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Anaerobic Growth, a Property Horizontally Transferred by an Hfr-Like Mechanism among Extreme Thermophiles

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Despite the fact that the extreme thermophilic bacteria belonging to the genus *Thermus* are classified as strict aerobes, we have shown that *Thermus thermophilus* HB8 (ATCC 27634) can grow anaerobically when nitrate is present in the growth medium. This strain-specific property is encoded by a respiratory nitrate reductase gene cluster (*nar*) whose expression is induced by anoxia and nitrate (S. Ramírez-Arcos, L. A. Fernández-Herrero, and J. Berenguer, Biochim. Biophys. Acta, 1396:215–1997). We show here that this *nar* operon can be transferred by conjugation to an aerobic *Thermus* strain, enabling it to grow under anaerobic conditions. We show that this transfer takes place through a DNase-insensitive mechanism which, as for the Hfr (high frequency of recombination) derivatives of *Escherichia coli*, can also mobilize other chromosomal markers in a time-dependent way. Three lines of evidence are presented to support a genetic linkage between *nar* and a conjugative plasmid integrated into the chromosome. First, the *nar* operon is absent from a plasmid-free derivative and from a closely related strain. Second, we have identified an origin for autonomous replication (*oriV*) overlapping the last gene of the *nar* cluster. Finally, the mating time required for the transfer of the *nar* operon is in good agreement with the time expected if the transfer origin (*oriT*) were located nearby and downstream of *nar*.

Most extreme thermophiles that live in geothermal environments are strict anaerobes (3, 11) as a consequence of the adaptation to the low solubility of oxygen at these temperatures. However, members of the genus *Thermus* constitute an exception to this general rule, being described taxonomically as strictly aerobic chemorganotrophs (2).

However, we recently showed that one of the most thermophilic isolates of this genus, *Thermus thermophilus* HB8, was able to grow anaerobically when nitrate was present in the medium. Biochemical and genetic evidence demonstrated that this ability was related to the synthesis of a membrane-bound respiratory nitrate reductase complex whose protein components, the α (NarG; 136 kDa), β (NarH; 57 kDa), and γ (NarI; 28 kDa) subunits, were homologous (about 48 to 50% sequence identity) to those from mesophilic facultative anaerobes (e.g., *Escherichia coli*). The genes encoding these subunits were located within a single operon (*nar*) that was induced under low oxygen concentrations when nitrate was present (21). In contrast to those described for most nitrate reducers, the product of nitrate respiration was secreted to the growth medium through an unknown transporter.

We also observed that even a closely related strain, such as *T. thermophilus* HB27, was unable to grow under such anaerobic conditions (21). Since the main difference between strains HB8 and HB27 of *T. thermophilus* is the absence of plasmids from the latter, the possibility that the *nar* operon could be encoded by a transferable genetic element, such as a plasmid, was considered.

In this article, we analyze this possibility and demonstrate that the ability to grow by nitrate respiration can be transferred to the aerobic strain *T. thermophilus* HB27 by conjugation. We also relate this ability to the integration of a *nar*-carrying conjugative plasmid into the chromosome of *T. thermophilus* HB8. Moreover, we show that, as for the Hfr strains of *E. coli*, this integrated plasmid can also mobilize other chromosomal genes in a time-dependent way.

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MATERIALS AND METHODS

Bacterial strains, phages, plasmids, and growth conditions. *T. thermophilus* HB8 (ATCC 27634) and a *Thermus* sp. strain (ATCC 27737) were obtained from the American Type Culture Collection (Rockville, Md.). *T. thermophilus* HB27, *Thermus aquaticus* YT1 (ATCC 25104), and *T. thermophilus* BPL7 were generously provided by Y. Koyama, M. Bothe, and J. Fee, respectively. The mutant strains *T. thermophilus* HB8 *slrA*::*kat* (9), *T. thermophilus* HB8 *slpM*::*kat* (9), and *T. thermophilus* HB8 *narGH*::*kat* (21) were used for interrupted-mating experiments. *E. coli* JM109 [K-12 *supE44* Δ(*lac-proAB*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* F′ (*traD36 proAB*$^+$ *lacI*^{q}*ZΔM15*)] and TG1 [*supE* Δ(*nsdM-mcrB*)*5* (r_K^- McrB$^-$) *thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q}*ZΔM15*)] were used for cloning. Plasmids pUC119 (27) and pKT1 (15), which contains a gene cassette encoding a thermostable kanamycin nucleotidyltransferase (*kat*), were used for subcloning and for the identification of *oriV*, respectively. Plasmid pMK18 (6) is a bifunctional *E. coli-Thermus* shuttle vector used as a control in transformation experiments.

For *nar* induction assays, static (microaerophilic) or stirred (aerobic) cultures of *T. thermophilus* HB8 were obtained by use of a rich medium (TB) containing 8 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 4 g of yeast extract (Oxoid, Hampshire, England), and 3 g of NaCl per liter of water, adjusted to pH 7.5 (8) and supplemented with 20 mM KNO_3 when necessary. Anaerobic cultures were obtained by growing cells in the same medium in 10-ml tubes filled to the top with mineral oil (21). Kanamycin (30 μg/ml) or chloramphenicol (20 μg/ml) was added for selection when needed.

