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Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate

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Abstract Anaerobic ammonium oxidation with nitrite to N_2 (anammox) is a recently discovered microbial reaction with interesting potential for nitrogen removal from wastewater. We enriched an anammox culture from a rotating disk contactor (near Kölliken, Switzerland) that was used to treat ammonium-rich leachate with low organic carbon content. This enrichment led to a relative population size of 88% anammox bacteria. The microorganism carrying out the anammox reaction was identified by analysis of the 16S rDNA sequence and by fluorescence in situ hybridization (FISH) with 16S-rRNA-targeting probes. The percentage sequence identity between the 16S rDNA sequences of the Kölliken anammox organism and the archetype anammox strain Candidatus *Brocadia anammoxidans* was 90.9%, but between 98.5 and 98.9% with Candidatus *Kuenenia stuttgartiensis*, an organism identified in biofilms by molecular methods. The Kölliken culture catalyzed the anaerobic oxidation of ammonium with nitrite in a manner seemingly identical to that of Candidatus *B. anammoxidans*, but exhibited higher tolerance to phosphate (up to 20 mM) and to nitrite (up to 13 mM) and was active at lower cell densities. Anammox activity was observed only between pH 6.5 and 9, with an optimum at pH 8 and a temperature optimum at 37 °C. Hydroxylamine and hydrazine, which are intermediates of the anammox reaction of Candidatus *B. anammoxidans*, were utilized by the Kölliken organisms, and approximately 15% of the nitrite utilized during autotrophic growth was converted to nitrate. Electron microscopy showed a protein-rich region in the center of the cells surrounded by a doughnut-shaped region containing ribosomes and DNA. This doughnut-shape region was observed with FISH as having a higher fluorescence intensity. Similar to Candidatus *B. anammoxidans*, the Kölliken anammox organism typically formed homogenous clusters containing up to several hundred cells within an extracellular matrix.

Keywords Anaerobic ammonium oxidation · Anammox · Identification · Planctomycete · Fluorescence in situ hybridization · Electron microscopy · Physiology · Wastewater · Intermediates

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Introduction

Nitrogen removal is an important aspect of wastewater treatment often accomplished by microbial processes such as nitrification and denitrification. These reactions have been known for a long time (Winogradsky 1890; Kluyver and Donker 1926; Beijerinck and Minkman 1910) and have been successfully applied in most modern wastewater treatment plants. Recently, a new microbial process for nitrogen removal was observed in a fluidized bed-reactor in Delft (The Netherlands) (Mulder et al. 1995). The process, called anaerobic ammonium oxidation (anammox), involves oxidation of ammonium to N_2 with nitrite as electron acceptor under strictly anoxic conditions (Van de Graaf et al. 1995, 1996; Strous et al. 1997). ^{15}N-studies have shown that one N-atom of the produced N_2 originates from nitrite, whereas the other originates from ammonium. Hydroxylamine (NH_2OH) and hydrazine (N_2H_4) were identified as intermediates of the anammox process (Van de Graaf 1997).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (1)$$

The existence of bacteria capable of catalyzing the anammox reaction (Eq. 1) had already been predicted in 1977 by Broda (1977) based on thermodynamic calculations. Further studies have shown that the anammox process is carried out by an autotrophic bacterium (Van de Graaf et al. 1996), which has recently been shown to belong to the order *Planctomycetales* and has been named Candidatus *Brocadia anammoxidans* (Strous et al. 1999a). This organism was also postulated to couple the oxidation of nitrite to nitrate for generating reducing equivalents for CO_2 fixation (Van de Graaf et al. 1997). Candidatus *B. anammoxidans* grows slowly and has been reported to have a doubling time of 11 days at pH 8 and 40 °C (Jetten et al. 1999).

The advantages of the anammox process over the traditional combination of nitrification and denitrification for wastewater treatment are lower oxygen demand, which is needed by nitrifiers for partial oxidation of ammonium to nitrite, and no requirement for external carbon sources because the process is autotrophic. However, process startup could be hindered by the relatively low growth rate of anammox bacteria. Implementing the anammox process as a manageable wastewater treatment technology will also require a better understanding of the range of permissible nitrite, ammonium, and organic carbon loads, and oxygen and pH regimes.

The basic physiological aspects of the anammox process have been studied in depth mostly by a single research group in Delft (Jetten et al. 1999). However, anammox activity has been suspected for other wastewater treatment plants with uncharacterized high ammonium losses (Helmer and Kunst 1998; Siegrist et al. 1998; Schmid et al. 2000). To understand the anammox process and its importance in natural or engineered systems, it is desirable to identify other anammox bacteria besides the archetype strain Candidatus *B. anammoxidans*. Unfortunately, anammox organisms have been extremely difficult to cultivate in pure culture. Even Candidatus *B. anammoxidans* has only been purified to apparent homogeneity by Percoll density centrifugation (Strous et al. 1999a). Here, we describe the enrichment of anammox activity from biofilm material from a rotating biological contactor (RBC). Emphasis was placed on characterizing the anammox organism in this enrichment and on comparing its physiological characteristics to those of Candidatus *B. anammoxidans*.

Material and methods

Biomass and mineral medium

The culture was enriched from biofilm material from a nitrifying RBC in Kölliken near Aarau, Switzerland. This reactor exhibited 40–70% removal of nitrate at very low organic carbon concentrations (Siegrist et al. 1998). Anammox bacteria were enriched in 2.25-l Schott flasks containing approximately 2 l mineral medium with ammonium as electron donor, nitrite as electron acceptor and bicarbonate as carbon source. The bottles were closed with gas-tight butyl septa and incubated in an anaerobic chamber filled with nitrogen gas and traces of H_2. The initial pH was 7 and was adjusted regularly with HCl.

