Characterization of Microbial Communities Removing Nitrogen Oxides from Flue Gas: the BioDeNOx Process

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BioDeNOx is an integrated physicochemical and biological process for the removal of nitrogen oxides (NOx) from flue gases. In this process, the flue gas is purged through a scrubber containing a solution of Fe(II)EDTA²⁻, which binds the NOx to form an Fe(II)EDTA·NO²⁻ complex. Subsequently, this complex is reduced in the bioreactor to dinitrogen by microbial denitrification. Fe(II)EDTA²⁻, which is oxidized to Fe(III)EDTA⁻ by oxygen in the flue gas, is regenerated by microbial iron reduction. In this study, the microbial communities of both lab- and pilot-scale reactors were studied using culture-dependent and -independent approaches. A pure bacterial strain, KT-1, closely affiliated by 16S rRNA analysis to the gram-positive denitrifying bacterium *Bacillus azotoformans*, was obtained. DNA-DNA homology of the isolate with the type strain was 89%, indicating that strain KT-1 belongs to the species *B. azotoformans*. Strain KT-1 reduces Fe(II)EDTA·NO²⁻ complex to N₂ using ethanol, acetate, and Fe(II)EDTA²⁻ as electron donors. It does not reduce Fe(III)EDTA⁻. Denaturing gradient gel electrophoresis analysis of PCR-amplified 16S rRNA gene fragments showed the presence of bacteria closely affiliated with members of the phylum *Deferribacteres*, an Fe(III)-reducing group of bacteria. Fluorescent in situ hybridization with oligonucleotide probes designed for strain KT-1 and members of the phylum *Deferribacteres* showed that the latter were more dominant in both reactors.

Nitrogen oxides (NOx), i.e., nitric oxide (NO) and nitrogen dioxide (NO₂), are important greenhouse gases causing air pollution. The NOx act as indirect greenhouse gases by producing the tropospheric greenhouse gas ozone during their breakdown in the atmosphere. Over the long time scale, they contribute to climate changes (36). Moreover, nitric oxide is highly toxic. It affects neurons and plays a role in the development of several diseases, such as Parkinson's disease and asthma (27). Therefore, removal of NOx from flue gases, which are responsible for 17 to 20% of the anthropogenic NOx emission (29), is of paramount importance for a clean and healthy environment.

To remove NOx from flue gas, several chemical and biological processes have been developed (9, 18, 28). However, a promising process, in which the advantages of chemical and biological processes are combined, is the BioDeNOx process (6). In this process, the NOx is absorbed by the Fe(II)EDTA²⁻ chelating complex in a scrubber and subsequently removed by microbial denitrification in a reactor. Due to the high temperature of flue gases, the process operates at a temperature of between 50 to 55°C.

The BioDeNOx process comprises four reactions, i.e., two chemical reactions in the scrubber and two microbiological reactions in the bioreactor (Fig. 1). The first reaction is the absorption and complexation of NOx with Fe(II)EDTA²⁻. This chemical reaction is fast and leads to an increased mass transfer rate of NOx (9). The second reaction is the oxidation

of Fe(II)EDTA²⁻ to Fe(III)EDTA⁻ by oxygen in the flue gas. This reaction is an unwanted chemical reaction as it lowers the availability of Fe(II)EDTA²⁻ for NO absorption. In the bioreactor, NOx are reduced to N₂ by denitrifying bacteria by using an organic substrate, e.g., ethanol, as electron donor. Subsequently, iron reducers reduce Fe(III)EDTA⁻ to Fe(II)EDTA²⁻, using, e.g., ethanol as an electron donor.

So far, nothing is known about the species composition of the microbial communities and their involvement in the BioDeNOx process. Therefore, the aim of this research was to study the structure and dynamics of the microbial community involved in the BioDeNOx process. For this purpose, we used a combination of culture-dependent and -independent approaches (5, 19). In the first approach, we isolated and characterized microorganisms from the BioDeNOx process. In addition, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments was used to study the species composition of the reactors removing NOx and to monitor population shifts over time. The phylogenetic affiliations of predominant microorganisms were inferred by sequencing of separated DGGE bands. Fluorescence in situ hybridization (FISH) with specific oligonucleotide probes was used to determine the abundances of the particular populations in the reactors.

