Review

Electron transport chains and bioenergetics of respiratory nitrogen metabolism in *Wolinella succinogenes* and other Epsilonproteobacteria

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ABSTRACT

Recent phylogenetic analyses have established that the Epsilonproteobacteria form a globally ubiquitous group of ecologically significant organisms that comprises a diverse range of free-living bacteria as well as host-associated organisms like *Wolinella succinogenes* and pathogenic *Campylobacter* and *Helicobacter* species. Many Epsilonproteobacteria reduce nitrate and nitrite and perform either respiratory nitrate ammonification or denitrification. The inventory of epsilonproteobacterial genomes from 21 different species was analysed with respect to key enzymes involved in respiratory nitrogen metabolism. Most ammonifying Epsilonproteobacteria employ two enzymic electron transport systems named Nap (periplasmic nitrate reductase) and Nrf (periplasmic cytochrome *c* nitrite reductase). The current knowledge on the architecture and function of the corresponding proton motive force-generating respiratory chains using low-potential electron donors are reviewed in this article and the role of membrane-bound quinone/quinol-reactive proteins (NapH and NrfH) that are representative of widespread bacterial electron transport modules is highlighted. Notably, all Epsilonproteobacteria lack a *napC* gene in their *nap* gene clusters. Possible roles of the Nap and Nrf systems in anabolism and nitrosative stress defence are also discussed. Free-living denitrifying Epsilonproteobacteria lack the Nrf system but encode cytochrome cd_1 nitrite reductase, at least one nitric oxide reductase and a characteristic cytochrome *c* nitrous oxide reductase system (*c*NosZ). Interestingly, *c*NosZ is also found in some ammonifying Epsilonproteobacteria and enables nitrous oxide respiration in *W. succinogenes*.

1. Introduction

The reactions of the biogeochemical nitrogen cycle are essential in sustaining life on Earth [1–3]. Three distinct types of anaerobic respiration have been recognized in the nitrogen cycle that depend on either nitrate and/or nitrite as electron acceptor substrate(s) (Fig. 1). These are denitrification, anaerobic ammonium oxidation (anammox) and respiratory ammonification of nitrate or nitrite [4–6]. Depending on the organism, denitrification yields different gaseous products (NO, N_2O, N_2) in various amounts whereas, in the ammonification pathway, nitrite is reduced to ammonia without releasing intermediate products. In the anammox process, ammonium is used as electron donor for denitrifying, i.e. N_2-producing, nitrite reduction. Currently,

no organism is known that catalyses more than one of these three respiratory modes.

Over the last decade, many high-resolution structures of terminal reductases involved in respiratory nitrogen metabolism of various denitrifying or ammonifying bacteria have been obtained. Such enzymes include the membrane-bound nitrate reductase complex NarGHI, the periplasmic nitrate reductase NapA and its redox partner NapB, the cytochrome cd_1 nitrite reductase NirS, Cu-containing nitrite reductase NirK, N_2O reductase NosZ and the ammonifying cytochrome *c* nitrite reductase NrfA (reviewed by [5,7–9]). These enzymes are membrane-bound or periplasmic metalloproteins (or protein complexes) that regularly exchange electrons with the membranous quinone/quinol pool. This is achieved either by direct interaction or via additional electron transport proteins like cytochromes or iron–sulfur proteins. In contrast to the detailed knowledge on individual respiratory enzymes, the enzymic composition of complete electron transport chains as well as the involved protein–protein and protein–quinone interactions are less well understood. Such information, however, is crucial for the elucidation of the mechanism by which the electron transport from an electron donor to an electron acceptor substrate is coupled to the generation of a proton motive force (*pmf*) across the bacterial membrane. There is currently only a limited understanding of the detailed function of quinol dehydrogenases
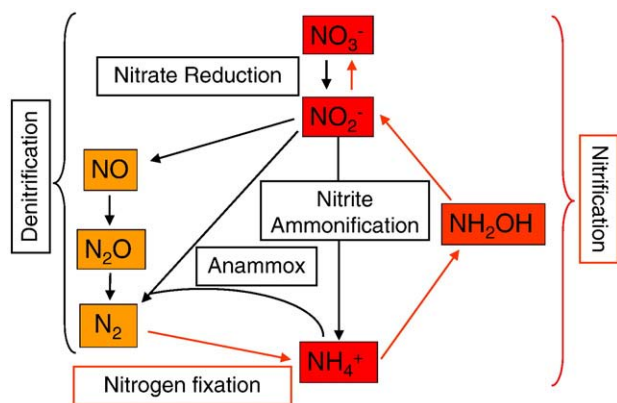

Fig. 1. Conversion of microbial substrates in the biological nitrogen cycle. Anaerobic respiratory processes are indicated by black arrows. Nitrogen fixation and nitrification (marked by red arrows) are not within the scope of this article.

involved in electron transport to the various terminal reductases of anaerobic respiration and models of the corresponding electron transport chains often rely on the arrangement of the respective gene clusters (e.g. *nar*, *nap*, *nrf*, *nir* and *nos* loci) encoding the terminal reductases mentioned above.

Many proteobacteria of the gamma, delta and epsilon classes including pathogenic species of *Escherichia*, *Haemophilus*, *Salmonella*, *Pasteurella* and *Campylobacter*, catalyse the respiratory reduction of nitrate to nitrite and/or nitrite to ammonium [5,10,11]. Increasing interest has been attracted recently to this metabolic pathway since this mode of respiration might play an important role in host invasion and colonization by enabling ATP synthesis in micro-oxic environments such as the intestinal tract (see e.g. [12]). In addition, the nitrate- and nitrite-dependent respiratory systems are involved in the

production or reductive detoxification of reactive or mutagenic nitrogen species such as nitric oxide (NO) and hydroxylamine [13–16]. In contrast to the ammonification pathway, the denitrification process appears to be mainly associated with free-living bacteria but these also include some clinically relevant species like *Pseudomonas aeruginosa* [4].

This article focuses on the respiratory nitrogen metabolism of Epsilonproteobacteria. During the last decade, the well known host-associated heterotrophic *Campylobacter*, *Helicobacter* and *Wolinella* species were accompanied by an ever-growing number of free-living Epsilonproteobacteria isolated from a diverse range of often sulfidic terrestrial and marine habitats where their ecological importance might have been grossly underestimated in the past [17]. In addition, many (meta)genomic data on uncultured species were obtained. Especially noteworthy is the dominant role of (thermophilic) Epsilonproteobacteria in deep-sea hydrothermal vents, the vent fauna and deep-sea marine subsurfaces [17–19]. Such bacteria often use hydrogen, formate, sulfide or thiosulfate as electron donors in order to fuel their autotrophic respiratory metabolism and reduce nitrate, elemental sulfur or, under microaerobic conditions, oxygen. Recently, complete genome sequences from free-living Epsilonproteobacteria were obtained [20–22], thus enabling the prediction of metabolic pathways and networks [17,18].