The medium contained initially in demineralized water: 3 mM $(NH_4)_2SO_4$, 6 mM $NaNO_2$, 25 mM $KHCO_3$, 1 mM K_2HPO_4, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2 ml of trace element solution 1 and 1 ml trace element solution 2 per liter. Trace element solution 1 contained (per liter demineralized water) 10 g $Na_2EDTA \cdot 2H_2O$ and 5 g $FeSO_4$. Trace element solution 2 contained (per liter demineralized water) 15 g $Na_2EDTA \cdot 2H_2O$, 0.43 g $ZnSO_4 \cdot 7H_2O$, 0.24 g $CoCl_2 \cdot 6H_2O$, 0.99 g $MnCl_2 \cdot 4H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 0.22 g $NaMoO_4 \cdot 2H_2O$, 0.19 g $NiCl_2 \cdot 6H_2O$, 0.08 g Na_2SeO_3, 0.014 g H_3BO_4.

Physiological experiments

All experiments were performed in 60-ml serum bottles filled with 43 ml medium and inoculated with 2 ml of an anammox bacteria suspension from the enrichment culture. The bottles contained a 15-ml headspace and were closed with butyl rubber stoppers (Maagtechnic, Dübendorf, Switzerland) and aluminum crimp seals. After inoculating the anammox culture and before starting incubations, the headspace was exchanged by applying several cycles of vacuum and overpressure (1.5 bar) or by flushing with a gas mixture containing 20% (mol% or mol-ppm) CO_2 (99.998% purity) and 80% He (99.9990%). The initial gas pressure of the headspace was 1.5 bar. All experiments were performed in triplicate at 37 °C and pH 7, unless stated otherwise. To study the effect of pH, a 20 mM phosphate buffer was used for experiments between pH 6 and 8, and a 50 mM Tris/HCl-buffer was used for the range between pH 8 and 9. The initial pH of the medium was adjusted by mixing different amounts of KH_2PO_4 and K_2HPO_4 and remained constant during the experiment. In the pH experiments, the headspace was flushed only with He (99.996%), and only 1 mM instead of 25 mM $KHCO_3$ was added. In control experiments without ammonium or nitrite, $(NH_4)_2SO_4$ or $NaNO_2$ were replaced with 2 mM Na_2SO_4. In experiments testing oxygen sensitivity, initial oxygen concentrations were set at 0.25, 0.5, 1 and 18%. Different amounts of oxygen gas were added to reach the different concentrations. The bottles containing 18% O_2 were flushed after 64 h with aligal62 [=20% CO_2 (99.5%) and 80% Ar (99.996%)]. All gases were purchased from Carbagas (Rümlang, Switzerland).

Nucleic acid extraction

Genomic nucleic acids were isolated from a 15-ml sample from the enrichment culture after four rounds of subculturing. The cells were pelleted and resuspended in a screw-capped vial in 1 ml of homogenization buffer (200 mM Tris-HCl pH 8.5, 1.5% SDS, 10 mM EDTA, 1% sodium desoxycholate, 1% nonidet-P40, 5 mM urea and 10 mM dithiothreitol). Glass beads (0.5 g, 0.1 mm in diameter) were added and the mixture was shaken twice for 1 min at 4,000 rpm in a Braun Cell Homogenizer (Inotech, Dottikon, Switzerland) with a 1-min interval on ice. The glass beads were allowed to settle without centrifugation, and the supernatant was transferred to a fresh Eppendorf tube. This sample was mixed 1:1 with acidic phenol (pH 5.3) and incubated in a water bath at 65 °C for 5 min (Aiba et al. 1981). After vortexing and centrifugation for 5 min at 15,000×*g* and 4 °C, the water phase was transferred to a new Eppendorf tube and mixed 1:1 (v/v) with Tris-HCl-buffered phenol (pH 8.0). After vortexing, chloroform/isoamylalcohol (24:1 v/v) was added, vortexed and centrifuged for 5 min as before. The water phase was again recovered and extracted with 1 ml of chloroform/ isoamylalcohol as before. The supernatant was transferred into a new Eppendorf tube, and DNA and RNA were pelleted with 0.7 volumes isopropanol and 0.1 volumes 3-M sodium acetate (pH 5.2) for 1 h at –80 °C. The tube was subsequently centrifuged for 15 min at 15,000×*g* to recover nucleic acids. The pelleted nucleic acids were washed with a solution of 70% (vol/vol) ethanol and 30% of a solution of 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) dissolved in RNase-free water.

Amplification of 16S rDNA

16S rDNA fragments from isolated total DNA of the enrichment culture were amplified with Taq DNA polymerase (GIBCO BRL, Life Technologies, Gaithersburg, Md.). The following eubacterial primers were used: 16S 6F (5′-ggagagttagatcttggctcag-3′) and 16S 1510R (5′-gtgctgcagggttaccttgttacgact-3′). As anammox-specific primers we used Pla46rc (5′-ggattaggcatgcaagtc-3′) and Amx820 (5′-aaaaccctctacttagtgccc-3′). Dimethylsulfoxide was added at 5%. Conditions for PCR were as suggested by the supplier, but with a cycling regime of: 4 min at 95 °C, then 35 cycles each of 45 s at 95 °C, 45 s at 56 °C, and 1 min at 70 °C. Final extension was carried out for 3 min at 72 °C. The 16S rDNA PCR products were cloned in *Escherichia coli* DH5α in vector pGEM-T-easy (Promega, Wallisellen, Switzerland) by established procedures (Sambrook et al. 1989). Plasmid DNAs for sequencing were isolated by boiling preparation (Sambrook et al. 1989). Plasmid inserts were sequenced on both strands by using a Thermosequenase Kit (Amersham, Little Chalfont, UK) with IRD-800 and IRD-700 labeled primers (MWG Biotech, Ebersberg, Germany). Universal vector-located primer sequences were used and, in addition, two primers which targeted a conserved region around position 785 of the cloned 16S rDNA fragment: EUB785R (5′-ggattagataccctg-gtag-3′) and EUB785F (5′-ctaccagggtatctaatc-3′). Sequence transcripts were separated and analyzed on a LiCOR 4200L IR^2 automated DNA sequencer (LiCOR, Lincoln, Neb.). The nucleotide sequence of the 16S rRNA gene of the Kölliken anammox organism was deposited in the GenBank database under accession no. AJ250882. Sequence comparisons were calculated by direct FastA alignments (Pearson and Lipman 1988).