DNA isolation and labeling. Standard methods were used to purify, analyze, and manipulate DNA (22). Uniformly ^{32}P-labeled DNA probes were obtained by use of random hexanucleotide primers, [α-^{32}P]dCTP (3,000 Ci/mmol) (Amersham Ibérica, Madrid, Spain), and the Klenow fragment of DNA polymerase I. Essentially, 150 to 200 ng of DNA probe was denatured by boiling and added to a 50-μl reaction mixture containing the hexanucleotides (6 units of optical density at 260 nm [OD_{260}]/ml), dATP, dGTP, and dTTP (0.1 mM each), β-mercaptoethanol (6 mM), $MgCl_2$ (20 mM), and bovine serum albumin (0.4 mg/ml) in 50 mM Tris-HCl–200 mM HEPES buffer (pH 7). The Klenow fragment (2 U) and 25 mCi of [α-^{32}P]dCTP (3,000 Ci/mmol) were added to this mixture, and the labeling reaction was developed at 30°C for 10 h. After this period, unincorporated nucleotides were removed by chromatography in a Sephadex G-50 column. The radiactive probes used were as follows: probe A is a 1.2-kbp *Bam*HI fragment containing 3′ and 5′ regions of *narG* and *narH*, respectively (21), and probe B is a 3.5-kbp *Kpn*I/*Sal*I fragment from plasmid pNIT9kat (this work).

PFGE. After *T. thermophilus* cells were placed in 1% (wt/vol) agarose, intact DNA was obtained by the method described by Marin et al. (17). Plasmids were removed from these agarose plugs by pulsed-field gel electrophoresis (PFGE) for

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36 h at 170 V/cm and a pulsing time of 150 s in 1% (wt/vol) agarose (SeaKem LE agarose; FMC) in TBE buffer (100 mM Tris, 100 mM boric acid, 0.2 mM EDTA [pH 8]) by use of a contour-clamped homogeneous electric field system (Pulsaphor apparatus; LKB) (4). Plugs containing only chromosomal DNA were then treated in situ with restriction enzyme *Nde*I (New England BioLabs) as described previously (17, 24), and the fragments obtained were subsequently separated by PFGE for 36 h at a field strength of 10 V/cm and a pulsing time of 40 s. After ethidium bromide staining, the sizes of the DNA fragments were calculated by comparison to lambda phage concatemers (50 kbp per monomer).

Southern blot analysis. Essentially we followed the protocol described by Sambrook et al. (22). DNA fragments separated by agarose gel electrophoresis were capillary transferred for 16 h to a nylon membrane (Hybond N; Amersham) in 20× SSC buffer (1× SSC is 0.15 M NaCl plus 20 mM sodium citrate). After UV cross-linking, hybridization was carried out for 16 h at 42°C with ~2×10^6 cpm of an appropriate ^{32}P-labeled DNA probe (see above) in 10 ml of hybridization solution (6× SSC, 0.1% sodium dodecyl sulfate [SDS], 100 mg of denatured salmon sperm DNA per ml). Finally, nonspecifically bound probe was removed by washing in 2× SSC–0.1% SDS at 62°C, and the remaining radioactivity was detected by autoradiography at −70°C.

Construction of pNITkat plasmids and transformation. The *kat* gene, encoding the thermostable kanamycin nucleotidyltransferase, was isolated from plasmid pKT1 and inserted into the *Pst*I restriction site of the *narI* gene (21) to obtain plasmid pNIT9kat. From this plasmid, a 3.5-kbp *Kpn*I/*Hin*dIII DNA fragment containing regions downstream of *nar* was cloned into the corresponding restriction sites of pUC119 to obtain plasmid pNIT5. Then, plasmids pNIT5kat1 and pNIT5kat2 were obtained by partial digestions with *Sma*I, followed by replacement with the *kat* gene cassette. Later, plasmids pNIT5kat1XSI and pNIT10kat were obtained from pNIT5kat2 by deletion of 0.6-kbp *Xho*I/*Sal*I and 1.4-kbp *Eco*RI/*Hin*dIII DNA fragments, respectively.

Natural competence in *T. thermophilus* was induced by growing cells at 70°C with aeration to an OD_{550} of 0.5 in TB containing 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Then, 0.5-ml aliquots of the culture were made, and plasmid DNA (1 μg) was immediately added. After 2 h of incubation, cells were plated on kanamycin (30 μg/ml)-containing plates and further incubated for 24 to 48 h at 70°C to obtain colonies. *E. coli* cells were made competent and transformed as described previously (5, 19).

DNA sequencing and computer analysis. Plasmid DNA was sequenced by the dideoxy chain termination method (23). For the manual method, we used 7-deaza-dGTP, modified T7 DNA polymerase (Sequenase 2.0; U.S. Biochemicals), and [α-^{35}S]dATP (1,000 Ci/mmol) (Amersham). For the automated method, sequences were obtained with an Applied Biosystems sequencer. Universal primers for M13mp and pUC vectors and oligonucleotides synthesized from partial sequences (Isogen Bioscience, Maarshen, Holland) were used for priming. Partial sequences were overlapped and analyzed with Wisconsin Genetics Computer Group software (7). Both strands of the template DNA were completely sequenced.

Horizontal transfer of the *nar* operon. A chloramphenicol-resistant strain (*T. thermophilus* HB27Cam^r) was isolated directly by plating 10^8 *T. thermophilus* HB27 cells on plates containing chloramphenicol (20 μg/ml). For mating experiments, the donor (*T. thermophilus* HB8) and recipient (*T. thermophilus* HB27Cam^r) strains were mixed in a 100:1 (receptor/donor) ratio in 100 ml of TB medium containing KNO_3 (20 mM) and incubated for 8 h at 70°C with low-speed stirring (100 rpm). A 500-μl sample of the culture was then inoculated into 100 ml of medium containing KNO_3 (20 mM) and chloramphenicol (20 μg/ml) and incubated overnight at 70°C without stirring to allow the selection of exconjugants. Finally, cells from this culture were inoculated into capped test tubes containing 10 ml of nitrate medium overlaid with mineral oil up to the screw cap and incubated for 24 h at 70°C. The latter process was repeated five times to guarantee the isolation of an anaerobic culture. Parallel negative controls in which both donor and recipient strains were incubated separately and subjected to the same selective procedure were developed.

