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Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations ☆

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ABSTRACT

Nitrogen is an essential element of life that needs to be assimilated in its most reduced form, ammonium. On the other hand, nitrogen exists in a multitude of oxidation states and, consequently, nitrogen compounds (NCs) serve as electron donor and/or acceptors in many catabolic pathways including various forms of microbial respiration that contribute to the global biogeochemical nitrogen cycle. Some of these NCs are also known as reactive nitrogen species able to cause nitrosative stress because of their high redox reactivity. The best understood processes of the nitrogen cycle are denitrification and ammonification (both beginning with nitrate reduction to nitrite), nitrification (aerobic oxidation of ammonium and nitrite) and anaerobic ammonium oxidation (anammox). This review presents examples of the diverse architecture, either elucidated or anticipated, and the high degree of modularity of the corresponding respiratory electron transport processes found in Bacteria and Archaea, and relates these to their respective bioenergetic mechanisms of *proton motive force* generation. In contrast to the multiplicity of enzymes that catalyze NC transformations, the number of proteins or protein modules involved in connecting electron transport to and from these enzymes with the quinone/quinol pool is comparatively small. These quinone/quinol-reactive protein modules consist of cytochromes *b* and *c* and iron-sulfur proteins. Conclusions are drawn towards the evolutionary relationships of bioenergetic systems involved in NC transformation and deduced aspects of the evolution of the biogeochemical nitrogen cycle are presented. This article is part of a Special Issue entitled: The evolutionary aspects of bioenergetic systems.

1. Introduction

The redox transformation of nitrogen compounds (NCs) is the basis of nature's biogeochemical nitrogen cycle (Fig. 1). The involved reactions and pathways are manifold and serve in nitrogen assimilation and nitrogen-based catabolism including respiration as well as detoxification of harmful NCs ("reactive nitrogen species"; see Table 1 for some general properties of the biologically most relevant NCs) [1–3]. In particular, the participation of a diverse range of microorganisms from the bacterial and archaeal domains is crucial for the maintenance of the nitrogen cycle. Important reactions of the nitrogen cycle include (i) anaerobic reduction of nitrate (NO_3^-) to nitrite (NO_2^-), (ii) reduction of nitrite to produce nitric oxide (NO) and nitrous oxide (N_2O) (during both anaerobic "classic" denitrification and aerobic "nitrifier" denitrification) and dinitrogen (during classic denitrification): (iii) reduction of nitrite to ammonium (ammonification pathways), (iv) aerobic ammonium oxidation to yield hydroxylamine, nitrite and eventually nitrate (nitrification), (v) dinitrogen (N_2) fixation, (vi) anaerobic ammonium oxidation (anammox) to produce N_2 and (vii) a recently described reaction of nitric oxide dismutation resulting in N_2 and O_2 production (Fig. 1). The processes (i), (ii), (iii), (iv), (vi) and (vii) belong to different modes of respiratory energy metabolism that drive ATP generation in the corresponding cells. In general, examined organisms that live under anoxic conditions are capable to catalyze only one of the denitrification, ammonification, anammox or NO dismutation processes.

Respiratory NC transformation is catalyzed by a diverse range of metalloproteins that are organized in electron transport chains

Abbreviations: Amo, ammonium monooxygenase; *Ca.*, *Candidatus*; Cu-MMO, copper-dependent membrane monooxygenase family; DMSO, dimethyl sulfoxide; ETC, electron transport chain; Fe/S, iron-sulfur center; *GOE*, *Great Oxygenation Event*; Hao, hydroxylamine oxidoreductase; HCO, heme-copper oxidase family; Hzo, hydrazine oxidoreductase; Hzs, hydrazine synthase; Hyd, hydrogenase; Fdh, formate dehydrogenase; MCC, multiheme cytochrome *c* family; Mo-*bis*-MGD, molybdenum *bis* molybdopterin guanine dinucleotide; MK/MKH_2, menaquinone/menaquinol; N, nitrogen; Nap, periplasmic nitrate reductase; Nar, membrane-bound nitrate reductase; NC, nitrogen compound; NirK, copper nitrite reductase; NirS, cytochrome cd_1 nitrite reductase; Nod, nitric oxide dismutase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase; Nrf, cytochrome *c* nitrite reductase; Nxr, nitrite oxidoreductase; pMmo, particulate methane monooxygenase; *pmf*, proton motive force; Q/QH_2, quinone/quinol (unspecified); QRP, quinone/quinol-reactive protein; rET, reverse electron transport; Tat, twin arginine translocation; TMAO, trimethylamine *N*-oxide; UQ/UQH_2, ubiquinone/ubiquinol

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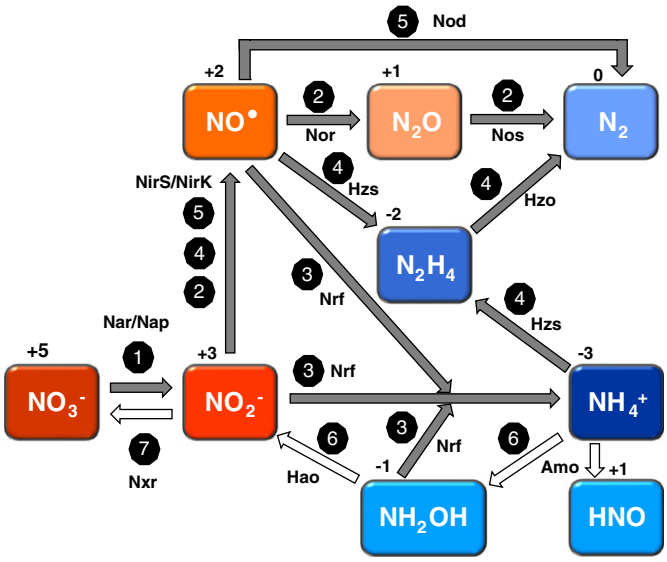

Fig. 1. Conversion of NCs serving as substrates in nitrogen cycle processes relevant to this article. Anaerobic respiratory pathways and aerobic nitrification are indicated by grey and white arrows, respectively. The oxidation state of nitrogen atoms is indicated above the NC. Numbers in black decagons refer to the following processes: 1, respiratory nitrate reduction to nitrite; 2, denitrification of nitrite to N_2; 3, Nrf-dependent ammonification; 4, anaerobic ammonium oxidation (anammox, i.e. comproportionation of nitrite and ammonium to form dinitrogen); 5, Nod-dependent nitrite reduction to dinitrogen; 6, ammonium oxidation to nitrite; 7, nitrite oxidation to nitrate. These metabolic pathways are catalyzed by distinct respiratory enzyme systems that are designated by the NC-converting enzyme: Nar, membrane-bound nitrate reductase; Nap, periplasmic nitrate reductase; NirS, cytochrome cd_1 nitrite reductase; NirK, copper nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase; Nrf, cytochrome *c* nitrite reductase; Hzs, hydrazine synthase; Hzo, hydrazine oxidoreductase; Nod, nitric oxide dismutase; Amo, ammonium monooxygenase; Hao, hydroxylamine oxidoreductase; Nxr, nitrite oxidoreductase.

(ETCs) and networks (resulting from interconnected ETCs). These proteins and protein complexes are either bound to membranes, for example the cytoplasmic ("inner") or the anammoxosome membrane, or they are soluble proteins that often reside in compartments outside the cytoplasm such as the periplasm or the lumen of intracellular membrane stacks of Gram-negative bacteria or inside the anammoxosome of the *Brocadiaceae*, the anammox bacteria. In microbes capable of using catabolic transformations of NCs to support growth, electron transport to and from NCs is designed to generate a *proton motive force* (*pmf*), i.e. a transmembrane electrochemical proton potential that drives ATP synthesis via the membrane-bound ATP synthase. In addition, there are cohorts of microbes capable of NC transformations that contribute to known N-cycle processes including N-respiration but cannot support their growth; for instance many methane-oxidizing bacteria can facilitate processes (i) to (v) and (vii) although they do not grow chemolithotrophically using nitrogen compounds [4].

A key redox mediator of the underlying electron transport chains is the membranous quinone/quinol pool that consists of one or more benzo- or naphthoquinones, usually ubi- and/or menaquinones, depending on the organism and its environmental conditions. Quinones are small, freely diffusible lipophilic molecules that are able to take up two electrons and two protons while converted from the oxidized quinone to the reduced quinol state. Various quinone/quinol-reactive proteins (QRPs) are known to contribute to the highly diverse ETCs that catalyze NC conversion. QRPs are divided into donor:quinone dehydrogenases and quinol:acceptor reductases as they catalyze redox reactions situated at either the oxidative or the reductive side during electron transport from a reduced electron donor substrate (the reductant) to an oxidized electron acceptor substrate (the oxidant). Depending on their oxidation state, NCs are functional in both processes (Fig. 1; Table 1).

Many high-resolution structures of NC-converting enzymes and also of a few QRPs have been obtained over the past two decades. On the other hand, the detailed enzymic composition and function of complete ETCs and networks is less well understood. Section 2 of this article introduces some prominent model organisms of respiratory NC turnover and considers the bioenergetic foundations of their catabolic lifestyles. The key enzymes of NC turnover and their connection to one or more distinct QRPs are described in Section 3. Emphasis is placed on ETC modularity and the mode(s) of *pmf* generation in order to separate protonmotive and non-protonmotive steps, which are comparatively addressed in Section 4. Key protein families in respiratory nitrogen metabolism and their assumed evolutionary relationships are presented in Section 5 whereas Section 6 draws conclusions on the evolution of bioenergetic systems involved in NC turnover, mainly on the basis of their modularity. Impacts on the evolution of the extant biogeochemical nitrogen cycle are also discussed.

Note that enzymes having solely assimilatory functions like soluble cytoplasmic nitrate and nitrite reductases as well as dinitrogen-fixing nitrogenases are not discussed in this article as their function is not directly coupled with respiratory metabolism. Furthermore, a detailed structural and functional description of individual enzymes is considered beyond

Table 1
Properties of nitrogen compounds relevant to this article.

Nitrogen compound	Oxidation state	Toxicity to microbial cells	Other properties
Nitrate (NO_3^-)	+5	Non-toxic in physiological concentrations.	Anion of strongly oxidizing and toxic nitric acid (HNO_3; $pK_a = -1.4$).
Nitrogen dioxide (NO_2)	+4	Toxic orange gas. Arises from the oxidation of nitric oxide by oxygen.	In equilibrium with the colorless gas dinitrogen tetroxide (N_2O_4).
Nitrite (NO_2^-)	+3	Toxic; binds to cellular iron atoms, for example in hemoglobin.	Forms nitrosonium in acidic conditions according to $HNO_2 + H^+ \rightarrow NO^+ + H_2O$. Anion of unstable nitrous acid (HNO_2; $pK_a = 3.4$).
Nitric oxide (NO)	+2	Highly reactive toxic radical. Binds to heme iron atoms and Fe/S centers and forms dinitrosyl iron complexes [$Fe(NO)_2$]. Causes nitrosation of thiol groups to form *S*-nitrosothiols (−S−N=O).	Generation of nitrogen dioxide (NO_2) or peroxynitrite ($ONOO^-$) in the presence of oxygen and superoxide radicals, respectively. Redox-related reactive species are the nitroxyl (NO^-) and nitrosonium (NO^+) ions.
Nitrous oxide (N_2O)	+1	Chemically inert and non-toxic in physiological concentrations.	-
Nitroxyl (HNO)	+1	Reactive towards nucleophiles, especially thiols.	Dimerizes to hyponitrous acid ($H_2N_2O_2$), which can be dehydrated to N_2O.
Dinitrogen (N_2)	0	Chemically inert and non-toxic.	–
Hydroxylamine (NH_2OH)	−1	Toxic by binding to heme groups. Irreversible inhibitor of the oxygen-evolving complex in photosystem II.	Mutagenic by uracil-forming cytosine deamination, thus causing G/C→T/A transitions in DNA.
Hydrazine (N_2H_4)	−2	Highly toxic.	Rocket fuel, forms monohydrate.
Ammonium (NH_4^+)	−3	Non-toxic in physiological concentrations.	Cation of ammonia (NH_3), which is a toxic uncoupling agent ($pK_a = 9.25$).

the scope of this article. Instead, the main focus is set on the modular design and evolutionary relationships of extant bioenergetic systems.

2. Metabolic lifestyles of model organisms and consideration of bioenergetic principles

Microbial growth using NCs as respiratory substrates is widespread in heterotrophic and autotrophic members of the domains Bacteria and Archaea. However, compared to the overwhelming amount of data from genomes and environmental metagenomes, only a tiny number of such organisms have been characterized in detail. Taxonomically, most of the well-understood bacteria belong to the phylum Proteobacteria, which is divided into the classes of Alpha-, Beta-, Gamma-, Delta-, Epsilon- and Zetaproteobacteria. Of those, the first three classes form the most recent phylogenetic group that comprises many well-known model bacteria. However, even in these closely related classes, an intriguing variety of respiratory electron transport chain design has been described and the same holds true for the phylogenetically older Delta- and Epsilonproteobacteria. Other bacterial (super) phyla whose study provided significant insight into NC respiration are Nitrospirae, Planctomycetes-Verrucomicrobia, Deinococcus-Thermus, Firmicutes, the NC10 phylum as well as the Cren-, Eury- and Thaumarchaeota. In fact, many recently discovered enzymes and even entire catabolic lifestyles are attributed to microorganisms from one of these less studied phyla, for some of which general microbiological knowledge is rather rudimentary (see Section 3). In the absence of biochemical data, metabolic models are often deduced from genomic information (most importantly by inspecting gene clusters encoding respiratory enzymes and their biogenesis systems), which allows the hypothetical reconstruction of ETCs. It should be kept in mind, however, that this deductive metabolic reconstruction approach might result in unproven ecological predictions, especially in the light of pronounced substrate promiscuity of some NC-converting enzymes.

Microbial respiration is designed for energy conservation, that is to generate ATP by ETC-level phosphorylation, and the organization of the corresponding ETCs generally reflects the energetic constraints of a catalyzed redox reaction. In principle, the free energy change (ΔG) of a given redox reaction is stepwise transduced into an electrochemical potential difference (the *pmf*) across the membrane in order to drive ATP synthesis [5]. This fact represents the unifying theory of bioenergetics introduced as the *chemiosmotic hypothesis* by Peter Mitchell in the 1960s. The standard free energy change at pH 7.0 ($\Delta G_0'$) of a respiratory process depends on standard redox potentials at pH 7.0 (E_0') of the electron donor and acceptor substrates (and their respective reaction products) according to the equation $\Delta G_0' = n \cdot F \cdot \Delta E_0'$, wherein n is the number of electrons transferred and F is the Faraday constant (see Table 2 for $\Delta E_0'$ values of selected NC redox pairs). Due to their rather positive E_0' values, the diverse range of NCs is well-suited to serve as terminal electron acceptor of respiratory ETCs, especially when low-potential substrates like formate, hydrogen gas, diverse sulfur compounds or NADH are used as electron donors in chemoorganotrophic or chemolithotrophic energy-conserving pathways. On the other hand, ammonium and nitrite are poor electron donors, explaining the fact that obligate chemolithotrophic nitrifying organisms usually use oxygen as the sole electron acceptor. In addition, operating a nitrifying electron transport chain by a chemolithoautotroph necessitates *reverse electron flow* in order to produce NAD(P)H for assimilatory CO_2 reduction.

Different kinds of quinone/quinol pairs are characterized by distinct E_0' values. High-potential ubiquinones [E_0' (UQ/UQH$_2$) ~ +110 mV] are predominantly used by aerobic organisms whereas low-potential menaquinones [E_0' (MK/MKH$_2$) ~ −80 mV] are better suited for anaerobic respiration when the cellular state is more reduced. From the E_0' values of different NCs presented in Table 2, it becomes clear that quinol oxidation (in particular menaquinol oxidation) in anaerobic respiration using nitrate, nitrite, nitric oxide, nitrous oxide or hydroxylamine as terminal electron acceptor is a strongly exergonic reaction. Therefore, in principal, *pmf* generation due to an electrogenic (protonmotive) ETC is energetically possible. Nonetheless, such reactions might be employed as electroneutral (non-protonmotive) steps due to the interchangeable modular architecture of the pertinent ETC components.