Analytical measurements

Concentrations of O_2 and N_2 were measured with a gas chromatograph (GC) (type GC-8A; Shimadzu, Tokyo, Japan). A gas volume of 1 ml (at the pressure existing in the sample flask) was injected with a gas-tight syringe (GC syringe series A2; Supelco, Switzerland). The carrier gas was helium (99.999%). The GC was equipped with a thermal conductivity detector and two parallel packed columns. One column was packed with molecular sieve 5A 80/100 (Brechbühler, Schlieren, Switzerland), the other with Porapack Q 80/100 (Brechbühler).

The concentration of nitrate was quantified by ion-exchange chromatography using a DX500-apparatus with IONPAC-ATC1 (anion trap column), IONPAC-AG11 guard column, an analytical IONPAC-AS11–4-mm column, ASRSII-4-mm suppressor, and CD20 conductivity detector (Dionex, Olten, Switzerland).

The concentration of NO_2^- was determined either with ion chromatography as described above or colorimetrically. For the colorimetric quantification, cells were removed from the sample by centrifugation. Fifty µl of the sample was mixed with 950 µl of the reagent, which contained 1% sulfanilic acid, 0.05% *N*-(1-naphthyl)-ethylene diammoniumdichloride (Riedel-deHaën, purchased from Fluka, Switzerland) and 1 M of H_3PO_4. After 5 min of incubation at room temperature, the color development was complete and the absorbance at 540 nm was determined.

Ammonium was analyzed spectrophotometrically as described elsewhere (Scheiner 1976). Phenol reagent (0.4 ml) and hypochlorite reagent (0.6 ml) were added to a 1 ml sample, followed by incubation at room temperature in the dark for at least 2 h. The absorbance was measured at 635 nm.

The concentration of hydroxylamine (NH_2OH) was determined according to the method of Frear and Burrel (1955). The following reagents were used: 1 g 8-quinolinol dissolved in 100 ml absolute ethanol, 1 M sodium carbonate, 12% (w/v) trichloroacetic acid and 0.05 M sodium phosphate buffer pH 6.8. Sodium phosphate-buffer pH 6.8 (0.3 ml) and trichloroacetic acid solution (60 µl) were added to the sample (50 µl), which was followed by the addition of 8-quinolinol solution (0.3 ml). The solution was mixed, and the tube was closed and placed in a boiling water bath for 1 min. After cooling for 15 min at room temperature, the absorbance was measured at 705 nm.

The concentration of hydrazine (N_2H_4) was measured according to Watt (1952). A 50-µl sample was incubated for 10 min at room temperature with 0.95 ml of a reagent containing 0.4 g *p*-dimethylaminobenzaldehyde, 20 ml ethanol and 2 ml of 37% HCl. The absorbance was measured at 458 nm. The absorbance increased linearly with hydrazine up to concentrations of 0.4 mM.

All spectrophotometric measurements were performed with a Uvikon 860 spectrophotometer (Kontron, Switzerland).

In situ hybridization

Cells were recovered by centrifugation and resuspended in phosphate-buffered saline (PBS), pH 7.4, consisting of 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per liter distilled water. The samples were fixed by adding three volumes of 4% paraformaldehyde (in PBS), mixing and incubating for 3 h on ice. Afterwards, the cells were washed twice with PBS. The fixed cells were suspended in a solution of 50% PBS and 50% ethanol, and the mixture was stored at –20 °C. Glass slides (Huber and Co., Reinach, Switzerland) were coated with gelatin (Amann et al. 1990). Between 2 and 10 µl of a fixed sample was applied on a well of the coated slide, dried for 3 h at 45 °C and sequentially dehydrated in solutions of 50%, 80% and 100% ethanol (vol/vol) in PBS for 3 min each. To start hybridization, 9 µl of hybridization buffer (containing the appropriate NaCl and formamide concentration for the used probe, Table 1) and 1 µl of a fluorescently labeled probe (at a concentration of 50 ng/µl) were added to a well. The hybridization was conducted for 90 min at 45 °C in a humidified chamber.

Following hybridization, a stringent washing step was performed for 15 min at 48 °C in a buffer with the appropriate NaCl concentration (Table 1), 20 mM Tris-HCl, pH 7, 5 mM EDTA and 0.01% SDS. The samples were counterstained with DAPI (4,6-diamidino-2-phenylindole) at a final concentration of 10 mg/l for 5 min at room temperature and mounted in Citifluor (Citifluor, London, UK).

Table 1 Probes used for fluorescence in situ hybridization

Probe	Sequence (5′→3′)	Formamide concentration	Wash buffer NaCl (mM)	Reference
EUB338	gctgcctcccgtaggagt	0%	900	Amann et al. (1990)
Alf1b	cgttcgytctgagccag	20%	225	Manz et al. (1992)
Amx820	aaaaccctctacttagtgccc	25%	159	Strous (2000)
Amx1240	tttagcatccctttgtaccaacc	60%	14	Strous (2000)
Bet42a	gccttcccacttcgttt	35%	80	Manz et al. (1992)
Kst1273	tcggctttataggtttcgca	25%	159	Schmid et al. (2000)
NIT3	cctgtgctccatgctccg	35%	80	Wagner et al. (1996)
Ntspa662	ggaattccgcgctcctct	35%	80	Daims et al. (2000)
Pla46	gacttgcatgcctaatcc	30%	112	Neef et al. (1998)

All oligonucleotide probes were obtained from Microsynth (Balgach, Switzerland). Microscopy was performed on an Olympus BX50 microscope, equipped with filters HQ-CY3, HQ-FITC and HQ-DAPI (all from AF Analysentechnik). Digital images were taken with a CCD camera (type Sensys, Photometrics, Tuscon, Ariz., USA) and acquired in the program METAVIEW (Visitron, Puchheim, Germany). The relative population size of the anammox bacteria in the enrichment was determined by confocal laser scanning microscopy by M. Schmid at the Technical University of Munich on paraformaldehyde-fixed samples, stained with probe Amx820 and counterstained with SYBR Green I, according to Schmid et al. (2000).

Electron microscopy

Freeze-fraction and transmission electron microscopy were carried out by E. Wehrli from the Laboratory for Electron Microscopy I, the central electron microscopy facility of the Biology Department of the Swiss Federal Institute of Technology (ETH-Zürich). Samples were prepared as described elsewhere (Wehrli and Egli, 1988).

