Chapter 12

NITRIFYING BACTERIA

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1. NITRIFICATION AS PART OF THE NITROGEN CYCLE

Nitrogen is one of the most important elements of life. In nature, several redox reactions of nitrogen are carried out almost exclusively by microorganisms (Figure 1). The most important nitrogen component on earth is dinitrogen (N_2), a highly stable gas that can only be used as a nitrogen source by a restricted group of microorganisms, the nitrogen-fixing bacteria (Figure 1, pathway 1). Biological nitrogen fixation is an important process because it provides ammonia (NH_3) for plants and animals. Nitrogen in form of ammonia can be assimilated into organic material (Figure 1, pathway 2). In modern agriculture, additional ammonia is supplied by mineral fertilizers derived from industrially fixed N_2. Conversely, ammonia is released from organic nitrogen compounds by microbial activity called ammonification (Figure 1, pathway 3). In soils, ammonia is mainly bound to clay particles primarily in the form of ammonium (NH_4^+), where it is available for utilization. Ammonia/ammonium is the most frequently found form of nitrogen in the biosphere and ammonia is transferred efficiently over long distances *via* volatilization.

Under oxic conditions, ammonia is not stable and is converted to nitrate by nitrifying bacteria in soil, freshwater, and marine environments (Figure 1, pathways 4-5). The microbial oxidation of ammonia to nitrite (NO_2^-) and nitrate (NO_3^-) is called nitrification. Nitrification is catalyzed by two physiological groups of bacteria. The aerobic ammonia-oxidizing bacteria produce nitrite (Figure 1, pathway 4), which is further metabolized by nitrite-oxidizing bacteria to nitrate (Figure 1, pathway 5). In contrast to ammonia/ammonium, nitrite is usually found only in trace amounts in aerobic habitats and it rarely accumulates at low partial

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pressures of O_2, *e.g.*, either in soils with high water potential or in alkaline environments. Due to the toxicity of nitrite for living organisms, the maintenance of a low nitrite concentration in aerobic habitats is essential. In general, nitrite is immediately consumed by nitrite oxidizers. The end product of nitrification, nitrate, is mobile and can readily leach into both ground water used for drinking and surface waters to cause eutrophication. Instead of or in combination with ammonia, some organisms can use nitrate as a nitrogen source for growth (Figure 1, pathway 6).

Nitrate also serves as a substrate for denitrification and is used for anaerobic respiration (Figure 1, pathways 7-9). The denitrifying bacteria generally produce N_2 *via* the intermediate, nitrite, and the greenhouse gases, nitric oxide (NO) and nitrous oxide (N_2O). Therefore, denitrification is involved in the destruction of stratospheric ozone, global warming, and the loss of ammonia fertilizer to the atmosphere as N_2. In addition to aerobic nitrification, anaerobic ammonia oxidation (Anammox) has been recently described (Jetten, 2001). The, so far, unisolated planctomycetes combine ammonia and nitrite to produce N_2 (Figure 1, pathway 10).

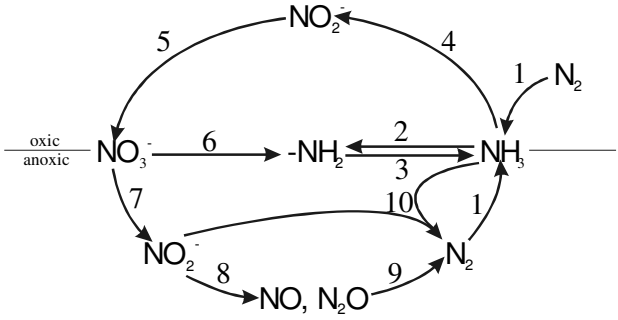

Figure 1. Nitrogen cycle. (1) Dinitrogen (N_2) fixation; (2) assimilation of ammonia (NH_3) into the amino group (-NH_2) of protein; (3) ammonification; (4) ammonia oxidation; (5) nitrite (NO_2^-) oxidation; (6) assimilation of nitrate (NO_3^-); (7, 8, 9) denitrification via *nitrite, nitric oxide (NO) and nitrous oxide (N_2O); (10) anaerobic ammonia oxidation.*

Nitrification, the biological oxidation of ammonia to nitrate *via* nitrite, occupies a central position within the nitrogen cycle (Figure 1, pathways 4-5). Nitrifying bacteria are the only organisms capable of converting the most reduced form of nitrogen, ammonia, to the most oxidized form, nitrate. The nitrification process has various direct and indirect implications for natural and man-made systems. It increases the loss of soil nitrogen due to leaching of nitrate, volatilization of nitrogen gases by denitrification, and chemodenitrification (loss of nitrite under acid conditions) and, therefore, influences the fixed nitrogen supply to plants. Furthermore, in unbuffered soils, the oxidation of ammonia to nitric and nitrous acid results in acidification, which allows the formation of aluminum ions, which are toxic to roots of trees, from insoluble aluminates (Mulder *et al.*, 1989). Consequently, nitrification is not beneficial in agricultural soils. Therefore, anhydrous ammonia is

used as nitrogen fertilizer and chemicals are occasionally added to the fertilizer to inhibit the nitrification process. However, plants can benefit from nitrifying activity. In particular, many trees prefer nitrate instead of ammonia as a nitrogen source. The production of acids by nitrifiers also contributes to the bio-deterioration of calcareous stone, for example, those of historical buildings (Mansch and Bock, 1996; 1998).

In contrast, nitrification is highly desirable in the treatment of sewage for the efficient removal of ammonium (Painter, 1986). Nitrifiers oxidize ammonium, which is, together with urea, the most frequently found nitrogen compound in sewage, to nitrate. Nitrate can subsequently be removed by denitrifying bacteria *via* anaerobic respiration predominantly in form of dinitrogen gas. This treatment is an integral part of modern nutrient removal of waste-water treatment plants protecting environments from high amounts of ammonia. In addition, ammonia oxidizers have potential applications in the bioremediation of polluted soils and waters that are contaminated with chlorinated aliphatic hydrocarbons, *e.g.*, trichloroethylene (TCE) (Hyman et al., 1995), which is currently the most widely distributed carcinogenic organic groundwater pollutant.