MATERIALS AND METHODS

Bioreactor characteristics. Biomass samples for enrichment and molecular analysis were obtained from lab- and pilot-scale BioDeNOx reactors operated by Paques B.V. The media in the reactors were not completely cellular, as there were some iron precipitates. The lab-scale reactor was run with an artificial flue gas containing an NO concentration of between 1,200 and 2,000 ppm and an oxygen percentage of 2.5 to 3.0%. The rest of the gas was N₂. The pilot-scale

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FIG. 1. Schematic drawing of the BioDeNOx process. NOx is a mixture of NO and NO₂ (6).

reactor was used to treat the flue gas from a fluidized catalytic cracking unit. The NOx concentration in the pilot-scale reactor was between 100 and 150 ppm, and the oxygen concentration was 3 to 5% of total flue gas. The flue gas was first treated for sulfur dioxide removal by using a gypsum scrubber and then taken for NOx treatment. Ethanol was used as an electron donor in both the lab- and the pilot-scale reactors. Table 1 gives the operational characteristics of both the reactors. In the lab-scale reactor, the scrubber and reactor were integrated in one vessel of 5 liters. In the upper 20% of this reactor, the gas containing NO was scrubbed. In the pilot-scale setup, the scrubber, with a volume of 450 liters, and the bioreactor, with a volume of 1,200 liters were separated, and biomass was not prevented from entering the scrubber.

Samplings for microbial analysis were done from the lab-scale reactor when the temperature of the reactor was changed (from 40°C to 55°C). A sample from the pilot-scale reactor was obtained when the reactor was at its maximal efficiency for NOx removal.

Medium and cultivation methods. Anoxic sodium bicarbonate (5 g/liter)-buffered media were used, with 0.28 g NH₄Cl, 0.25 g K₂HPO₄, 0.1 g MgSO₄·7H₂O, 0.01 g CaCl₂·2H₂O, and 0.1 g yeast extract per liter. After autoclaving and cooling under argon, 1 ml of trace element solution (42) was added per liter of medium, and the pH of the medium was adjusted to 7.0. Serum bottles (100-ml volume) were filled with 30 ml mineral medium. Subsequently, 1 ml of inoculum was added anaerobically. Substrates were injected aseptically with syringes to final concentrations of 25 mM Fe(II)EDTA²⁻, 25 mM Fe(III)EDTA⁻, 5 mM nitrate, 2 mM nitrite, 2 mM ethanol, 2 mM acetate, and 2 mM succinate. EDTA, nitrite, nitrate, and acetate were added as sodium salts. Fe(II)EDTA²⁻ and Fe(III)EDTA⁻ stock were prepared using equal concentrations (100 mM) of sodium-EDTA and FeCl₂ or FeCl₃ under anoxic condition (41). Hydrogen, NO, and N₂O were flushed in the headspace when they were used as the substrates. The bottles were incubated at 55°C.

Growth experiments for determining the optimal pH and temperatures for the isolate and the type strain were performed in mineral medium containing nitrite as an electron acceptor and ethanol as an electron donor. A pH range from 5 to 9 and a temperature range from 20°C to 60°C were used, and growth was estimated by protein determination after 24 and 48 h.