Protein measurements

Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Cell suspensions (2 ml) were pelleted, resuspended in the same amount of 0.1 M NaOH and incubated for 1.5 h at 80 °C. An 0.8 ml-aliquot of this suspension was incubated with 0.2 ml of Bio-Rad dye-reagent for 15 min. The absorbance was measured at 595 nm. A standard curve was prepared from known concentrations bovine serum albumin (Fraction V, Sigma).

Results

Enrichment of anammox bacteria

Anammox activity was previously observed in an RBC operated at the landfill site in Kölliken (near Aarau, Switzerland) (Koch et al. 2000). The RBC was used to treat ammonium-rich leachate with low organic carbon content. Cultures incubated under anoxic conditions with approximately 0.5 g of biofilm material scratched from the RBC in 1 l of mineral medium immediately converted nitrite (6 mM) and ammonium (5.2 mM). After several additions of nitrite and ammonium to the same culture flask (in each case to a final concentration of 6 and 5.2 mM, respectively), stable production rates of dinitrogen and nitrate were obtained (data not shown) and the culture density increased slowly. The culture had a brownish-red color, probably due to a high cytochrome content (Jetten et al. 1999). After reaching an OD_{600nm} of 0.08, the enrichment culture was diluted tenfold and used to inoculate a new flask. This dilution and enrichment procedure was repeated three more times within 6 months and led to an increase of the relative population size of anammox bacteria in the culture.

The enrichment culture was subsequently analyzed for the presence of anammox bacteria by cloning amplifiable 16S rDNA fragments, and by fluorescence in situ hybridization (FISH). Total DNA was isolated from the enrichment culture and 16S rDNA fragments were amplified by PCR using conserved eubacterial primers for almost the entire 16S rDNA length or using the primers Pla46rc and Amx820 (Table 1). The fragments were cloned, sequenced and compared to entries in the GenBank database. Among eleven plasmid inserts in the full-length 16S rDNA library, six were identical and revealed 90.9% sequence identity to the 16S rDNA sequence of Candidatus *B. anammoxidans* (Strous et al. 1999a, GenBank accession number AJ131819). On the basis of such a high percentage of sequence identity with the archetype anammox bacterium, we suspected this 16S rDNA fragment to be representative of possible anammox bacteria in the Kölliken enrichment culture. Furthermore, the percentages of sequence identity of this 16S rDNA fragment of the Kölliken enrichment culture with 16S rDNA fragments derived from biofilm material of a trickling filter operating in Stuttgart were between 98.5% and 98.9% (from a 1407-nucleotide overlap), as determined by direct FastA alignments (GenBank accession numbers AF202655-AF202663, Schmid et al. 2000). The 16S rDNA fragments analyzed from the reactors in Stuttgart have been proposed to originate from anammox microorganisms that were classified as Candidatus *Kuenenia stuttgartiensis* (Schmid et al. 2000). Therefore, the organisms enriched from the biofilm material from Kölliken seem more similar to Candidatus *K. stuttgartiensis* and less similar to Candidatus *B. anammoxidans*. We verified whether the near-full length 16S rDNA sequence of the Kölliken enrichment was representative for anammox bacteria in the culture by reamplifying a specific part of the 16S rDNA using one primer targeting a region conserved for *Planctomycetales* (Pla46rc) and one for all known anammox bacteria (Amx820). Eleven cloned 16S rDNA fragments were sequenced, which were all identical to the previously determined, near-full length 16S rDNA sequence.

Based on the cloned 16S rDNA sequence from Kölliken, we verified the presence of anammox bacteria in the enrichment culture by FISH. Two oligonucleotide probes (Amx820 and Amx1240), which previously were found to specifically detect Candidatus *B. anammoxidans* in Delft enrichments (Strous 2000), reacted positively with most organisms in our enrichment culture (Fig. 1). The probe Kst1275, which was developed for the anammox bacteria in Stuttgart (Schmid et al. 2000), reacted positively as well. Several other oligonucleotide probes specific for Candidatus *B. anammoxidans* (i.e., Amx156, Amx223 and Amx1154, Schmid et al. 2000) did not react with the Kölliken organism, and the target sequences for these probes were not conserved in the 16S rDNA sequence derived from the Kölliken organism (not shown).

Other general probes that reacted positively with most cells in the Kölliken biofilm enrichment were the ALF1b probe for α-Proteobacteria, the EUB338 general eubacterial probe, and the Pla46 probe for the order *Planctomycetales* (Neef et al. 1998). Similar to Candidatus *B. anammoxidans* (Strous 2000), FISH-stained cells typically had an inner area with very low fluorescence intensity (Fig. 1). By counting FISH-stained cells with different specific probes, we estimated that the enrichment culture con-

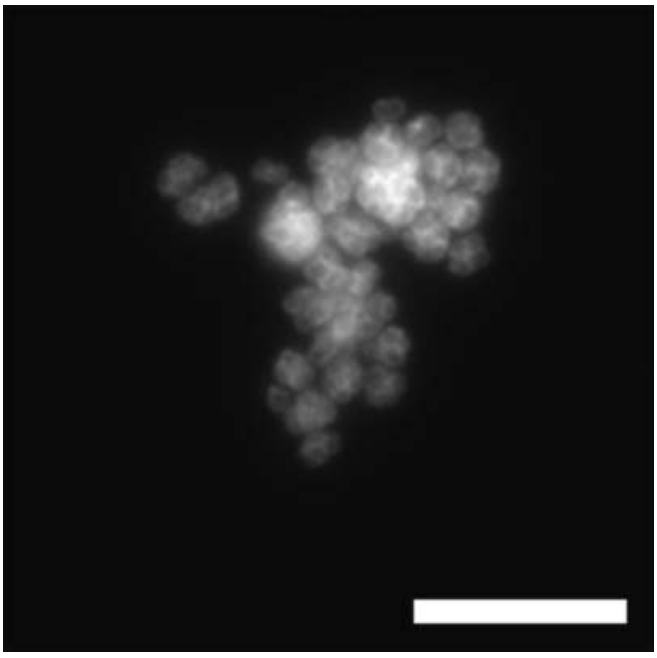

Fig. 1 Sample from the Kölliken anammox enrichment culture, stained with the probe Amx820. Note the optically lighter inside of the cells which results in their "doughnut" appearance. *Bar* 10 μm