For interrupted mating, donors (different *T. thermophilus* HB8::*kat* derivatives) and recipients (*T. thermophilus* HB27Cam^r) were mixed as described above in a medium containing 6 mM $MgCl_2$ and DNase I (100 μg/ml) to exclude the possibility of natural competence-mediated transfer. Aliquots (300 μl) of the mixtures were incubated at 70°C for different times before being vortexed vigorously. Then, 200 μl from each tube was added to 300 μl of prewarmed medium containing chloramphenicol (30 μg/ml) and incubated for 3 h at 70°C under strong aeration. Finally, 200 μl of this mixture was spread on kanamycin (30 μg/ml) and chloramphenicol (20 μg/ml) agar plates and incubated for 48 h at 70°C.

SDS-PAGE and Western immunoblotting. Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (14). For Western blotting, an antiserum raised against a C-terminal fragment of NarG was used (21). Specifically bound antibodies were detected with the enhanced chemiluminescence Western blotting analysis system from Amersham International.

Nitrate reductase activity. The nitrate reductase activity of triplicate cell samples corresponding to an OD_{550} of 0.6 was measured after permeabilization with tetradecyltrimethylammonium (20% [wt/vol]) with methyl viologen as the electron donor (21, 25). One enzyme unit under these conditions was defined as the amount that produced 1 nmol of nitrite per min at 80°C.

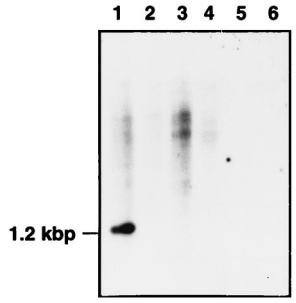

FIG. 1. The *nar* operon is strain specific. Southern blot of *Bam*HI-digested total DNA of *T. thermophilus* HB8 (lane 1), *T. thermophilus* HB8 *narGH*::*kat* (lane 2), *T. thermophilus* BPL7 (lane 3), *T. thermophilus* HB27 (lane 4), *Thermus* sp. strain ATCC 27737 (lane 5), and *T. aquaticus* YT1 (ATCC 25104) (lane 6). The *nar* operon was detected with ^{32}P-probe A.

Nucleotide sequence accession number. Accession number AJ225043 has been assigned by the EMBL gene bank to the nucleotide sequence of the *oriV* region that we studied (see Fig. 6).

RESULTS

The *nar* operon is absent from most *Thermus* strains. In order to check if the nitrate respiration ability shown by *T. thermophilus* HB8 was present in other strains of this genus, we inoculated the isolates *Thermus* sp. strain ATCC 27737, *T. aquaticus* YT1, *T. thermophilus* HB27, and *T. thermophilus* BPL7 and the *nar* mutant *T. thermophilus* HB8 *narGH*::*kat* (21) into TB medium containing nitrate and incubated them for 48 h at 70°C under anaerobic conditions. The results of such experiments demonstrated that none of these strains was able to grow under these conditions, while the control strain *T. thermophilus* HB8 grew to an OD_{550} of 0.8 (data not shown).

To check whether these results were related to the absence of the *nar* operon or were due to problems in its expression, we developed a parallel Southern blot of *Bam*HI-digested total DNA from these strains by using ^{32}P-probe A as a marker for the presence of the *nar* operon. As shown in Fig. 1, a 1.2-kbp labeled fragment detected in *T. thermophilus* HB8 (lane 1) was absent from all the other strains analyzed. As a negative control, we included the *narGH*::*kat* strain (Fig. 1, lane 2), an insertional derivative of *T. thermophilus* HB8 from which the fragment used as a probe was deleted (21).

The *nar* operon is horizontally transferred. The results reported above, especially the absence of the *nar* operon from *T. thermophilus* BPL7, a plasmid-free derivative of *T. thermophilus* HB8 (18), suggested the likely location of the *nar* operon to be in a plasmid and prompted us to test its putative transfer to other aerobic *Thermus* strains.

To test this possibility in conjugation experiments, a chloramphenicol-resistant derivative of *T. thermophilus* HB27 (*T. thermophilus* HB27Cam^r) was used as a recipient and *T. thermophilus* HB8 was used as a donor (see Materials and Methods). After the 8-h mating period, nitrate-respiring and chloramphenicol-resistant strains were isolated by growing the cells consecutively under microaerophilic and completely anaerobic conditions. Parallel controls in the absence of the recipient were developed to check the possible selection of chloramphenicol-resistant derivatives of strain HB8 during this process.

As shown in Fig. 2A, the chloramphenicol-resistant organism selected by this experiment (hereafter referred to as *T. thermophilus* HB27Cam^r::*nar*) was able to grow under anaerobic conditions with nitrate, while the parental recipient organism, *T. thermophilus* HB27Cam^r, was not.