The *pmf* has both a chemical (ΔpH, dimensionless) and an electrical ($\Delta\psi$, dimension mV) component and the formula $pmf\ (\mathrm{mV}) = \Delta\psi - 59\ \Delta\mathrm{pH}$ describes this correlation in its simplest form. $\Delta\psi$ is defined as the electrical potential difference between the positively and negatively charged side of the membrane (usually a positive value) whereas the ΔpH is defined as the pH difference between both membrane sides (usually negative). Since the *pmf* has a value in the range of about 150 to 200 mV, a redox potential change (ΔE) of at least this magnitude is required during electron transfer from a donor to an acceptor substrate if this step is to be coupled to *pmf* generation. The ratio of protons (transported across the membrane) and electrons (transported from donor to acceptor), i.e. the H^+/e^- value, corresponds to the quotient of ΔE and the *pmf* (at a hypothetical 100% thermodynamic efficiency). There are different possibilities to generate the *pmf*: (i) proton pumping as exemplified by NADH:ubiquinone oxidoreductase (complex I-type NADH dehydrogenase) or complex IV-type heme-copper oxidases (HCOs), (ii) movement of protons across the membrane via the so-called "Q-cycle" as catalyzed by the cytochrome bc_1 complex (ubiquinol:cytochrome *c* oxidoreductase; complex III) and (iii) the electrogenic redox loop mechanism in which a separation of positive and negative charges across the membrane is achieved by different locations of the active sites for substrates and quinones in respiratory enzymes (or in respiratory electron transport chains) [6]. Classically, a redox loop consists of two quinone/quinol-reactive (multi-subunit) enzymes and a quinone species that serves as redox mediator between them. Bacterial redox loops are commonly found in anaerobic respiratory systems that are driven by ΔG values smaller than in chemoorganoheterotrophic aerobic respiration and, consequently, shorter ETCs with a lower number of coupling sites are employed in such cases [6].

3. Enzymes and protein modules involved in extant respiratory nitrogen compound transformations

Based on the diversity of NC conversions depicted in Fig. 1, a compilation of the involved respiratory key enzymes is presented in Table 2. Throughout this section, emphasis is laid on the various modes of how such enzymes are connected with the membranous quinone/quinol pool and how NC turnover is envisaged to be coupled to *pmf* generation by the respective ETC. In particular, the taxonomic distribution, modularity and substrate specificity of such ETCs are pointed out.

3.1. Nitrate reductases

Respiratory nitrate reductase complexes belong to either the Nar- or the Nap-type (enzymes 1 and 2 in Table 2). Both nitrate-reducing subunits (NarG or NapA) produce nitrite in an energetically favorable reaction using a quinol as electron donor (Table 2). However, the enzymic composition of the corresponding electron transport chains as well as their contribution to *pmf* generation is highly diverse in bacterial and archaeal species (Figs. 2 and 3) [7–10]. The common feature of the membrane-bound nitrate reductase, the Nar-type, is a heterodimeric NarGH complex that is attached to the membrane by at least one other protein, which is quinol-reactive. The NarGH complex is similar and evolutionarily related to the nitrite-oxidizing NxrAB complexes from anammox bacteria and aerobic nitrifiers (see Sections 3.5, 3.7 and 5.2.2) [11]. The NarG protein binds a molybdenum *bis* molybdopterin guanine dinucleotide (Mo-*bis*-MGD) cofactor at the active site of nitrate reduction and a 4Fe–4S cluster while the NarH subunit binds four further iron sulfur clusters (one 3Fe–4S and three

Table 2
Selected enzymes and enzyme complexes involved in respiratory nitrogen compound turnover.

Enzyme designation and corresponding standard potential of the relevant redox pair (oxidized/reduced) at pH 7 (E_0') [a]	Physiological function	Redox partner	Selected model organism(s) [b]
Nitrate reductases: $NO_3^- + 2\,e^- + 2H^+ \rightarrow NO_2^- + H_2O$; E_0' (NO_3^-/NO_2^-) = +0.43 V			
1a. nNar/NarGHI (Mo-bis-MGD, Fe/S, heme *b*; dimeric)	Cytoplasmic nitrate reduction	Quinol (oxidized by NarI)	***Escherichia coli*** (γ), *Paracoccus denitrificans* (α)
1b. *c*Nar/NarGHIC (Mo-bis-MGD, Fe/S, heme *b*, heme *c*)	Cytoplasmic nitrate reduction	Ubiquinol and periplasmic cytochrome *c* pool via NarC	*Thermus thermophilus* (Deinococcus–Thermus)
1c. pNar/NarGH in a complex with other proteins (see text) (Mo-bis-MGD, Fe/S)	Nitrate reduction at the outside of the cytoplasmic membrane	Quinones and possibly small periplasmic electron transfer proteins	*Haloferax mediterranei*, *Haloarcula marismortui* (both Euryarchaeota), *Pyrobaculum aerophilum* (Crenarchaeota)
2a. NapAB (Mo-bis-MGD, Fe/S, heme *c*)	Periplasmic nitrate reduction	Quinol dehydrogenases NapC and/or NapGH	***Rhodobacter sphaeroides*** (α), *Escherichia coli* (γ), *Paracoccus denitrificans* (α), *Bradyrhizobium japonicum* (α), *Shewanella oneidensis* (γ), *Wolinella succinogenes* (ε)
2b. NapA (Mo-bis-MGD, Fe/S)	Periplasmic nitrate reduction	Not known, potentially NapM.	***Desulfovibrio desulfuricans*** (δ)
Nitrite-reactive enzymes			
(i) NO-genic nitrite reduction: $NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O$; E_0' (NO_2^-/NO) = +0.36 V			
3. NirS, cytochrome cd_1 (hemes *c* and d_1; dimeric)	Nitrite reduction	Monoheme cytochromes *c* (cyt. c_{550} or c_{551}), NirT or cupredoxin	***Paracoccus pantotrophus*** (α), ***Pseudomonas aeruginosa*** (γ), *Pseudomonas stutzeri* (γ)
4a. NirK (Cu_I and Cu_{II} centers; trimeric)	Nitrite reduction	Monoheme cytochromes *c* (cyt. c_{550} or c_{551}) or cupredoxin	*Bradyrhizobium japonicum* (α), ***Achromobacter cycloclastes*** (β), ***Achromobacter xylosoxidans*** (β)
4b. *c*NirK (heme *c*; Cu_I and Cu_{II} centers)	Nitrite reduction	Monoheme cytochromes *c* (cyt. c_{550} or c_{551}) or cupredoxin	*Burkholderia* sp. (β), *Bdellovibrio bacteriovorus* (δ)
4c. CuNirK (three Cu centers)	Nitrite reduction	Monoheme cytochromes *c* (cyt. c_{550} or c_{551}) or cupredoxin	*Hyphomicrobium denitrificans* (α)
(ii) Ammonifying nitrite reduction: $NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2\,H_2O$; E_0' (NO_2^-/NH_4^+) = +0.34 V			
5a. NrfA (5 heme *c*; one CX_2CK motif; dimeric)	Periplasmic nitrite ammonification	Pentaheme cytochrome *c* NrfB	***Escherichia coli*** (γ)
5b. NrfA (5 heme *c*; one CX_2CK motif)	Periplasmic nitrite ammonification	Tetraheme cytochrome *c* quinol dehydrogenase CymA	*Shewanella oneidensis* (γ)
5c. NrfHA complex (NrfA: 5 heme *c*; one CX_2CK motif; NrfH: four heme *c*; Dimers of $NrfHA_2$ assembly in crystal structure)	Periplasmic nitrite ammonification	Quinol (oxidized by NrfH)	***Wolinella succinogenes*** (ε), ***Desulfovibrio vulgaris*** (δ)
5d. NrfA (5 heme *c*; five CH_2CH motifs)	Periplasmic nitrite ammonification	Quinol (oxidized by NrfH)	*Campylobacter jejuni* (ε)
6. Onr (8 heme *c*; hexameric)	Not known. Onr reduces nitrite, hydroxylamine, sulfite and hydrogen peroxide	Not known	***Thioalkalivibrio nitratireducens*** (γ)
7. Otr (8 heme *c*)	Not known. Otr reduces nitrite and hydroxylamine and interconverts tetrathionate and thiosulfate	Not known	*Shewanella oneidensis* (γ)
8. εHao (8 heme *c*)	Most likely nitrite reduction	Cytochrome *c* of the NapC/NrfH family (?)	*Campylobacter concisus*, *Campylobacter curvus*, *Campylobacter fetus*, *Nautilia profundicola* (all ε)
(iii) Nitrite oxidoreductase: $NO_2^- + H_2O \rightarrow NO_3^- + 2\,e^- + 2H^+$; E_0' (NO_3^-/NO_2^-) = +0.43 V			
9a. nNxr/NxrABIC (Mo-bis-MGD, Fe/S, hemes *b* and *c*)	Cytoplasmic nitrite oxidation	Quinone pool and cytochrome aa_3 oxidase	*Nitrobacter hamburgensis* (α)
9b. pNxr/NxrAB (Mo-bis-MGD, Fe/S, possibly heme *b*)	Periplasmic nitrite oxidation	Quinone pool and cytochrome *bd*-type oxidase	*Ca.* Nitrospira defluvii (Nitrospirae)
9c. aNxr/NxrAB (might form large complex with several other subunits, see text)	Nitrite oxidation in anammoxosome	Quinone	*Ca.* Kuenenia stuttgartiensis (Planctomycetes)
Nitric oxide-reactive enzymes			
(i) N_2O-genic nitric oxide reductases: $2\,NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$; E_0' (NO/N_2O) = +1.18 V			
10a. *c*Nor/NorBC (hemes *b* and *c*, Fe; dimeric)	Nitric oxide reduction	Monoheme cytochromes *c* (cyt. c_{550} or c_{551}) or cupredoxin	*Paracoccus denitrificans* (α), ***Pseudomonas aeruginosa*** (γ)
10b. qNor/NorB (heme *b*, Fe)	Nitric oxide reduction at the outside of the cytoplasmic membrane	Quinol (MKH_2)	*Pyrobaculum aerophilum* (Crenarchaeota), ***Geobacillus stearothermophilus*** (Firmicutes), *Ralstonia eutropha* (β), *Neisseria meningitidis* (β)
10c. Cu_A-qNor (heme *b*, Fe, Cu_A)	Nitric oxide reduction at the outside of the cytoplasmic membrane	Menaquinol	*Bacillus azotoformans* (Firmicutes)
10d. sNor, eNor, gNor	Periplasmic nitric oxide reduction	Monoheme cytochromes *c*	Diverse Proteobacteria
(ii) Hydrazine synthase (comproportionation of nitric oxide and ammonium): $NO + NH_4^+ + 3e^- + 2H^+ \rightarrow N_2H_4 + H_2O$; $E_0' = +0.06$ V			
11. Hzs (heme *c*)	Comproportionation of nitric oxide and ammonium in anammoxosome	Cytochromes *c* in anammoxosome	*Ca.* Kuenenia stuttgartiensis (Planctomycetes)
(iii) Nitric oxide dismutase (Nod): $2\,NO \rightarrow N_2 + O_2$			
12. Nod (enzyme yet to be identified)	Nitric oxide reduction in oxygenic denitrification of nitrite by methanotrophs	Not known	*Ca.* Methylomirabilis oxyfera (NC10 phylum)

(continued on next page)

Table 2 (*continued*)

Enzyme designation and corresponding standard potential of the relevant redox pair (oxidized/reduced) at pH 7 (E_0') [a]	Physiological function	Redox partner	Selected model organism(s) [b]
Nitrous oxide reductase: $N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O$; E_0' (NO/N_2O) = +1.35 V			
13a. NosZ (Cu_A, Cu_Z; Dimer)	Periplasmic nitrous oxide reduction	Monoheme cytochromes *c* (cyt. c_{550} or c_{551}), cupredoxin, or NosR/NosXR	***Pseudomonas stutzeri*** (γ), *Paracoccus denitrificans* (α), *Marinobacter hydrocarbonoclasticus* (γ)
13b. *c*NosZ (heme *c*, Cu_A, Cu_Z)	Periplasmic nitrous oxide reduction	Monoheme cytochromes *c* or NosGH quinol dehydrogenase	*Wolinella succinogenes* (ε)
Ammonia monooxygenase: $NH_4^+ + O_2 + 2e^- + H^+ \rightarrow NH_2OH + H_2O$; E_0' (NH_2OH/NH_4^+) = +0.74 V			
14. AmoABC (Cu, Fe; trimeric)	Periplasmic ammonia oxidation	Quinone pool (assumed)	*Nitrosomonas europaea* (β)
Hydroxylamine oxidoreductase: $NH_2OH + H_2O \rightarrow NO_2^- + 4\,e^- + 5H^+$; E_0' (NO_2^-/NH_2OH) = +0.10 V			
15. Hao (8 heme *c*; trimeric)	Periplasmic hydroxylamine oxidation	Cyt. c_{554} (assumed)	***Nitrosomonas europaea*** (β)
Hydrazine oxidoreductase: $N_2H_4 \rightarrow N_2 + 4\,e^- + 4H^+$; E_0' (N_2H_4/N_2) = −0.75 V			
16. Hzo (8 heme *c*)	Hydrazine oxidation in anammoxosome	Cytochromes *c* in anammoxosome	*Ca.* Kuenenia stuttgartiensis (Planctomycetes)

[a] Individual enzymes or enzyme complexes are consecutively numbered and their metal/cofactor content as well as their typical multimerization status is provided if reported.
[b] Names in bold face indicate that a high-resolution enzyme structure from the respective organism is available. Phyla and proteobacterial classes (Greek letters) are given in parentheses. *Ca.*, *Candidatus*.

4Fe–4S clusters) [12]. In Proteobacteria (and also in some Gram-positive bacteria), the NarGH complex is attached to the cytoplasmic side of the membrane by the quinol-oxidizing diheme cytochrome *b* membrane anchor NarI that contains five transmembrane helices (Fig. 2A). The two heme groups of NarI are oriented towards opposite sides of the membrane. The NarGHI complex is also called the nNar system since nitrate is reduced at the potential-negative cytoplasmic face of the membrane, the n-side (enzyme 1a in Table 2). Bioenergetically, nNar contributes to *pmf* generation via a redox loop mechanism [6,13]. As depicted in Fig. 2A, quinol oxidation by NarI is catalyzed at the periplasmic side of NarI. Hence, two protons are released into the periplasmic space whereas two electrons are moved across the membrane where they eventually serve in cytoplasmic nitrate reduction coupled to the take-up of two cytoplasmic protons. Overall, quinol oxidation by nitrate using the nNar system results in a transmembrane electrochemical proton gradient with a H^+/e^- ratio of one. It is notable that such a system requires nitrate import (and usually also nitrite export), which, in Proteobacteria, is achieved by several distinct transporters (uniport and nitrate/nitrite antiport) [14,15]. Interestingly, bacterial NarG proteins contain a so-called remnant twin arginine translocation (Tat) signal peptide suggesting that primordial bacterial NapGH complexes have been exported and were functional at the periplasmic face of the cytoplasmic membrane [16].

A variation of the nNar system was described in the hyperthermophilic bacterium *Thermus thermophilus* (phylum Deinococcus–Thermus) [17]. In this case, a periplasmic diheme cytochrome *c* (NarC) was reported to interact with the NarGHI complex that itself seems to form a supercomplex with a membrane-bound NADH dehydrogenase, the Nrc complex (Fig. 2B). The NarGHIC assembly, here designated *c*Nar, presumably catalyzes electrogenic nitrate reduction using ubiquinol as electron donor (enzyme 1b in Table 2). NarC is envisaged to donate electrons to periplasmic cytochrome cd_1 nitrite reductase (NirS) and membrane-bound nitric oxide reductase (Nor), thus functionally bypassing a cytochrome bc_1-type complex of proteobacterial denitrifiers (see below) [17].

Several archaeal denitrifiers also possess a NarGH complex (enzyme 1c in Table 2) [18]. In contrast to extant bacterial systems, however, the archaeal NarG protein carries an intact Tat signal sequence suggesting that a cofactor-containing NarGH complex is exported to the potential-positive outside of the cytoplasmic membrane, the p-side [7,19,20]. The genetic context of the corresponding gene clusters implies that NarGH is part of a membrane-bound nitrate reductase system referred to as pNar. The proteins of this putative complex in several haloarchaea such as *Haloferax mediterranei* and *Haloarcula marismortui* include a transmembrane diheme cytochrome *b* whose primary structure resembles the cytochrome *b* of cytochrome bc_1 complexes (also termed NarC though unrelated to NarC from *T. thermophilus*), a Rieske-type iron sulfur protein (NarB) and three other potential membrane proteins including a monoheme cytochrome *b* (Fig. 2C). Therefore, it cannot be excluded that an electrogenic Q-cycle-type mechanism is driven by nitrate reduction coupled to ubiquinol oxidation [19]. The proposed Nar supercomplex might also donate electrons to other denitrifying enzymes such as copper nitrite reductase (NirK), a complex IV HCO-type nitric oxide reductase (*c*Nor) and nitrous oxide reductase (NosZ) (Fig. 2C). An alternative membrane anchor (NarM, a monoheme cytochrome *b* with only two proposed transmembrane domains) was identified in case of the pNar enzyme from the hyperthermophile *Pyrobaculum aerophilum* [20]. Since the singular heme *b* group is likely to be oriented to the outside of the cytoplasmic membrane, menaquinol oxidation by nitrate is thought to be electroneutral in this organism.