tained approximately 90% anammox organisms (of all DAPI-stained cells). Very precise determination, however, was difficult since the anammox organisms tended to form stable and relatively large clusters (Figs. 1 and 2). By using confocal laser scanning microscopy on FISH-stained samples, a relative biovolume for the anammox population of 88%±5% could be determined (M. Schmid, personal communication). Since the probes Amx820, Kst1275, Amx1240 and Pla46 all stained the majority of the cells in the enrichment culture, and since positively reacting cells were all of the same type, we concluded that the derived 16S rDNA sequence originated from the majority of cells in the enrichment. These cells most likely represent anammox bacteria; however, they differed from the Delft archetype strain *B. anammoxidans*. Furthermore, no positively stained cells were observed with the probes Bet42a (Manz et al. 1992) for the β-Proteobacteria, indicative of the genus *Nitrosomonas*, Ntspa662 for *Nitrospira* (Daims et al. 2000) and Nit3 (Wagner et al. 1996) for the genus *Nitrobacter*.

Thin sections of fixed enrichment samples were also analyzed by transmission and freeze-fraction electron microscopy (Fig. 2). Typically, clusters of different size were

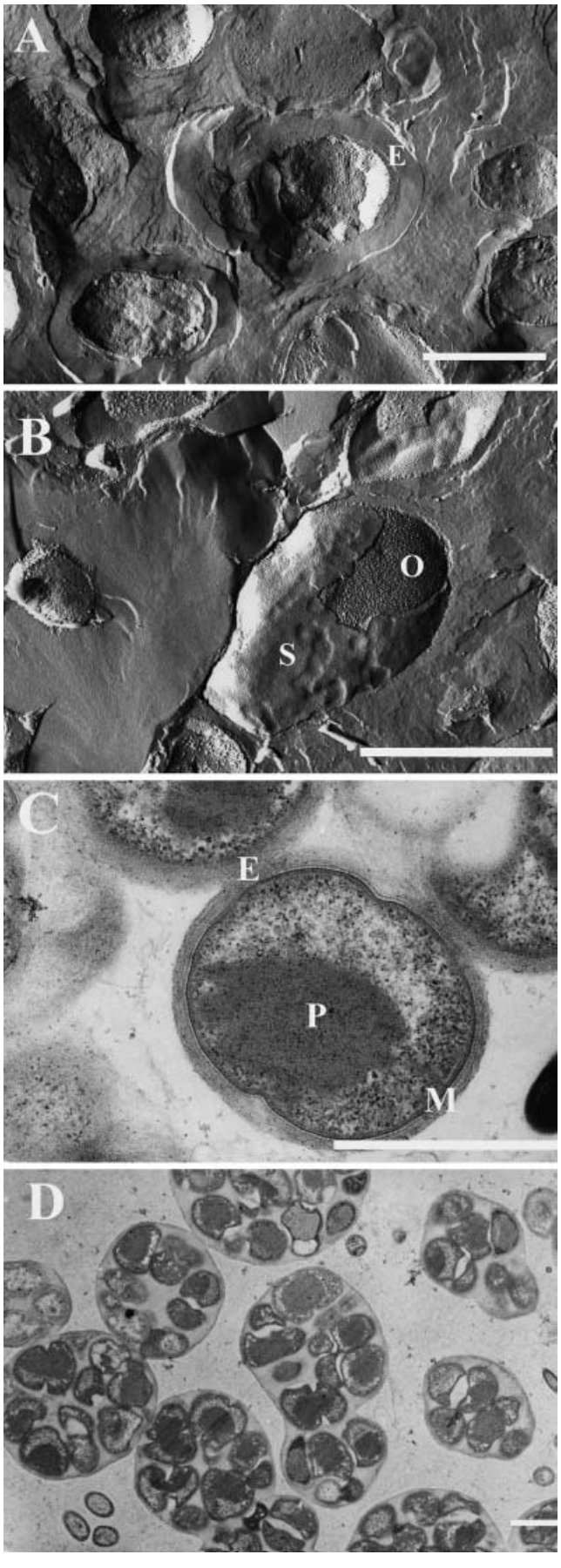

Fig. 2A–D Electron micrographs of Kölliken anammox cells. Freeze-fractions show cells with a large extracellular polymer matrix (*E*, panel **A**) and the S-layer (*S*) and the outer membrane (*O*, panel **B**). With transmission electron microscopy a protein-rich area (*P*) within the cells (*dark region*, panel **C**), the (now reduced) extracellular polymer layer (*E*) and the membranes (*M*) are to be seen, panel **D**) Small compact clusters of anammox cells. *Bar* 1 μm