The current taxonomic framework groups the Epsilonproteobacteria into two orders, namely the Campylobacterales (representative genera are *Arcobacter*, *Campylobacter*, *Helicobacter*, *Sulfurimonas*, *Sulfurospirillum* and *Wolinella*) and the Nautiliales (*Caminibacter*, *Lebetimonas* and *Nautilia*). In addition, there are several genera of as yet unclassified Epsilonproteobacteria like *Nitratiruptor*, *Sulfurovum* and *Thioreductor* species. Respiratory nitrate reduction in Epsilonproteobacteria results in the production of either ammonium (ammonification) or dinitrogen gas (denitrification). Section 2 of this article gives an overview on the respiratory and assimilatory nitrogen metabolism of ammonifying and denitrifying Epsilonproteobacteria with

Table 1
Presence and composition of gene clusters encoding the respiratory Nap, Nrf, Nir, Nor and cNos systems in Epsilonproteobacteria whose complete genome sequences are available

Phylogenetic order and organism	Nap	Nrf[a]	Nir (Cyt. cd_1)	Nor[b]	cNos[c]
Order Campylobacterales					
Wolinella succinogenes DSMZ 1740[e]	AGHBFLD	HAIJ (CXXCK)	–	–	Present
Arcobacter butzleri RM4018	AGHBFLD1[d]	HAI (CXXCK)	–	qNor	–
Campylobacter concisus 13826	AGHBFLD	–	–	qNor	Present
Campylobacter curvus 525.92	AGHBFLD	–	–	qNor	Present
Campylobacter fetus subsp. *fetus* 82-40	AGHBFLD	HA (CXXCH)	–	–	Present
Campylobacter hominis ATCC BAA-381	AGHBFLD	HA (CXXCH)	–	–	–
Campylobacter jejuni[e]	AGHBLD	HA (CXXCH)	–	–	–
Campylobacter jejuni subsp. *doylei*	–	HA (CXXCH)	–	–	–
Campylobacter coli RM2228	AGHBLD	HA (CXXCH)	–	–	–
Campylobacter lari RM2100	AGHBLD	HA (CXXCH)	–	–	–
Campylobacter upsaliensis RM3195	AGHBLD	HA (CXXCH)	–	–	–
Helicobacter hepaticus ATCC 51449	AGHBLD	HA (CXXCH)[f]	–	–	–
Sulfurimonas denitrificans DSMZ 1251	AGHBFL1D23[d]	–	Present	cNor, gNor	Present [g]
Order Nautiliales					
Caminibacter mediatlanticus TB-2	AGHBFLD	–	–	–	–
Unclassified Epsilonproteobacteria					
Nitratiruptor sp. SB155-2	AGHBFLD1[d]	–	Present	cNor	Present
Sulfurovum sp. NBC37-1	AGHBF4LD2[d]	–	Present	cNor, gNor	Present

A total of 21 genome sequences were taken into account. The bacterial species are classified according to the phylogenetic system based on 16S rRNA sequences. Proteins of the Nap and Nrf systems are given according to the gene order in the corresponding *nap* and *nrf* gene clusters. The genomes of the following *Helicobacter* species contain neither of the gene clusters shown in the table: *H. acinonychis* Sheeba, *H. canadiensis* MIT98-5491, *H. cinaedi* CCUG18818, *H. pullorum* MIT98-5489, *H. pylori* 26695, *H. pylori* HPAG1, *H. pylori* HPKX 438 AG0C1, *H. pylori* HPKX 438 CA4C1, *H. pylori* J99 and *H. pylori* Shi470. –, not encoded in the genome.

[a] The haem *c* binding motif given in parentheses is that of the active site haem *c* group of NrfA.

[b] cNOR, cytochome *c*:nitric oxide reductase (NorCB complex); qNor, quinol:nitric oxide reductase; gNor, quinol-dependent nitric oxide reductase of the haem copper oxidase family, distantly related to cNor [20].

[c] All corresponding gene clusters contain adjacent *nosG*, *-C1*, *-C2* and *-H* genes that are surrounded by the accessory *nos* genes *nosD* (upstream) and *nosF* (downstream).

[d] Each number denotes the presence of a hypothetical open reading frame. Identical numbers in different organisms indicate the prediction of similar proteins.

[e] The denoted properties refer to each of the following strains: *C. jejuni* RM1221, *C. jejuni* subsp. *jejuni* 260.94, 81-176, 81116, 84-25, CF93-6, CG8421, CG8486, HB93-13 and NCTC11168.

[f] Due to a stop codon, an otherwise *Campylobacter*-typical NrfA is disrupted into two separate open reading frames encoding proteins HH0216 and HH0217.

[g] The genome contains two similar copies of the *nosZ* gene [20].

emphasis on those organisms whose genomes have been completely sequenced. Section 3 introduces the molecular and genetic basis of respiratory nitrate ammonification in the model organisms *Wolinella succinogenes* and *Campylobacter jejuni* and in Sections 4 to 6 the respiratory Nap, Nrf and *c*Nos systems that respectively catalyse nitrate reduction to nitrite, nitrite ammonification and nitrous oxide reduction to dinitrogen are reviewed in more detail. The arrangement of the *nap*, *nrf* and *nos* gene clusters encoding the respiratory electron transport chains that connect the quinol pool and periplasmic terminal reductases are presented and the function of individual gene products are discussed. Finally, Section 7 presents some implications of the respiratory systems in nitrosative stress defence.

2. The respiratory and assimilatory nitrogen metabolism of Epsilonproteobacteria

Many proteobacterial model organisms for both denitrification and nitrate/nitrite ammonification have been described and there is a wealth of information on their physiology and enzymology of respiratory nitrogen metabolism as well as on gene loci encoding the respective key enzymes [1,4,5,23,24]. In this regard, however, information on Epsilonproteobacteria is rather scarce. Table 1 shows a compilation of gene clusters that encode the respiratory reductases of the nitrogen metabolism in Epsilonproteobacteria with completely sequenced genomes. In order to catalyse nitrate reduction, these organisms encode the periplasmic nitrate reductase system (Nap) whereas genes encoding the subunits of the membrane-bound nitrate reductase complex (NarGHI) are absent. The NarGHI enzyme complex is abundant in other proteobacteria (e.g. in *E. coli*) and catalyses the electrogenic oxidation of a quinol coupled to nitrate reduction [24].

Many ammonifying Epsilonproteobacteria employ the NrfHA system to reduce nitrite to ammonium (Table 1). Interestingly, some ammonifying species also contain gene clusters encoding a putative quinol-dependent NO reductase (qNor) or the unusual cytochrome *c*

N_2O reductase (*c*Nos). On the other hand, they all lack cytochrome cd_1 nitrite reductase (NirS) and Cu-dependent nitrite reductase (NirK) (Table 1). There are several more Epsilonproteobacteria for which nitrate reduction to ammonium has been either described or predicted, e.g. *Sulfurospirillum deleyianum*, *Caminibacter hydrogenophilus*, *Caminibacter profundus*, *Caminibacter mediatlanticus*, *Hydrogenimonas thermophila* and *Thioreductor micantisoli* [25–30].

Ammonifying Epsilonproteobacteria do not encode homologs of typical assimilatory nitrate and nitrite reductases that are employed in other proteobacteria and therefore seem to rely on the periplasmic Nap and Nrf systems to produce ammonium [10]. Accordingly, mutants of *W. succinogenes* lacking either the *nap* or *nrf* genes were not able to grow with nitrate as sole nitrogen source in minimal media [5,31,32]. Ammonium is likely to be taken up from the periplasm by an Amt-type ammonium transporter and cytoplasmic ammonium can then be assimilated by glutamate dehydrogenase and glutamine synthetase [33]. This scenario is thought to hold true also for the ammonifying species of the genera *Arcobacter*, *Campylobacter* and *Helicobacter* listed in Table 1. All these species lack the genetic information for prototypic nitrate and nitrite uptake systems (like NarK, NarU and NirC) homologous to those found in several other proteobacteria [34–36]. Interestingly, *Caminibacter mediatlanticus*, for which nitrate ammonification has been shown [28], appears to lack the Nrf system but encodes homologs of NarK and NirC as well as a cytoplasmic assimilatory ferredoxin-dependent nitrite reductase (NirB). This suggests a different way of nitrite ammonification in a bacterium of the order Nautiliales.

