Hypothesis

The unprecedented *nos* gene cluster of *Wolinella succinogenes* encodes a novel respiratory electron transfer pathway to cytochrome *c* nitrous oxide reductase

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Abstract The ε-proteobacterium *Wolinella succinogenes* grows anaerobically by respiratory nitrite ammonification but not by denitrification. Nevertheless, it is capable of N_2O reduction to N_2. Recently, the genome sequence of *W. succinogenes* revealed a *nos* gene cluster with intriguing features encoding a new type of N_2O reductase. The predicted enzyme is similar to other N_2O reductases exhibiting conservation of all residues ligating the two multinuclear copper centers but carries an unprecedented C-terminal monoheme cytochrome *c* domain. Notably, the N_2O reductase pre-protein is synthesized with a Sec-dependent signal peptide, rather than the usually observed twin-arginine signal sequence, implying that the copper and heme cofactors are both incorporated in the periplasm. The *nos* gene cluster further consists of four adjacent open reading frames which are predicted to encode two monoheme *c*-type cytochromes as well as homologs of NapG and NapH. The latter proteins are thought to function in quinol oxidation coupled to cytochrome *c* reduction in electron transport to periplasmic nitrate reductase. While the accessory genes *nosD*, *-F*, *-Y* and *-L* are present in *W. succinogenes*, homologs of *nosR* and *nosX* are absent from the genome. We hypothesize that the *nos* gene cluster of *W. succinogenes* encodes a complete electron transport chain catalyzing N_2O reduction by menaquinol, a pathway which might also be relevant to other bacteria.

Keywords: Cytochrome *c* nitrous oxide (N_2O) reductase (Nos); Periplasmic nitrate reductase (Nap); Menaquinol dehydrogenase; Respiratory nitrite ammonification; Denitrification; *Wolinella succinogenes*

1. Introduction

Within the biological nitrogen cycle sustained by prokaryotes, respiratory nitrite reduction occurs either by denitrification or nitrite ammonification [1]. In denitrification, N_2 is generated by reduction of nitrite via nitric oxide (NO) and nitrous oxide (N_2O) catalyzed by cytochrome cd_1- or Cu-containing nitrite reductase, NO reductase and N_2O reductase, respectively [2]. Respiratory nitrite ammonification catalyzed by cytochrome *c* nitrite reductase yields ammonium as the only product [1]. Organisms that carry out both respiratory nitrite ammonification and complete denitrification have not been described so far. Nevertheless, *Wolinella succinogenes*, an ε-proteobacterium that grows by nitrate respiration as well as by respiratory nitrite ammonification is also known to grow by N_2O respiration but not by NO reduction [3,4]. The pathway of nitrate ammonification in *W. succinogenes* involves periplasmic nitrate reductase (Nap) and cytochrome *c* nitrite reductase (Nrf). The respective electron transport chains from electron donor substrates like H_2 or formate to nitrate and nitrite have been thoroughly examined [5–7]. Recently, the *W. succinogenes* genome sequence revealed an unprecedented *nos* gene cluster but absence of genes coding for an NO-producing nitrite reductase and a respiratory NO reductase [8]. The *nos* gene cluster encodes a unique cytochrome *c* N_2O reductase and displays unusual features that prompt us to suggest a novel electron transfer route from electron donor substrates to cytochrome *c* N_2O reductase.

2. Organization of the *nos* gene cluster of *W. succinogenes*

The *nos* gene cluster comprises 12 open reading frames (Fig. 1) [8]. The gene arrangement within this cluster is unique as compared to other known *nos* gene clusters [9]. The previously purified cytochrome *c* N_2O reductase of *W. succinogenes* is encoded by the *nosZ* gene [10]. The *W. succinogenes nos* locus includes conventional *nosD*, *-F*, *-Y* and *-L* genes although their arrangement is atypically interrupted by several open reading frames. Particularly noteworthy is an insertion between the *nosD* and *-F* genes that encodes homologs of the putative NapGH quinol dehydrogenase and two monoheme *c*-type cytochromes. A databank search revealed a similar four-gene cluster only in the ε-proteobacterium *Campylobacter fetus* (Cf0040–Cf0043 in GenBank Accession No. AY211269), here adjacent to a partially sequenced upstream *nosD* homolog and downstream *nosF* and *-Y* genes. It is possible that a *nosZ* gene