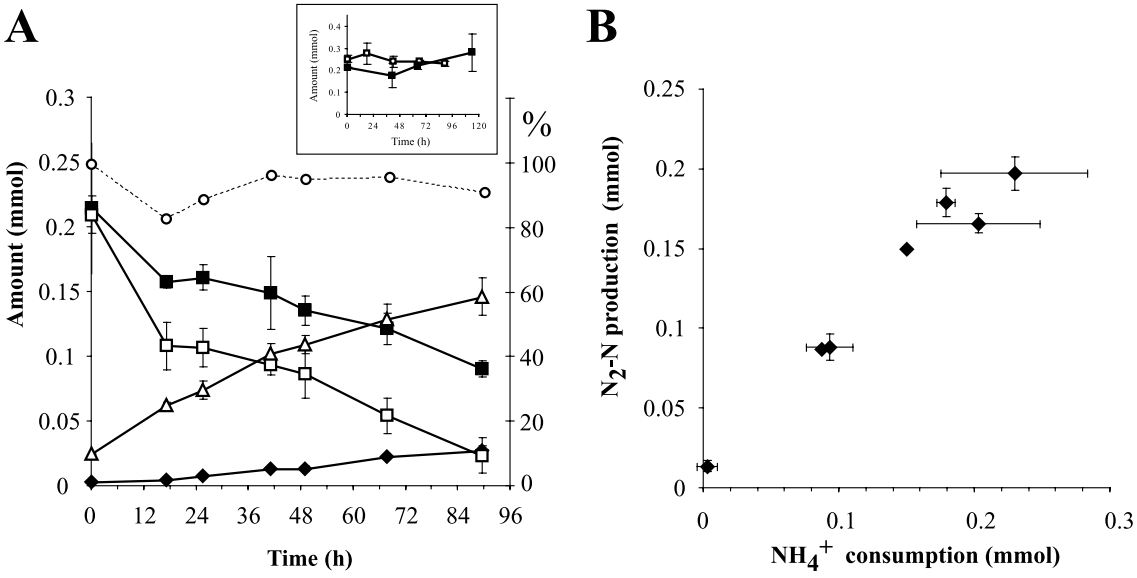

Fig. 3A, B Anammox activity of the enrichment culture at pH 7.5 and 37 °C. **A** Disappearance of NH_4^+ and NO_2^- and concomitant formation of N_2 and NO_3^-. Note that the *y-axis* shows the total amount of NH_4^+, NO_2^-, NO_3^- and N_2 in mmol per incubation flask (=45 ml liquid volume, 15 ml headspace). Total mass balance for N is given on the secondary *y-axis* as percentage from the beginning. **B** Correlation of N_2-production with NH_4^+-consumption in flasks with constant NO_2^- concentrations but with increasing amounts of NH_4^+. N_2-production and NH_4^+-consumption were measured after 4 days. *Error bars* represent±one standard deviation from the mean of triplicate incubations. ❑ Nitrite amount, ■ ammonium amount, Δ amount of N_2, ◆ amount of nitrate, ❍ total N

observed (up to approximately 17 μm in diameter, judged from electron micrographs). Cells within the clusters seemed embedded in an extracellular matrix, apparently holding the cells tightly together. The clusters in the electron micrographs consisted only of one type of bacterium, similar to the observations with fluorescence microscopy. Cells imaged in electron microscopy on freeze fractions and in thin sections displayed both an inner and an outer membrane, and a putative S-layer (Fig. 2). It was not possible to elucidate from freeze-fraction electron microscopy whether there were crateriform structures as described for Candidatus *B. anammoxidans* (Strous et al. 1999a). A high density, presumably protein-rich compartment was observed in the center of the cells (Fig. 2). This structure might be similar to the „anammoxosome“ described for Candidatus *B. anammoxidans* (Strous 2000). Some cells displayed larger white areas that looked like membranes which had collapsed during fixation. Although there was a protein-rich area, the nitrogen content of dry mass of the anammox enrichment was only about 10%. Approximately 46% of the dry weight was carbon, indicating no unusual carbon or nitrogen content (Egli 2000).

Physiology of anammox activity

To determine whether the enrichment culture from Kölliken was indeed performing the anammox reaction, several parameters in anoxic batch cultures were tested and changes in ammonium, nitrite, nitrate and N_2-gas concentrations were measured. A decrease of ammonium and nitrite concentrations and a concomitant production of nitrate and N_2 were observed only when both ammonium and nitrite were present (Fig. 3A). The rate of N_2 production was approximately 24 nmol N_2 min^{-1} (mg protein)$^{-1}$. When only nitrite or ammonium was added to the enrichment culture, no N_2 was produced and there was no change in nitrite or ammonium concentrations (Fig. 3A, inset). This indicates that classical nitrification and denitrification reactions did not play a significant role during anoxic incubations.

When the anammox culture was incubated with different ammonium concentrations (0, 2, 4 and 6 mM), but with the same nitrite concentration (5 mM), the production of nitrate and N_2 increased linearly with ammonium utilization (Fig. 3B). The overall stoichiometry of mol N_2-N production per mol of ammonium utilized was not significantly different from 1. In addition, nitrate production increased with nitrite utilization at an overall stoichiometry of 0.15 mol nitrate per mol nitrite, which is in agreement with the stoichiometries predicted for the anammox process by Candidatus *B. anammoxidans* (Strous et al. 1998).

To determine the optimal temperature for the Kölliken anammox organisms, we carried out incubations at 11, 20, 25, 30, 37 and 45 °C. The highest anammox activity (as rate of N_2 production) was observed at 37 °C. No anammox activity was observed at 45 °C and the activity could not be restored by reducing the temperature to 37 °C. Anammox activity at 11 °C was approximately 24% of that at 37 °C.

To investigate any inhibitory effects of nitrite, batch cultures with different nitrite concentrations were incu-

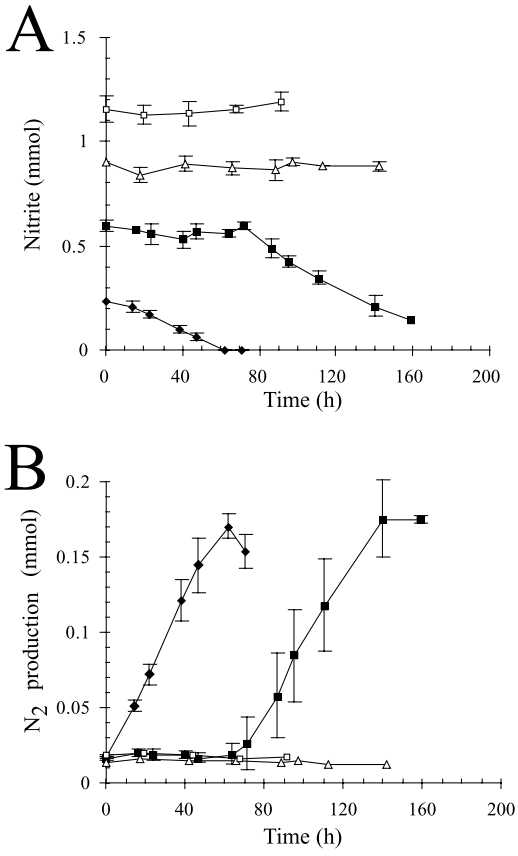

Fig. 4A, B Effect of increasing nitrite concentrations on the anammox activity of the enrichment culture incubated at the same ammonium concentration (3 mM). **A** Nitrite concentrations and **B** N_2 production during incubation. ◆ 6 mM Nitrite (corresponding to 0.25 mmol per flask), ■ 12 mM nitrite, Δ 18 mM nitrite, ❑ 24 mM nitrite. *Error bars* represent ±one standard deviation from the mean of triplicate incubations

