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Molecular analysis of ammonia oxidation and denitrification in natural environments

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Abstract

This review summarizes aspects of the current knowledge about the ecology of ammonia-oxidizing and denitrifying bacteria. The development of molecular techniques has contributed enormously to the rapid recent progress in the field. Different techniques for doing so are discussed. The characterization of ammonia-oxidizing and -denitrifying bacteria by sequencing the genes encoding 16S rRNA and functional proteins opened the possibility of constructing specific probes. It is now possible to monitor the occurrence of a particular species of these bacteria in any habitat and to get an estimate of the relative abundance of different types, even if they are not culturable as yet. These data indicate that the composition of nitrifying and denitrifying communities is complex and apparently subject to large fluctuations, both in time and in space. More attempts are needed to enrich and isolate those bacteria which dominate the processes, and to characterize them by a combination of physiological, biochemical and molecular techniques. While PCR and probing with nucleotides or antibodies are primarily used to study the structure of nitrifying and denitrifying communities, studies of their function in natural habitats, which require quantification at the transcriptional level, are currently not possible. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Nitrification (the oxidation of ammonia to nitrate via nitrite) and denitrification (the reduction of nitrate to molecular nitrogen via nitrite, nitric oxide and nitrous oxide) are essential steps in the global nitrogen cycle. In many ecosystems, these processes counteract natural and man-made eutrophication, and they are used to reduce N concentrations in sewage treatment plants. The importance of both processes is reflected by numerous reviews: ecology of denitrification and dissimilatory nitrate reduction [1]; autotrophic nitrification in bacteria [2]; aspects of denitrification in soil and sediment [3]; factors controlling denitrification [4]; probes for phylogenetic and functional genes of nitrification and denitrification [5]; enzymology of ammonia oxidation [6]; cell biology and molecular basis of denitrification [7]; enzymology of the nitrogen cycle [8]; nitrifying bacteria and aquaculture [9]; anaerobic ammonia oxidation [10]; nitrogen cycling in coastal marine ecosystems [11]; dissimilatory nitrate reductases in bacteria [12]; and inorganic nitrogen metabolism in bacteria [13].

For both nitrifying and denitrifying bacteria, substantial knowledge has accumulated on the physiology, biochemistry and the molecular regulatory mechanisms of only a few selected species (e.g. *Nitrosomonas europaea* or *Pseudomonas stutzeri*). This has provided the initial information from which to develop molecular approaches for environmental studies. However, the current state of information on the ecology of these organisms is inadequate. Only a few thousand bacterial species have been characterized and deposited in type culture collections, yet there is evidence that 1 g of soil contains 4×10^3–10^4 different bacterial genomes [14,15]. Only about 1–10% of soil bacteria [16] or even less have been cultivated so far and are thus available for taxonomic characterization. Molecular techniques may be powerful tools to investigate bacteria in their natural environment, especially those that are not yet culturable (see [17]). We are starting to recognize that for nitrifiers and denitrifiers the real bacterial world is also much more complex and diverse than ever thought. Those bacteria which have been studied extensively in pure cultures are probably not the major 'players' in most natural habitats. Thus, molecular techniques have given access to a whole world of yet unknown nitrifying and denitrifying bacteria.

The scope of this review is to stress the recent achievements in the field of nitrification and denitrification obtained by applying molecular techniques, with emphasis on soil and natural aquatic environments. Engineered systems, like sewage treatment plants, are mentioned only occasionally, when they provide examples of recent, innovative approaches.

2. Nitrification

2.1. Taxonomy and distribution of nitrifying bacteria

Nitrifying bacteria are ubiquitous in soil, freshwater and marine environments. They have been found even in extreme habitats such as building sandstone [18], extremely alkaline soda biotopes [19], Antarctic ice [20], hot springs [21], or in association with marine sponges [22]. They are fascinating and ideal study objects, partly because most of them are of monophyletic origin and have a unique metabolism, with many enzymes that have been found only in this group of organisms.

Never since their discovery by S.N. Winogradsky at the end of the 19th century has this group of bacteria received so much attention as in the last 5–7 years. Even today only a few experts worldwide are able to isolate pure cultures and maintain them indefinitely.

Nitrification is a two-step process which involves two different groups of bacteria. Ammonia-oxidizing bacteria (AOB) oxidize ammonia to nitrite, and nitrite-oxidizing bacteria (NOB) oxidize nitrite to nitrate. So far, no autotrophic bacterium is known to oxidize ammonia directly to nitrate. Traditionally, AOB were classified by cell morphology into the five different genera: *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosovibrio* and *Nitrosolobus* [23]. Recently, on the basis of 16S rRNA sequence homology, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus* were proposed to be combined into one common genus *Nitrosospira* [24]. With the exception of *Nitrosococcus*, all genera represent closely related organisms of the β subclass of Proteobacteria [25]. The genus *Nitrosococcus* is phylogenetically not homogeneous. *N. mobilis* is a β subclass organism, while *N. oceani*, and probably also *N. halophilus*, are γ-Proteobacteria [25]. So far, only a few ammonia-oxidizing γ-Proteobacteria have been isolated from marine habitats, and none from soil or freshwater habitats. In general, AOB are obligatory chemolithoautotrophs, although some of them can use organic compounds (acetate, pyruvate) for mixotrophic growth. NOBs will not be discussed in this article.

Some heterotrophic bacteria and fungi [26] can also oxidize ammonia and/or reduced nitrogen from organic compounds to hydroxylamine, nitrite and nitrate. Whilst N oxidation is the only energy-yielding process in autotrophic nitrifiers, nitrification in heterotrophic organisms seemingly does not contribute significantly to their energy metabolism. Heterotrophic nitrifiers are known for their ability to nitrify and denitrify simultaneously [27]. More recently, it was shown that the autotrophic AOB *N. europaea* and *N. eutropha* could also simultaneously nitrify and denitrify when grown under oxygen limitation [28].

2.2. Physiological aspects of ammonia oxidation

Until recently, ammonia oxidation was thought to be a strictly aerobic process, requiring molecular O_2. Usually, AOB can cope better with low O_2 than with low ammonia concentrations. In a chemostat fed with water from a highly eutrophic wastewater reservoir, nitrification was observed at O_2 concentrations down to 0.05 mg l^{-1} [29]. Under anoxic conditions *N. eutropha* and *N. europaea*, and probably also some other AOB, can oxidize ammonia in the presence of pyruvate, and with nitrite as electron acceptor [30] or with NO_2 gas [31]. Thus, AOB can denitrify endogenously produced nitrite to N_2O, NO and N_2. Both groups of nitrifiers can survive under anoxic conditions for months. Therefore, high numbers of nitrifiers can be observed in anoxic hypolimnia [32] or sediment [33] of eutrophic freshwaters or wastewater basins [34] even under conditions that do not permit active nitrification. In soil and sediments of eutrophic freshwaters with areas of periodically low O_2 concentration, chemolithotrophic AOB are probably adapted to nitrify under microoxic conditions [35]. It seems that this is due to physiological adaptations, such as higher affinity for oxygen or alternative electron acceptors, rather than to a change in the composition of the nitrifying community.