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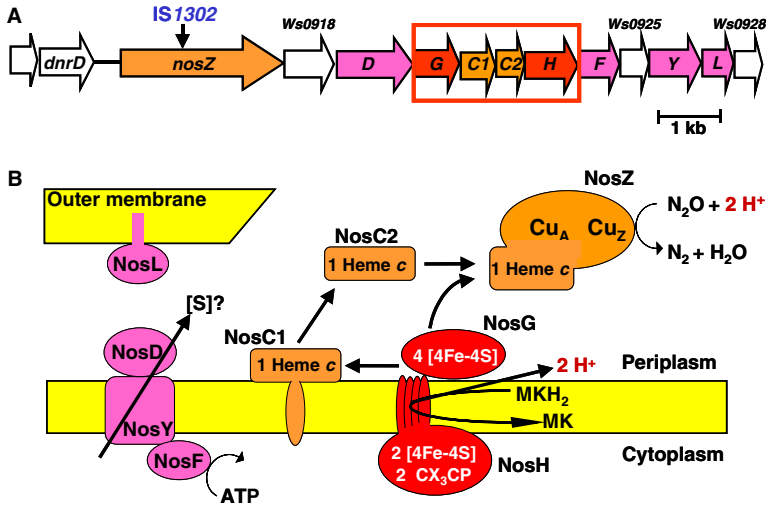

Fig. 1. Physical map of the *W. succinogenes nos* gene cluster (A) and model of the function of individual *nos* gene products (B). The insertion site of IS*1302* within *nosZ* of the published genome sequence [8] is marked by a vertical arrow. Cytochromes *c* are shown in orange, components of the putative menaquinol-oxidizing complex (NosGH) in red and proteins likely to be involved in NosZ maturation in purple. The same color code applies to the corresponding genes. Monomeric NosZ is shown for clarity as the presence of homodimeric NosZ is highly probable. The four open reading frames that are proposed to encode electron transfer components from the menaquinone pool to N_2O reductase are boxed. The topologies of NosG and NosH are drawn according to that of NapG and NapH [23,24]. The localization of NosC1 and NosC2 in the membrane or the periplasmic space is speculative. Drawing of NosF, -Y, -D and -L was adopted from [20].

exists upstream of *nosD* enabling *C. fetus* to reduce N_2O [4]. Other ε-proteobacteria with known genome sequences (*Helicobacter pylori*, *Helicobacter hepaticus* and *Campylobacter jejuni*) do not possess *nos* genes. The *W. succinogenes nos* locus does not encode homologs of the otherwise commonly present NosR and NosX proteins, and corresponding genes are absent from its genome [8].

In the genome sequence of *W. succinogenes* DSMZ1740 [8], the *nosZ* gene is inactivated by a copy of the insertion element IS*1302* [11]. However, with genomic DNA of strain DSMZ1740 and appropriate primers hybridizing to regions upstream and downstream of the insertion site, two amplification products were obtained. The dominant band was the larger IS*1302*-containing *nosZ* fragment and the weaker band corresponded to a *nosZ* fragment lacking the copy of IS*1302* (J. Simon, unpublished). Comparison of the two sequences revealed that an AGC nucleotide triplet was duplicated upon IS*1302* insertion. The result implies that a minor portion of the cells carries an intact *nosZ* gene. The primary structure of the re-assembled full-length NosZ is shown in Fig. 2 and the corresponding nucleotide sequence has been deposited in the GenBank data bank under the Accession No. AJ640086. The amino acid sequence (864 residues) comprises the proteins Ws0914 (residues 1–392) and Ws0916 (residues 396–864) as deduced from the genome sequence [8].

Three conserved hypothetical open reading frames are part of the *W. succinogenes nos* gene cluster (Ws0918, Ws0925 and Ws0928) (Fig. 1). A protein similar to Ws0918 (32% identical residues) is encoded in a genome contig of the α-proteobacterium *Magnetospirillum magnetotacticum* (protein Acc. No. Magn8042) upstream of a sequence predicting homologs of *W. succinogenes* NosD, -G, -H, -F, Ws0925 and NosY (Magn8041–8036). Another homolog of Ws0918 (26% identity) is encoded in the gram-positive bacterium *Desulfitobacterium hafniense* (Desu0380) where it is flanked by a partially sequenced *nosZ* gene (encoding Dh_NosZC in Fig. 2) and followed by a gene arrangement encoding proteins similar to *W. succinogenes* NosD, Ws0925, NosY and NosF (Desu0379–0376). Ws0925 and Ws0928 share 26% identical residues and have some similarity to NosL proteins.