Examples of denitrifying Epsilonproteobacteria are free-living organisms that belong to the genera *Sulfurimonas*, *Nitratiruptor*, *Sulfurovum* or *Nitratifractor* [20,37,38]. The genomes of *Sulfurimonas denitrificans* DSMZ 1251, *Nitratiruptor* sp. SB155-2 and *Sulfurovum* sp. NBC37-1 have been reported [20,21]. These three organisms lack the Nrf system and the Cu-dependent nitrite reductase NirK but encode cytochrome cd_1 nitrite reductase, at least one NO reductase and the
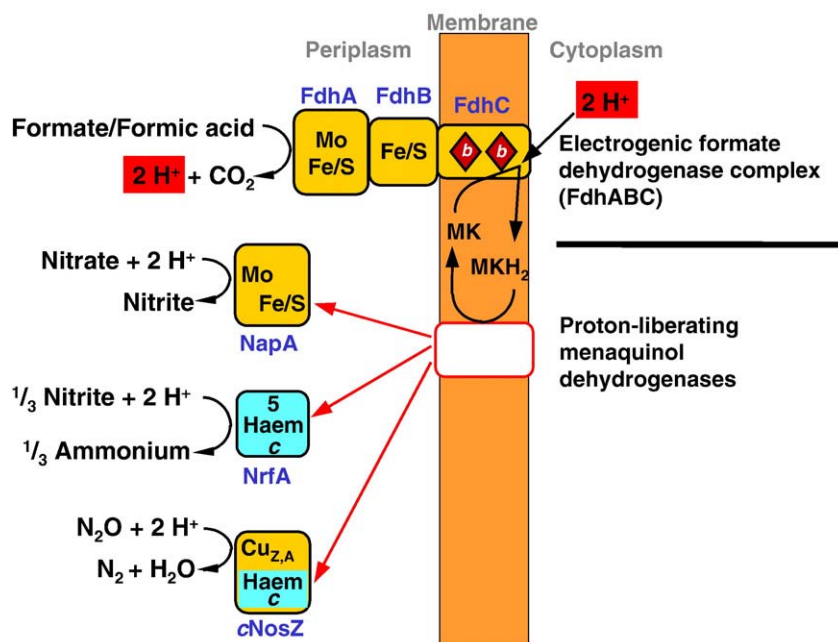
Fig. 2. Electrogenic menaquinone reduction by formate and electron transport to nitrate, nitrite and N_2O in the bacterial periplasm. The *pmf* is generated across the membrane by the formate dehydrogenase complex FdhABC according to the redox loop mechanism. The catalytic centres for formate and menaquinone are situated on different sides of the coupling membrane and the resulting charge separation is indicated by protons highlighted in red. Formate dehydrogenase may be substituted by heterotrimeric Ni/Fe-hydrogenase that couples hydrogen oxidation to menaquinone reduction, thereby also generating a *pmf* [24]. This type of membrane-bound uptake hydrogenase contains a dihaem cytochrome *b* membrane anchor (HydC) which is similar to FdhC [50], an iron–sulfur protein (HydA) and a catalytic subunit (HydB) that contains a typical Ni/Fe centre. In order to replenish the menaquinone pool, menaquinol has to be oxidized by a membrane-bound quinol dehydrogenase (white box) that delivers electrons to the proton-consuming periplasmic terminal reductases NapA, NrfA and *c*NosZ. See Figs. 3–6 for further details on the architecture of the respiratory chains in *W. succinogenes*. $Cu_{Z,A}$, binuclear Cu_A and tetranuclear Cu_Z centres of *c*NosZ; Fe/S, iron–sulfur centre; Mo, molybdenum-*bis*-molybdopterin guanine dinucleotide cofactor.

*c*Nos system for N$_2$O reduction (Table 1). In addition to the Nap system, the above-mentioned *Nitratiruptor* and *Sulfurovum* species encode a putative assimilatory nitrate reductase (NarB, also present in *C. mediatlanticus*) as well as homologs of NarK and NirB. *S. denitrificans* contains NarK, NirC and NirB homologs but lacks NarB suggesting cytoplasmic ammonium assimilation from nitrite.

The only Epsilonproteobacterium whose genome predicts a functional nitrogenase system is *W. succinogenes* [39]. However, there are no reports available on nitrogen fixation or expression of the *nif* gene cluster.

3. The ammonifying model Epsilonproteobacteria *W. succinogenes* and *C. jejuni*

With respect to the physiology and enzymology of respiratory nitrate ammonification, *W. succinogenes* is arguably the best characterized member of the Epsilonproteobacteria [5,24,40,41]. *W. succinogenes* is a non-fermentative commensal rumen bacterium that can easily be cultivated in defined media. The cells preferentially use formate or hydrogen gas as electron donor substrates to drive *pmf*-generating reduction of various electron acceptors like fumarate, nitrate, nitrite, nitrous oxide, dimethyl sulfoxide or polysulfide [42–47]. The current knowledge on the *W. succinogenes* electron transport chains from formate (or hydrogen gas) to either nitrate, nitrite or nitrous oxide is presented in more detail in the following three sections. Any of these reactions is highly exergonic due to the positive standard redox potentials of the redox pairs nitrate/nitrite, nitrite/ammonium and nitrous oxide/dinitrogen [4,5]. According to the chemiosmotic theory of energy transduction, the involved electron transport chains produce a *pmf* across the membrane that is used for ATP synthesis. The membrane-bound formate dehydrogenase (FdhABC) and Ni/Fe-hydrogenase (HydABC) complexes of *W. succinogenes* couple the oxidation of formate or hydrogen to the reduction of membraneous menaquinone-6 thereby generating an electrochemical proton gradient ($H^+/e^- = 1$) according to the redox loop mechanism (Fig. 2; [5,31,48–50]; see also [24] for a recent review on this particular mode of *pmf* generation). Therefore, in principal, the

participation of such an enzyme complex is sufficient to sustain growth irrespective of whether or not the menaquinone-replenishing oxidation of menaquinol by an appropriate electron acceptor is catalysed in an electrogenic fashion. In fact, each of the menaquinol-oxidizing systems that transfer electrons to the terminal reductases NapA, NrfA and *c*NosZ appear to catalyse electroneutral reactions (see the following sections). Such electron transport systems are regarded as prototypical for ammonifying Epsilonproteobacteria.

Formate and hydrogen are common electron donor substrates for both ammonifying and denitrifying Epsilonproteobacteria [17]. Accordingly, gene clusters encoding heterotrimeric formate dehydrogenases and Ni/Fe-hydrogenases are regularly present in epsilonproteobacterial genomes with the notable absence of *fdhABC* genes in *Helicobacter* species apart from *Helicobacter hepaticus*. The latter organism is also the only known *Helicobacter* species that contains *nap* and *nrf* gene clusters which are regularly found in *Campylobacter* strains (Table 1).

C. jejuni is the leading cause of food-borne acute gastroenteritis in humans but appears to be a commensal of the gastrointestinal tracts in poultry [51,52]. The genome of *C. jejuni* encodes a complex network of donor:quinone dehydrogenases and quinol:acceptor reductases involved in both anaerobic and microaerobic respiration which is quite similar to that of *W. succinogenes* [53,54]. On the other hand, *C. jejuni* is more fastidious in its nutrient requirements and there is some controversy whether or not *C. jejuni* is able to grow when kept strictly anaerobic [12,54,55]. Besides *W. succinogenes*, *C. jejuni* is the only other Epsilonproteobacterium for which mutants lacking a respiratory system of the nitrogen metabolism have been characterized [12,56,57]. *C. jejuni* is representative for many other species of *Campylobacter* in terms of the enzymology of respiratory nitrate ammonification (Table 1). Notable exceptions are *C. concisus* and *C. curvus* which lack the Nrf system (and also do not contain a NirB homolog).