2.3. Enzymes involved in ammonia oxidation

In autotrophic ammonia oxidizers, two key enzymes are necessary for energy conservation during the oxidation process, ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). In vivo both enzymes are co-dependent because they generate the substrate and electrons, respectively, for each other. AMO catalyzes the oxygenation of ammonia to hydroxylamine:

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$$
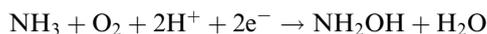
The two electrons required in this process are derived from the oxidation of hydroxylamine to nitrite by HAO:

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$$
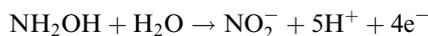
One of the oxygen atoms in NO_2^- derives from O_2, the other one from water. Two of the four electrons generated by HAO are transferred via the tetra-heme cytochrome c_{554} [36], either to AMO, or diverted into an electron transport chain [37,38], where many of the carriers involved and intermediates have not been completely characterized [6]. As these reactions have been studied mainly with *N. europaea*, alternatives to this pattern may be discovered when a broader range of organisms from diverse habitats are examined.

2.3.1. Ammonia monooxygenase

Indirect evidence suggests that the enzyme AMO in *N. europaea*, and probably some other autotrophic AOB of the β and γ subclasses of Proteobacteria, has three subunits, AMO-A, AMO-B and AMO-C, with different sizes, structures and arrangements within the membrane/periplasmic space of the cells. The enzyme catalyzes the oxygenation of a broad range of substrates [6]. Due to its essential function in the energy metabolism of AOB, the enzyme is probably constitutive.

The only purification of an AMO as active enzyme has been achieved from the heterotrophic nitrifier *Paracoccus denitrificans* [39]. This enzyme consists of only two subunits (not three as the AMO from autotrophic nitrifiers) and has several features in common with the enzyme family which includes not only AMO, but also the particulate methane monooxygenase (pMMO) from methanotrophs. From another heterotrophic nitrifier, *Pseudomonas putida*, a DNA region has been sequenced which showed partial homology to the *amoA* gene of *N. europaea* [40]. There were also indications that AMO is expressed in this organism [40].

The three subunits of AMO from autotrophic AOB are encoded by the genes *amoC, amoA* and *amoB* of the *amo* operon [41,42]. All three AMO genes have been cloned and sequenced from several AOB [43–47].

PCR primers used to amplify sequences of the *amo* operon from different environments have been designed to target *amoA*, encoding the subunit that carries the active site of this enzyme [48–50]. Within *amoA*, the region encoding the C-terminus appears to be a suitable target site for primers and probes to discriminate between AOB of the β and γ subclasses of the Proteobacteria as well as between ammonia and methane oxidizers within the γ subclass [42].

Between the three *amo* genes, intergenic spacer regions of variable, but strain-specific length have been observed [42]. A spacer between *amoA* and *amoB* was observed only in the γ subclass proteobacterium *N. oceani* [42]. This feature may be used to screen for the survival rate of a specific strain or to describe the population dynamics in environmental samples by PCR with primers flanking the variable intergenic regions [42]. Because of the key function of AMO in the energy metabolism of these bacteria, all three genes encoding the subunits of the enzyme must have evolved under high functional pressure, whereas the intergenic spacers may provide a selectively neutral region.

2.3.2. Homology of AMO and pMMO

The enzymes AMO of nitrifying bacteria and pMMO of methanotrophic bacteria are homologous and share many features [51,52]. The structures of their operons are almost identical. The high similarity of the nucleotide sequence of the genes is indicative of a common evolutionary origin [53,54]. At the DNA and amino acid sequence level, similarities between *amo*/AMO and *pmo*/pMMO of the γ subclass of the Proteobacteria are higher than within the *amo*/AMO of the γ and β subclass AOB [42,53]. It has been proposed that *amo* from the β and γ subclass AOB and

pmo from the γ subclass methane-oxidizing bacteria separated into three lineages early in evolution [42].

Methanotrophic bacteria can oxidize ammonia to nitrite by the combined action of pMMO and a unique HAO, which is different from that in AOB [55,56]. Methanotrophic and ammonia-oxidizing bacteria occupy similar niches in soil and aquatic habitats, at counteracting gradients of O_2 and methane or ammonia, respectively. Their interactions are complex and poorly understood so far.

2.3.3. Hydroxylamine oxidoreductase

HAO is an unusual enzyme with a highly complex structure, located as a soluble enzyme in the periplasmic space, but anchored in the cytoplasmic membrane. Each subunit of the trimeric enzyme contains, as probable components of the active site, seven *c*-type hemes and one residue of the unusual heme P_{460} [57,58]. The molecular and biochemical characterization is given in [59–61]. Soluble, non-heme-containing hydroxylamine oxidases from the heterotrophic nitrifying bacteria *P. denitrificans* and *Pseudomonas* strain PB16 [62,63] are very different from the HAO of autotrophic AOB and may be responsible for the inability of these heterotrophs to grow autotrophically by nitrification.

2.3.4. Multiple copies of genes encoding electron transport proteins

Several of the genes involved in ammonia oxidation are present in multiple copies per genome. It has been suggested that the ratio of the number of copies of different genes could be responsible for maintaining a certain ratio of the gene products [64]. Between two and four copies of the *amo* gene have been observed in the AOB of the β subclass [41–43,45,65]. Additional copies of *amoC* sometimes occur outside the operon. *N. oceani* (ATCC 19707), a member of the γ subclass, and another unnamed strain (C-113) have only one copy of the entire operon [42]. All of the multiple copies described so far are highly similar, and it is not yet clear whether they have different functions. Studies with insertional mutants of *N. europaea*, in which each copy of *amoA* alone was inactivated, showed that both copies were expressed. Inactivation of only one copy resulted in slower growth, less full-sized mRNA, and reduced AMO activity (measured as ammonia-dependent O_2 consumption and [^{14}C]acetylene incorporation). Thus, each copy had a distinct metabolic function, but was not essential for growth [66]. As most of the multiple gene copies are nearly identical whilst much less similarity exists between *amo* from different species, it is unlikely that they originated from horizontal gene transfer, they probably arose by a gene duplication event [65,67] that occurred relatively early in the evolution of this lineage. It is of particular interest for evolutionary ecology that the highest multiplicity of *amo* genes is found in organisms of the *Nitrosospira*/*Nitrosolobus* group, whereas the two AOB investigated so far from the γ subclass have only one copy.

Three copies of the genes for HAO (*hao*) and cytochrome c_{554} (*cycA*) have been identified in the genome of *N. europaea* [44]. No other proteins with substantial similarities to HAO were found in a database search [64,68,69].

2.3.5. Regulation of enzyme function

Ammonia regulates the activity of AMO at the transcriptional [70], translational [71,72] and post-translational [72] levels. Using RT-PCR, products of all three *amo* genes were detected in the same transcript, indicating that *amoC* is part of the *amo* operon [41,47]. In *N. europaea*, transcription of *amo* as well as *hao* was induced by ammonia [70] but mRNA of these genes disappeared within 8 h under conditions of ammonia starvation.

In *N. europaea* cells that had been starved of ammonia, HAO remained stable for up to 72 h, as indicated by Western blot analysis and activity measurements [73–75]. In nature the key enzymes AMO and HAO should remain stable even under conditions of fluctuating ammonia concentrations. No indications have been found for the differential expression of the *amo* operon.

Ammonia limitation caused specific inhibition of the AMO but not of the HAO activity within 24 h, whereas little change in the ammonia oxidation activity occurred in a medium without ammonia [74]. Under long-term (342 days) ammonia starvation of *N. europaea*, the activity of AMO and HAO remained stable, and the cells maintained a high level of the enzyme. After the addition of ammonia or hydroxylamine, there was an immediate response, measured as nitrite production, without initial protein synthesis [76].

2.4. Analysis of the community structure

Due to their special requirements, which are often unknown, and low growth rates, autotrophic AOB are difficult to isolate in pure cultures. This, together with usually low numbers (less than 0.1% of direct counts), hampered our knowledge on the distribution and relative abundance of these bacteria in nature until molecular approaches became available.

