

Chapter 2

Nitric Oxide is a Bioproduct in Prokaryotes

2.1 Prokaryotes are NO Producer Organisms

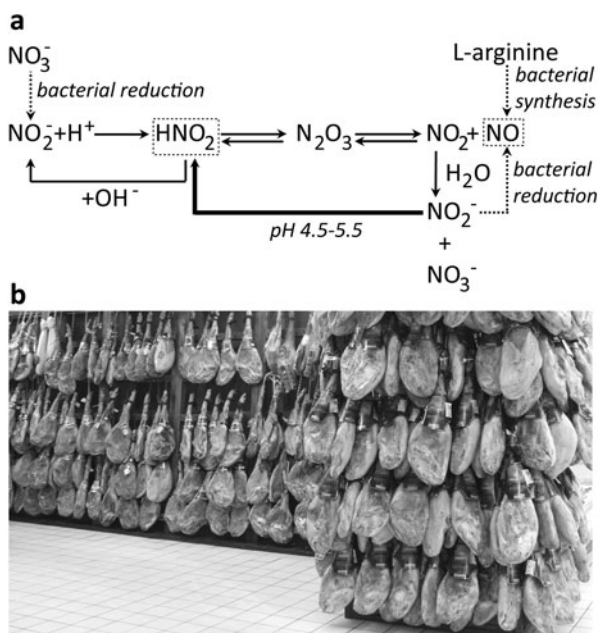
It was known since the 1960's that various denitrifying bacteria generate NO as an intermediate product of their dissimilatory NO_3^- metabolism (Barbaree and Payne 1967; Payne et al. 1971; Balderston et al. 1976). Today several prokaryote species are known as NO producers; many of them synthesize NO by reduction of NO_2^- , while others contain NOS-like enzymes and show oxidative NO generation from L-arginine or N ω -hydroxy-L-arginine (Zumft 1993; Sudhamsu and Crane 2009; Crane et al. 2010). Various ecological niches house these NO producer prokaryotes: marine environments (Baumann et al. 1983; Romanenko et al. 2005; Weon et al. 2006; Marinho et al. 2009; Santos et al. 2010), poorly ventilated or flooded soils (Zumft 1997), contaminated and eutrophized waters (Shapleigh 2006; Kampschreur et al. 2007, 2008), fermented meat or milk products (Morita et al. 1997; Gündoğdu et al. 2006; Gotterup et al. 2007) and the surface of mucosal barriers (Salzman 1995; Cuzzolin et al. 1997) are all colonized by NO synthesizing bacteria.

Better understanding of prokaryote NO production has agricultural, biotechnological and medical impact. Importantly, mitochondria and chloroplasts preserved some key features of prokaryote NO synthesis, giving an evolutionary vista to the NO synthesizing prokaryote world. Although NO was recognized as a bacterial bioproduct much before the discovery of the physiological impact of NO in mammals, the importance of bacterial NO synthesis gained attention only in very recent years (Sudhamsu and Crane 2009).

2.2 Bacteria Synthesize NO and Contribute to Chemical NO Release from Nitrogen Oxides

Bacteria may produce NO endogenously, but their ability to metabolize nitrogen oxides also contributes to abiotic NO release in their surroundings. Of biotechnological impact, the fermentation process of milk products or cured meat involves both of these two distinct mechanisms: NO can be synthesized by the bacteria, or the

Fig. 2.1 Chemical and bacterial NO liberation in the fermentation process. *Top:* Meat fermentation starts with the addition of NO_3^- and NO_2^- containing salts, which at the prevailing pH of the meat are chemically transformed to nitrous acid (HNO_2) and NO. Bacterial reduction of NO_2^- or oxidation of L-arginine also contributes to the NO production. In fermented milk products, the bacterial NO synthesis is more prominent than the chemical NO release. In meat, the generated HNO_2 has an antimicrobial effect, while NO forms a complex with myoglobin and develops the characteristic red color of cured meat. *Bottom:* Iberian ham is an example of cured meat



bacterial NO_3^- metabolism can facilitate chemical NO release from nitrogen oxides (Fig. 2.1).

Chemical NO release occurs during the meat curing process, when NO_3^- and NO_2^- containing salts are added to the meat (Møller et al. 2003). This treatment leads to the replacement of a predominantly Gram-negative flora of aerobic saprophytes by Gram-positive bacteria of the lactic acid group, which convert NO_3^- to NO_2^- (Gündoğdu et al. 2006). In the slightly acidic environment of the meat (pH 5.5–6.5) NO_2^- forms nitrous acid (HNO_2) and NO (Shank et al. 1962). As a result NO establishes a pentacoordinate complex with the hem-group of meat myoglobin, producing nitrosylmyoglobin, the stable red pigment of cured meat (Møller et al. 2003). The presence of nitrogen oxides (especially HNO_2) in the meat also exerts a bacteriostatic effect, which determines the quality of the bacterial flora and is required for the proper curing and preservation of the meat (Hu et al. 2007). In this process, the bacterial activity only provides a substrate (NO_2^-) supply and maintains the appropriate conditions (e.g. pH and O_2 saturation) for chemical NO elaboration (Fig. 2.1).

However, various isolates of lactic acid bacteria from fermented milk and meat products also produce NO enzymatically and thereby are capable of converting myoglobin to nitrosylmyoglobin (Møller et al. 2003; Gündoğdu et al. 2006). The most important NO synthesizing bacteria in fermented products are *Pediococcus acidilactici* S2, *Pediococcus acidilactici* S3, *Lactobacillus plantarum* T119, and *Lactobacillus fermentum* (Møller et al. 2003; Gündoğdu et al. 2006; Hu et al. 2007).

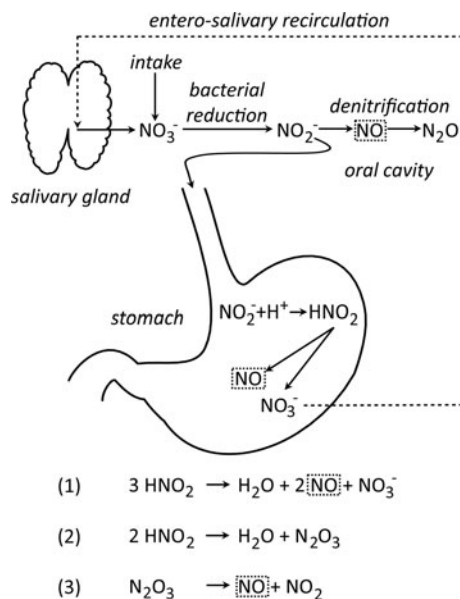
Similarly, various isolates of cured meat-associated staphylococci are also NO producers (Gotterup et al. 2007). These bacteria elaborate NO as a byproduct of their dissimilatory NO_3^- metabolism, by reducing NO_2^- . Interestingly, *Lactobacillus fermentum* produces NO in the absence of NO_2^- or NO_3^- , using L-arginine as a substrate (Khouw and McCurdy 1969). In this way, NO also occurs as a bacterial product in the fermentation process (Fig. 2.1).

