrS protein was expected. In fact, expression of DnrS required the presence of DnrT (see below).

Parallel growth experiments revealed that neither the *dnrS* nor the *dnrT* null mutant was able to grow under complete anaerobic conditions with nitrate (not shown). As all the *nrc::kat* null mutants are still able to grow anaerobically with nitrate (Cava *et al.*, 2004), the inability of the *dnrS* and the *dnrT* null mutants to grow under such conditions suggests that the expression of DnrT and DnrS is required for the synthesis of the nitrate reductase, the only component of the respiratory machinery which is fully required for anaerobic growth (Ramirez-Arcos *et al.*, 1998a). This was confirmed by the results of Fig. 2C and D, which show that NarG was not expressed in any of the mutants under inducing conditions. However, there was a clear difference between both mutants with respect to the expression of NrcD: whereas the *dnrT* null mutant was unable to express it (Fig. 2D), in the *dnrS::kat* mutant the NrcD protein was expressed anaerobically (Fig. 2C) and even during the aerobic growth (Fig. 2B). It is then noteworthy that the expression of NrcD is concomitant to that of DnrT, which is expressed constitutively in *dnrS::kat* mutants. A final conclusion from these assays was that the DnrS protein was not expressed in the *dnrT* null mutant, supporting that DnrT is also required for the expression of its own operon (Fig. 2D).

To confirm these results, complementation experiments of each mutant were carried out. For this we used pWUR112/77-1 (pWUR thereafter) derivatives, which



confer a thermostable resistance to Bleomycin (Brouns *et al.*, 2005) that is compatible with the resistance to Kanamycin of the *dnrS* and *dnrT* null mutants (this work). As shown in Fig. 3A, transformation of the wild-type strain or the *dnrT* null mutant with pWURdnrT results in constitutive expression of DnrT during aerobic growth (A). Concomitant to the constitutive synthesis of DnrT, the NrcD protein is strongly expressed. In contrast, constitutive expression of DnrT does not guarantee the expression of NarG in the wild type or in the *dnrT* null mutant during aerobic growth, and its synthesis was still dependent on oxygen depletion and nitrate presence (An).

On the other hand, constitutive expression of DnrS from pWURdnrS in a *dnrS::kat* genetic background, in which DnrT is also constitutively expressed (Fig. S2B), does not result in the expression of significant amounts of NarG under aerobic conditions (Fig. 3B, lane O<sub>2</sub>). However, it is interesting to note the existence of a partial expression of NarG under anoxic conditions in the absence of nitrate when both, the DnrT and the DnrS proteins, are constitutively expressed (Fig. 3B, lane -O<sub>2</sub>). Nevertheless, full expression of NarG still requires nitrate in addition to anoxia.

These results allowed us to conclude that: (i) DnrT is required for the synthesis of NarG, NrcD and DnrS, (ii) the DnrT-dependent activation of the NrcD synthesis is insensitive to oxygen and does not require nitrate, (iii) DnrS is

**Fig. 3.** Constitutive expression of DnrS and DnrT, and the role of the HTH motif of DnrT. A. Western blots to detect DnrT, NarG and NrcD were carried out on aerobic (A) or on nitrate/anoxia-induced (An) cultures of the NAR1 (Wt) and the *dnrT* null strains (*dnrT*) transformed, either with the pWUR control plasmid, or with its derivatives that express the wild-type DnrT (pWURdnrT) protein or its SR/AL mutant (pWURdnrTSR).

B. Cultures of the *dnrS::kat* null mutant transformed with plasmid pWURdnrS were grown aerobically, or incubated overnight under anoxic conditions in the presence or in the absence of nitrate (40 mM) before immunodetection of DnrS and NarG. The nitrate reductase activity of the cultures was also measured (nmol of nitrite produced per minute and per milligram of protein).

C. Detection of the indicated proteins on nitrate/anoxia-treated cultures of the aerobic HB27 strain (27), the facultative NAR1 strain (Wt), the *dnrT* null mutant, and a mutant in which the *dnrT* gene was replaced by that encoding the SR/AL DnrT mutant (SR).

required for the expression of NarG but not for that of NrcD, and (iv) expression of NarG is still dependent on nitrate and anoxia, although DnrT and DnrS were present.

#### *The HTH motif of DnrT is required to activate the expression of the three NCE operons*

In order to analyse *in vivo* if this activator role of DnrT on the synthesis of the NarG, NrcD and DnrS was related to its hypothetical ability to bind to the respective gene promoters, the complementation experiment of the *dnrT* null mutant was repeated with a DnrT site-directed mutant in which two highly conserved residues of its putative HTH motif (S185 and R186) were changed by A and L respectively (SR/AL mutant thereafter). Figure 3A shows how the constitutive expression of this SR/AL DnrT protein from plasmid pWURdnrTSR does not allow the synthesis of NarG or NrcD in a *dnrT* null mutant background. As the amount of the SR/AL mutant protein detected by Western blot in cells transformed with pWURdnrTSR was similar to the amount of wild-type DnrT observed in cells transformed with pWURdnrT, the differences in their effects on the expression of NarG and NrcD were necessarily the consequence of a mutation-associated function loss, and not due to putative differences in their stability. Thus, the DnrT-dependent synthesis of NarG and NrcD is most likely a consequence of the transcription activation of the

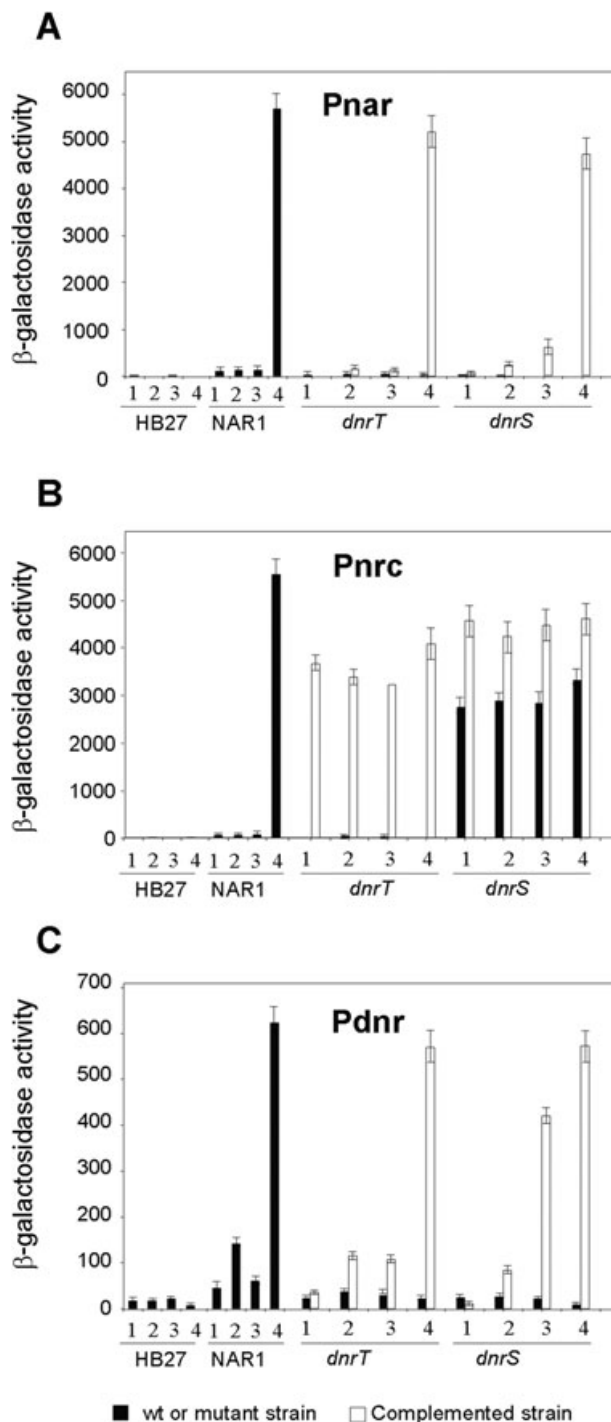
respective gene promoters after the binding of DnrT through its HTH motif. It is also noteworthy that the expression of the SR/AL mutant from pWURdnrTSR interferes with the synthesis of NarG under nitrate and anoxia in the wild-type strain (Fig. 3A).

