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Respiratory and dissimilatory nitrate-reducing communities from an extreme saline alkaline soil of the former lake Texcoco (Mexico)

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Abstract The diversity of the dissimilatory and respiratory nitrate-reducing communities was studied in two soils of the former lake Texcoco (Mexico). Genes encoding the membrane-bound nitrate reductase (*narG*) and the periplasmic nitrate reductase (*napA*) were used as functional markers. To investigate bacterial communities containing *napA* and *narG* in saline alkaline soils of the former lake Texcoco, libraries of the two sites were constructed (soil T3 with pH 11 and electrolytic conductivity in saturated extract (EC_{SE}) 160 dS m^{-1} and soil T1 with pH 8.5 and EC_{SE} 0.8 dS m^{-1}). Phylogenetic analysis of *napA* sequences separated the clone families into two main groups: dependent or independent of NapB. Most of *napA* sequences from site T1 were grouped in the NapB-dependent clade, meanwhile most of the *napA* sequences from the extreme soil T3 were affiliated to the NapB-independent group. For both sites, partial *narG* sequences were associated with representatives of the *Proteobacteria*, *Firmicutes* and *Actinobacteria* phyla, but the proportions of the clones were different. Our results support the concept of a specific and complex nitrate-reducing community for each soil of the former lake Texcoco.

Keywords Dissimilatory nitrate reduction · Saline alkaline soil · *napA* gene · *narG* gene

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Introduction

One of the most important biogeochemical cycles is the nitrogen (N) cycle, which includes the biological transformation of different N species (Stolz and Basu 2002). Nitrate (NO_3^-) is the most oxidized form of N in the biosphere and serves as a nutrient and as an important oxidant for the conservation of metabolic energy (Eisle and Kroneck 2004). Prokaryotes that use nitrate as an alternative electron acceptor, constitute a wide group with members among the *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, some members of the phylum *Firmicutes*, and even halophilic and hyperthermophilic Archaea (Zumft 1997).

The reduction of NO_3^- to NO_2^- in a non-assimilatory pathway can be catalyzed by two different prokaryotic nitrate reductases, the membrane-bound nitrate reductase (Nar) and the periplasmic nitrate reductase (Nap) (Moreno-Vivian et al. 1999; Stolz and Basu 2002). Nar enzymes are associated with denitrification and anaerobic nitrate respiration, participating in the generation of proton motive force (PMF) (Berks et al. 1995a). Nar consists of a cytoplasmic (NarGH) complex and a membrane anchor subunit (NarI). The catalytic subunit of Nar (NarG) is encoded by the gene *narG* (~3,800 bp) (Fig. 1). Nap is located in the periplasm and presents a dilemma as it can be involved in redox balancing, respiration [either denitrification or dissimilatory nitrate reduction to ammonia (NH_3)] and NO_3^- uptake (Berks et al. 1995b; Carter et al. 1995; Castillo et al. 1996; Potter et al. 1999). Nap is believed not to contribute directly to PMF (Moreno-Vivian et al. 1999). However, *Pseudomonas sp.* strain G-179 and *Desulfovibrio desulfuricans*, two nitrate respiring bacteria possess only this enzyme (Bursakov et al. 1997; Bedzyk et al. 1999). The energy

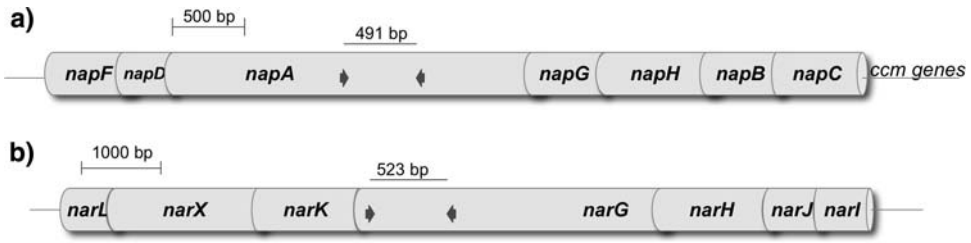

Fig. 1 General organization of the periplasmic (*nap*) and respiratory (*nar*) nitrate reductase loci in *E. coli* W3110 (AP009048). *Arrows* indicate the position of primers for *napA* and *narG* fragments PCR amplification, and *lines* above show the size of the obtained amplicons. *Scale bars* are shown and genes are drawn approximately to scale, the *nap* cluster is 5.8 Kb and the *nar* cluster is 11.3 Kb

generation mechanism by Nap needs thus still to be understood. The *napA* gene (~2,500 bp) encodes for the catalytic subunit (NapA) of Nap, and is widely distributed among *Proteobacteria* (Fig.2). The results of genome sequencing projects have shown that some *Firmicutes*, such as *Symbiobacterium thermophilum* and *Desulfitobacterium hafniense*, retained the Nap nitrate reductase gene cluster (Ueda et al. 2004; Nonaka et al. 2006).