This combined chemical and bacterial NO generation also has physiological relevance, as it has been pointed out in the antimicrobial defense mechanism of the mammalian stomach. Physiologically the salivary glands secrete NO_3^- (derived from the dietary NO_3^- of green vegetables), which is concentrated in the saliva. Although NO_2^- is not secreted by the saliva, it is measurable in the oral cavity (Fritsch et al. 1985). This NO_2^- is a product of bacterial reduction of dietary NO_3^- . This $\text{NO}_3^-/\text{NO}_2^-$ reduction is missing from germ free rats, and reduced by the administration of wide-spectrum antibiotics in humans (Dougall et al. 1995), confirming that microbial activity is responsible for NO_2^- production (Duncan et al. 1995). The posterior dorsal surface of the tongue (dorsum linguae) harbours large numbers of facultative anaerobic bacteria (*Staphylococcus spp.*, *Streptococcus spp.*, *Rothia spp.*, *Veillonella spp.*) which reduce NO_3^- to NO_2^- under hypoxic conditions (Li et al. 1997; Doel et al. 2005). Various denitrifier bacteria of the oral cavity further reduce NO_2^- to NO and N_2O (Mitsui and Kondo 1998; Mitsui and Kondo 1999; Bayindir et al. 2005). These nitrogen oxides display antimicrobial activity; however, the more potent microbicidal effect evolves when the NO_2^- enriched saliva reaches the stomach (Benjamin et al. 1994; Duncan et al. 1995; McKnight et al. 1997). In the acidic environment of the stomach NO_2^- is being protonated and forms various nitrogen oxides (dominantly NO), which act as antimicrobial agents and facilitate the elimination of pathogens ingested with the food (Benjamin et al. 1994; McKnight et al. 1997) (Fig. 2.2). For example *Candida albicans* and *Escherichia coli* are much more susceptible to the combined effect of nitrogen oxides and gastric acid than the acidic destruction alone (Benjamin et al. 1994). High NO_2^- intake can lead to NO-evoked injury of the stomach epithelia at the esophageal-gastric junction, underscoring the role of salivary NO_2^- levels in the control of intragastric chemical NO release (Asanuma et al. 2005) (Fig. 2.2).

2.3 NO-Generating Microbes: Health, Biotechnological and Ecological Impact

Denitrifying bacteria, which generate NO as a byproduct are present in various mucosal barriers (e.g. in the airways and the upper alimentary tract) (Salzman 1995) and in the dental plaques (Bayindir et al. 2005). Under pathological conditions NO releasing bacteria are abundant in infected areas of mucosal layers (Genc et al. 2006). Importantly, NO production increases the antibiotic resistance and stress adaptation of certain pathogenic bacteria (Gusarov et al. 2009). However, we are still far from definitely understanding the physiological and pathological roles of

Fig. 2.2 Combined bacterial and chemical NO generation in the upper digestive system. NO_3^- is either secreted by the saliva or derived directly from dietary uptake. Bacterial reduction of NO_3^- to NO_2^- or NO and N_2O takes place in the oral cavity. Within the acidic environment of the stomach NO_2^- forms nitrogen oxides—including NO—by reactions (1–3), providing an antimicrobial defense mechanism. The acidic pH of the stomach also favors NO_3^- generation, which is absorbed and then concentrated again in the saliva (Fritsch et al. 1985). This entero-salivary pathway (Duncan et al. 1995) recirculates approximately 25% of the dietary NO_3^-



microbial NO synthesis. It is already known that NO synthesis by the gingival eNOS and iNOS has a protective role in the oral mucosa, since NO is an important agent against *Porphyromonas gingivalis*, the main periodontal pathogen (Gyurko et al. 2003; Skaleric et al. 2006). Consequently, altered NO homeostasis in the oral mucosa is accompanied with periodontal disease (Ohashi et al. 1999; de Sa Siqueira et al. 2010; Parwani et al. 2011) and inhibition of eNOS (Sun et al. 2010). Although the iNOS expression is increased in the *P. gingivalis* infected mucosa (Sun et al. 2010), the salivary NO_2^- level is lower in patients with periodontitis than in healthy subjects (Aurer et al. 2001). This may reflect the increased NO_2^- consumption of bacterial communities of the oral cavity, although the impact of the microbial NO emission in this pathology is still elusive.

Recently, the relevance of bacterial NO emission in industrial biotechnology has also been defined, showing that it impacts the biological degradation of waste materials (Kampschreur et al. 2008) and the production of renewable fuels (Ahmed and Lewis 2007). In wastewater treatment systems, ammonia-oxidizing (nitrosifying), NO_2^- oxidizing (nitrifying) and denitrifying bacteria produce NO and NO_2^- availability and O_2 limitation generally favors their NO emission (Stüven and Bock 2001; Kampschreur et al. 2007, 2008, 2009) (Fig. 2.3). The negative effects on some biodegradation processes from bacterial generation of NO have been shown (Tas and Pavlostathis 2008; Okutman Tas and Pavlostathis 2010). Microbial conversion of biomass-generated synthesis gas to ethanol, a candidate renewable fuel, is also affected by NO production of microbes (Ahmed and Lewis 2007).

Soil bacteria are also NO producers: in acidic forest soil (pH 4.0) denitrifying bacteria are responsible for NO release (Krämer and Conrad 1991). In the slightly alkaline agricultural soil (pH 7.8) reduced ventilation (low O_2 availability) and soil



Fig. 2.3 Examples of ecological niches occupied by NO-synthesizing bacteria. Various NO producer prokaryotes are present in wastewater treatment systems, contaminated and eutrophized waters, cultivated agricultural soils, sandstones, poorly ventilated or flooded soils. NO may be generated in the major conversions of the biogeochemical nitrogen cycle: denitrification, nitrification and ammonium oxidation. Some N_2 -fixing bacteria and cyanobacteria under hypoxia also reduce NO_2^- to NO and NOS-containing bacteria may generate NO by L-arginine oxidation. Local NO concentrations in bacterial communities suggests that NO is an important bioactive compound in natural environments. The production and consumption of NO is shared by separate regions of stratified microbial communities. (Schreiber et al. 2008)

fertilization (excess NO_3^- and NO_2^-) favors NO generation (Krämer and Conrad 1991) by both denitrifier and NO_2^- reducing nitrifier bacteria (Remde and Conrad 1991; Martinez-Espinosa et al. 2011) (Fig. 2.3). Bacterial NO production is also involved in the establishment of symbiotic interaction between rhizobiont bacteria and plant roots (Del Giudice et al. 2011). Soil bacteria with the ability to reduce NO_2^-

to NO have been detected even at 6000 m heights of the Himalayas (Henry et al. 2006). Anaerobic microniches within the generally aerobic uppermost soil layer (0.05–0.1 m) are the sites of this reductive NO synthesis (Remde et al. 1993). Bacterial NO_2^-/NO reduction therefore affects NO_2^- content and thereby determines the quality of the upper soil layer (Martinez-Espinosa et al. 2011). Moreover, bacterial NO emission has more general environmental impact since NO is an air pollutant gas (Jaegle et al. 2004; Martinez-Espinosa et al. 2011). Its release from the soil shows that seasonal periodicity and environmental factors (e.g. rain) affect the rate of NO emissions (Jaegle et al. 2004). Of global importance, rainforest soils exhibit high NO emission rates. For example, the total NO_x emission of the soils in tropical Africa is $\sim 3 \text{ TgN}$ ($10^{12} \text{ g biomass}$)/year, although the majority of NO_x is scavenged and consumed within this ecosystem (Jaegle et al. 2004).