The understanding of the metabolism of both ammonia and nitrite oxidizers will facilitate attempts both to reduce their negative effects and to make use of their beneficial effects.

2. TWO PHYSIOLOGICAL GROUPS OF BACTERIA CONTRIBUTE TO NITRIFICATION

Under oxic conditions, the most important group of organisms involved in nitrification are aerobic chemolithoautotrophic nitrifying bacteria, the ammonia and nitrite oxidizers. For these organisms, the oxidation of inorganic nitrogen compounds serves as their characteristic energy source. They can derive all cellular carbon from carbon dioxide (CO_2). No chemolithotroph is known that can carry out the complete oxidation of ammonia to nitrate. Under anoxic conditions, it has been recently indicated that representatives of the order Planctomycetes are involved in the chemolithotrophic nitrification (Strous et al., 1999). These organisms seem to combine ammonia and nitrite directly into dinitrogen gas (Figure 1, pathway 10). Unfortunately, pure cultures of the involved bacteria have not been obtained so far.

In addition to lithotrophic nitrification, various heterotrophic bacteria, fungi, and algae are capable of oxidizing ammonia to nitrate in the presence of O_2 (Kilham, 1986). However, in contrast to lithotrophic nitrification, heterotrophic nitrification is not coupled to energy generation. Consequently, the growth of heterotrophic nitrifiers is dependent on the oxidation of organic substrates. During heterotrophic nitrification, either ammonia or reduced nitrogen from organic compounds is co-oxidized. The rate of heterotrophic nitrification is much slower than that accomplished by the chemolithotrophic nitrifying bacteria and may not be ecological significant. In the following discussion, the aerobic lithoautotrophic nitrifying bacteria will be described exclusively. For more details about nitrifiers, see Bock and Wagner (2001).

Originally, the lithoautotrophic nitrifying bacteria were collected together within one family, named *Nitrobacteracea,* where ammonia oxidizers are characterized by

the prefix *Nitroso-* and the nitrite oxidizers are characterized by the prefix *Nitro-* (Watson *et al.,* 1989). However, phylogenetic investigations have made it evident that ammonia and nitrite oxidizers are not closely related (Teske *et al.,* 1994; Woese *et al.,* 1984, 1985). Several genera are recognized on the basis of both morphology and gene-sequence analyses. So far, five genera of ammonia oxidizers, including *Nitrosomonas, Nitrosospira, Nitrosovibrio, Nitrosolobus*, and *Nitrosococcus*, and four genera of nitrite oxidizers, including *Nitrobacter, Nitrospira, Nitrospina*, and *Nitrococcus*, have been described.

According to comparative 16S rDNA sequence analyses, all recognized ammonia oxidizers are members of two lineages within the β- and γ-subclasses of the Proteobacteria (Koops and Pommerening-Röser, 2001). The marine species of the genus *Nitrosococcus* cluster together in the γ-subclass of Proteobacteria (Woese *et al.*, 1985). The four other genera of ammonia oxidizers, *Nitrosomonas* (including *Nitrosococcus mobilis*, which belongs phylogenetically to *Nitrosomonas*), *Nitrosospira, Nitrosovibrio*, and *Nitrosolobus*, form a monophyletic assemblage within the β-subclass of Proteobacteria (Woese *et al.*, 1984).

Nitrite oxidizers are phylogenetically more distinct. Among the nitrite oxidizers, the genera *Nitrobacter* and *Nitrococcus* were assigned to the α- and γ-subclass of Proteobacteria, respectively (Teske *et al.,* 1994). Nitrite oxidizers of the genus *Nitrospira* are affiliated with the recently described phylum *Nitrospira*e, which represents an independent line of decent within the domain Bacteria (Ehrich *et al.,* 1995; Spieck and Bock, 2001). Because of limited sequence data, *Nitrospina* was first aligned with the δ-subclass of Proteobacteria (Teske *et al.,* 1994), however, in the latest release of ribosomal database project, it is listed in its own subdivision (http://rdp.cme.msu.edu/cgis/treeview.cgi).

All genera of nitrifiers can be initially distinguished by their cell shape. They are rod-shaped, ellipsoidal, spherical, spirillar, vibrioid, or lobular. All species have a typical Gram-negative multilayered cell wall. Many of the nitrifying bacteria have complex intracytoplamic membranes, which may occur as either flattened lamellae or randomly arranged tubes. Cells may be either motile or non-motile and the flagella of motile cells are polar to lateral or peritrichous. Glycogen and polyphosphate inclusions have been observed. In some species, polyhedral cellular inclusions, called carboxysomes, occur and these contain the key enzyme of carbon-dioxide fixation. Cells occur singly, in pairs and, occasionally, in short chains. Extracelluar polymeric substances (EPS), which cause cell aggregation, have been observed both in cultures as well as in natural habitats.

3. ECOLOGY AND DETECTION OF NITRIFYING BACTERIA

Nitrifying bacteria are widely distributed in nature. They are found in most aerobic environments where organic matter is mineralized and, therefore, considerable amounts of ammonia are present. They occur both in lakes and streams, which receive inputs of ammonia, and at the thermocline of lakes, where both ammonia and O_2 are present. However, in accordance with their ecophysiological

characteristics, several species have been observed to occur either predominantly or exclusively at special sites, *e.g*., in rivers, freshwater lakes, salt lakes, oceans, brackish waters, sewage disposal plants, rocks or natural stone of historical buildings, and acid soils (de Boer *et al.,* 1991; Mansch and Bock, 1998; Koops and Pommerening-Röser, 2001). Some species have an obligate salt requirement; others prefer either eutrophic or oligotrophic environments and may tolerate high or low temperatures (Golovacheva, 1976; Koops *et al*., 1991). Furthermore, nitrifying bacteria are found in niches with either a low O_2 concentration or under anoxic conditions, like in Antarctic lakes and at a depth of 60m in permafrost soils (Voytek *et al*., 1999; Wagner *et al*., 2001). Especially in aquatic environments, ammonia-oxidizing bacteria often occur attached as flock or a biofilm (Stehr *et al.,* 1995).