The soil of the former lake Texcoco is "extreme" saline alkaline with pH >10 and electrolytic conductivity (EC) in saturation extracts >150 dS m^{-1} (Valenzuela-Encinas et al. 2008). It is located in the valley of Mexico City (Mexico) at an altitude of 2,240 m above sea level with a mean annual temperature of 16.7°C and annual precipitation of 705 mm, is formed from volcanic ash deposited in situ in a lacustrine environment and covered recently by colluvial materials (Luna-Guido et al. 2000). The aquifer is near to the surface (80–150 cm) and the groundwater is highly saline, Na^+, Cl^-, HCO_3^- and CO_3^{2-} being dominant. The structure is granular in the topsoil and prismatic in the subsoil and the organic matter contents range from 20 to 50 g kg^{-1} (dry soil). Natural drainage is poor and roots are restricted by a compact ash layer 5–20 cm thick to depths of 16–40 cm (Luna-Guido et al. 2001). Dynamics of C and N have been investigated intensively, but no information exists about enzymes involved in the respiratory and dissimilatory reduction of NO_3^- (Conde et al. 2005; Castro-Silva et al. 2008).

Our aim is to study the dissimilatory and the respiratory nitrate reduction communities in a saline alkaline soil of the former lake Texcoco, and compare it with five different salt-affected soil communities using molecular tools. Libraries of *napA* and *narG* amplicons from a saline alkaline soil were compared with a drained soil and grouped in a phylogenetic tree. The denitrifying community composition in soil samples was determined with a denaturing gradient gel electrophoresis (DGGE) using suitable primers, i.e., fragments smaller than 500 bp.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli HB101, *Pseudomonas aeruginosa* and one strain (*MS* 5.2) containing the *napA* gene isolated from a saline alkaline soil were used as controls for *napA* gene fragment amplification. *E. coli* HB101 and a isolated strain from a saline alkaline soil matching *B. subtilis*, were used as positive amplification controls, for *narG* amplicons.

E. coli was grown in Luria Broth medium at 37°C, *P. aeruginosa* in nutrient broth at 37°C, *MS* 5.2 in a low-salt mineral medium at 28°C (Sorokin et al. 2006) and *B. subtilis* in R2A medium. The cells were harvested by centrifugation (15,550*g*, 20°C, 5 min), washed twice in TE buffer [Tris–HCl 100 mM (pH 8) and EDTA 3 mM] and stored in 15% glycerol at −80°C.

Environmental samples

Five soil samples were used to test the designed primers and use them in a DGGE analysis. Three soil samples were taken from the former lake Texcoco (19°30′N, 98°59′W). A first soil sampled at Texcoco had a low EC_{SE} 0.68 (T1), a second had a medium EC_{SE} of 10.1 (T2) and a third was extreme saline alkaline with EC_{SE} 159 dS m^{-1} (T3) (Table 1). Additionally a saline rich-organic-matter soil was sampled at Xochimilco (Xo) and an arable soil at Acolman (A1). Soil samples were taken from the 2–10 cm top-layer, sieved (<5 mm), mixed and stored at −80°C pending analysis. Soil physicochemical characteristics were determined as described in Conde et al. (2005).

DNA extraction

Total DNA from pure strains was extracted by enzymatic and chemical methods. Colonies grown in solid medium (~10–20 mg) were suspended in 180 µl lysis buffer [20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 1.2% Triton X-100] containing lysozyme (20 mg ml^{-1}). After

Table 1 General soil characteristics

Site	pH_w^a	EC[b] dS m^{-1}	Organic C g kg^{-1}	Inorganic C g kg^{-1}	Total N g kg^{-1}	Clay content g kg^{-1}	USDA soil classification
Acolman (A)	7.8	0.93	13.7	ND[c]	0.81	220	Loam
Texcoco 1 (T1)	8.5	0.68	32.1	32	1.74	160	Loamy Sand
Xochimilco (X)	8.6	43.6	90.4	1.3	3.1	90	Sandy loam
Texcoco 2 (T2)	10.1	56.0	53.0	11	6.7	220	Sandy clay loam
Texcoco 3 (T3)	11.8	159	23.5	7.4	0.63	540	Clay

[a] pH in aqueous soil suspension 2:1

[b] EC the electrolytic conductivity in saturated soil extract

[c] ND not detectable

Table 2 Primers sequences used to amplify *napA* and *narG* fragments

Primer	Position[a,b]	Primer sequence (5′ → 3′)[c]	T_m^d (°C)	PCR fragments expected size (bp)	Target amino acid sequence
napA_F1	1107–1129	C TGG ACI ATG GGY TTI AAC CA	60.2	492	WTMGFN
napA_R1	1579–1598	CC TTC YTT YTC IAC CCA CAT	57.2		MWVEKE
narG_F1	145–164	ACI CAY GGI GTI AAC TGY AC	54.3	523	THGVNC
narG_R1	649–668	TC GSM RTA CCA GTC RTA RAA	56.1		FYDWYC
M13 R		CAG GAA ACA GCT ATG AC	50.6		
M 13 F		GTA AAA CGA CGG CCA	56.18		