Interestingly, NO producing bacteria may grow in the soil around buildings and may also colonize corroding surfaces of buildings or sandstones of historical monuments in air polluted regions (Bock 1987; Meincke et al. 1989) (Fig. 2.3). These bacteria metabolize nitrogen compounds derived from the contaminated air and the generated NO is released to the atmosphere ($\sim 0.4\text{--}4 \text{ ng/h/g}$) (Baumgärtner et al. 1990) or forms nitric acid and leads to the corrosion of the mineral content of the sandstones (Meincke et al. 1989). Although the growth of these bacteria is rather slow, their endolithic NO production may have impact on the deterioration of our technical environment (Sand and Bock 1991) and at a greater time scale on the preservation of archeological objects (McNamara et al. 2006).

2.4 Mechanisms of Reductive NO Synthesis in Prokaryotes

2.4.1 Denitrifying Bacteria Reduce NO_2^- to NO: NO Synthesis in Anaerobiosis

Denitrification is a form of dissimilatory NO_3^- metabolism (Fig. 2.4), in which NO_3^- is being sequentially reduced to NO_2^- , NO, nitrous oxide (N_2O) and dinitrogen (N_2) by four nitrogen oxide reductases (NO_3^- , NO_2^- , NO, and N_2O reductases, respectively) (Zumft 1997). Among prokaryotes, the denitrifying organisms are mostly facultative anaerobic, chemolithotrophic, phototrophic, diazotrophic or organotrophic bacteria and archaea¹ (Zumft 1997). Denitrification allows anaerobic nitrate-respiration and may also be a mechanism to dispose of excess reducing equivalents and modulate intracellular redox state (Shapleigh 2006).

The ability of NO production by a prokaryote was first shown in the marine denitrifying bacterium *Pseudomonas perfectomarinus* (Barbaree and Payne 1967) [its taxonomic name has been revised and corrected to *Pseudomonas perfectomarina*

¹ Mitochondria of fungi also contain denitrifying enzymes.

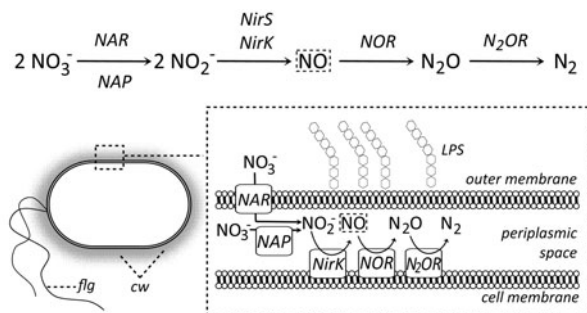


Fig. 2.4 Generation of NO by the denitrification system. Denitrification is a form of dissimilatory NO_3^- metabolism, in which NO_3^- is being sequentially reduced to NO_2^- , NO, N_2O and N_2 by four nitrogen oxide reductases. NAR: NO_3^- -reductase, NAP: periplasmic NO_3^- -reductase, NirK, NirS: dissimilatory NO_2^- -reductases, NOR: NO-reductase, N_2OR : N_2O -reductase. In Gram-negative bacteria, these enzymes are associated with the cell membrane, the outer membrane and the periplasmic space. *flg* flagellum, *cw* cell wall, *LPS* lipopolysaccharide

(Baumann et al. 1983), and more recently, it is considered a member of the *Pseudomonas stutzeri* subgroup (Lalucat et al. 2006)]. This facultative anaerobe species is a Gram-negative, rod-shaped bacterium, first isolated from marine sediments (Zobell and Upham 1944). It belongs to the class of γ -proteobacteria within the phylum of proteobacteria (Lalucat et al. 2006). Under low oxygen tension and in the presence of NO_3^- or NO_2^- it is able to respire anaerobically by means of denitrification (Liu et al. 1983), and thereby produces NO. Several closely related *Pseudomonas* species are known from marine sediment [e.g. *P. stutzeri*, *P. nautica*, *P. doudoroffii* (Baumann et al. 1983)] although these species are non-denitrifiers (Baumann et al. 1983) thus they possibly lack the ability to generate NO. Recently, several novel denitrifying *Pseudomonas* species, (*P. balearica*, *P. migulae*, *P. brenneri*, *P. pohangensis*, *P. pachastrellae* and a close relative of *P. segetis*), have been described from aerated seawater, the seashore and in association with marine sponges (Romanenko et al. 2005; Weon et al. 2006; Gao et al. 2010), suggesting that NO-producing bacteria may be more widespread in the marine environment than it was previously expected.

In *Pseudomonas perfectomarina* (*stutzeri*), the NO synthesis is detectable in cells grown on NO_3^- or NO_2^- media under anoxic conditions, and NO liberation is associated with the NO_2^- reducing cell fraction (Payne et al. 1971). The responsible NO-generating enzyme is a membrane-bound NO_2^- reductase (NiR), which catalyzes the one-electron reduction of NO_2^- to NO (Balderston et al. 1976; Liu et al. 1983). The mechanism of NO generation by denitrification has been studied in various *Pseudomonas* species (Matsubara and Zumft 1982), the α -proteobacteria *Rhodobacter sphaeroides* and *Paracoccus denitrificans*, the β -proteobacteria *Achromobacter cycloclastes* and *Ralstonia eutropha* and some representatives of archaea (Risgaard-Petersen et al. 2006). In these species, NO occurs as an intermediate product of denitrification (Shapleigh 2008) and the reduction of NO_2^- to NO is catalyzed by hem-containing cytochrome-cd₁ NiR (NirS or type 1 NiR) or copper-containing

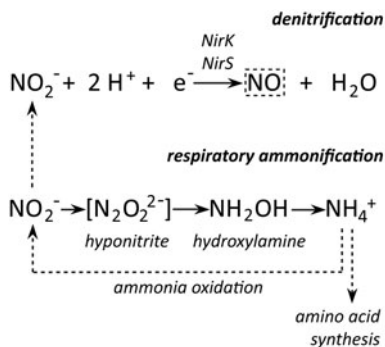


Fig. 2.5 Two distinct ways of NO_2^- reduction: denitrification and respiratory ammonification. Reductive NO synthesis occurs in the denitrification process, catalyzed by dissimilatory NO_2^- reductases (e.g. NirK or NirS). Similar NO_2^-/NO reduction may also occur in eukaryote cells (e.g. by a NirK-like mitochondrial enzyme in fungi). In respiratory ammonification, NO_2^- may undergo a six-electron reduction to NH_4^+ , catalyzed by cytochrome c NiR (NrfA). In certain bacteria, ammonia oxidation may generate NO_2^- which can be reduced by NirK to NO

NiR (NirK or type 2 NiR) (Liu et al. 1983; Coyne et al. 1990; Hallin and Lindgren 1999; Moreno-Vivian et al. 1999; Cabello et al. 2004). These NiRs are expressed in cells grown anaerobically under denitrifying conditions (in the presence of nitrogen oxides) and activity of NirK is inhibited by O_2 (Liu et al. 1983).

This form of reductive NO synthesis occurs in denitrifiers of hypoxic niches, such as marine sediments, flooded or waterlogged soils and the aerobic/anaerobic interface of eutrophized waters. In non-denitrifier bacteria, NO_2^- may undergo a six-electron reduction to ammonia catalyzed by cytochrome-c NiR (NrfA) (Simon 2002). This process is the so-called respiratory ammonification (Fig. 2.5), which takes place in the cytoplasm and is required for anabolic purposes (Simon 2002). Reduction of NO_2^- in respiratory ammonification fails to produce NO.