4. Nitrate reduction to nitrite by the respiratory Nap system

NapA is a periplasmic nitrate reductase produced by many different Gram-negative bacteria [10,11,23]. The corresponding *nap*
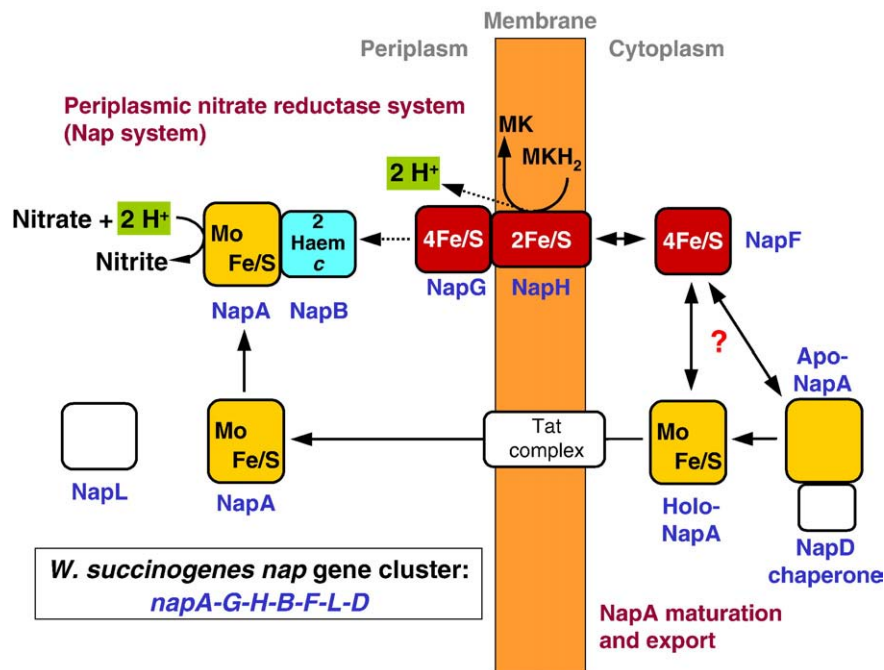
Fig. 3. The respiratory Nap system of *W. succinogenes*. Nitrate is reduced by a periplasmic NapAB complex that is likely to be reduced by the membrane-bound menaquinol dehydrogenase complex NapGH (*cf.* Fig. 4). The molybdenum cofactor (Mo) and the iron–sulfur centre of NapA are attached in the cytoplasm by specific maturation systems prior to Holo-NapA export by the twin-arginine translocation (Tat) complex. The chaperone NapD is required to coordinate maturation and export of NapA. The cytoplasmic interaction between NapF and NapA has not been shown experimentally in *W. succinogenes*. See text for details.

gene clusters vary immensely and it seems that the *napA* and *napD* genes are the only open reading frames that are present throughout [41,58]. This is not surprising as NapD was shown to be a chaperone that is required for directing cytoplasmic NapA to the twin-arginine translocation (Tat) complex [59]. The NapC protein is a membrane-bound tetrahaem cytochrome *c* that serves in quinol oxidation and the prototype of the so-called NapC/NrfH quinol dehydrogenase family [40]. NapC is an essential component of the *E. coli* Nap system and thought to reduce the periplasmic dihaem cytochrome *c* NapB that forms a complex with NapA [60]. In *E. coli*, the putative iron–sulfur proteins NapG and NapH have been postulated to form a ubiquinol dehydrogenase complex that donates electrons via NapC to NapAB [61,62]. This model was deduced mainly from growth experiments performed with mutants that produced only one type of quinone in combination with either NapC or NapGH. The structure and detailed function of the NapGH proteins, however, remained unclear as these have not been purified from *E. coli* nor from any other organism.

NapG and NapH proteins are encoded in every known epsilonproteobacterial *nap* gene cluster (Table 1) and homologous proteins (NosG and NosH) are also predicted by the corresponding genes in *nos* loci (see Section 6). However, epsilonproteobacterial *nap* gene clusters generally lack a *napC* gene, and the Nap system of *W. succinogenes* was indeed shown to be functionally independent of any NapC-type cytochrome [32]. NapA is the only nitrate reductase in *W. succinogenes* and is encoded by the first gene of the *napAGHBFLD* gene cluster (Fig. 3) [32].

Similar *nap* gene arrangements are present in other Epsilonproteobacteria but even within this class some variations exist: (I) the *napF* gene is absent in several, but not all, *Campylobacter* species as well as in *H. hepaticus* and (II) the *nap* clusters from *S. denitrificans*, *Arcobacter butzleri*, *Nitratiruptor* sp. SB155-2 and *Sulfurovum* sp. NBC37-1 contain additional open reading frames whose function is not known (Table 1). Recently, the role of individual *nap* genes situated downstream of *napA* was assessed in *W. succinogenes* by characterizing non-polar gene inactivation mutants [41,63]. It turned out that both NapB and NapD are essential components for growth by nitrate respiration, with NapD being required for the production of mature NapA [63]. The absence of NapG and NapH almost abolished growth without affecting the formation and activity of NapA [63]. The function of NapL is not known and there are no conserved sequences in NapL primary structures that would suggest the presence of metal-containing cofactors. Inactivation of *napL* in *W. succinogenes* did only slightly affect the growth behaviour of mutant cells in minimal nitrate medium although the NapA-dependent nitrate reductase activity was clearly reduced. A similar phenotype was also reported for a NapL-deficient mutant of *C. jejuni* [57]. *W. succinogenes* NapL was found to be located in the periplasm which is in line with the presence of a Sec-dependent signal peptide (M. Kern and J. Simon, unpublished data). At present, there are only two known NapL-encoding bacteria outside the Epsilonproteobacteria: *Hydrogenivirga* sp. 128-5-R1-1 and *Sulfurihydrogenibium* sp. YO3AOP1. Both belong to the class Aquificae and contain a *napAGHBFLD* gene cluster.

The NapG and NapH proteins were proposed to form a cytochrome *c*-independent quinol dehydrogenase module [40] and the composition of the epsilonproteobacterial *nap* gene clusters suggests that NapGH generally replaces the function of NapC in other Proteobacteria. As a matter of fact, the *W. succinogenes* NapG and NapH proteins were shown recently to form a membrane-bound protein complex that is likely to catalyse menaquinol oxidation and electron transport to the periplasmic NapAB nitrate reductase complex (Figs. 3 and 4) [41]. Both NapG and NapH are predicted iron–sulfur proteins that contain typical ferredoxin-like polycysteine clusters (Fig. 4). In *W. succinogenes*, NapG was detected in the periplasmic cell fraction of a mutant that lacked NapH [41] and both adjacent arginine residues of the signal peptide of NapG were shown to be essential for nitrate respiration suggesting that mature NapG is exported by the Tat system
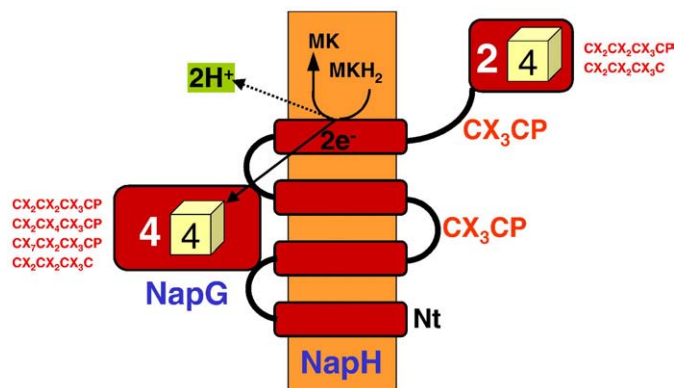

Fig. 4. Model of the NapGH complex from *W. succinogenes*. The orientation of the four transmembrane segments of NapH is depicted according to experimental evidence obtained for *E. coli* NapH using a series of alkaline phosphatase and β-galactosidase fusion proteins [62]. The number and location of the menaquinol-binding site(s) is not known. Menaquinol oxidation by nitrate would be electroneutral if the protons (green background) were released on the periplasmic side (*cf.* Fig. 3). The conserved poly-cysteine motifs of NapG and NapH are shown in red.