The experiments above supported the hypothesis that the activating effect of DnrT on Pnar and Pnrc was dependent on its HTH motif, but did not inform us about the putative role of DnrT on its own expression. To analyse this, the chromosomal wild-type *dnrT* gene was replaced by the mutant one encoding the SR/AL DnrT protein (*Experimental procedures*). The results of Fig. 3C show that neither the DnrT SR/AL mutant protein nor the NarG or the NrcD protein was induced under conditions in which the three proteins were expressed in the wild-type strain. Consequently, DnrT most likely acts as a transcription activator by binding through its HTH motif to the promoters of the *nar*, *nrc* and *dnr* operons.

#### Effects of DnrS and DnrT on the activity of the Pnar, Pnrc and Pdnr promoters

To analyse and to quantify the putative effect of DnrS and DnrT on the transcription of the *nar*, *nrc* and *dnr* operons, the promoter region of each of them was cloned upstream of a reporter gene (*bgaA*) encoding a thermostable beta-galactosidase (*Experimental procedures*). The constructs were then inserted into pMH184, a new cloning vector that confers a thermostable resistance to Hygromycin B (Nakamura *et al.*, 2005; this work), which is compatible with the thermostable resistances to Kanamycin and Bleomycin. In this way, it was possible to quantify the beta-galactosidase activity expressed from each promoter even in Kanamycin-resistant mutants (*dnrT* and *dnrS*) transformed with plasmids conferring Bleomycin resistance (pWUR-derivatives). The beta-galactosidase activities were measured under four different conditions: presence or absence of oxygen, and presence or absence of nitrate. The results are shown in the Fig. 4.

As expected, none of these promoters were expressed under any condition in the aerobic strain *T. thermophilus* HB27, which lacks the NCE (< 20 units). In contrast, in the wild-type facultative strain (NAR1), the Pnar and the Pnrc promoters were strongly induced under anoxic conditions with nitrate ( $5\text{--}6 \times 10^3$  units), whereas the Pdnr promoter was induced at a much weaker level ( $6\text{--}7 \times 10^2$  units), as it could be expected for genes encoding regulatory proteins. These data represents for Pnar, Pnrc and Pdnr, a 56-fold, 97-fold and 14-fold induction of their respective basal levels (40–100 units) under aerobic growth without nitrate. Neither nitrate nor anoxia was able to induce the Pnar or Pnrc promoters independently in a significant way (less than 2-fold), although a slight expression of Pdnr (3- to 4-fold) was observed with nitrate alone (Fig. 4C). The



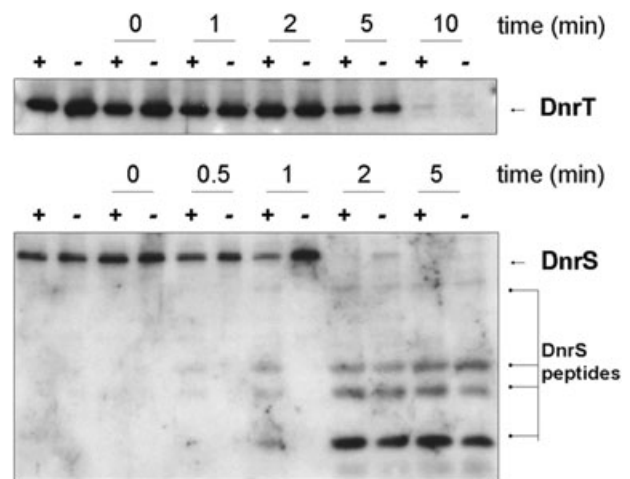
**Fig. 4.** Quantification of the transcriptional activity of the *nar*, *nrc* and *dnr* operon promoters. The indicated strains carrying the promoter-probe plasmids pMHnarbgaA (A), pMHnrcbgaA (B) or pMHdnrbgaA (C) were transformed either with pWUR (black bars) or with a pWUR derivative that complemented the strains by expressing the corresponding DnrS or DnrT proteins constitutively (white bars). Then the beta-galactosidase activity of cultures grown aerobically without (1), or with nitrate (2), or anaerobically without (3) or with nitrate (4) was measured (*Experimental procedures*). The represented data correspond to the mean values of three independent experiments.

results also showed that none of these promoters was expressed in a *dnrT* null mutant, reinforcing the view of DnrT as a central transcription activator during nitrate respiration. We also observed that constitutive expression of DnrT (from pWURdnrT) induced transcription from Pncr (60- to 70-fold), independently of the presence or absence of oxygen and/or nitrate (Fig. 4B). In contrast, constitutive expression of DnrT did not affect the nitrate and anoxia dependence of Pdnr and Pnar. These results support that the activator effect of DnrT on the Pncr promoter is signal-insensitive, and that DnrT and one or more additional signal-sensitive transcription factors are required to activate the transcription from the Pnar and Pdnr promoters.

On the other hand, the absence of DnrS in the *dnrS::kat* mutant abolished transcription from Pnar and Pdnr (Fig. 4A and C). In contrast, Pncr was constitutively expressed (47- to 70-fold) in the *dnrS::kat* mutant, as expected from the polar effect of the mutation on *dnrT* (Fig. 2B). Interestingly, simultaneous expression of DnrS from the complementation pWURdnrS plasmid and of DnrT (because of the polar effect of the *dnrS::kat* mutation, Fig. S2B) does not make the transcription from Pnar and Pdnr signal-independent. In such conditions, anoxia by itself has a minor (sixfold) stimulating effect on Pnar expression, but combination with nitrate is still required to get a strong induction (47-fold). This is in agreement with the data from Fig. 3B showing a minor induction of the NarG protein and of the nitrate reductase activity under anoxic conditions. In contrast, Pdnr was significantly induced by anoxia alone (10-fold, a 70% of the maximum detected in the wild-type strain), whereas nitrate had only a minor effect on its expression (Fig. 4C) in these complemented *dnrS::kat* mutants. Thus, we deduced that DnrS functions as an oxygen-sensitive transcription factor that works in cooperation with DnrT for the activation of Pnar and Pdnr. However, a yet unknown nitrate-sensitive component of the induction apparatus is apparently also required, in special to stimulate transcription from the Pnar promoter.

#### *DnrS changes its folding in the presence of oxygen*

The data above supported that DnrS requires anaerobic conditions to allow transcription from the Pdnr promoter. Thus, we wondered if oxygen-induced inactivation of DnrS could affect its folding. To check this, the DnrS protein was expressed from vector pWURdnrS in two cultures of *T. thermophilus* grown under aerobic conditions or under anoxic conditions with nitrate, before its sensitivity to trypsin was compared and detected by Western blot (*Experimental procedures*). As shown in Fig. 5, the DnrS protein expressed under anaerobic conditions remained undigested by the protease after 1 min of treatment, whereas most of the DnrS protein