Enrichment cultures of nitrifying bacteria are obtained by using selective media containing either ammonia or nitrite as electron donor and bicarbonate as sole carbon source. Nitrifying bacteria are slow-growing organisms because their cell growth is inefficient. The shortest generation times, as measured in laboratory experiments, were *ca*. 7 hours for *Nitrosomonas* and 10 hours for *Nitrobacter.* For cell division in natural environments, most nitrifying species need from several days to weeks, depending on substrate and O_2 availability, as well as on the temperature and pH-value. Because of the inefficiency of growth of these organisms, visible turbidity will not develop even after extensive nitrification has occurred, so that the best means of monitoring growth is to assay for either the production or disappearance of nitrite.

The slow growth rates of nitrifiers have severely hampered cultivation-dependent approaches to investigate the number as well as the community composition and dynamics of nitrifiers in different environments. The number of nitrifiers in complex systems has been traditionally determined by the most-probable-number (MPN) technique (Matulewich *et al.,* 1975) and selective plating. However, these techniques are time-consuming, often underestimate the number of nitrifiers, and do not allow discriminations at the species level (Belser, 1979; Konuma *et al*., 2001). Depending on the culture medium and incubation conditions, only a fraction of the total nitrifying community can ever be measured by viable counting methods. Therefore, other methods have been developed for *in situ* identification and enumeration based on the specificity of antibodies and nucleic-acid sequences in order to avoid the limitations of the MPN-technique.

Nitrifiers can be detected in environmental samples, independent of their culturability, by using either antibodies or 16S rRNA-targeted oligonucleotide probes. The fluorescent antibody technique can be applied for direct microscopic enumeration of nitrifiers in complex environmental samples (Belser and Schmidt, 1978; Stanley and Schmidt, 1981; Völsch et al., 1990; Sanden et al., 1994). But for antibodies, which recognize epitopes of the cell wall, the target cells have to be isolated first as pure cultures and the produced antibodies often recognize only a few strains of a given species. Recently, antibodies that target the key enzymes of ammonia oxidizers and nitrite oxidizers were developed (Aamand *et al*., 1996; Pinck *et al.,* 2001), which overcome the problems of antibodies recognizing only epitopes of the cell wall. They were successfully applied for the detection of nitrifiers in environmental samples (Bartosch *et al.,* 1999, 2002; Fiencke, pers. com.) as well as

for physiological studies of the key enzyme (Pinck *et al*., 2001). Alternatively, nitrifiers can be detected in environmental samples by using a variety of different PCR-techniques, which in general use 16S rRNA (Degrange and Bardin, 1995; Hiorns *et al.,* 1995; McCaig *et al.,* 1999) as well as key enzyme sequence information (Rotthauwe *et al.,* 1997; Nold *et al.,* 2000). Recently, investigations demonstrated that fatty acid profiles of nitrite oxidizers reflect their phylogenetic heterogeneity and could, therefore, be used to for the differentiation and allocation of new isolates (Lipski *et al.,* 2001). Quantitative population structure analysis of nitrifying bacteria can be performed by applying oligonucleotide probes for fluorescence *in situ* hybridization (FISH) (Wagner *et al.,* 1995, Mobarry *et al.,* 1996; Voytek *et al*., 1999).

4. METABOLISM OF NITRIFYING BACTERIA

Nitrifiers are chemolithotrophic bacteria that are characterized by the ability to grow on reduced inorganic nitrogen compounds. They are autotrophic bacteria, which can derive all cellular carbon from carbon dioxide (CO_2) that is fixed *via* the Calvin/Benson-Cycle. In the past, they were described as obligate chemolithoautotrophic. Nitrifiers seemed incapable of growth with other carbon or energy sources and organic compounds seemed to be toxic to them. This restricted capability is surprising because both ammonia and nitrite are poor energy sources (Hooper, 1989). However, it has now been demonstrated that several nitrifiers are able to grow mixotrophically with either ammonia or nitrite as electron donors and a combination of carbon dioxide and organic compounds as carbon source (Clark and Schmidt, 1966; Martiny and Koops, 1982). Compared to purely autotrophic growth, the addition of organic compounds stimulated cell growth and increased cell yield (Steinmüller and Bock, 1976; Krümmel and Harms, 1982). Furthermore, the nitrite oxidizer, *Nitrobacter*, can grow heterotrophically and organotrophically with pyruvate, formate, and acetate, which serve as both energy and carbon sources (Bock, 1976). However, for these organisms, organotrophic growth was always slower than lithotrophic growth.

Recently, heterotrophic growth of *Nitrosomonas* with fructose and pyruvate as sole carbon source has also been detected (Hommes *et al.,* 2003). But, in contrast to nitrite oxidizers, organotrophic growth of ammonia oxidizers with organic compounds as energy source and O_2 as electron acceptor has not been detected so far (Matin, 1978; Krümmel and Harms, 1982). The reasons proposed for obligate chemolithotrophy of ammonia oxidizers are: (i) the incompleteness of the tricarboxylic acid cycle; (ii) the inability to regulate the levels of enzymes to effectively utilize organic substrates; and (iii) the inability to oxidize NADH at a rate sufficient to utilize organic substrate (Hooper, 1969).

Nevertheless, the metabolism of nitrifiers is more heterogeneous than originally expected. Under O_2 limitation and anoxic conditions, *Nitrosomonas* and *Nitrobacter* are able to denitrify concomitantly producing nitric oxide, nitrous oxide, and dinitrogen (Freitag *et al*., 1987; Stüven *et al.,* 1992; Bock *et al.,* 1995). Moreover, *Nitrosomonas* has been shown to grow anaerobically with nitrogen

dioxide/dinitrogen tetraoxide (NO_2/N_2O_4) as oxygen donor instead of O_2 (Schmidt and Bock, 1997; 1998).

4.1. Biochemistry of Ammonia Oxidizers

Ammonia oxidizers derive their energy from the oxidation of ammonia to nitrite, which is catalyzed by two key enzymes, ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). AMO oxidizes NH_3 to the intermediate hydroxylamine (NH_2OH) (equation 3). This oxidation is subdivided into two parts (equations 1 and 2). The exergonic reduction of O_2 to water (equation 2) enables the endergonic ammonia oxidation (equation 1). Consequently, the oxidation of ammonia needs two exogenously supplied electrons (Wood, 1988).

