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

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A B S T R A C T
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.

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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 catalysis 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 (NO2−), (ii) reduction of nitrite to produce nitric oxide (NO) and nitrous oxide (N2O) (during both aerobic “classic” denitification and anaerobic “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) denitrogen (N2) fixation; (vi) anaerobic ammonium oxidation (anammox) to produce N2 and (vii) a recently described reaction of nitric oxide dismutation resulting in N2 and O2 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
Anaerobic respiratory pathways and aerobic nitrification are catalyzed by distinct respiratory enzyme systems that are designated by the oxidation state of nitrogen atoms.

**Table 1**

<table>
<thead>
<tr>
<th>Nitrogen compound</th>
<th>Oxidation state</th>
<th>Toxicity to microbial cells</th>
<th>Other properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate (NO₃⁻)</td>
<td>+5</td>
<td>Non-toxic in physiological concentrations.</td>
<td>Anion of strongly oxidizing and toxic nitric acid (HNO₃, pKₐ=1.4).</td>
</tr>
<tr>
<td>Nitrogen dioxide (NO₂)</td>
<td>+4</td>
<td>Toxic orange gas. Arises from the oxidation of nitric oxide by oxygen.</td>
<td>In equilibrium with the colorless gas dioxygen (O₂).</td>
</tr>
<tr>
<td>Nitrite (NO₂⁻)</td>
<td>+3</td>
<td>Toxic; binds to cellular iron atoms, for example in hemoglobin.</td>
<td>Forms nitrosamines in acidic conditions according to HNO₂ + H⁺ → NO⁻ + H₂O. Anion of unstable nitrous acid (HNO₂, pKₐ=3.4).</td>
</tr>
<tr>
<td>Nitric oxide (NO)</td>
<td>+2</td>
<td>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 dinitrosyl iron complexes and forms dinitrosyl iron complexes [Fe(NO)₂]. Causes nitrosation of thiol groups to form dinitrosyl iron complexes.</td>
<td>Dimerizes to byronitrosylic acid (H₂[N₄O₃]), which can be dehydrated to N₂O.</td>
</tr>
<tr>
<td>Nitrous oxide (N₂O)</td>
<td>+1</td>
<td>Chemically inert and non-toxic in physiological concentrations.</td>
<td>Mutagenic by uracil-forming cytosine deamination, thus causing G/C → T/A transitions in DNA.</td>
</tr>
<tr>
<td>Nitrosoyl (HNO)</td>
<td>+1</td>
<td>Chemically inert and non-toxic.</td>
<td>-</td>
</tr>
<tr>
<td>Dinitrogen (N₂)</td>
<td>0</td>
<td>Chemically inert and non-toxic.</td>
<td>-</td>
</tr>
<tr>
<td>Hydroxylamine (NH₂OH)</td>
<td>-1</td>
<td>Toxic by binding to heme groups. Irreversible inhibitor of the oxygen-evolving complex in photosystem II.</td>
<td>-</td>
</tr>
<tr>
<td>Hydrazine (N₂H₄)</td>
<td>-2</td>
<td>Highly toxic.</td>
<td>Rocket fuel, forms monohydride.</td>
</tr>
<tr>
<td>Ammonium (NH₄⁺)</td>
<td>-3</td>
<td>Non-toxic in physiological concentrations.</td>
<td>Cation of ammonia (NH₃), which is a toxic uncoupling agent (pKₐ=9.25).</td>
</tr>
</tbody>
</table>

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 ubiqui- 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 initiated at either the oxidative or the reductive side during electron transport from a reduced electron donor substrate (the reduc- tant) 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 catalytic 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 protonotive and non-protonotive 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...
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 (ΔG°) of a respiratory process depends on standard redox potentials at pH 7.0 (E°) of the electron donor and acceptor substrates (and their respective reaction products) according to the equation ΔG° = n · F · ΔE°, wherein n is the number of electrons transferred and F is the Faraday constant (see Table 2 for ΔE° values of selected NC redox pairs). Due to their rather positive E° 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 chemooxygenotrophic or chemolithothrophic energy-conserving pathways. On the other hand, ammonium and nitrite are poor electron donors, explaining the fact that obligate chemolithothrophic nitrifying organisms usually use oxygen as the sole electron acceptor. In addition, operating a nitrifying amino- or nitrite-oxidizing complex (i.e. H⁺/e− coupled oxygen as the sole electron acceptor). In addition, operating a nitrifying complex (i.e. H⁺/e− coupled oxygen as the sole electron acceptor)

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 enzymatic 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 molybdenopterin 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 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-protonotive) steps due to the interchangeable modular architecture of the pertinent ETC components.