[a] Position in the *napA* gene of *Escherichi coli* O6, Genbank Accesion number Q8CVW4

[b] Position in the *narG* gene of *Escherichi coli* W3110, Genbank Accesion number BAA36094.1

[c] Sequence according to the IUPAC nomenclature, I means Inosine

[d] Oligo T_m calculated by Sigma Life Science using the nearest neighbor method with values of 50 mM for cation concentration and 0.5 micromolar for the strand concentration

incubation at 37°C for 60–120 min, 20 µl proteinase K (20 mg ml^{-1}) and 20 µl SDS (10%) were added, and the mixture was incubated at 56°C for 60–120 min. 500 µl TE solution [10 mM Tris–HCl (pH 8.0), 1 mM EDTA] and 20 µl NaCl (5 M) were added and the solution was gently mixed.

Two 1 h phenol gently extractions were performed (500 µl, buffer saturated phenol, pH 7.6–8.0) followed by an extraction with chloroform:isoamyl alcohol (500 µl, 24:1). Extractions were performed using a rotary devise. Residual phenol in the aqueous phase was removed by extracting three times with 1 ml water-saturated ether, and residual ether was removed incubating the sample at 65°C for 15 min. Nucleic acids were precipitated with 500 µl isopropanol at room temperature and gently mixed until all DNA fibres acquired a cotton-appearance. The sample was centrifuged (10,000*g*, 30 seg), the pellet was washed with ethanol 70% (v/v), dried and dissolved in 40 µl sterile water.

Total DNA from the soil samples was extracted from approximately 250 mg of wet soil using the PowerSoil™ DNA Kit (MO BIO Laboratories, Solana Beach, CA, USA) according to the manufacturer's protocol with a small modification as total precipitated DNA was washed twice with ethanol 70% before eluting in 50 µl H_2O.

Primer design and PCR amplification of *napA* and *narG* genes

Primers for the detection of *napA* and *narG* genes were designed using reported sequences from microorganisms that might be present in the extreme soil of the former lake Texcoco. Sixteen *napA* amino acidic sequences of α, β, γ, δ and ε-*Proteobacteria* were aligned (Q7WIQ1, CAA50507, NP_356246, ABA81591, Q6LTV9, AAZ26086.1, AAG04563, ABA42173, AAZ48244, CAG74799, NP_416710, AAL55891, Q9PPD9, AAM21158, CAI72603, NP_901899) and conserved regions that could provide a suitable primer target sites were investigated. A second alignment was done using the nucleotide sequences to verify the sequence of the PCR primers obtained by reverse translation of the conserved protein target region (Table 2 and Fig. 1). The same methodology was used for the design of *narG* amplification primers. In this case, *narG* sequences belonging to the phyla of *Proteobacteria*, *Euryarchaeota* and *Firmicutes* were used (BAB84312, AAT47523,

YP_573384, YP_335281, AAZ97356, YP_937282, YP_055221, ZP_01127888, YP_318678, P42175, YP_018768, YP_501143, EDX42303, BAD63254, BAE82123, ACG68137, CAF21906, CAB89111, EDN48928).

The PCR amplification was done in a total volume of 25 µl. Amplification protocol of *napA* and *narG* gene fragment consisted of a initial denaturalization at 93°C for 4 min, followed by 25 cycles at 93°C (1 min), at 52°C (1 min) and at 72°C (1 min), with a final extension step at 72°C (7 min). The PCR mixture for amplification from total DNA obtained from pure strains contained 2.5 µl 10 × PCR buffer, 1.5 mM $MgSO_4$, 200 µM of each deoxynucleotide triphosphate, 0.65 U Taq polymerase (Invitrogen Life Technologies, Sao Paulo, Brazil), 1.0 mM of each primer, 60 µg BSA and 10–100 ng DNA. An additional 1.0 mM $MgSO_4$ was added for the amplification of the environmental samples. PCR products were detected by agarose (2.0%) gel electrophoresis and UV translumination after ethidium bromide staining. Thermal cycling was done with an iCycler thermal cycler (Bio-Rad Laboratories, Inc.).

Cloning PCR products, sequencing and computer analysis

narG and *napA* PCR products from soil T1 and T3 were inserted into the vector pCR®2.1-TOPO® and clone libraries were constructed by means of a TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The obtained clones contained ~500 bp putative *napA* or *narG* gene amplicon.

For *napA* libraries, 44 clones were sequenced for site T1 and 78 for site T3. Clones were sequenced once and for the phylogenetic analysis the deduced amino acidic sequences from 480 nucleotides were used. For *narG* libraries, 45 clones were sequenced for T1 and 32 for T3. Clones were sequenced two times with the forward and reverse primers, the deduced amino acidic sequences from 519 nucleotides were used.

