A New Type of NADH Dehydrogenase Specific for Nitrate Respiration in the Extreme Thermophile Thermus thermophilus*

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A four-gene operon (nrcDEFN) was identified within a conjunctive element that allows Thermus thermophilus to use nitrate as an electron acceptor. Three of them encode homologues to components of bacterial respiratory chains: NrcD to ferredoxins; NrcF to iron-sulfur-containing subunits of succinate-quinone oxidoreductase (SQRO); and NrcN to type-II NADH dehydrogenases (NDHs). The fourth gene, nrcE, encodes a membrane protein with no homologues in the protein data bank. Nitrate reduction with NADH was catalyzed by membrane fractions of the wild type strain, but was severely impaired in nrc::kat insertion mutants. A fusion to a thermophilic reporter gene was used for the first time in Thermus spp. to show that expression of nrc required the presence of nitrate and anoxic conditions. Therefore, a role for the nrc products as a new type of membrane NDH specific for nitrate respiration was deduced. Consistent with this, nrc::kat mutants grew more slowly than the wild type strain under anaerobic conditions, but not in the presence of oxygen. The oligomeric structure of this Nrc-NDH was deduced from the analysis of insertion mutants and a two-hybrid bacterial system. Attachment to the membrane of NrcD, NrcF, and NrcN was dependent on NrcE, whose cytoplasmic C terminus interacts with the three proteins. Interactions were also detected between NrcN and NrcF. Inactivation of nrcF produced solubilization of NrcN, but not of NrcD. These data lead us to conclude that the Nrc proteins form a distinct third type of bacterial respiratory NDH.

Many facultative anaerobes adapt their respiratory electron-transport chains to changing environmental conditions by synthesizing specific primary dehydrogenases and final reductases (or oxidases) (1). As a paradigmatic example, Escherichia coli encodes up to 15 primary dehydrogenases and 10 final reductases, many of which appear to be biochemically redundant (2). However, these enzymes actually show differences, such as substrate affinity, or the ability to couple electron transport to proton or sodium extrusion (3), which are adapted to specific growth conditions. Therefore, there is a sophisticated regulatory network of transcriptional controls that finely tunes their expression according to environmental signals and leads to the coexistence of a limited number of dehydrogenase-reductase pairs in a given cell. Regulatory elements responding to dioxygen and to diverse electron acceptors are the major environmental factors responsible for such transcriptional tuning, allowing a hierarchical expression of final reductases and, in some cases, appropriate dehydrogenase(s), even when different electron acceptors are simultaneously present (3).

Two types of NADH dehydrogenases (NDHs) can function as the first component of respiratory chains in E. coli and other Enterobacteria. Type I NDH is a H+-translocating NDH: quinone reductase, homologous to the 42-subunit mitochondrial respiratory complex I (4). In E. coli, this enzyme consists of 14 subunits and a large number of prosthetic groups and redox centers (5). These 14 subunits are cotranscribed in a single mRNA, which is preferentially expressed under “energy-limited” growth conditions (anaerobic or microaerophilic) to provide a higher H+/e− ratio. Under aerobic conditions with highly reduced substrates (”high energy”), an alternative NDH:ubiquinone oxidoreductase, or type II NDH, plays the main role in supplying electrons to the respiratory chain. With the exception of the mitochondria from certain yeast and fungi, type II NDH homologues are absent from eukaryotes. Type II NDHs are monomeric enzymes (47 kDa) that do not couple NADH oxidation to proton pumping, thus keeping a lower H+/e− ratio under high energy growth conditions. Functionally, these enzymes are peripheral membrane proteins containing FAD as a cofactor, which can feed the electron transport chains by direct reduction of the quinone pool (5).

Most isolates of the ancient thermophilic bacteria Thermus spp. require oxygen to grow (6), despite the presence in their genome of genes encoding homologues to proteins involved in the anaerobic energy metabolism of other bacteria. In the recently sequenced HB27 strain of the aerobe Thermus thermophilus, a single type I NDH and a succinate dehydrogenase seem to be the major electron providers for respiratory chains toward either low (caa3) or high (bba3) affinity cytochrome c oxidases (7).

By contrast with the aerobic character of the HB27 strain, the closely related isolate T. thermophilus HB8 can grow anaerobically with nitrate as the electron acceptor (8). Such dissipative nitrate reduction ends with nitrite as the final product, and depends on the presence of a seven-gene operon (narCGHJK2), which encodes a respiratory nitrate reductase (NarGHI), a dedicated chaperone (NarJ), two proteins

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The abbreviations used are: NDH, NADH dehydrogenase; kbp, kilobase pair; NR, nitrate reductase; RT, reverse transcriptase; SQRO, succinate-quinone oxidoreductase; HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide.