The pmf has both a chemical (ΔpH, dimensionless) and an electrical (ΔΨ, dimensionless) component and the formula pmf (mV) = ΔΨ − 59 ΔpH describes this correlation in its simplest form. ΔΨ 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 “o-cycle” as catalyzed by the cytochrome bc1 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 chemooxygenotrophic aerobic respiration and, consequently, shorter ETCs with a lower number of coupling sites are employed in such cases [6].
Table 2
Selected enzymes and enzyme complexes involved in respiratory nitrogen compound turnover.

<table>
<thead>
<tr>
<th>Enzyme designation and corresponding standard potential of the relevant redox pair (oxidized/reduced) at pH 7 (E°)</th>
<th>Physiological function</th>
<th>Redox partner</th>
<th>Selected model organism(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate reductases: NO3– + 2 e– + 2H+ → NO2– + H2O; E° (NO3–/NO2–) = +0.43 V</td>
<td>Cytoplasmic nitrate reduction</td>
<td>Quinol (oxidized by NarI)</td>
<td>Escherichia coli (γ), Paracoccus denitrificans (α), Thermus thermophilus (Deinococcus–Thermus)</td>
</tr>
<tr>
<td>1a. nNar/NarGHI (Mo-bis-MGD, Fe/S, heme b; dimeric)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b. Nar/NarGCH (Mo-bis-MGD, Fe/S, heme b, heme c)</td>
<td>Cytoplasmic nitrate reduction</td>
<td>Ubiquinol and periplasmic cytochrome c pool via NarC</td>
<td>Haloflexus mediterranei, Haloarcula marismortui (both Euryarchaeota), Pyrobaculum aerophilum (Crenarchaeota)</td>
</tr>
<tr>
<td>1c. pNar/NarGH in a complex with other proteins (see text) (Mo-bis-MGD, Fe/S)</td>
<td>Nitrate reduction at the outside of the cytoplasmic membrane</td>
<td>Quinones and possibly small periplasmic electron transfer proteins</td>
<td>Rhodobacter sphaeroides (α), Escherichia coli (γ), Paracoccus denitrificans (α), Bradyrhizobium japonicum (α), Shewanella oneidensis (γ), Wolinella succinogenes (ξ)</td>
</tr>
<tr>
<td>2a. NapAB (Mo-bis-MGD, Fe/S, heme c)</td>
<td>Periplasmic nitrate reduction</td>
<td>Quinol dehydrogenases NapC and/or NapCH</td>
<td>Desulfovibrio desulfitrovorum (ξ)</td>
</tr>
<tr>
<td>2b. NapA (Mo-bis-MGD, Fe/S)</td>
<td>Periplasmic nitrate reduction</td>
<td>Not known, potentially NapM</td>
<td></td>
</tr>
<tr>
<td>Nitrite-reactive enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) NO–genic nitrite reduction: NO2– + e– + H2O → NO3–; E° (NO2–/NO3–) = +0.36 V</td>
<td>Nitrification</td>
<td>Monoamine oxidases (Cyt. c550 or cyanide oxidase)</td>
<td>Paracoccus pantotrophus (α), Pseudomonas aeruginosa (γ), Pseudomonas stutzeri (γ), Bradyrhizobium japonicum (α), Achromobacter cycloclastes (β), Achromobacter xylosoxidans (β), Burkholderia sp. (β), Bdellovibrio bacteriovorus (η)</td>
</tr>
<tr>
<td>3. NirS, cytochrome c4 (heme c and d1; dimeric)</td>
<td>Nitrification</td>
<td>Monoamine oxidases (Cyt. c550 or cyanide oxidase)</td>
<td></td>
</tr>
<tr>
<td>4a. NirK (C4a and C4b centers; trimeric)</td>
<td>Nitrification</td>
<td>Monoamine oxidases (Cyt. c550 or cyanide oxidase)</td>
<td></td>
</tr>
<tr>
<td>4b. cNirK (heme c; C4a and C4b centers)</td>
<td>Nitrification</td>
<td>Monoamine oxidases (Cyt. c550 or cyanide oxidase)</td>
<td></td>
</tr>
<tr>
<td>5c. NirB complex (NirDK: 5 heme c; one C4a/C4b motif; dimeric)</td>
<td>Periplasmic nitrite ammionification</td>
<td>Pentaheme cytochrome c; NirB</td>
<td>Escherichia coli (γ), Wolinella succinogenes (ξ), Desulfovibrio vulgaris (η)</td>
</tr>
<tr>
<td>5b. NirF (5 heme c; one C4a/C4b motif)</td>
<td>Periplasmic nitrite ammionification</td>
<td>Tetraheme cytochrome c quinol dehydrogenase CymA</td>
<td></td>
</tr>
<tr>
