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Missing Lithotroph Identified as New Planctomycete

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With the increased use of chemical fertilizers in agriculture, many densely populated countries face environmental problems associated with high ammonia emissions. The process of anaerobic ammonia oxidation (‘anammox’) is one of the most innovative technological advances in the removal of ammonia nitrogen from waste water[1,2]. This new process combines ammonia and nitrite directly into dinitrogen gas[3]. Until now, bacteria capable of anaerobically oxidizing ammonia had never been found and were known as “lithotrophs missing from nature”[4]. Here we report the discovery of this missing lithotroph and its identification as a new, autotrophic member of the order *Planctomycetales*, one of the major distinct divisions of the Bacteria[5]. The new planctomycete grows extremely slowly, dividing only once every two weeks. At present, it cannot be cultivated by conventional microbiological techniques. The identification of this bacterium as the one responsible for anaerobic oxidation of ammonia makes an important contribution to the problem of unculturability.

For over a century, microbiologists have generally acknowledged Koch’s postulate that to prove that a process such as the anaerobic oxidation of ammonia is mediated by a bacterium, this bacterium should be isolated in pure culture and still be able to reproduce the process. But for the past ten years, the anammox process has resisted such meaningful microbiological characterization.

Electron microscopy provided an incentive to continue pursuing the quest for the bacterium responsible for anammox, because a peculiar morphotypical microorganism dominated the multispecies biofilms that mediated the process. Thin sectioning of cryosubstituted material revealed that cells of this morphotype were characteristically compartmented in their internal ultrastructure (Fig. 1a). Such membrane-bounded cell compartments were so far found only in cultured strains of the order *Planctomycetales* [6,7]. When cell surfaces were examined by negative staining (Fig. 1b), the anammox morphotype displayed uniformly distributed crateriform structures, circular electron-dense areas known to be characteristic of planctomycetes[8].

It would be surprising if a planctomycete was responsible for anammox. The order *Planctomycetales* - comprising a separate major division of the Bacteria - has been represented so far only by a few organotrophs[8]. Formerly the planctomycetes were considered to be of limited environmental importance. But this view is changing rapidly as molecular microbial ecology repeatedly provides new evidence showing that these bacteria are ubiquitous and make up a substantial portion of the natural bacterial population [9-13]. The recognition of the planctomycetes as a major bacterial division may change our notion of what bacteria are. Planctomycetes have single- or double-membrane-bounded compartments (see above and Fig. 1) separating their chromosome from the remainder of the cytoplasm[6,7]. They lack peptidoglycan in their cell walls and are insensitive to ampicillin[14,15].