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involved in nitrate/nitrite transport (NarK₁, NarK₂), and a di-heme cytochrome c (NarC), which is required for the synthesis of active enzyme (9, 10). The nar operon is actually encoded as part of a genetic element that could be transferred by conjugation to the aerobic strain HB27, allowing the exconjugant (HB27::nar) to grow anaerobically (11). Pulse-field gel electrophoresis revealed that a DNA fragment with a minimum size of 30 kb was transferred from the chromosome of the donor strain (HB8) to that of the receptor (HB27) during conjugation. As the size of the nar operon (~12 kb) was around a third of this, it seemed likely that genes related to regulation and/or the use of nitrate as an electron acceptor could also be encoded within this mobile DNA element. Therefore, we wondered whether anaerobically expressed genes could also form part of this element to render the receptor cells optimally suited for anaerobic growth.

In this work we describe the presence of a four-gene operon (nrcDEFN) within this mobile DNA element that confers on the receptor cells the ability to grow anaerobically. We demonstrate that the nrc operon encodes a dedicated new type of primary dehydrogenase that is synthesized in parallel with the nitrate reductase (NR), leading to a complete respiratory chain with nitrate as the final electron acceptor. We present evidence to support the hypothesis that the nrc operon makes the organism capable of a more efficient anaerobic growth by using nitrate as an electron acceptor.

### EXPERIMENTAL PROCEDURES

#### Strains, Plasmids, and Growth Conditions

Table I sets out the details of the description and origin of the bacterial strains and plasmids used in this work. E. coli was grown in LB (12) at 37 or 30 °C. Kanamycin (30 mg/liter), ampicillin (100 mg/liter), and/or chloramphenicol (20 mg/liter) were used as required. Aerobic growth of T. thermophilus was conducted at 70 °C with shaking (150 rpm) in TB (8). For plasmid and mutant selection, kanamycin (30 mg/liter) was added. For anaerobic growth, cells grown under aerobic conditions were inoculated in 10-ml cultures of TB with KNO₃ (40 mM), and subsequently incubated in 15-ml tubes filled to the top with mineral oil and screw-capped.

For induction of nitrate/anoxia-dependent promoters, cells were grown aerobically in TB without nitrate up to an A₅₅₀ of 0.4, and transcription was activated by the addition of KNO₃ (40 mM) and the simultaneous cessation of shaking. Because of the low solubility of oxygen at high temperatures, and its consumption by growing bacteria, unshaken cultures rapidly become anaerobic. Cells were kept in such conditions for 4–7 h before being processed.

T. thermophilus transformation was achieved on naturally competent cells (13). Transformation of E. coli was carried out as described (14). Plates containing the appropriate antibiotic(s) and concentrations were used for selection.

#### Nucleic Acid Techniques

DNA isolation, plasmid purification, restriction analysis, and plasmid construction were developed as described (15). Automatic methods (Applied Biosystems) were used for sequencing. RNA was purified with the Tri-Regent-Ls kit (Molecular Research Center, Inc., Cincinnati, OH). PCR and RT-PCR of the experiment shown in Fig. 3 were performed with the DNA polymerase from T. thermophilus, and the Retrotools kit as described by the manufacturer.
turer (BIOTTOOLs B&K, Madrid, Spain). For long mRNA, 2 ng of purified single-stranded DNA binding protein from T. thermophilus were added to improve the RT-PCR (16). For semiquantitative RT-PCR purification of single-stranded DNA binding protein from