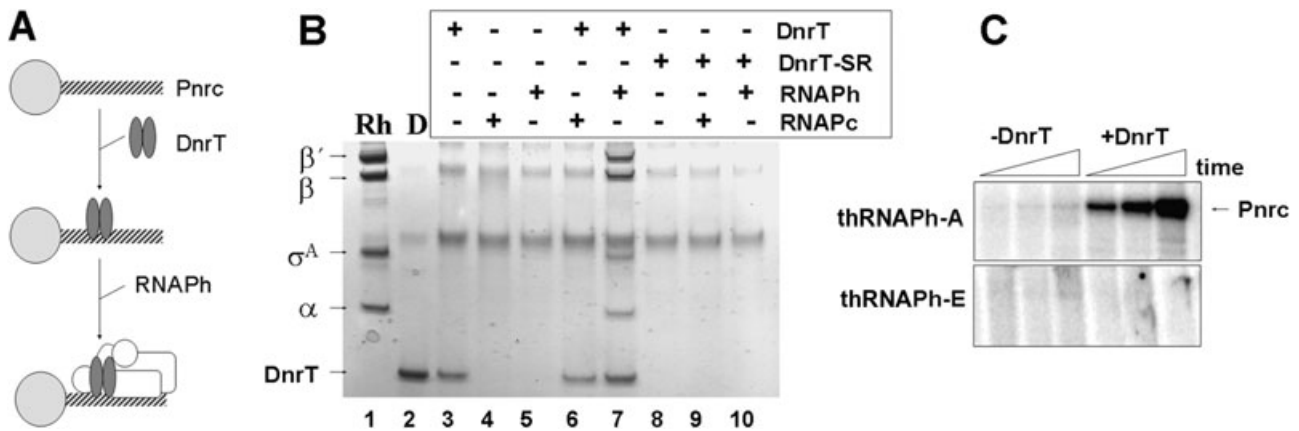
**Fig. 5.** Sensitivity of DnrT and DnrS to oxygen. The cytoplasmic fraction of aerobic (+) and anaerobic (-) cultures of the wild-type mutant transformed with pWURdnrT or with pWURdnrS was treated during the indicated periods with trypsin (*Experimental procedures*), and the remaining protein and resulting peptides were immunodetected by Western blot with the respective antisera.

from the aerobic culture was digested in the same period. In contrast, the DnrT protein expressed from pWURdnrT showed a lower sensitivity to the protease irrespective of the conditions used to express the protein. It is interesting to note that DnrS degradation products were similar in the protein expressed under both conditions, suggesting that similar oxygen-mediated folding change is taking place during the protease treatment.

#### *Purified DnrT activates transcription on Pncr*

The results from the Fig. 4 showed the relevance of DnrT as a common transcription activator required for the expression of the *nar*, *nrc* and *dnr* operons, and revealed its post-translational signal-insensitive character, at least when acting on the Pncr promoter. Taking advantage of this apparently insensitive nature, we expressed in *E. coli* and purified the DnrT wild-type protein and its SR/AL mutant (*Experimental procedures*). During this purification, size-exclusion chromatography revealed a retention time for DnrT corresponding to 45–50 kDa, around twice its theoretical size, suggesting that DnrT forms a dimer (data not shown).

Subsequent *in vitro* experiments with the purified protein showed the ability of the wild-type DnrT protein to bind to the Pncr promoter. In particular, DNase I footprint assays at 50°C revealed that DnrT was able to protect a large sequence (CCTTCACCTTACTCCTTGACCCCG-GTCAT) creating a hypersensitivity site at position -43 (underlined) with respect to the transcription start point of the Pncr promoter (Fig. S3). Further recruitment experi-



**Fig. 6.** Activation of Pnrc by DnrT.

A. Scheme of the recruitment experiment. The Pnrc promoter (grey-black helix) bound to streptavidin-agarose beads (grey ball) was incubated with DnrT or its SR/AL mutant, and subjected to a further incubation with thRNAP core or holoenzyme. Proteins bound to the beads were recovered by centrifugation and analysed by PAGE.

B. Results of the recruitment experiment. Samples of the immobilized Pnrc were incubated in the presence (+) or absence (–) of DnrT (DnrT), its SR/AL mutant (DnrT-SR), the thRNAPh (RNAPh) or the thRNAPc (RNAPc), and proteins bound to the beads were analysed through a 4–20% NuPAGE Bis-Tris gradient gel (lanes 3–10, see the text for details). Standards corresponding to purified thRNAPh (Rh, 1) and DnrT (D, 2) were run in parallel, and positions of each protein subunit are indicated. Common bands in all the lanes correspond to the BSA fraction present in the buffer.

C. *In vitro* transcription on Pnrc by the thRNAPh carrying either the  $\sigma^A$  (thRNAPh-A) or the  $\sigma^E$  (thRNAPh-E) in the presence or absence of 5 pmol of DnrT. Samples were taken after 2, 5 and 15 min. The full transcript corresponds to a 47-mer run-off of the Pnrc promoter.

ments were carried out to determine if DnrT was required or not for the binding of the *T. thermophilus* RNA polymerase (thRNAP) to the Pnrc promoter. Figure 6A shows a scheme of the procedure followed. In this, biotin-labelled Pnrc promoter was bound to streptavidin-agarose beads and used as a bait to bind the wild-type DnrT protein or its SR/AL derivative at 50°C. Then the samples were incubated with the RNA polymerase core (thRNAPc) or its SigA ( $\sigma^A$ )-bearing holoenzyme (thRNAPh), and the bound proteins were recovered by centrifugation and analysed by SDS-PAGE. The results are shown in Fig. 6B. As expected, the wild-type DnrT was recovered bound to the Pnrc promoter (lane 3), whereas its SR/AL mutant did not (lane 8). Neither thRNAPc nor thRNAPh was able to bind to the promoter by themselves (lanes 4 and 5). However, the presence of DnrT efficiently recruited thRNAPh (lane 7) but not thRNAPc (lane 6). As expected, the SR/AL mutant protein was able to recruit neither thRNAPc nor thRNAPh (lanes 9 and 10), because of its inability to bind itself to the promoter. Thus, we concluded that DnrT binds to the Pnrc promoter through its HTH domain and recruits the thRNAPh.

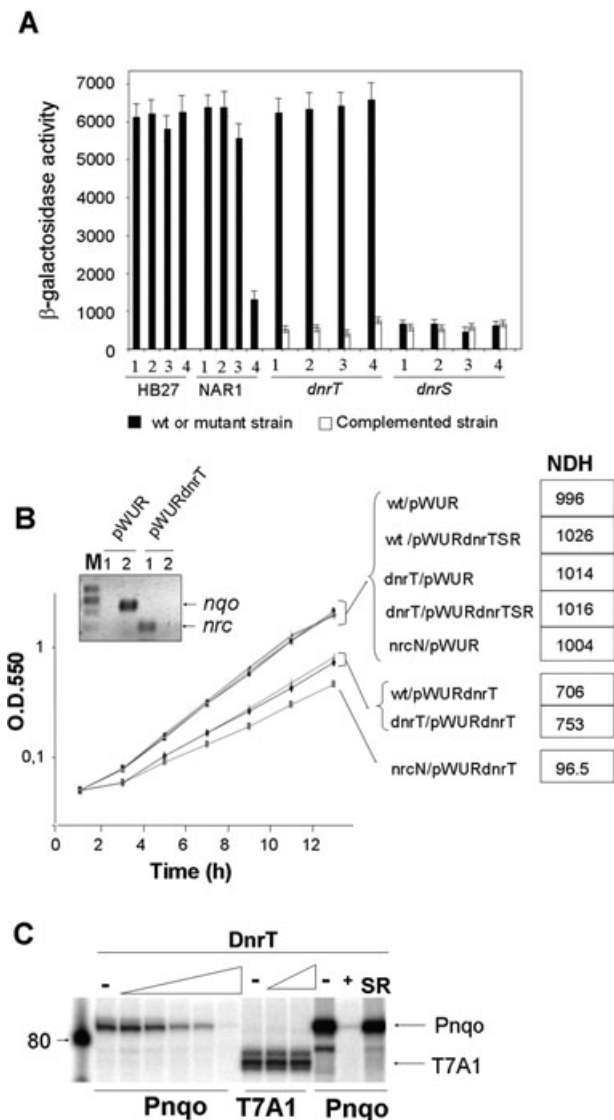
Further evidence of the role of DnrT on the activation of the transcription from Pnrc by the thRNAPh was obtained by *in vitro* experiments at near physiological (60°C) temperatures. As shown in Fig. 6C, thRNAPh alone exhibits only trace transcriptional activity on Pnrc, whereas in the presence of increasing concentrations of the DnrT protein, it efficiently generates a full-length transcript. Interestingly, a thRNAPh carrying the  $\sigma^E$  (SigE)

factor instead of the  $\sigma^A$  (SigA) was unable to initiate transcription in the presence of DnrT. Therefore, *in vitro* transcription from Pnrc requires only DnrT and its cognate  $\sigma^A$ -thRNAPh, is insensitive to oxygen, and does not require nitrate. In contrast, transcription experiments on the Pnar and Pdnr promoters with the DnrT and thRNAPh were unsuccessful (not shown), in agreement with our *in vivo* data showing a requirement for at least DnrS in addition to DnrT (Fig. 4). Addition of purified DnrS protein (from *E. coli*) to the *in vitro* transcription reactions was also unsuccessful.