<td>5a. NirF (5 heme c; one C4a/C4b motif; dimeric)</td>
<td>Periplasmic nitrite ammionification</td>
<td>Quinol oxidized by NirH</td>
<td></td>
</tr>
<tr>
<td>6. NirB complex (NirDK: 4 heme c; Dimers of NirB, assembly in crystal structure)</td>
<td>Periplasmic nitrite ammionification</td>
<td>Not known. Or reduces nitrite, hydroxylamine, sulfite and hydrogen peroxide</td>
<td></td>
</tr>
<tr>
<td>7. OtA (8 heme c)</td>
<td>Not known. Or reduces nitrite, Hydroxylamine and interconverts tetrothionate and thiosulfate</td>
<td>Quinol oxidized by NirH</td>
<td></td>
</tr>
<tr>
<td>8. iNao (8 heme c)</td>
<td>Most likely nitrite reduction</td>
<td>Cytochrome c of the Nap/NirH family (?)</td>
<td>Campylobacter concisus, Campylobacter curvus, Campylobacter fetus, Neutalia profundisolaris (all ε)</td>
</tr>
<tr>
<td>(ii) Nitrite oxidoreductase: NO2– + H2O → NO2– + 2 e– + 2H+; E° (NO2–/NO2–) = +0.43 V</td>
<td>Cytoplasmic nitrite oxidation</td>
<td>Quinone pool and cytochrome oxidase</td>
<td>Nitrobacter hamburgensis (α)</td>
</tr>
<tr>
<td>9a. mNox/NxABC (Mo-bis-MGD, Fe/S, hemes b and c)</td>
<td>Cytoplasmic nitrite oxidation</td>
<td>Quinone pool and cytochrome oxidase</td>
<td></td>
</tr>
<tr>
<td>9b. gNao/NxABC (Mo-bis-MGD, Fe/S, possibly heme b)</td>
<td>Periplasmic nitrite oxidation</td>
<td>Quinone pool and cytochrome oxidase</td>
<td>Cu. Nitrosira defluviis (Nitrosirae)</td>
</tr>
<tr>
<td>9c. aNao/NxABC (might form large complex with several other subunits, see text)</td>
<td>Nitrit oxidation in anammoxosome</td>
<td>Quinone oxidase</td>
<td>Cu. Kuenenia stuttgartiensis (Planctomycetes)</td>
</tr>
<tr>
<td>Nitric oxide–reactive enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) N2O–genic nitric oxide reductases: 2 NO + 2e– + 2H+ → N2 + H2O; E° (NO/N2) = +1.18 V</td>
<td>Nitric oxide reduction</td>
<td>Monoheme cytochrome c (Cyt. c550 or Cyt. c551) or cupredoxin</td>
<td>Paracoccus denitrificans (α), Pseudomonas aeruginosa (γ), Pyrobaculum aerophilum (Crenarchaeota), Geobacillus stearothermophilus (Firmicutes), Ralstonia eutropha (γ), Neisseria meningitidis (β), Bacillus azotoformans (Firmicutes)</td>
</tr>
<tr>
<td>10a. cNor/NorBC (hemes b and c, Fe; dimeric)</td>
<td>Nitric oxide reduction</td>
<td>Monoheme cytochrome c (Cyt. c550 or Cyt. c551) or cupredoxin</td>
<td></td>
</tr>
<tr>
<td>10b. qNor/NorB (heme b, Fe)</td>
<td>Nitric oxide reduction at the outside of the cytoplasmic membrane</td>
<td>Quinol (MKH2)</td>
<td></td>
</tr>
<tr>
<td>10c. gNor/qNor (heme b, Fe, Cyt. c4)</td>
<td>Nitric oxide reduction at the outside of the cytoplasmic membrane</td>
<td>Menaquinol</td>
<td></td>
</tr>
<tr>
<td>10d. sNor, sNor, gNor</td>
<td>Periplasmic nitric oxide reduction</td>
<td>Monoheme cytochrome c (Cyt. c550 or Cyt. c551) or cupredoxin</td>
<td>Diverse Proteobacteria</td>
</tr>
<tr>
<td>(ii) Hydrazine synthase (complementation of nitric oxide and ammonium): NO + 3H2 + 3e– + 2H+ → N2H4 + H2O; E° = +0.06 V</td>
<td>Complementation of nitric oxide and ammonium in anammoxosome</td>
<td>Cytochromes c in anammoxosome</td>
<td>Cu. Kuenenia stuttgartiensis (Planctomycetes)</td>
</tr>
<tr>
<td>11. Hzs (heme c)</td>
<td>Complementation of nitric oxide and ammonium in anammoxosome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(iii) Nitric oxide dismutase (Nod): 2 NO → N2 + O2</td>
<td>Nitric oxide reduction in oxygenic</td>
<td>Not known</td>
<td>Cu. Methylomirabilis oxyfera (NC10 phylum)</td>
</tr>
<tr>
<td>12. Nod (enzyme yet to be identified)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(continued on next page)
4Fe–4S clusters) [12]. In Proteobacteria (and also in some Gram-