2.4.1. Immunofluorescence approaches

AOB were counted directly by application of fluorescent polyclonal antibodies (FA). The production of FA depends on the availability of pure cultures. A considerable serological diversity was noticed within the isolates from the same sample of soil [77]. FA developed for selective counting of several *Nitrosomonas* spp. allowed the species-specific counting of attached and suspended bacteria [78]. On the other hand, FA produced against several marine ammonia or nitrite oxidizers reacted with a broad spectrum of isolates of the same physiological type [79]. About 70% of 30 AOB isolates and eight of nine nitrite oxidizers reacted with at least one of the FA raised against ammo-

nia or nitrite oxidizers, respectively. These bacteria represented only 0.1–0.8% of the total bacterial counts in oceanic waters. As a consequence, extensive counting times are needed to get statistically significant numbers. Another drawback is the variability of the clonal composition of the antibodies as discussed in Section 3.2. Nevertheless, compared to the most-probable-number (MPN) technique the use of FA provides results within hours and has a higher resolution and sensitivity.

For a long time, *Nitrosomonas* spp. were believed to be the dominant [80], or at least the most common, [81] ammonia oxidizers in aquatic environments, whereas *Nitrosospira* spp. were most frequent in soil [82]. *Nitrosomonas* spp. were the dominant nitrifiers in the lower part of the river Elbe, as determined from high MPN dilutions [83], by immunofluorescence microscopy [84], as well as DNA hybridization and partial sequence analysis of 16S rRNA partial gene sequences [83]. However, more recent investigations of other locations have changed this view (see below).

2.4.2. Phylogenetic approaches

The fact that nitrifying bacteria (ammonia and nitrite oxidizers) seem to have only one operon for 16S rRNA genes [85,86] greatly facilitates community analyses by molecular approaches. The knowledge of these sequences allows different approaches for studying the community composition of nitrifying bacteria. Most of the work has been done for the monophyletic group of the AOB within the β-Proteobacteria.

2.4.2.1. Fluorescent in situ hybridization (FISH).

Several oligonucleotide probes for detecting AOB of the β-Proteobacteria have been described [87–89], but there are only few applications of FISH for estimating the abundance of nitrifying bacteria in natural environments. The probe NEU [87] was designed to be specific for *Nitrosomonas* spp. At present the probes NSO190 and NSO1225 have the broadest specificity and are therefore the most suitable for this approach. According to a recent BLAST search, both probes match sequences of 16S rRNA in AOB and a few unidentified clones. They could be applied simultaneously (or separately as controls for one another) because their specificity is slightly different.

Voytek et al. [90] reported the detection and quantification of β subclass Proteobacteria using FISH in samples collected from several permanently ice-covered lakes in the Taylor Valley of Antarctica. Abundances derived from FISH compared well with those estimated by immunofluorescence (maximum of about 2000 cells ml^{-1}) and slight apparent differences in cell distribution were attributed to the slightly different specificities of the antibodies versus the DNA probes. In samples from the Baltic Sea, FISH yielded cell numbers near the detection limit, when a control with a nonsense probe was used (S. Bauer and G. Jost, unpublished). Therefore, the application of more specific probes that distinguish different groups of AOB might be feasible only in habitats with high AOB abundances, such as sewage treatment plants. So far, no probes for AOB of the γ-Proteobacteria have been described, which would be especially useful for enumerating AOB in marine samples.

2.4.2.2. PCR-based techniques.

From the 16S rRNA gene sequences known up to 1997, 30 oligonucleotides with known specificity for AOB of the β-Proteobacteria were suggested (for review see [91]) for PCR or hybridizations with extracted DNA/RNA or whole cells. Nearly all knowledge about the diversity of AOB communities in natural environments has been obtained using these oligonucleotides. Oligonucleotide primers for *N. oceani* were reported [92] but have not been widely applied [93].

PCR with primer combinations of known specificity (for all AOB of the β-Proteobacteria or subgroups thereof) was used to amplify 16S rDNA of the ammonia-oxidizing community from natural samples. Either direct PCR [94–97] or nested PCR (first PCR with primers for eubacteria) [98–101] was conducted. In some cases, nested PCR revealed positive results from samples where direct PCR failed, which was mainly attributed to low abundances of AOB [102,103].

During investigations of different environmental samples (water and soil, nutrient-poor and enriched) it became evident that *Nitrosospira*-like AOB are nearly ubiquitous [98,99,101,104–106]. From different soils, a variety of sequences were isolated that grouped into four clusters related to *Nitrosospira*, and three clusters related to *Nitrosomonas* [107]. Clones from marine and soil samples grouped into different clusters [94,96,108].

Nitrosospira-like AOB seemed to be ubiquitously present and also the dominant AOB in most natural environments. Nevertheless, *Nitrosomonas*-like AOB could also be detected in many environmental samples. *N. europaea*-type 16S rDNA was present in different depths of three lakes in northern Germany [103]. Cloned 16S rDNA sequences formed a strong monophyletic cluster together with the sequences from *N. ureae* [95]. Significant differences in the community structure between free-living and attached AOB have been found in marine samples. Whereas the majority of the partial sequences obtained from planktonic samples were related to cluster 1 within the *Nitrosospira* spp. nearly all sequences from particle-associated material were related to cluster 7 within the *N. eutropha/europaea* lineage [100]. It has long been known that at least some, if not all, of the AOB tend to adhere to surfaces [2]. Due to their ability to produce extracellular polymers [84], they can form aggregates, attach to soil, sediments, suspended particles in water, or surfaces of culture vessels. As a survival strategy, exopolymers may help recovery after desiccation stress in soil [109], survival in starvation conditions [29,34] or facilitate nitrification at low pH [110]. However, strong adsorption to particles may

influence the extraction of cells from soil [111] as well as the permeability of the cells for oligonucleotide probes in FISH, or the quantitative recovery of DNA for PCR.

The probability of detection of members of the *N. eutrophaleuropaea* lineage rises with increasing nutrient concentrations [97,100,101,104]. Sometimes, even if *Nitrosomonas*-like sequences could not be detected in environmental DNA samples, enrichment cultures prepared from the same sampling site revealed *Nitrosomonas* spp. [98,99,112]. Whitby et al. [101] provided evidence that even within different sediment types (littoral and profundal sediments) distinct groups related to either *N. europaea* or *N. eutropha* occurred. 16S rRNA sequences most similar to those from *N. europaea* and *N. eutropha* were recovered from the highly saline waters of Mono Lake, California [93].

Interestingly, within a steadily increasing number of environmental studies sequences belonging to similar clusters of the AOB were retrieved from very different locations (water and sediment samples from freshwater and an estuary) with different composition of their 'total' microbial communities. On the other hand, the data give the impression of great diversity within a group for which only a few strains are in culture and are not physiologically well characterized.

In fertilized soil a drastic increase in nitrification rate, but not in population size, was estimated by competitive PCR based on 16S rRNA and *amoA* genes. This suggested phenotypic changes within the AOB community, while the population size increased 6 weeks later [106]. In a study using 16S rDNA fragments to characterize the community of AOB in the root zone and bare sediment of a shallow lake in the Netherlands, no evidence was found that any particular taxonomic/phylogenetic group was specific for these periodically anoxic environments [113]. It would be interesting to see if such adaptations could be monitored when targeting mRNAs of key enzymes (AMO, HAO) of ammonia oxidation.