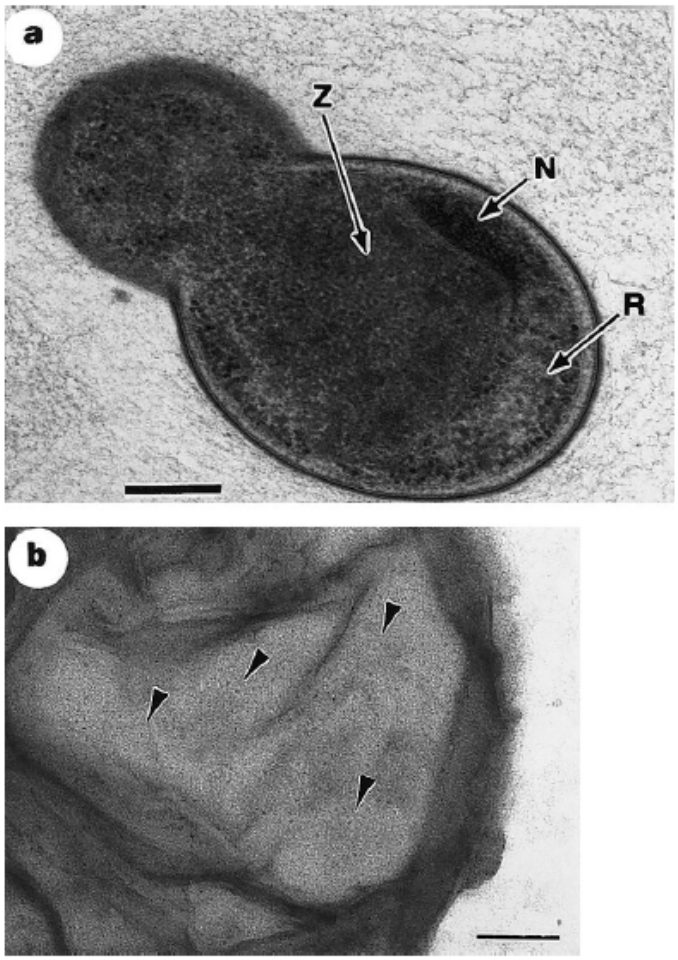

Figure 1 Transmission electron micrograph of anammox biofilms. **a**, Thinsectioned cell of dominant morphotype from the anammox biofilm enrichment culture, demonstrating unusual compartmentalized internal organization and budding reproduction. The mother cell of the budding cell shown possesses a centralmembrane-bounded ribosome-free zone (Z), surrounded by a membranebounded ribosome-rich region (R) in which the fibrillar nucleoid (N) is situated. Bar marker is 0.2 μm. **b**, Portion of the surface of a negatively stained cell of dominant morphotype from the same culture, displaying uniformly distributed, circular crateriform structures, examples of which are indicated by arrows. Bar marker is 100 nm.

As conventional purification using single-cell isolation had failed, we physically purified the morphotypical microorganism from the multispecies biofilms[16] by density-gradient centrifugation. The purification procedure consisted of three steps: (1) disruption of the biofilms by mild sonication; (2) separation of the single cells obtained and the remaining biofilm fragments; and (3) purification of the single cells using Percoll density-gradient centrifugation (Table 1).

We showed that the purified cells were responsible for anaerobic ammonium oxidation by incubating the obtained cell suspensions (99.6% pure) with the substrates ammonium and nitrite (5mM each) and measuring substrate consumption over time.

Surprisingly, it seemed that both the purified and unpurified cells were only active when the cell concentration was higher than 10^{10}-10^{11} cells ml^{-1}. This was not noticed previously because we had studied anammox only in biofilms, where the cell density is naturally very high. One might argue that the high concentrations of purified cells were required to attain a sufficient amount of contaminating microorganisms required for anammox activity. However, this possibility could be eliminated because control experiments showed that the activity of unpurified cells was also density dependent (Table 1). Therefore, we concluded that the cell density of the anammox morphotype was important, and not the amount of contaminants. It is as yet unclear whether the densitydependent activity of these cells results from cell-to-cell communication through homoserine lactone autoinducers (quorum sensing)[17] or whether a different mechanism is responsible for the density dependency in this case.

Because the Percoll purification procedure yielded only a small number of cells (Table 1), the anaerobic activity test was miniaturized to 30-40 µl to attain high cell densities. In addition to the required high cell concentration, the cells needed to be supplied with a trace amount of one of the anammox intermediates hydrazine or hydroxylamine (100 µM) before they became active, and the nitrite concentration needed to be lower than 5mM to prevent substrate inhibition.

Table 1 Purification and cell-density-dependent activity of anammox cells from anammox biofilms

Purification step	Purity* (%) cells (mg protein)	Total yield of bacterial per mg protein per min)	Specific activity* (nmol NH^{+4} (cells per ml)	Cell density threshold
Mixed culture biofilms	70 ± 10	50	25 ± 5	10^{7}-10^{9}[2]
Single cells from sonicated biofilms	85 ± 5	2	20 ± 4	10^{10}-10^{11}
Purified cell suspension after density gradient centrifugation	99.6 ± 0.2	0.5	18 ± 3	10^{10}-10^{11}

*Values are expressed as mean 6 standard deviation.
[2]Average of biofilms and culture liquid. Inside the biofilms, cells were densely packed, leading to local cell densities of at least 10^{10}-10^{11} ml^{-1}.