Nucleotide sequences were determined by automated sequencing with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using M13F and M13R-vector-targeted sequencing primers. *napA* and *narG* protein sequences were obtained by translating the nucleotide sequence using the BCM Search Launcher tool (http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.htm). Nucleotide sequences were compared to entries in GenBank, EMBL, DDBJ and PDB using blastx (Altschul et al. 1997). Protein sequences were first aligned using ClustalW to reconstruct phylogenetic trees (Chenna et al. 2003), then molecular sequence data were analyzed by maximum likelihood using the model of substitution WAG (Whelan and Goldman 2001) with the computer program TREE-PUZZLE (Schmidt et al. 2002). Phylogenetic trees were drawn using Tree View (Page 1996).

The determined *napA* and *narG* gene partial sequences are available in GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers EU495650-EU495848.

DGGE

The melting profiles of the *napA* and *narG* fragments were analyzed by DGGE. A 39-pb GC-clamp (5′ CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG 3′) was attached to the 5′ end of the napA_F1 and narG_F1 primers to modify the melting profile and avoid complete denaturing of the amplified products. One µl of the amplification product obtained with the same primers was used as template for PCR amplification, and thermocycling conditions were the same as earlier described.

One-mm thick, 16 by 16 cm polyacrilamide gels [7.0% (v/v) acrylamide-bisacrylamide (37.5/1); denaturant (urea/formamide)] were poured using a gradient maker (Bio-Rad Laboratories Inc., USA). Forty µl of the PCR products that had been amplified with the primers were run on denaturing gradients of 40–80%. A mixture of 7 M urea and 40% formamide was defined as 100% denaturant. The gels were run in 0.5 × TAE (40 mM Tris–acetate and 1 mM EDTA) at 45 V for 20 h for *napA* amplicons and at 130 V for 16 h for *narG* amplicons, both at 60°C. Migration patterns were visualised by staining with 1:10,000 (v/v) SYBR Green 1 Nucleic Acid Gel stain (SIGMA-ALDRICH Co.) under UV using a SYBR photographic filter (S7569, Invitrogen Life Technologies Carlsbad, CA).

Results and discussion

Polymerase chain reaction (PCR) amplification of *napA* and *narG* genes from different saline and saline alkaline soils

Oligonucleotides used to detect *napA* and *narG* genes in nitrate respiring bacteria and in community DNA have been reported before (Flanagan et al. 1999; Gregory et al. 2000; Philippot et al. 2002). However, given the research aims, i.e., PCR fragments ~500 bp and no nested PCR protocols, primers were designed to amplify partial sequences of the *napA* and *narG* genes using DNA extracted from extreme saline alkaline soils of the former lake Texcoco.

Designed primers were used to detect and amplify *napA* and *narG* genes from genomic DNA of control strains. These PCR fragments were of expected size (*napA* ~492 bp, *narG* ~523 bp) and a BLAST analysis of the sequences confirmed the amplification of the desired target

zones. For the environmental samples, positive amplifications were obtained when total DNA extracted from five different types of soil was used as template (Table 1).

All the analyzed sequences obtained from the libraries of the *napA* and *narG* partial sequences genes were associated with those reported in databases. In a further analysis, the deduced amino acid fragments from partial *napA* nucleotide sequences contained portions of domains II and III of periplasmic nitrate reductases. A conserved region (Ser-Leu-Thr-Gly-Gln-Pro), and charged residues, such as Arg^{354} and Asp^{355} (positions according to the sequence of *Desulfovibrio desulfuricans*, PBD 2NAP), responsible for the transit of substrates were also identified among the sequences (Dias et al. 1999). The same was found in partial *narG* sequences from the libraries. The analysis of the translated region of the 77 sequences showed that they included segments of the domain I and II of the membrane-bound nitrate reductase. A histidine and three cysteine residues in charge of holding the [4Fe–4S] cluster were detected (His^{50}, Cys^{54}, Cys^{58} and Cys^{93} in *E. coli* B7A, *narG,* ZP_00716715.1). Characteristic residues of the segment II, such as arginine, proline and tryptophan and a conserved region present in molybdoproteins were identified in all sequences (Blasco et al. 2001).

The BLAST analysis and identification of conserved residues among NapA and NarG deduced protein fragments suggested that the primers napA_F1/napA_R1 and narG_F1/narG_R1 can be used for the selective amplification of this genes from environmental samples, such as, extreme saline alkaline soils.

Phylogenetic analyses of *napA* fragments

To study *napA*-community composition in soils of the former lake Texcoco, two libraries were constructed: one for soil T1 and one for soil T3. A phylogram was constructed from the deduced amino acid sequences by a maximum likelihood method considering 160 amino acids. The phylogenetic analysis was done with representative sequences from both libraries and related ones obtained from the Genbank database (Fig. 2). Even when the analysis was done with partial *napA* sequences (160 aa, ~20% of the complete gene), the topology of the obtained phylogenetic tree agreed with the one described by Jepson et al. (2006). The phylogram showed two main clades, the Clade I that possessed the heterodimeric (NapAB) periplasmic nitrate reductases and the Clade II grouping monomeric (NapA) periplasmic nitrate reductases (Fig. 2).