Type strains used in this study. *Deferribacter thermophilus* (DSM 14813), *Denitrovibrio acetophilus* (DSM 12809), *Bacillus infernus* (DSM 10277), *Bacillus simplex* (DSM 1321), *Bacillus thermodenitrificans* (DSM 465), and *Bacillus azotoformans* (DSM 1046) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Enrichment and isolation of NO-reducing bacteria. Enrichment of NO-reducing microorganisms was performed using Fe(II)EDTA²⁻ as an electron donor and nitrosyl complex [i.e., Fe(II)EDTA·NO²⁻] as an electron acceptor. The medium was supplied with 100 mg/liter yeast extract as a carbon source. The nitrosyl complex at a concentration of 5 mM was formed with 5 mM nitrite and 25 mM Fe(II)EDTA²⁻ (41) according to the following stoichiometry: 2Fe(II)EDTA²⁻ + NO₂⁻ + 2H⁺ ⇌ Fe(II)EDTA·NO²⁻ + Fe(III)EDTA⁻ + H₂O. After all the nitrite reacted with Fe(II)EDTA²⁻ to form Fe(II)EDTA·NO²⁻ complex, the free nitrite concentration in the medium was checked and the bottles were inoculated with 1 ml of inoculum from both the lab- and pilot-scale reactors to enrich nitrosyl complex-reducing microorganisms. The

enrichment cultures were transferred every 3 to 4 days. Growth was observed by turbidity and measured by the increase in the protein concentration.

Pure cultures were isolated from the enrichment described above by the agar dilution method (32). Cells from the enrichment of the lab-scale reactor were diluted in the same medium containing 2% (wt/vol) agar in Hungate tubes and incubated at 55°C. Individual colonies were picked and transferred to the same medium. The purity of the isolate was further substantiated microscopically and by the use of PCR-DGGE (40).

Analytical and growth procedures. Ferrous iron was quantified photometrically at 510 nm after chelating with 6 mM o-phenanthroline in 1.5 M sodium acetate buffer in a test volume of 1 ml (a modification of method described in reference 10). Enzymatic bioanalysis kits (R-Biopharm AG, Germany) were used to measure ethanol and acetate. Nitrate (4) and nitrite (17) were measured using standard colorimetric assays. The growth yield was measured by protein estimation (20). DNA-DNA hybridization and subsequent determination of G+C content were performed as described by Marmur (24), Gillis et al. (14) and De Ley et al. (8).

The headspace gas of the enrichments and batch cultures were analyzed for N₂ and N₂O qualitatively using on-line gas chromatography as described previously by Otte et al. (30).

DNA extraction. Samples were taken at different time points from both the lab- and pilot-scale reactors. The DNA from the bacterial biomass was extracted using a soil DNA extraction kit (MOBIO Laboratories) according to the procedure described by the manufacturer. The quality and yield of the extracted DNA were determined by agarose gel electrophoresis. Subsequently, the extracted DNA was used as template DNA in the PCR.

PCR amplification of 16S rRNA gene fragments. Fragments carrying the 16S rRNA gene of the domain *Bacteria* were amplified by using the primers 341F with a GC clamp and 907R (37) for DGGE analysis. Primers GM3 and GM4 were used to produce a nearly complete 16S rRNA gene fragment of the isolated strain (5). PCR amplifications were performed in 100-μl volumes containing 5 to 100 ng of template DNA, 10 μl of 10-times-concentrated *Taq* buffer, 250 μM of each deoxynucleoside triphosphate, 25 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (Amersham Biosciences, Roosendaal, The Netherlands). PCR products were determined by electrophoresis in 1% (wt/vol) agarose gels, which were stained with ethidium bromide and destained with water.

DGGE analysis of PCR products. DGGE was performed as described previously, using the D-Code system (Bio-Rad Laboratories, Veenendaal, The Netherlands) with 1-mm-thick gels (37). PCR products were applied directly onto 6% polyacrylamide gels with denaturing gradients from 20 to 70% denaturant (100% denaturant is 7 M urea and 40% [vol/vol] formamide). Individual bands were excised from the gel, reamplified using the same primer pair, and again checked for purity by DGGE. Subsequently, the PCR products were purified with a QIA-Quick gel extraction kit (QIAGEN Laboratories) and sequenced.