**TABLE II**

| Name            | Sequence (5’-3’)                          | Purpose                   |
|-----------------|-------------------------------------------|----------------------------|
| ferred.ndeI     | ggtgcatactcggcgccagcgt                   | pET22narC, RT-PCR         |
| ferreda.rev     | ctactctccgaacggt                   | pET22narC, RT-PCR         |
| Orf F.dir       | gaggagacctatagggggtgtt                | RT-PCR                     |
| Orf F. rev      | teagagcctccgaacggt                   | RT-PCR                     |
| Orf N.dir       | gasagattggaaggagcgt                  | RT-PCR                     |
| Orf N.rev       | etaggggaggaacggt                  | RT-PCR                     |
| gdh1.nde1       | etaggggaggaacggt                  | PCR                        |
| gdh1.not        | etaggggaggaacggt                  | PCR                        |
| csaF2           | gtaggggaggaacggt                  | PCR                        |
| csaRInt         | gtaggggaggaacggt                  | PCR                        |
| 227-55          | ctaggggaggaacggt                  | Northern blot              |
| N371 Tt Kpn     | tggtagtatcgggaactcactatgtagt          | THS, pT18narC forward (KpnI) |
| N371 Tt Hind    | ttagatatcgggaactcactatgtagt          | THS, pT18narC reverse (HindIII) |
| H5              | gggctactcgggaactcactatgtagt          | THS, pT18narC forward (PstI) |
| H6              | ggtagactcgggaactcactatgtagt          | THS, pT25narC forward (BamHI) |
| H11             | aaaagagctcgggaactcactatgtagt          | THS, pT25narC reverse (EcoRV) |
| H12             | ggtgtagctcgggaactcactatgtagt          | THS, pT25narC reverse (EcoRV) |
| H13             | ctagggtgcagctcggtaactcactatgtagt      | THS, pT18narC forward (KpnI) |
| H14             | ctagggctgctcggtaactcactatgtagt        | THS, pT18narC reverse (EcoRV) |
| H15             | ctagggctgctcggtaactcactatgtagt        | THS, pT18narC forward (EcoRV) |
| H16             | ctagggctgctcggtaactcactatgtagt        | THS, pT18narC reverse (EcoRV) |
| H22             | ctagggctgctcggtaactcactatgtagt        | THS, pT18narC forward (PstI) |
| H23             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (PstI) |
| H24             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| H25             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |
| H28             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| H31             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |
| H32             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| H33             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |
| H34             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| H35             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |
| H36             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| H37             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |
| H38G            | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| H39             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |
| H40             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| H41             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |
| H42             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| H43             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |
| H44             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| H45             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |
| H46             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| H47             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |
| H48             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| H49             | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |
| NQOEcORI        | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC forward (PstI) |
| NQOBamHI        | ctagggctgctcggtaactcactatgtagt        | THS, pT25narC reverse (EcoRV) |

* THS, Two-hybrid system.

Cloning of the nrc Operon—The nrc operon was cloned from a T. thermophilus HB8 gene library constructed in AEMBL3 (17). **32P-Labeled probes from the 5’ extremity of the nar operon (narC gene) were used for screening. Positive clones were analyzed by enzyme restriction,** and adequate DNA restriction fragments were subcloned into pUC19 for sequencing purposes. The plasmid pUP3P (Table I), which contains the whole nrc operon, was selected.

Isolation of nrc Mutants—nrc::kat mutants were obtained by insertion of the **kat** gene, which encodes a thermostable resistance to kanamycin (18) at appropriate restriction sites of the target gene, always maintaining the same transcription direction of the operon to allow the expression of downstream genes. Previous work on the role of **narC** allowed us to ensure the transcription of downstream genes after insertion of the **kat** cassette (10). To inactivate **nrcF**, the **kat** gene was inserted into a XhoI site, which led to the truncation of the **NrcF** protein at amino acid position 151. **nrcN** was inactivated by inserting the **kat** gene at a PstI site, which caused truncation of the **NrcN** sequence at amino acid position 304. **nrcE** required the insertion of **kat** gene at a NcoI site, leading to a truncated **NrcE** protein of 310 amino acids that included the whole membrane domain. Plasmids containing the inserted **kat** genes were used to transform T. thermophilus HB8 to obtain the expected replacement after homologous recombination. Those colonies that grew after 48 h at 70 °C on plates containing kanamycin were analyzed for the presence of the expected mutation by PCR and Southern blot.

The suicidal plasmid pUPRB3gal was used for the insertion of the bgaA gene into nrcN. This plasmid is a pK18 (Table I) derivative that carries a DNA fragment for recombination that includes the whole **nrcF** gene and a region from +1 to 492 of the **nrcN** coding sequence, followed by the **bgaA** gene. This gene encodes a thermostable β-galactosidase from Thermus sp. T2 (19), preceded by the Shine-Dalgarno sequence of the **spA** gene, which codes for the S-layer protein of T. thermophilus HB8 (20). After transformation with this suicidal vector, those clones resistant to kanamycin were tested for the presence of the expected transcriptional fusion.

**Overproduction of NrcD and Antiserum Preparation**—To produce NrcD, its coding region was amplified by PCR (primers ferred.ndeI and ferred.rev, Table II) and cloned into the NdeI and EcoRI restriction sites of plasmid pET22b (Novagen) to render pET22nrcD. After induction of exponential cultures of transformed E. coli BL21/D3 cells with isopropyl-1-thio-D-galactopyranoside (1 mM), the soluble fraction was subjected to heat denaturation to yield a NrcD-enriched soluble fraction. Slices of 10% SDS-PAGE containing the overexpressed protein were sent to a private company (Charles River Laboratories, Chalaronne, France) for immunization of New Zealand rabbits. Antiserum was incubated with E. coli cells (DH5α) for 1 h and centrifuged before use.