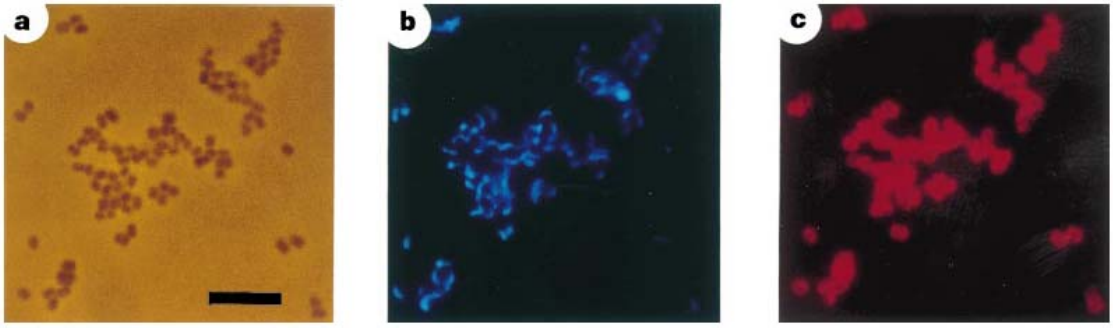

Figure 2 Fluorescent in situ hybridization of purified anammox cells. Identical field viewed by: **a**, phase contrast microscopy; **b**, epifluorescence microscopy with cells stained with the general DNA stain DAPI; and **c**, epifluorescence after hybridization with anammox organism 16S rRNA-sequence based, Cy3-labelled probe S-G-Amx-1015-a-A-18 (5'-GAT ACC GTT CGT CGC CCT-3') at 20% (v/v) formamide. Bar marker is 5µm.

The high cell density and the low substrate concentration allowed the purified cells only a brief burst of activity, little more than 100 catabolic cycles (each cell consumed only a small amount of substrate, every individual enzyme turning over 100 times at most). Nevertheless, the activity of the purified cells was only slightly lower than the activity of the biofilms (Table 1) and the stoichiometry of the conversion was identical: 1.3 moles of nitrite were consumed and 0.2 moles of nitrate were produced per mole of ammonium consumed. Even assimilation of CO_2 by the anaerobic ammonium-oxidizing cells could be measured. During the experiment, the stoichiometry of ^{14}C-CO_2 incorporation increased rapidly from 0 to 20 millimoles of CO_2 per mole of ammonium consumed. In control experiments with unpurified cell suspensions prepared from enrichment culture biofilms, the rate of CO_2 incorporation was always less. The pathway of CO_2assimilation is not yet resolved but the recorded ribulose bisphosphate carboxylase activity in the biofilm enrichment culture was less than 50% of the activity required to account for the measured CO_2 incorporation. Alternatively, one of the other four known autotrophic pathways might be present in this case. Up until now, planctomycetes were known as organotrophic organisms (that is, incapable of autotrophic CO_2 fixation) and their biochemical pathways are so far unexplored.

Because the responsible bacterium was purified but not isolated, contaminating organisms might contribute to the observed activity. However, from kinetic and stoichiometric perspectives it is extremely unlikely that the contaminating organisms (1 in 200) contribute significantly to anammox catabolism or anabolism. However, it is quite possible that other organisms are required for growth of the anammox organism. Possible symbionts might, for example, supply growth factors and scavenge by-products of anammox anabolism.

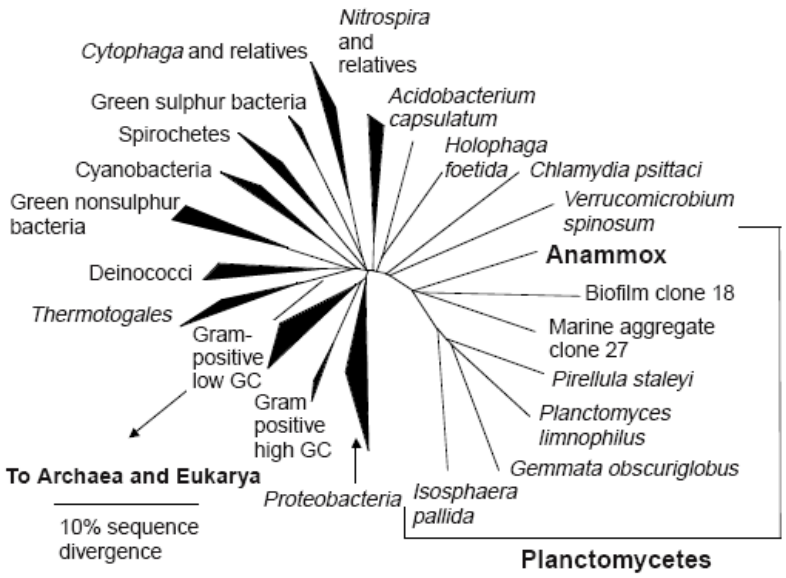

Figure 3 Phylogenetic position of the lithotroph responsible for anaerobic ammonium oxidation within the domain Bacteria, based on 16S rRNA phylogeny. The anammox bacterium represents a new, deep branch inside the order *Planctomycetales*.