2.4.3. Comparison of the community analysis by either 16S rRNA or functional genes

As mentioned above, AOB of the β subclass of the Proteobacteria form a distinct group within this subclass. Their 16S rRNA sequence has high similarity with that of the phototrophic *Rhodocyclus purpureus* and the iron-oxidizing bacterium *Gallionella ferruginea*. Therefore, a slight lack of specificity in the PCR reaction with 'specific' primers may shift the spectrum of sequences that is obtained towards phylogenetically related but physiologically and ecologically different organisms. As none of the primer combinations described for amplification of β-proteobacterial AOB seems to be absolutely specific [91,95,96,107], there is a risk of erroneous results. Our own experiences (J. Höppner and K.-P. Witzel, unpublished) showed that the proportion of non-nitrifier sequences in cloned PCR products from freshwater samples increased dramatically

when the PCR was not performed under high stringency. Many of these unspecific clones were identified as *Gallionella* or *Rhodocyclus* spp.

Important physiological differences may exist within bacteria of a monophyletic 16S rDNA group with high sequence identity [95]. It is questionable whether 16S rDNA sequences are optimally suited for the specific detection of AOB even if the sequence homology is high [46,48,86]. The highly variable, selectively neutral, intergenic spacer regions between the 5S and 16S or 16S and 23S rRNA genes [86,114] might provide better targets. At present, there is not enough information available on this subject for comparative studies.

Under functional or ecological aspects, genes encoding key enzymes may be a better target for fine-scale resolution because they are under high selection pressure [48]. For a marine isolate of AOB, belonging to the γ subclass, the 16S rDNA sequence was nearly identical (only one and two bases out of 976 were different) to the 16S rDNA from two strains of *N. oceani*. In contrast, a comparison of all three *amo* genes of these bacteria revealed a much lower identity of around 88–90% [42]. AMO and HAO, which are crucial for the existence of these bacteria, must have been optimized during evolution for the prevailing conditions in the natural habitat. Therefore, it may be speculated that different ecotypes of these enzymes must have evolved for optimal function in different habitats.

For community structure analyses, it seems rather unlikely that other parts of the bacterial genome could fully replace the 16S rDNA gene as target sites, not only due to insufficient sequence information for comparisons, but also because AOB have only one copy of the rRNA operon. More important than an enhanced resolution within the AOB is information about ecological requirements and physiological capacities in relation to environmental factors of the organisms to which the retrieved sequences belong. The next step is probably a comparison between mere presence and activity, for example, by comparing *amoA* gene sequences with the related mRNA analyses.

3. Denitrification

3.1. Introduction

Denitrification (dissimilatory nitrate reduction, nitrate respiration) is characterized by consecutive steps starting from nitrate via nitrite, nitric oxide (NO), nitrous oxide (N_2O) to the dinitrogen gas (N_2). The ability to denitrify is widespread among bacteria of unrelated systematic affiliations, most likely due to lateral gene transfer in evolution [115,116]. Denitrification has been described for some Archaea [7,116] and even for the mitochondria of some fungi [117]. While the NO reductase from the fungus *Fusarium oxysporum* has been crystallized and its structure described [118], molecular details and the ecological significance of

Table 1
Enzymes of nitrification and denitrification[a]

Enzyme	EC number	Reaction performed	Genes	Prosthetic group	Function of gene product	Gene size (bp)	Bacteria from which genes were sequenced[c]
NO$_3^-$ reductase[b]	1.7.99.4	NO$_3^-$ +2e$^-$ +2H$^+$ → NO$_2^-$ +H$_2$O	narG	[4Fe–4S], molybdopterin	catalytic center	3588–3786	1, 2, 3, 5, 6, 7; 12 others partly
			narH	3 [4Fe–4S]+[3Fe–4S]	confers e$^-$ from NarI to NarG	1464–1677	1, 2, 3, 4, 5, 6, 7, partly 8
			narJ		function unknown	516–741	1, 2, 3, 4, 5, 6, 7
			narI	2 cyt b	membrane anchor, QH$_2$ oxidation	672–741	1, 2, 3, 4, 5, 6, 7
Heme NO$_2^-$ reductase	1.7.2.1	NO$_2^-$ +e$^-$ +2H$^+$ → NO+H$_2$O	nirS	cyt cd_1/subunit (α_2)	catalytic center	1665–1791	4, 9, 10; four others partly
Cu NO$_2^-$ reductase		NO$_2^-$ +e$^-$ +2H$^+$ → NO+H$_2$O	nirK	Cu(I), Cu(II) (α_2)	catalytic center	1092–1140	11, 12, 13, 14, 15, 16, 17; crystal structure with 2.3 Å resolution available
NO reductase	1.7.99.7	2NO+2e$^-$ +2H$^+$ → N$_2$O+H$_2$O	norB	cyt b	catalytic center	1341–1542	13, 18, 4, 19, 5, 10, 15, 17, partly 20
			norC	cyt c	catalytic center	441–453	13, 2×4, 19, 5, 10, 2×17, 15
N$_2$O reductase	1.7.99.6	N$_2$O+2e$^-$ +2H$^+$ → N$_2$+H$_2$O	nosZ	4 Cu/subunit (α_2)	catalytic center	1905–1959	11, 13, 2×4, 5, 10, 9, 21; 43 others partly
Ammonia monooxygenase	1.13.12	NH$_3$+2[H]+O$_2$ → NH$_2$OH+H$_2$O	amoA		active site	744–825 (1530)	22, 23, 24, 25, 26, 27, 28; 69 others partly
			amoB		involved in activity	1250–1262	23, 24, 26; two others partly
			amoC		function unknown	813–880	26, 29, 30; two others partly
Hydroxylamine oxidoreductase	1.7.3.4	NH$_2$OH+H$_2$O → HNO$_2$+4[H]	hao	7 cyt c, heme P$_{460}$ (α_3)	catalytic center	1713	26, 31; one partly

[a] The table was compiled by C. Rösch (Cologne) from data bank information available in March 2000.

[b] E. coli has a second membrane-bound nitrate reductase (encoded by narZYWV), and a further, periplasmic dissimilatory enzyme (napFDAGHBC).

[c] The numbers represent the following bacteria: (1) Bacillus subtilis, (2) Escherichia coli, (3) Mycobacterium tuberculosis, (4) Paracoccus denitrificans, (5) Pseudomonas aeruginosa, (6) Staphylococcus carnosus, (7) Thermus thermophilus, (8) Pseudomonas fluorescens, (9) Ralstonia eutropha, (10) Pseudomonas stutzeri, (11) 'Achromobacter cycloclastes', (12) Alcaligenes faecalis, (13) Bradyrhizobium japonicum, (14) Pseudomonas chlororaphis, (15) Pseudomonas sp., (16) Rhizobium hedysari, (17) Rhodobacter sphaeroides, (18) Nitrobacter hamburgensis, (19) Paracoccus halodenitrificans, (20) Synechocystis sp., (21) Sinorhizobium meliloti, (22) Pseudomonas putida, (23) Nitrosococcus oceani, (24) Nitrosospira multiformis, (25) Nitrosospira sp. C-113, (26) Nitrosomonas europaea, (27) Nitrosospira briensis, (28) Nitrosospira tenuis, (29) Nitrosospira sp. NpAV, (30) Nitrosomonas sp. TK794, (31) Nitrosomonas sp. ENI-11.

denitrification in these organisms still need to be characterized in more detail. In nitrate respiration, some bacteria reduce nitrate only to nitrite or N_2O. In addition, species have been described which grow anaerobically with nitrite or N_2O as the sole respiratory electron acceptor. The expression of denitrification genes is subject to complex regulation. Anaerobic conditions and NO are major factors for the expression of denitrification genes which are under control of the transcription factor FNR, and also by a phosphorylated NarL protein in dissimilatory nitrate reduction of *Escherichia coli* [119]. For details on this subject, the reader is referred to recent reviews [7,8,13, 120,121].