$NH_3 + ½\ O_2$	$\rightarrow NH_2OH$	$\Delta G_0' = +17$ kJ.mol^{-1}	(1)
$½\ O_2 + 2\ H^+ + 2e^-$	$\rightarrow H_2O$	$\Delta G_0' = -137$ kJ.mol^{-1}	(2)
$NH_3 + O_2 + 2\ H^+ + 2e^-$	$\rightarrow NH_2OH + H_2O$	$\Delta G_0' = -120$ kJ.mol^{-1}	*(3)

*The $\Delta G_0'$ values of the reaction were calculated using the assumption that the reducing equivalents for AMO are energetically near the ubiquinone level (+110 mV) (Schmidt and Bock, 1998).

Hydroxylamine is further oxidized by periplasmatic HAO to nitrite (NO_2^-) (see equation 4).

$NH_2OH + H_2O$	$\rightarrow HNO_2 + 4\ H^+ + 4e^-$	$\Delta G_0' = +23$ kJ.mol^{-1}	(4)
$½\ O_2 + 2\ H^+ + 2e^-$	$\rightarrow H_2O$	$\Delta G_0' = -137$ kJ.mol^{-1}	(5)
$NH_2OH + ½\ O_2$	$\rightarrow HNO_2 + 2\ H^+ + 2e^-$	$\Delta G_0' = -114$ kJ.mol^{-1}	(6)

This second oxidation is also composed of several parts in which enzyme bound nitroxyl (NOH) and nitric oxide (NO) are formed (Hooper and Terry, 1979; Hooper and Balny, 1982). NOH can be released as nitrous oxide (N_2O) and NO (Anderson, 1965; Anderson and Levine, 1986). Two electrons from hydroxylamine oxidation (equation 4) are transferred to ammonia oxidation (equation 3) and the other two electrons pass to the respiratory chain for energy generation (equation 5). The overall reaction shows that biogenic ammonia oxidation causes nitrous acid production (equation 7).

$NH_3 + 1½\ O_2$	$\rightarrow HNO_2 + H_2O$	$\Delta G_0' = -234$ kJ.mol^{-1}	(7)

Under anoxic conditions, *Nitrosomonas eutropha* can grow with nitrogen dioxide/dinitrogen tetraoxide (NO_2/N_2O_4) as oxygen source instead of O_2 for ammonia oxidation to hydroxylamine (Schmidt and Bock, 1997, 1998). Further, the isolated AMO can oxidize ammonia with NO_2 under anoxic conditions (Fiencke, unpublished data). In both cases, NO is formed. Based on these findings, it was postulated that NO_2/N_2O_4 might also be used as oxygen donor under oxic conditions and the produced NO reacts with O_2 to regenerate NO_2/N_2O_4 (Zart and Bock, 1998).

This speculative nitrogen-oxide cycle is supported by the formation of NO and NO_2/N_2O_4 during aerobic ammonia oxidation (Stüven and Bock, 2001).

Ammonia can be used directly as substrate or can made available by hydrolization of urea (Koops *et al.*, 1991). The capability of using urea as an ammonia source might be important for ammonia oxidizers in acid soils, where the actual ammonia concentration is low as a result of the low pH (de Boer and Kowalchuk, 2001).

4.1.1. Ammonia Monooxygenase (AMO)

Because AMO is an important enzyme of nitrification, efforts have been initiated to isolate it. However, AMO is not stable once isolated from the cells and many attempts to purify the enzyme have failed (Suzuki *et al.,* 1981; Ensign *et al*., 1993). Only recently have some attempts succeeded (Fiencke, unpublished data). Therefore, little is known about its structure and function. Most of the breakthroughs in our understanding of the molecular properties of AMO have been deduced from studies using intact cells of *Nitrosomonas europaea*. Using $^{18}O_2$, AMO was shown to be a monooxygenase (Dua *et al*., 1979; Hollocher *et al*., 1981). The K_m values and pH optima point to ammonia, rather than ammonium, as substrate (Drozd, 1976). The low K_m values for both ammonia (2-61 µM) (Koops and Pommerening-Röser, 2001) and O_2 (0.5-7.5 µM) (Laanbroek and Gerards, 1993) allow ammonia oxidation under low substrate concentrations.

AMO has a broad range of substrates. Besides ammonia, a huge number of compounds can be oxidized; these include carbon monoxide (CO) (Tsang and Suzuki, 1982), methane (CH_4) (Jones and Morita, 1983), alkyl and acryl (Hyman *et al*., 1988), halogenated (Vanelli *et al*., 1990), and aromatic hydrocarbons (Hyman *et al*., 1985). This conspicuously broad substrate range offers potential application for bioremediation of sites contaminated with chlorinated aliphatic hydrocarbons. Unfortunately, the oxidation of these alternative substrates cannot supply energy for growth. On the contrary, these substrates act as reversible inhibitors of AMO (Keener and Arp, 1993). The inhibition of AMO by nonpolar compounds points towards a nonpolar catalytic site on the enzyme.

In addition to the reversible inhibition, AMO can be inhibited irreversibly by either acetylene (Hynes and Knowles, 1982; Hyman and Wood, 1985) or trichloroethylene (Hyman *et al*., 1995). Acetylene is only inhibitory when AMO is catalytically active and high concentrations of ammonium protect the cells (Hyman and Arp, 1992). Therefore, it is assumed that acetylene is oxidized at the catalytic site. he oxygen reactive intermediates bind irreversible to AMO. When cells of *Nitrosomonas europaea* are incubated with radioactive acetylene ($^{14}C_2H_2$), AMO activity is lost and a membrane bound 27-kDa polypeptide, called AmoA, is labeled (Hyman and Wood, 1985). Therefore, AmoA is thought to contain the catalytic site for NH_3 oxidation (Hyman and Arp, 1992).