[63]. According to the model of the NapGH complex depicted in Fig. 4, NapH traverses the membrane four times. Interestingly, two conserved CX_3CP motifs (which could bind a redox-active metal centre or undergo thiol/disulfide chemistry) as well as two tetracysteine motifs predicted to ligate two [4Fe–4S] centres are expected to be located in the cytoplasm. Together with the four tetracysteine motifs of NapG, this implies that the NapGH complex harbours iron–sulfur centres at opposite sides of the cytoplasmic membrane which is an exceptional feature for a quinone-reactive protein complex [40]. The bioenergetic implications of NapGH-dependent menaquinol oxidation by nitrate are yet to be experimentally addressed but, minimally, only a peri-plasmic quinol-binding site in NapH is required to initiate electron transport via NapG to NapAB which would result in an electroneutral Nap system (Figs. 3 and 4) [24]. If true, this situation would resemble the bioenergetics of the Nrf system (see Section 5) and is in line with the similar cell yield observed for formate-dependent nitrate and nitrite respiration [42]. However, each of the four polycysteine motifs in NapH proved essential for nitrate respiration as cysteine to alanine mutations adversely affected either electron transport or the assembly of the NapGH complex [41]. Similarly, all four tetracysteine clusters of NapG were found to be essential for nitrate respiration [41].

There are two hypotheses with respect to the function of the proposed cytoplasmic metal centres of NapH. The first suggestion implies electrogenic nitrate reduction by menaquinol which is line with the fact that there is enough free energy between the menaquinone/menaquinol and nitrate/nitrite redox couples (standard redox potential difference of about 400 mV) to allow for this. Such a scenario would assume a second quinol-binding site at the cytoplasmic side of the membrane. Furthermore, it cannot be excluded that some rather hydrophobic stretches of NapH might enable the iron–sulfur centres of NapG and NapH to come sufficiently close to allow electron transport between them. These assumptions suggest a Q-cycle-like mechanism for the NapGH complex in which two molecules of menaquinol are oxidised at the periplasmic side and one molecule of menaquinone is reduced at the cytoplasmic side of the membrane giving net translocation of two positive charges per menaquinol oxidised. To date, however, there is no experimental evidence that menaquinol oxidation by nitrate generates an electro-chemical proton potential across the membrane. A second conceptual possibility comes from the fact that certain redox centres in newly synthesized proteins, including molybdenum enzymes and N_2O reductase, undergo reductive activation steps (e.g. [64,65]). It is possible that such a step is involved in cytoplasmic NapA maturation, possibly catalysed by the polyferredoxin-type protein NapF that

harbours four conserved polycysteine motifs. NapF from *E. coli* has been shown to interact with NapA in the cytoplasm [66] and *Rhodobacter sphaeroides* NapF has been proposed to play a role in incorporating the iron–sulfur cluster into NapA [67]. *W. succinogenes* NapF has been recently shown to be located in the membrane fraction of nitrate-grown wild-type cells. In contrast, NapF was found in the cytoplasmic cell fraction of a mutant that lacked the *napH* gene, thus indicating interaction between NapH and NapF (Fig. 3) (M. Kern and J. Simon, unpublished data). A *W. succinogenes napF* deletion mutant was apparently retarded in NapA maturation as the unprocessed, i.e. cytoplasmic form of NapA became clearly visible in immunoblot analysis [63]. Furthermore, the *napF* mutant showed a lower nitrate reductase activity as compared with wild-type cells [63]. On the other hand, NapA maturation and activity was not affected in the absence of NapH [63]. In this case, cytoplasmic NapF might be reduced by another as yet unknown electron delivery route. Taken together, the model outlined above suggests that the arrangement of redox centres allows the NapGH complex to be multifunctional in electron transport to NapA and in NapA maturation (Fig. 3). The existence of a heterotrimeric NapGHF complex is conceivable.

The Nap system of *C. jejuni* is likely to be functionally similar to that of *W. succinogenes* although NapF is absent. A *C. jejuni napA* mutant was shown to be incapable of nitrate reduction to nitrite and NapG from *C. jejuni* was demonstrated to play a dominant role in electron transport of nitrate respiration [57]. In this case, however, it appears that the NrfH protein (see below) initiates an alternative, albeit less-efficient, route of electron delivery to the Nap system that accounts for minor residual growth by nitrate respiration in the *napG* mutant [57]. A NapA-deficient mutant of *C. jejuni* NCTC 11168 was shown to colonize the caecum of one-week-old chicks at slightly lower levels than the wild-type strain [12].

5. Nitrite reduction to ammonium by the respiratory Nrf system

The nitrite reductase of the Nrf system is the periplasmic pentahaem cytochrome *c* NrfA [5,68]. Two different electron transport pathways have been described that connect the quinol pool with NrfA, namely the tetrahaem cytochrome NrfH (a member of the NapC/NrfH family [40,69]) and the NrfB, -C and -D proteins. The latter system is present in enteric bacteria and comprises a pentahaem cytochrome *c* (NrfB, not related to NrfH), an iron–sulfur protein (NrfC) and a membraneous protein (NrfD) [5,70]. NrfD is an assumed quinol dehydrogenase of the so-called NrfD/PsrC family [40]. The available data indicates that ammonifying Epsilonproteobacteria only use the NrfH quinol dehydrogenase since *nrfB*, -C and -D genes are generally absent from their *nrf* gene clusters (Table 1). The *nrfH* gene is always located upstream of *nrfA*.

The NrfH protein from *W. succinogenes* was shown to form a complex with NrfA that catalyses quinol oxidation by nitrite (Fig. 5) [31]. Reconstitution of the purified enzyme in proteoliposomes confirmed that menaquinol oxidation coupled to nitrite reduction is an electroneutral reaction [31]. Non-polar inactivation of the *nrfH* gene in *W. succinogenes* abolished growth by nitrite respiration and resulted in NrfA being located exclusively in the periplasmic space of the mutant strain [71]. Primary structure alignments and site-directed modification experiments suggested different axial haem *c* iron ligation patterns between the related NapC- and NrfH-type proteins [40,72]. Crystals of the NrfHA complex from *W. succinogenes* have been obtained [73] but the only known high-resolution structure of a NapC/NrfH-type cytochrome *c* is that of *Desulfovibrio vulgaris* NrfH, a subunit of the (NrfHA$_2$)$_2$ crystal complex [74]. As predicted, *D. vulgaris* NrfH forms a likely N-terminal transmembrane helix and contains a menaquinone-binding site close to the first haem *c* group [74,75]. The four haem *c* groups form a linear four-haem *c* electron transfer wire that feeds electrons into the NrfA subunit(s) suggesting that each haem *c* group is functionally essential. This assumption is supported

Fig. 5. The respiratory Nrf system of *W. succinogenes*. NrfHA is a membrane-bound menaquinol:nitrite oxidoreductase complex. Protons deriving from menaquinol oxidation are liberated on the periplasmic side of the membrane thus rendering the overall reaction electroneutral (see protons with green background). NrfI is a cytochrome *c* haem lyase that specifically attaches haem to the CXXCK haem *c* binding motif of NrfA [83]. The *nrfI* gene is absent in *nrf* gene clusters that encode a NrfA homolog with five CXXCH haem *c* binding motifs (Table 1).

by the finding that individual replacement of every cysteine and histidine residue within the four CXXCH haem *c* binding motifs of *W. succinogenes* NrfH abolished electron transport from formate to nitrite [69,76]. Two of the four haem *c* groups in *D. vulgaris* NrfH were found to deviate from the standard *bis*-histidine haem *c* iron ligation proposed for NapC-like proteins. Haem 1 has an unusual proximal methionine ligand that derives from a conserved CXXCHXM sequence and the corresponding methionine residue was shown to be functionally essential in *W. succinogenes* NrfH [72]. Haem 4 is histidine/lysine-coordinated, with the distal lysine ligand being provided by an NrfA subunit in the *D. vulgaris* nitrite reductase crystal structure [74]. Several conserved residues in close vicinity of the menaquinol-binding site of *D. vulgaris* NrfH were replaced in *W. succinogenes* NrfH and the phenotypes of the mutants indicated that the quinone-binding site is conserved in NrfH-type cytochromes [69]. This view is also supported by a homology model of *W. succinogenes* NrfH that underlines the overall conservation of tertiary structure in spite of a low sequence homology (the NrfH proteins from *W. succinogenes* and *D. vulgaris* share only 23% identical residues) [69].