The obtained sequences were first compared to sequences stored in the GenBank database by using the BLAST algorithm. Subsequently, the phylogeny of the community members was inferred using the software program ARB (21). Briefly, the sequences were imported into ARB and aligned using the Automatic

TABLE 1. Operational characteristics of lab- and pilot-scale reactors

Parameter	Value in:	
	Lab-scale reactor	Pilot-scale reactor
Temp (°C)	41–55	40–60
pH	6.8–7.5	7–8
Redox potential (mV)	–200 to –50	–250 to –50
Hydrolytic retention time (days)	2–3.5	4–17
Overall Fe-EDTA (mM)	25	30
Gas flow rate (m ³ /h)	0.09	123
NOx (NO + NO ₂) (ppm)	1,200–1,500	100–150
Oxygen (%)	2.5–3	3 to 6
NOx removal (%) (at the time of sampling)	99	90–99
Ethanol dosed (mol/h)	0.065	0.87
% Ethanol used for NO reduction	30–40	20–30
% Ethanol for used Fe(III)EDTA ⁻ reduction	60–70	70–80

TABLE 2. FISH probes used in this study

Probe name	Target organism(s)	Probe sequence (5' to 3')	Length (nucleotides)	Formamide concn (%)	Reference(s)
EUB 338	<i>Eubacteria</i>	GCT GCC TCC CGT AGG AGT	18	0–80	1, 7
ALF968	Alpha <i>Proteobacteria</i>	GGT AAG GTT CTG CGC GTT	18	20	22
BET42A	Beta <i>Proteobacteria</i>	GCC TTC CCA CTT CGT TT	17	35	22
GAM42A	Gamma <i>Proteobacteria</i>	GCC TTC CCA CAT CGT TT	17	35	22
DeltaSRB385	Sulfate-reducing delta <i>Proteobacteria</i>	CGG CGT CGC TGC GTC AGG	18	35	2
CF319a	<i>Cytophaga</i>	TGG TCC GTG TCT CAG TAC	18	35	23
HGC69a	Gram-positive, high-G+C bacteria	TAT AGT TAC CAC CGC CGT	18	25	34
ARCH915	<i>Archaea</i>	GTG CTC CCC CGC CAA TTC CT	20	20	38
BDN 584	<i>Deferribacteres</i> group	GCG GUG CGG UAA GUC AGG G	19	30	This study
BDN 174	KT-1	CCG GAU AAC UCU UUG GAU CG	20	35	This study

Aligner function. Subsequently, a neighbor-joining tree was created using the Jukes-Cantor correction mode. The statistical significance of the phylogenetic groups in the tree was tested using bootstrap analysis (1,000 replicates), which was performed with the software program PAUP.

Oligonucleotide probes used in this study. Specific probes for the isolated strain KT-1 and for members of the phylum *Deferribacteres* were designed using the Probe Design function in the ARB software (21). The complete 16S rRNA sequence of strain KT-1 was used for designing probes. In case of the *Deferribacteres* probe, sequences obtained from excised DGGE bands were used. Fixed cells of type strains (see above) were used as reference strains for testing the specificities of the probes. Table 2 gives an overview of the probes used in the study.

FISH. Samples from the reactors and from pure cultures were washed with 10 mM phosphate buffer (pH 7.2) containing 130 mM NaCl and were resuspended in the same buffer. Subsequently, the cells were fixed with paraformaldehyde for the fixation of gram-negative bacteria or with ethanol for the fixation of gram-positive bacteria. Hybridization was carried out according to the protocol described by Pernthaler et al. (31), using formamide concentrations as specified in Table 2. For gram-positive bacteria, an intermediate lysozyme incubation step of 30 min on ice was carried out, followed by another dehydration step through 50%, 80%, and 96% (vol/vol) ethanol as described previously (11, 25). The slides were embedded in Vectashield (Vector Laboratories, Burlingame, CA) and observed with a Zeiss Axioplan 2 epifluorescence microscope. Images were acquired with Leica FW4000 software.

Cell counts of strain KT-1, *Deferribacter*, and other bacteria were done by semiquantitative evaluation as described by Neef et al. (26). The hybridized samples were analyzed by two observers for determining the fraction of probe-positive cells relative to all cells visualized with the general bacterial probe Eub338 or DAPI (4',6'-diamidino-2-phenylindole) stain. The hybridization experiments were repeated three-times to confirm the results, and different microscopic fields of the slides were photographed and analyzed.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank database under accession numbers AY920317 to AY920325.