Subcellular Localization and Biological Effects of Reductive NO Synthesis Immunogold labeling with colloidal-gold probes has shown that bacterial NiRs are associated with the periplasmic space or with the cell membrane (Fig. 2.4) (Coyne et al. 1990). Accordingly, NO_3^- reductases (the membrane-bound NO_3^- reductase NAR and the periplasmic NO_3^- reductases NAP), moreover, NO-reductase (NOR) and nitrous oxide reductase (N_2OR)², are all enriched in the cell membrane of denitrifying bacteria (Balderston et al. 1976; Matsubara and Zumft 1982; Zumft and Frunzke 1982; Korner and Mayer 1992; Hendriks et al. 1998; Pohlmann et al. 2000; Richardson et al. 2001; Gonzalez et al. 2006; Hino et al. 2010; Simpson et al. 2010). The cell membrane with the periplasmic space thus represents the major subcellular pool of NO synthesis.

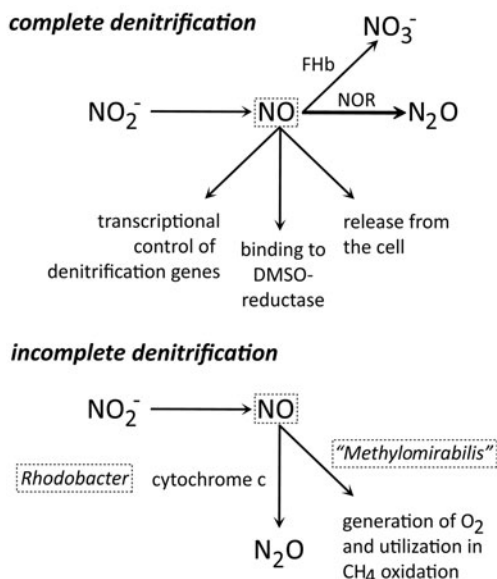
² The literature uses the "Nos" abbreviation to indicate nitrous oxide reductase. However we have reserved the "NOS" acronym for NO-synthase in this book. To avoid the confusion of these two distinct enzymes, we have applied the " N_2OR " abbreviation for nitrous oxide reductase.

Fig. 2.6 Possible fates of NO generated by denitrification

Top: In bacteria with a complete denitrification enzyme chain, the majority of NO is converted to N₂O (by NORs). NO may also increase the transcription of genes encoding denitrification enzymes, may bind to membrane proteins (e.g. DMSO-reductase) or may release from the cell.

Bacterial flavohemoglobins (Fhb) scavenge excess NO and convert it to NO₃⁻.

Bottom: In bacteria lacking NORs, NO may be reduced by cytochrome c or may be consumed for O₂ generation



Since NO occurs transiently as a byproduct of NO₃⁻ dissimilation, one can debate that NO would gain functional impact in denitrifier bacteria. Some studies however, suggest that the locally produced NO affects distinct functions of the cell membrane and the periplasmic space. For instance, in the denitrifier 2.4.3. strain of *Rhodobacter sphaeroides*, one possible target of NO is dimethyl sulfoxide (DMSO) reductase (Fig. 2.6). This enzyme is located in the periplasmic space (McEwan et al. 1991), near the site of reductive NO synthesis. DMSO reductase is a molybdenum (VI) containing enzyme, and functions as a terminal reductase in anaerobic respiration by using DMSO as a terminal electron acceptor. In *Rhodobacter*, the DMSO respiratory pathway has a role in redox homeostasis and it also enables the cells to utilize a variety of carbon sources during anaerobic growth (Kappler et al. 2002). It has been shown that NO carries out a one electron reduction of molybdenum in DMSO reductase and stabilizes molybdenum (V) without inhibiting enzyme activity (Bastian et al. 1995). It is possible that the molybdenum (V)-NO complex is a transition state analog of the enzyme-substrate complex, however the functional impact of NO on DMSO reductase still remains elusive (Bastian et al. 1995).

Another candidate function of reductive NO synthesis has been proposed in a recently discovered anaerobic methane-oxidizing bacterium, "*Candidatus Methyloirabilis oxyfera*" (Ettwig et al. 2010; Wu et al. 2011). This prokaryote reduces NO₂⁻ to NO by its denitrifying system, however NO is not converted further to N₂O, instead a yet unidentified enzyme metabolizes NO to produce O₂ and N₂ (Ettwig et al. 2010) (Fig. 2.6). The O₂ produced in this unique "bypassed" denitrification system is utilized in methane (CH₄) oxidation (Ettwig et al. 2010; Wu et al. 2011). Enzymes of the methane oxidation pathway are associated with intracellular membranes and the periplasm (Brantner et al. 2002), thus the periplasmic NO pool may be consumed

locally. In this organism, anaerobic NO synthesis thereby provides an endogenous O₂ supply for CH₄ oxidation (Wu et al. 2011). The finding that an anaerobic bacterium is capable of producing O₂ from NO, using NO₂⁻ as a substrate opens the possibility that O₂ may be generated by other means in addition to the canonical O₂-yielding pathways, i.e. photosynthesis, chlorate respiration and the conversion of reactive oxygen species (Ettwig et al. 2010).

Denitrifying enzymes and a set of proteins required for the assembly of the denitrification system are expressed in response to hypoxic shift and in the presence of nitrogen oxides (NO₃⁻, NO₂⁻, NO, N₂O) (Sabaty et al. 1999). It has been shown that among these nitrogen oxides, NO is a key signal, which acts at the level of gene expression of the denitrification system (Fig. 2.6). In denitrifying bacteria NO controls the transcription of genes encoding NiRs and NORs (e.g. *nirS/JF* and *norZ*, *qnorB*, *cnorB*, respectively). These NO-target genes are associated with various NO-responsive transcriptional regulators in proteobacteria. The NO-activated signal pathway in the genera *Pseudomonas*, *Paracoccus* and *Rhodobacter* involves regulators of the FNR (fumarate and nitrate reductase regulatory protein) family of transcription factors (Zumft 1993). In *Ralstonia eutropha* the sigma-54 dependent transcriptional regulator NorR controls NOR transcription in a NO-dependent manner (Pohlmann et al. 2000; Cramm et al. 2006).

How Denitrifiers Avoid and Benefit from NO Cytotoxicity The amount of NO produced by denitrification may reach the micromolar range, which can evoke nitrosative stress. However, denitrifier bacteria convert NO instantly to N₂O by NORs. This reduction step combines two NO molecules to N₂O and H₂O. The two reducing equivalents necessary for the two NO/N₂O conversions are delivered by Fe²⁺/Fe³⁺ transitions of the hem and non-hem iron of the NORs. In prokaryotes with incomplete denitrification systems, the NOR-like activity of cytochrome-c is responsible for reducing excess NO to N₂O (Cross et al. 2001) (Fig. 2.6). Increased NO₂⁻ and NO availability and anaerobic conditions increase the expression of NOR, which is the most effective NO-converting enzyme under anaerobiosis. Under aerobic or microaerophilic conditions, the bacterial flavohemoglobin (Fhb) is responsible for NO elimination. Fhb is a NO-dioxygenase, which converts NO and O₂ into NO₃⁻ with concomitant oxidation of Fe²⁺ to Fe³⁺ heme within the Fhb molecule (Gardner et al. 1998; Hausladen et al. 1998; Forrester et al. 2011). Bacteria expressing NORs and Fhb are thereby able to avoid NO-toxicity (Hausladen et al. 1998) (Fig. 2.6).