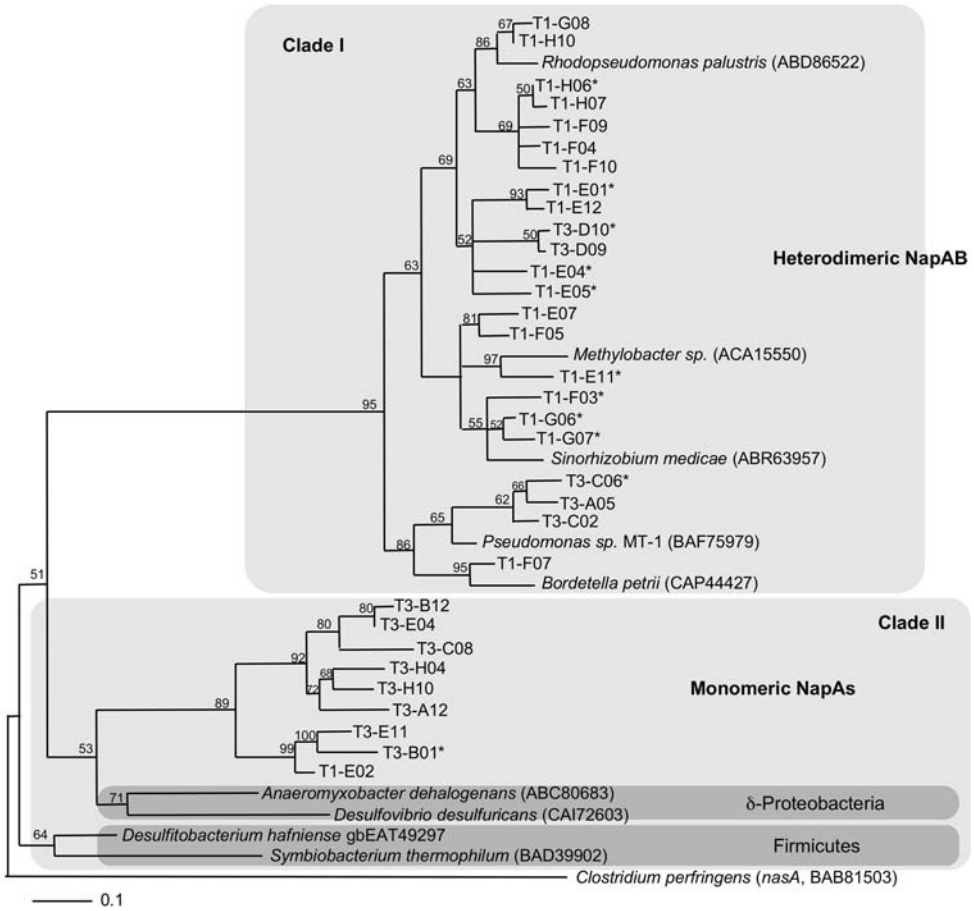

Fig. 2 Phylogram for *napA* based on partial protein fragments. The maximum likelihood phylogram was constructed from deduced protein sequences, clones from two soil libraries were analyzed: an extreme saline alkaline soil (T3) and a soil of the former lake Texcoco soil drained >10 years (T1). Reported sequences from cultured bacteria were included, the *nas*A sequence from *Clostridium perfringens* was used as outgroup. The percentage support values above 50%, a *scale bar* representing 10% sequence divergence, and clusters I and II are indicated. Clone designations T1 and T3 are according the soil; *asterisk* (*) indicates sequences found more than once in the library

Clade I included NapAs of the type NapB-dependent, i.e., heterodimeric NapAB reported for *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*. Most of the clones (97%) from site T1 were grouped in this clade and shared high levels of amino acidic identity (>80%) with *napA* sequences from cultured bacteria. These results agree with reported studies where most of the translated sequences were affiliated with these classes (Flanagan et al. 1999; Smith et al. 2007). Clones T1-E04* and T1-E05* were frequently found and both represented 12% of the library sequences. A BLAST analysis of these showed that T1-E04* shared 87% amino acids with a NapA fragment from a *Ralstonia eutropha* H16 (CAA50507.1) and T1-E05* had 84% identity with a NapA sequence from *Shewanella amazonensis* SB2B (YP_927499.1).

Sequences from the library of the extreme saline alkaline soil T3 were also included into this clade (32% of the T3 library clones). The clone T3-C06* was often found (12%) and a BLAST analysis of this sequence showed 83% identity with the NapA sequence from *Pseudomonas sp.* (BAF75979).

Bacteria that contained the monomeric class of periplasmic nitrate reductases, such as *Desulfovibrio desulfuricans, Symbiobacterium thermophilum, Desulfitobacterium hafniense* and *Anaeroxyxobacter dehalogenans* were located in the Clade II (Fig. 2). This clade was composed mainly by clones from the extreme soil T3 and shared less than 60% identity with reported sequences. Sequence T3-B01 shared 98–99% identity with almost half of the T3 library clones. A protein alignment showed that T3-B01 had 58% identity with a MopB_CT_Nitrate-R-NapA-like from *Anaeromyxobacter dehalogenans* 2CP-C (YP_464120.1). Just one sequence from library T1 was included in Clade II.