RESULTS

Reactor operation. The nitrogen oxide removal efficiency in both lab- and pilot-scale reactors was between 90 and 99% within a temperature range from 45°C to 60°C (see Table 1 for the operational details of the reactors). In the pilot-scale system, the sulfur dioxide removal efficiency in the gypsum scrubber was 99% (data not shown). Sulfate concentrations of 20 to 70 mg/liter were measured in the pilot-scale reactor. Calculation of the stoichiometry of ethanol oxidation for NO reduction and Fe(III)EDTA[−] reduction (based on the concentration of NO removed, the oxygen concentration in the influent gas, and the amount of ethanol consumed) showed that 60 to 80% of the ethanol was used for Fe(III)EDTA[−] reduction and 20 to 40% was used for NO reduction (Table 1). In both reactors an approximate biomass concentrations of 1 g/liter was measured by the industrial operators.

Enrichment and isolation of NO-reducing bacteria. Enrichment of microorganisms reducing Fe(II)EDTA·NO^{2−} was performed with inocula from both the lab-scale and pilot-scale reactors. The cultures showed a change in medium color from green to reddish brown after 2 to 3 days of incubation. The chemical analysis of controls and inoculated bottles confirmed the reduction of Fe(II)EDTA·NO^{2−} complex to N₂ with Fe(II)EDTA^{2−} as the electron donor. After six successive transfers (with a 1:10 dilution in every transfer), the enrichment culture obtained from the lab-scale reactor was used for isolation of pure cultures. Pure cultures were obtained in 2% (wt/vol) agar incubated at 55°C. Colonies appeared after 3 to 4 days and showed a similar morphology. Five of the colonies were picked and inoculated in different bottles with the same culture medium used for the enrichment. One of the bottles showed growth and was taken for indefinite serial liquid dilution to obtain a pure culture. The purity of the culture was checked by microscopic observation. In addition, PCR-DGGE analysis showed one band (Fig. 2, lane 7), indicating a pure culture.

Characterization of strain KT-1. After the purity was confirmed, the nearly complete 16S rRNA gene sequence of the isolate, named strain KT-1, was determined. Comparative analysis with sequences stored in the GenBank database showed a similarity of 95% with the 16S rRNA sequences of *Bacillus azotoformans* (33, 39). A neighbor-joining tree was created to infer the phylogenetic affiliation of strain KT-1 (Fig. 3). Strain KT-1 clustered with *B. azotoformans* (DSM 1046), as confirmed by a bootstrap value of 90%. DNA-DNA homology between strain KT-1 and *B. azotoformans* was 89%. Strain KT-1 and *Bacillus azotoformans* are gram-positive sporeformers and were checked for growth at different temperatures and pHs. Both strains showed optimum growth at pH 7 to 7.5. Strain KT-1 can grow within a temperature range of 40°C to 60°C, with an optimal temperature of 50°C to 55°C. *B. azotoformans* (DSM 1046) can grow between 30°C and 40°C, with 30°C as the optimal temperature for growth. Ethanol, acetate, succinate, and yeast extract were used by both strains as organic electron donors. Fe(II)EDTA^{2−} can be used as an inorganic electron donor by both strains, with the supplement of an organic substrate (i.e., 100 mg/liter yeast extract) as a carbon source. The electron acceptors nitrate, nitrite, Fe(II)EDTA·NO^{2−}, and oxygen can be used by both strains, whereas Fe(III)EDTA[−] was not used as an electron acceptor. Growth was observed for both strains when NO was flushed in

FIG. 2. DGGE profiles of PCR-amplified 16S rRNA gene fragments obtained from biomass taken at different time points from the lab-scale (LS) and pilot-scale (PS) BioDeNOx reactors. Lane 1, lab-scale reactor, August 2001, 55°C; lane 2, lab-scale bioreactor, February 2002, 50°C; lane 3, lab-scale reactor, October 2001, 55°C; lane 4, lab-scale reactor, April 2002, 40°C; lane 5, lab-scale reactor, May 2002, 40°C; lane 6, pilot-scale reactor, May 2002, 50°C; lane 7, KT-1. Labeled bands were excised, reamplified, and sequenced.

the headspace and ethanol was used as an electron donor [the medium did not have Fe(II)EDTA^{2-}].