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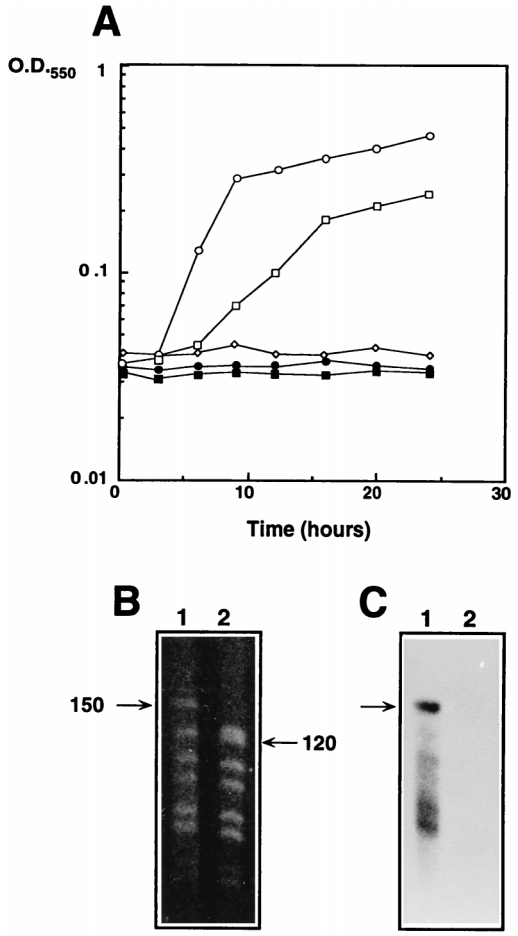

FIG. 2. Horizontal transfer of the *nar* operon. (A) Growth under anaerobic conditions of *T. thermophilus* HB8 (circles), *T. thermophilus* HB27Cam^r (diamonds), and the exconjugant *T. thermophilus* HB27Cam^r::*nar* (squares) in the presence (open symbols) or absence (closed symbols) of KNO_3 (20 mM). (B) PFGE of *Nde*I-digested chromosomal DNA from *T. thermophilus* HB27Cam^r::*nar* (lane 1) and *T. thermophilus* HB27Cam^r (lane 2). (C) Southern blot of the PFGE sample shown in panel B labeled with probe A. Lanes are as in panel B. Numbers beside lanes in panel B are in kilobase pairs.

Aiming to identify the transferable genetic element carrying the *nar* operon, we analyzed by conventional agarose gel electrophoresis and Southern blotting the plasmid fractions of donor, recipient, and exconjugant strains. Unexpectedly, we did not detect any specific labeling with a *nar* probe (probe A) for these plasmid fractions (data not shown). Consequently, we tried to locate the *nar* operon in the chromosomes of these strains. To do this, we used two consecutive PFGE runs. The first one included long pulses to allow the plasmids to enter the agarose gel, while the chromosome remained in the origin (17). A parallel Southern blot showed that the *nar* operon was actually located in the chromosome of both the donor (*T. thermophilus* HB8) and the exconjugant (*T. thermophilus* HB27Cam^r::*nar*) strains. For the second run, agarose plugs containing only chromosomal DNA were treated in situ with *Nde*I and then subjected to PFGE to separate the resulting restriction fragments, followed by Southern blotting with probe A.

The results of this experiment (Fig. 2B) demonstrated that as a consequence of the transfer of the *nar* operon, a 120-kbp *Nde*I restriction fragment from recipient strain *T. thermophilus* HB27Cam^r (lane 2) changed its mobility up to 150 kbp in the exconjugant strain (lane 1). As this 150-kbp DNA fragment hybridized with the *nar* probe (Fig. 2C), we concluded that a DNA genetic element of at least 30 kbp containing the *nar* operon was mobilized from the chromosome of the donor strain to that of the recipient strain. Furthermore, the similarity between the *Nde*I digestion fragment profiles of the recipient and the exconjugant clearly identified the latter as a derivative of the former.

Expression of the *nar* operon in the exconjugant *T. thermophilus* HB27Cam^r::*nar*. As shown in Fig. 3A, SDS-PAGE protein patterns of *T. thermophilus* HB8 (donor, lanes 2 to 4) were easily distinguishable from those of *T. thermophilus* HB27Cam^r (recipient, lane 5) and *T. thermophilus* HB27Cam^r::*nar* (exconjugant, lanes 6 and 7). In fact, the protein profiles of the recipient (Fig. 3A, lane 5) and the exconjugant (lanes 6 and 7) were almost identical, thus confirming their genetic relationship. However, a 140-kDa protein specifically detected in the exconjugant (Fig. 3A, lanes 6 and 7) was absent from the parental recipient (lane 5) but apparently present in *T. thermophilus* HB8 cells grown under microaerophilic (lane 3) and anaerobic (lane 4) conditions. A parallel Western blot (Fig. 3B) clearly identified this 140-kDa protein as NarG, the α subunit of the thermophilic nitrate reductase (21).

Apart from demonstrating the transfer of the *nar* operon, these experiments also showed that its expression in *T. thermophilus* HB27Cam^r::*nar* was increased under anaerobiosis (Fig. 3, lane 7) compared to microaerophilic conditions (lane 6). In contrast, there were no differences in the *nar* induction