Table 2 (continued)

<table>
<thead>
<tr>
<th>Enzyme designation and corresponding standard potential of the relevant redox pair (oxidized/reduced) at pH 7 (E°)</th>
<th>Physiological function</th>
<th>Redox partner</th>
<th>Selected model organism(s) a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrous oxide reductase: N₂O + 2e⁻ + 2H⁺ → N₂ + H₂O; E° (N₂O/N₂) = +1.35 V</td>
<td>Periplasmic nitrous oxide reduction</td>
<td>Monoheme cytochromes c (Cyt. c₅₅₃ or c₅₅₅), cupredoxin, or NosR/NosOR</td>
<td>Pseudomonas stutzeri (α), Marinobacter hydrocarbonoclasticus (γ), Wolinella succinogenes (ε)</td>
</tr>
<tr>
<td>13a. NosZ (Cua₅, Cua₆; Dimer)</td>
<td>Periplasmic nitrous oxide reduction</td>
<td>Monoheme cytochromes c or NosGH quinol dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>13b. cNosZ (heme c, Cua₅, Cua₆)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonia monooxygenase: NH₃ + O₂ + 2e⁻ + H⁺ → NH₂OH + H₂O; E° (NH₃/NH₂OH) = +0.74 V</td>
<td>Periplasmic ammonia oxidation</td>
<td>Quinone pool (assumed)</td>
<td>Nitrosomonas europaea (β)</td>
</tr>
<tr>
<td>14. AnnOR (Cu, Fe; trimeric)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxylamine oxidoreductase: NH₂OH + H₂O → N₂ + H₂O; E° (NO₂⁻/NO) = +1.35 V</td>
<td>Periplasmic hydroxylamine oxidation</td>
<td>Cyt. c₅₅₃ (assumed)</td>
<td>Nitrobacter europaeus (β)</td>
</tr>
<tr>
<td>15. Hao (heme b; trimeric)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrazine oxidoreductase: N₂H₄ → N₂ + 4H⁺; E° (N₂H₄/N₂) = −0.75 V</td>
<td>Hydrazine oxidation in anammoxosome</td>
<td>Cytochromes c in anammoxosome</td>
<td>Ca. Kuenenia stuttgartiensis (Planctomycetes)</td>
</tr>
<tr>
<td>16. Hzo (heme c)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
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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.
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 electrochemical 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 (NirS, containing the unusual cytochrome *d*₄ at the active site) or copper-dependent enzymes (NirK) that typically contain two copper centers (Cu₈ and Cu₉). 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*₃₅₃, or *c*₅₅₅ (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*₃ 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 NrfH 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 NrfH complex was shown to catalyze electroneutral menaquinol oxidation by nitrite in the Epsilonproteobacterium *Wolinella succignocens* (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. 2. Model of electrogenic ETs 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 eNar enzyme. Note that the Nrc and cNar complexes are likely to form a supercomplex. C. Electron transport from quinol to nitrite in halobacterial 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.](image-url)
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 de-restricted 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 N2 and O2 (Nod; enzyme 12). Moreover, several evolutionarily and structurally unrelated soluble microbial enzymes were reported to convert nitric oxide to either nitrite [flavohemoglobin-NO-dioxygenase (Hmp)], nitrite [hydroxylamine oxidoreductase; cytochrome P460 (CytP)] or nitrous oxide [flavobiredoxin proteins (Fdp), flavobiredoxin (NorVW), cytochrome ε554 (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 (cnor 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 b56:F2 active site that is reduced by another heme b group bound by the same protein (named NorB). In cnor 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 cnor and Geobacillus stearothermophilus
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*, pseudooazurin or cytochrome c₅₅₉ were found to donate electron to the NorC subunits of a heterotetrameric (NorBC)₂ complex [69]. There is no indication that the cNor 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 cNor enzymes. Genes encoding “ccNorC” proteins with a second heme-binding domain fused to the usually monoheme cytochrome c₂-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 cNor, qNor enzymes are reactive with ubiquinol and/or menaquinol and contain an N-terminal extension that is absent from NorB in the cNor 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 (Cu₄-qNor), exemplified by the enzyme from *Bacillus azotoformans* (enzyme 10c in Table 2), contains NorB in a complex with a subunit harboring a Cu₄ site (typically found in oxygen-reducing HCOs), which makes this enzyme competent in receiving electrons from membrane-bound cytochrome c₅₅₉ 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 chemolithothrophic bacteria [71]) and gNor (predominantly in sulfur-catabolic chemolithothrophic bacteria [30,72], see also Section 5.2.3).