All molecular studies on the ecology of denitrifying bacteria are based on functional genes and their products. Genes involved in denitrification (*nar, nor, nirS, nirK* and *nos*, see Table 1) contain highly conserved DNA regions which can be successfully exploited for developing gene probes. From a mechanistic point of view, NO reductase is a very interesting enzyme, because it catalyzes the formation of the dinitrogen bond in N_2O. Little is known about this process. The conversion of nitrite to nitric oxide (or nitrous oxide) is the crucial step in the reaction sequence because it leads to gas formation. Therefore, most of the ecological work has been done with probes for both types of nitrite reductases [122,123]. Denitrifying bacteria possess either a cytochrome cd_1 (cd_1 NIR) encoded by *nirS*, or a Cu-containing enzyme for nitrite reduction (Cu NIR) encoded by *nirK*. The *nirK* gene encoding Cu NIR from *Pseudomonas aureofaciens* can be transferred and functionally expressed in a mutationally cd_1 NIR-free background of *P. stutzeri* [124]. It is believed that *nirS* is more widespread, but less conserved among bacteria than is *nirK*. Therefore, it is more difficult to design broad-range primers or probes for *nirS* than for *nirK* [125,126]. The *nirK* gene occurs in bacteria of totally unrelated systematic affiliation but is apparently reasonably conserved throughout the bacterial world. A general probe recognizing both *nirK* and *nirS* cannot be developed, since these structurally different enzymes are encoded by genes which do not share sequence homology. Recently, the distribution and diversity of denitrifiers in marine sediment has been investigated by the use of N_2O reductase (*NosZ*) gene-specific primers and sequence analysis [127–129].

Denitrification is related to nitrate ammonification and to the newly discovered anaerobic ammonia oxidation (anammox) reaction. Nitrate ammonification is typical for the Enterobacteriaceae, and e.g. *Wolinella succinogenes* and *Sulforospirillum deleyianum*. The first step, the reduction of nitrate to nitrite, is catalyzed by a nitrate reductase with properties similar to that enzyme in denitrifying bacteria. *E. coli* contains at least two different membrane-bound nitrate reductases (encoded by *narGHJI* and *nar-ZYWV*) and a periplasmic nitrate reductase (*napF-DAGHBC*). Recent work with a mutant defective in

both membrane-bound enzymes showed that nitrate respiration solely via the periplasmic enzyme is possible [130]. Nitrite is then directly reduced to ammonia by a cytochrome *c*-dependent nitrite reductase of which two forms have been isolated [131]. The enzyme from *S. deleyianum* has been crystallized [132], and at least five different sequences for the *napC/nirT* gene family (see Table 1) have been published. Thus, it should now be possible to develop specific probes for this enzyme to assess its distribution in bacteria of different ecological habitats. Remarkably, *E. coli* can even convert N_2O to N_2 [133].

Recently, an anammox reaction in biofilms has been described for bacteria whose closest relative is a planctomycete [120,134]. Ammonia and nitrite are comproportionated mainly to dinitrogen gas. However, nitrate is also formed to some extent in this reaction. Such bacteria may be ubiquitous in nature and were claimed to account for a substantial proportion of the bacterial population of the wastewater bioreactor in this study [134].

3.2. Serological approaches to study denitrification

Immunological techniques have been used mainly to study the distribution of the cd_1 NIR and Cu NIR amongst different bacteria. Körner et al. [135] raised a polyclonal antiserum against the nitrous oxide reductase and cd_1 NIR from *Pseudomonas perfectomarina* (renamed *P. stutzeri*) and screened different pseudomonads and other selected denitrifying bacteria for these enzymes using Ouchterlony double immunodiffusion tests. While almost all tested *Pseudomonas* strains showed reactivity with the anti-N_2O reductase antiserum, cross-reactivity with the anti-cd_1 NIR antiserum was limited to one strain of *P. stutzeri*. The same antiserum was used to investigate the onset and cessation of denitrification enzyme production by *P. stutzeri* in continuous cultures at defined concentrations of dissolved O_2 covering the full range of transition from air saturation to complete anaerobiosis [136]. A polyclonal antiserum against the cd_1 NIR of the same *P. stutzeri* strain was raised by Ward et al. [137]. Using Western blot analysis, the serum reaction was almost strain-specific. These results clearly demonstrated that the antigenic character of cd_1 NIR is variable even among closely related strains. Therefore, antisera which show cross-reactivity with a broader range of bacteria containing cd_1 NIR are difficult to produce. A DNA probe encoding cd_1 NIR showed a broader range of reactivity with different cd_1 NIR-containing bacteria than did the serological results [137]. Michalski and Nicholas [138] used highly specific polyclonal antisera against the nitrate, nitrite, and nitrous oxide reductases of a photosynthetic, denitrifying bacterium (*Rhodobacter sphaeroides* sp.f. *denitrificans*) to investigate the distribution of bacterial denitrifying enzymes. ELISA and Western blot analysis revealed that the molybdenum-containing nitrate reductase and the multi-copper nitrous oxide reductase appear to be common proteins

Table 2
Characterization of polyclonal antisera (1–4) and monoclonal antibodies (5,6) raised against Cu NIR and cd_1 NIR

Bacterial strain		NIR type	Antibody[a]					
			1	2	3	4	5	6
Achromobacter cycloclastes	ATCC 15466	Copper	positive	n.d.	n.d.	n.d.	n.d.	n.d.
	ATCC 21921	Copper	n.d.	positive	n.d.	n.d.	n.d.	n.d.
Achromobacter xylosoxidans	NCIB 11015	Copper	n.d.	positive	n.d.	n.d.	n.d.	n.d.
	DSM 30026	Copper	n.d.	n.d.	n.d.	n.d.	positive	negative
Agrobacterium tumefaciens	DSM 30205	Copper	n.d.	n.d.	n.d.	n.d.	positive	negative
Alcaligenes denitrificans	ATCC 27061	Copper	positive	n.d.	n.d.	n.d.	n.d.	n.d.
Alcaligenes faecalis	ATCC 8750	Copper	n.d.	n.d.	n.d.	negative	n.d.	n.d.
	DSM 30030	Copper	n.d.	n.d.	n.d.	n.d.	positive	negative
Aquaspirillum itersonii	ATCC 11331	cd1	n.d.	n.d.	negative	n.d.	n.d.	n.d.
Azospirillum lipoferum	ATCC 29707	cd1	n.d.	negative	n.d.	n.d.	n.d.	n.d.
Bacillus azotoformans	ATCC 29788	Copper	n.d.	positive	n.d.	n.d.	n.d.	n.d.
Corynebacterium nephridii	ATCC 11425	Copper	n.d.	positive	n.d.	n.d.	n.d.	n.d.
Flavobacterium sp.	ATCC 33514	cd1	n.d.	n.d.	negative	n.d.	n.d.	n.d.
Ochrobactrum anthropi	LMG 18952	Copper	n.d.	n.d.	n.d.	n.d.	positive	positive
Ochrobactrum tritici	LMG 18957	Copper	n.d.	n.d.	n.d.	n.d.	positive	negative
Ochrobactrum grignonense	LMG 18954	Copper	n.d.	n.d.	n.d.	n.d.	positive	negative
Paracoccus denitrificans	ATCC 19367	cd1	n.d.	negative	n.d.	n.d.	n.d.	n.d.
Pseudomonas aeruginosa	DSM 50071	cd1	n.d.	n.d.	negative	n.d.	n.d.	n.d.
	ATCC 10145	cd1	n.d.	negative	n.d.	n.d.	n.d.	n.d.
	DSM 10	cd1	n.d.	n.d.	n.d.	n.d.	negative	negative
Pseudomonas alcaligenes	ATCC 14909	Copper	positive	n.d.	n.d.	n.d.	n.d.	n.d.
Pseudomonas aureofaciens	ATCC 13985	Copper	n.d.	positive	n.d.	negative	n.d.	n.d.
Pseudomonas denitrificans	ATCC 13543	Copper	positive	n.d.	n.d.	n.d.	n.d.	n.d.
	ATCC 13867	Copper	n.d.	positive	n.d.	negative	n.d.	n.d.
Pseudomonas fluorescens	ICPB 2708-401	cd1	n.d.	n.d.	negative	n.d.	n.d.	n.d.
	DSM 50415	cd1	n.d.	n.d.	negative	n.d.	n.d.	n.d.
	ATCC 17802	cd1	n.d.	negative	n.d.	n.d.	n.d.	n.d.
	ATCC 33512	cd1	n.d.	n.d.	n.d.	negative	n.d.	n.d.
Pseudomonas mendocina	DSM 50017	cd1	n.d.	n.d.	negative	n.d.	n.d.	n.d.
Pseudomonas nautica	DSM 50418	cd1	n.d.	n.d.	negative	n.d.	n.d.	n.d.
Pseudomonas putida	ATCC 12633	cd1	n.d.	n.d.	n.d.	negative	n.d.	n.d.
Pseudomonas stutzeri	ATCC 17588	cd1	n.d.	n.d.	positive	n.d.	n.d.	n.d.
	ATCC 14405	cd1	n.d.	n.d.	positive	positive	n.d.	n.d.
	ATCC 11607	cd1	n.d.	n.d.	n.d.	positive	n.d.	n.d.
	ATCC 17588	cd1	n.d.	n.d.	n.d.	positive	n.d.	n.d.
Rhodobacter sphaeroides	ATCC 17023	Copper	positive	n.d.	n.d.	n.d.	n.d.	n.d.