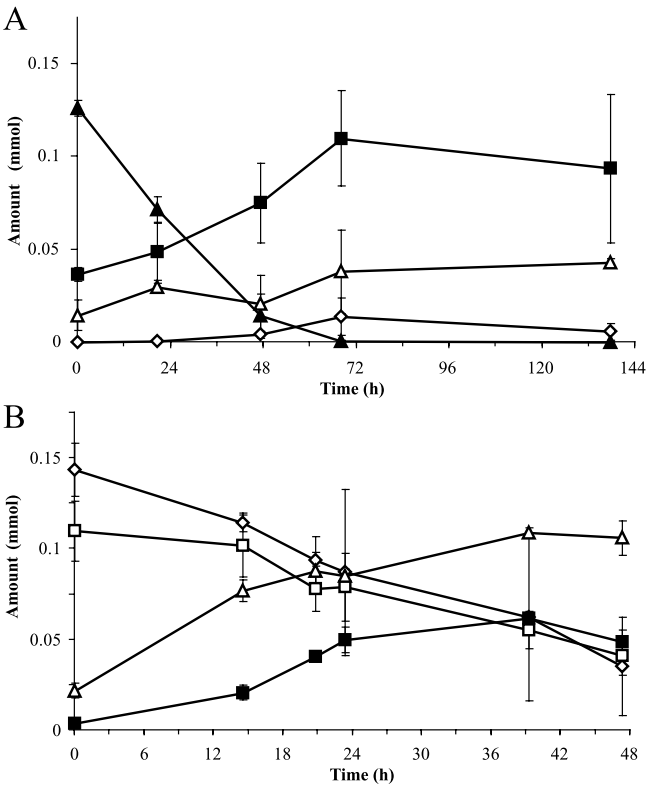

Fig. 5A, B Addition of possible intermediates to the enrichment culture. **A** Conversion of hydroxylamine (▲ NH_2OH) with ammonium (■). **B** The parallel decrease of hydrazine (N_2H_4, *open diamonds*) and nitrite (❑). *Error bars* represent±one standard deviation from the mean of triplicate incubations. Δ represent amount of N_2 produced

bated, keeping the initial ammonium concentration constant. At 5.3 mM nitrite, anammox activity started immediately (Fig. 4), and, as expected, nitrogen and nitrate production stopped when all nitrite was consumed. At 13.2 mM nitrite, anammox activity did not start until after 3 days (Fig. 4). Until then, no changes in the nitrite, ammonium and nitrate concentrations took place, and no N_2 was produced. Anammox activity stopped when all ammonium was depleted. At higher initial nitrite concentrations, no anammox activity was observed within the tested period. The specific N_2 production at 5.3 and 13.2 mM nitrite and pH 7 was similar at around 13 nmol min^{-1} (mg protein)$^{-1}$. The mean biomass concentration in all incubations was 65 μg protein per ml culture and did not change significantly during the experiments. Therefore, practically no growth occurred during this 1-week experiment, which reflects the slow growth of anammox bacteria.

To test the effects of pH of the medium on anammox activity, batch cultures at 37 °C were incubated with nitrite and ammonium at the following pH values: 6, 6.5, 7, 7.5, 8, 8.5 and 9. No anammox activity was observed at pH 6 and 6.5; ammonium and nitrite concentrations remained constant and neither N_2 nor nitrate was produced (not shown). The highest activity was observed at pH 7.5 and 8 [24 and 26.5 nmol N_2 min^{-1} (mg protein)$^{-1}$, respectively]. The N_2-production rate at pH 7 was 56% of the maximum. Anammox activity was also observed at pH 8.5 and 9. There was no significant difference in protein levels of cultures incubated at pH 7, 7.5 and 8 compared to those at pH 6 and 6.5 (mean level: 20.9 μg protein per ml culture), again indicating that practically no growth occurred during the batch incubations. The effect of pH was tested in buffer containing 20 mM sodium phosphate. The N_2-production rates with 20 mM phosphate (at pH 7) were essentially the same as with bicarbonate buffer (at pH 7), therefore, the anammox reaction was not inhibited by phosphate.

The sensitivity to oxygen was tested by adding different amounts of oxygen to the anoxic headspace in batch incubations. Initially, 0.25, 0.5, 1 or 18% of oxygen saturation was present in the headspace. Within 16 h, oxygen at the lowest concentrations (0.25 and 0.5%) had disappeared, probably due to any remaining aerobic microorganisms, and anammox activity started subsequently (data not shown). Anammox activity was also observed in bottles with 1% initial oxygen concentration, but again only after oxygen had disappeared. No anammox activity oc-

curred within 64 h in bottles with 18% initial oxygen, and anammox activity could not be restored after oxygen was removed. This indicates a reversible inhibition of the anammox process at low oxygen concentrations, but irreversible inhibition at higher oxygen concentrations.

Metabolic pathway for anammox conversion

To confirm that the anammox organisms from the RBC in Kölliken were carrying out a reaction similar to that of Candidatus *B. anammoxidans* (Van de Graaf et al. 1997), we tested whether hydroxylamine and hydrazine were possible intermediates in anaerobic ammonium oxidation. In regular incubations with ammonium and nitrite (both at 5 mM), no hydroxylamine or hydrazine was detected at a concentration above the detection limit (i.e. 25 μM and 10 μM). However, in incubations with 2.8 mM hydroxylamine and 0.8 mM ammonium, hydroxylamine was indeed disappearing (Fig. 5A). Hydrazine was transiently produced (up to 0.31 mM), which is consistent with the hypothesis that ammonium is oxidized with hydroxylamine to hydrazine (Schalk et al. 1998). The concentrations of ammonium and N_2 also increased during this experiment, possibly due to hydrazine disproportionation.