The Nap system of periplasmic nitrate reduction is found almost exclusively in the phylum Proteobacteria. Depending on the species, the Nap system is employed under anoxic and/or (micro)oxic conditions and fulfills several distinct functions, for example in anaerobic respiration or as electron sink during aerobic (photo)organoheterotrophic growth on reduced carbon sources to ensure redox homeostasis, i.e. to dissipate excess reductant [21,22]. Many Proteobacteria encode both Nar and Nap systems whose production is intimately regulated in response to ambient nitrate and oxygen concentrations. Similar to NarG, NapA binds Mo-*bis*-MGD and a 4Fe–4S cluster. In the majority of known cases NapA forms a complex with the diheme cytochrome *c* NapB (enzyme 2a in Table 2; see Fig. 3 for models on different Nap systems from representative organisms). Generally, mature NapA is transported across the membrane by the Tat apparatus and this process requires the cytoplasmic chaperone NapD, which is encoded in all known *nap* gene clusters [23]. At least two independent membrane-bound quinol-oxidizing systems have evolved that are specific to Nap systems (Fig. 3). These are (i) the tetraheme cytochrome *c* NapC, a member of the NapC/NrfH family (see Section 5.1.4) and (ii) the NapGH complex which consists of two proposed Fe/S proteins (see Section 5.1.2). NapH is a membrane-bound quinol dehydrogenase containing four transmembrane domains while NapG is a periplasmic electron transfer adapter protein. In many, though not all cases, NapGH is accompanied by another iron sulfur protein, NapF, which is thought to bind to NapH at the cytoplasmic side of the membrane (Fig. 3). The function of NapF is unclear but it was hypothesized that it provides reductive power for NapA maturation in the cytoplasm

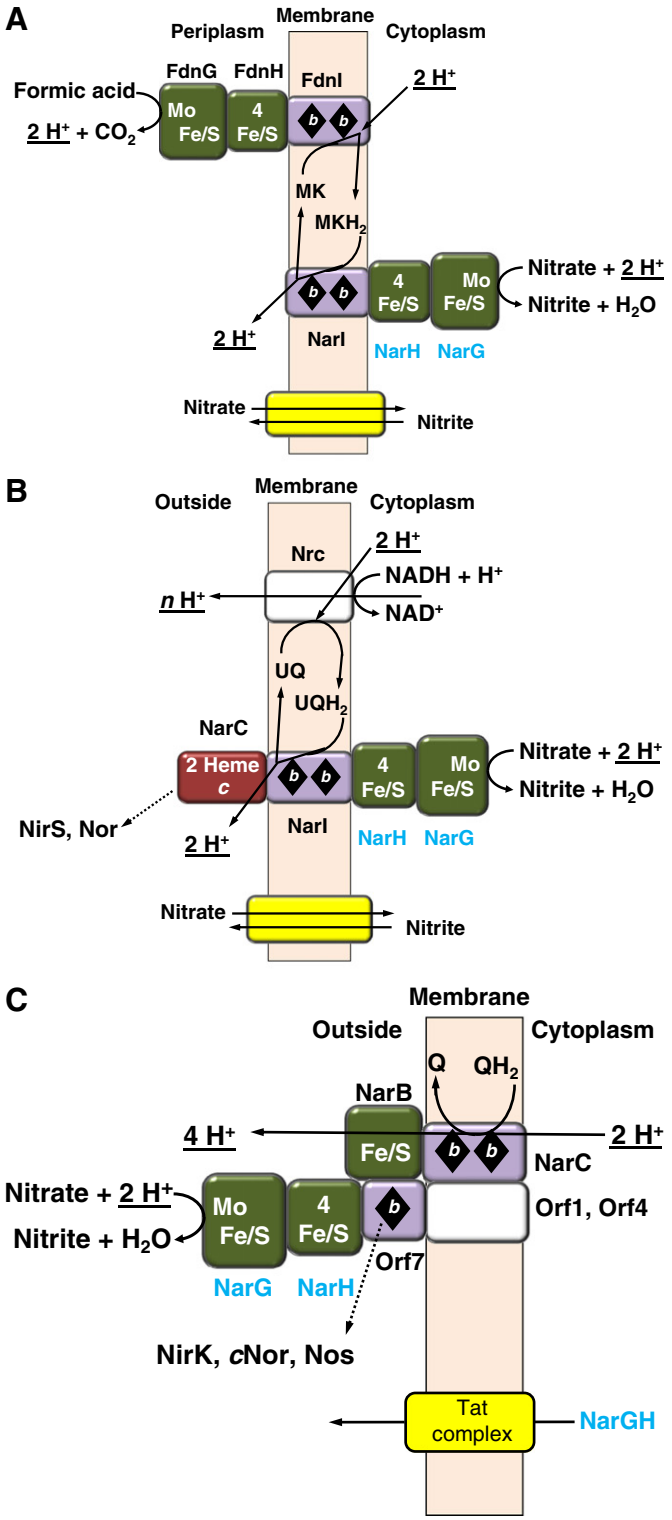

Fig. 2. Model of electrogenic ETCs in Nar-dependent nitrate reduction. A. ETC from formate (formic acid) to nitrate in *E. coli* using the nNar enzyme. B. ETC from NADH to nitrate in *T. thermophilus* using the *c*Nar enzyme. Note that the Nrc and *c*Nar complexes are likely to form a supercomplex. C. Electron transport from quinol to nitrate in haloarchaeal species using the pNar enzyme. See text and Table 2 for details. Electrogenic protons are underlined. Dashed arrows denote speculative reactions. For simplicity, only monomeric enzyme forms are shown. Mo, molybdenum-*bis*-molybdopterin guanine dinucleotide cofactor; Fe/S, iron-sulfur center; *b*, heme *b*.

[24–27]. For *Escherichia coli*, it was shown that NapC acts as a menaquinol dehydrogenase whereas the NapGH complex functions as ubiquinol dehydrogenase [28]. Notably, a *napC* gene is usually absent from epsilonproteobacterial *nap* gene clusters [29,30]. *Shewanella oneidensis* is likely to use CymA, an analogue of NapC, as electron donor to NapAB (see also Section 5.1.4). However, CymA is not specific for the Nap system as it feeds electrons into numerous other respiratory systems including the Nrf system of nitrite reduction (see Section 3.2) [31]. Independently of the architecture of a particular Nap system, it appears that all these assemblies work in a non-protonmotive fashion. In other words, there is no experimental support that Nap-dependent quinol oxidation by nitrate generates a *pmf*, in contrast to what has been shown or predicted for any of the three Nar systems described above. If true, the main function of the Nap system is nitrate-dependent regeneration of quinones that are reduced in the course of one or more electrogenic reactions (see Section 4). In addition, the Nap system has been implicated in N assimilation [32,33] and it is not clear whether the quinol oxidation function or the assimilatory function was the primary evolutionary pressure (see Sections 5 and 6).

3.2. Nitrite reductases

Respiratory nitrite reductases are categorized here according to their reaction product, which either is nitric oxide (enzymes 3 and 4 in Table 2) or ammonium (enzymes 5–8) (Fig. 1). Both classes usually reside outside the cytoplasm (e.g. in the periplasm in Gram-negative bacteria or between the cell membrane and the cell wall/S-layer in Archaea). NO-genic nitrite reductases are either cytochromes cd_1 (NirS, containing the unusual cytochrome d_1 at the active site) or copper-dependent enzymes (NirK) that typically contain two copper centers (Cu_I and Cu_{II}). In most cases, these enzymes draw electrons from a periplasmic pool of electron-transferring proteins that consists of (i) various forms of small copper-containing proteins named azurin, pseudoazurin or cupredoxin and (ii) numerous monoheme and diheme cytochromes *c* like cytochromes c_{550}, c_{551}, c_{552} or c_{553} (Fig. 4) [34]. A complex of *Achromobacter xylosoxidans* NirK and cytochrome c_{551} demonstrated the presence of a hydrophobic electron transport pathway at the docking interface of both proteins [35]. The redox mediator pool is reduced by the cytochrome bc_1 complex, which makes electron transport from quinol to nitrite electrogenic. An exception is *Pseudomonas stutzeri* that is thought to employ the tetraheme cytochrome *c* NirT protein, a member of the NapC/NrfH family, as the quinol dehydrogenase proposed to directly reduce NirS [36]. In this set-up, it is thought that quinol oxidation by nitrite is electroneutral. Variations of the NirK enzyme were reported that either contained a C-terminal monoheme cytochrome *c* domain of about 160 to 190 residues or an N-terminal cupredoxin domain (enzymes 4b and 4c in Table 2) [37]. Both domains are likely to serve in electron transfer to the core portion of NirK (Fig. 4B).

The best-known ammonium-generating nitrite reductase is the pentaheme cytochrome *c* nitrite reductase, NrfA, of which several forms exist (enzymes 5a–d in Table 2) [38]. Depending on the organism, NrfA was described to be a soluble periplasmic protein (for example in the Gammaproteobacteria *E. coli* and *S. oneidensis*) or as a subunit of a membrane-bound menaquinol-reactive complex formed by NrfA and a tetraheme cytochrome *c* of the NapC-type called NrfH (Fig. 5) [39–41]. Such NrfHA complexes are present in Delta- and Epsilonproteobacteria. NrfA catalyzes the six-electron reduction of nitrite to ammonium but commonly also converts alternative substrates like nitric oxide, hydroxylamine, hydrogen peroxide and sulfite [38,42]. This reactive promiscuity of NrfA appears to be beneficial under corresponding stress conditions, indicating that NrfA has a detoxifying function in cell physiology [43–46]. The NrfHA complex was shown to catalyze electroneutral menaquinol oxidation by nitrite in the Epsilonproteobacterium *Wolinella succinogenes* (Fig. 5A) [39]. In the absence of a NrfH-type quinol dehydrogenase, however, nitrite ammonification in *S. oneidensis* depends on CymA, the structural and functional analogue of NrfH mentioned above [31]. On the other hand, enteric bacteria like *E. coli* employ an assembly consisting of the proteins NrfB, NrfC and NrfD to communicate with

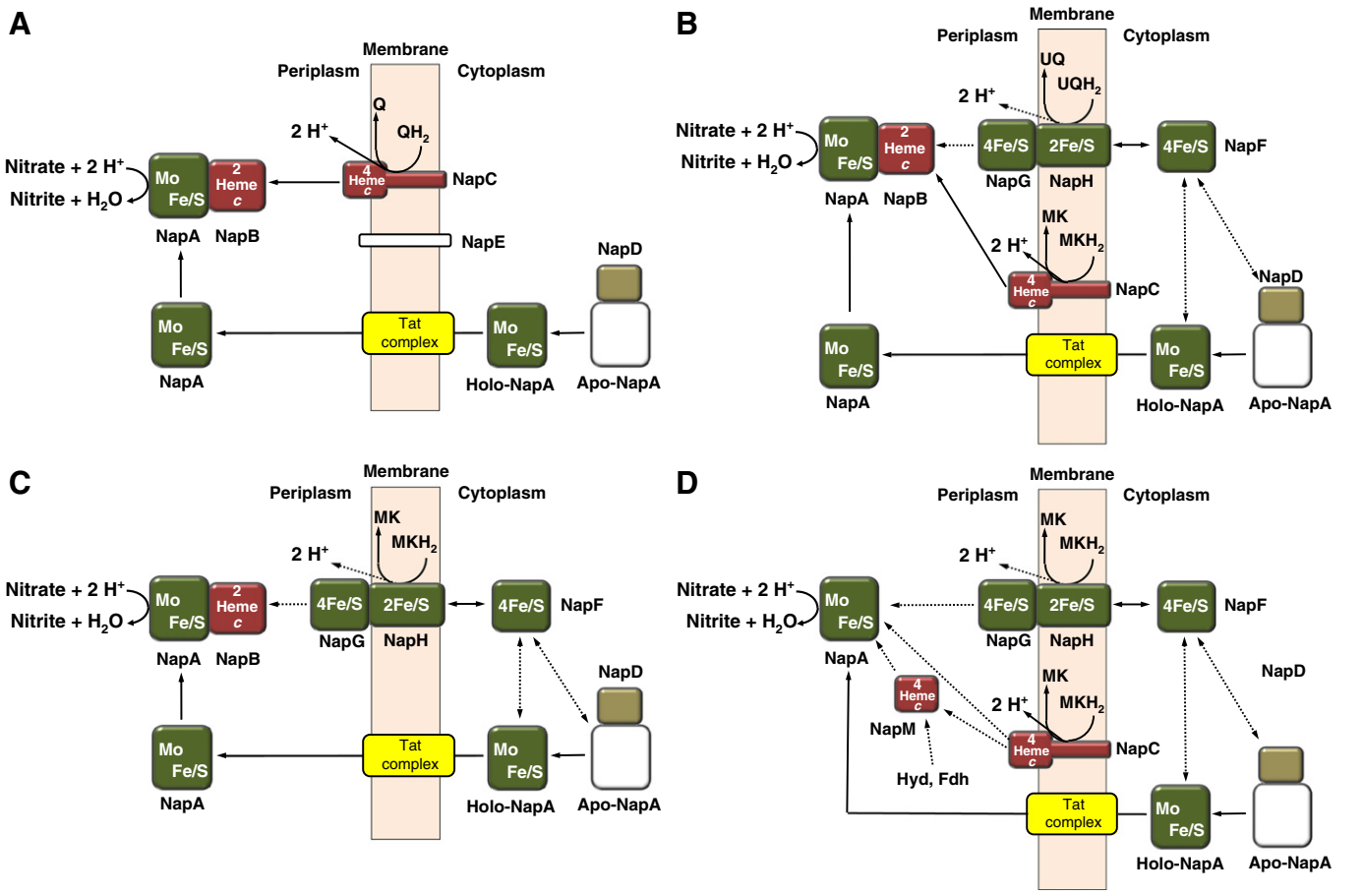

Fig. 3. Model of respiratory Nap systems. A. Nap system of *P. pantotrophus*. B. Nap system of *E. coli*. C. The Nap system of *W. succinogenes* which is representative for NapC-lacking Epsilonproteobacteria. D. Nap system of *Desulfovibrio desulfuricans* lacking NapB [201]. See text and Table 2 for details. For further explanations see legend of Fig. 2.

the menaquinol pool (Fig. 5B). Deduced from genetic studies, a membrane-bound menaquinol-oxidizing NrfCD complex was postulated, which is similar to the structurally resolved PsrBC subcomplex of a potential polysulfide reductase from *T. thermophilus* (see Section 5.1.3) [47]. This structure established that a member of the NrfD/PsrC family contains a quinone/quinol binding site near the periplasmic membrane surface [48]. To date, there is no experimental evidence that a member of the NrfD/PsrC family is involved in *pmf* generation. The electron transfer between NrfCD and NrfA in *E. coli* is mediated by the pentaheme cytochrome *c* NrfB, which is distantly related to NrfH (Fig. 5B) [49–51].

Apart from NrfA, several octaheme cytochromes (enzymes 6–8 in Table 2) have been described that, amongst other substrates, convert nitrite to ammonium ([38] and references therein). These cytochromes *c* are part of a taxonomically widely distributed multiheme cytochrome *c* (MCC) superfamily that includes NrfA, hydroxylamine oxidoreductase (Hao) and hydrazine oxidoreductase (Hzo) (see Sections 3.6 and 5.2.1) [52,53]. Especially intriguing is the so-called εHao (enzyme 8 in Table 2) that is encoded in some epsilonproteobacterial genomes that were described as ammonifiers despite lacking the *nrfA* gene [8,32,54]. Some, but not all, εHao enzymes are encoded within gene clusters that also encode a member of the NapC/NrfH family.