The monomeric NapA of *D. desulfuricans* is the closest evolutive link between the periplasmic nitrate reductases and the cytoplasmic nitrate reductases, most similar to the one found in cyanobacteria and typified by the NarB enzyme from *Synechococcus* (BAA17488). The current consensus is that, during evolution, these cytoplasmic enzymes acquired a signal peptide and were translocated to the periplasm (Jepson et al. 2006). In the phylogram, the *nas*A sequence encoding the catalytic subunit of a cytoplasmic nitrate reductase (NasA) from *Clostridium perfringens* was closely related to NapB-indepent NapAs (Fig. 2).

A BLAST analysis indicated that the sequences contained in Clade II shared a 58–59% identity with the partial sequence of *napA* from *Desulfitobacterium hafniense* Y51 ($E \sim 1 \times 10^{-52}$) and a 48% identity with a fragment of the assimilatory nitrate reductase from *Synechocystis sp.* PCC 6803 ($E \sim 2 \times 10^{-41}$).

The distribution of the sequences between these clades revealed different *napA*-containing communities for the two soils. Common sequences were not found in the libraries of the sites T1 and T3. These groupings suggest that different sequences in the *napA* dissimilatory gene may reflect physiological adaptative responses to particular soil conditions, i.e., N deficiency, high pH and high salt concentrations (Luna-Guido et al. 2000). It must to be remembered, however, that other soil characteristics, such as clay content, might have affected the soil communities.

Phylogenetic analyses of *narG* fragments

The respiratory nitrate community composition was analyzed in sites T1 and T3 of the former lake Texcoco. Libraries containing partial *narG* sequences were constructed. Representative amino acidic sequences from databases were considered for the construction of the phylogenetic tree, and a total of 36 sequences derived from the libraries were used. The phylogram was constructed from the deduced amino acid sequences by a maximum likelihood method considering 177 amino acid sites.

The constructed phylogenetic tree showed major lineages, such as *Proteobacteria*, *Actinobacteria* and *Firmicutes*, and the archaeal linage containing sequences from Euryarchaeota and Crenarchaeota (Fig. 3). In the proteobacterial clade, well-defined subclades containing *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* were found. A clade with unaffilated sequences was also found, grouping the *narG* sequence from *Anaeromyxobacter dehalogenans*, a *Deltaproteobacteria*, and *Thermus thermophilus,* an extremophile belonging to the *Deinococcus-Thermus* phylum. This topology is similar to the one found by Stolz and Basu (2002).

Sequences derived from both libraries were found in all clades with the exception of the archaeal lineage. These results agree with other studies where the *narG* gene is used as a functional marker (Philippot et al. 2002; Chèneby et al. 2003; Gregory et al. 2003; Mounier et al. 2004; Philippot et al. 2006). The results of these investigations indicated that diverse unknown groups dominate the soil nitrate-reducing community and strengthened the hypothesis that each environment has its own specific *narG*-containing community (Nijburg and Laanbroek 1997). The membrane associated nitrate reductase is typically involved in nitrate respiration under anoxic conditions and probably plays an important role in the environmental nitrogen cycle (Richardson et al. 2001). Bacterial groups such as *Proteobacteria*, *Actinobacteria* and *Firmicutes*, are the most representative phyla in the environmental nitrate respiring communities.

Sequences from site T1 and T3 were grouped into the *Actinobacteria*/*Firmicutes* and the unaffiliated clade.