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**RESULTS**

Cloning the nrc Cluster

The use of a probe against the narC gene in a λEMBL3 gene library of the facultative anaerobe *T. thermophilus* HB8 allowed the cloning of a large DNA region upstream of the *nar* operon. Its sequence revealed the presence of a type II NDH homologue and components of electron transport chains in the same DNA strand as the *nar* operon. A ~3500-bp DNA fragment from this region, for which the 3‘ extremity was located 4.6 kbp upstream from *narC*, was subcloned into pUC119 to yield pUP91b, and sequenced. Based on their physical proximity to the *nar* operon and their similarity to electron transport components (see below), the genes were named *nrc* (nitrate respiration) (Fig. 1). When the sequence of the *nrc* cluster (accession number AJ858200) was compared with the genome of the aerobic strain *T. thermophilus* HB27 (accession numbers AE017221 and AE017222), no positive hits were found, implying that this cluster was specific to facultative anaerobic strains of *T. thermophilus*.

The nrc Cluster Is Transferred by Conjugation Along the nar Operon

The putative genetic linkage between the *nrc* gene cluster and the *nar* operon was checked by PCR. As shown in Fig. 2, amplification products corresponding to the first (*nrcD*) and the last (*nrcN*) genes of the *nrc* cluster were identified in the facultative anerobe HB27::nar, and HB8 (lanes 2 and 3), but not in the aerobic HB27 (lanes 1). Identical results were obtained when a gene from the *nar* operon (*narH*) was assayed. By contrast, the *gdh* (glutamate dehydrogenase) and *csaB* (cell-surface anchoring) genes, which are not involved in energy metabolism, were identified in the three strains. Therefore, the *nrc* cluster was part of the conjugative genetic element transferred by conjugation along the *nar* operon during the isolation of the facultative anaerobic strain HB27::nar.

The Four nrc Genes Are Cotranscribed

The sequence and organization of the *nrc* genes suggested that they were cotranscribed into a single mRNA. To check this, we isolated total RNA from aerobically grown and nitrate/anoxia-induced cultures (“Experimental Procedures”) of *T. thermophilus* HB8, and tested for the presence of *nrc* mRNA by Northern blot and RT-PCR.

As shown in Fig. 3A, an mRNA of around 3.2 kb was detected in cells subjected to anoxic conditions with nitrate, but not in cells grown aerobically. Data from RT-PCR (Fig. 3B) confirmed these results and revealed the cotranscription of the four genes.

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*J. L. Ruiz, F. Cava, J. Ferrer, J. Berenguer, and M. J. Bonete, unpublished results.*
expression of the reporter gene in the nrcN::bgaA mutant (aerobic cultures). Under anoxia (anaerobic cultures) the presence of nitrate (N) was required for induction. The inducer effect of nitrate could not be mimicked either by fumarate (F) or by nitrite (Ni). The use of a combination of nitrate and fumarate or nitrite did not improve the effects of nitrate alone (not shown).

Similarly, induction under anoxia and nitrate was not affected by the presence of glucose or succinate. Consequently, we inferred that transcription of the nrc operon was dependent on nitrate and anoxia, but that it did not respond to the carbon source. Therefore, a specific role for the nrc-encoded proteins in nitrate respiration was concluded.

Analysis of the Proteins Encoded by the nrc Cluster

The four open reading frames encoded by the nrc cluster (Fig. 1) were preceded by respective Shine-Dalgarno sequences (GGA/GA/GA/GA) located between positions −11 and −9 with respect to their ATG start codons. With the exception of NrcN, the distance between the stop codon of one gene and the ATG start codon of the next was quite small, even overlapping by one base in the case of nrcE-nrcF. However, in nrcN, this distance was apparently longer, but the presence of an in-frame GTG codon preceded by a putative Shine-Dalgarno sequence close to the end of NrcF suggests that this could be the actual translational start point of the protein.

Computer predictions suggest a cytoplasmic location for the translation products of all these genes, except for NrcE, for which an integral membrane protein containing seven putative transmembrane spanning helices is strongly suggested (see below). This putative membrane protein has no homologues in the protein data banks, unlike the significant sequence similarities of NrcD, NrcF, and NrcN and proteins involved in electron transport and NADH oxidation (Table III). Phylogenetic analysis situates these Nrc proteins at the root of the corresponding phylogenetic trees. The essential sequence information about these proteins is provided below.

NrcD—NrcD encodes an 87-amino acid acid-long protein (9917 Da) highly similar (86% identity) to the soluble seven-iron ferredoxin from _T. thermophilus_ (FdT, 79 amino acids long, accession number P03942), whose three-dimensional structure is known (26). Sequence alignment of both proteins revealed a complete conservation of the cysteine residues that coordinate the [3Fe-4S] and [4Fe-4S] clusters of FdT. The major difference between them is the presence of a 10-amino acid C-terminal sequence in NrdC that is absent from FdT.