Denitrifiers also emit some amount of NO to their surroundings (Fig. 2.6), which may limit the growth of nondenitrifying bacteria in heterogenous bacterial communities (Choi et al. 2006). Bacterial growth is inhibited by NO, although the NO sensitivity shows species variation (Shank et al. 1962). Derivatives of NO (such as HNO₂) and NO itself may also kill bacteria (Shank et al. 1962; Cuzzolin et al. 1997). It is possible, that NO liberation of denitrifying prokaryotes provides an adaptive advantage by limiting the spread of competitor bacteria within the same niche. Various marine *Pseudomonas* species form biofilms and are associated with sponge species. In this bacterial-sponge symbiotic relationship, the *Pseudomonas* cells generate antimicrobial substances and provide an “immunological” defense mechanism

for the poriferan cell colony (Marinho et al. 2009; Santos et al. 2010). Denitrifier bacteria and their NO_2^-/NO converting activity have been found in the alimentary tract of earthworms (Matthies et al. 1999), where it contributes to the defense against microbes.

2.4.2 *An Apparent Paradox: Nitrogen Fixation and NO Synthesis by Denitrification may be Present in the Same Bacterium*

Interestingly, reduction of NO_2^- to NO may also occur in N_2 -fixing bacteria. Fixation of N_2 and denitrification are antagonistic processes; however reductive NO synthesis by means of denitrification can be present in rhizobiont N_2 fixing bacteria. For instance, *Azospirillum brasilense* contains a plasmid-encoded NirK (Petrova et al. 2010) and elaborates NO by the reduction of NO_2^- (Molina-Favero et al. 2008). A set of genes encoding denitrification enzymes has also been found in the bacterial plasmid DNA, although the main function of the denitrification system may solely be the production of NO in this bacterium (Petrova et al. 2010). It is possible that denitrification genes (including the NO producing NirK) spread with horizontal transfer among rhizospheric N_2 -fixing bacteria (Petrova et al. 2010). Since root development involves NO as a signal molecule in vascular plants (Chap. 3), the bacterial NO emission increases root branching and helps establish plant-bacteria rhizobial symbiosis in various legumen species (Molina-Favero et al. 2008; Del Giudice et al. 2011). The NO_2^- supply for reductive NO synthesis is maintained by the activity of both plant and bacterial NO_3^- -reductase (NR) in the legumen root nodule (Horchani et al. 2011). Interestingly, NR is also capable of reducing NO_2^- to NO (with $\sim 1\%$ effectivity) if the NO_3^- supply is limited and NO_2^- is available in excess (Shapiro 2005). In N_2 -fixing bacteria, the NO production has an important adaptive benefit since bacterial NO emission facilitates root development of the host plant and stabilizes rhizobial plant-bacteria symbiosis by forming root nodules (Del Giudice et al. 2011). Possibly, this is the only one example of NO-mediated intercellular communication in the bacterial world.

2.4.3 *Anaerobic Ammonia Oxidation (“anammox”) Also Generates NO*

Ammonia can also be oxidized anaerobically, by using NO_2^- as a terminal electron acceptor (van der Star et al. 2008; Martinez-Espinosa et al. 2011). Only the members of the Planctomycetales order are able to catalyze this unique process (van der Star et al. 2008), in which they combine two nitrogen compounds (NH_4^+ and NO_2^-) to generate N_2 . This is the so-called anammox process, which occurs in anoxic aquatic environments (Lam et al. 2009), including wastewater treatment systems (Kampschreur et al. 2009).

The presence of a gene encoding NirS (EMBL accession number CAJ74898) has been shown in the anammox bacterium “*Candidatus Kuenenia stuttgartiensis*” (Strous et al. 2006; Kartal et al. 2007). It means that similar to the denitrifiers and aerobic ammonia oxidizers, anammox bacteria reduce NO_2^- to NO by NiRS (Fig. 2.5). To date however, the role of NO production in anammox bacteria is unexplored. It is known that the anammox bacterium “*Candidatus Brocadia anammoxidans*” tolerates high NO doses which otherwise inhibit nitrogen metabolism in other NO-forming denitrifiers or aerobic ammonia oxidizers (Schmidt et al. 2002; Kartal et al. 2010). This suggests that anammox bacteria have a rather effective NO-detoxifying mechanism. Supporting this possibility, genes encoding NOR (reduces NO to the less toxic N_2O) and bacterial hemoglobin (sequesters NO) have been identified in “*Candidatus Kuenenia stuttgartiensis*” (Strous et al. 2006). A recent study also shows that NO has metabolic impact on these bacteria, since the excess NO is used for ammonia oxidation and N_2 production in a still hypothetical pathway (Kartal et al. 2010).

2.4.4 Aerobe Bacteria are Also Capable of Reducing NO_2^- to NO

Reductive NO Synthesis in Ammonia Oxidizing and Nitrite Oxidizing Bacteria Until recently, the non-denitrifying bacteria were considered unable to catalyze NO_2^-/NO reduction. Unexpectedly, some ammonia oxidizing (nitrifier) and NO_2^- oxidizing (nitrite oxidizer) bacteria show reductive NO synthesis, using NO_2^- as a substrate (Remde and Conrad 1990).

Representatives of these aerobic ammonia oxidizer bacteria are *Nitrosomonas europaea*, *Nitrosovibrio* spp. and *Nitrospira* spp. They colonize soil, various solid surfaces (e.g. walls of buildings) and aquatic environments and they are important players in mineralization (Meiklejohn 1950; Meincke et al. 1989; Remde and Conrad 1990). *Nitrosomonas europaea* is also used in bioremediation since it is capable of metabolizing various organic waste materials and—at least partially—this bacterium is responsible for NO emission of wastewater treatment systems (Stüven and Bock 2001). These nitrifying bacteria produce NO_2^- by oxidation of ammonia, and then reduce NO_2^- to NO by NiRs. A NiR protein has been purified from *Nitrosomonas europaea* (Ritchie and Nicholas 1974) and a NirK coding gene has also been identified within its genome (Beaumont et al. 2005). It is likely that NirK, the “classic” denitrifier enzyme is responsible for NO_2^-/NO conversion (Remde and Conrad 1990). Importantly, increasing the NO_2^- supply enhances the expression of NirK through a unique NO_2^- sensitive transcription factor (Beaumont et al. 2004). The ability of these bacteria to reduce NO_2^- to NO ensures the removal of toxic NO_2^- (product of their nitrification process) and increases their NO_2^- tolerance (Cantera and Stein 2007b). However, mutant *N. europaea* cells lacking NirK are still capable of NO_2^-/NO reduction (Beaumont et al. 2002), suggesting that as yet undefined alternative mechanisms may also be responsible for NO synthesis.

Aerobe nitrifier bacteria oxidize NO_2^- to NO_3^- and thereby contribute to the transformation of soil NO_2^- to NO_3^- , the most important nitrogen source for vascular

plants. The α -proteobacter *Nitrobacter winogradskyi* is a representative of these NO_2^- oxidizer prokaryotes. Recently, a putative NirK-encoding gene (Nwin_2648) has been identified within its genome (Starkenbourg et al. 2008). Low O_2 availability (0–4% O_2) and the presence of NO_2^- increase its transcription and the putative NirK can reduce NO_2^- to NO (Starkenbourg et al. 2008). Similar NirK-encoding sequences are known from other *Nitrosomonas* and *Nitrospira* isolates (Cantera and Stein 2007a). However, to date the possible function of the NO synthesis in these bacteria is unknown.