3.3. Nitric oxide reductases and nitric oxide dismutase

Genomic analyses of microbes implicated in NC transformations have revealed that the molecular diversity of inventory within the nitric oxide conversion module is by far the greatest among all nitrogen cycle modules and this central role of nitric oxide in the nitrogen cycle will be further discussed in Sections 5.2 and 6 [1,55,56]. Respiratory nitric oxide-reducing enzymes either convert nitric oxide to nitrous oxide (Nor; enzymes 10a–d in Table 2) or comproportionate nitric oxide and ammonium into hydrazine (Hzs; enzyme 11) or dismutate two molecules of NO into N_2 and O_2 (Nod; enzyme 12). Moreover, several evolutionarily and structurally unrelated soluble microbial enzymes were reported to convert nitric oxide to either nitrate [flavohemoglobin-NO-dioxygenase (Hmp)], nitrite [hydroxylamine oxidoreductase; cytochrome P460 (CytL)] or nitrous oxide [flavodiiron proteins (Fdp), flavorubredoxin (NorVW), cytochrome c_{554} (CycA), cytochrome *c*′-beta (CytS) and cytochrome *c*′-alpha (CytP)] [45,57–61]. These enzymes are thought to mediate nitrosative stress defense, thus serving primarily in detoxification [44,62]. Notably, cytochrome *c* nitrite reductase NrfA was reported to reduce nitric oxide to ammonium or nitrous oxide (see Section 3.2), a capability that might be widespread in the MCC family [38,63].

Membrane-bound nitrous oxide-generating NO reductases (Nor enzymes) catalyze nitric oxide reduction at the outside of the cytoplasmic membrane and several such enzymes from denitrifying Proteobacteria, Firmicutes and Archaea have been characterized [64–66]. The best known NO reductases (*c*Nor and qNor; enzymes 10a and 10b in Table 2) either use cytochrome *c*/cupredoxins (Fig. 4A) or quinones as immediate redox partners and both belong to the superfamily of HCOs (see Section 5.2.3). The catalytic site of nitric oxide reduction harbors a dinuclear heme b_3::Fe_B active site that is reduced by another heme *b* group bound by the same protein (named NorB). In *c*Nor enzymes, NorB receives electrons from the monoheme cytochrome *c* subunit NorC while qNor enzymes are quinol-reactive single-subunit enzymes that resemble NorB. High-resolution crystal structures of *Pseudomonas aeruginosa* *c*Nor and *Geobacillus stearothermophilus*

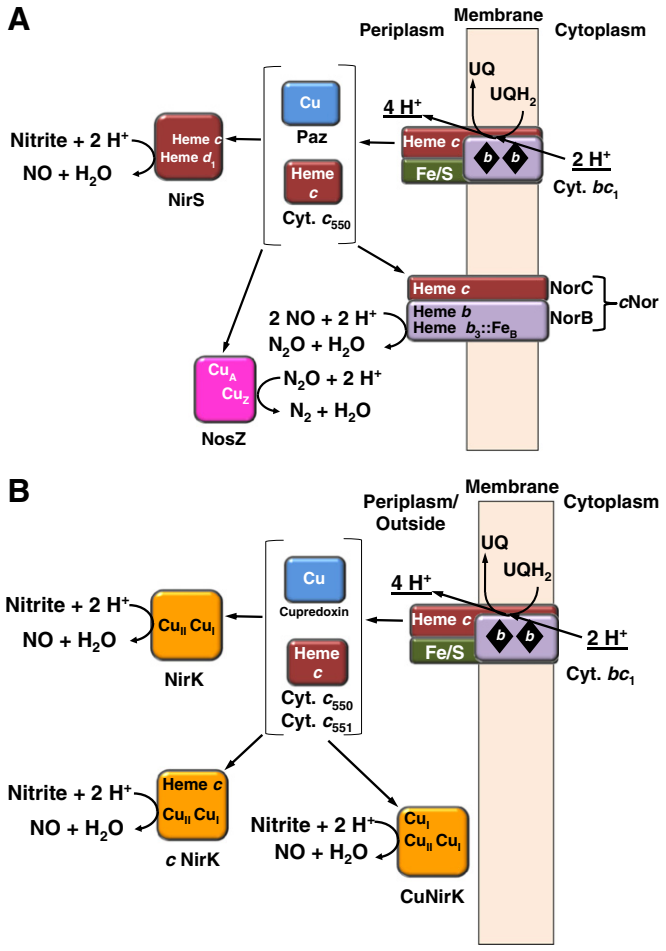

Fig. 4. Model of ETCs in denitrifying bacteria. A. ETC from ubiquinol to nitrite, nitric oxide and nitrous oxide in *P. denitrificans*. B. ETC from ubiquinol to different NirK-type nitrite reductases. Ubiquinol is reduced by NADH dehydrogenase, succinate dehydrogenase and/or acyl CoA dehydrogenase. Cu_Z and Cu_A denote the binuclear Cu_A and tetranuclear Cu_Z centres of NosZ while the distinct copper centers of NirK are abbreviated Cu_I and Cu_{II}. Heme b_3::Fe_B refers to the dinuclear NO-reducing reaction center of NorBC. Paz, pseudoazurin. See text and Table 2 for details. For further explanations see legend of Fig. 2.

qNor have been reported recently [67,68]. NorB proteins contain 12 transmembrane helices while NorC is anchored to the membrane by a single membrane-spanning segment. In *Paracoccus denitrificans*, pseudoazurin or cytochrome c_{550} were found to donate electron to the NorC subunits of a heterotetrameric $(NorBC)_2$ complex [69]. There is no indication that the *c*Nor complex contributes to *pmf* generation, i.e. that it takes up protons from the cytoplasmic side of the membrane. The so-called D- and K-channels that serve this function in oxygen-reducing HCOs are absent in *c*Nor enyzmes. Genes encoding "*cc*NorC" proteins with a second heme-binding domain fused to the usually monohome cytochrome c_2-type NorC protein have been identified in genomes of *Acidovorax* sp. JS42, *Anaeromyxobacter* sp. Fw109-5, *Bdellovibrio bacteriovorus* HD100, *Nitrosospira multiformis* ATCC 25196 and *Thiobacillus denitrificans* ATCC 25259, but no biochemical studies with these proteins have been reported.

In contrast to *c*Nor, qNor enzymes are reactive with ubiquinol and/or menaquinol and contain an N-terminal extension that is absent from NorB in the *c*Nor complex. While this N-terminal extension shows similarity to NorC, a heme *c*-binding motif is lacking. The crystal structure of *G. stearothermophilus* qNor revealed a water channel from the cytoplasm that might serve in proton delivery [68]. Thus, the possibility that qNor might catalyze electrogenic quinol oxidation coupled to nitric oxide reduction cannot be excluded. An unusual qNor subgroup (Cu_A-qNor), exemplified by the enzyme from *Bacillus azotoformans* (enzyme 10c in Table 2), contains NorB in a complex with a subunit harboring a Cu_A site (typically found in oxygen-reducing HCOs), which makes this enzyme competent in receiving electrons from membrane-bound cytochrome c_{551} in addition to the menaquinol pool [70]. Versions of complex IV HCO-type enzymes with NO reduction activity that differ in critical residues in their active sites have been described (enzymes 10d in Table 2), two of which have been termed sNor (predominantly in nitrogen- and sulfur-catabolic chemolithotrophic bacteria [71]) and gNor (predominantly in sulfur-catabolic chemolithotrophic bacteria ([30,72], see also Section 5.2.3).

Hydrazine synthase (Hzs; enzyme 11 in Table 2) is a key player is the anammox process. Hzs reductively combines nitric oxide (generated by a nitrite reductase) and ammonium to produce hydrazine, which is a central intermediate in the catabolism of anammox bacteria (Fig. 6) [73]. A heterotrimeric complex displaying Hzs activity was purified from the soluble extract of *Candidatus* Kuenenia stuttgartiensis cells [73]. Two of the three proteins (HzsABC, named kuste2859, kuste2860 and kuste2861) are predicted diheme cytochromes *c* and it is thought that the HzsABC complex is located in the anammoxosome lumen, alongside with NirS and hydrazine oxidoreductase (Hzo; see Section 3.6) (Fig. 6). In this scenario, *pmf* generation by anammox bacteria solely relies on the cytochrome bc_1 complex and the electron flow is solely cyclic (see also Section 3.6) [56,73]. The genome of *Candidatus* Scalindua profunda contains a fusion gene that corresponds to the genes encoding kuste2859 and kuste2860 in *Candidatus* K. stuttgartiensis [74].

Finally, a novel nitric oxide-converting enzyme was proposed to act as a crucial enzyme in anaerobic denitrifying methanotrophs [75–77]. These organisms oxidize methane with nitrite as electron acceptor according to the equation $3\,CH_4 + 8\,NO_2^- + 8\,H^+ \rightarrow 3\,CO_2 + 4\,N_2 + 10\,H_2O$. In corresponding enrichment cultures a dominant bacterium belonging to the NC10 phylum was identified and named *Candidatus* Methylomirabilis oxyfera [78]. This organism is assumed to produce N_2 from nitric oxide (produced from nitrite by either a NirS-type or an εHao-type nitrite reductase) using the suggested enzyme nitric oxide dismutase (Nod, enzyme 12 in Table 2; Fig. 7), thus bypassing nitrous oxide as a denitrification intermediate [76]. The Nod enzyme would produce N_2 and O_2 in equal amounts from nitric oxide ($2\,NO \rightarrow N_2 + O_2$). Three quarters of the internally produced oxygen is thought to serve in methane oxidation to CO_2 while the remaining oxygen is probably reduced to water by a cytochrome *bo*-type quinol oxidase [77]. To date, the nature of the Nod enzyme has not been revealed but it is striking that the genome of *Candidatus* M. oxyfera encodes three putative

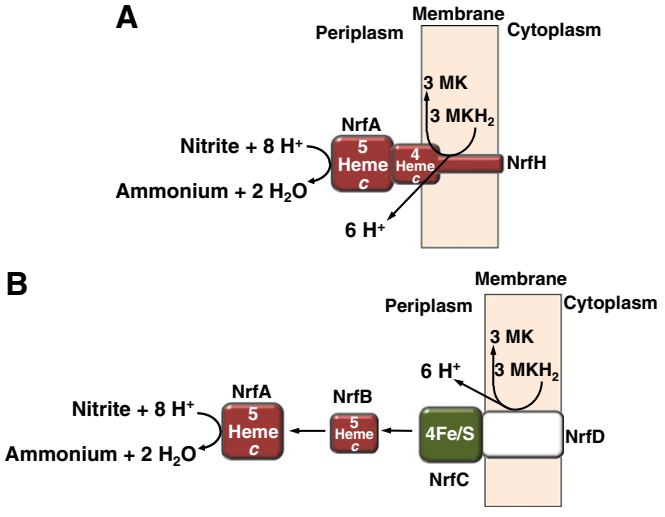

Fig. 5. Model of respiratory Nrf systems. A. Nrf system of *W. succinogenes*. B. Nrf system of *E. coli*. See text and Table 2 for details. For further explanations see legend of Fig. 2.

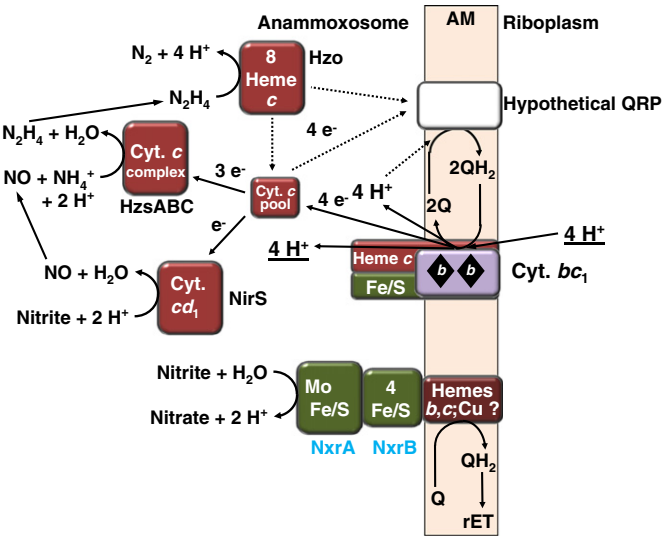

Fig. 6. Model of electron transport in the anammox process of *Candidatus* K. stuttgartiensis. The composition of the cytochrome bc_1 complex is speculative. See text and Table 2 for details. For further explanations see legend of Fig. 2. AM, anammoxosome membrane; rET, reverse electron transport.

membrane-bound qNor-like enzymes that are expected to be associated with nitric oxide turnover in this organism [76]. It is assumed that NirS and Nod are not directly involved in *pmf* generation (Fig. 7). Instead, the genome of *Candidatus* M. oxyfera encodes a membrane-bound NADH dehydrogenase (complex I), a cytochrome bc_1 complex (complex III) and several complex IV-type terminal oxidases of the HCO family, thereby employing classic electrogenic electron transport modules [76,77].

3.4. Nitrous oxide reductases

The canonical nitrous oxide reductase of denitrifiers is a homodimeric copper protein designated NosZ (enzyme 13a in Table 2). The periplasmic NosZ receives electrons from the cytochrome *c*/cupredoxin pool in an electroneutral process, thus resembling nitrite and nitric oxide reduction (Fig. 4A). From a bioenergetic point of view, it is therefore not surprising that many denitrifiers reduce nitrate or nitrite only to the level of nitrous oxide due to the absence of a *nosZ* gene [79]. NosZ contains two redox-active copper centers, termed Cu_A (dinuclear copper site) and Cu_Z (tetranuclear copper site) [80–82]. In the functional homodimer, the

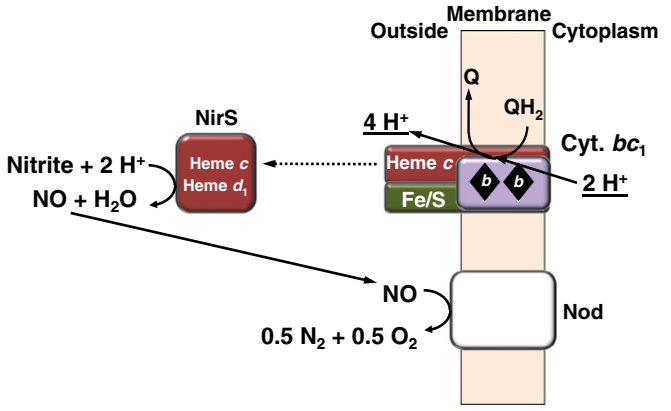

Fig. 7. Model of respiratory nitrogen metabolism during oxygenic denitrification of nitrite by the methanotroph *Candidatus* M. oxyfera. Oxygen produced by Nod supposedly serves in methane oxidation (75%) as well as in respiratory reduction/detoxification by a cytochrome *bo*-type quinol oxidase (25%). See text and Table 2 for details. For further explanations see legend of Fig. 2.

Cu_A electron input site of one monomer is in close contact with the catalytic Cu_Z center of the partner subunit.

The nitrous oxide reductase from *Wolinella succinogenes* and other Epsilonproteobacteria contains a C-terminally fused monoheme cytochrome *c* domain, which is thought to donate electrons to the Cu_A site (enzyme 13b in Table 2) [83,84]. Such *c*NosZ enzymes are encoded in gene clusters that also contain *nosG*, *-C1*, *-C2* and *-H* genes which were postulated to encode a putative menaquinol dehydrogenase pathway to *c*NosZ alternative to the conventional cytochrome bc_1 complex (Fig. 8A). This pathway comprises a NapGH-type menaquinol dehydrogenase (NosGH, see Section 5.1.2) and two monoheme cytochromes *c* (NosC1 and NosC2) [84]. Several *nos* gene clusters from α-, β- and γ-Proteobacteria encode two FMN-binding flavoproteins (NosR and NosX) that might constitute yet another electron transport pathway from the quinone pool to NosZ (Fig. 8B). NosR resembles NosH but contains an additional periplasmic FMN-binding domain [85]. Despite these variations, no ETC that connects the quinone/quinol pool and NosZ/*c*NosZ has been reported to be electrogenic.