Batch experiments were conducted with strain KT-1 in two different culture media, and kinetic data were obtained. In the first experiment (Fig. 4A), 5 mM nitrosyl complex, with Fe(II)EDTA^{2-} (25 mM) and ethanol (1 mM), were provided. The Fe(II)EDTA^{2-} oxidation to Fe(III)EDTA^- with the oxidation of ethanol and reduction of nitrosyl complex showed that Fe(II)EDTA^{2-} was used as the electron donor and ethanol as the carbon source. The biomass yield obtained was 4 mg protein/mol of nitrite. In the second experiment, ethanol was provided in surplus (i.e., 3 mM) with the same concentrations of nitrosyl complex and Fe(II)EDTA^{2-} (Fig. 4B). The results showed ethanol oxidation and nitrosyl complex reduction without significant iron oxidation, indicating that ethanol was used both as an electron donor and as a carbon source. The yield obtained was 5.6 mg protein/mol of nitrite.

PCR-DGGE analysis of the BioDeNOx microbial community. The microbial communities of the bioreactors from the lab- and pilot-scale reactor were studied by DGGE analysis of PCR-amplified 16S rRNA gene fragments (Fig. 2). All reactor samples showed a relatively low complexity, with a maximum of five bands. The microbial community in the lab-scale reactor changed over time and with temperature, and the communities

of the lab- and pilot-scale reactors were only slightly different (Fig. 2). There were DNA bands with melting behavior similar to that for strain KT-1 (Fig. 2, bands PS1 and LS1). To identify the microorganisms in the reactors and to substantiate the presence of strain KT-1, the bands were excised and sequenced.

Identification of bacteria in the BioDeNOx process. A neighbor-joining tree was generated using the sequences of the excised DNA fragments, showing the phylogenetic affiliation of BioDeNOx community members (Fig. 3). Both the lab- and pilot-scale reactors showed the presence of DNA fragments at the same position in the gel as for strain KT-1. Nevertheless, comparative analysis of the sequences of these fragments (Fig. 2, bands PS1 and LS1) showed that they were more closely related to members of the *Deferribacteres* phylum, i.e., *Deferribacter thermophilus* (16) and *Flexistipes sinusarabici* (12).

The sequence from another DNA fragment of the pilot-scale reactor (Fig. 2, band PS2) was closely related to the genus *Desulfotobacterium*, a thermophilic sulfite- and Fe(III) -reducing bacterium (13). Furthermore, sequences of bacteria affiliated with members of the β subdivision of the *Proteobacteria*, such as *Hydrogenophilus* spp. and *Alcaligenes* spp., were identified in the lab-scale reactor sample (Fig. 2, band LS2). Sequences affiliated with the phylum *Bacteroidetes*, which includes members of the *Cytophaga*, *Flavobacteria*, and *Bacteroides* (Fig. 2, bands LS5, LS6, and LS7), and with the phylum *Spirochaetes* (Fig. 2, band LS4) were also identified in the lab-scale reactor samples.

In situ detection of members of the *Deferribacteres* group. Probes specific for members of the phylum *Deferribacteres* were designed from the sequences of the DGGE-separated DNA fragments. Probe BDN_584, which belongs to accessibility class IV (3), was tested with the type species *Deferribacter thermophilus*, *Denitrovibrio acetophilus*, and KT-1 as reference strains. The probe gave positive signals with *D. thermophilus* and *D. acetophilus* but not with strain KT-1. The probe was tested on samples from both the lab-scale (May 2002, 40°C) and pilot-scale (May 2002, 50°C) reactors. Approximately 65% of all cells were stained with this probe, using Eub338 as a general probe targeting all bacteria (Fig. 5A).

Of the other probes tested, only the probe specific for members of the β subdivision of the *Proteobacteria* gave a positive signal with some of the bacterial cells in the reactor samples (Fig. 5A).