The periplasmic NrfA protein is the only ammonifying respiratory nitrite reductase and several crystal structures of NrfA proteins have been solved [74,77–80]. The enzyme was proposed to reduce nitrite via bound NO and hydroxylamine intermediates [81]. Apparently, the periplasmic NO-producing nitrite reductases from many Gram-negative denitrifying bacteria (cytochrome *cd*$_1$ and Cu-dependent nitrite reductases) cannot enter this pathway due to unfavourable, i.e. rather positive, midpoint potentials of their cofactors. The ammonifying nature of many NrfA proteins is thought to be related to a special active site haem 1 group. This haem *c* is axially ligated by a lysine ligand, which originates from a CXXCK haem *c* binding motif in the NrfA proteins from the Epsilonproteobacteria *W. succinogenes*, *S. deleyianum* and *A. butzleri* (Table 1) as well as in many enteric bacteria and sulfate reducers [5]. The lysine residue of the CXXCK motif was shown to be required for full enzymic activity in *E. coli* and *W. succinogenes* NrfA [82,83]. In *W. succinogenes*, the replacement of the lysine residue by histidine resulted in a dramatic decrease (95%) in specific electron transport activity (measured with intact cells using formate as electron donor) although a haem *c* group was still attached to the standard CXXCH haem *c* binding motif [83]. This effect might be explained by alteration of the haem 1 midpoint potential due to an extended distance between the central haem *c* iron atom and its proximal nitrogen ligand.

The incorporation of the CXXCK-ligated haem *c* group requires dedicated cytochrome *c* haem lyase isoenzymes in both *E. coli* and *W.*

succinogenes [82–84]. In *W. succinogenes*, this haem lyase was designated NrfI and belongs to the so-called system II of cytochrome *c* maturation [85,86]. In the absence of NrfI, an inactive tetrahaem NrfA was formed that lacked the active site haem *c* group [83]. On the other hand, NrfI was dispensable in *W. succinogenes* when a NrfA variant with five CXXCH motifs was produced indicating that NrfI specifically handles the attachment of haem 1 to NrfA [83]. The *nrfI* gene is also present downstream of *A. butzleri nrfA* (Table 1). This organism, however, lacks a *nrfJ* gene which is the distal gene of the *nrfHAIJ* gene cluster in *W. succinogenes*. A *nrfJ* gene deletion mutant of *W. succinogenes* was not impaired in respiratory nitrite ammonification [31]. The *nrfJ* gene might be a pseudogene in *W. succinogenes* as it is not present in any other *nrf* gene cluster.

Intriguingly, some NrfA proteins from Epsilonproteobacteria, especially *Campylobacter* species, genuinely contain five CXXCH motifs and therefore lack the CXXCK motif (Table 1). This feature is also found in the predicted NrfA proteins from the Deltaproteobacteria *Bdellovibrio bacteriovorus* and *Myxococcus xanthus*, the Planctomycetes *Rhodopirellula baltica* and *Planctomyces maris* as well as from *Chtho-niobacter flavus* (phylum Verrucomicrobia) but there is no report on the purification of NrfA from any of these bacteria. Recently, however, the heterologous expression of a codon-optimized *C. jejuni nrfA* in *W. succinogenes* yielded a highly active NrfA that could be successfully purified by affinity chromatography (M. Kern and J. Simon, unpublished data). It remains to be seen whether there are major structural rearrangements in this type of NrfA protein that would allow for efficient nitrite reduction even in the absence of the lysine ligand at the active site. Furthermore, variations in the substrate range, e.g. towards the reduction of NO and hydroxylamine (see below) are possible consequences of a slightly altered active site architecture. At present, it also cannot be excluded that another lysine residue located elsewhere in the primary structure is used as the haem *c* iron ligand at the active site, similar to the situation in the tetrathionate- and nitrite-reducing octahaem cytochrome *c* (Otr) from *Shewanella oneidensis* [87,88]. NrfA was shown to be the only nitrite reductase in *C. jejuni* and NrfH appears to be the only electron donor to NrfA [57]. There are conflicting reports on the ability of Nrf-deficient mutants to colonize the chicken gut [12,57].

NrfA from *W. succinogenes* and *S. deleyianum* are known to reduce both nitric oxide and hydroxylamine and crystal structures with these substrates bound to the active site haem *c* group have been reported [81,89,90]. Using a high-potential electron donor, *W. succinogenes* NrfA was also shown to produce N_2O from NO and whole cells of *W. succinogenes* were reported to catalyse this reaction in the presence of acetylene that probably inhibited the *c*Nos system (see Section 6) [91,92]. The NO and hydroxylamine reducing activities of cytochrome *c* nitrite reductase were also observed with *E. coli* NrfA [13,93]. In addition, NrfA was shown to reduce sulfite to sulfide, thus revealing a connection to the biogeochemical sulfur cycle and suggesting that NrfA might have evolved from a sulfite-reducing enzyme [89,94,95]. It was also argued that the NrfA-type nitrite reductase represents an evolutionary ancient enzyme within the nitrogen cycle [3,95]. Conceivably, the NrfHA complex evolved in ancestors of the Epsilonproteobacteria with NrfA carrying five conventional, i.e. CXXCH-bound, haem *c* groups. Later, the lysine ligand was established that possibly imparted an as yet unresolved evolutionary advantage in terms of NrfA activity or substrate range. However, the invention of the CXXCK motif necessitated the co-evolution of a dedicated cytochrome *c* haem lyase (NrfI) since the standard haem lyase was not suitable to deal properly with the CXXCK motif [83]. When the CXXCK-containing NrfA was then transferred to other organisms, e.g. Gammaproteobacteria like *E. coli*, the host organism had to establish a dedicated haem lyase system as well as an electron transfer pathway from the quinol pool to NrfA. In extant Gammaproteobacteria, these tasks are accomplished by the respective NrfEFG and NrfBCD systems that are unrelated to the much simpler NrfI and NrfH proteins from

W. succinogenes [5]. Notably, *E. coli* and *W. succinogenes* use the unrelated cytochrome *c* maturation systems I and II, respectively [85,86].