NrcE—NrcF is predicted to be a 26,223-Da protein similar in sequence to the iron-sulfur subunit (B subunit) from succinate-quinone oxidoreductases (SQRs) and quinol-fumarate reductases. The cysteine residues involved in the coordination of the [2Fe-2S], [4Fe-4S], and [3Fe-4S] clusters of the B subunit from

Nitrate and Anoxia Are Required for Transcription of nrc

To check if nitrate or anoxia were independently able to induce expression of the nrc operon, and to quantify the induction levels achieved, we developed a new suicidal vector that carries a reporter gene (bgaA) encoding a thermophilic β-galactosidase preceeded by its own Shine-Dalgarno sequence. A fragment of nrcN was placed upstream of the reporter as a recombination target. Insertion of this construction into the chromosome was selected by the thermostable resistance to kanamycin conferred by the bga gene located in the suicidal plasmid (“Experimental Procedures”). The mutant strain nrcN::bgaA, the reporter gene was located at position 483 of nrcN (Fig. 1), giving rise to a transcriptional fusion nrcN::bgaA.

As shown in Fig. 4, the presence of oxygen repressed the
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TABLE III

Comparison of Nrc protein sequences

| Product | Size (aa) | Homologue | E value | Identity |
|---------|-----------|-----------|---------|----------|
| NrcD    | 87        | Ferredoxin (P03942) from *T. thermophilus* | 1e-37   | 65/75 (56%) |
| NrcE    | 362       | ND⁴       | 1e-70   | 128/217 (58%) |
| NrcF    | 232       | Iron-sulfur subunit of succinate dehydrogenase/fumarate reductase from several organisms | 6e-75   | 176/406 (43%) |
| NrcN    | 395       | NADH dehydrogenase from *Xanthomonas axonopodis* |         |          |

⁴ Number of amino acids in the protein.
⁵ Only the protein with the highest similarity is indicated.
⁶ Only the highest scores are shown.
⁷ Protein without homologues in any data bank.

both enzymes are fully conserved in NrcF, as does residue Asp⁶¹, which is involved in the coordination of the [2Fe-2S] cluster. NrcE—NrcN is homologous to type II NDH (EC 1.6.99.3) of different origins. This protein family contains a pyridine nucleotide-oxidoreductase motif consisting of a small NADH binding domain within a larger FAD-binding domain. As is frequently the case with other thermophilic proteins, NrcN (395 amino acids) is smaller than its mesophilic counterparts (450 to 465 amino acids).

NrcE—The nrcE gene encodes a 362-amino acid-long protein (40,388 Da) without homologues in the protein data banks. Topology predictions suggest the presence of seven-transmembrane α-helices, with a small N-terminal region facing the periplasm, and a larger (110 amino acids) C-terminal cytoplasmic domain, which contains four cysteine residues that could coordinate a redox center. Unexpectedly for a thermophilic protein, a fifth cysteine residue is present within the sequence of the first transmembrane α-helix.

**Location of Nrc Proteins**

Immunodetection of NrcD revealed lower electrophoretic mobility (~30 kDa) than expected from its predicted size (~10 kDa), independently of the bacterial host in which it was expressed (Fig. 5). As no signal was detected in the aerobic strain (HB27, not shown) the specificity of the antisera for NrcD was guaranteed.

As shown in Fig. 5, NrcD was overproduced as a soluble protein in *E. coli* (Ec), as predicted by computer. Moreover, as it remained soluble after 30 min of incubation at 70 °C, its thermostability was also assessed. By contrast, NrcE was associated with the membrane fraction of nitrate/anoxia-induced cultures of *T. thermophilus* (lanes M). Therefore, the existence of an interaction with a membrane component in the thermophile was concluded. As the membrane location of NrcD was not affected in nrcF::kat and nrcN::kat mutants, neither NrcF nor NrcN could be responsible for attachment of NrcD to the membrane. Conversely, NrcD was not detected in the membrane fractions of nrcE::kat mutants, and only faint signals of NrcD and of small proteins were detected in its soluble fraction. Therefore, we concluded that NrcE anchors NrcD to the membrane, and that solubilization of NrcD results in degradation of the protein.

Oxidation of NDH provided some clues concerning the location of NrcN and NrcF. Membrane fractions of nitrate/anoxia-induced cultures exhibited significant NDH oxidative activity in the presence of nitrate (Fig. 6B). In the absence of nitrate, NADH oxidation by these membrane fractions was negligible. By contrast, the presence of nitrate did not affect NADH oxidation by membrane fractions of aerobically grown cells (data not shown).

Interestingly, parallel assays revealed low NADH oxidation activity (~1/10 of that of the membrane) in soluble fractions of nitrate/anoxia-induced wild type cells. This soluble activity was not nitrate-dependent and disappeared completely in nrcN::kat and nrcN::bgaA mutants. By contrast, there was a 3-fold in-

**Nitrate Reduction by Membrane Fractions of nrc Mutants**

To test whether the Nrc proteins were directly involved in electron transfer from NADH to the nitrate reductase, membrane fractions from cultures of the wild type strain and the nrc mutants grown for 7 h under anaerobiosis in TB medium containing nitrate were tested for the production of nitrite with NADH as the electron donor. Reduced methyl viologen, a direct electron donor to the αβ complex of the NR (27), was used as control for the amount of active NR in the membrane fractions.