In situ detection of the strain KT-1. Specific oligonucleotide probes were designed from the complete 16S rRNA sequence of strain KT-1 and used to study the relative abundance of this strain in the lab- and pilot-scale reactors. Probe BDN_174, which belongs to accessibility class III (3), was tested at different formamide concentrations with strain KT-1, *Bacillus thermodenitrificans*, *Bacillus simplex*, and *Bacillus infernus*. The probe gave a positive result with strain KT-1 and no results with the other bacteria. Subsequently, the probe was tested on different samples of the lab-scale (May 2002, 40°C) and pilot-scale (May 2002, 50°C) bioreactors. Approximately 20% of the total number of bacterial cells, as determined with DAPI, were stained with the probe in both samples, indicating a substantial presence of strain KT-1 (Fig. 5B).

FIG. 3. Neighbor joining tree based on 16S rRNA sequences, showing the phylogenetic affiliation of strain KT-1 and other microbial community members in the lab- and pilot-scale BioDeNO_x reactors. The scale bar represents an estimated 10% sequence divergence. Names in boldface refer to sequences obtained in this study. Numbers on the branches are bootstrap values; only values higher than 90% are given.

DISCUSSION

Characterization of strain KT-1. KT-1 is a moderately thermophilic bacterium, which grows at a broad range of temperatures. The batch experiments show that strain KT-1 could reduce nitrosyl complex at a concentration of 5 mM with both Fe(II)EDTA²⁻ and ethanol as electron donors. However, the results showed that ethanol is the preferred electron donor over Fe(II)EDTA²⁻, and Fe(II)EDTA²⁻ oxidation occurs only when ethanol is limited in concentration. The electron donors and acceptors tested showed that the strain is a facultative anaerobe.

The initial results of 16S rRNA sequencing of strain KT-1 showed only 95% similarity with the type species *Bacillus azotoformans*. This suggested that strain KT-1 could be a new *Bacillus* species belonging to the phylum *Firmicutes* of the low-G+C gram-positive bacteria, since the similarity percentage was lower than 97% (standard threshold percentage for new species description). However, DNA-DNA hybridization analysis of both strains gave a result of 89% homology. Clearly, this is far above the species common boundary (50 to 70%) described by Rosello-Morá and Amann (35) in their article discussing the comparison of DNA-DNA homology and 16S

FIG. 4. (A) Growth of strain KT-1 with 25 mM Fe(II)EDTA²⁻, 5 mM nitrite, and 1 mM ethanol. (B) Growth of strain KT-1 with 25 mM Fe(II)EDTA²⁻, 5 mM nitrite, and 3 mM ethanol.

FIG. 5. (A) In situ detection of bacteria in the pilot-scale bioreactor (May 2002, 50°C) with a Cy3-labeled (red) probe specific for members of the phylum *Deferribacteres* (i.e., probe BDN_584) and a Cy5-labeled (green) probe specific for the β subdivision of the *Proteobacteria*. DAPI (blue) was used to stain all bacteria. Bar, 20 μ m. (B) In situ detection of strain KT-1 in the pilot-scale bioreactor with a Cy3-labeled (red) probe specific for strain KT-1 (i.e., probe BDN_174). DAPI (blue) was used to stain all bacteria. Bar, 20 μ m.

rRNA similarities of 180 strains. The same article also mentioned that around 10% of these microorganisms, which fall inside the species common boundary, have a 16S rRNA similarity lower than 97%. In addition, high similarity of the whole-cell protein profile (results not shown) and physiological characteristics (except the optimum temperature for growth) of strain KT-1 and *B. azotoformans* (in this study) suggested that strain KT-1 is a new strain of the species *B. azotoformans*.

Analysis of diversity and dynamics in BioDeNOx reactors. DGGE analysis showed that the microbial community in the BioDeNOx reactors was changing with time, temperature, and flue gas composition (Fig. 2). In the pilot-scale reactor, apart from oxygen, some traces of untreated SO_2 in the flue gas could also have been the reason for the difference in the banding pattern. The detection of SO_4^{2-} (20 to 70 mg/liter) in the bioreactor can be explained because of SO_2 oxidation.