Hydrazine synthase (Hzs; enzyme 11 in Table 2) is a key player in 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 bc₁ 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 CH₄ + 8 NO₂⁻ + 8 H⁺ → 3 CO₂ + 4 N₂ + 10 H₂O. In corresponding enrichment cultures a dominant bacterium belonging to the NC10 phylum was identified as *Candidatus* *Methylophilalis oxyfera* [78]. This organism is assumed to produce N₂ from nitric oxide (produced from nitrite by either a NirS-type or an eHao-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 N₂ and O₂ in equal amounts from nitric oxide (2 NO → N₂ + O₂). Three quarters of the internally produced oxygen is thought to serve in methane oxidation to CO₂ 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

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**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. Cu₃g and Cu₄ denote the binuclear Cu₃g and tetranuclear Cu₂g centres of NosZ while the distinct copper centers of NirK are abbreviated Cu₄ and Cu₄. Heme b₃:Fe₃g 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.

**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.
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 complex (complex III) and several complex IV-type terminal oxidases of the HCO family, thereby employing classic electron-transport 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. 8A). From a bioenergetic point of view, it is therefore not surprising that many denitrifiers reduce nitrate or nitric oxide only to the level of nitrous oxide due to the absence of a nosZ gene [79]. NosZ contains two redox-active copper centers, termed CuA (dinuclear copper site) and CuB (tetranuclear copper site) [80–82]. In the functional homodimer, the CuA electron input site of one monomer is in close contact with the catalytic CuB 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 CuA site (enzyme 13b in Table 2) [83,84]. Such cNosZ 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 cNosZ alternative to the conventional cytochrome b/c 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/cNosZ 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 heterotrimetric (AmoABC) 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
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 $\Delta$$\mu$F 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 genes.

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.
singletons [101] whereas singleton amoA and amoB genes have not yet been found in any AOB genome. Interestingly, orthologues of amoB (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 amoB 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 methene 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 thaumarcheal amoA, amoB and amoC genes in the genome varies between individual thaumarcheal 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 HaoA3 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 c552 (CyCA) by Hao [111], modeling of this interaction [112,113] as well as demonstration of quinone reductase function of cytochrome c552 (CyCB) [50], an early linear model of electroneutral electron flow from hydroxylamine to quinone (Hao→c554→c552) had been established (Fig. 9A). The observed clustering of Hao- and cytochrome c554- and c552-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 c554 and c552 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 c552 cannot be excluded (Fig. 9A). Cytochrome c552 is related to the NapC/NrfH QPR 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 c552 has a mild NO reductase activity although it is unclear whether this is physiologically relevant [61]. Nevertheless, clustering of cytochrome c552 (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 Verrucomicrobacteria 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 oceanii 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 the fact that M. capsulatus lacks cycAB genes and thus HURM. Analysis of hao gene expression in N. europaea strain EN1–11 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 biochemical 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 (kuste0694; named hydrazine oxidoreductase or Hzo; enzyme 16 in Table 2) was dedicated to hydrazine oxidation; an enzyme sensitive to hydrazine and nitric oxide [73]. However, it is unknown how electrons derived from hydrazine oxidation ultimately reach the cytochrome bc complex and whether this process is electrogenic, e.g. by involving a quinone species (Fig. 6). This would leave the cytochrome bc complex as the main coupling site in anammox metabolism. However, the Candidatus K. stuttgartiensis genome encodes three different cytochrome bc-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 c1 of Proteobacteria might be functionally replaced by di-, hexa- or octaheme cytochromes c in different Candidatus K. stuttgartiensis bc1-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 aa3-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 cNar 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 c550, the cytochrome bc1 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 through the catalysis by NirK and a so far unidentified nitric oxide reductase (or by sNOR as found in *Nitrobacter hamburgensis* [133]).

In *Candidatus Nitrospira defluvii* (phylum Nitrospirae), the nitrite-oxidizing site of the NxrAB assembly is likely to be oriented...
to the outside of the cytoplasmic membrane [\textit{pNrr}; enzyme 9b in Table 2; Fig. 10B] [111]. In this case, it is unknown how electrons are transported to the oxygen-reducing enzyme (probably a terminal oxidase of the cytochrome \textit{bd}-type) or fed into the reverse ETC (Fig. 10B). The involvement of cytochrome(s) \textit{b} and \textit{c} cannot be excluded [111].