3.5. Ammonia monooxygenase

The ammonia monooxygenase of aerobic obligate chemolithotrophic bacteria and thaumarchaeota is a complex of three membrane proteins: the heterotrimeric $(AmoABC)_3$ complex (referred to as Amo in Fig. 9) [86–88]. The active site of the bacterial Amo complex contains non-heme iron and at least one copper center that facilitate the oxidation of ammonia to hydroxylamine, a highly reactive NC that is released into the periplasm and oxidized by the multiheme cytochrome *c* hydroxylamine oxidoreductase Hao (see Section 3.6) [89]. When compared to the bacterial AmoB protein, the thaumarchaeal Amo has a slightly different structure as its AmoB protein, which harbors the catalytic site, lacks one cupredoxin domain and the C-terminal transmembrane spanning domain [90]. In addition, thaumarchaeal genomes do not encode known

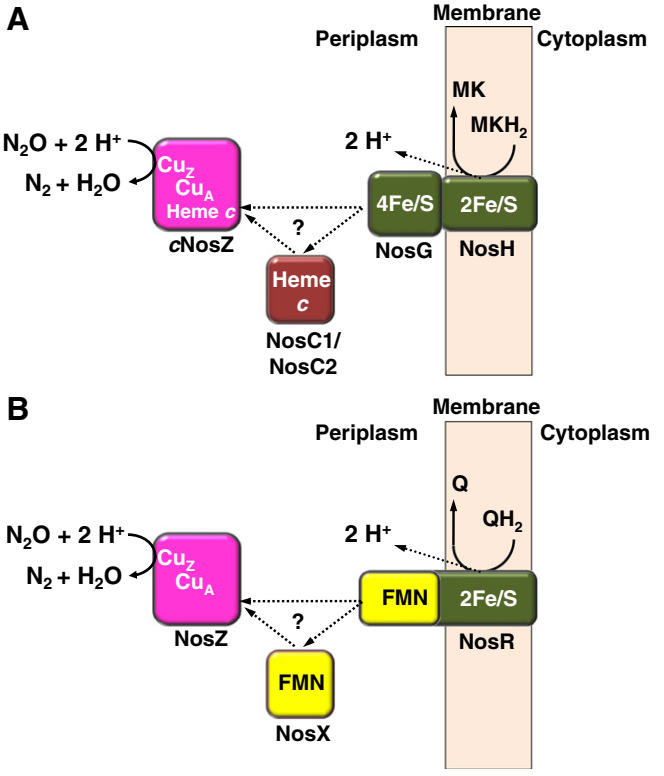

Fig. 8. Model of alternative electron transport routes to nitrous oxide reductase. A. NosGH-dependent ETC from menaquinol to *c*NosZ in *W. succinogenes*. B. NosR-dependent ETC from quinol to NosZ in *P. stutzeri*. See text and Table 2 for details. For further explanations see legends of Figs 2 and 4.

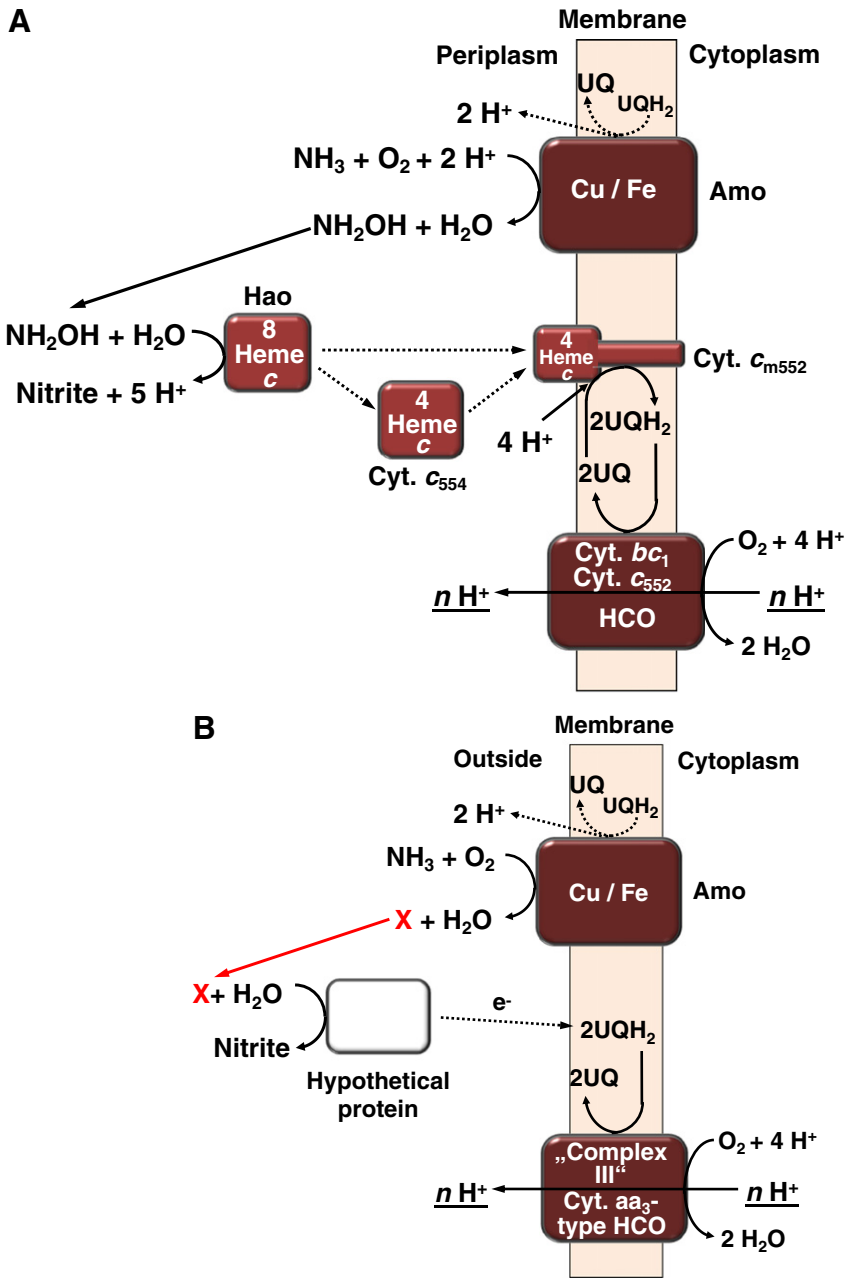

Fig. 9. Model of ETCs in ammonium-oxidizing bacteria (genus *Nitrosomonas*; A) and ammonium-oxidizing archaea (genus *Nitrosopumilus*; B). The red "X" in subpanel B denotes a speculative intermediate which might be hydroxylamine or nitroxyl (HNO). See text and Table 2 for details. For further explanations see legend of Fig. 2.

hydroxylamine detoxifying enzymes and they apparently lack the ability to produce cytochromes *c*. It is thus not clear whether thaumarchaeal Amo produces hydroxylamine or another reactive NC such as nitroxyl (HNO) (Fig. 9B) [90,91]. It has been proposed that the Amo protein complex initiates ammonia oxidation to hydroxylamine when oxygen is activated with two electrons and it has been proposed that these electrons are obtained from the quinol pool whereby electrogenic electron flow was not excluded [89]. However, these authors also stated that there was no experimental evidence for a quinol dehydrogenase function of the Amo complex nor a contribution to *pmf* and to this day, experimental evidence for either proposal is still elusive. Newer models suggest an alternative in form of monooxygenase activation by NO, which is based on observations that relate expression and activity of NO-genic NirK to ammonia monooxygenation in Bacteria [4,92] and Archaea [93,94].

The clustered *amoCAB* genes encoding the subunits of the bacterial Amo complex were believed necessary and sufficient for Amo synthesis and function [86,95–97]; however, the *amoC*, *-A* and *-B* genes were recently found as members of co-regulated overlapping operons (*amoCABD*, *amoCAB*, *amoAB*, *amoC*, *amoED*), which differ in number [55] and regulation between beta- [98,99] and gammaproteobacterial [100] ammonia-oxidizing bacteria (AOB). In Beta-AOB, the *amoD* gene (which encodes a potentially monotopic membrane protein of unknown function) is found in tandem with a likely duplicated orthologue (*amoE*) downstream of the *amoCAB* genes [101] and first expression experiments suggest that *amoED* is co-regulated and not part of the same operon as *amoCAB* [102]. In Gamma-AOB, the *amoD* gene is expressed only as a member of the *amoCABD* operon and significantly higher transcript levels of *amoC* as compared to *amoCABD* and *amoAB* are achieved by differential regulation (initiation and termination) of the *amoCABD* gene cluster [100]. In contrast, genomes of Beta-AOB encode multiple copies of non-operonal *amoC* genes [55,97] that have been implicated in recovery from ammonia starvation [102]. All AOB encode also *amoD*

singletons [101] whereas singleton *amoA* and *amoB* genes have not yet been found in any AOB genome. Interestingly, orthologues of *amoD* (but not *amoE*) are also encoded by genomes of aerobic nitrifying methanotrophs where they reside either downstream of the gene cluster encoding particulate methane monooxygenase (pMmo) in Alphaproteobacteria (type-II methanotrophs) or in proximity of a gene tandem encoding blue copper oxidases in Gammaproteobacteria (type-I and -X methanotrophs). Homologues of bacterial *amoD* and *amoE* genes have only been found in genomes of nitrifying bacteria. While it is known for quite some time that the Amo and pMmo complexes are encoded by homologous genes [103], it is only clear since recently that both enzymes are members of a superfamily of copper-dependent membrane monooxygenases (Cu-MMOs) with significant promiscuity for reduced carbon and nitrogen compounds (see Section 5.2.4). Based on this homology, it is likely that ammonia oxidation facilitated by Amo operates identical to methane oxidation facilitated by pMmo, whose crystal structure has been resolved and whose biochemistry has thus been extensively studied [104,105]. To this day, Amo has not been crystallized.

The thaumarchaeal Amo differs from its bacterial homologue not only in structure and, maybe, in function, its genetic basis is also significantly different. While detailed *amo* gene expression studies are not yet available, the organization of thaumarchaeal *amoA*, *amoB* and *amoC* genes in the genome varies between individual thaumarchaeal lineages and they are not clustered in the canonical bacterial order *amoCAB* or are present in more than one copy [90,91,106].

3.6. Hydroxylamine and hydrazine oxidoreductases

The capacity of AOB to aerobically catabolize ammonia as their sole source of energy and reductant requires another specialized protein complex in addition to Amo: hydroxylamine oxidoreductase (Hao) as well as redox-active cytochromes *c*, which relay the electrons extracted from hydroxylamine to the quinone pool (Fig. 9A) [86,89,107]. The oxidation of hydroxylamine to nitrite is catalyzed in the periplasm by Hao, which consists of three octaheme cytochrome *c* monomers (HaoA) cross-linked between a tyrosine residue and the active site heme *c* group of the neighboring monomer [89,108]. The circular symmetric arrangement of the $HaoA_3$ complex is regarded as a prerequisite for the oxidative chemistry of the enzyme [109,110]. Hao lacking the critical tyrosine residue failed to oxidize hydroxylamine to nitrite [89].

Based on experiments that evidenced *in vitro* reduction of cytochrome c_{554} (CycA) by Hao [111], modeling of this interaction [112,113] as well as demonstration of quinone reductase function of cytochrome c_{m552} (CycB) [50], an early linear model of electroneutral electron flow from hydroxylamine to quinone ($Hao \rightarrow c_{554} \rightarrow c_{m552}$) had been established (Fig. 9A). The observed clustering of Hao- and cytochrome c_{554}- and c_{m552}-encoding genes in genomes of all AOB [55] along with the proposed interaction of their products led to a designation of the "Hydroxylamine Ubiquinone Redox Module" (HURM), even though a redox interaction between cytochromes c_{554} and c_{m552} *in vitro* or during ammonia-oxidation to nitrite in AOB as well as the functionality of the redox chain in the absence of either cytochrome has never been experimentally established [56]. Therefore, a direct interaction of HaoA and cytochrome c_{m552} cannot be excluded (Fig. 9A). Cytochrome c_{m552} is related to the NapC/NrfH QRP family (see Section 5.1.4) and it is therefore likely that quinone reduction is achieved using protons taken up from the periplasmic space. If true, this would make HURM-catalyzed hydroxylamine oxidation by quinone an electroneutral process (Fig. 9A). Interestingly, cytochrome c_{554} has a mild NO reductase activity although it is unclear whether this is physiologically relevant [61]. Nevertheless, clustering of cytochrome c_{554} (*cycA*)-like genes with homologs of the *napC/nrfH* gene family in genomes of Bacteria that are not AOB suggest potential for such interaction in form of a quinol-reactive nitric oxide reduction module [114].

It is significant to mention that HURM, which contains a QRP as an essential module element, has been identified thus far only in those nitrifying bacteria that can support growth based on catabolism of ammonia as the sole source of energy and reductant. All other nitrifying bacteria such as methane-oxidizing Proteobacteria and Verrucomicrobia lack HURM (in particular, a homologue of *cycB*) and they need additional sources of energy and reductant [114]. The ammonia-based HURM genes are organized in a conserved gene cluster, *haoAB-cycAB* [51,114], however as in the case of Amo-encoding genes, HURM-encoding genes are differentially expressed in Beta- and Gamma-AOB. Previous studies with the Beta-AOB *N. europaea* suggested that the *haoA* gene and the *cycAB* genes are expressed independently, and no evidence for the transcription of *haoB* (orf2) was found [115,116]. In contrast, studies of the transcriptional response of the Gamma-AOB such as *Nitrosococcus oceani* ATCC 19707 to ammonia suggested the presence of a steady-state mRNA that included all four genes; nevertheless, basal expression produced independent *haoAB* and *cycAB* transcripts [117]. Interestingly, ammonia and hydroxylamine, although both sources of energy and reductant, differentially induced gene expression in AOB including *amo* and *hao* genes [118]. Ammonia also induced expression of an *haoAB* gene tandem in the nitrifying methanotroph, *Methylococcus capsulatus* Bath [119], which provided the initial designation of the first two genes in the HURM gene cluster as *haoAB* despite thefact that *M. capsulatus* lacks *cycAB* genes and thus HURM. Analysis of *haoA* gene expression in *N. europaea* strain ENI-11 revealed differential regulation and identified the one *haoA* gene copy not located in the vicinity of the two *amoCAB* operons as being expressed at the highest level and as the sole *haoA* gene in cells denied an energy source [120].

Anaerobic ammonia-oxidizing (anammox) bacteria also harbor significant Hao activity and proposals in the pre-genomics era suggested that Hao participated in the anammox process with a dual function by also oxidizing hydrazine [121,122]. The first genome sequence of an anammox bacterium did not only lead to a correction of the initially proposed biochemistry of the anammox process, which established NO as the critical intermediate, but also unraveled a wealth of unexpected inventory with dedicated functions in hydrazine synthesis and oxidation as well as nitrite and nitric oxide transformations (Fig. 6) [123–125]. Ongoing genome analysis and subsequent biochemical and physiological experiments confirmed the production of active Hao enzymes and it turned out that only one of the many encoded octaheme cytochromes *c* encoded in the *Candidatus* K. stuttgartiensis genome (kustec0694; named hydrazine oxidoreductase or Hzo; enzyme 16 in Table 2) was dedicated to hydrazine oxidation; an enzyme sensitive to hydroxylamine and nitric oxide [73]. However, it is unknown how electrons derived from hydrazine oxidation ultimately reach the cytochrome bc_1 complex and whether this process is electrogenic, e.g. by involving a quinone species (Fig. 6). This would leave the cytochrome bc_1 complex as the main coupling site in anammox metabolism. However, the *Candidatus* K. stuttgartiensis genome encodes three different cytochrome bc_1-type complexes whose biochemical properties await investigation (e.g. with respect to quinone reactivity) [123]. One of those (kuste4569-4574) was reported to be highly expressed and was speculated to function as a *pmf*-generating complex in the anammoxosome membrane [73]. It is noteworthy that the monoheme cytochrome c_1 of Proteobacteria might be functionally replaced by di-, hexa- or octaheme cytochromes *c* in different *Candidatus* K. stuttgartiensis bc_1-type complexes.

In contrast to *hao* genes in aerobic AOB, most genes encoding functional Hao and Hzo proteins in anammox bacteria are not clustered with genes encoding other cytochromes *c* or proteins that have been implicated in other redox and electron flow functions [73]. However, specific genetic differences provided tools for discriminating detection of *hzo* and *hao* genes in the environment, a difficult approach because *hzo* and *hao* genes are homologous [52,126]. Both Hao and Hzo belong

to the MCC family already mentioned in Section 3.2. The evolutionary history of the MCC family will be discussed in Section 5.2.1.