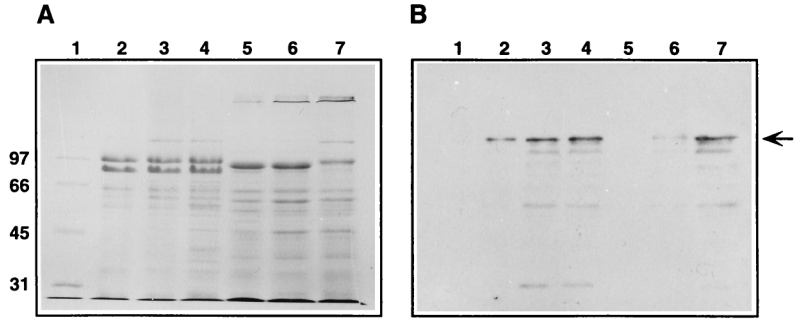

FIG. 3. Expression of the *nar* operon in *T. thermophilus* HB27Cam^r::*nar*. (A) Coomassie blue-stained SDS-PAGE sample showing proteins from the particulate fractions of *T. thermophilus* HB8 (lanes 2, 3, and 4), *T. thermophilus* HB27Cam^r (lane 5), and *T. thermophilus* HB27Cam^r::*nar* (lanes 6 and 7). Cells were grown for 24 h under microaerophilic (lanes 2, 3, 5, and 6) or anaerobic (lanes 4 and 7) conditions with nitrate (lanes 3 to 7). The sample in lane 2 was grown without nitrate. Lane 1 represents markers whose sizes (in thousands) are labeled at the left. (B) Parallel Western blot with antiserum against NarG. A 140-kDa protein which corresponds to NarG is indicated by an arrow. Lanes are as in panel A.

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TABLE 1. Time-dependent horizontal transfer[a]

Donor strain	Level of Km^r Cam^r colonies for a transfer time (min) of:									
	10	20	30	40	50	60	70	80	90	100
slrA::*kat*	−	+	++	++	++	++	++	++	++	++
slpM::*kat*	+	++	++	+++	+++	+++	+++	+++	+++	+++
narGH::*kat*	−	−	−	−	+	++	++	++	++	++

[a] After the mating experiment (see Materials and Methods), we detected kanamycin- and chloramphenicol-resistant colonies at the following levels: −, none; +, less than 20; ++, 20 to 100; +++, more than 100.

level between microaerophilic and anaerobic conditions for *T. thermophilus* HB8 (Fig. 3, compare lanes 3 and 4).

Furthermore, despite the difference in expression observed (Fig. 3, lanes 6 and 7), the nitrate reductase activities were similar under microaerophilic and anaerobic conditions (560 and 510 U, respectively, per identical cell mass) in the exconjugant. Therefore, some of the NarG protein copies synthesized under anaerobiosis were inactive in the exconjugant. In fact, similar levels of NarG (Fig. 3B, compare lanes 4 and 7) yielded activities about threefold higher in *T. thermophilus* HB8.

Transfer of the *nar* operon occurs through an Hfr-like mechanism. The results of the experiments shown in Fig. 2 and 3 suggested that the *nar* operon was transferred from the donor chromosome to that of the recipient to obtain the exconjugant *T. thermophilus* HB27Cam^r::*nar*. Since we could not detect the presence of DNA in the conjugation media (as would be expected from the use of a bacteriostatic antibiotic as a selective criterion), the mechanism most likely responsible for this transfer was conjugation. However, the natural competence capability described for *Thermus* spp. (12) still remained a less likely explanation.

To distinguish between these two possibilities, we used a classic interrupted-mating experiment (see Materials and Methods) to check whether there was a time dependence for the transfer of different genes. If the transfer were ordered, a conjugative mechanism could be inferred. In contrast, random transfer would indicate transformation as the most likely mechanism.

For these experiments and to make the selection easier (kanamycin resistance), we used as donors different *T. thermophilus* HB8::*kat* mutants, including the *narGH*::*kat* derivative (9, 21). As shown in Table 1, transfer of the *slpM*::*kat* mutation required less than 10 min, followed shortly afterward by the *slrA*::*kat* mutation. Nevertheless, about 50 min of mating was required for transfer when the *narGH*::*kat* mutant was used as a donor. Thus, we concluded that there was a time dependence for the transfer of each mutation assayed.

To obtain further arguments supporting conjugation, these experiments were repeated in the presence of DNase I (100 μg/ml) with identical results. Controls for enzyme activity were carried out after the mating period to demonstrate that the remaining enzyme was still able to digest a 1-μg/μl solution of pUC119 in 10 min at 70°C.

We concluded that the transfer of the *nar* operon occurs through a conjugative mechanism which can also mobilize other chromosomal genes, as for the Hfr (high frequency of recombination) strains of *E. coli.*

Identification of a replicative origin (*oriV*) downstream of the *nar* operon. During the construction of *narI*::*kat* insertion mutants of *T. thermophilus* HB8 (unpublished data), we detected a high transformation efficiency with the circular form of plasmid pNIT9kat, which contains part of the *nar* operon and its downstream region (Fig. 4). In fact, the transformation efficiency observed (Table 2) was similar to that obtained with the bifunctional *E. coli-Thermus* shuttle vector pMK18 (6). Therefore, this result suggested the possible presence downstream of the *nar* operon of sequences encoding an origin for autonomous replication (*oriV*) in *Thermus* strains.

If this hypothesis were true, we should have been able to isolate plasmid pNIT9kat from *Thermus* transformant colonies. As shown in Fig. 5A, a DNA fragment of the size expected for pNIT9kat (6.7 kbp) was detected in the plasmid fraction of *T. thermophilus* HB8 (lane 2), and this fragment hybridized with a specific DNA probe (probe B) in a parallel Southern blot (Fig. 5B). Furthermore, when this plasmid fraction was used to transform competent cells of *E. coli* TG1, a high number of transformants (Table 2) contained pNIT9kat (confirmed by restriction analysis of plasmids from several colonies). These data revealed the ability of pNIT9kat to replicate autonomously in *Thermus* and, consequently, supported the existence of a replicative *oriV* downstream of the *nar* operon.