As shown in Fig. 6, NADH was a highly efficient electron donor for nitrate reduction (286 ± 36 nmol of nitrite/min mg of protein). Reduction of nitrate with NADH was decreased 10-fold in nrcE::kat, nrcN::kat, and nrcN::bgaA mutants. Nitrate reduction in nrcF::kat mutants decreased by a factor of five (Fig. 6A). A similar pattern was observed for the oxidation of NADH: membranes from nitrate/anoxia-induced cultures of the wild type exhibited NADH-oxidation activity (353 ± 11 nmol of NADH consumed/min mg of protein) in accordance with the amount of nitrate produced (296 ± 36 nmol of nitrite/min mg of protein), whereas NADH consumption was effectively undetectable in all but the nrcE::kat mutant, in which it decreased by a factor of 10. These findings imply that the Nrc proteins constitute a membrane-bound NDH required for nitrate reduction. It is also clear from these results that a quantitatively less important Nrc-independent pathway for nitrate reduction is also present on the membranes of *T. thermophilus* HB8 during anaerobic respiration. Interestingly, membranes isolated from cell cultures of each of the mutants grown for shorter times (2–4 h) under nitrate/anoxia presented a higher percentage of NDH and nitrate reduction residual activities (data not shown), thus showing that this Nrc-independent pathway decreases in relevance during anaerobic growth. This fact is related with the repression of the transcription of the *ngo* operon, encoding type I NDH, detected by RT-PCR (Fig. 6C).
Panel C

The percentage of nitrite production (B) with respect to that of the wild type strain were represented. Panel C, RT-PCR on 20 ng of total RNA from an aerobic culture (1 and 2) and from a culture grown for 7 h under nitrate/anoxia conditions (3 and 4). The mRNA encoding the subunit 8 of type I NDH (1 and 3) was detected with primers NQOEcoR1 and NQOBamH1. The mRNA coding for NrcD (2, 4) was detected with primers ferred.nde1 and ferrede.rev. The sizes of the amplified fragments are indicated in bp.

Quinones Mediate Electron Transfer between the Nrc and NR Complexes

To establish whether electron transfer between NADH and nitrate was mediated by the respiratory quinones, different concentrations of inhibitors such as rotenone, diuron, and HQNO were used, and the corresponding inhibitory concentrations compared with that required to inhibit NADH oxidation by membrane fractions of cells grown aerobically (type I NDH). Similar concentrations of diuron and HQNO (0.2 μM) were required for complete inhibition of the membrane-bound NDH activity from aerobically grown cells (type I NDH) and from cells grown for 7 h under nitrate/anoxia conditions (Nrc-NDH). In contrast, a small difference in rotenone sensitivity was found: at 0.1 μM, NDH from aerobic membranes (type I) was completely inhibited whereas 50% NADH oxidation was still detected in nitrate/anoxia-induced membranes (Nrc). At 0.2 μM, rotenone completely inhibited NADH oxidation by aerobic membranes, but a 20% of activity was still detected with “anaerobic” membranes (Fig. 6B). At 2 μM, both type I and Nrc NDH were completely inhibited. On the other hand, nitrate reduction was insensitive to such inhibitors when reduced methyl viologen, which donates the electrons directly to the NDH complex, was used as electron donor (data not shown).

Interactions between the Nrc Proteins

We used a bacterial two-hybrid system (23) to test for putative interactions between Nrc proteins. Fig. 7 shows the mean results of six measurements of β-galactosidase activity in cells transformed with pairs of plasmids derived from pT18 and pT25 that express the assayed Nrc proteins as fusions to the C-terminal (Nrc-T18) and N-terminal (T25-Nrc) domains of the adenylate cyclase from B. pertussis (23). All the nrc genes were cloned in both plasmids to avoid specific effects because of their positions at the N or C terminus of the fusion proteins. Negative controls (<100 units) were performed for each of the Nrc fusions and the complementary T18/T25 fragment fused to a leucine zipper domain (Zip). Three kinds of positive control were used: the leucine zipper fusions to the T18 and T25 fragments (~1000 units), and two pairs of thermophilic proteins from T. thermophilus HB8, the NarG subunit of NR and its chaperone NarJ (~280 units), and the glutamate dehydrogenase, an homo-oligomeric enzyme (~400 units).