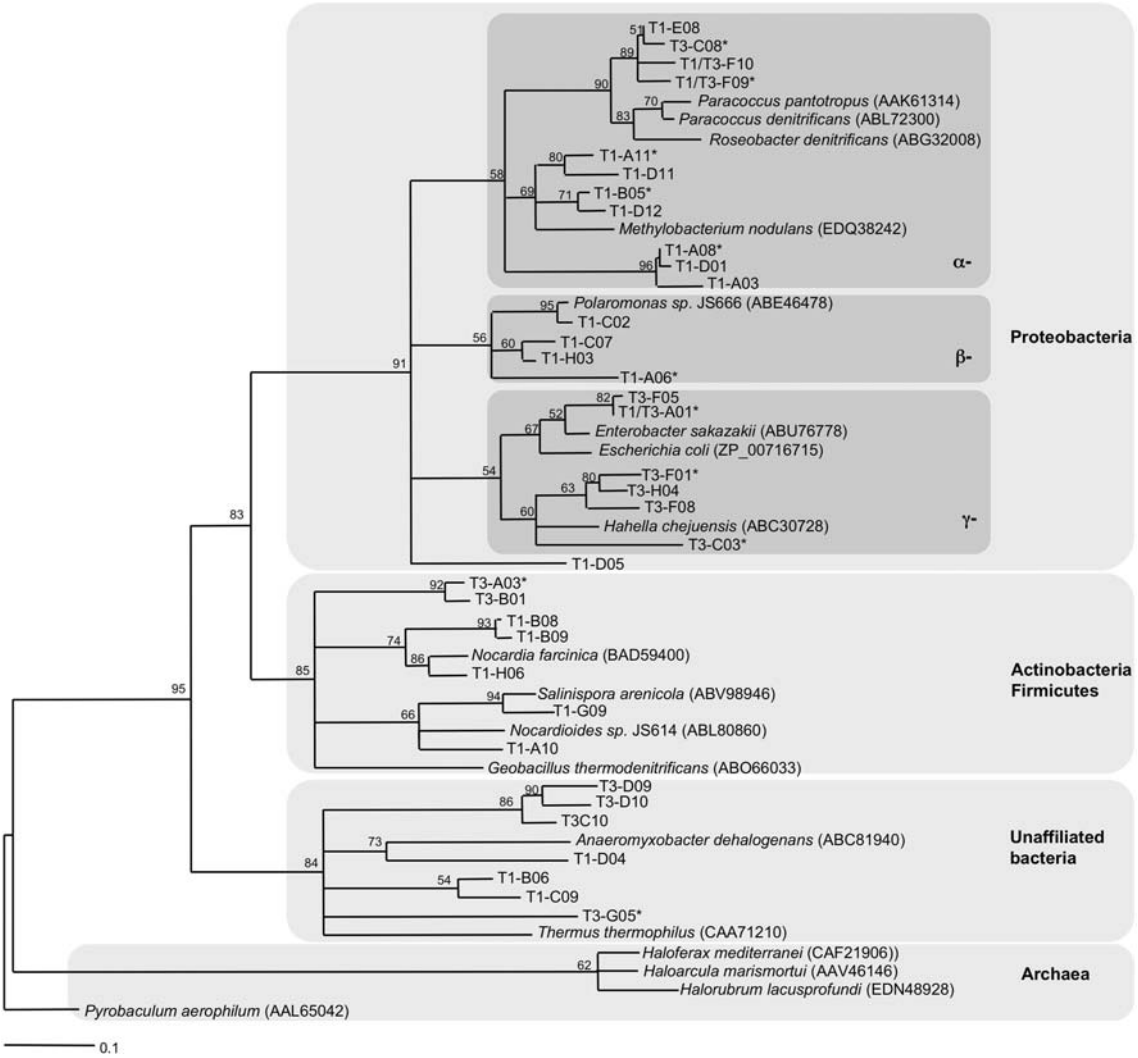

Fig. 3 Phylogram for *narG* based on partial protein fragments. The maximum likelihood dendrogram was constructed from deduced protein sequences, clones from two soil libraries were analyzed: an extreme saline alkaline soil (T3) and a soil of the former lake Texcoco soil drained >10 years (T1). Reported sequences from cultured bacteria were included, the *narG* sequence from *Pyrobaculum aerophylum* was used as outgroup. The percentage support values above 50%, and a *scale bar* representing 10% sequence divergence are indicated. Clone designations T1 and T3 are according the soil; T1/T3 is used to describe clone sequences found in both libraries; *asterisk* (*) indicates sequences found more than once in the library

The clones affiliated within the *Proteobacteria* group were the largest fraction of the *narG*-containing communities in the two soils of the former lake Texcoco, representing 86% of the sequences from the library of T1 and 62% from the library of T3 (Fig. 3). Similar results were found by Chèneby et al. (2003). The clones affiliated with the *Gammaproteobacteria* subgroup were mainly from site T3. Clones such as T3-F01*, T3-H04, T3-F08 and T3-C03* were related to a NarG fragment from *Hahella chejuensis* (Fig. 3), one of the most prevalent prokaryotic species found in marine environments (Giovannoni and Rappé 2000; Venter et al. 2004). Clone T3-C03* represented 15% of the T3 library clones.

In contrast to *napA* libraries, common sequences (T1/T3-A01, T1/T3-F10 and T1/T3-F09) were found at sites T1 and T3. These sequences were also grouped in the proteobacterial clade. One of these clones, T1/T3-A01, represented 26% of the T1 library clones and was also found in 18% of the clones derived of soil T3. A BLAST analysis showed that T1-A01 shared 92% amino acids with a fragment of a hypothetical MopB_Nitrate-R-NarG-like protein from *Enterobacter sakazaki* (YP_001437614). This common sequence found at both sites might indicate that one species was dominant. However, it has been shown, that some strains have identical partial *narG* sequences, but are well separated by 16S rRNA phylogeny (Gregory et al. 2003). As a consequence, we could talk more of group dominance than of species abundance. This dominance of one or two groups has been previously observed in culture and independent-culture based studies (Nijburg and

Laanbroek 1997; Nijburg et al. 1997; Chèneby et al. 2003; Mounier et al. 2004). Horizontal transfer of the *narG* gene could explain the occurrence of very similar sequences in distantly related bacteria (Stolz and Basu 2002; Gregory et al. 2003). As a consequence, the *narG* gene cannot be used to infer taxonomic information and the lack of congruence between the *narG* and 16 rDNA trees suggests that this cannot be done with sufficient accuracy (Gregory et al. 2003).