When the exconjugant *T. thermophilus* HB27Cam^r::*nar* was used as the host in parallel experiments, identical results were obtained (Table 2). Furthermore, we could repeat several transformation cycles between *Thermus* and *E. coli*, always recovering plasmid pNIT9kat. Surprisingly, when *T. thermophilus* HB27Cam^r (the recipient strain in the conjugation experiments) or *T. thermophilus* BPL7 (a plasmid-free derivative of *T. thermophilus* HB8) was used, no transformant colonies appeared. This result suggests the requirement for a replication factor present only in *nar*-carrying strains (Table 2).

To locate more precisely *oriV*, we developed a group of derivative plasmids which contained different DNA fragments of the *nar* downstream region (Fig. 4) and used them to transform the four *T. thermophilus* strains. Except for pNIT10kat, all the derivatives showed results similar to those obtained with pNIT9kat, transforming *T. thermophilus* HB8 and *T. thermophilus* HB27Cam^r::*nar* but failing to transform *T. thermophilus* HB27Cam^r and *T. thermophilus* BPL7 (Table 2). As for pNIT9kat, we repeated with these plasmids the cycles of transformation between *T. thermophilus* HB27Cam^r::*nar* and *E. coli* and always recovered them from both organisms.

On the basis of these results, the replicative *oriV* is located within an 0.8-kbp *Sma*I/*Xho*I fragment downstream of the *kat* gene of pNIT5kat2 (Fig. 4). The sequence of this region (Fig. 6) revealed the existence of an open reading frame encoding the C-terminal part of a protein homologous (29% identity) to the C terminus of the nitrite extrusion protein NarK from *E. coli*. Immediately downstream of this open reading frame is a region which contains two inverted repeats, of 12 bp (ir1) and 10 bp (ir2), the latter of which is followed by a T-rich sequence. Two groups of A-rich sequences are also located around ir1. Direct repeats of different sizes (dr1) were also found both downstream of and overlapping the *narK* homolog.

DISCUSSION

Two important conclusions arise from the results presented in this article. First, we describe for the first time for strains of the genus *Thermus* a conjugative mechanism which is also able to mobilize chromosomal genes in a time-dependent manner. The second conclusion is more general, since we show that the ability to grow under anaerobic conditions may be encoded within a mobilizable element, thus making doubtful the relevance of the "aerobic" character of an organism in formal taxonomy (2).

The transfer of the *nar* operon from the facultative anaerobe

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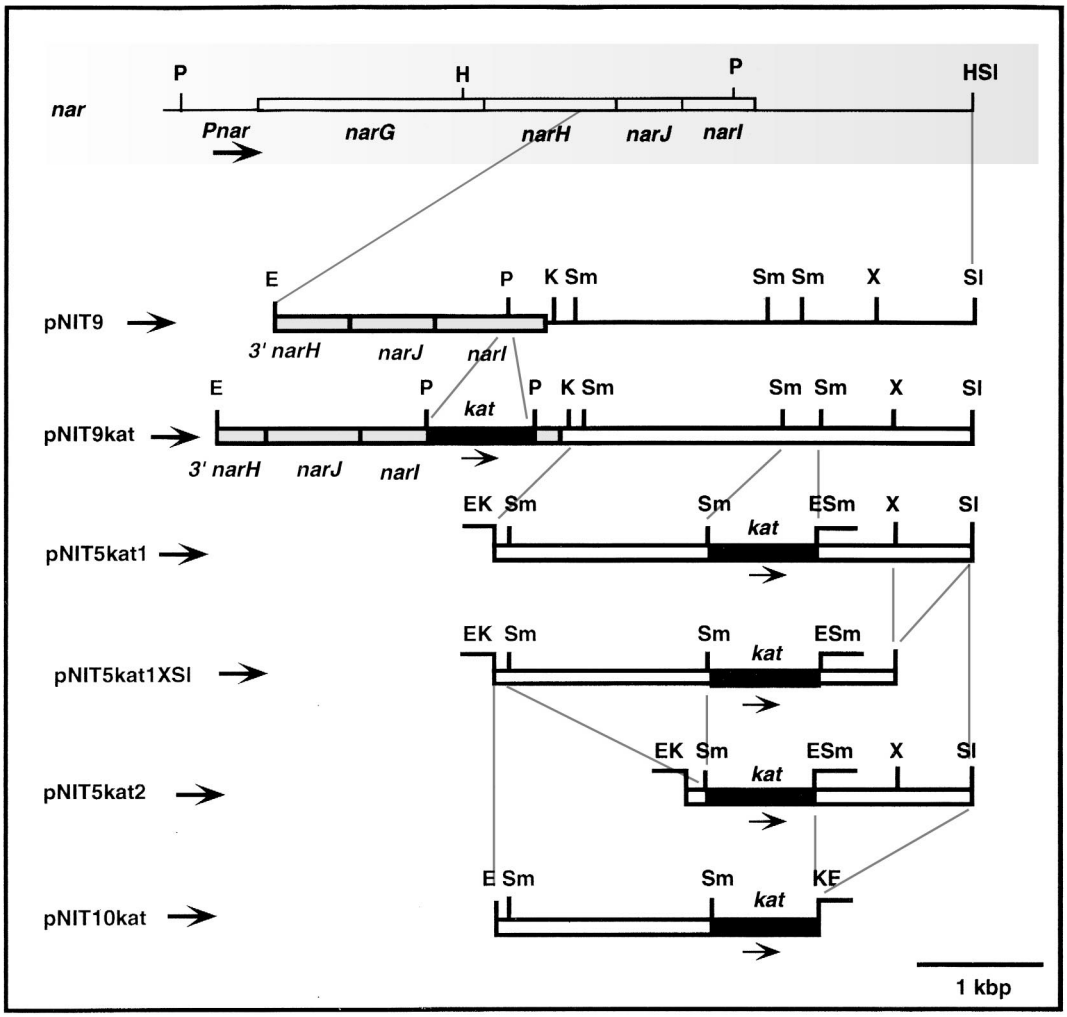