Other inhibitors of AMO are nitrapyrin (2-chloro-6-trichloromethyl pyridine) (Vannelli and Hooper, 1993) and chelating agents, like thiourea and allylthiourea (Hooper and Terry, 1973). Because these chelating agents preferentially bind

copper (Cu) and the ammonia-oxidizing activity of cell-free extracts increased in the presence of added Cu, it is suggested that Cu is a cofactor for AMO (Bédard and Knowles, 1989; Ensign *et al.,* 1993).

The AMO enzyme likely consists of three polypeptides. In addition AmoA, a second polypeptide, called AmoB, copurified with AmoA (McTavish *et al*., 1993). The evidence for AmoC, a third polypeptide of AMO, is indicated by the mRNA of AmoC being co-transcribed with the mRNA of AmoA (Sayavedra-Soto *et al*., 1998). None of the subunits have been purified in an active state yet but the corresponding genes, *amoA, amoB, amoC*, have been identified (McTavish *et al*., 1993; Bergmann and Hooper, 1994; Klotz *et al*., 1997). The gene *amoA* codes for a hydrophobic 32-kDa protein consisting of four-to-five transmembrane sequences (McTavish *et al*., 1993; Hooper *et al*., 1997). In the same operon and adjacent to *amoA* is *amoB*, which codes for a less hydrophobic 43-kDa protein consisting of only two-to-three transmembrane domains and a hydrophobic leader sequence (McTavish *et al*., 1993; Bergmann and Hooper, 1994). The *amoC* gene is located upstream of *amoA* and *amoB* and encodes a hydrophobic 31-kDa protein with six transmembrane domains (Klotz *et al*., 1997).

The number of copies of the *amo* operon may be genus specific. Two nearly identical copies are present in strains of both *Nitrosomonas* and *Nitrosovibrio* and three copies are found in strains of *Nitrosospira* and *Nitrosolobus* (Norton *et al*., 1996; 2002; Klotz and Norton, 1998; Sayavedra-Soto *et al*., 1998), whereas only a single copy could be detected in marine *Nitrosococcus* strains of the γ-subclass of *Proteobacteria* (Alzerreca *et al*., 1999). The proteins, AmoA, AmoB and AmoC, of the γ-ammonia oxidizers clearly differ from the AMO proteins of the β-ammonia oxidizers. There is only 37-43% amino-acid sequence similarity for the two groups, whereas the amino-acid sequences of AMO proteins of the β-ammonia oxidizers are 73-85 % similar (Alzerreca *et al*., 1999).

Polyclonal antibodies have been developed against AmoA and AmoB (Pinck *et al*., 2001; Fiencke, unpublished data). Quantitative immunoblot analysis, using polyclonal antibodies against AmoB, revealed that total cell protein of *Nitrosomonas eutropha* consisted of *ca*. 6% AmoB, when cells were grown under standard conditions (Pinck *et al*., 2001). The specific amount of AMO in cells of *Nitrosomonas eutropha* was regulated by the ammonium concentration. At high ammonium concentrations, less AMO was found than under ammonium-limiting conditions. Furthermore, AMO seems to be strongly protected from degradation. Cells that were starved for one year for ammonia still contained high amounts of AMO, although they showed far less ammonia-oxidation activity than growing cells. Hence, the amount of AMO does not directly correlate with the activity of ammonia oxidation.

AMO is similar to particulate methane monooxygenase (pMMO) in the methane-oxidizing bacteria in its putative subunit composition, the inhibitor profiles, the broad substrate range, and the DNA sequences of the genes encoding the proteins (Bédard and Knowles, 1989; Semrau et al., 1995). It is postulated that AMO and pMMO are phylogenetically related and have evolved divergently in correlation with their different physiological roles (Klotz and Norton, 1998). Recently, a third

member of this class of monooxygenases, one which oxidizes butane, was recognized (Hamamura *et al*., 2001).

4.1.2. Hydroxylamine Oxidoreductase (HAO)

In contrast to AMO, HAO has been isolated and characterized. This second key enzyme of ammonia oxidation is located in the periplasmic space, but might be anchored in the cytoplasmic membrane (Olson and Hooper, 1983; McTavish *et al*., 1995). HAO is a multi-heme enzyme, a complex homodimer or homotrimer of identical 63-kDa subunits, which contain seven c-type hemes and a novel heme, P460, per monomer (Arciero and Hooper, 1993; Sayavedra-Soto *et al*., 1994; Iverson *et al*., 1998; Hendrich *et al*., 2001). The P460 heme is probably the catalytic site of hydroxylamine oxidation (Hooper *et al*., 1983; Arciero *et al*., 1993). HAO constitutes *ca*. 40% of the c-type heme of *Nitrosomonas europaea* (DiSpirito *et al*., 1985a). The crystal structure of HAO revealed the orientation of the hemes in each subunit and suggested potential pathways of electron flow through the enzyme (Igarashi *et al*., 1997). The oxidation of hydroxylamine to nitrite was postulated to be a two-step reaction with enzyme-bound nitroxyl (HNO) and NO as intermediates (Andersson and Hooper, 1983).

The detailed coupling between ammonia oxidation and hydroxylamine oxidation is not established. Addition of hydroxylamine to ammonia-oxidizing cells in the lag-phase increases the growth yield, however, all attempts to grow ammonia oxidizers on hydroxylamine alone have failed (Nicholas and Jones, 1960; Böttcher and Koops, 1994). The toxicity of hydroxylamine in the absence of ammonia might be due to a surplus of electrons at the AMO under these conditions. With O_2 present, this surplus at the AMO might cause the formation of peroxynitrite (ONO_2), which damages the cells. This suicidal activity of ammonia oxidizers might also cause nitrification breakdown in waste-water treatment plants if plenty of organics is available as an additional electron donor.