Fueled by early suggestions that proteobacterial nitrite oxidoreductase (\textit{NxrAB}) and nitrate reductases (\textit{NarGH}) have close genetic relationships and similar inhibitor profiles [136], recent in silico and experimental studies revealed that the \textit{Nxr} complex in anammox bacteria (\textit{aNrr}; enzyme 9c in Table 2; Fig. 6) facilitates anaerobic nitrite oxidation to nitrate, which provides energy and reduces carbon needed for growth [73,74]. Similar to the closely related \textit{Nxr} enzyme in \textit{Candidatus N. defluvii} [111], the extracted electrons could flow via cytochromes to the quinol pool [123,124]. It has been proposed that the \textit{Nxr} complex in anammox bacteria may also function reversely as a true nitrate reductase when oxidizing small organic molecules (e.g. propionate) with nitrate as electron acceptor [137]. The genes proposed to encode the required soluble complement of monoheme and diheme cytochromes \textit{c} have been identified in \textit{Candidatus K. stuttgartiensis} and in \textit{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/quinol and (ii) cytochromes \textit{b} and/or \textit{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 \textit{c} and kupredoxins. With the exception of the \textit{nNor}, \textit{cNar} and \textit{Nrr} 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 plasmid), in the anaamnosome 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 \textit{nNrr}/\textit{cNar} nitrate reductase complexes and the Q-cycle that operates in cytochrome \textit{b} complexes and alternative complex III-type structures (see Section 5.1.3) [138]. Additional protonotive 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 \textit{NADH}:quinone oxidoreductase and electrogenic HCOs, or redox loop enzymes other than \textit{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 \textit{nNrr} 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 \textit{b} membrane anchor subunit FdhC [6]. As with \textit{nNrr}, the H+/e" ratio of this mechanism is one. Heterotrophic membrane-bound \textit{Ni}/Fe-hydrogenases work according to the same principle [6,139–141].

Takahashi, 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 \textit{nNrr} and \textit{cNar} 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 Nar system of nitrite 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.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Bioenergetic coupling of electron transport chains involved in respiratory NC transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic entry and corresponding nitrogen compound transforming enzyme a</td>
<td>Involved enzymes or modules (pmf-generating enzymes in bold)</td>
</tr>
<tr>
<td>1. Nitrile reduction by formate; \textit{nNrr} (1a)</td>
<td>FDH complex (FdhGHI) → Q/QH2 → \textit{nNrr} (NarGHBC)</td>
</tr>
<tr>
<td>2. Nitrile reduction by NADH; \textit{cNar} (1b)</td>
<td>\textit{NADH dehydrogenase} – Q/QH2 → \textit{cNar} (NarGHBC)</td>
</tr>
<tr>
<td>3. Nitrile reduction by quinol; \textit{pNar} (1c)</td>
<td>QH2 → \textit{pNar} (NarC)</td>
</tr>
<tr>
<td>4. Nitrile reduction by quinol; \textit{NapA} (2a, 2b)</td>
<td>QH2 → \textit{NapA} (NarPHG) → \textit{NapM} → \textit{NapA}/\textit{NapAB}</td>
</tr>
<tr>
<td>5. NO-gene nitrite reduction by quinol; \textit{NiS} or \textit{NiK} (3, 4)</td>
<td>\textit{NapA}/\textit{NapBC} → \textit{cytochrome b}\textsubscript{c1} → ET → \textit{NiS}/\textit{NiK}</td>
</tr>
<tr>
<td>6. Ammonofying nitrite reduction by quinol; members of the MCC family (5–8)</td>
<td>\textit{NiC} → Member of the \textit{Nap}/\textit{NrfH} family or \textit{NrfBCD} → cytochrome \textit{c} nitrite reductase of the MCC family</td>
</tr>
<tr>
<td>7. N2O-gene nitric oxide reduction by quinol; \textit{cNrr} (10a)</td>
<td>\textit{NapA}/\textit{NapBC} → \textit{cytochrome b}\textsubscript{c1} → ET → \textit{cNrr}</td>
</tr>
<tr>
<td>8. N2O-gene nitric oxide reduction by quinol; \textit{qNrr} (10b)</td>
<td>QH2 → \textit{ET}/\textit{Nos}/\textit{Nos}(X) → \textit{Nos}/\textit{Nos}</td>
</tr>
<tr>
<td>9. N2-gene nitrous oxide reduction by quinol; \textit{NoS} (13a, 13b)</td>
<td>QH2 → \textit{cytochrome b}\textsubscript{c1} → ET → \textit{Nos}/\textit{H2}/\textit{H2} → ET → Q</td>
</tr>
<tr>
<td>10. Anamnogenesis; \textit{NiS}, \textit{H2S}, \textit{H2} (3, 11, 16)</td>
<td>AmoABC → Q → cytochromes c → \textit{Hao}; QH2 → \textit{cytochrome b}\textsubscript{c1} – Oxidase</td>
</tr>
<tr>
<td>11. Ammonium oxidation by oxygen; \textit{Amo}, \textit{Hao} (14, 15)</td>
<td>\textit{Nrr} → \textit{cytochrome b} (and c) – Oxidase</td>
</tr>
</tbody>
</table>

EPT, electron transfer proteins forming a redox mediator pool. These proteins comprise monoheme cytochromes \textit{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 The exception is the \textit{cNrr} complex carried out by specific \textit{cNrr} systems (see Section 5.1.3).