6. Reduction of nitrous oxide by the unconventional *c*Nos system

N_2O reduction is catalysed by the soluble periplasmic Cu-containing enzyme nitrous oxide reductase (NosZ) which is characteristic of various denitrifying organisms [96]. The homodimeric NosZ binds a binuclear Cu_A and a tetranuclear Cu_Z centre, the latter being located at the active site of N_2O reduction to N_2. Although NosZ has been extensively studied, the nature of its electron-delivering redox partner protein(s) remained unclear although it has been assumed that small (monohaem) *c*-type cytochromes or low-molecular mass cupredoxins such as (pseudo)azurin carry out this function [96]. In the late 1980s, an unusual cytochrome *c* N_2O reductase (*c*NosZ) was purified from *W. succinogenes* and this organism is known to grow by formate-dependent N_2O respiration in addition to nitrate and nitrite ammonification [45,97]. Later, the *W. succinogenes* genome sequence revealed an unprecedented *nos* gene cluster that confirmed the presence of *c*NosZ (Fig. 6) [98]. The predicted primary structure of *c*NosZ is that of a typical N_2O reductase but it carries a C-terminal monohaem cytochrome *c* domain that contains a CXXCH haem *c* binding motif [98]. Several other genes encoding *c*NosZ enzymes appeared recently in the protein data banks and all of them derived from epsilonproteobacterial species (Table 1). One of these bacteria is *C. fetus* subsp. *fetus* which is known to grow by N_2O respiration [92]. Each epsilonproteobacterial *nos* gene cluster is similar to that of *W. succinogenes* and they all contain a unique assembly of *nosG*, -*C1*, -*C2* and -*H* genes surrounded by the *nosD* and -*F* genes (Fig. 6). To date, the *nosG*, -*C1*, -*C2* and -*H* gene arrangement was found exclusively in Epsilonproteobacteria. Notably, *S. denitrificans* contains an additional copy of a *c*NosZ-encoding enzyme situated downstream of another open reading frame that predicts a cytochrome c_{553} homolog. Fig. 6 illustrates the deduced model of what appears to be a typical epsilonproteobacterial electron transport chain that connects the quinol pool with periplasmic *c*NosZ. NosG and NosH are highly similar to NapG and NapH and therefore expected to form a NosGH complex that should be functionally equivalent to NapGH (see Section 4) [40,98]. NosC1 and NosC2 are monohaem *c*-type
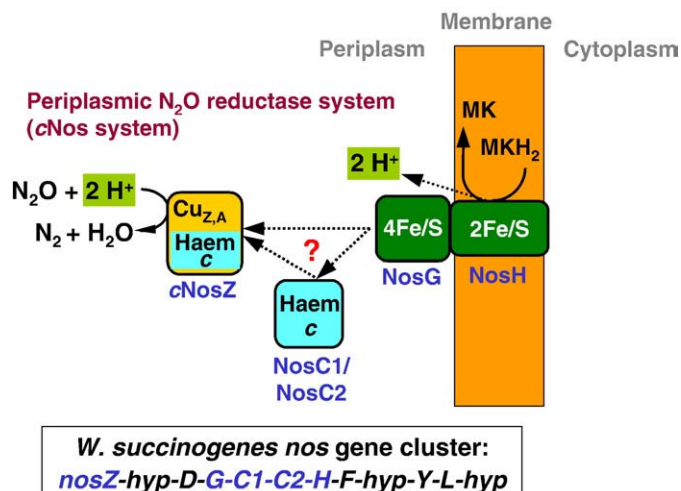

Fig. 6. Model of the respiratory *c*Nos system of *W. succinogenes*. The function of the menaquinol-oxidizing NosGH complex and the electron transfer pathway to cytochrome *c* nitrous oxide reductase (*c*NosZ) via NosC1 and NosC2 is speculative. The *nosF*, -*Y*, and -*D* genes encode a membrane-bound ABC transporter involved in the maturation of the Nos system [96]. The hypothetical open reading frame (*hyp*) situated between *nosZ* and *nosD* in *W. succinogenes* is conserved in all Epsilonproteobacteria listed in Table 1 whereas those located downstream of *nosF* and *nosL* are not present throughout. Note that *napL* is absent from the *nos* clusters in *C. concisus* and *C. curvus*. $Cu_{Z,A}$, binuclear Cu_A and tetranuclear Cu_Z centres of *c*NosZ.

cytochromes that are located either in the periplasm or attached to the membrane via an N-terminal helix (note that such a helix cannot be accurately distinguished from a Sec-dependent signal peptide by standard bioinformatic protocols). Recently, chimeric *nap/nos* operons were expressed in *W. succinogenes* indicating that a NapG–NosH complex is functional in nitrate respiration whereas NosG was apparently not capable to interact with the NapAB system when expressed in either a *nosGH* or *nosG–napH* arrangement [41]. The NapG–NosH assembly was found to be less stable than the NapGH complex since NapG could be easily washed off the membrane by applying low concentrations of sodium chloride [41]. These results suggest that NapG and NosG represent adaptor proteins that determine the specificity of periplasmic electron transport to either the Nap or the Nos system as shown in Figs. 3 and 6. Remarkably, the polycysteine motif arrangement of NapH/NosH proteins is also present in NosR which is encoded in *nos* gene clusters outside the Epsilonproteobacteria [96]. NosR from *Pseudomonas stutzeri* was shown to contain an additional N-terminal periplasmic flavin-binding domain that is essential for N$_2$O respiration [99].

It is unclear why various ammonifying Epsilonproteobacteria contain the *c*Nos system which is assumed to be a typical component of denitrifying species (Table 1). In *W. succinogenes*, the *c*Nos system might be advantageous in reducing N$_2$O that might be produced by NrfA (see Section 5) or Fdp (see Section 7).

7. Nitrosative stress defence in ammonifying Epsilonproteobacteria

Considering the enzymic promiscuity described in Section 5, a role for NrfA in periplasmic nitrosative stress defence and hydroxylamine detoxification was suggested [13–15]. Exerting nitrosative stress is an antimicrobial mechanism in macrophages and there are several studies on nitrosative stress defence in ammonifying Epsilonproteobacteria like *C. jejuni* and *Campylobacter coli* [57,100,101]. The *C. jejuni nrfA* mutant showed a hypersensitive phenotype to nitrosative stress mediated by various compounds that release nitric oxide radicals or NO$^+$ [57]. Based on these results, NrfA was postulated to represent a constitutive periplasmic detoxification mechanism towards reactive nitrogen species. In addition to NrfA, *C. jejuni* was shown to synthesize haemoglobin-type proteins referred to as single-domain haemoglobin (Cgb) and truncated haemoglobin (Ctb) [100,102]. The production of

Cgb in *C. jejuni* is induced under nitrosative stress conditions and a *cgb* mutant is hypersensitive to S-nitrosoglutathione (GSNO) and nitric oxide [100]. Within the Epsilonproteobacteria, however, the presence of Cgb and Ctb seems to be limited to certain *Campylobacter* species (Table 2). Interestingly, the *nrf*-lacking *Campylobacter* species *C. concisus* and *C. curvus* also lack genes encoding haemoglobins suggesting a different sort of nitrosative stress defence in these organisms. Furthermore, Epsilonproteobacteria generally do not encode flavohaemoglobin (Hmp) which is present in many bacterial classes (e.g. in enteric bacteria like *E. coli*) where it decomposes NO both aerobically and anaerobically to nitrate and N$_2$O respectively [14].

There are some more enzymes that have been shown to be implicated in bacterial NO turnover or become up-regulated in response to nitrosative stress [14,103]. Flavodiiron proteins (Fdp, related to enterobacterial flavorubredoxin NorV) catalyse cytoplasmic NO reduction to N$_2$O [104,105]. However, Fdp is not an abundant protein in Epsilonproteobacteria and NorV homologs are generally absent (Table 2). Nonetheless, *W. succinogenes* mutants lacking either Fdp or the Nrf system showed a significantly increased sensitivity to GSNO- and spermine NONOate-induced nitrosative stress in disc diffusion assays under both nitrate- or fumarate-respiring conditions (M. Kern, J. Volz and J. Simon, unpublished data). Hybrid-cluster proteins (Hcp) have also been proposed to play some role in oxidative and/or nitrosative stress defence or hydroxylamine detoxification in proteobacteria [106–108]. Again, Hcp homologs are not regularly encoded in epsilonproteobacterial genomes (Table 2). Preliminary results from work with *W. succinogenes* suggest that solely the Nrf system accounts for hydroxylamine degradation while deletion of the *hcp* gene neither altered the rate of hydroxylamine conversion nor the sensitivity toward reactive oxygen and nitrogen species (M. Kern, J. Volz and J. Simon, unpublished data). Some Epsilonproteobacteria encode multihaem *c*-type cytochromes whose function is not known. Examples are a homolog of hydroxylamine oxidoreductase from nitrifying bacteria (Hao) or the octahaem cytochrome *c* MccA that displays unconventional ligation of a single haem *c* group [109] (Table 2). Possible roles for these cytochromes in epsilonproteobacterial nitrogen metabolism remain to be investigated.