The four electrons originating from the oxidation of hydroxylamine are probably channeled from HAO to cytochrome c_{554}, a 26-kDa tetraheme protein (Figure 2) (Andersson *et al*., 1986; Arciero *et al*., 1991; Bergmann *et al*., 1994). Cytochrome c_{554} is a positively charged (pI 10.7) periplasmic enzyme, which can bind to the negatively charged membrane (Yamanaka and Shinra, 1974; McTavish *et al*., 1995). This two-electron carrier might form ionic complexes with negatively charged electron donors and acceptors, like HAO (pI 3.4) (Iverson *et al*., 1998) and the 10-kDa monoheme cytochrome c_{552} (pI 3.7) (Fujiwara *et al*, 1995). Cytochrome c_{554} represents a branching point in the electron-transport chain. Two of the four electrons coming from HAO are passed through cytochrome c_{554}, the membrane-associated tetraheme cytochrome c_{m552} (Cyt c_B), and then the ubiquinone-cytochrome bc_1 complex to AMO (Wood, 1986; Whittaker et al., 2000). In addition to tri- and tetramethylhydroquinone, both hydroxylamine and NADH could be probable electron donors to AMO (Suzuki *et al*., 1981; Shears and Wood, 1986). The remaining electrons from HAO are channeled through cytochrome c_{554} *via* cytochrome c_{552} to either the terminal cytochrome oxidase aa_3 (DiSpirito *et al*.,

1986) or the periplasmic nitrite reductase (Miller and Nicholas, 1985). On the electron pathway, the ubiquinone-cytochrome bc_1 complex might be a mediator between cytochrome c_{554} and cytochrome c_{552} (Wood, 1986). Ubiquinone and membrane-bound cytochromes of the b and c types were detected in *Nitrosomonas europaea* (DiSpirito *et al*., 1985 a; Whittaker *et al*., 2000) and other electron carriers have been identified whose functions are unknown (Hooper *et al*., 1997; Whittaker *et al*., 2000).

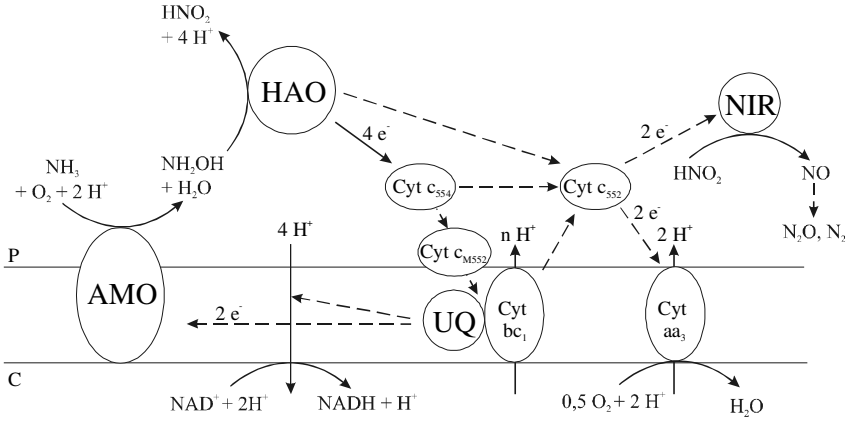

Figure 2. Model of electron transport chain of Nitrosomonas europaea modified from Whittaker et al. (2000) and Poughon et al. (2001).
AMO = ammonia monooxygenase; C = cytoplasmic side of the membrane; Cyt = cytochrome; HAO = hydroxylamine oxidoreductase; NIR = nitrite reductase; P = periplasmic side of the membrane; UQ = ubiquinone.

The first step of ammonia oxidation to hydroxylamine by AMO cannot be used for energy conservation. Thus, hydroxylamine is the real energy-conserving substrate. The oxidation of hydroxylamine to nitrite builds up a proton gradient across the cytoplasmic membrane (Hollocher *et al*., 1982; Kumar and Nicholas, 1982). The gradient results in a proton motive force, which drives the synthesis of ATP (Drozd, 1976). The proton/oxygen (H^+/O) ratio depends on the substrate. The ratios are 3.4 for ammonia oxidation and 4.4 for hydroxylamine oxidation (Hollocher *et al*., 1982). A portion of the electrons are used for the reduction of pyridine nucleotides (NAD(P)), which are necessary for carbon-dioxide fixation. Reducing power in form of NAD(P)H might be formed by reversed electron transport, using energy from the proton motive force (Aleem, 1966) (Figure 2). Fifty percent of the electrons circulating in the chain are used to oxidize ammonia, only 6% are involved in the reverse electron chain, and 44% reach the terminal oxidase (Poughon *et al*., 2001).

In addition to the above-mentioned reactions, ammonia oxidizers show denitrifying activity. Instead of O_2, nitrite can be used as the terminal electron acceptor (Anderson *et al*., 1993; Bock *et al*, 1995). Under these conditions, NO,

N_2O, and N_2 are produced (Poth and Focht, 1985). The nitrite reductase is induced under O_2 limitation (DiSpirito *et al.*, 1985 b; Miller and Nicholas, 1985). Neither an NO- nor a N_2O-reductase has been identified yet (Hooper *et al.*, 1997) although, in the genome of *Nitrosomonas europaea*, a probable gene for NO-reductase was detected (Whittaker *et al.*, 2000). Recently, anaerobic growth of pure cultures of *Nitrosomonas eutropha* with H_2 and pyruvate as energy source and nitrite as terminal electron acceptor has been described (Bock *et al.,* 1995).

Most studies have focused on *Nitrosomonas europaea*, which possesses a genome size of 2.8 Mbp, within which the genes of the electron-transport chain were identified (Chain *et al.*, 2003). These genes are frequently found in similar or identical copies, like the genes for AMO. In the genome, three nearly identical copies of genes for HAO (*hao*) were found (McTavish *et al.*, 1993; Hommes *et al.*, 2001). There is a copy of the gene that codes for cytochrome c_{554} (*cycA* or *hcy*) that is located downstream of each copy of *hao* (Sayavedra-Soto *et al.*, 1994; Hommes *et al.*, 1996). The membrane-bound tetraheme c-type cytochrome c_{m552} is encoded by genes contiguous with two of the three copies of *cycA* (Bergmann *et al.*, 1994). The genes for cytochrome c_{m552} are co-transcribed with the genes for cytochrome c_{554}.