#### *DnrT* represses *nqo*

As commented before, the *nqo* operon encodes the type I NDH that constitutes the main electron donor during aerobic growth. As *nqo* transcription is repressed during anaerobic growth with nitrate (Cava *et al.*, 2004), we decided to test whether DnrT or DnrS was implicated in such repression. As a first approach, we used promoter-probe constructs to analyse the transcription from the Pnqo promoter under the same conditions and strains used in the experiment shown in Fig. 4. As it can be deduced from Fig. 7A, the Pnqo promoter was constitutively active in the aerobic strain HB27 ( $6\text{--}7 \times 10^3$  units) in every condition assayed (1–4). In contrast, in the facultative NAR1 strain, this promoter was repressed (to around one-fifth of its activity) upon incubation with nitrate and anoxia for 4 h (4). Interestingly, this repression did not occur in a *dnrT* null mutant, supporting the responsibility





of DnrT on this effect. This was confirmed by expressing DnrT in a constitutive way from pWURdnrT, which resulted in Pnqo repression (1/10 of its normal activity) even under aerobic growth. Moreover, a similar repression was also found in *dnrS::kat* mutants (they express DnrT constitutively), reinforcing the idea that DnrT but not DnrS was responsible for the observed Pnqo repression.

To test this directly, DnrT was constitutively expressed in *T. thermophilus* from pWURdnrT during aerobic growth (without nitrate) and the amount of mRNA synthesized from *nqo* and *nrc* operons was subsequently analysed by semi-quantitative RT-PCR. The results showed that the expression of DnrT leads to the repression of Pnqo and to the concomitant activation of Pnrc even during aerobic growth (Fig. 7B, inset). Interestingly, when the growth of the wild-type and the *dnrT* null mutant strains bearing

**Fig. 7.** DnrT represses Pnqo.

A. The transcriptional activity of the Pnqo promoter was measured under the four conditions used in Fig. 4 on the indicated strains carrying the pMHnqobgaA plasmid and complemented (white bars) or not (black bars) with the pWUR derivatives expressing either DnrS or DnrT.

B. Inset shows the result of a semi-quantitative RT-PCR performed to detect the transcripts from *nrc* (1) or *nqo* (2) in aerobic cultures of the NAR1 strain carrying pWUR or pWURdnrT. M: DNA size markers (759, 611, 453 and 273 bp). The graph shows the growth curves at 60°C of aerobic cultures of the NAR1 strain (wt) and of its *dnrT* and *nrcN* null mutants, transformed either with pWUR control plasmid or with pWURdnrT or pWURdnrTSR derivatives. The NDH activity of membrane fractions corresponding to 8 h of growth of each culture is indicated (nmol of NADH oxidized per minute and per milligram of protein).

C. *In vitro* transcription on Pnqo in the presence of thRNAPh and increasing concentrations (0, 5, 10, 20, 40, 80 pmol) of DnrT. Parallel control experiments were carried out with the T7A1 promoter and DnrT (0, 40, 80 pmol) to show the absence of repression by DnrT on this unrelated promoter. An independent experiment is also shown in which transcription from Pnqo was carried out without (-) or with 80 pmol of DnrT (+) or its DnrT-SR/AL mutant (SR). The run-off transcript from Pnqo and PTTA1 correspond to 90 mer and 50 mer respectively. All reactions were performed at 60°C for 10 min.

or not the pWUR derivatives were compared, it was observed that expression of DnrT (from pWURdnrT) decreased the growth rate in comparison with the same strain carrying the control plasmid pWUR, or expressing the DnrT SR/AL mutant (from pWURdnrTSR). We concluded that DnrT was actually decreasing the respiratory efficiency during aerobic growth due to the replacement of type I Nqo-NDH by the Nrc-NDH. Accordingly, the NDH activity from membrane fractions isolated from the cultures (time 8 h) revealed that the expression of DnrT results in a decrease in the activity of 25–30% (700–750 units) compared with those expressing the SR/AL mutant or carrying the control pWUR plasmid (around 1000 units). To identify which part of this activity was actually due to the Nrc-NDH and which was due to the Nqo-NDH remaining in the membrane, we included the *nrcN::kat* mutant in the experiment (Cava *et al.*, 2004). As shown in Fig. 7B the *nrcN::kat* mutant grows aerobically as the wild-type strain when carrying the pWUR control plasmid or when expressing the SR/AL mutant, and shows similar NDH activity (around 1000 units). In contrast, expression of DnrT in this *nrcN::kat* mutant resulted in a very low NDH activity (around 100 units) and in a further decrease of the growth rate of the strain in comparison with that shown by the wild-type strain expressing DnrT. This residual NDH activity most likely corresponds to the Nqo-NDH still remaining in the membrane, which is in agreement with the repression of the *nqo* promoter by the pWURdnrT plasmid detected in Fig. 7A. Additionally, these data show that the Nrc complex is active as NDH in the wild-type strain when expressed under aerobic conditions, but not as efficient for growth as the Nqo type I NDH is.

A final approach to ascertain the repression of *nqo* operon by DnrT was an *in vitro* transcription assay with the thRNAPh. As shown in Fig. 7C, Pnqo was transcribed by the thRNAPh, and the addition of increasing concentrations of DnrT to the reaction mix resulted in decreased transcription until complete repression. In contrast, in control experiment, transcription from unrelated T7A1 promoter was not affected, even by the highest concentration of DnrT assayed. As expected, transcription from Pnqo was not repressed by the SR/AL mutant in an independent experiment (lane SR). These results support our conclusion that the observed repression of Pnqo by DnrT was promoter-specific and dependent on its HTH motif.

## Discussion

The ability of some strains of *T. thermophilus* to use nitrate as electron acceptor during anaerobic growth requires the replacement of the aerobic respiratory chain, whose main electron donor is the Nqo type I NDH, by a specific respiratory chain made of the heterotetrameric enzymes NDH (Nrc) and nitrate reductase (Nar) (Cava *et al.*, 2004). Here we identify a new operon of the NCE that encodes two transcription factors implicated in this replacement of respiratory chains. In the following paragraphs we discuss their specific roles according to our data.

### The DnrS protein

The analysis of null *dnrS::kat* mutants, in which the DnrT protein is constitutively expressed, and the complementation assays with plasmid pWURdnrS revealed the requirement of the DnrS protein for the expression of Nar but not for the expression of Nrc. The development of a new system of promoter-probe vectors, compatible with other thermostable antibiotic resistances, allowed us to observe that this effect took place at the transcription level. Moreover, we could observe that DnrS was required also to activate transcription from its own promoter. Having in mind that DnrS has a C-terminal BTAD domain, found in the DNRI/REDD/AFSR family of regulators of secondary metabolism of Actinobacteria, we concluded that DnrS most likely acts as a transcription activator on the Pnar and Pdnr promoters.

However, constitutive expression of DnrS from plasmid pWURdnrS in a *dnrS::kat* mutant, in which DnrT is constitutively expressed, did not allowed transcription from Pnar or Pdnr under aerobic growth. Therefore, either the activities of DnrS or DnrT were sensitive to nitrate or to oxygen, or alternatively, an unknown factor necessary for the transcription from these promoters was not expressed or inactive under such conditions. The analysis of the separate effects of these two signals in cells expressing

DnrS and DnrT constitutively revealed that maximum activity required both signals. Nevertheless, oxygen depletion was more relevant than nitrate on the transcription of the Pnar and Pdnr promoters. This was specially relevant for the Pdnr promoter, in which oxygen depletion produced a significant transcription increase (73% of its maximum) in the absence of nitrate. Having in mind the apparently insensitive character of DnrT on the activation of Pnrc (see below), these results lead us to propose DnrS as an O<sub>2</sub>-sensitive component of the induction system.