3.7. Nitrite oxidoreductase

Early work with nitrifying bacteria unraveled the critical function of a membrane protein complex termed nitrite oxidoreductase in obligate aerobic Proteobacteria able to grow on energy derived from the oxidation of nitrite to nitrate [127]. Initial genetic and biochemical experiments identified that these specialized nitrifiers required fairly high substrate concentrations due to the need for nitrite uptake and that the nitrite oxidoreductase complex was part of a simple respiratory electron flow involving an electrogenic cytochrome *c* oxidase HCO of the aa_3-type (Fig. 10A) [128]. Later discoveries of other aerobic nitrite oxidizers with different nitrite oxidation kinetics and taxonomic affiliations led to the discovery of new inventory, a clean-up in gene terminology (establishing Nxr as the designated term for nitrite oxidoreductase) and mind-boggling evolutionary relationships between this inventory (assumed to specifically operate in obligate aerobic bacteria) and the NC-transforming inventory in anammox bacteria [11,129–134]. It has been proposed for *Nitrobacter* species that nitrite is oxidized by a membrane-bound Nxr complex consisting of four subunits (NxrABIC; Fig. 10A; enzyme 9a in Table 2) in an arrangement similar to the *c*Nar complex (Fig. 2B) [132,133]. The NxrAB assembly contains molybdenum and iron and is thought to be located at the cytoplasmic surface of the membrane (therefore called nNxr here). NxrI is a membrane-bound diheme cytochrome *b* and NxrC is a diheme cytochrome *c*, which might act as an electron donor to the terminal oxidase (Fig. 10A). Given the positive standard potential of the nitrate/nitrite redox pair, it is unlikely that a quinone is involved in the respiratory ETC from nitrite to oxygen or that this process is electrogenic. Cytoplasmic nitrite oxidation necessitates nitrite import and nitrate export, both of which might be catalyzed by specific transporters of the proteobacterial NarK-type. Furthermore, a reversed ETC from the quinone pool (possibly reduced by nitrite through NxrI) to NADH via cytochrome c_{550}, the cytochrome bc_1 complex and a NADH:ubiquinone oxidoreductase (complex I) is predicted by the inventory of *Nitrobacter* genomes [132,133]. A quinone reductase activity of NxrI could also explain why some *Nitrobacter* species are able to grow anaerobically with nitrate as electron acceptor [135]. Produced nitrite is thought to ultimately end up in nitrous oxide trough the catalysis by NirK and a so far unidentified nitric oxide reductase (or by sNOR as found in *Nitrobacter hamburgensis* [133]).

In *Candidatus* Nitrospira defluviii (phylum Nitrospirae), the nitrite-oxidizing site of the NxrAB assembly is likely to be oriented

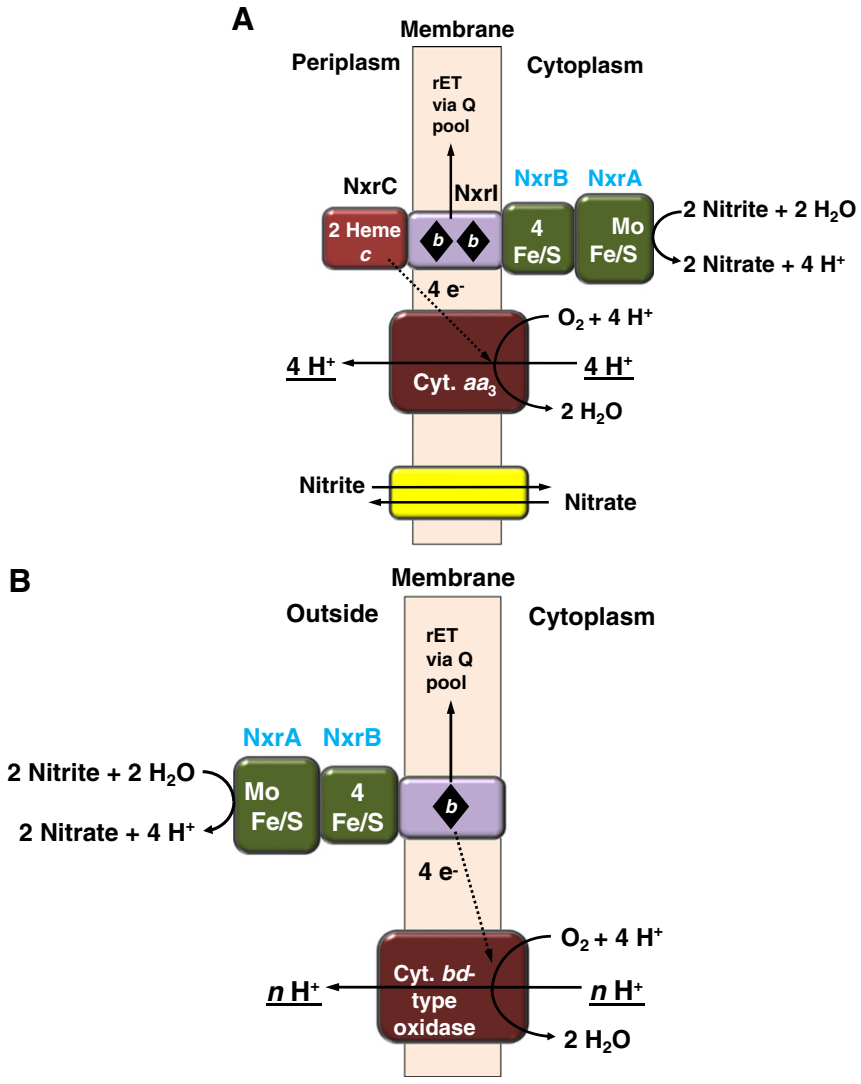

Fig. 10. Model of ETCs in different nitrite-oxidizing bacteria. A. Bacteria of the genus *Nitrobacter* (phylum Proteobacteria). B. Bacteria of the genus *Nitrospira* (phylum Nitrospirae). See text and Table 2 for details. For further explanations see legend of Fig. 2. rET, reverse electron transport.

to the outside of the cytoplasmic membrane (pNxr; enzyme 9b in Table 2; Fig. 10B) [11]. In this case, it is unknown how electrons are transported to the oxygen-reducing enzyme (probably a terminal oxidase of the cytochrome *bd*-type) or fed into the reverse ETC (Fig. 10B). The involvement of cytochrome(s) *b* and *c* cannot be excluded [11].

Fueled by early suggestions that proteobacterial nitrite oxidoreductase (NxrAB) and nitrate reductases (NarGH) have close genetic relationships and similar inhibitor profiles [136], recent *in silico* and experimental studies revealed that the Nxr complex in anammox bacteria (aNxr; enzyme 9c in Table 2; Fig. 6) facilitates anaerobic nitrite oxidation to nitrate, which provides energy and reductant needed for growth [73,74]. Similar to the closely related Nxr enzyme in *Candidatus* N. defluviii [11], the extracted electrons could flow via cytochromes to the quinol pool [123,124]. It has been proposed that the Nxr complex in anammox bacteria may also function reversely as a true nitrate reductase when oxidizing small organic molecules (e.g. proprionate) with nitrate as electron acceptor [137]. The genes proposed to encode the required soluble complement of mono- and diheme cytochromes *c* have been identified in *Candidatus* K. stuttgartiensis and in *Candidatus* S. profunda [74,134]. The phylogenetic relationship of the NarGH-NxrAB family is discussed in Section 5.2.2.

4. A comparative view on the design of bioenergetic systems in respiratory nitrogen metabolism

Despite the numerous differences concerning individual enzymes and electron transport pathways displayed in Figs 2–10, Section 3 has revealed some common elements of the bioenergetic systems in question. The most important fact is that almost all of them appear to depend on the presence of (i) quinones/quinols and (ii) cytochromes *b* and/or *c*. Furthermore, iron-sulfur centers are obligatory to the vast majority of the discussed metabolic processes and many different fusion proteins have been found that originated from the genetic combination of otherwise separate electron transfer modules like monoheme cytochromes *c* and cupredoxins. With the exception of the nNar, *c*Nar and nNxr systems, NC conversion takes place at the outside of the cytoplasmic membrane such as in the periplasm or in the lumen of stacks of intra-cytoplasmic membranes of Gram-negative bacteria (Proteobacteria, members of the NC10 phylum), in the anammoxosome of the Brocadiaceae or between the plasma membrane and the cell wall/S-layer in Archaea. Proven mechanisms of *pmf* generation discussed in Section 3 are the redox loop of nNar/*c*Nar nitrate reductase complexes and the Q-cycle that operates in cytochrome bc_1 complexes and alternative complex III-type structures (see Section 5.1.3) [138]. Additional protonmotive steps are frequently present, especially in microorganisms that grow chemoorganotrophically on carbohydrates, lipids, fatty acids, proteins and/or amino acids or by aerobic respiration (with externally present or internally produced dioxygen). Such cells usually contain proton-pumping enzyme complexes, such as NADH:quinone oxidoreductase and electrogenic HCOs, or redox loop enzymes other than Nar-type nitrate reductases [6]. A prominent example is the membrane-bound formate dehydrogenase complex of enteric bacteria that oxidizes formate, a common bacterial fermentation product, to yield carbon dioxide, a proton and two electrons (Fig. 2A). The architecture of this enzyme resembles that of nNar but with an "inverse" orientation resulting in periplasmic formate oxidation [13]. Therefore, protons are released in the periplasm and this process is coupled to proton uptake in the course of menaquinone reduction taking place at the cytoplasmic surface of the diheme cytochrome *b* membrane anchor subunit FdhC [6]. As with nNar, the H^+/e^- ratio of this mechanism is one. Heterotrimeric membrane-bound Ni/Fe-hydrogenases work according to the same principle [6,139–141].

Taken together, Nar complexes, i.e. enzymes 1a, 1b and 1c in Table 2, appear to be the only experimentally proven NC-transforming enzymes that couple substrate conversion directly to *pmf* generation (Table 3; see footnotes of Table 3 for possible exceptions to this rule). As described above and considering evolutionary history, the nNar and *c*Nar enzymes are thought to carry out a relatively recently invented redox loop mechanism due to the conversion of NarGH orientation from the outside to the inside of microbial cells (possibly combined with membrane attachment and the resulting direct link to quinol oxidation). All other bioenergetic systems presented in Table 3 rely on proton-pumping, the Q-cycle or redox loop enzymes that operate independent of, but are driven by, NC conversion. In these cases, maybe surprisingly, NCs serve as non-protonmotive electron sources or sinks or, in other words, in electroneutral redox balancing of, for example, the quinone/quinol pool. The Nap system of nitrate reduction and its versatile function in microbial physiology is a prime example for this fact (see Section 3.1). Since electroneutrally operating systems are unable to conserve the free energy of a redox reaction (which rather is lost as heat) and assuming that the non-protonmotive character of particular redox reactions was preserved during evolution, it appears that *pmf* generation (and hence ATP generation) was not a driving force during the evolution of such bioenergetic systems. In

Table 3
Bioenergetic coupling of electron transport chains involved in respiratory NC transformation.

Metabolic property and corresponding nitrogen compound transforming enzyme [a]	Involved enzymes or modules (*pmf*-generating modules in bold)	Is NC transformation directly coupled to *pmf* generation? [b]
1. Nitrate reduction by formate; nNar (1a)	**FDH complex (FdhGHI) → Q/QH_2 → nNar (NarGHI)**	**Yes** (redox loop; Fig. 2A)
2. Nitrate reduction by NADH; *c*Nar (1b)	**NADH dehydrogenase → Q/QH_2 → *c*Nar (NarGHIC)**	Presumably **yes** (redox loop; Fig. 2B)
3. Nitrate reduction by quinol; pNar (1c)	**QH_2 → pNar**	Presumably **yes** (potential Q-cycle; Fig. 2C)
4. Nitrate reduction by quinol; NapA (2a, 2b)	QH_2 → NapC/NapGH → (NapM) → NapA/NapAB	**No** [c] (Fig. 3)
5. NO-genic nitrite reduction by quinol; NirS or NirK (3, 4)	QH_2 → **cytochrome bc_1** [d] → ETP → NirS/NirK	**No** (Fig. 4)
6. Ammonifying nitrite reduction by quinol; members of the MCC family (5–8)	QH_2 → Member of the NapC/NrfH family or NrfBCD → cytochrome *c* nitrite reductase of the MCC family	**No** [e] (Fig. 5)
7. N_2O-genic nitric oxide reduction by quinol; *c*Nor (10a)	QH_2 → **cytochrome bc_1** → ETP → *c*Nor	**No** (Fig. 4A)
8. N_2O-genic nitric oxide reduction by quinol; qNor (10b)	QH_2 → qNor	Presumably **no** [f]
9. N_2-genic nitrous oxide reduction by quinol; Nos/*c*Nos (13a, 13b)	QH_2 → ETP/NosGH/NosR(X) → Nos/*c*Nos	**No** (Figs. 4A and 8)
10. Anammox reaction; NirS, Hzs, Hzo (3, 11, 16)	QH_2 → **cytochrome bc_1** → ETP → NirS/Hzs/Hzo → ETP → Q	**No** (Fig. 6)
11. Ammonium oxidation by oxygen; Amo, Hao (14, 15)	AmoABC → Q ← cytochromes *c* ← Hao; **QH_2 → cytochrome bc_1 → Oxidase**	**No** (Fig. 9A)
12. Nitrite oxidation by oxygen; NxrAB (9a, 9b)	NxrAB → cytochrome *b* (and *c*) → **Oxidase**	**No** (Fig. 10)

ETP, electron transfer proteins forming a redox mediator pool. These proteins comprise monoheme cytochromes *c* and/or small copper redox proteins (cupredoxins).
[a] Numbers in parentheses refer to enzyme designations given in Table 2.
[b] See Sections 3 and 4 for details.
[c] Unless the NapGH complex catalyzes electrogenic quinol oxidation (see Section 5.1.2).
[d] Exceptionally, NirT replaces the cytochrome bc_1 complex in *P. stutzeri* which would make this step electroneutral.
[e] Unless the NrfCD complex catalyzes electrogenic quinol oxidation (see Section 5.1.3).
[f] Unless qNor is a proton pump (see Section 3.3).

accordance with this assumption, it seems that bioenergetic systems are not optimized to obtain maximal H^+/e^- ratios.

It was recently suggested that selection for high-throughput redox systems that generate reactive and toxic intermediates was highly unlikely unless the genome already encoded significant detoxification capacity [142]. Following this line of thought, it is likely that many of the extant respiratory enzymes primarily served in detoxification of harmful NCs described in Table 1 (see also Section 6). Later on, higher concentrations of these substances and lateral acquisition of high-throughput production modules might have favored the adoption of such enzymes in catabolic metabolism in order to facilitate the function of already present protonmotive, i.e. ATP-producing mechanisms (like membrane-bound hydrogenases and formate dehydrogenases, NADH dehydrogenases, cytochrome complexes of the *bc*-type and terminal HCO-type oxidases).

5. The phylogeny of electron transport modules essential to bioenergetic systems of NC conversion

From the previous sections it is inferred that NC conversion depends on a finely tuned arrangement of suitable electron transport modules that likely have been interchanged during evolution of extant ETCs. In this section, knowledge on some prominent modules is reviewed and the phylogeny of distinct protein families is discussed.

5.1. QRP families

As described above, the recruitment of an NC converting enzyme is insufficient to drive a bioenergetic system unless the enzyme is connected to an electron transport network that commonly involves the quinone/quinol pool. It is generally agreed upon that quinones (in particular low-potential menaquinones), cytochromes (predominantly cytochromes *b* and *c*) and Fe/S cluster proteins have evolved early in the history of life [143–145]. Interestingly, functional analogues of evolutionary progenitors of the quinone-reactive cytochrome bc_1 complex are also thought to represent ancient protein assemblies that have always been functionally located at the core of bioenergetic systems [56]. Examples are a highly expressed gene cluster encoding a putative complex III in the anammoxosome membrane (proteins kuste4569-4574) ([73,123] and references therein) or the alternative complex III that evolved from the NrfD/PsrC-type QRP module (see Section 5.1.3) [138] Therefore, one of the key questions regarding the formation of NC-converting systems is how QRPs have evolved in order to create the versatile cyclic and/or linear electron transport-facilitating modules that they represent today. It is indisputable that genes encoding QRP modules have been shuffled during evolution to create new genetic contexts (module tinkering) and that these have probably been transferred extensively between individual genomic entities by horizontal gene transfer. Abstraction of bioenergetic systems involved in microbial NC transformations reveals four important QRP modules: the diheme cytochromes *b*, the NapH/NosH and NrfD/PsrC protein families and the NapC/NrfH family comprising several subfamilies of multiheme cytochromes *c* (see Table 4 for a compilation). Interestingly, the era of genomics has afforded us to recognize that these QRPs exist both as stand-alone modules and as parts of bioenergetically relevant protein complexes. The combination of QRPs was likely driven by both the evolution of the quinone pool as well as some prominent enzyme families involved in NC turnover, which will be discussed in Section 5.2.

5.1.1. Quinone/quinol-reactive diheme cytochromes b

Membrane-bound diheme cytochrome *b* QRPs are key components of several enzyme complexes serving in aerobic and anaerobic respiratory ETCs of many bacterial and archaeal species although detailed information on them usually derived from studies with proteobacterial model organisms. The reactivity of such cytochromes with both menaquinone and the evolutionary younger ubiquinone has been shown and many quinone-binding sites have been structurally resolved. Prominent examples are the cytochrome bc_1 complex as well as quinone-reactive membrane anchor subunits of nNar, *c*Nar, Ni/Fe-hydrogenase, formate dehydrogenase, succinate dehydrogenase and fumarate reductase (Table 4) ([6] and references therein). In addition, such cytochromes *b* also occur in cytochrome bo_3 and cytochrome *bd* quinol oxidases. The two heme *b* groups are located within the membrane plane, usually oriented to different sides of the membrane and axially ligated by histidine side chains that are provided by at least two different transmembrane helical regions (note that the distribution of histidine ligands is not conserved [139,146]). Redox partner proteins of diheme cytochromes *b* are mainly iron-sulfur proteins or *c*-type cytochromes (Table 4; see also Figs 2 and 4 for examples). In many cases, electron transport catalyzed by diheme *b*-type cytochromes is coupled to the generation of a *pmf* across the membrane, either by proton pumping, the Q-cycle or the redox loop mechanism (Table 3) [6].