3. Cytochrome *c* N_2O reductase

N_2O reductase is a homodimeric protein with two multinuclear copper centers and was first identified as a periplasmic protein in *Pseudomonas stutzeri* [2,12]. The recent determination of the crystal structures of the N_2O reductases from *Pseudomonas nautica* and *Paracoccus denitrificans* provided the arrangement of the two copper centers Cu_A and Cu_Z, the latter representing the catalytic site of N_2O reduction [13–15]. The deduced primary structure of *W. succinogenes* NosZ shows all conserved ligands of the Cu_A and Cu_Z centers that have been identified in the crystal structures with the exception of a tryptophan residue located between the two Cu_A cysteine ligands (Fig. 2). The ligands of two calcium atoms and one chloride ion found in the structures are less conserved.

W. succinogenes NosZ is unique in exhibiting a C-terminal extension of about 200 residues that carries a single heme *c*-binding motif (CNGCH). This is in line with the previous characterization of the purified *W. succinogenes* enzyme as a cytochrome *c* N_2O reductase [10]. The cytochrome *c* domain might function as an electron donor to the Cu_A center. This is reminiscent of the interaction between cytochrome *c* and the Cu_A center of cytochrome *c* oxidase in aerobic respiration. The Cu_A centers of cytochrome *c* oxidase and N_2O reductase are structurally similar. The fact that *W. succinogenes* NosZ carries a covalently attached heme *c* implies that the machinery responsible for heme export and cytochrome *c* maturation is also required for this particular N_2O reductase [16].

Since the N-terminal residue of the mature *W. succinogenes* NosZ is Ser-25 (O. Einsle and P.M.H. Kroneck, unpublished),