To determine the phylogenetic identity of the purified cells, we extracted DNA and RNA from the purified suspensions and amplified the 16S ribosomal RNA gene directly using polymerase chain reaction or indirectly after reverse transcription of RNA (RTPCR) from complementary DNA obtained from the 16S rRNA itself. Although the cell suspensions were more than 99% pure, only the RT-PCR-derived clone libraries were dominated (80%) by a single sequence. Based on this sequence we designed 18 specific oligonucleotide probes, labelled with Cy3 fluorochrome for fluorescent in situ hybridization (FISH). Ten of these probes gave bright and specific hybridization signals with the purified morphotypical microorganism (Fig. 2). Combined FISH and 4,6-diamidino-2-phenylindole (DAPI) staining confirmed the internal cell compartmentalization. The FISH signal was ring shaped, indicating the absence of ribosomes in the inner cell compartment. The DAPI signal, staining the genomic DNA, was concentrated in a half-ring, which is consistent with the localization of the nucleoid in Fig. 1a.

The 16S rRNA sequence of the anammox organism (1,453 base pairs, from position 7 to 1,390; *Escherichia coli* numbering) was aligned and analysed phylogenetically with other bacterial 16S rRNA sequences[18]. The anammox organism was identified as a deep-branching planctomycete (Fig. 3), most closely related to a sequence amplified from a marine snow aggregate[19] (sequence identity 80.2%). Sequence identity with other planctomycetes was 74-77%. The new sequence of the 16S rRNA gene shared most but not all signature positions with those of the other planctomycetes[8,20,21], confirming its position as a deep-branching but still related sequence. Finally, the missing link in the biogeochemical nitrogen cycle seems to have been found; it is an organism in the order *Planctomycetales*, a distinctive division of the domain Bacteria, with unique cell

organization and cell walls, large evolutionary distances and few cultivated representatives.

Methods

Purification and activity. Biofilms[16] were washed and concentrated in 40 ml HEPES/bicarbonate buffer (70/10mM, pH 7.8), sonicated in 5 ml portions (150 W, 30 s, 18-mm tip width) and, after centrifugation (10,000*g*, 5 min), an upper orange pellet containing undisrupted biofilm fragments was separated from the lower red pellet containing the single cells. After washing in the same buffer, the single-cell fraction was purified using Percoll centrifugation (6.9 ml Percoll (Pharmacia) and 3.1 ml cell suspension; centrifugation at 10,000g for 60 min at 5 º C; Sorval SS34 fixed angle rotor). A broad, red band of target cells in the lower half of the tube was extracted and washed in sterile buffer (see above). Anammox activity was tested in a 30-µl activity test in anaerobic containers (same buffer containing 4.5 mg protein ml^{-1} (10^{10}-10^{11} cells ml^{-1}), 5mM ammonium and nitrite, 100 mM hydrazine) and consumption of ammonium and nitrite and production of nitrate and incorporation of ^{14}C-CO_2 was measured as described previously[16,22]. Purity was assessed by microscopic counting, electron microscopy and FISH.

Electron microscopy. Pelleted cell flocs were processed by a cryosubstitution protocol before embedding, sectioning and section staining, all using methods described in ref. 7. Cells were negatively stained using 1% uranyl acetate + 0.4% sucrose, after dispersal of flocs. Fluorescent microscopy. FISH and DAPI staining were done as described10. Formamide concentration versus specificity of all probes was determined with the appropriate reference organism for each probe and with the biofilm enrichment culture and several undefined, heterogeneous environmental samples. All probes were at least 18 nucleotides long.

Phylogeny. Treeing and phylogenetic analysis was done using the ARB software package[18]. The sequence was obtained after DNA isolation, PCR amplification (primers 27 or 519 forward and 1,390 reverse; annealing at 46 º C, melting at 94º C, 30 cycles), ligation of the product in vector pGEMT (Promega) and transformation into competent DH5α *E. coli* cells. The clone library was screened using restriction digestion, and representative clones of each restriction pattern were sequenced. The anammox sequence (GenBank accession number AJ131819) always grouped with the other planctomycete sequences, independent of treeing algorithm (neighbour joining, distance matrix or parsimony), inclusion of other bacterial phyla in the tree or the choice of the outgroup (*Thermotoga, Aquifex* or the Archaea).

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