Table 4
Compilation of Qrp families relevant to this article. Note that QRPs of the first three families appear to be solely reactive with iron-sulfur proteins. Families 2 to 4 are independent of cytochrome *b*.

Qrp family and typical members. The corresponding respiratory system is given in parentheses.	Redox partner protein (and its cofactor) other than quinone/quinol
1. Diheme cytochromes *b*	
1.1 Cytochrome *b* of the cytochrome bc_1 complex	Rieske protein (Fe/S) and cytochrome c_1
1.2 FdhC/HydC (formate dehydrogenase/hydrogenase)	FdhAB (MGD, Fe/S)/HydAB (Ni/Fe, Fe/S)
1.3 NarI (nitrate reductase)	NarGH (MGD, Fe/S)
1.4 NarC (haloarchaeal nitrate reductase)	NarB (?) (Fe/S) (?)
2. NapH/NosH family	
2.1 NapH (nitrate reductase)	NapG (Fe/S)
2.2 NosH (nitrous oxide reductase)	NosG (Fe/S)
2.3 NosR (nitrous oxide reductase)	Not known
3. NrfD/PsrC family	
3.1 NrfD (cytochrome *c* nitrite reductase)	NrfC (Fe/S)
3.2 PsrC (polysulfide reductase)	PsrB (Fe/S)
3.3 TtrC (tetrathionate reductase)	TtrB (Fe/S)
3.4 MccD (cytochrome *c* sulfite reductase)	MccC (Fe/S)
3.5 ActC (alternative complex III)	ActB (Fe/S)
3.6 QrcD (quinone reductase complex from sulfate reducers)	QrcC (Fe/S)
4. Multiheme cytochromes *c* of the NapC/NrfH family	
4.1 NapC (nitrate reductase)	Multiheme cytochromes *c* NapB (2 heme *c*) or NapM (4 heme *c*)
4.2 NrfH (cytochrome *c* nitrite reductase)	NrfA (5 heme *c*)
4.3 NirT (cytochrome cd_1 nitrite reductase)	Cytochrome cd_1 (hemes *c* and d_1)
4.4 CymA (multifunctional)	Various heme *c* proteins
4.5 Cyt. c_{m552} (hydroxylamine oxidoreductase)	Cytochrome c_{554} (4 heme *c*)
4.6 FccC (methacrylate reductase)	FccB (4 heme *c*)
4.7 TorC [a] (TMAO reductase)	TMAO reductase TorA (MGD, Fe/S)
4.8 DorC [a] (DMSO reductase)	DMSO reductase DorA (MGD, Fe/S)

[a] Fusion proteins between a core tetraheme cytochrome *c* and a C-terminal monoheme cytochrome *c* domain.

5.1.2. Quinol dehydrogenases of the NapH/NosH family

Prototypic NapH/NosH-type proteins contain four hydrophobic segments that are likely to form transmembrane domains, two conserved CX_3CP signatures and two four-cysteine clusters typically involved in [4Fe–4S] center formation. The NosR protein mentioned in Section 3.4 also belongs to this family (Table 4). Prototypic NapH/NosH proteins

are thought to form stable complexes with specific periplasmic redox partner proteins called NapG or NosG that are predicted to be polyferredoxins with four [4Fe–4S] iron-sulfur centers (Figs 3B–D and 8). Recently, the formation of a NapGH complex has been shown in the case of *W. succinogenes* [147]. NapGH/NosGH complexes are assumed to catalyze ubi- or menaquinol oxidation and to transfer electrons to cognate periplasmic *c*-type cytochromes in electron transport chains leading to either periplasmic nitrate reductase (NapA) or cytochrome *c* nitrous oxide reductase (*c*NosZ) (see Sections 3.1 and 3.4). The expression of chimeric *nap*/*nos* operons in *W. succinogenes* indicated that a NapG-NosH complex is functional in nitrate respiration whereas NosG was apparently not capable of interacting with the NapAB system when expressed in either a *nosGH* or *nosG-napH* arrangement [147]. The NapG-NosH assembly was found to be less stable than the NapGH complex since NapG could be easily washed off the membrane by applying low concentrations of sodium chloride. Thus, it seems conceivable that NapG and NosG represent adaptor proteins that determine the specificity of periplasmic electron transport to the corresponding terminal reductase.

The orientation of the four transmembrane segments of NapH was determined experimentally for *E. coli* NapH using a series of alkaline phosphatase and β-galactosidase fusion proteins and implying that all four poly-cysteine motifs are located at the cytoplasmic side of the membrane [148] where they might interact with a cytoplasmic protein like NapF [27] (see Section 3.1). The architecture and localization of the quinol-reactive site in NapH/NosH is not known and it cannot be excluded that more than one such site is present. Furthermore, the function of the cytoplasmic, potentially metal-binding CX_3CP motifs and the putative iron-sulfur centers has not been elucidated. The potential participation of NapH/NosH-type proteins in *pmf* generation has not been examined.

Interestingly, *napH*-like genes were also discovered in genetic contexts other than nitrate or nitrous oxide reductase gene loci. These genes are *mauN*, *rdxA* and *ccoG* that are present in methylotrophs (methylamine dehydrogenase gene cluster), photosynthetic and aerobic bacteria (cytochrome cbb_3 oxidase accessory gene cluster) respectively [48].

5.1.3. Quinol dehydrogenases of the NrfD/PsrC family

NrfD is a polytopic membrane protein encoded in the *nrf* gene cluster of enteric bacteria. It was shown to be essential for formate-dependent nitrite reduction of *E. coli* and was predicted to traverse the membrane eight times with both the N- and C-termini located in the periplasm [149]. NrfD forms a complex with NrfC, which is predicted to bind four [4Fe–4S] clusters (Fig. 5B). The NrfD homologue PsrC was experimentally shown to form a complex with the NrfC-type protein PsrB and the catalytic subunit PsrA in the membrane-bound polysulfide reductase complex of *W. succinogenes* [150]. PsrC is the membrane anchor of the complex and the two hydrophilic subunits PsrB and PsrA are oriented toward the periplasm. The crystal structure of a NrfD/PsrC-type QRP from *T. thermophilus* confirmed the predicted presence of eight transmembrane helices and was found to contain a single quinol-binding site at the periplasmic membrane surface [47]. Comparative studies revealed that NrfD-type proteins are widespread in bacterial anaerobic electron transport chains with homologous proteins present, for example, in polysulfide, tetrathionate and sulfite reductase systems (Table 4). Furthermore, such proteins were reported as subunits of "alternative complex III" (ActC) and a quinone reductase complex from sulfate reducers (QrcD) [151,152]. The corresponding genes are found in a variety of different genetic contexts, usually in combination with a gene encoding a polyferredoxin similar to NrfC/PsrB.

5.1.4. The NapC/NrfH family of multiheme cytochromes c

The proteins of this family are membrane-bound tetra- or pentaheme cytochromes *c* that comprise an N-terminal membrane-spanning helix and a globular cytochrome *c* domain situated at the outside of the bacterial membrane (Table 4) [153]. Most members are tetraheme cytochromes *c* consisting of about 175 amino acid residues while some proteins of the TorC clade contain a C-terminal monoheme cytochrome *c* domain that very likely resulted from a gene fusion [154]. The NapC/NrfH family was previously referred to as the NapC/NirT family since NirT from *P. stutzeri* was the first such protein to which a function had been assigned (see Section 3.2) [36]. Within their respective functional modules, NapC and NrfH donate electrons, either directly or indirectly, to periplasmic nitrate reductase (NapA) and periplasmic cytochrome *c* nitrite reductase (NrfA), respectively, whereas another family member, cytochrome c_{m552}, was proposed to feed electrons derived from hydroxylamine oxidation into the ubiquinone pool of nitrifying organisms ([50,51,155–157] and references therein) (Fig. 9A). In each case, multiheme cytochromes *c* act as redox partner proteins (Table 4). In *Shewanella* species, CymA acts as an electron transport hub in several respiratory chains (see Section 3.1). Recently, CymA from *Shewanella oneidensis* was functionally characterized and found to contain a bound menaquinone molecule [158,159]. The crystal structure of NrfH from *Desulfovibrio vulgaris* was solved as part of a quinol oxidizing $(NrfHA_2)_2$ complex [40,160]. A menaquinol binding site was shown to be present at the periplasmic side of the membrane, situated in the vicinity of heme 1, which serves as an entrance to a densely packed electron-transferring heme *c* wire [156]. It is therefore very likely that protons are exchanged with the periplasmic space during quinone/quinol turnover and this would rule out the participation of NapC/NrfH-type and c_{m552}-type cytochromes in *pmf* generation and consumption, respectively (Fig. 5A and Table 3). Notably, the axial heme *c* ligands of the NrfH and NapC families differ considerably [157,161].

Prior phylogenetic analyses of proteins in the NapC/NrfH QRP superfamily already reported relationship between the membrane-bound QRP, NrfH, and the soluble periplasmic NrfB protein, the pentaheme cytochrome *c* protein that relays electrons from the NrfCD complex to the catalytic NrfA protein in formate-oxidizing ammonifying proteobacteria (see Section 3.2) [51]. Unexpectedly, a recent analysis revealed that members of the NapC/NrfH QRP superfamily may interact with a great diversity of functional redox partners and that the explosion over evolutionary time into the diversity of the extant superfamily likely started from the NrfH-type ([51] and our unpublished results). While extant NrfH and NapC appear to interact solely with menaquinol in predominantly anaerobic bacteria and cytochrome c_{m552} interacts solely with ubiquinone in obligate aerobic bacteria (note the reversal of electron flow direction), NirT and TorC are assumed to interact with both quinol types in aerobic, facultative anaerobic and anaerobic bacteria. It is thus most parsimonious to predict that an ancestral tetraheme cytochrome *c* QRP-encoding *nrfH*-type gene has emerged in a sulfur-, formaldehyde- or hydrogen-oxidizing anaerobic respiratory bacterium.

5.1.5. Concluding remark on the presented QRP families

The data presented in Sections 5.1.1 to 5.1.4 (see also Table 4) suggest the evolution of four independent QRP families based on common respiratory cofactors such as heme *b*, heme *c* and iron-sulfur centers. In general, the functional crosstalk between members of different QRP modules is assumed to be scarce despite the fact that an exceptional interaction of NapH and NapC has been reported in the *E. coli* Nap system [148]. All four QRP modules seem to be early inventions during evolution (see also Section 6) although the origin of individual QRP families remains elusive.

5.2. Enzyme families involved in nitrogen compound turnover

Similar to the QRP families discussed above, Section 3 has revealed the existence of enzyme (super)families involved in NC transformation, the most prominent of which are presented in Sections 5.2.1 to 5.2.4. The significantly increased number of members of such (super)families has rekindled the search for ancestral variants. This research has either

led to identification of an ancient prototype such as NrfH in the NapC/NrfH superfamily (see Section 5.1.4) or it has identified gene superfamilies that encode highly substrate-promiscuous enzymes recruited in diverse metabolic modules without answering the question regarding the ancestral type. Phylogenetic reconstructions are sensitive to uneven population of divergent branches with representatives and subject to misrepresentation of extant diversity due to limitations in the discovery process, which largely relies on nucleic acid primers and probes. This calamity was only just recently overcome by significant progress in studies of NC-processing enzymes.

5.2.1. The MCC family

Amongst others, the MCC family comprises the NrfA, Hao and Hzo enzyme families mentioned in previous sections (enzymes 5–8, 15 and 16 in Table 2). Some of its individual members have been investigated repeatedly in the last decade whenever either new functional details or new members were discovered ([38] and references therein). A first comprehensive analysis identified direct evolutionary links between pentaheme (NrfA) and octaheme cytochrome *c* nitrite reductases (Onr and εHao), octaheme tetrathionate reductase (Otr), hyroxylamine (Hao) and hydrazine (Hzo) oxidoreductases and a large number of unidentified octaheme cytochrome *c* proteins, whose substrate and mechnism of transformation (reduction or oxidation) is still enigmatic, thereby predicting a gene extension model along diversification [52]. This, recent reports on octaheme cytochrome *c* sulfite reductase [53,162] and numerous new sequences provided reason and opportunity for an extensive new analysis that not only established the new boundaries of a large MCC superfamily (for example by including the tetraheme cytochrome c_{554} from nitrifiyers) but revised the evolutionary direction along the lines of gene/protein contraction (from octaheme to penta- and tetraheme proteins) and a context for proposing the family's functional origin in the sulfur cycle [53].

5.2.2. The NarGH–NxrAB family

As indicated in Section 3.7, it has been suggested earlier that proteobacterial nitrite oxidoreductase (NxrAB) and nitrate reductases (NarGH) have close genetic relationships and exhibit similar inhibitor profiles [136]. More recent *in silico* and experimental studies revealed that the process of anaerobic oxidation of nitrite to nitrate in anammox bacteria is facilitated by a Nxr complex (aNxr; enzyme 9c in Table 2), which provides energy and reductant needed for growth [73,74]. A phylogenetic analysis by Lücker et al. [11] demonstrated that the NxrA (homolog of NarG) and NxrB (homolog of NarH) proteins from *Nitrobacter* species and from *Candidatus* N. defluvii cluster on opposite branches in the respective subunit protein trees of the type II DMSO reductase superfamily of molybdoproteins. Interestingly, that tree showed that the Nxr proteins from *Candidatus* N. defluvii cluster with respective proteins from the anammox bacterium *Candidatus* K. stuttgartiensis in a way that suggests that the enzymes present in extant aerobic Nitrospirae are more delineated over evolutionary time. In a recent article, Sorokin et al. [163] reported the unexpected discovery of an aerobic nitrite-oxidizing bacterium in the phylum Chloroflexi, whose NxrAB proteins clustered with those from *Nitrobacter* and *Nitrococcus* albeit most closely with the NarGH proteins from the anaerobic methanotroph, *Candidatus* M. oxyfera in the NC10 phylum. The phylogenetic trees in both studies suggest that nitrite oxidoreductase has evolved at least twice in diverse genomic backgrounds of bacteria adapted to anoxic environments and in reversible metabolic contexts of nitrite oxidation and nitrate reduction. The studies further suggest that the respective genes were likely laterally transferred into genomic backgrounds of different phyla where they assumed key catabolic functions in the chemolithotrophic context of aerobic nitrification.

5.2.3. The Nor/HCO family

Members of the heme copper-oxidase (HCO) superfamily are also known in the literature as terminal cytochrome *c* oxidases or complex IV oxygen reductases (OR) [164]. Based on extensive comparative genomic, phylogenetic and biochemical analyses, it has been proposed recently that the high-affinity oxygen reductases in the so-called B-, H- and C-families evolved after the low affinity A-family [165]. Numerous papers also established the evolutionary relationship between HCO subunit I and the corresponding subunit of membrane-bound nitric oxide reductases, NorB (see Section 3.3; [166] and references therein). While most authors agree that A-type HCOs are phylogenetically older than the other families, including Nor enzymes, their discussions usually end in speculations when considering that NO is the primordial oxidant and that oxygen at best was sufficiently present locally (maybe as a result of an intra-oxygenic metabolism as it was discovered recently in NC10 bacteria [76,77]) but still at low concentrations before the *Great Oxygenation Event* (see Section 6). This conundrum is likely the result of oxygen-centric thinking and the assumption that branched electron flow has arisen within a very narrow window of evolutionary time, is constrained to the quinone:cytochrome *c* oxidoreductase of the Rieske bc_1-type (complex III) and closed by existence of energy conserving complex IV HCOs. In contrast, this review has identified the many different membrane-bound and evolutionarily ancient soluble periplasmic protein complexes that transform nitric oxide by either reduction or oxidation (see Section 3.3). It is thus most parsimonious to predict that early NO reduction was facilitated by soluble, cytochrome *c* or flavocytochrome based inventory that interacted with the quinone/quinol pool via multi cytochrome *c* complexes such as those that are functional as QRP complexes in extant anammox bacteria [73,123] and that NO-mediated electron transfer evolved from circular to linear flow (see also Section 6) and branched off the membrane (quinol pool) without return to a second membrane-bound and potentially energy-conserving module. With this premise, applying the results of phylogenetic analyses (namely that HCO-Nor evolved from HCO-ORs early) and extant metabolic scenarios [165] is without conflict and fits into the overall scheme of that detoxification mechanisms needed to be in place before sophisticated redox (electron flow) systems with high throughput capacity evolved. Hence the absence of Cu_B centers in Nor enzymes compared to HCO-ORs can be explained as an adaptation by reduction of complexity rather than a reflection of metal availability in different geochemical eras (see Section 6). Interestingly, the high-affinity oxygen reductases in the C-family of HCOs (the cytochrome cbb_3 oxidases) have also, albeit a lower, affinity to NO, which has been implicated as an additional NO detoxification function in wastewater-adapted ammonia-oxidizing bacteria [71].