Recently ammonia-oxidizing archaea have been found in terrestrial and marine environments. The genome of two ammonia-oxidizer archaeon (*Nitrosopumilus maritimus* and *Cenarchaeum symbiosum*) also contains NirK homologs, suggesting that reductive NO synthesis can be widespread in aerobic microbes (Bartossek et al. 2010).

2.4.5 Reductive NO Synthesis Without NiRs: Cyanobacterial NO Production

Cyanobacterial hemoglobin (cyanoglobin) also reduces NO_2^- to NO under anoxia (Thorsteinsson et al. 1999; Sturms et al. 2011), thereby cyanobacteria are capable of producing NO in the lack of NiRs (Busch et al. 2002). Both NO_2^- or HNO_2 , which are in equilibrium under acidic conditions react with Fe^{2+} in the hem group of deoxyhemoglobin, which leads to the release of NO, as shown by eq. 2.1 (Sturms et al. 2011).



Since cyanoglobins are peripheral membrane proteins and they are components of the membrane-associated terminal cytochrome oxidase (Hill et al. 1996), the reductive NO generation is possibly restricted to the cell membrane.

Since NO_2^- is toxic for most microorganisms (Martinez-Espinosa et al. 2011), the primary function of cyanobacterial NO_2^- reduction is the efficient elimination of NO_2^- when this nitrogen oxide is abundant in the environment and the oxygen supply is limited (e.g. in eutrophized waters) (Sturms et al. 2011). The byproduct NO may exert some secondary biological functions. For instance, a heme NO/ O_2 binding protein has been identified in a *Nostoc* species, which has ~35% sequence identity and high structural homology to the beta subunit of soluble guanylyl cyclase and may function as a NO sensor in the cyanobacterium (Tsai et al. 2010). Moreover NO alleviates oxidative damage induced by UV-B irradiation in the cyanobacterium *Spirulina platensis* 794 strain (Xue et al. 2007). The administration of NO increases superoxide dismutase, catalase and glutathione levels and activities, consequently reducing superoxide concentration in the cell (Xue et al. 2007). However, it is still unknown if NO release from deoxy-cyanoglobin would be sufficient to evoke this antioxidant effect. The elimination of excess NO is catalyzed by NORs (Busch et al. 2002).

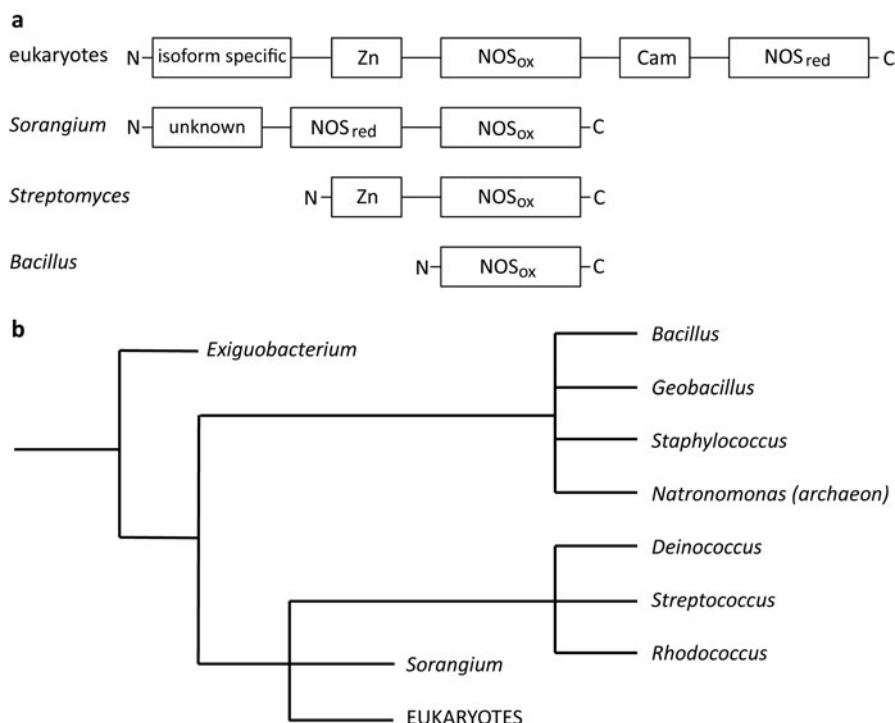


Fig. 2.7 Schematic representation of NOS domain structure and the possible phylogenetic tree of prokaryote NOS-encoding genes. Recent studies have identified open reading frames encoding homologs of the mammalian oxygenase NOS domain (NOS_{ox}) in the genome of several gram-positive bacteria. These bacterial NOS molecules show similarities to the eukaryote-type NOS_{ox}. Bacterial NOS molecules lack the covalently attached reductase domain (NOS_{red}), with the exception of the *Sorangium* NOS (**a**). Zn Zn-containing domain, Cam CaM-binding domain. A simplified phylogenetic tree of various prokaryote NOSs (Sudhamsu and Crane 2009) (**b**)

against human iNOS (Cohen and Yamasaki 2003). However, the known *Nocardia* genomes do not contain any NOS-homolog genes and in the other species the genes encoding the responsible NOS molecules are similarly undefined (Crane et al. 2010).

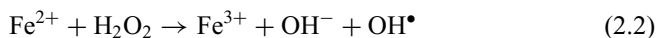
2.5.2 Characterization of Bacterial NOS Molecules

Recent studies have identified open reading frames encoding homologs of the mammalian oxygenase NOS domain (NOS_{ox}) in the genome of several gram-positive bacteria (representatives of the Bacillales, Actinobacteria and Deinococcus orders) and an archaeon (Fig. 2.7). These bacterial NOS_{ox}-like proteins have been characterized in *Bacillus subtilis* [bsNOS], *Bacillus anthracis* [baNOS], *Bacillus cereus* [bcNOS], *Staphylococcus aureus* [saNOS], *Streptomyces turgidiscabies*

[stNOS], *Geobacillus (Bacillus) stearothermophilus* [gsNOS], *Sorangium cellulosum* [scNOS] and *Deinococcus radiodurans* [drNOS] (Sudhamsu and Crane 2009; Crane et al. 2010; Montgomery et al. 2010). These bacterial NOS molecules catalyze the oxidation of L-arginine or ω -hydroxy-L-arginine and produce NO. Structures of these proteins show high similarities to the mammalian NOS_{ox}, and their catalytic activity resembles the mammalian-type NO synthesis (some differences exist, e.g. bacterial NOS utilizes either BH₄ or tetrahydrofolate). However, bacterial NOS molecules lack the covalently attached reductase domain (Fig. 2.7) and they receive electrons from various reductase partners (e.g. *B. subtilis* flavodoxin) (Crane et al. 2010). There is only one bacterial NOS, *Sorangium cellulosum* scNOS, which contains a reductase domain (Agapie et al. 2009; Crane et al. 2010).