e Unless the \textit{NrfCD} complex catalyzes electrogenic quinol oxidation (see Section 5.1.3).

f Unless \textit{qNrr} 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 catalytic metabolism in order to facilitate the function of already present protonotive, 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 bc₁ 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 tinkingering) 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 bc₁ complex as well as quinone-reactive membrane anchor subunits of nNar, cNar, Ni/Fe-hydrogenase, formate dehydrogenase, succinate dehydrogenase and fumarate reductase (Table 4) ([16] and references therein). In addition, such cytochromes b also occur in cytochrome bo₃ 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 luop mechanism (Table 3) [6].

Table 4

<table>
<thead>
<tr>
<th>Qrp family and typical members.</th>
<th>Redox partner protein (and its cofactor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Diheme cytochromes b</td>
<td></td>
</tr>
<tr>
<td>1.1 Cytochrome b of the cytochrome bc₁ complex</td>
<td>Rieske protein (Fe/S) and cytochrome c₁ complex</td>
</tr>
<tr>
<td>1.2 FdH/CHy/C (formate dehydrogenase/hydrogenase)</td>
<td>FdhAB (MCD, Fe/S)/HydAB (Ni/Fe, Fe/S)</td>
</tr>
<tr>
<td>1.3 Nar (nitrate reductase)</td>
<td>NarGH (MGD, Fe/S)</td>
</tr>
<tr>
<td>1.4 NarC (halorhodan nitrate reductase)</td>
<td>NarB (?) (Fe/S) (?)</td>
</tr>
<tr>
<td>2. NapH/NosH family</td>
<td></td>
</tr>
<tr>
<td>2.1 NapH (nitrate reductase)</td>
<td>NosC (Fe/S)</td>
</tr>
<tr>
<td>2.2 NosH (nitrous oxide reductase)</td>
<td>Not known</td>
</tr>
<tr>
<td>2.3 NosR (nitrous oxide reductase)</td>
<td>Not known</td>
</tr>
<tr>
<td>3. NrfD/PsrC family</td>
<td></td>
</tr>
<tr>
<td>3.1 NrfD (cytochrome c nitrite reductase)</td>
<td>NrfC (Fe/S)</td>
</tr>
<tr>
<td>3.2 PsrC (polysulfide reductase)</td>
<td>TrfB (Fe/S)</td>
</tr>
<tr>
<td>3.3 TrfC (tetrathionate reductase)</td>
<td>MccC (Fe/S)</td>
</tr>
<tr>
<td>3.4 MccD (cytochrome c sulfite reductase)</td>
<td>MccD (Fe/S)</td>
</tr>
<tr>
<td>3.5 ActC (alternative complex III)</td>
<td>ActB (Fe/S)</td>
</tr>
<tr>
<td>3.6 QrdC (quinone reductase complex from sulfate reducers)</td>
<td>QrdC (Fe/S)</td>
</tr>
<tr>
<td>4. Multiheme cytochromes c of the NapC/NrfH family</td>
<td></td>
</tr>
<tr>
<td>4.1 NapC (nitrate reductase)</td>
<td>Multiheme cytochromes c NapB (2 heme c) or NapM (4 heme c)</td>
</tr>
<tr>
<td>4.2 Nrh (cytochrome c nitrite reductase)</td>
<td>NrfA (5 heme c)</td>
</tr>
<tr>
<td>4.3 NrfH (cytochrome c₅ nitrite reductase)</td>
<td>Cytochrome c₅ (hemes c₁ and d₁)</td>
</tr>
<tr>
<td>4.4 CymA (multifunctional)</td>
<td>Various heme c proteins</td>
</tr>
<tr>
<td>4.5 Cyt. c₅₅₄ (hydroxylamine oxidoreductase)</td>
<td>Cytochrome c₅₅₄ (4 heme c)</td>
</tr>
<tr>
<td>4.6 FccC (methacrylate reductase)</td>
<td>FccB (4 heme c)</td>
</tr>
<tr>
<td>4.7 Tonc (TMAO reductase)</td>
<td>TMAO reductase TorA (MGD, Fe/S)</td>
</tr>
<tr>
<td>4.8 DorC (DMSO reductase)</td>
<td>DMSO reductase DorA (MGD, Fe/S)</td>
</tr>
</tbody>
</table>