The nitric oxide induced expression of the *C. jejuni cgb* and *ctb* genes is mediated by the NO-sensitive transcriptional regulator NssR [101,110]. NssR is related to Dnr (dissimilatory nitrate respiration regulator) proteins of the Crp-Fnr regulator superfamily and is

Table 2
Genomic prediction of proteins that might be implicated in nitrosative stress defence in Epsilonproteobacteria

Phylogenetic order and organism	Fdp	Cgb	Ctb	Hcp	Hao	MccA
Order Campylobacterales						
Wolinella succinogenes DSMZ 1740	Present	–	–	Present	–	Present
Arcobacter butzleri RM4018	–	–	–	–	–	–
Campylobacter concisus 13826	–	–	–	Present	Present	Present
Campylobacter curvus 525.92	–	–	–	Present	Present	Present
Campylobacter fetus subsp. *fetus* 82-40	–	–	Present	Present	Present	–
Campylobacter hominis ATCC BAA-381	Present	–	–	Present	–	–
Campylobacter jejuni[a]	–	Present	Present	–	–	Present[a]
Campylobacter jejuni subsp. *doylei*	–	Present	Present	–	–	–
Campylobacter coli RM2228	–	Present	Present	–	–	–
Campylobacter lari RM2100	–	Present	Present	–	–	Present
Campylobacter upsaliensis RM3195	–	–	Present	–	–	–
Helicobacter hepaticus ATCC 51449	–	–	Present	–	–	–
Sulfurimonas denitrificans DSMZ 1251	Present[b]	–	Present	–	–	–
Order Nautiliales						
Caminibacter mediatlanticus TB-2	–	–	–	Present	Present	–
Unclassified Epsilonproteobacteria						
Nitratiruptor sp. SB155-2	–	–	–	–	–	–
Sulfurovum sp. NBC37-1	–	–	–	–	–	–

See Table 1 for further explanations. Neither of the following *Helicobacter* species contains any of the six proteins shown here: *H. acinonychis* Sheeba, *H. canadiensis* MIT98-5491, *H. cinaedi* CCUG18818, *H. pullorum* MIT98-5489, *H. pylori* 26695, *H. pylori* HPAG1, *H. pylori* HPKX 438 AG0C1, *H. pylori* HPKX 438 CA4C1, *H. pylori* J99 and *H. pylori* Shi470. –, not present in the genome.

[a] Footnote [e] from Table 1 applies except for MccA which is encoded only on the genomes of the *C. jejuni* subsp. *jejuni* strains 81116, 260.94 and HP93-13.

[b] The *fdp* gene is accompanied by a rubredoxin-encoding gene suggesting the presence of a flavorubredoxin complex [20].

therefore distinct from known NO-responsive regulatory systems in enteric bacteria or in *Neisseria gonorrhoeae* [103,111,112]. Epsilonproteobacteria commonly contain at least one homolog of *C. jejuni* NssR indicating the possibility of a conserved nitrosative stress regulon. Putative palindromic NssR binding sites (consensus sequence TTAAC-N$_4$-GTTAA) were described in *C. jejuni* [101]. Notably, *W. succinogenes* encodes three NssR-type regulators. One of these (*dnrD*) is situated directly upstream of the *nos* gene cluster while a second one is located in the region between the *nap* and *hcp* loci [98]. NssR-type binding sites are located upstream of the *W. succinogenes nap*, *nrf* and *nos* genes in reasonable distances to the experimentally determined transcriptional start sites (C. Winkler and J. Simon, unpublished data).

It is possible that NssR regulators directly bind NO in order to trigger a transcriptional response of target genes. The high-resolution structure of the related Dnr protein from *P. aeruginosa* has been solved and it was argued that this protein could bind a haem cofactor that might respond to NO coordination [113]. It is intriguing that Epsilonproteobacteria do not contain nitrate/nitrite-sensing systems homologous to the NarXL/NarQP two-component systems from enteric bacteria [114]. An obvious question is therefore whether NO might be sensed instead of nitrate and nitrite as small amounts of NO might be present under nitrate-respiring conditions [115]. In *C. jejuni*, nitrate, nitrite or GSNO induced the NssR-dependent expression of the *cgb* gene [57,110]. In contrast, a Nap-deficient mutant did not induce *cgb* expression in response to nitrate, but only in the presence of nitrite indicating that Nap-catalysed nitrate reduction is required for an NssR-mediated response [57]. Recently, the Nar-type nitrate reductase from *S. enterica* has been shown to reduce nitrite to NO in the absence of nitrate [16] and it will be interesting to see if the Nap enzyme catalyses a similar reaction. Furthermore, NrfA itself might be NO-genic in the presence of high nitrite concentrations [116]. In *W. succinogenes*, transcription of the *nap*, *nrf* and *nos* gene clusters is regulated in response to nitrate, nitrite and N$_2$O, respectively [97,117]. Experiments designed to investigate the involvement of NO and the three NssR homologs in the underlying regulatory responses are currently carried out in our laboratory.

8. Concluding remarks and perspective

The ammonifying and denitrifying members of the Epsilonproteobacteria demonstrate a remarkably diverse nitrogen metabolism within the same phylogenetic class (Table 1). Nevertheless, some characteristic features of the involved enzymes and their gene loci are conserved within the class but distinct from other Proteobacteria. One of the most extensive enzymic inventories is displayed by *W. succinogenes* and the concluded model of its nitrogen metabolism is summarized in Fig. 7. We hope that this article provides a framework for the interpretation of future epsilonproteobacterial genome sequences (e.g. from the order Nautiliales) and sequences obtained from metagenomic studies, especially from habitats in which Epsilonproteobacteria dominate. Much of the molecular work on Epsilonproteobacteria is still in its infancy, especially on the detailed investigation of the mentioned bioenergetic mechanisms, enzyme structures and electron transport chain composition. In particular, purification and structural analysis of a NapH/NosH protein or a NapGH/NosGH complex would help to understand the function of this widespread class of membrane-bound quinone-reactive proteins. Such electron transport modules are potential pathogenicity factors of, for example, *Campylobacter* species and therefore conceivable pharmaceutical targets due to their role in survival under micro-aerobic or anoxic conditions during host invasion and colonization. A more detailed knowledge of the respiratory chain of denitrification in Epsilonproteobacteria is also desirable as denitrification causes emission of NO and N$_2$O which is of major concern for atmospheric chemistry and climate change. N$_2$O is the most potent greenhouse gas with a global-warming potential about 300-times greater than that of
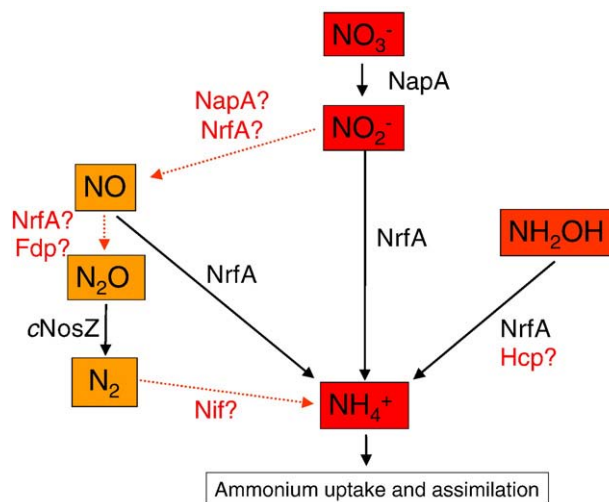

Fig. 7. Schematic model of nitrogen compound conversion in *W. succinogenes*. Reactions depicted by red arrows are hypothetical. The question marks denote that the involvement of the proteins in the given reactions is speculative. See text for details.

carbon dioxide. It accounts for 9% of total greenhouse gas emissions and is indirectly involved in the depletion of the stratospheric ozone layer.

In this context, ammonifying Epsilonproteobacteria that also respire N$_2$O are unusual organisms as they appear to act as sink (but not as source) of both NO and N$_2$O. Therefore, they appear to be excellent model systems for the microbial conversion of N$_2$O to environmentally harmless N$_2$.

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