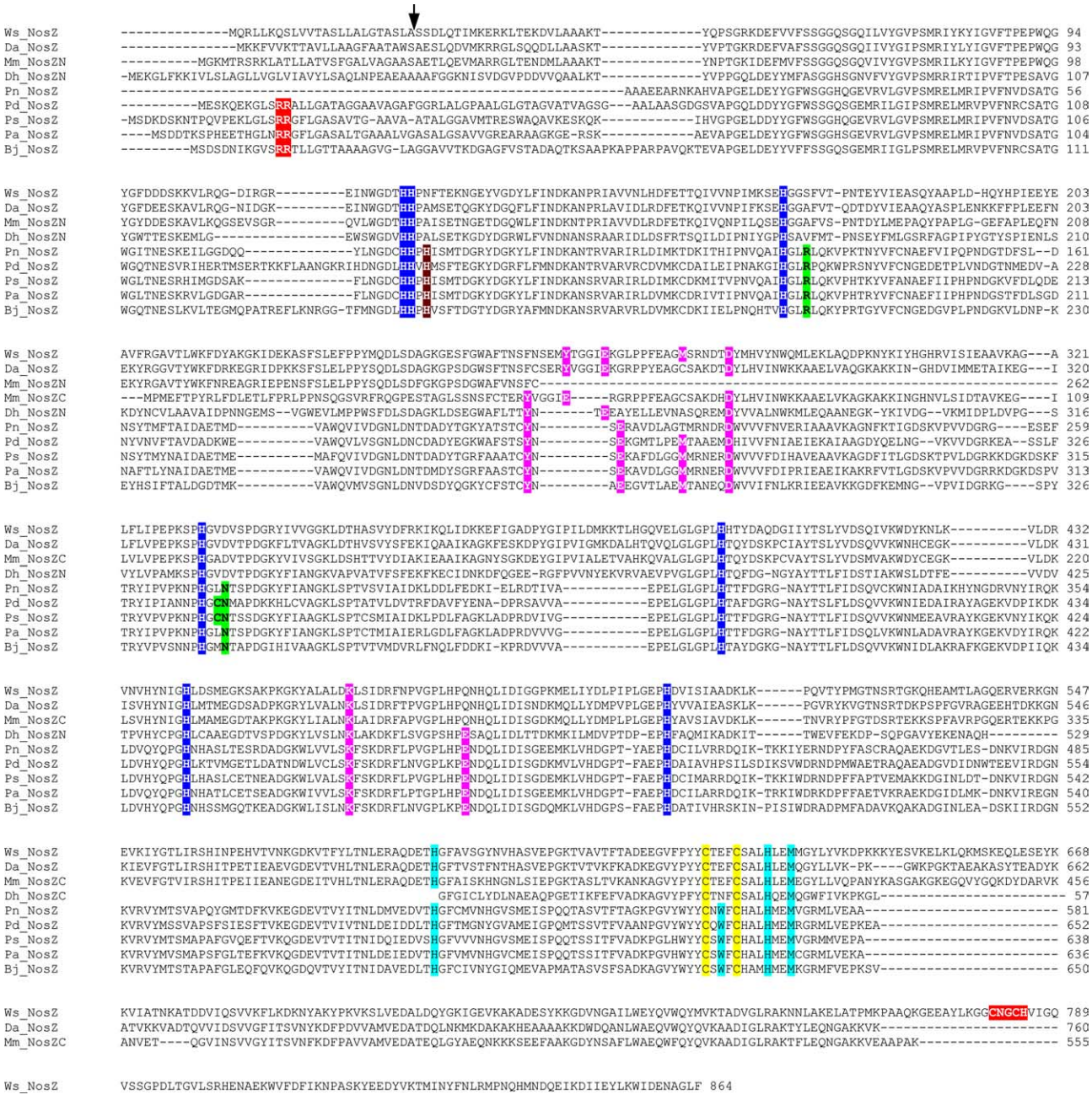

Fig. 2. Alignment of NosZ primary structures from *W. succinogenes* and other bacteria. The experimentally determined cleavage site of *W. succinogenes* pre-NosZ is marked by an arrow. Conserved histidine ligands of the Cu_Z center are highlighted in dark blue; cysteine and other ligands of the Cu_A center in yellow and turquoise, respectively. The partially conserved histidine residue is highlighted in brown. Calcium atom and chloride ion ligands, as identified by the known crystal structures of N_2O reductases, are shown in magenta and green, respectively. The conserved twin-arginine motif within signal peptides and the heme *c*-binding motif of *W. succinogenes* NosZ are marked in red. Two putative NosZ sequences originating from the termini of different *M. magnetotacticum* genome sequence contigs are referred to as Mm_NosZN and Mm_NosZC. Although both sequences have a non-identical overlap in a middle region of the alignment, it might be possible that they belong to the same protein. There are also two partial sequences of *D. hafniense* NosZ which are denoted Dh_NosZN and Dh_NosZC. For *P. nautica* no signal sequence is shown since the gene has not been identified. The alignment was generated with CLUSTAL W 1.82 and manually adjusted. Ws, *Wolinella succinogenes*; Da, *Dechloromonas aromatica*; Mm, *Magnetospirillum magnetotacticum*; Dh, *Desulfitobacterium hafniense*; Pn, *Pseudomonas nautica*; Pd, *Paracoccus denitrificans*; Ps, *Pseudomonas stutzeri*; Pa, *Pseudomonas aeruginosa*; Bj, *Bradyrhizobium japonicum*.

the protein is predicted to be synthesized with a typical Sec-dependent signal peptide of 24 residues (Fig. 2). With this cleavage site and the covalent binding of one heme group, a molecular mass of 95 424 is calculated. This value is in close proximity to $95\,540 \pm 39$ determined previously by mass spectrometry [17]. The signal peptide of *W. succinogenes* NosZ does not contain the two conserved arginine residues that are commonly found in other NosZ signal peptides (Fig. 2). This type of signal peptide is characteristic of proteins exported by the twin-arginine transport (Tat) system and

its presence suggests that NosZ proteins are commonly transported across the membrane in a folded state [18]. As pre-proteins of *c*-type cytochromes are generally exported by the Sec system, it is not surprising to find a Sec- rather than a Tat-dependent signal peptide in *W. succinogenes* NosZ. Over the years evidence has been accumulated for metal cofactor insertion into NosZ occurring in the periplasm despite the fact that this reductase is transported by the Tat system [19,20]. The NosZ protein thus represents a clear exception to the view generally held that Tat-transported proteins are supplied with their cofactors in the cytoplasm [18]. Sec dependence of NosZ transport in *W. succinogenes* provides indirect support for periplasmic cofactor insertion from an independent and quite unexpected side.

NosZ sequences most similar to that of *W. succinogenes* are those from *Dechloromonas aromatica*, *M. magnetotacticum* and *D. hafniense* as deduced from the respective preliminary genome sequences (Fig. 2). The NosZ proteins of the latter three organisms lack typical twin-arginine signal peptides but carry reasonable Sec signal peptides. The sequences from *D. aromatica* and *M. magnetotacticum* have C-terminal extensions similar to that of *W. succinogenes* NosZ but do not exhibit heme *c*-binding motifs (Fig. 2). It is striking that the primary NosZ structures of *W. succinogenes*, *D. aromatica*, *M. magnetotacticum* and *D. hafniense* are clearly divergent from those of the other sequences shown in Fig. 2, especially between the third and fourth Cu_Z histidine ligand. Furthermore, only in these four sequences is the otherwise conserved histidine residue, following the first two Cu_Z histidine ligands, replaced by asparagine or alanine (Fig. 2).