Reported *narG* sequences from archaea were located in a deep branch of the dendogram, and the Nar enzymes of the unaffiliated bacteria were closely related to the crenarchaeote *Aeropyrum pernix*. T3 is an extreme saline alkaline soil of the former lake Texcoco from which the archaeal community has been described (Valenzuela-Encinas et al. 2008), but sequences associated with the archaeal clade of the parcial *narG* dendogram were not detected.

napA and narG fragments DDGE

DGGE analyses were done to explore the *napA* and *narG*-containing community diversity across salinity and pH in soil (Table 1, Fig. 4).

The DGGE analysis of partial *narG* genes resulted in few bands and poor resolution for environmental samples.

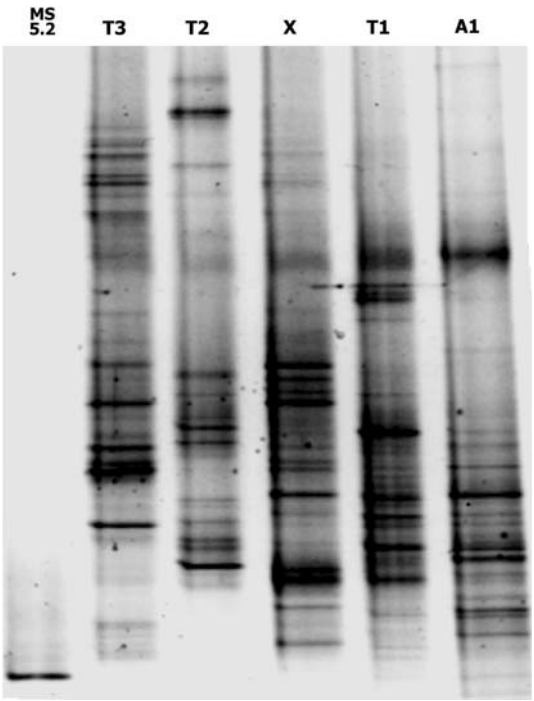

Fig. 4 DGGE analysis of dominating dissimilatory nitrate-reducing communities in an arable soil from Acolman (*A1*), a soil of the former lake Texcoco drained >10 years (*T1*), a saline rich-organic-matter soil from Xochimilco (*Xo*), a saline alkaline soil (*T2*) and a extreme saline alkaline soil (*T3*), both from Texcoco. *napA* gene fragment from a bacteria isolated from T3 (*MS 5.2*)

Although many experimental conditions were essayed, the resolution could not be improved and the number of *narG*-containing bacteria could not be estimated with this technique (Data not shown). The poor resolution might be due to multiple melting domains presented in these particular fragments (Kisand and Wikner 2003).

Well-defined bands were detected in the polyacrylamide gel of the partial *napA* amplicons. The number of bands of the *napA* gene was similar for the five different sites: 22 visible bands were detected for soil A1, 23 for T1, 24 for X, 19 for T2 and 23 for T3 indicating a similar number of *napA*-containing organisms at each site (Fig. 4). The fragment distribution, was different for each soil and dominant *napA*-containing species were generally not shared between soils. However, six common bands were detected in soil X and T3. A *napA* fragment was successfully amplified when DNA extracted from a strain isolated from the soil T3 of the former lake Texcoco (*MS* 5.2) was used as a template. The DGGE analysis of this amplicon resulted in a single band (Fig. 4, Lane 1). This analysis proved that normally only one *napA* copy per genome is found in a strain (Philippot 2002). Consequently, the diversity of the sequences can be an indicator of the *napA*-community diversity. A similar number of bands was detected in the five soil samples collected from the diverse habitats. Bru et al. (2007) also found that the number of proteobacterial *napA* gene copies was similar in five different soil samples. The abundance of *napA*-containing bacteria cannot be associated with a single physicochemical soil characteristic given that the periplasmic nitrate reductase has a variety of physiological roles in different organisms (Richardson et al. 2001). For example, in environments were the oxygen concentration is low, as in the rizosphere, *napA*-containing bacteria possess an adaptive advantage in using nitrate as an alternative acceptor of electrons (Philippot et al. 1995; Ghiglione et al. 2000). Nitrate reduction by periplasmic nitrate reductase can also be important in soils with large amounts of organic matter where the denitrification process is stimulated (Christensen et al. 1990; D'Haene et al. 2003).

Conclusion

Differences in composition of *napA*- and *narG*-containing soil communities were evident when libraries from soil T1 and T3 were analyzed. The *narG* and *napA* genes might provide a good model to study saline alkaline environments due to the great diversity of prokaryotes that use nitrate as an alternative electron acceptor in saline environments.

The composition of the respiratory and dissimilatory nitrate-reducing community was different in the salt-affected soils. Differences were evident when *napA* was used as functional marker in DGGE analysis. However,

DGGE analysis of the *narG* partial sequences did not evidence these differences.

Further studies will be needed to understand the function and biochemical impact of dissimilatory and respiratory nitrate reducers in salt-affected environments, and especially in saline alkaline soils where knowledge about N cycle is limited.

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