In agreement with its apparent O<sub>2</sub>-sensitive character, the protein DnrS has a GAF domain at its N-terminus. Such domains are found in several cytoplasmic sensory proteins, either associated to other signalling domains such as histidin kinases, adenylate cyclases, diguanilate cyclase/phosphodiesterases and protein phosphatases, or associated to output domains (Galperin, 2004), like the BTAD domain found in DnrS. The signal receptor role of the GAF domains is usually associated to their capability to bind small molecules. In fact, there are precedents of oxygen and/or NO sensing by the GAF domains of the DosS protein of *Mycobacterium tuberculosis* (Sardiwal *et al.*, 2005) and of the NorR protein of *E. coli* (D'Autreaux *et al.*, 2005). The way by which the signal is transmitted from the GAF to the output domains is not yet understood, but it is generally assumed that it implies a conformational change, which affects the folding state and, concomitantly, the activity of the regulatory domain (Galperin, 2004). In this sense, we have detected a higher protease sensitivity of the DnrS protein exposed to oxygen during its synthesis than when expressed under oxygen depletion, supporting that an oxygen-dependent conformational change that unstabilizes the protein is actually taking place. Unfortunately, we cannot associate this conformational change to a loss of function in *in vitro* assays (DNA binding, for example) because the DnrS protein purified from *E. coli* was inactive in all the assays carried out. Thus, we hypothesize that native DnrS has an oxygen-sensitive cofactor at its GAF domain that under reducing conditions allows the protein to bind to specific sequences on the Pdnr and Pnar promoters, but that upon oxidation makes the protein inactive through a conformational change. In this way, the DnrS protein could mimic the role that the FNR factor plays as oxygen sensor during nitrate respiration in *E. coli* (Korner *et al.*, 2003). A detailed molecular analysis of the way by which DnrS is inactivated by O<sub>2</sub> will have to wait until active DnrS could be purified from anaerobic cultures of *T. thermophilus*.

### DnrT as transcription activator

DnrT is required for the synthesis of the Nar and Nrc enzymes, and also for its own expression, so it behaves

as a central regulator of the nitrate respiratory system. It is also clear from the experiments with the promoter-probe vectors that DnrT acts at the transcription level on the corresponding operon promoters. However, the form by which these three promoters are activated by DnrT differs: while it has to act in co-ordination with DnrS to stimulate transcription from Pdnr and Pnar under nitrate respiration conditions, its sole presence allows Pnrc to be transcribed, independently of the presence or absence of nitrate or oxygen. This behaviour supports that DnrT, in contrast to DnrS, is insensitive to any of these signals, in such a way that it could be considered as a constitutively active transcription factor, whose function depends basically on the concentration reached and not on a putative conformational change. In agreement to this, we found no differences in the sensitivity to trypsin between DnrT proteins produced either aerobically or anaerobically (Fig. 5). This signal-insensitive behaviour contrasts with the modular structure of the CRP family of transcription activators to which DnrT belongs. Actually, most CRP-like factors contain sensory modules at the N-terminal half of the protein that upon binding of molecules such as cAMP (CRP), 2-oxoglutarate (NtcA), or upon detection of signalling molecules such as O<sub>2</sub> (FNR), CO (CooA) or NO (Dnr, Nnr) by specific protein-bound sensory cofactors, suffer a conformational change that allows the protein to interact with a target sequence on specifically induced (or repressed) promoters (Korner *et al.*, 2003). In those modules evolved to detect O<sub>2</sub>, either iron-sulphur centres or haem groups are present (Korner *et al.*, 2003), but none of them have been detected in the purified DnrT protein. However, besides iron-sulphur centres or haem groups, we cannot rule out formally the possibility that small molecules, common enough to be expressed in *E. coli*, could bind to the 'cAMP-binding domain' present in the DnrT sequence. In fact, a model for DnrT conserves the two pockets that in the CRP structure contain the cAMP molecules (Fig. S4A).

In any case, the DnrT protein purified from overproducing strains of *E. coli* was able to bind *in vitro* to the Pnrc promoter through its HTH motif (the SR/AL mutant did not bind), and to recruit purified thRNAPh in a SigA ( $\sigma^A$ )-dependent way, allowing the transcription to proceed under thermophilic conditions (Fig. 6C). As neither the thRNAPc nor the thRNAPh was able to bind to Pnrc by themselves under these experimental conditions, and having in mind that DnrT binds to the Pnrc promoter at a position (-43) similar to CRP on type II promoters (Fig. S3), these results support the existence of specific DnrT-thRNAPh interactions on Pnrc similar to those observed on such promoters between activation region AR3 of CRP and the C-terminal domain of  $\sigma^{70}$  (Busby and Ebright, 1999; Lawson *et al.*, 2004). To check if similar interactions in the *T. thermophilus* pro-

teins were likely, and having in mind the availability of high-resolution structures of thRNAPh, we used docking programs to define a putative DnrT-thRNAPh interaction model by using the DnrT AR3 region deduced from the model, and the structure of  $\sigma^A$  in the thRNAPh crystal (Fig. S4B). It is noteworthy that automatic docking, without any hand-conducted refinement, resulted in a DnrT-thRNAPh complex similar to modelled open promoter complex proposed by Lawson *et al.* (2004) and by Artsimovitch *et al.* (2004) (Fig. S4C). In this automatic DnrT-thRNAPh model, the AR2 region of DnrT and its putative target in the  $\alpha$ -thRNAP are very close to each other, supporting that interactions through them take place in the DnrT-thRNAPh complex also. On the other hand, it was possible to model a nicely complementary set of interactions between the putative AR1 region of the DnrT model and the C-terminal region of the  $\alpha$ -thRNAP on the basis of the co-crystal between CRP and the  $\alpha$ -ecoRNAP of structure 1LB2 (Benoff *et al.*, 2002) (Fig. S4D). Although yet a model, these data support the existence of specific DnrT-thRNAPh interactions on the Pnrc promoter similar to those produced between CRP and ecoRNAPh on type II promoters (Busby and Ebright, 1999). The specificity of such interactions deduced from our model explains the absence of transcription when the thRNAPh containing  $\sigma^E$  was used in the transcription experiments on Pnrc (Fig. 6C).

DnrT is also required to activate transcription on Pnar and Pdnr, but comparison of their sequence with that of the region protected by DnrT on the Pnrc promoter did not reveal clear similarities, probably as a consequence of the complexity of both promoters, which also require the presence of DnrS to be activated. In spite of having no *in vitro* evidence for the binding of DnrS to DNA, our data suggest that this is most likely the way by which it helps DnrT to activate transcription. In this sense, the promoter of the operon encoding the nitrate reductase A of *E. coli* requires the binding of three proteins to get active transcription in *in vitro* assays: promoter-proximal binding of reduced dimers of FNR; promoter-distal binding of several copies of the phosphorylated nitrate-response regulator NarL; and binding of the IHF (integration host factor) protein in an intermediate region (Schroder *et al.*, 1993). Therefore, it is not surprising to find a similar complex expression requirement for the transcription of the thermophilic Pnar counterpart. In this sense, our results suggest the existence of DnrT-DnrS interactions on the Pnar promoter, not just due to the obvious requirement for both proteins to activate transcription, but also because of the inhibitory effect that the presence of DnrT SR/AL mutant has on the expression of NarG in the wild-type strain under normally inducing conditions (Fig. 3A). The most likely explanation for this result is that the mutant and the wild-type DnrT proteins

compete for interaction with DnrS, and due to the higher concentration of the first, few DnrS proteins remain available to activate transcription. In contrast, this effect does not affect expression of NrcD because of its only dependence on DnrT.