[a](1) Michalski and Nicholas [138], (2) Coyne et al. [139], (3) Körner et al. [135], (4) Ward et al. [137], (5) Metz and Schloter [140], (6) Metz and Schloter [140].

among denitrifying bacteria. The pattern of immunological cross-reactivity for nitrite reductase, however, confirmed that there are two different kinds of the enzyme, namely cd_1 NIR and Cu NIR, commonly distributed amongst bacteria [138]. The Cu NIR type from *R. sphaeroides* was also detected in *Alcaligenes denitrificans*, two *Pseudomonas* spp. and *Achromobacter cycloclastes*. Coyne et al. [139] raised two different polyclonal antisera to identify cd_1 NIR and Cu NIR in 100 isolates of denitrifying bacteria from diverse environments, mainly agricultural soils. Using Western blotting, the Cu NIR type was identified in the species *Alcaligenes* (renamed *Ralstonia*) *eutrophus*, *Bacillus azotoformans* and *Corynebacterium nephridii* exclusively, while *Aquaspirillum itersonii*, *Flavobacterium* spp. and *Pseudomonas fluorescens* contained the cd_1 NIR type. Coyne et al. [139] concluded from their results that Cu NIR can be found in taxonomically more unrelated strains.

Until now immunological studies concerning denitrification have only been performed with bacterial isolates. However, the ex situ measurement of the functions of selected microorganisms provides only a measure of potential activity. Recently, an immunological test system for in situ detection of actually denitrifying bacterial communities in different habitats was described on the basis of the in situ distribution of the Cu NIR [140], which is widely distributed among different bacterial taxa (see above).

Polyclonal antisera, which were used in pure culture studies, have three main limitations for in situ use: (1) they are mixtures of antibodies with different specificities and it is therefore difficult to validate the exact cross-reactivities in complex ecosystems; (2) the available amount is limited by the blood volume of the animal used for immunization; and (3) the exact composition of antibodies cannot be reproduced by immunizing a second animal [141]. Monoclonal antibodies (mAbs) [142], as used
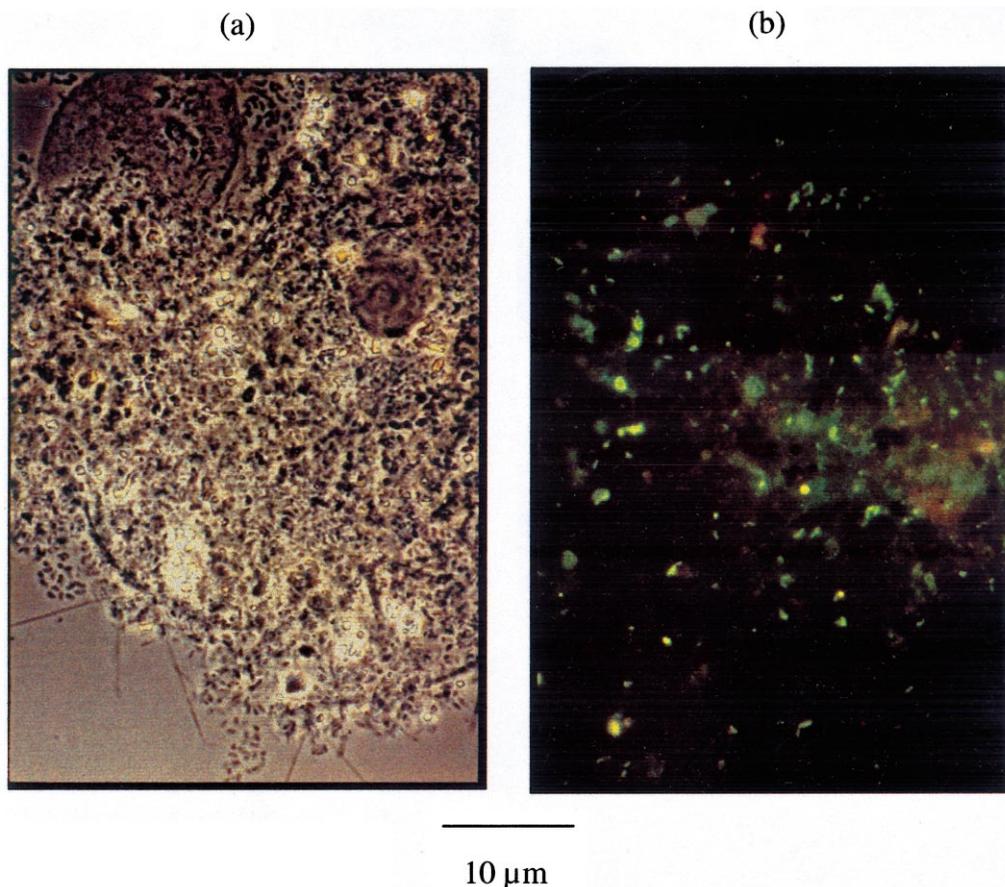
(a)   (b)

10 µm

Fig. 1. Phase contrast (a) and epifluorescence (b) microscopic pictures of a biofilm with denitrifying bacteria from an aerated nitrification–denitrification basin of industrial wastewater treatment plant. The denitrifying bacteria were labeled with a monoclonal antibody against cd_1 NIR, labeled with FLUOS®. The green fluorescence was excited at 488 nm.

by the authors, do not show these limitations. To obtain the Cu NIR protein in high amounts and purity suitable for immunization, two different ways were chosen. (1) Cu NIR from *Ochrobactrum anthropi*, a typical denitrifying soil bacterium [143], was purified using a classical protein isolation and purification approach [144]. (2) The whole Cu NIR gene from *Alcaligenes faecalis* S6, which shows very high homology to the Cu NIR gene from *O. anthropi* [145], was amplified by PCR, cloned and expressed in *E. coli* IM105. The recombinant enzyme, which had a $6 \times$ His affinity tag at the N-terminal end, was purified using immobilized metal affinity chromatography.