4.2. Biochemistry of Nitrite Oxidizers

Nitrite oxidizers derive their energy from the oxidation of nitrite to nitrate (equation 8). Only a single step is involved in this oxidation, which is carried out by the enzyme nitrite oxidoreductase in *Nitrobacter*. In the genera *Nitrococcus, Nitrospina* and *Nitrospira*, the key enzyme is called the nitrite-oxidizing system. For the oxidation of nitrite, no O_2 is consumed because the oxygen atom in the nitrate molecule is derived from water. The two electrons released during oxidation are transported *via* cytochromes to O_2 for energy conservation (equation 9). There is no acid production when nitrite is oxidized to nitrate (equation 10).

$NO_2^- + H_2O$	$\rightarrow NO_3^- + 2\,H^+ + 2e^-$	$\Delta G_0' = +83$ kJ.mol^{-1}	(8)
$\frac{1}{2}\,O_2 + 2\,H^+ + 2e^-$	$\rightarrow H_2O$	$\Delta G_0' = -157$ kJ.mol^{-1}	(9)
$NO_2^- + \frac{1}{2}\,O_2$	$\rightarrow NO_3^-$	$\Delta G_0' = -74$ kJ.mol^{-1}	*(10)

*The $\Delta G_0'$ values of the reactions were calculated under the assumption that the reducing equivalents for the nitrite oxidoreductase are energetically near cytochromes of the a- and c-type (+/- 0 mV).

4.2.1. Nitrite Oxidoreductase (NOR)

Most biochemical investigations of nitrite oxidation have been performed with members of the genus *Nitrobacter* and, thus, cannot be generalized for the other genera of nitrite oxidizers. An active form of the membrane-bound nitrite oxidoreductase of *Nitrobacter* was isolated and characterized (Meincke *et al.*, 1992). It consists of 2-3 subunits depending on the isolation procedure (Sundermeyer-Klinger *et al.*, 1984). A membrane-associated α-subunit, NorA (115-130 kDa), and β-subunit, NorB (65 kDa), can be solubilized by heat treatment. An additional

protein with a molecular mass of 32 kDa co-purified with the α- and β-subunits when using sodium deoxycholate and subsequent isolation by sucrose (Sundermeyer-Klinger *et al*., 1984). This protein was described as the γ-subunit of nitrite oxidoreductase. Cytochromes of the a- and c-type were present when the enzyme was solubilized with Triton X-100 and purified by ion-exchange and size-exclusion chromatography (Tanaka et al., 1983). This purified nitrite oxidoreductase was composed of the three subunits of 55, 29, and 19 kDa. All preparations of NOR contained both molybdenum and iron-sulfur clusters (Meincke *et al*., 1992). Molybdenum is essential for nitrite oxidation and occurs in form of molybdopterin.

NorA might contain the catalytic site of NOR and NorB might function as an electron-channeling protein between NorA and the membrane-integrated electron-transport chain. Using three monoclonal antibodies recognizing NorA and NorB of *Nitrobacter*, this key enzyme was found to be homologous in all four known species of *Nitrobacter* (Aamand *et al*., 1996). Furthermore, the antibodies detected the NorB of the genera *Nitrococcus, Nitrospina* and *Nitrospira* (Bartosch *et al*., 1999). The molecular masses of NorB of *Nitrobacter* and *Nitrococcus* were identical (65 kDa), whereas NorB of both *Nitrospina* (48 kDa) and *Nitrospira* (46 kDa) differed significantly.

The nitrite-oxidizing enzyme occurs as characteristic membrane-associated two-dimensional crystals in all nitrite oxidizers. In *Nitrobacter,* these regularly arranged particles, which appear as dimers with a size of 8-10 nm, are located at the inner surface of the cytoplasmic and intra-cytoplasmic membranes (Sundermeyer-Klinger *et al*., 1984; Meincke et al., 1992; Spieck et al., 1996a). The integrity of a structure that extends between two neighboring particles was assumed to be necessary for conservation of activity (Tsien and Laudelout, 1968). The molecular weight of a single particle was 186 kDa suggesting that it represents a αβ-heterodimer (Spieck *et al*., 1996a). The location of the particles correlated with immuno-gold labeling of the key enzymes, using antibodies against the NorA and NorB (Spieck *et al*., 1996b). As in *Nitrobacter*, the nitrite-oxidizing system of *Nitrococcus* is located on the inner side of the cytoplasmic and intracytoplasmic membranes and the particles are arranged in rows (Watson and Waterbury, 1971). In cells of *Nitrospina* and *Nitrospira*, which do not possess intracytoplasmic membranes, the nitrite-oxidizing system was found in form of hexagonal particle patterns in the periplasmic space (Spieck et al., 1998). With regard to the location and molecular masses of the nitrite-oxidizing enzymes, those for the genera *Nitrobacter* and *Nitrococcus* are similar, whereas those for *Nitrospina* and *Nitrospira* are different and form a second coherent group reflecting the phylogeny of the genera.

The genes for both NorA and NorB (*norA* and *norB*) of *Nitrobacter hamburgensis* were identified and sequenced (Kirstein and Bock, 1993; Degrange, personal comm.). The genes cluster together with an additional open reading frame (*norX*) of unknown function in the order *norA*, *norX* and *norB*. Both subunits, NorA and NorB, show significant sequence similarities to several dissimilatory nitrate reductases of several chemoorganotrophic bacteria, *e.g*., of *Escherichia coli*. NorA is similar to the catalytic α-subunits and NorB is similar to the β-subunits of

dissimilatory nitrate reductases. NorB contains four cysteine clusters with striking homology to those of iron-sulfur centers of bacterial ferredoxins. Further, a close functional similarity between the nitrite oxidoreductase and dissimilatory nitrate reductases was suggested by Hochstein and Tomlinson (1988).

The pH optimum of the isolated enzyme was 8.0 and the K_m value for nitrite was 0.5-2.6 mM (Tanaka *et al*., 1983) and 3.6 mM (Sundermeyer-Klinger *et al*., 1984). Besides catalyzing nitrite oxidation to nitrate, the NOR of *Nitrobacter* can act as nitrate reductase under anoxic conditions and, therefore, also catalyze the reversible denitrifying process (Sundermeyer-Klinger *et al*., 1984). During this type of growth, nitrate can be used as the acceptor for electrons derived form organic compounds. For the reduction of nitrate, a K_m value of *ca*. 0.9 mM was calculated.