On the other hand, transcription from P<sub>dnr</sub> presents some differences with respect to the expression from P<sub>nar</sub>, despite the common requirement for DnrT and DnrS. On this promoter the putative nitrate-response regulator has a less relevant role once both DnrS and DnrT have been expressed. A putative 'sequential induction' model could explain this (Fig. 1A). In this model, a yet unknown nitrate-response regulator could be required to get an increase of the basal expression level of P<sub>dnr</sub> as suggested by the results from Fig. 4C. A parallel or further decrease in oxygen concentration could subsequently stabilize DnrS in an active conformation (Fig. 5), allowing the autoamplification of the regulators in an anoxic environment (Fig. 4C). In this way, P<sub>nrc</sub> could be induced by DnrT to provide an appropriate electron donor for nitrate (Fig. 4B) and, putatively, also for the whole denitrification pathway, whereas expression from P<sub>nar</sub> would be more dependent on the continuous presence of nitrate (Fig. 4A). Supporting this hypothesis, we have preliminary evidences showing that the Nrc-NDH also constitutes the main electron donor in complete denitrifying strains of *T. thermophilus* growing on nitrite (in preparation).

#### *DnrT as repressor*

In addition to its role as transcription activator of the *nrc*, *nar* and *dnr* operons from the NCE, a main observation of this work is that DnrT also acts as repressor of the chromosomal *nqo* operon, which encodes the aerobic type I NDH. For this repression to be detected *in vitro*, a greater concentration of DnrT was required compared with that needed to activate transcription on *nrc* (Fig. 7C), thus suggesting that the amount of Nqo decreases after enough Nrc is synthesized, allowing a progressive replacement of the respiratory chains.

This replacement of Nqo by Nrc is apparently contradictory in terms of energy supply. In fact, in mesophiles like *E. coli* the proton-extruding Nqo homologue (Nuo) is induced during anaerobic growth with nitrate as a way to compensate the loss of energy that the use of nitrate instead of oxygen implies, whereas the non-proton-pumping type II NDH is expressed in the presence of oxygen, when the cells do not require the use of a high-yield energy metabolism (Unden and Bongaerts, 1997). Interestingly, this replacement of respiratory enzymes in *E. coli* is under the control of FNR, a DnrT homologue. Although Nrc is inhibited by Rotenone as Nqo does, its subunit architecture, similar to the non-proton-pumping succinate:quinone oxidoreductases (Cava *et al.*, 2004),

suggests that it lacks the ability to pump protons upon NADH oxidation. Moreover, the observation that the replacement of Nqo by Nrc in cells growing aerobically (strains carrying pWUR<sub>dnrT</sub>) results in a much lower growth rate, and the relatively minor effect on these growth rates of a *nrcN* mutation (Fig. 7B) supports a lower energy yield for Nrc than for Nqo per mol of NADH oxidized when oxygen is the final electron acceptor. Therefore, the energy yield seems not to be the driving force that had selected for the adaptation of DnrT to its repressor role on *nqo*, unless some kind of electron channelling between respiratory complexes exists that could limit the energy yield after the partial replacement (Nqo by Nrc) of components. In this sense, there are growing evidences of the formation of respiratory supra-complexes in bacteria (Megehee *et al.*, 2006) and yeasts (Cruciat *et al.*, 2000). Alternatively, one can imagine DnrT as a mechanism evolved by a selfish mobile element, like NCE, to ensure its integrity upon selection after horizontal transference: repression of Nqo could make the cell more energetically dependent on the presence of NCE.

#### *Concluding remarks and perspectives*

This work has shown the DnrT protein as the direct responsible for the replacement of respiratory chains that takes place during adaptation of *T. thermophilus* to nitrate respiration, showing for the first time *in vitro* and *in vivo* the direct activation and repression of thRNAP<sub>H</sub> transcription activity by a thermophilic CRP homologue. However, our data also opened several questions about how the absence of oxygen and the presence of nitrate are sensed by this organism. We have presented evidences that suggest that *T. thermophilus* uses for this purpose completely different mechanisms from that shown by *E. coli* to detect both signals. Instead of using a FNR homologue as oxygen sensor, *T. thermophilus* apparently uses DnrS, a protein containing a GAF domain, but the actual O<sub>2</sub>-sensory cofactor remains unidentified. On the other hand, the mechanism by which nitrate affects the expression of the *dnr* and *nar* operons remains unsolved. In most bacteria, nitrate as a non-permeable solute is sensed at the periplasm by homologues of NarX, the membrane partner of a two-component regulatory system of *E. coli*, and the signal is transmitted by phosphorylation of homologues of NarL, the corresponding response regulator. However, neither the NCE nor the genomes of the sequenced strains of *T. thermophilus* encode homologues of such a nitrate-sensory transmission system. This suggests that as it apparently happens with O<sub>2</sub> detection, a different mechanism for nitrate detection and transcription activation has evolved in this ancient bacterial lineage. The availability of new genetic tools for this extreme ther-

mophile, described and used in this work for the first time, will help to find answers to these questions.

## Experimental procedures

### Strains and growth conditions

The *E. coli* strains DH5 $\alpha$  [*supE44*,  $\Delta$ *lacU169* ( $\phi$ 80 *lacZ* $\Delta$ M15), *hsdR17*, *recA*, *endA1*, *gyrA96*, *thi-1*, *relA1*] and BL21DE3 [*hsdS*, *gal* ( $\lambda$ clts857, *ind1*, *Sam7*, *nin5*, *lacUV5-T7* gene 1)] were used for plasmid construction and protein expression respectively. *T. thermophilus* NAR1 is the facultative anaerobic wild-type strain in which the presence of the NCE was first described. This strain was identified as HB8 in previous works (Ramirez-Arcos *et al.*, 1998a), but sequence comparisons with the recently available genome of the type strain revealed that they are highly related but different strains. The derivative *nrcN::kat* mutant of this strain was also used (Cava *et al.*, 2004). *T. thermophilus* HB27 is a wild-type aerobic strain provided by Dr Koyama. *T. thermophilus* HB27c is a facultative anaerobe derived from HB27 to which the NCE was transferred by conjugation (Ramirez-Arcos *et al.*, 1998b).

Aerobic growth of *T. thermophilus* was routinely carried out on TB at 60°C or 70°C under mild stirring (150 r.p.m.) in one-fifth of the volume of the flask (Ramirez-Arcos *et al.*, 1998b). Induction by nitrate and anoxia (nitrate/anoxia conditions) was achieved by arresting the shaker and the simultaneous addition of potassium nitrate (40 mM) to aerobic cultures grown to an OD<sub>550</sub> of 0.3. Under these conditions, rapid consumption of O<sub>2</sub>, along its low solubility at high temperature, makes the culture anoxic in a very short time. Completely anaerobic growth was carried out in screw-capped tubes containing 10 ml of TB and potassium nitrate (40 mM), overlaid by mineral oil (Cava *et al.*, 2004). Agar (1.5% w/v) was added to the TB medium for growth on plates. When required for selection, Kanamycin (30 mg l<sup>-1</sup>), Bleomycin (15 mg l<sup>-1</sup>) or Hygromycin B (100 mg l<sup>-1</sup>) were added to the growth medium.

*Escherichia coli* was grown at 37°C on liquid or solid LB medium. Kanamycin (30 mg l<sup>-1</sup>), Ampicillin (100 mg l<sup>-1</sup>), Bleomycin (3 mg l<sup>-1</sup>) and/or Hygromycin B (100 mg l<sup>-1</sup>) were also used when needed.

### DNA and RNA methods

DNA and RNA isolation, plasmid purification, restriction analysis, plasmid construction and DNA sequencing were carried out by standard methods (Sambrook *et al.*, 1989). PCR was performed with the DNA polymerase from *T. thermophilus* as described by the manufacturer (BIOTOOLS B & M, Madrid, Spain). For semi-quantitative RT-PCR the Ready-To-Go™ kit (Amersham Biosciences) was used. Directed mutagenesis was carried out by PCR. The primers used are described in Table S1.