Interaction was also detected between NrcE and NrcD (~600 units). Weaker but positive signals were detected between NrcE and NrcF (~200 units) and between NrcE and NrcN (~300 units). However, no interactions were detected between NrcD and NrcF or NrcN (~100 units). On the other hand, NrcN and NrcF (~300 units) were also shown to interact to a similar extent as do NarG and NarJ. All these results were independent of the direction used in the assays (i.e. Nrc proteins expressed as N- or C-terminal parts of the fusion proteins). As NrcE seemed to interact with all the other Nrc proteins, we decided to check whether such interactions were dependent on its C-terminal domain, which is predicted to face the cytoplasm (amino acids 264 to 362). For this, the C-terminal domain of NrcE (c-NrcE) was fused to plasmids pT25 and pT18 and used in the same assays. As shown in Fig. 7, the results for c-NrcE were almost identical to those obtained for the entire protein, indicating that the interactions between NrcE and the other Nrc proteins take place through its cytoplasmic C-terminal domain.

The Role of the nrc Operon in Nitrate Respiration

To analyze the role of nrc in vivo, the growth of mutants in nrcE (nrcE::kat), nrcF (nrcF::kat), and nrcN (nrcN::kat) under anaerobic or aerobic conditions was assayed. As illustrated in Fig. 8D, all mutants grew anaerobically with nitrate after 24 h, implying that the nrc cluster was not an absolute requirement for anaerobic growth. However, when each mutant was cocultivated with the wild type strain, the latter outgrew each of them (~15% of mutants remaining in the culture) after 24 h of anaerobic growth. This growth bias was the consequence of a slower growth rate of all mutants compared with the wild type under such anaerobic conditions (Fig. 8C).

Conversely, when the competition experiments were developed under aerobicosis, no significant population bias was detected (Fig. 8A). Therefore, the nrc operon confers a selective advantage only during anaerobic growth with nitrate.
units that can couple quinone reduction to proton extrusion during respiration. In the genome of the aerobic strain *T. thermophilus* HB27, a single type I NDH is encoded, and is currently used as a model for the structural analysis of this type of enzyme (7, 28). In many other bacteria, but not in aerobic strains of *T. thermophilus*, the so-called alternative or type II NDH can also reduce the membrane quinones under specific growth conditions (3). These are simple, monomeric enzymes, which interact directly with the membranes and do not extrude protons (3, 29). What we describe in this article is a new type of respiratory NDH that is expressed concomitantly with nitrate reductase, leading to the production of a complete respiratory chain. The presence of their respective encoding operons in a conjugative plasmid incorporated into the chromosome (11) implies that they constitute the first description of a mobile “respiratory island.” Specific points reinforcing these conclusions are discussed below.

**NrcN Is the Catalytic Subunit of a Multimeric NDH**—Although it was not possible to produce NrcN in an active form in *E. coli* or to assay the activity of the recombinant protein directly (not shown), the role of NrcN as the catalytic subunit of a NDH was first inferred from the presence of a pyridine nucleotide-oxidoreductase motif in its sequence, and from its similarity to monomeric respiratory type II NDH sequences. Further confirmation of this role was provided by the analysis of the NADH-oxidative capability of the membrane and soluble fractions of cells grown with nitrate under anoxic conditions, where our data showed a clear relationship between NADH oxidation and NrcN integrity.

It is interesting to note that NADH oxidation by membrane fractions of nitrate/anoxia-induced cells of the wild type strain was completely dependent on the presence of nitrate and proportional to the amount of nitrite produced. Given that mutations in *nrcN*, the final gene of the operon, made NADH oxidation undetectable and severely decreased nitrite production, it was concluded that NrcN is responsible for this membrane-associated NDH activity.

The nature of NrcN as a subunit of a multimeric enzyme was deduced from the effect of *nrcF* and *nrcE* mutations. In both cases, NADH oxidation by membrane fractions was severely decreased, leading to the conclusion that NrcE and NrcF are proteins associated to NrcN that are required for its membrane-bound NDH activity.

Interestingly, an increase in nitrate-independent NADH oxidation was found in the soluble fraction of *nrcE* and *nrcF* mutants. As there was no such activity in *nrcN* mutants and was very weak in the wild type strain, we concluded that it was associated with a solubilized NrcN protein. Therefore, NrcN has its own NADH oxidative capability that is specific for nitrate as the electron acceptor when bound to the membrane through NrcE and NrcF, but which becomes nitrate-independent upon mechanical (in the wild type) or genetic (*nrcE* and *nrcF* mutants) solubilization.