2.5.3 Functions of Bacterial NOS

The available studies show that NOS is not associated with the cell membrane, thus it is supposed to be a cytosolic protein in the bacterial cell (Fig. 2.8). This cytosolic NO production is essential for oxidative stress protection: NO inhibits the generation of free radicals (OH⁻, OH[•]) within the bacterial cell (Gusarov and Nudler 2005). Exposure of bacteria to hydrogen peroxide (H₂O₂) leads to the production of OH⁻, OH[•] which cause DNA damage (Sudhamsu and Crane 2009). The generation of OH⁻, OH[•] is attributable to the Fenton-reaction of H₂O₂ with free cellular Fe²⁺. This process is shown by eq. 2.2 (Prousek 2007).



The free reduced Fe²⁺, which is required for the Fenton reaction, is depleted instantly with H₂O₂ exposure. However, the presence of cellular reductants (such as cysteine) reduces Fe³⁺ to Fe²⁺ and this recycling of Fe³⁺/Fe²⁺ sustains the Fenton reaction and leads to cell death (Prousek 2007). Synthesis of NO suppresses the Fenton reaction by transiently inhibiting cysteine reduction (Gusarov and Nudler 2005). Moreover, NO increases the expression of catalase (CAT) in *Bacillus subtilis*, which detoxifies H₂O₂ and mitigates oxidative stress (Gusarov and Nudler 2005).

Recently, it has been shown that NOS-derived NO ensures resistance of *Bacillus subtilis* to certain antibiotics (quinolone, acridine, amynoglicoside, cephalosporin, phenothiazine and lactam-type antimicrobials) (Gusarov et al. 2009). The underlying mechanism may be a chemical reaction of NO with the antibiotics (Gusarov et al. 2009). In the plant pathogen *Streptomyces* strains, NOS activity is required for the production of thaxtomin-A, a bacterial toxin, which interferes with the plant cell wall synthesis. Bacterial NOS is essential for the nitration of the tryptophanyl moiety of thaxtomin-A. This nitro group of thaxtomin-A is derived from the terminal guanidinium nitrogen of L-arginine. To date NOS is the only known enzyme, which catalyzes the oxidation of terminal guanidinium nitrogen of L-arginine (Crane et al. 2010) (Fig. 2.8). The NOS-derived NO also affects gene expression: transcriptional changes during the recovery from radiation damage and cell proliferation in *Deinococcus* are associated with NO synthesis (Crane et al. 2010).

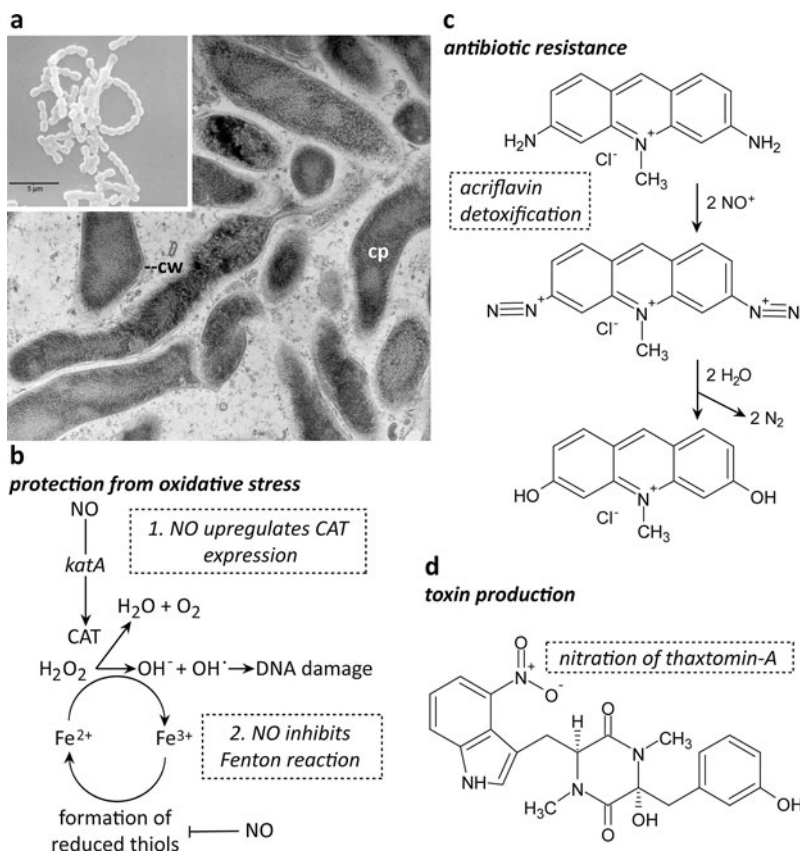


Fig. 2.8 Biological functions of NOS activity in bacteria. *Streptomyces* (a) and other bacteria contain NOS in their cytoplasm (SEM and TEM images with the courtesy of Dr. Iván Schmelcz; *cw* cell wall, *cp* cytoplasm). The cytosolic NO production is essential for oxidative stress protection (b), since NO inhibits the generation of OH⁻ and OH[•] by interruption of the Fenton reaction. NO also increases the expression of CAT in *Bacillus subtilis*, which detoxifies H₂O₂ and mitigates oxidative stress. NOS-derived NO ensures resistance of *Bacillus subtilis* to certain antibiotics, possibly by chemical modification of the antibiotics, e.g. acriflavin (c). In the plant pathogen *Streptomyces* strains, NOS activity is required for the nitration of the tryptophanyl moiety in thaxtomin-A (d), a bacterial toxin, which interferes with the plant cell wall synthesis

2.6 Subcellular NO Synthesis: Fruit or Root in the Tree of Phylogeny?

NO synthesis is widespread among the prokaryotes so we may assume that NO generating organisms were present in the archaeal biosphere. The generation of NO requires the reduction of NO₂⁻ or oxidation of guanidino nitrogens of L-arginine; thereby the evolutionary driving force for the development of NO-synthesizing

pathways is the large-scale production of NO_2^- and L-arginine in the biosphere. Logically, NO production could become possible when these substances were available in the ancient Earth.

Today, it is commonly accepted that cellular life originated from a last universal common ancestor (LUCA) which occurred in the Hadean ocean of the ancient Earth, around 4.5–5 Ga (gigaannum, 10^9 years) ago (Nisbet and Sleep 2001). This hypothetical LUCA gave rise to the diversification of prokaryotic life and the evolution of all cellular organisms (Prokaryota [Archaea and Bacteria] and Eukaryota). LUCA and its immediate descendants could gain NO-synthesizing ability only if substrates were available. There is a great debate, however, regarding the chemical composition and physical characteristics of the environment in which the early diversification and proliferation of cellular life took place.

In the case of NO_2^- , the substrate of reductive NO synthesis, there are two major hypotheses (Vlaeminck et al. 2011). The so-called “ON” scenario on the timing of nitrogen metabolism hypothesizes that the primordial atmosphere contained CH_4 and less than 50% CO_2 and oxygenic photosynthesis evolved before the biological generation of nitrogen oxides. In this model, LUCA and the first prokaryotes were unable to generate NO since NO_2^- could be present only in negligible amounts. The estimated development of NO_2^- reduction could be ~ 3.8 – 2.5 Ga, when oxygenic photosynthesis was already evolved (Vlaeminck et al. 2011).