### Gene cloning, plasmids and transformation

The *dnrS* and *dnrT* genes were identified within the sequence of plasmid pUP1B, a pUC119 derivative from a gene library

(Fernández-Herrero *et al.*, 1997) selected by colony blot with a 3' probe of the *nar* operon. *E. coli*-*Thermus* sp. shuttle vectors pMK184 (this work), pWUR112/77-1 (Brouns *et al.*, 2005) and pMH184 (this work) are derivatives of pMK18 (de Grado *et al.*, 1999) encoding compatible thermostable resistances to Kanamycin, Bleomycin and Hygromycin B (Nakamura *et al.*, 2005) respectively. Plasmids pMHnrbgaA, pMHnrcbgaA, pMHdnrbgaA and pMHnqobgaA are derivatives of pMH184 in which the thermostable beta-galactosidase reporter gene *bgaA* (Moreno *et al.*, 2003) is expressed under the control of DNA fragments containing the promoter regions of the *nar*, *nrc*, *dnr* and *nqo* operons respectively. The sizes of the promoters included in these plasmids with respect to the translation start codon of their respective first genes are: -784 to +6 (*nar*), -249 to +3 (*dnr*), -343 to +315 (*nrc*) and -448 to +3 (*nqo*). In all the constructs, the *bgaA* gene was preceded by a Shine-Dalgarno sequence (GGAGG).

Plasmids pWURdnrT, pWURdnrSR and pWURdnrS are derivatives of pWUR112/77-1 used for constitutive expression of the wild-type DnrT, its S185A/R186L mutant and the DnrS protein respectively. For this purpose, the respective genes were amplified by PCR (Table S1) and cloned between the NdeI and the EcoRI sites of plasmid pET22b (Novagen) to further proceed to their isolation by XbaI and EcoRI digestion and cloning in the same sites of pWUR112/77-1. In these constructs, transcription from the constitutive PslpA promoter of the Bleomycin resistance cassette continues through the cloned genes, allowing their constitutive expression.

Transformation of *T. thermophilus* with linear or circular DNA was achieved by natural competence (Cava *et al.*, 2004), whereas standard protocols were used to transform *E. coli*.

### Isolation and complementation of mutants

Plasmid pK18 is a derivative of pUC18 used as integrative vector in *T. thermophilus* because of the presence of the *kat* gene (Laptenko *et al.*, 2006). Null *dnrT* mutants were isolated by recombinative insertion of a pK18 derivative (pK18 $\Delta$ NtCt-dnrT) carrying a central fragment of *dnrT* (positions +101 to +548) obtained by PCR with the primers dnrTEcoRI and dnrTBamHI (Table S1). The S185A/R186L-directed mutant was obtained by standard PCR protocols by using primers dnrTmutSR, dnrTEcoR1rev and dnrTNde1 (Table S1). The replacement of the wild-type *dnrT* gene by its derivative encoding the S185A/R186L double mutant was carried out by using a construct in which the mutant gene was cloned into pK18 from positions +101 to the final stop codon of the *dnrT* sequence to direct its integration by homologous recombination. Insertional *dnrS::kat* mutants were obtained by transformation with a linear DNA fragment carrying the *dnrS* gene interrupted by the *kat* gene at a PstI site at position +1255 with respect to its translation start, and flanked by appropriate upstream and downstream homologous regions for recombination. In this *dnrS::kat* mutant, the PslpA promoter of the *kat* gene provides constitutive transcription to *dnrT* because of the absence of a transcription terminator. In all instances, the nature of the isolated mutants was checked by PCR and by immunodetection with anti-DnrT or anti-DnrS. Complementation experiments of these mutants were carried

out by transformation with the pWUR derivatives described above.

### Promoter induction assays

Quantitative measurements of the transcription of the *nar*, *nrc*, *dnr* and *nqo* operons were tested in cultures of wild-type or mutant strains of *T. thermophilus* transformed with promoter-probe plasmids pMHnrbgaA, pMHnrcbgaA, pMHdnrbgaA or pMHnqobgaA respectively. The effect of the absence or of the constitutive expression of DnrS or DnrT on these promoters was assayed on *dnrT* or *dnrS* null mutants transformed with the control pWUR plasmids or its corresponding derivatives.

For aerobic expression, cells were grown for 8 h to the exponential phase ( $OD_{550}$  between 0.3 and 0.6) under stirring (150 r.p.m.) in a 1/10 volume of the flasks to allow maximum oxygen diffusion. For anaerobic induction, aliquots of these cultures were subsequently transferred to complete the total volume of 2 ml screw-capped tubes, which were incubated for 16 more hours. Nitrate effect was assayed by providing potassium nitrate at 40 mM to the TB medium. All these experiments were performed at 60°C to limit the putative toxicity of the constitutive expression of DnrS or DnrT.

The beta-galactosidase activities of soluble cell extracts were assayed in triplicate experiments on the chromogenic substrate ONPG (ortho-nitrophenyl-galactopyranoside) at 70°C. The activities were expressed in arbitrary units as described by Miller (1992).

### Sensitivity to trypsin

Cultures of the NAR1 wild-type strain bearing the pWURdnrS or pWURdnrT plasmids were grown at 70°C on nitrate-free TB medium, either under aerobic conditions (180 r.p.m.) in one-fifth volume of the flask to reach an  $OD_{550}$  of 0.5, or in static bath overnight to limit oxygen diffusion. Then cells were harvested, and subsequently disrupted by sonication (Braun Labsonic; 1 min in 0.5 s pulses, maximum power) in TS buffer (50 mM Tris-HCl, 50 mM NaCl, pH 7.5), before separation of soluble and particulate fractions by ultracentrifugation (150 000 g, 15 min at 4°C).

Sensitivity to trypsin was assayed in 80  $\mu$ l of cell fraction samples containing 2 mg ml<sup>-1</sup> of protein. Then, 2  $\mu$ l of trypsin solution (5 mg ml<sup>-1</sup> in HCl 1 mM) was added to the samples, which were incubated at room temperature for the indicated times. Digestion was stopped by boiling in Laemmli loading buffer, and the proteins were separated by SDS-PAGE (Sambrook *et al.*, 1989). DnrS and DnrT were identified by Western blot with specific rabbit antisera and detected by chemiluminescence with the ECL detection kit (Amersham).

### Purification of DnrT, DnrS and thRNAP

The genes encoding DnrT, its S185A/R186L mutant, and DnrS, were amplified with appropriate primers (Table S1) and cloned between the NdeI and EcoRI sites of pET22b and

pET28b (Novagen) to allow their expression in *E. coli* BL21DE3, either as the native protein sequences or with N-terminal 6x-Histidine-tag fusions. Specific rabbit anti-DnrT and anti-DnrS antisera were prepared by a private company (Charles River Laboratories, Chalaronne, France) from the respective proteins purified by SDS-PAGE for their further use in immunodetection assays.

Both proteins were purified by affinity chromatography on Ni-NTA agarose (Qiagen) followed by thermal denaturation (70°C, 15 min) of remaining *E. coli* proteins. DnrT was further purified through a size-exclusion chromatography on Superdex-200 and stored in aliquots at -20°C with glycerol (40%) until use. The thRNAPc was purified from *T. thermophilus* as in (Vassilyeva *et al.*, 2002). Quantitative *in vitro* transcription assays (Vassilyeva *et al.*, 2002; Laptenko and Borukhov, 2003) revealed that the thRNAPc preparation contained 80% of catalytically active molecules. Reconstituted thRNAPh (holoenzyme) was obtained by combining 0.5 mg of thRNAPc with purified 0.15 mg of recombinant SigA ( $\sigma^A$ ) (Nishiyama *et al.*, 1999) or SigE ( $\sigma^E$ ) factors followed by holoenzyme purification by size-exclusion chromatography using Superose 6 column (Vassilyeva *et al.*, 2002). Recombinant SigE was a generous gift from Dr Jookyung Lee.