When hydrazine and nitrite were added to the enrichment culture, both compounds simultaneously disappeared at similar rates (Fig. 5B), which is consistent with the proposed reduction reaction of hydrazine and nitrite to form hydroxylamine. Ammonium and molecular nitrogen were also produced. The hydrazine concentration remained constant in sterile tubes (not shown), indicating that hydrazine disproportionation is a biologically mediated process.

Discussion

The results presented in this work show that microorganisms that carry out the anammox process could be enriched from biofilm material of the RBC in Kölliken. Although the experiments were not performed with a pure culture, we established that about 90% of the population was an anammox organism functionally similar to but taxonomically different than Candidatus *B. anammoxidans*. The 16S rDNA sequence obtained for the Kölliken anammox organism showed highest percentage of identity (between 98.5% and 98.9%) to the 16S rRNA gene sequences of Candidatus *K. stuttgartiensis*, a putative anammox organism in biofilm reactors in Stuttgart (Schmid et al. 2000). Other sequences from GenBank showed much lower percentages of identity (less than 86%) to the sequence of the Kölliken organism. Based on several specific probe sequences developed for Candidatus *B. anammoxidans* and Candidatus *K. stuttgartiensis*, and based on other group-specific probes, we showed that 90% of the organisms in the Kölliken enrichment culture possessed the 16S rRNA gene identified in this work. Despite the significant difference in 16S rDNA sequence of *B. anammoxidans* and the Kölliken organism (9.1%), their principal morphological properties were similar. Within the cells, a protein-rich region with lower content of ribosomes than the surrounding area was detected with electron microscopy and by FISH. In addition, the anammox organisms clumped in aggregates of up to 350 cells (judged from the electron micrographs and FISH pictures). The 16S rDNA sequence information indicates that the anammox organisms from the Kölliken biofilm are most similar to Candidatus *K. stuttgartiensis* (Schmid et al. 2000) and therefore might be addressed with the same Candidatus *Kuenenia* genus name. Presently, it is not clear whether the few sequence differences found between the 16S rDNAs of Candidatus *K. stuttgartiensis* and the Kölliken organism represent true phylogenetic differences. However, since Candidatus *K. stuttgartiensis* was not further enriched to high relative population sizes (relative abundance in the biofilms 49%), any physiological differences between the Kölliken and the Stuttgart anammox bacteria remain to be determined.

The physiological experiments confirmed that the Kölliken culture indeed performed the anammox process. N_2 production took place when both ammonium and nitrite were present and oxygen was absent, but not when ammonium or nitrite was added alone. Still, the anoxic oxidation of ammonium with nitrite could also have been catalyzed by „aerobic“ ammonium oxidizers of the genus *Nitrosomonas* (Bock et al. 1995), although the catalytic activity of these organisms is about 20-fold lower than that of Candidatus *B. anammoxidans* (Jetten et al. 1999). However, since no cells in the Kölliken enrichment stained positively in FISH with probes for β-Proteobacteria (which would detect *Nitrosomonas*), and for the genera *Nitrospira* and *Nitrobacter*, this strongly suggests that no classical ammonium- and nitrite-oxidizing bacteria were present at significant population sizes in the enrichment culture. Furthermore, when the nitrite concentration was kept constant, N_2 production increased linearly with increasing ammonium consumption. Therefore, nitrite removal and N_2 production could not be ascribed to denitrification, and ammonium removal could not be ascribed to nitrification at low oxygen levels. The concurrent removal of ammonium and nitrite with N_2 production can only be reasonably explained by the anammox reaction, catalyzed by anammox bacteria.

Approximately 15% of the nitrite removed was oxidized to nitrate, which is similar to observations for Candidatus *B. anammoxidans* and seems to be due to the need for electrons to reduce CO_2 for autotrophic growth (Van de Graaf et al. 1996). In addition, both hydroxylamine and hydrazine were converted by the enrichment culture, and a transient accumulation of hydrazine was observed when hydroxylamine was added, possibly due to condensation with ammonium. An increase in N_2 and ammonium concentrations was also observed when hydroxylamine was added, possibly due to its disproportionation. These results suggest that the anammox reaction mediated by the Kölliken organism proceeds as proposed for Candidatus *B. anammoxidans* (Van de Graaf et al. 1996).

Based on the relatively low percentage of identity of the 16S rDNA sequences of Candidatus *B. anammoxidans* and the Kölliken anammox organism, one might expect that the organisms would have physiological differences. However, our data showed only a few physiological differences. For example, the optimum pH and temperature of the two organisms were very similar. The Kölliken organism still showed activity at pH 9, whereas Candidatus *B. anammoxidans* has an activity range between pH 6.7 and 8.3 (Strous et al. 1999b). In addition, the Kölliken organisms exhibited higher tolerance to nitrite, up to 13 mM, compared to the 7 mM reported for Candidatus *B. anammoxidans* (Strous et al. 1999b).

The highest observed anammox activity of the Kölliken organism (at pH 8 and 37 °C) was 26.5 nmol N_2 min^{-1} (mg protein)$^{-1}$, which is lower than that reported for Candidatus *B. anammoxidans* [55 nmol N_2 min^{-1} (mg protein)$^{-1}$ at pH 8 and 40 °C, Jetten et al. (1999)]. However, the Kölliken anammox organisms were active at low cell densities (2×10^8 cells per ml or 20 µg protein per ml), whereas the optimal activity of Candidatus *B. anammoxidans* was reached only in suspensions with a density higher than 10^{10}–10^{11} cells per ml (Strous et al. 1999a). Finally, there was no inhibitory effect of phosphate (up to 20 mM tested) on the activity of the Kölliken culture compared to a loss of activity of Candidatus *B. anammoxidans* when incubated with more than 2 mM phosphate (Van de Graaf et al. 1996). Perhaps this is due to a higher affinity of the Kölliken organism for hydrazine, which may be lost from the metabolic pathway by autooxidation. Autooxidation rates of hydrazine were reported to increase with increasing phosphate buffer concentrations (Moliner and Street 1989).

In conclusion, converging lines of evidence show mainly phylogenetic and slight physiological differences between Candidatus *B. anammoxidans* and the Kölliken anammox organism, although the main nitrogen transformation reactions appear to be identical.

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