**A Tentative Model for Nrc-NDH**—A tentative model for the oligomeric structure of Nrc-NDH emerges from our data (Fig. 9), by which NrcE functions as a membrane anchor for the three other proteins. This is based on the computer-predicted presence of seven-membrane spanning helices within its sequence, and on the observed solubilization of NrcD and the NrcN-associated NDH activity in the absence of NrcE. The implication of the C-terminal cytoplasmic domain of NrcE in such subunit anchoring was first deduced from the position of the *kat* gene cassette close to the 3′ terminus of the gene in the *nrcE::kat* mutant, which should allow the synthesis of a truncated, 310-amino acid long NrcE protein. Further evidence was obtained from the results of the two-hybrid assays, which revealed specific interactions between this C-terminal domain of NrcE and NrcD, NrcF, and NrcN.

Concomitantly, several lines of evidence support the computer-predicted soluble nature of the other components of the Nrc complex. The soluble nature of NrcN was deduced from the increase in the aforementioned solubilization of NDH activity, and of NrcD, through their detection by Western blots in *nrcE* mutants. The soluble nature of NrcF can be deduced indirectly. First, NrcN is solubilized in *nrcF* mutants, suggesting an NrcN-NrcF interaction, which was subsequently confirmed by the two-hybrid assays. Second, NrcN is also solubilized in *nrcE* mutants. Finally, a complex between the A and B subunits of the respiratory SQR, homologues of NrcF and NrcN, respectively, can be solubilized in *E. coli* (Ref. 30, see below). Consequently, the most probable explanation of our results is that NrcF forms a soluble complex with NrcN in *nrcE* mutants. The presence of NrcD in this soluble complex cannot be ruled out by our data, but the absence of detectable interactions with NrcD, NrcF, and NrcN, and its rapid degradation in *nrcE* mutants, makes this unlikely.

This Nrc enzyme model (Fig. 9) is distinct from that of the multimeric (13–14 subunits) respiratory type I NDH (28) and, also from that of the monomeric alternative type II NDH (29). By contrast, it shares some sequence and structural similarities...
NrcE catalyzes the reduction of a membrane quinone at the cytoplasmic face of the membrane. Reduced quinones will then travel toward the NR, where they will be oxidized at the periplasmic face of the membrane. The absence of conserved sequence motifs for quinone binding sites among quinol reductases prevents us proposing a protein domain in NrcE as responsible for such quinone reduction. However, the observed inhibition of nitrate reduction and NADH oxidation by rotenone, diuron, and HQNO in our *in vitro* experiments strongly supports this model, according to which quinones shuttle electrons between the Nrc and NADH complexes (4).

The quinone most likely to be involved in this process is menaquinone-8, which is by far the most important respiratory quinone in *T. thermophilus* (32). In fact, no ubiquinone has been found in *T. thermophilus* (32), and homologues of UbiC and other enzymes implicated in the synthesis of ubiquinone are not encoded among the sequence of *T. thermophilus* HB27 (7), being also not found among the 22 kbp of the nar element so far sequenced. Therefore, the Nrc and the NDH-I complexes likely use the same quinones. In support of this conclusion is the fact that the amount of residual nitrate reduction with NADH as electron donor in nrcN::kat mutants, where only NDH-I is functional, decreases along the time when cells are grown under nitrate/anoxia conditions. Thus, it follows that the quinone reduced by NDH-I, essentially menaquinone-8 (28, 32), is oxidized by the NR. Consequently, it is unlikely that the specificity of nitrate reduction by Nrc-mediated NADH oxidation was based on the use of different quinones, but more probably on the repression of NDH-I expression, as revealed by the semiquantitative RT-PCR assays of Fig. 6C.

**Nrc Is Specific for Nitrate Respiration**—The model discussed above clearly shows that the Nrc complex constitutes a membrane-bound NDH that is structurally different from both type I and type II NDH dehydrogenases. Its involvement in nitrate respiration is based on three arguments: 1) its dependence on nitrate and anoxia for expression; 2) the NADH-dependent in *vitro* assays of nitrate reduction; and 3) the lower growth rates of nrc mutants under anaerobic conditions.

The insertion of a thermostable reporter gene in *nrcN* allowed us to define the requirements for transcription of the *Pnrc* promoter. In fact, as the NR can be easily detected by the production of nitrite (8), we were able to confirm that the expression of the *nrc* operon in *nrcN::bgaA* mutants was
parallel to the expression of the NR. Such coordinated synthesis probably depends on signal-transduction systems that respond to nitrate and anoxia, similarly to the FNR and NarL/NarX two-component systems described for E. coli (3), although neither FNR nor NarL binding sequences are found around the Parc and Pnar promoters. However, the genes coding for transcription regulators involved in the expression of the Pnar promoter are transferred by conjugation along the nar operon (33). Accordingly, we have identified a homologue to DNR transcription factors encoded between the nar and nrc operons, but no NarL/NarX homologues could be identified neither in the conjugative element nor in the genome of T. thermophilus HB27 (7). The DNR transcription factors constitute a subgroup of the CRP/FNR protein family that are specifically tuned for nitrate respiration.

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