FIG. 4. Identification of *oriV* downstream of the *nar* operon. Restriction maps of the *nar* region (shaded bars) and of the plasmids used to localize a replicative *oriV*. Genes *narG*, *narH*, *narJ*, and *narI* are labeled. The *kat* gene used for their selection in *Thermus* strains is shown by black bars. The approximate position of the *nar* promoter (*Pnar*) is shown. Restriction enzyme abbreviations: E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xho*I.

T. thermophilus HB8 (donor) to the aerobe *T. thermophilus* HB27Cam^r (recipient) resulted in the isolation of a Cam^r facultative anaerobe. The nature of this organism as a derivative of the recipient was assessed in several ways. First, parallel controls in the absence of the recipient did not allow the isolation of any Cam^r mutant from the donor. Second, the total protein pattern of the organism selected after the conjugation was identical to that of the recipient and clearly different from that of the donor (compare lanes 5 and 6 with lanes 2 and 3 of Fig. 3). Finally, the profiles of *Nde*I digestion fragments of chromosomal DNA from the recipient and the exconjugant were identical except for the insertion of a 30-kbp DNA fragment.

A second point concerns the mechanism of transfer. That the transfer of the *nar* operon was due to conjugation and not to the natural competence described for *Thermus* was sup-

TABLE 2. Identification of a replicative *ori* downstream of the *nar* operon[a]

Host	Level of Km^r colonies in the presence of plasmid:					
	pMK18	pNIT9kat	pNIT5kat1	pNIT5kat1XS	pNIT5kat2	pNIT10kat
HB8	+++	+++	++++	++++	++	−
HB27Cam^r::*nar*	++++	+++	++++	++++	++	−
HB27Cam^r	++++	−	−	−	−	−
BPL7	++	−	−	−	−	−
E. coli TG1	++++	+++	+++	+++	+++	

[a] Data represent the results of transformation of *Thermus* strains with plasmids purified from *E. coli* and the results of transformation of *E. coli* with the same plasmids isolated from *Thermus* strains. pMK18 was used as a control for transformation efficiency. Number of colonies: −, none; ++, 50 to 100; +++, 100 to 500; ++++, >500.

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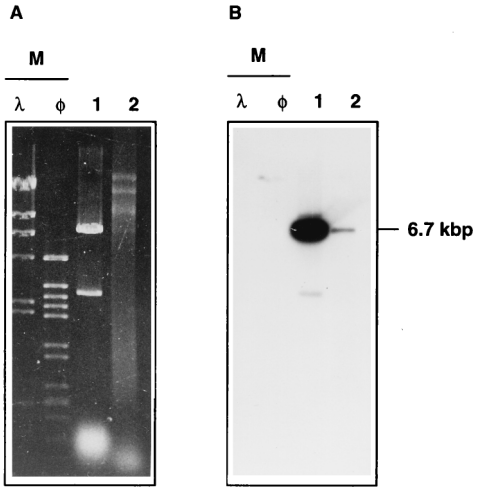

FIG. 5. pNIT9kat replicates in *Thermus* strains. (A) Agarose gel electrophoresis of *Eco*RI-digested pNIT9kat purified from *E. coli* (lane 1) and *Eco*RI-digested plasmid fraction from *T. thermophilus* HB8 transformed with pNIT9kat (lane 2). (B) Parallel Southern blot labeled with probe B. Lanes are as in panel A. *Hin*dIII-digested DNAs from λ and ϕ29 phages were used as size markers (lane M).

ported by different arguments. (i) The existence of external DNA during the mating period was unlikely due to the use of a bacteriostatic selective criterion (chloramphenicol) in the expression medium. In fact, we did not detect DNA in the medium after the conjugation. (ii) The transfer also took place in the presence of DNase I, an enzyme which was still active at 70°C even after the mating period. (iii) Different mating times were required to transfer different genes, a situation that would not occur if a transformation phenomenon were implicated.

The time-dependent transfer of chromosomal genes could be explained only on the basis of a mechanism similar to that of the Hfr strains of *E. coli*. In these strains, a plasmid (F) inserted into the chromosome provides the genes (*tra*) and an origin (*oriT*) required to drive the transfer of a single-strand copy of the whole chromosome to a recipient cell (16). As *oriT* is located within the plasmid sequence, plasmid-encoded genes located upstream of *oriT* are the last to be transferred, requiring about 100 min of mating in the *E. coli* system. Keeping in mind that the circular chromosome of *T. thermophilus* HB8 is about 1.740 kbp long (1) and assuming a rate of transfer similar to that in *E. coli* (chromosome size about 4.200 kbp), the 45 to 50 min of mating required to transfer the *narGH*::*kat* mutation could be expected if *oriT* were located immediately downstream of the *nar* operon. Unfortunately, the limited repertoire of gene markers makes impossible at the present time a detailed genetic analysis to check the putative association between the *nar* operon and *oriT*.