The low microbial diversity observed in DGGE may be due to the high temperature of 50 to 55°C at which the process is carried out. The high temperature might have eliminated mesophilic bacteria, which are commonly found in other processes and environments.

Microbial community composition in BioDeNOx reactors. Both the lab- and pilot-scale reactors showed sequences closely affiliated to type strains of the phylum *Deferribacteres*. The literature indicated that the members of the phylum *Deferribacteres* are moderately thermophilic bacteria that can reduce Fe(III), nitrate, arsenate, and sulfur by using organic substrates as electron donors (12, 16). *Deferribacter thermophilus* is a thermophilic dissimilatory iron-reducing bacterium isolated from a petroleum reservoir (16), and *Flexistipes sinusarabici* is a thermophilic bacterium isolated from hot brine water of the Red Sea (12). In addition to these bacteria, the pilot-scale reactor biomass showed sequences affiliated with the genus *Desulfitobacterium* (13), which can reduce sulfite as well as Fe(III). The remaining amount of SO_2 in the flue gas of the

pilot-scale reactor could be the reason for this bacterium to appear in the reactor.

Hybridization analysis with probe BDN_584, designed for the *Deferribacteres* group, confirmed their presence in large numbers both in the lab- and pilot-scale reactors (Fig. 5A). This is consistent with the DGGE results. FISH results with probe BDN_174, specific for strain KT-1, showed that the isolate is a representative bacterium in the samples from both reactors (approximately) but cannot be considered the most dominant species in either reactor (Fig. 5B). This is in contrast to the DGGE results, where sequences of strain KT-1 were not found. The reason for this difference could be a bias in the PCR or the relatively low number of cells. Also, the gram-positive cell wall structure would have been highly resistant to the cell wall disruption step of the DNA extraction methodology.

FISH experiments using general probes gave positive signals with bacteria belonging to the β subclass of the *Proteobacteria*. This is also consistent with PCR-DGGE results (Fig. 3). The positive result obtained with β -subclass-specific probes (20 to 25% of the biomass [Fig. 5A]) indicates that these bacteria might play a role in the BioDeNOx process.

The oligonucleotide probe for members of the phylum *Bacteroidetes* did not show a positive signal, in contrast to the DGGE sequencing results. This may be because these microorganisms were low in number or because the general probe for this phylum does not target them. However, *Cytophaga*-like bacteria cannot be directly related to the process function, as it is known that they are common in anaerobic microbial communities as fermentative chemoorganoheterotrophs (15). In addition, DGGE sequences closely related to *Spirochaeta* were detected in only one sample of the lab-scale reactor, indicating that this group does not play an important role in the process.

Conclusions. The combination of culture-dependent and -independent approaches gave important information on the

structure and function of the microorganisms involved in the BioDeNO_x process. Strain KT-1, isolated from the BioDeNO_x reactor, is a moderately thermophilic strain of the species *Bacillus azotoformans*. It can reduce Fe(II)EDTA·NO²⁻ to N₂ but does not reduce Fe(III)EDTA⁻. DGGE analysis of PCR-amplified 16S rRNA gene fragments showed sequences of community members closely affiliated with *Deferribacter thermophilus*, which is a moderately thermophilic iron-reducing bacterium. Moreover, fluorescence in situ hybridization with KT-1-specific and *Deferribacter*-specific probes showed that the former covered between 15 and 20% and the later around 65% of the overall microbial community. The process data showed that ethanol as an electron donor was used predominantly (60 to 70%) for the reduction of Fe(III)EDTA⁻ and only 20 to 30% for the reduction of the nitrosyl complex. This indicates that members of the phylum *Deferribacteres* are important players in the reduction of Fe(III)EDTA⁻. For a better insight into the dynamics of the microbial community, however, replicate samples from the bioreactors need to be taken more frequently. Future work should concentrate on this and on the isolation of community members involved in the reduction of Fe(III)EDTA⁻.

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