‡ Fusion proteins between a core tetraheme cytochrome c and a C-terminal monoheme cytochrome c domain.
are thought to form stable complexes with specific periplasmic redox partner proteins called NapG or NosG that are predicted to be poly-ferrredoxins with four [4Fe–4S] iron-sulfur centers (Figs 3B–D and 8). Recently, the formation of a NapG complex has been shown in the case of W. succinogenes [147]. NapG/NosG 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 (cNosZ) (see Sections 3.1 and 3.4). The expression of chimeric nap/nos operons in W. succinogenes indicated that a NapG-Nos complex is functional in nitrate respiration whereas NosG was apparently not capable of interacting with the NapAB system when expressed in complex is functional in nitrate respiration whereas NosG was apparent-
tions of sodium chloride. Thus, it seems conceivable that NapG and could be easily washed off the membrane by applying low concentra-
ners are gene

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 CxCxCP 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 mauA, rbdA and cocG that are present in methyloptrophs (methyamine dehydrogenase gene cluster), photosynthetic and aerobic bacteria (cytochrome cbb3, 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 cyt-
alysic 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 homologues 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 (QrC) [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 bacte-
ral membrane (Table 4) [153]. Most members are tetrahezemo cyto-
chromes c consisting of about 175 amino acid residues while some proteins of the Torc clade contain a C-terminal monoheme cyto-
ochrome 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 cmon552, 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 cyto-
chromes c act as redox partner proteins (Table 4). In Shewanella spe-
cies, 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 (NrfH2A2)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 NrfC/NrfH-type and cmon552-type cytochromes in pmf generation and consumption, respectively (Fig. 5A and Table 3). Notably, the axial heme c ligands of the NapC and Nap-family 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 cyto-

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) sug-
gest 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 NrfC 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 cHao), octaheme tetrathionate reductase (Otr), hyroxylamine (Hao) and hydrazine (Hzo) oxidoreductases and a large number of unidentified octaheme cytochrome c proteins, whose substate and mechanism 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 c554 from nitriﬁers) 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 nitrate to nitrammon bacteria is facilitated by a Nar complex (aNxr; enzyme 9c in Table 2), which provides energy and reductant needed for growth [73,74]. A phylogenetic analysis by Lücker et al. [111] demonstrated that the NxrA (homolog of NarG) and NxrB (homolog of NarH) proteins from Nitrobacter species and from Candidatus N. deﬂuviicola cluster on opposite branches in the respective subunit protein trees of the type II DMSO reductase superfamily of molydopeproteins. Interestingly, that tree showed that the Nxr proteins from Candidatus N. deﬂuviicola cluster with respective proteins from the anammox bacterium Candidatus K. stuttgartiensis in a way that suggests the enzymes present in extant aerobic Nitrosopirea 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 Nitrocosmus 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 nitrate 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 catalytic 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 Oxidation 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 bc1-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 QPc 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 Cu6 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 cbb3 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 Thaumarchaeota [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 “pMMo” 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 methanotroph. 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 Thaumarchaeota 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 eco-physiological 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 rRNA sequence trees) due to extensive horizontal gene transfer of the inventory of NC-converting enzymes is not predictable from the 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 Oxidation 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-enzyme 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 CuB 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 MCGs) 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 cNar); (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 in inactivated reactive nitrogen species (nitric oxide and nitrite) and possibly also toxic sulfur compounds like sulfate in the periplasm and outside the cell. In this respect, it is noteworthy that the heme of cytochrome cd1 nitrite reductase is synthesized from siroheme, the cofactor of several cytoplasmic enzymes such as respiratory sulfite reductase and assmilatory 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 principal redox mediators in the extracellular space and that the cytochrome bc complex as well as other QRPs like the NapH, NosH, NrF/D/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 biochemical evidence on record. Such an oxygen source might have provided significant amounts of oxygen locally prior to the GOE that could have served as a substrate for electron donors, 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, cNOR and qNOR (which evolved from the HCO C-family of cytochrome cbb3, 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 the 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 monoxygenases such as the soluble iron-dependant methane monoxygenase (that still operates in a number of extant methanotrophs) or a primordial copper-independent substrate-promiscuous membrane-bound monoxygenase 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 monoxygenases (Cu-MMO enzymes) and the recruitment of their hosts into nitrate inorganic 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 nitrogen oxides (NO and hydroxylamine (Nraf, Onr, cytochrome C55a, eHao) to ammonia 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 nitrite to nitrite and to nitric oxide by the molybdoenzymes NapA and NarG, by NirS (cyt bd) and to ammonia by NirK, Onr and NapC. The other main complement of early NC transformations were the reductions of nitrite to nitrite and to nitric oxide by the molybdoenzymes NapA and NarG, by NirS (cyt bd), which has also low activity 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 disproportionating NO and ammonium to hydrazine (hydrazine synthase) and ammonia. It has been proposed that anammox bacteria contain a NirK homolog [196], but 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 bio synthesis [123] and was likely the main biologically way of nitrate production before aerobic nitritation 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 evidence 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 Archean era. Despite all uncertainties, this article outlined some possible scenarios that are in line with biochemical and well as environmental and geological 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, eHao, 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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