Additional evidence which supported the association between the *nar* operon and a conjugative plasmid was the fortuitous identification of a replicative *oriV* downstream of the *nar* operon (Fig. 4) which was functional in *T. thermophilus* HB8 and in *T. thermophilus* HB27Cam^r::*nar* but not in *T. thermophilus* HB27Cam^r or *T. thermophilus* BPL7 (Table 2). This fact suggests the existence of a gene carried by the transferred fragment, whose expression is required for the replication of this *oriV*. It is possible that the role of this factor is the identification of specific sequences at *oriV*, allowing the melting of DNA and the subsequent recruiting of a replication complex, as is the situation with the Rep protein(s) from many other plasmids (13).

The sequence of the region containing *oriV* revealed the presence of inverted and direct repeats downstream of a sequence which encodes the C-terminal part of a protein homologous to the nitrite extrusion protein NarK (Fig. 6). Although the presence of downstream T-rich sequences suggests a role for ir2 as a Rho-independent transcription terminator, the other repeats found may be related to the binding of the putative Rep factor mentioned above. Nevertheless, their role in replication and/or transcription is not known at present, and further deletion analysis is required to determine their role.

The above results support the existence of a conjugative plasmid which has been integrated into the chromosome of *T. thermophilus* HB8. Two plasmids, pTT8 and pVV8, have been described for *T. thermophilus* HB8 (26). Of these, only pVV8 is a likely candidate to be conjugative because of its larger size (26), its ability to confer an aggregation phenotype, and its ability to integrate into the chromosome through homologous regions (18). However, Southern blot assays revealed that neither pVV8 nor pTT8 hybridized with a *nar* probe (data not shown). Consequently, the *oriV* that we identified downstream of the *nar* operon belongs to a different plasmid. In this sense, its absence from *T. thermophilus* BPL7, a derivative of HB8, means that the *nar*-carrying plasmid was lost during the complex procedure followed for its selection, which included long-term growth under 100% oxygen, nitrosoguanidine mutagenesis, and ampicillin enrichment (18). Such a complex selection could have induced excision from the chromosome and the loss of the *nar*-carrying integrated plasmid to yield *T. thermophilus* BPL7.

After its transfer to the recipient, the expression of the *nar* operon in the exconjugant *T. thermophilus* HB27Cam^r::*nar* was still regulated by nitrate and oxygen. Whether this result was due to the simultaneous transfer of both the oxygen and the

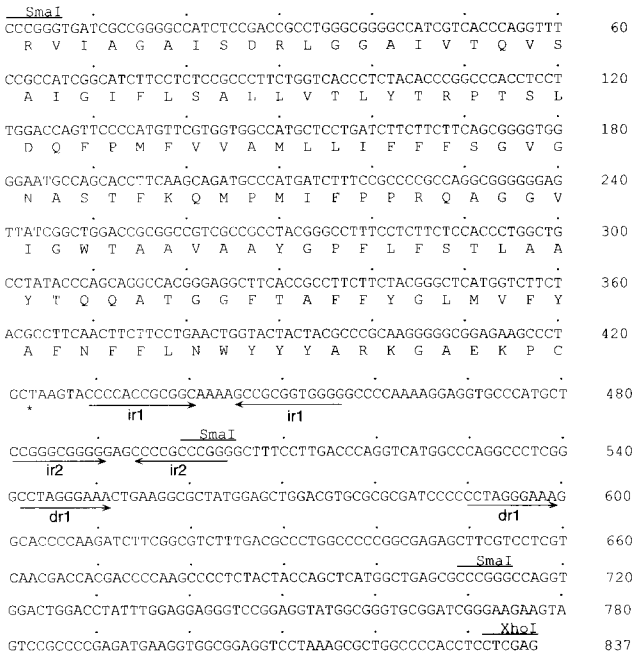

FIG. 6. Sequence of the minimal *oriV* region. The 837-bp *Sma*I-*Xho*I region contains an origin for autonomous replication in *nar*-carrying *Thermus* strains. The amino acid sequence shown below the DNA sequence corresponds to the C-terminal part of a protein homologous to the nitrite extrusion protein NarK. Inverted and direct repeats are labeled with arrows. Sites for restriction endonucleases *Sma*I and *Xho*I are also shown.

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nitrate sensors or to their previous presence in the recipient cannot be answered at present. However, the inability of the recipient to use nitrate (21) makes the last possibility most unlikely, at least for the nitrate sensor system.

It is noteworthy that *nar* expression was even more gradual in the exconjugant than in *T. thermophilus* HB8; although full induction in HB8 was reached under microaerophilic conditions, completely anoxic conditions were required for full expression in the exconjugant. Furthermore, in spite of the expression of similar amounts of NarG protein (Fig. 3, lanes 4 and 7, for the HB8 and HB27Cam^r::*nar* strains, respectively), the enzymatic activity in the exconjugant strain was three times lower than that in strain HB8, suggesting that part of the enzyme synthesized was inactive. Accordingly, the exconjugant strain yielded less growth under anaerobic conditions than did the parental donor strain (Fig. 2A).

All of these data support the fact that the ability of *T. thermophilus* HB8 to respire nitrate is encoded in a genetic element which can be transferred to aerobic strains of the same genus, changing an obligate character to facultative. In fact, the unexpected presence of *nar* operons in certain strains of supposedly obligate aerobes such as *Pseudomonas fluorescens* (20) and *Bacillus subtilis* (10) could be due to a process of horizontal transfer similar to that described here. If this were the case, the requirement for oxygen, commonly used in formal taxonomy as one of the main characteristics for the classification of microorganisms, could be viewed as meaningless.

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