5.2.4. The Cu-MMO family

We learned only recently that members of the copper-containing membrane-bound monooxygenase (Cu-MMO) family are substrate-promiscuous enzyme complexes that are encoded in diverse bacterial and archaeal genomic backgrounds [142]. While a common evolutionary history of two of its representatives, Amo and particular methane monooxygenase (pMmo), has been demonstrated for the first time already in 1998 [103] (the frequently cited reference [167] erroneously compared only PmoA sequences and not *N. oceani* AmoA), it were the very recent findings of Amo in Thaumarchaeaota [88], of pMmo in Verrucomicrobia [168] and NC10 bacteria [76], of ethane-MO (GenBank loci BAH22833 and BAH22839) and ethylene-MO [169] in Proteobacteria, of butane-MO [170] and hexane-MO [171] in Gram-positive bacteria as well as a "pXmo" of unknown substrate specificity in Proteobacteria [142] that led to the correction of a believed narrow taxonomic distribution and dedicated function of Cu-MMOs in bacterial nitrification and methanotrophy. Even though the evolutionary relationship between extant Cu-MMOs has been reconstructed, their origin is still elusive. Their operation is dependent on molecular oxygen and bioavailable copper and all substrates identified so far are reduced oxygen-free nitrogen and carbon compounds. The pMmo operating in strictly anaerobic but intra-oxygenic NC10- bacteria is copper-dependent and not ancestral [142]. Therefore, it is thinkable but yet without experimental

evidence that the Cu-MMO ancestor was actually copper-free and may have utilized NO as the source of oxygen in an environment free of molecular oxygen. Although a thermophilic thaumarchaeal origin of Amo as ancestral Cu-MMO has been hypothesized [90], the fact that Thaumarchaeaota lack cytochrome *c* proteins completely and that their redox protein complement is almost exclusively copper-dependent suggests that their present catabolic machinery is the product of an evolutionarily rather late origin. Additional discoveries will be required to identify genomic backgrounds that have more likely been suited for evolving ancestral Cu-MMO.

6. Conclusions on the evolution of bioenergetic systems involved in NC transformation

There is ample evidence supporting the existence of diverse microbial catabolic lifestyles in the anoxic past of planet Earth, many of which including anaerobic respiration [172]. Because of the high frequency of lateral transfer events, it is generally accepted that gains and losses of particular inventory (genes, proteins) or changes in the composition of ancient microbial genomes over evolutionary time is not traceable. It is possible, however, to establish the evolutionary histories of individual inventories found in extant organisms, establish functional associations between them and generate ecophysiological contexts with particular host organisms. Along the same line of thought, it will remain unclear whether primordial enzymes and ETCs that had an impact on nitrogen cycling (and these might have used other metal cofactors than today's enzymes) became extinct during evolution. Another problem arises from the fact that the inventory of NC-converting enzymes is not predictable from the phylogenetic position of a microorganism (usually based on 16S rRNA sequence trees) due to extensive horizontal gene transfer of the respective gene loci. Therefore, evolutionarily "ancient" enzymes are often found in various domains and phyla of extant microbes. In addition, recent discoveries have cemented earlier findings that conversion of particular NCs can be facilitated by multiple and evolutionarily unrelated enzyme complexes; there are, for instance, more than eight different enzyme complexes known to oxidize or reduce NO. Keeping all this in mind, this section discusses conceivable scenarios regarding the evolution of nitrogen cycle bioenergetics that are primarily based on the facts presented in the previous sections of this article, i.e. viewed from the perspective of extant ETC design. In this regard, the following questions are considered relevant:

(A) Which NCs and enzyme cofactors were present and biologically exploitable in different geochemical eras?
(B) Which of the presented systems and metabolic pathways are ancient and which are comparatively young on an evolutionary time scale?
(C) How did the bioenergetic systems in question contribute to the establishment of the global nitrogen cycle as we know it today?

It is conceivable that nitrogen gases such as ammonia, nitric oxide and nitrous oxide were present in the slightly reductive atmosphere of the Archaean era that ended about 2.4 billion years ago, i.e. prior to the *Great Oxygenation Event* (*GOE*) that by and large resulted from the emergence of oxygen production by water-splitting phototrophs [173–175]. There is a considerable debate, however, whether nitrogen oxyanions like nitrate and nitrite were available to drive anaerobic respiration before the *GOE* [143,176]. In principle, nitrate and nitrite might have been formed from nitric oxide under anoxic conditions in the Hadean and Archaean atmosphere [177]. Their concentrations in (micro)environments are difficult to estimate although values as high as in the micromolar range have been suggested [178]. On the other hand, it was argued that nitrate and nitrite only accumulated after their production by aerobic nitrification from ammonium [179], which is known today as an obligatory aerobic process facilitated by copper-dependent catalysis (see below for the bioavailability of copper ions). In this scenario, nitrate and nitrite respiration succeeded the *GOE* [180].

It is widely accepted that copper is unlikely to have played a biological role in the Archaean due to its extremely low solubility (and thus bioavailability) in a sulfidic environment [181]. It has to be assumed that copper only became bioavailable after the *GOE* when the "sulfidic lock" was abolished [182]. Following this geochemical reasoning, copper enzymes like NirK, NosZ, Amo, HCOs and cupredoxins are seemingly "younger" than those using iron (or porphyrins like heme), molybdenum or nickel as cofactors (Table 2). If true, nitrous oxide reduction and aerobic ammonium oxidation are relatively recent additions to the metabolic repertoire of NC-converting organisms. However, there might have been functionally similar but copper-independent enzymes that probably were abandoned when the biosynthetically less costly copper-dependent enzymes appeared. For instance, the function of the diiron cluster in Cu-MMOs in context with the reactive Cu_B sites is still not resolved [104,105] and leaves room for speculations of an "iron first, copper second" scenario in the evolution of this large enzyme superfamily. Consequently and according to metal availability predictions, NarGH, NapA, cytochromes *b* and *c* (including cytochrome *bc* complexes and MCCs) and all QRPs shown in Table 4 might have been formed in the pre-*GOE* era. Therefore, anaerobic processes like nitrate reduction, nitrite reduction to nitric oxide, nitrous oxide or ammonium and the anammox pathway could have been operational in the Archaean, and the only reasonable process to close the nitrogen cycle and return fixed nitrogen to the dinitrogen pool was the anammox process [56]. A few more hypotheses can be put forward with respect to assumed properties of "older" enzymes: (i) exported enzymes [requiring protein export by the ancient twin arginine translocation (Tat) system] are older than those that convert NCs in the cytoplasm, thus requiring substrate/product transporters (especially for toxic products like nitrite in the case of nNar of *c*Nar); (ii) enzymes with activities playing a role in the sulfur cycle (like PsrC and members of the MCC and Nap/NrfH families) are early inventions [183]; (iii) enzymes reacting directly with redox mediator pools (quinone/quinol and periplasmic cytochrome *c* pools) are older than those forming tight complexes with QRPs or cytochromes [56]; (iv) enzymes operational at low substrate concentrations are older, (v) enzymes that resulted from the fusion of otherwise separated protein domains are younger and their genes more likely prone to horizontal transfer; (vi) within enzyme families, proteins showing special active sites or requiring dedicated biogenesis systems (like some members of the MCC family) are younger inventions [184–191].

Early bioenergetic systems likely have evolved from detoxification systems that inactivated reactive nitrogen species (nitric oxide and nitrite) and possibly also toxic sulfur compounds like sulfite in the periplasm and outside the cell. In this respect, it is noteworthy that the d_1 heme of cytochrome cd_1 nitrite reductase is synthesized from siroheme, the cofactor of several cytoplasmic enzymes such as respiratory sulfite reductase and assimilatory nitrite reductase [192]. Moreover, nitric oxide transforming enzymes are present in Archaea and many different bacterial phyla and constitute an unprecedented high abundance and diversity [4,114]. According to the assumptions made above, the Nar, NirS and Nrf-type modules also qualify as ancient enzyme systems. Thus, denitrification of nitrate to yield nitrous oxide as well as nitrate ammonification appears to constitute significant processes in the primordial unclosed nitrogen cycle [56]. These pathways might have been electroneutral in ancestral microbes, using available high-potential NCs as electron sinks in order to replenish the quinone pool and employing ETCs that consisted of simple enzymes modules depending on hemes and iron-sulfur centers. It can thus be predicted that small cytochromes *c* and iron-sulfur proteins such as ferredoxins have been the principle redox mediators in the extracellular space and that the cytochrome bc_1 complex as well as other QRPs like the NapH/NosH, NrfD/PsrC and NapC/NrfH families represent the complement of early respiratory modules.

It cannot not be excluded that nitric oxide dismutase is also an ancient enzyme that may have made oxygen bioavailable intracellularly for respiratory processes, a situation that likely would not left any biogeochemical isotope record. Such an oxygen source might have provided significant amounts of oxygen locally prior to the *GOE* that could have served as substrate for electrogenic, low-affinity HCOs in the A-family (see Section 5.2.3) [165,179,193]. In contrast to the nitric oxide reductases that evolved from the high affinity oxygen reductases in the HCO B- and H-families, extant representatives of the two sister lineages of nitric oxide reductases, *c*Nor and qNor (which evolved from the HCO C-family of cytochrome cbb_3 high-affinity oxygen reductases), contain several non-heme irons and no copper in their active sites, which has provided the impetus for studying unusual representatives of the qNor family as candidates of the nitric oxide dismutase (see Section 3.3). For example, the genome of *Candidatus* M. oxyfera encodes one qNor and two versions of qNor-like proteins and sequence-similar variants have been detected in other bacteria; however, to date there is no physiological evidence that they perform that reaction (James Hemp, personal communication). In this context and provided that proper detoxification systems were in place, it is thinkable that microaerobic low-throughput oxidation of reduced carbon and nitrogen compounds by soluble monooxygenases such as the soluble iron-dependent methane monooxygenase (that still operates in a number of extant methanotrophs) or a primordial copper-independent substrate-promiscuous membrane-bound monooxygenase constituted the reductive branches of early supplementary catabolism in a largely anoxic environment [114,142]. The evolution of such an enzymic inventory occurred likely in the genomes of sulfur-dependent anaerobic bacteria [56] and an association with microbes with intra-oxygenic capacity may have locally increased the bioavailability of sulfur-trapped copper [181]. If true, the evolution of copper-associated membrane-bound monooxygenases (Cu-MMO enzymes) and the recruitment of their hosts into nitrification aggregates by Nxr-containing bacteria could have led to the production of nitrite and nitrate prior to the *GOE*, contrary to what was suggested by others [167,180,194] before the recent discovery of anaerobic nitrite oxidation to nitrate by anammox (see below).

It has been proposed that representatives of the MCC family have participated in early NC transformations before there was a closed nitrogen cycle [56], which occurred mainly in two capacities: the reduction of nitrite, NO and hydroxylamine (NrfA, Onr, cytochrome c_{554}, εHao) to ammonium and the oxidation of hydroxylamine (Hao) to NO or nitrite [32,52,53]. The other main complement of early NC transformations were the reductions of nitrate to nitrite and to nitric oxide by the molybdoproteins NapA and NarG, by NirS (cyt cd_1, which has also low affinity for oxygen [195]) as well as by soluble tetraheme cytochromes *c*′ (CytS, CytP). All of these modules likely evolved as means for detoxification, some of which with utility in anaerobic respiration. The major breakthrough in terms of employing these reactions for a productive catabolic lifestyle likely occurred in anammox bacteria that were able to functionally unite these modules by invention of a cytochrome *c* protein complex capable of comproportionating NO and ammonium to hydrazine (hydrazine synthase) and acquisition of NirS [74] (note that some anammox bacteria contain a NirK homolog [196]). This was possible because MCCs were capable of detoxifying hydrazine by oxidation to dinitrogen, which not only provided reductant for *pmf* generation but also established the first (closed) nitrogen cycle [56,137]. Nxr (Type II DMSO reductase family) was employed to oxidize nitrite to nitrate anaerobically [11,163], which provided anammox bacteria with the needed energy for assimilation and biosynthesis [123] and was likely the main biological way of nitrate production before aerobic nitrification emerged. Once oxygen concentrations rose past threshold levels that led to the emergence of copper-containing enzymes (NirK, cupredoxins with electron storage function, NosZ and Amo), some of the anammox modules were replaced by aerobic, high-throughput modules (i.e., soluble periplasmic NirS and hydrazine synthase were replaced with a Cu-MMO), which provided metabolic control and more efficient use of reductant thereby enabling linear electron flow and branched ETCs for better energy conservation [56]. Despite this adaptation and reliance on molecular oxygen as the main oxidant in oxic and hypoxic environments, both anaerobic and aerobic microbes with active roles in the extant nitrogen cycle have retained and express an arsenal of NO-transforming modules. It can therefore be stated that the reactive nitrogen detoxification mechanisms shown in Table 1 constituted not only the prerequisites for the evolution of nitrogen-based catabolic pathways but must continue to play important roles despite the presence of oxygen as the most powerful oxidant.

7. Perspective

One of the most astonishing features of the presented microbial ETCs is their high degree of modularity that appears to be a major prerequisite for optimized electron transport to and from available NCs. Interestingly, it turned out that the various dehydrogenase and reductase complexes are electronically connected by only a few types of soluble electron transfer proteins (mainly small *c*-type cytochromes and cupredoxins) and by a limited number of QRP modules. Furthermore, *pmf* generation seems to depend on the functional architecture of only a few of these modules while most of them merely serve in efficient electron transport and substrate turnover. Genomic studies clearly revealed that genes encoding NC-converting enzymes as well as their corresponding electron transport modules have been arranged in many different contexts during evolution and it is quite obvious that these genetic arrangements are well suited for the acquisition of new metabolic features by horizontal gene transfer (despite the fact that elaborate biogenesis systems are needed to achieve the maturation of some of the discussed enzyme systems). On the other hand, extensive module tinkering makes it very difficult to reconstruct the evolution of bioenergetic systems (rather than that of individual enzymes) since the Archaean era. Despite all uncertainties, this article outlined some possible scenarios that are in line with biochemical and well as environmental and geological contraints and concepts [180,197,198].

Unfortunately, many electron transfer reactions discussed in this article have not been demonstrated biochemically (for example using purified enzymes or by reconstitution experiments in case of QRP-quinone interaction) but were rather predicted from the individual characterization of potentially interacting enzymes or from the composition of corresponding gene clusters. Especially in light of the emerging wealth of genomic data, it is critical that modelling electron transfer chains or networks is not exclusively based on genetic information alone (despite the fact that many models established in this way proved to be correct later on). One also has to realize that only a very small proportion of the known (and cultured) bacteria have been investigated in depth and surely we can await many future surprises in terms of the biochemical characterization of ETCs involved in nitrogen metabolism. Furthermore, crystal structures of many key enzymes discussed in this article are still lacking, for example those of Amo, Nxr, εHao, Hzs, NapH or NapC.

One of the most discussed challenges in modern microbiology is the rational design of synthetic microorganisms that can be applied to preserve or restore human and/or environmental health. Regarding the nitrogen cycle, this is evident when considering global warming and the increases in atmospheric nitrous oxide and methane concentrations due to significant anthropogenic overloads of fixed nitrogen (i.e. Haber-Bosch process produced ammonium as fertilizer) to the nitrogen cycle [79,180,199,200]. Synthesizing new or changing existing organisms clearly also requires proper design of energy metabolism, for which more and in-depth knowledge of substrate conversion mechanisms and enzyme interactions that operate in extant microorganisms is necessary (not to mention the underlying sensing mechanisms and regulatory pathways). Furthermore, a more detailed knowledge on enzyme maturation processes, synthesis of co-factors and their insertion

into proteins as well as of transport processes of apo- or holoproteins across membranes is required. Progress in this field also depends on efficient heterologous production systems for metalloenzymes or enzyme complexes in suitable host bacteria. Ultimately, the combined knowledge may lead to the *de novo* construction of organisms whose energy metabolism meets favored requirements in industrial and environmental applications.

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