The hybridoma cell lines produced mAbs with different specificity. While the mAb from procedure (1) was very specific and showed only cross-reactivity with the Cu NIR from the *O. anthropi* strain used for immunization, most of the mAbs obtained from procedure (2) showed a broader range of cross-reactivities with the copper-containing nitrite reductase from bacteria of different taxa, e.g. *Achromobacter xylooxidans*, *Alcaligenes faecalis*, *Agrobacterium tumefaciens*, *Ochrobactrum* spp. These distinct reactivities of the monoclonal antibodies might be explained by the different epitopes which are recognized. The mAb raised via procedure (1) could bind to flanking region of

the Cu NIR protein, which show high variabilities, whereas mAbs raised via procedure (2) could react with the active part of the enzyme which is highly conserved. The data on cross-reactivities of both mAb types are listed in Table 2. The mAbs could detect 10 ng purified Cu NIR protein in ELISA procedures.

The mAbs raised via procedure (2) were used for in situ detection of denitrifying bacteria in different environments. Fig. 1 shows one example with FITC-labeled antibodies applied to bacteria which express the Cu NIR phenotype. The bacteria were detected in biofilms from an aerated nitrification–denitrification basin of an industrial wastewater treatment plant, which is known for its high loads of nitrate and its high number of denitrifying bacteria [146].

In future, double labeling with mAbs raised via procedure (2) could be combined with taxonomic oligonucleotide probes in order to identify the bacteria which show the Cu NIR phenotype in situ [147]. Together with the enrichment by flow cytometry of cells labeled with a fluorescent mAb [148] these techniques might have great potential to detect and enrich denitrifying bacteria from complex ecosystems and to analyze directly their genotype without further cultivation.

3.3. Detection of denitrifying bacteria in soils

Any investigation of soil is immediately faced with the problem that this substrate is highly variable on small scales, both horizontally and vertically in the soil layer. In the case of higher plants, it is impossible to explain and predict why one plant species occupies one soil spot but not the next one on a given soil type, because factors determining the competitiveness of a plant are many and complex. The same might also apply to bacterial communities in soils. Due to our inability to cultivate most soil bacteria and due to the impossibility of detecting individual genes present in single copy or low copy numbers in intact bacterial cells [149], recent attempts to analyze soil bacteria have concentrated mainly on characterizing soil DNA and the relative abundance of genes encoding denitrification and nitrification functions.

Such an approach requires the isolation of soil DNA in high yields and of high purity. Several protocols have been published [150–153], and each investigator seems to have optimized his/her own recipe for a particular application. The potential for widespread use of uniform techniques is not clear. DNA recoveries are determined by seeding experiments with culturable bacteria. The efficiency and reproducibility of the DNA extraction method can be evaluated by competitive PCR [154]. It is not known whether indigenous bacteria stick more tightly to soil particles or can as readily be retrieved from soils as the culturable microorganisms stirred into a soil suspension. Soils contain substances such as tannins, polyphenols and polysaccharides in variable amounts, and these interfere with DNA isolation and detection ([151], and references therein). In extractions of DNA directly from soils, factors such as the efficiency of bacterial cell wall lysis and the non-specific adsorption of DNA to clay strongly influence the yield [155]. In contaminated DNA preparations, restriction enzyme digestion and *Taq* DNA polymerase activity may be affected and the efficiency of DNA–DNA hybridizations may also be decreased [156]. Preparations often contain a large amount of sheared DNA and are often not amenable to restriction analysis. Commercially produced kits for rapid preparation of soil DNA of such quality are now available (e.g. Mo Bio Laboratories Inc, Solana Beach, CA, USA), but the details of the kit methods are not published for obvious reasons.

Instead of analyzing total DNA and its relative content it would be even more rewarding to perform transcript analysis of the respective genes. However, compared to DNA, mRNA is even more unstable. Transcript analysis of genes involved in denitrification has not yet been performed in soil samples.

When the experiments [157] started some 10 years ago, only a few sequences for denitrification genes were available, and the gene probes used at that time also contained other sequences not encoding denitrification genes. These probes, however, recognized denitrification genes in a large number of bacteria [157]. These results of DNA–DNA hybridization experiments (dot-blot analyses) with these gene probes were interpreted as indicating that culturable denitrifying bacteria are enriched in the upper soil layer and in the vicinity of plant roots. Using a similar hybridization approach [137,158], high abundances of putative denitrifying bacteria were detected in marine water columns and sediment environments. Since these initial reports, many further sequences of denitrification genes have been published (Table 1). Thus, it is not difficult to synthesize by PCR specific probes which recognize the target genes in a wide range of bacteria. With probes of 0.3–1.0 kb in size, the distribution of denitrification genes were assessed in different *Hyphomicrobium* species or strains occurring in a sewage plant and its incoming water [159,160]. Experiments with these gene probes also confirmed that the concentration of culturable denitrifying bacteria is highest in the upper 5 cm and steadily decreases with the depth of the soils investigated [161].

The two molecular approaches which have been applied to date to study the ecology of denitrification are PCR amplification using 15–25-oligomer primers and DNA–DNA hybridizations with 0.4–1.0-kb probes targeted against nitrite reductase genes. The use of a set of oligonucleotide primers makes it possible to amplify segments of *nirS* or *nirK*, and the products generally have the correct molecular mass and DNA sequence. However, the PCR approach is often not successful, particularly when the DNA template is not entirely free of contaminants. In some cases, such as two *Pseudomonas denitrificans* and one *Alcaligenes* (renamed *Ralstonia*) *eutrophus* strains [126], PCR products could not be obtained for *nirS*, although activity measurements and biochemical data did show the presence of this enzyme in these strains. To obtain quantitative data on the relative distribution of denitrification genes in bacterial genomes, quantitative PCR would be required. Despite the advertisements of companies selling instruments for quantitative PCR, this technique is seemingly still in its infancy and has not yet successfully been employed with soil DNA. Competitive PCR, or even competitive RT-PCR, offers a possible alternative.

Hybridization of extracted DNA with 0.4–1.0-kb probes for genes involved in denitrification gives positive scores more often than in the PCR approach using enrichment cultures. Distinct hybridization bands are often obtained, and the data from the DNA–DNA hybridization are usually in accordance with the results from activity measurements. However, *Azospirillum lipoferum* Sp59 has been described as expressing a cd_1 NIR and produces N_2O from nitrite (in the presence of acetylene and under anoxic conditions), but hybridizations with a *nirS* probe were always negative with DNA from this bacterium under all stringencies employed (A. Mergel and H. Bothe, unpublished). Faint signals are often obtained in DNA–DNA hybridization, even at high stringencies. This is the major problem when the occurrence of denitrification genes in a

bacterial isolate from a soil is to be assessed. In our studies [162,163], the *nir* probes used (for *nirS* and *nirK)* did hybridize with DNA isolated from the soils, in contrast to preparations used in other studies [164]. However, for this purpose, DNA extracted from soils must be highly purified (close to $OD_{260/280nm} = 1.8$). Experiments with cruder preparations yielded erratic data (A. Mergel and H. Bothe, unpublished).