In *Nitrobacter,* the NOR concentration varies with growth conditions and the enzyme is inducible by either nitrite or nitrate (Bock *et al*., 1991). The nitrite-oxidizing activity of mixotrophically grown cells was higher than that of autotrophically grown cells (Milde and Bock, 1985). Although many strains of *Nitrobacter* are able to grow heterotrophically, growth is very inefficient and slow (Bock, 1976). During heterotrophic growth, NOR was repressed by more than 90% (Wiesche and Wenzel, 1998). Except for *Nitrobacter*, all other isolated nitrite oxidizers are obligate lithotrophs with nitrite serving as the only energy source. The K_m values for nitrite oxidation (15-270 µM NO_2^-) normally exceed nitrite concentrations in natural environments, where it rarely accumulates (Prosser, 1989). Because, at low pH, the oxidation of nitrite is inhibited by non-dissociated substrate (HNO_2), which is chemically unstable, nitrite enriches only in alkaline sites or when O_2 is limited.

The electrons from nitrite oxidation are transported to the electron-transport chain. They are probably channeled from the molybdopterin and the iron-sulphur clusters of NOR *via* cytochrome a_1 and c to the terminal cytochrome aa_3 oxidase (Figure 3; Hooper, 1989; Bock *et al*., 1991).

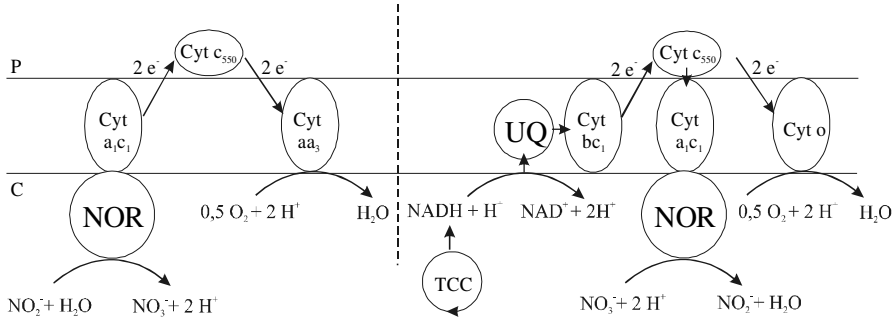

Figure 3. Model of electron-transport chain of Nitrobacter, with, on the left, lithotrophic growth with nitrite, and on the right, heterotrophic growth with organic substances. C = cytoplasmic side of the membrane; Cyt = cytochrome; NOR = nitrite oxidoreductase;P = periplasmic side of the membrane; TCC = tricarboxylic acid cycle; UQ = ubiquinone pool.

The reduction of cytochrome c is a thermodynamically unfavorable step because electrons derived from the high potential of the NO_3^-/NO_2^- redox couple (E_0' +420 mV) have to be transported to the low potential of the cytochrome c redox couple (E_0' +260 mV). A relatively high nitrite concentration would cause a lowering of the redox potential but, in natural habitats, high nitrite concentrations are rarely found. In fact, a highly active cytochrome aa_3 pushes nitrite oxidation by the removal of electrons from cytochrome c (O´Kelley *et al*., 1970). At constant NOR content, nitrite-oxidation activity is regulated by the concentration of cytochrome aa_3. In contrast to the mitochondrial terminal oxidase, purified terminal oxidase aa_3 consists of two subunits with molecular masses of 40 and 27 kDa and contains magnesium but no zinc (Yamanaka *et al*., 1981). Cells of *Nitrobacter* seem to possess different terminal oxidases depending on the growth conditions. During nitrite oxidation, cytochrome aa_3 was active, whereas a b-type cytochrome was used as terminal oxidase for heterotrophic growth (Kirstein *et al*., 1986).

Nitrite oxidation has been reconstituted in proteoliposomes using isolated nitrite-oxidoreductase, cytochrome c oxidase, and nitrite as substrate. O_2 was consumed in the presence of membrane-bound cytochrome c-550 (Nomoto *et al*., 1993). The electron-transport chain may differ between the genera because both *Nitrobacter* and *Nitrococcus* are rich in cytochromes c and a, whereas the other two genera *Nitrospina* and *Nitrospira* lack cytochromes of the a-type (Watson *et al*., 1986; Ehrich *et al*., 1995).

On heterotrophic growth of *Nitrobacter*, electrons pass from NADH via flavine mononucleotide and ubiquinone to a cytochrome bc_1-complex and finally to either the terminal oxidase with O_2 as electron acceptor or to the nitrate-reducing NOR with nitrate as electron acceptor (Figure 3; Kirstein *et al*., 1986). Under certain conditions, NOR co-purifies with a nitrite reductase (NiR), which reduces nitrite to nitric oxide (Ahlers *et al*., 1990). Because the activity of NiR is enhanced when the O_2 partial pressure is low, the enzyme is involved in denitrification. However, ATP generation has not been detected during nitrite reduction, which may, therefore, function as nitrite detoxification (Freitag and Bock, 1990).

Generation of membrane potential probably occurs for ATP synthesis and the reduction of pyridine nucleotides (NAD). However, the mechanisms of energy conservation are still unclear because proton translocation linked to the electron-transport chain has not been demonstrated yet (Hollocher *et al*., 1982). A purified ATPase from *Nitrobacter* has been characterized (Hara *et al*., 1991), but the primary energy product is NADH (Sundermeyer and Bock, 1981), which is used for ATP synthesis (Freitag and Bock, 1990). However, up to now, it is not clear how energy conservation occurs because the postulated reverse electron flow for the generation of NADH has not been confirmed. The reduction of cytochrome is thermodynamically unfavorable because of the high potential of the NO_3^-/NO_2^- redox couple, so the energy charge is extremely low (0.37) (Eigener, 1975). The low energy generation in *Nitrobacter* may be compensated by the large amount of nitrite oxidoreductase, which can represent 10-30% of total cell protein (Bock *et al*., 1991).

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