The contrary “NO” scenario implies that the ancient atmosphere had a significantly higher CO_2 concentration and various nitrogen oxides were generated abiotically (e.g. by electric discharges, volcanic activity and UV radiation, Fig. 2.9). This hypothesis postulates that NO was abundant in the primordial atmosphere and the Hadean ocean was rich in NO_2^- . Nitrogen oxides therefore were available substrates for the hypothetical LUCA and the first prokaryote organisms ~ 4 – 3.8 Ga ago (Vlaeminck et al. 2011) (Fig. 2.9). Indeed, it is also hypothesized that NO was the first strong oxidant in the archaean world (Ducluzeau et al. 2009). Recent phylogenetic analysis of respiratory O_2 reductases and NORs suggests that NORs could be present in LUCA. It is possible that the most primordial prokaryotic cells could reduce NO_2^- to NO and the mechanisms similar to the recent denitrification and anammox processes developed before the advent of O_2 in the atmosphere (Ducluzeau et al. 2009). The example of *Methylophilis oxyfera* also suggests that NO_2^- reduction could also contribute to O_2 generation in the ancient biosphere before the occurrence of the first photosynthetic organisms. The “NO” model also postulates that decreasing atmospheric CO_2 levels led to the decline of abiotic generation of nitrogen oxides. This event evoked a “nitrogen crisis” of the Archaean life ~ 3.8 Ga ago. Limitation of NO, NO_2^- and NO_3^- availability might lead to the development of N_2 fixation and could also provide evolutionary force to NORs to use O_2 as an alternative substrate instead of NO (Ducluzeau et al. 2009).

Oxidative synthesis of NO from L-arginine is less widespread in the prokaryote world than the reductive NO production from NO_2^- . Abiotic generation of basic amino acids, such as arginine is a debated issue, and possibly, arginine was absent from the prebiotic Earth (McDonald and Storrie-Lombardi 2010). Although it is hypothesized that amino acids could be delivered by extraterrestrial materials (Kvenvolden et al. 1970; Barbier et al. 1998), the notable lack of arginine has been

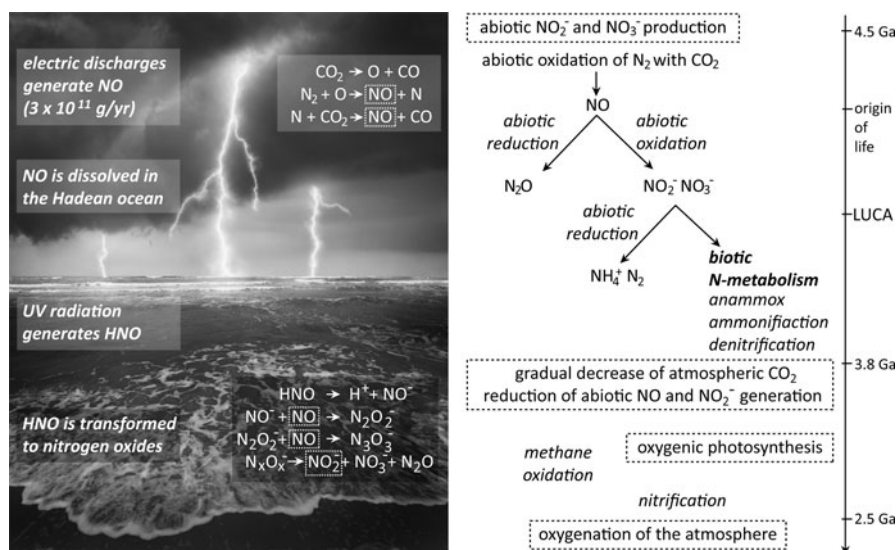


Fig. 2.9 Abiotic generation of NO and the possible development of reductive NO synthesis. The “NO” scenario implies that various nitrogen oxides—including NO—were generated abiotically (e.g. by *electric discharges*, volcanic activity and *UV radiation*) in the ancient atmosphere (Ducluzeau et al. 2009). This hypothesis postulates that NO was abundant in the primordial atmosphere and the Hadean ocean was rich in NO_2^- . It is possible that primordial prokaryotic cells could reduce NO_2^- to NO by mechanisms similar to the recent denitrification and anammox processes

found in meteorites that contain organic compounds (so-called carbonaceous chondrites) (Miller 1986; Engel and Macko 2001). Since amino acids of carbonaceous chondrites are synthesized abiotically, it is plausible that similar prebiotic synthesis took place on the primitive Earth (Miller 1986). However, the lack of arginine from the yet analyzed meteorites makes arginine’s presence in the most ancient biosphere unreliable. Accordingly, several bacterial proteins, e.g. bacterial flagellar proctins do not contain basic amino acids (arginine and lysine), supporting the idea that cellular life occurred in an arginine-free environment (McDonald and Storrie-Lombardi 2010). However, most bacteria have the ability to metabolize L-arginine (Hird 1986) and *de novo* arginine biosynthesis is present in a limited number of bacterial phyla (Xu et al. 2007). L-arginine provides a positively charged guanidino group, which affects secondary protein structures and allows establishment of DNA-protein interactions, thereby it could be involved in the development of eukaryotic chromatin structures (Hird 1986). Arginine metabolism, and particularly oxidative NO synthesis from L-arginine, requires a prevailing complexity of cellular life, suggesting that NOS-catalyzed L-arginine oxidation could evolve after the reductive NO synthesis.

As a milestone in the development of the eukaryotic world, the cell architecture became more complex and distinct biochemical microniches have evolved into the cell organelles (e.g. mitochondria, cell nucleus, endoplasmic membrane systems) and specific subcellular compartments (e.g. membrane microdomains). Some of the cell organelles are derived from endosymbiotic associations of ancient prokaryote

cells. Hence, several aspects of organelle-specific NO synthesis and function resemble bacterial-type NO homeostasis. For instance, the intermembrane space of mitochondria and the thylakoid lumen of chloroplasts are evolutionary descendents of the periplasmic space of bacteria (Herrmann et al. 2009). Accordingly, these subcellular compartments display reductive NO synthesis and bacterial-type NO scavenging (Castello et al. 2006, 2008; Poyton et al. 2009; Nakanishi et al. 2010). NOS-like enzymes in the chloroplast stroma and the mitochondrial matrix may be descendents of the prokaryotic NOS molecules. Organelles with endosymbiotic origins thereby have preserved the key features of ancient prokaryotic NO biology. However, further development of subcellular NO biology required the diversification of NOS molecules and the development of various mechanisms (e.g. dynamic post-translational modifications) which ensure the correct intracellular orientation of NOS in the complex eukaryotic cell.

2.7 Chapter Summary

Mechanisms of NO generation in prokaryotes

- NO_2^-/NO reduction is catalyzed by dissimilatory NO_2^- -reductases (NirK, NirS)
- Deoxygenated bacterial hemoglobin is also capable of reducing NO_2^- to NO
- Several bacteria contain NOSs, and synthesize NO from L-arginine
- Bacterial N-metabolism also contributes to chemical NO release from nitrogen oxides

Functions of NO in the prokaryote cell

- Within the bacterial cell, NO has antioxidant effects by the inhibition of Fenton chemistry. It increases the transcription of genes involved in stress adaptation, denitrification and NO-elimination
- NO synthesis is involved in bacterial toxin production and antibiotic resistance
- NO may release from the bacterial cell and inhibit the growth of competitor bacteria, or facilitate the development of bacterial-plant symbiosis

Origin of spatial organization of subcellular NO synthesis

- Reductive NO synthesis is associated with the bacterial cell membrane, while NOS activity is confined to the cytosol. Similar arrangement of NO synthesis is notable in homolog structures of the eukaryote cell
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