### ThRNAP recruitment

A scheme of the recruitment experiment is shown in Fig. 6A. Biotine-labelled Pnc (positions -207 to +28, 235 bp) was bound to ImmunoPure Immobilized Streptavidin Gel (Pierce) in binding buffer (20 mM HEPES, 50 mM NaCl, 10% glycerol, 0.1 mg ml<sup>-1</sup> Bovine seroalbumin, 5 mM 2-mercaptoethanol, pH 6.9). Then, the DnrT protein or its SR/AL mutant (1  $\mu$ g) was incubated for 15 min at 50°C with 0.2  $\mu$ g of Pnc-resine in 20  $\mu$ l samples containing 2  $\mu$ g of salmon sperm DNA. ThRNAPc or thRNAPh (4.7 pmol) was then added and incubated for 15 more minutes at the same temperature. After three washing steps with 300  $\mu$ l of binding buffer, the bound proteins were eluted with 1% SDS, separated in a 4–20% NuPAGE Bis-Tris gradient gel (Invitrogen), and Coomassie blue stained. Samples without DnrT were used as negative controls.

### In vitro transcription

*In vitro* transcription was carried out in 10  $\mu$ l samples of transcription buffer (50 mM MOPS, 40 mM KCl, 10 mM MgCl<sub>2</sub>, 0.1 mM Dithiothreitol, pH. 6.5) containing 0.3 pmol of the promoter DNA (Pnar, PT7A1, Pnc or Pnqo), 1.75 pmol of thRNAPh, bearing either  $\sigma^A$  or  $\sigma^E$ , and the specified amounts of wild-type DnrT or its SR/AL mutant. Transcription started at 60°C by adding a NTP mix (0.8 mM GTP, 0.8 mM ATP, 0.1 mM CTP, 0.2 mM UTP and [ $\alpha$ -<sup>32</sup>P]-CTP) and continued for different times before analysis. The sizes for the promoter regions amplified by PCR (Table S1) used in these assays were: -207 to +28 for Pnc (235 bp), -784 to +6 (790 bp) and -211 to +1 (212 bp) for Pnar, and -448 to +3 for Pnqo (458 bp). The 270 bp T7A1 promoter was used as positive transcription control in different experiments (Nudler *et al.*, 1997).

### Amino acid sequence alignment and molecular modeling of DnrT

The domain identification on DnrS and DnrT was carried out through the InterPro Scan Sequence search program at the EMBL-EBI. DnrT homologues obtained with BLAST (Altschul *et al.*, 1995) were aligned using CLUSTALW (Thompson *et al.*, 1994) and T-COFFEE (Notredame *et al.*, 2000) algorithms. The structural model of DnrT was built on CRP–DNA complex structure (PDB entry 1O3R; Chen *et al.*, 2001) by using the SWISS-MODEL server facilities (Schwede *et al.*, 2003) and its quality examined with WHAT-CHECK procedure (Hooft *et al.*, 1996) of the WHAT IF program (Vriend, 1990). The model was further refined by subjecting it to three steps of 50 cycles of the steepest descent minimization method of the DEEVIEW program (Guex and Peitsch, 1997). The model for interaction of thRNAP  $\alpha$ -C-terminal domain with AR2 of DnrT was built using the protein–protein rigid docking method implemented in HEX program (Ritchie and Kemp, 2000), setting the initial position of the putative complex on the CRP- $\alpha$ -ecoRNAP C-domain structure (PDB entry 1LB2; Benoff *et al.*, 2002). The model for DnrT–sigma A interaction was carried out by the same method using the deduced DnrT model structure and the co-ordinates of thRNAP structure, which includes the position of  $\sigma^A$  in the complex (PDB 1SMY; Artsimovitch *et al.*, 2004). To reduce the translational-rotational search, the initial positioning of the putative complex was set by an initial generation of  $5 \times 10^3$  different docking solutions for the complex, using the low-resolution docking algorithm GRAMM (Vakser, 1995), and by selecting solution that minimizes the average distance between the DnrT AR3 site and its binding site on  $\sigma^A$ , as described (D'Autreaux *et al.*, 2005).

### Data deposition

The sequence of the *dnr* operon was deposited to EMBL GenBank with the identification No. AM161043.

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### Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** DnrT amino acid sequence alignment. Sequences of DnrT and other members of the CRP family were compared and aligned using CLUSTALW program. Identical residues are shaded in grey, and residues subjected to site-directed mutagenesis in DnrT are marked with asterisks. Secondary structure elements (H, alpha helix; E, beta strand) of *E. coli* CRP (CRP-Eco, PDB entry 1O3R), as well as two structural motifs, the dimerization helix and the helix-turn-helix DNA-binding motif are shown in the lower row (CRP-Eco (ss)). Known sites of CRP interaction with RNAP (activation regions AR1, AR2 and AR3) are shown as coloured boxes above the DnrT sequence. Schematic diagram showing the spatial relationship of AR1-AR3 regions of CRP and RNAP subunits is presented at the bottom. Protein accession numbers: THET2-TtheH – TTC1072 from *T. thermophilus* HB27; FLP-Llac – Q9S393 from *Lactococcus lactis*; NarR-Ppan – Q93PW2 from *Paracoccus pantotrophus*; DnrE-Pst – Q9X7J7, DnrD-Pst – Q9X7J4, and FnrA-Pst – P7200 from *Pseudomonas stutzerii*; Dnr-Pae – Q51441 and ANR-Pae – P23926 from *P. aeruginosa*; FNR-Eco – P0A9E6 and CRP-Eco – P0ACJ8 and from *E. coli*.

**Fig. S2.** A) Immuno-detection of the indicated amounts (ng) of DnrS and DnrT purified from over-expressing strains of *E. coli*. B) Western blot of total protein extracts from *T. thermophilus* showing the DnrS and DnrT proteins expressed in the facultative wild type strain (NAR1) grown aerobically (A) or treated for 4 hours under anoxia with nitrate (An), and in its *dnrS::kat* mutant grown aerobically before (–) or after transformation with pWUR (pW) or with pWURdnrS (pWdnrS). The aerobic strain HB27 (27) is shown as control. Note that constitutive expression of DnrT in the *dnrS::kat* mutant during aerobic growth is independent of the presence of pWURdnrS.

**Fig. S3.** DNase I footprinting analysis on triplicate samples of the Pnrc promoter with (+DnrT) or without (-DnrT) the DnrT protein. Samples (20  $\mu$ l) of Pnrc (7 pmol), labelled in the template strand by PCR with primers [<sup>32</sup>P]-FPpncRev and FPpnc47rev, were incubated for 5 min at 50°C with DnrT (5,

10, 50 pmol) in the presence of 2  $\mu$ g of [dl-dC], and digested with DNase I for 1 min at 50°C in reaction buffer (8 mM HEPES, 6 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml BSA, pH 7.5). Samples were analyzed by denaturing PAGE and visualized by autoradiography. The sequence protected by DnrT is shown.

**Fig. S4.** Structural model for DnrT. A) Ribbon plot of the proposed structure for DnrT dimer interacting to a DNA molecule, represented as a surface coloured according to electrostatic properties. Atoms of residues located in the interaction areas AR1 (yellow), AR2 (blue) and AR3 (green) are depicted as spheres. Model shows the location of the large dimerization helices of both molecules in the dimer interface, as well as the HTH motif in close contact to the major groove of DNA. The location of the cAMP molecules in the CRP structure is shown for clarity. B) Detail of modeled AR3- $\sigma^A$  – DNA interaction. Position of residues in AR3 domain, represented as ‘sticks’, indicates the location of side chains of R63 and K64 (blue) pointing to the negatively charged surface of DNA phosphate backbone. Acidic chains of D59 and E61 (red) are faced to the positive residues of K403, R406, and K407 of  $\sigma^A$ . Residues R396, and R398 appear to bind phosphate groups in the backbone of DnrT-bound DNA. C) Modelled interaction of DnrT to  $\alpha$ -thRNAP. Proteins, as well as DNA bound to DnrT, are represented as atomic surfaces. AR2 site in DnrT and its proposed recognition site in  $\alpha$ -thRNAP have been highlighted. D) Model for the interaction of the C-terminal domain of the  $\alpha$ -thRNAP (green ribbon) with DnrT through the AR1 domain. Atoms of residues in contact in both proteins are illustrated as spheres. DNA surface has been omitted for clarity. Plots were generated using PyMOL (DeLano Scientific, San Carlos, CA).

**Table S1.** Oligonucleotides used in this work and their purpose.

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