In principle, probing soil DNA provides information about the relative abundance of the target gene in a soil layer or habitat. The intensity of the signal is, however, different from bacterium to bacterium, mainly depending on the sequence alterations with each bacterium. Therefore, signal intensities cannot be transformed to cell numbers, as has also been pointed out for rRNA dot-blot hybridizations [165]. Apart from this, signal intensities of the bands can normally be compared only on a given membrane. Many factors determine the signal intensity in DNA–DNA hybridizations (the hybridization stringency; the quality of the membrane, which is often not uniform; the labeling intensity of the probe with any label being variable from preparation to preparation, etc.). Thus, it is advisable to use the same membrane first for the DNA probe developed for a functional gene, then to strip the membrane and to rehybridize it with a general probe recognizing all bacterial DNA (e.g. a general 16S rRNA probe). Signal intensities can be quantified densitometrically.

This approach was used to study the distribution of denitrifying bacteria in acid soil (humisol type) under an oak–hornbeam forest in the vicinity of Cologne [162]. The hybridization data indicated that most bacteria of the total population lived in the upper (5 cm) soil layer. Hybridizations with all denitrification probes (with the exception of *nirK)* also showed that denitrifying bacteria are enriched in the vicinity of plant roots compared to the bulk, root-free soil. Unexpectedly, denitrifying bacteria were not enriched in the deeper soil layer ($\sim$ 25 cm depth) where the concentration of nitrate was still not growth limiting and where the O_2 partial pressure might be low. Altogether, it was estimated that less than 5% of the total bacterial population possess the denitrification genes. With all soil samples assayed, denitrification activity (N_2O formation) was only detected when the assays were supplemented with high amounts of nitrate.

Bacteria living in such soils may not gain a selective advantage from possessing the denitrification genes. Such a soil is rarely waterlogged, so that the concentration of O_2 in the upper 5 cm may always be high enough to suppress the expression of denitrification genes. It remains to be shown whether any aerobic denitrification occurs in such habitats. The ability to respire with nitrate as an electron acceptor in the presence of oxygen is seemingly widespread among bacteria [166]. It has been estimated that 10^4–10^7 bacteria per g of soil or sediment are able to respire with nitrate under oxic conditions [167]. In ad-

dition, anoxic microsites may also occur in such soils, and some evidence for denitrification in such microsites has been published [168–170]. Exact oxygen concentrations in densely packed soils cannot be determined by microelectrodes. Measurements have been reported for soil aggregates and fir litter [168,171]. As in the soil of the oak–hornbeam forest near Cologne, enrichment of denitrifying bacteria was observed in the upper layer of a Norway spruce forest in southern Germany [163] where the N input to the soil from the air was marginal due to low industrial activity in this area. In this study [163], the number of culturable bacteria, determined by the MPN method, and of denitrifying bacteria showed large seasonal fluctuations during the investigation period December 1994–August 1998. Seasonal fluctuations in the bacterial population hybridizing with the nitrogenase probe *nifH* were also observed at a Douglas fir forest site in the Oregon Cascade mountain range [172]. Such fluctuations may be responsible for the peak activities in N_2O formation often observed in spring in field studies [173,174]. Fertilization of one area of the Norway spruce stand in southern Germany with a high load of ammonium nitrate did not cause a shift in the population of denitrifying bacteria [163]. The nitrate content and the presence of plants in habitats may, however, determine the composition of the nitrate-respiring bacterial community at other locations [175].

In the long run, an analysis of the complete population of denitrifying and nitrifying bacteria in soils is required if their potential denitrification activities (NO_x fluxes) are to be predicted. Quantification of specific genes and gene products in complex microbial communities by in situ PCR has been employed [149,176]. Such techniques have not been used very widely so far, probably because they suffer from non-specific amplification of non-target DNA and from non-specific binding of fluorescence probes to DNA, particularly when the preparation is not entirely free of contaminants. A laborious and tedious, yet rewarding avenue is to construct clone libraries of PCR products from soil DNA using primers for bacterial 16S rRNA gene sequences [177]. The use of separation methods such as DGGE [113,178] or TGGE [179] largely helps to separate the products obtained. However, only a limited number, perhaps approx. 100 clones, which represent only a small percentage of the roughly 10^4 ribotypes per g soil, can be sequenced in one study. The first investigations indicated that sequences of a large number of uncultured bacteria with unknown phylogenetic affiliations are obtained by this approach [177].

3.4. Denitrification in aquatic habitats

Several molecular studies on denitrifiers in aquatic environments have been published and only a few can be mentioned here. Experiments in lakes, particularly in biofilms, are facilitated by the fact that microelectrodes are

available to monitor the changes in the concentrations of oxygen, nitrate, ammonia, and other compounds. Specific amplification by PCR indicated the occurrence of *Alcaligenes xylosoxidans* in freshwater lakes [145]. Hybridization with the *nir* specific probe developed from *P. stutzeri* indicated that 0.1-1% of the total bacterial population possess this gene in seawater samples [137]. In sediments of a Danish Fjord estuary denitrification activity determined by ^{15}N isotope measurements and by the acetylene inhibition technique showed that denitrification capacity was highest in the upper 1 cm of the sediment and declined with depth. Denitrification showed peak activities in spring and autumn, whereas nitrate ammonification rates were highest in summer [180]. To our knowledge, a study combining electrode measurements and ^{15}N isotope techniques with new molecular approaches for determining denitrification in aquatic habitats has not yet been thoroughly performed. Results for marine sediments, in which nitrate supplementation was required to detect potential denitrification activity as assayed by the acetylene inhibition technique [158], are consistent with observations in soils (above). High abundances of *nirS* were detected by DNA hybridization in these sediments, but individual strains of denitrifying bacteria, enumerated by immunofluorescence, were present at very low abundance.

4. Conclusions and future developments

Molecular approaches have made the study of unknown microorganisms and their behavior in diverse environments much more accessible. However, we are far from understanding the complexity of bacterial life in any natural habitat. As mentioned above, about 10^4 different genomes per gram occur in soils, and the number may be very high in aquatic habitats as well. Target DNA (sequences encoding 16S rRNA or functional genes) can be amplified by PCR, cloned and sequenced. Techniques such as DGGE, TGGE or T-RFLP might help to screen large sample sets for a comparison of local and seasonal variations. However, only a limited number (10^2–10^3, the amount depending on the sequence length and the enthusiasm of the group involved) can be cloned and sequenced. From the functional genes, e.g. *amoA* for nitrification or *nirS* for denitrification, only few sequences have been deposited. Thus most sequences obtained for functional genes will be new. This situation is not so dramatic in the case of the 16S rRNA sequences, but clearly the characterization of any new habitat will add new sequence information to the richness of the 16S rRNA data banks. When the 16S rRNA sequence is new, it does not provide information on the physiological capabilities of the organism. Due to the monophyletic origin of most AOB known so far, it can at least be guessed whether a detected 16S rRNA gene sequence belongs to a nitrifier. This does not apply at all to denitrifiers or heterotrophic nitrifiers. This

means that sequences of each of the desired target genes, like 16S rDNA, *nirK*, *amoA* etc., have to be obtained from the same bacterial population. New techniques, like microarrays (DNA/RNA chips) with diverse copies of all nitrification and denitrification genes, will likely facilitate screening of natural communities for their specific metabolic potential in the N cycle. When a large percentage of the genomes in soil or water have been examined for their nitrification and denitrification traits, it may help to obtain a better understanding of factors which regulate NO_x fluxes. Such guesses are necessary for any prediction of the impact of global NO_x emissions on climate changes caused by nitrifying and denitrifying bacteria. Information is needed not only about the microbial community structure, but also about their activities. As a first step, this requires the development of protocols to isolate and identify stable transcripts (mRNA) for the key enzymes of the processes. Antibodies may help to quantify enzyme concentrations.

Besides all the excitement about the molecular approaches that enable us to study microorganisms directly in their natural habitats, we need more attempts to cultivate and characterize those organisms that are still unknown.

Acknowledgements

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