4. A predicted electron transport chain from menaquinol to NosZ

In *W. succinogenes*, H_2 and formate are oxidized by membrane-bound heterotrimeric hydrogenase and formate dehydrogenase complexes, respectively [1,21]. Both enzymes couple substrate oxidation to reduction of menaquinone-6 which is the only quinone found in nitrate-grown *W. succinogenes* cells. We hypothesize that the predicted proteins NosG, NosH, NosC1 and NosC2 are involved in electron transfer from menaquinol to the periplasmic NosZ in *W. succinogenes* (Fig. 1). The grouping of the corresponding genes within the *nos* gene cluster is not unusual for functionally interacting components of bacterial electron transport chains. Proteins that transfer electrons directly to NosZ in vivo have not been identified so far, but it is likely that *c*-type cytochromes carry out this function [2].

W. succinogenes NosG and NosH are homologous to NapG and NapH, respectively, which are predicted by numerous *nap* gene clusters encoding the periplasmic nitrate reductase complex, NapAB [7,22]. Both proteins are predicted to be polyferredoxins binding iron–sulfur centers through conserved cysteine motifs. *W. succinogenes* NosG as well as typical NapG proteins carry a Tat signal peptide and are predicted to bind four [4Fe–4S] centers. The conserved third cysteine motif of NapG ($CX_7CX_2CX_3CP$), however, is altered to $CX_{40}CX_2CX_3CP$ in *W. succinogenes* NosG and *C. fetus* Cf0043, and to $CX_{43}CX_2CX_3CP$ in *M. magnetotacticum* Magn8040. Typical NapH sequences exhibit two conserved CX_3CP and two conserved $CX_2CX_2CX_3C$ motifs which are also present in *W. succinogenes* NosH and *C. fetus* Cf0040. In contrast, the second cysteine residue in the first four-cysteine-motif of *M. magnetotacticum* Magn8039 is apparently replaced by glutamate.

The functions of NapG and NapH have been investigated in detail only in *Escherichia coli* where the two proteins transfer electrons from ubiquinol to NapAB [23,24]. NapH is a hydrophobic protein that is probably anchored in the membrane by four conserved transmembrane domains, while NapG is thought to be transported across the cytoplasmic membrane where it might form a complex with NapH [24]. Such a quinol dehydrogenase complex is presumed to transfer electrons to a *c*-type cytochrome, either the tetraheme protein NapC or the diheme protein NapB. *W. succinogenes* NosG and NosH are similar to *W. succinogenes* NapG and NapH and the latter two proteins are likely to be involved in the NapC-independent electron transport from menaquinol to NapAB [7].

The predicted primary structures of the monoheme *c*-type cytochromes NosC1 and NosC2 of *W. succinogenes* are 35% identical. Both sequences have hydrophobic N-terminal regions that may represent Sec-dependent signal peptides or function as membrane anchors. Proteins similar to *W. succinogenes* NosC1 and NosC2 are encoded in the genome of *C. fetus* (Cf0042 and Cf0041). These data suggest that a *c*-type cytochrome transfers electrons to NosZ. Zhang and Hollocher [25] examined a *W. succinogenes* cytochrome *c* which in vitro rapidly transfers electrons to NosZ. This cytochrome, however, is not identical to NosC1 or NosC2 but corresponds to the monoheme cytochrome *c* Ws0700 [8]. Monoheme *c*-type cytochromes unrelated to *W. succinogenes* NosC1 and NosC2 are encoded in or adjacent to *nos* loci in *D. hafniense*, *P. denitrificans* and various *Ralstonia* species [9]. The partial *nosZ* gene of *M. magnetotacticum* (Magn5537) is accompanied by two genes which both encode proteins with two heme *c*-binding motifs each.

It remains to be determined which of the NosG, NosH, NosC1 and NosC2 proteins are essential for N_2O respiration in *W. succinogenes*. There might be convergent pathways channeling electrons to the monoheme domain of NosZ. The NosG and NosH proteins are of particular interest as they may form a membrane-bound quinol oxidation module interacting with *c*-type cytochromes that participate in various electron transport chains. Interestingly, a portion of *W. succinogenes* N_2O reductase seems to be associated with the membrane (U. Hole and P.M.H. Kroneck, unpublished), which is also the case for the periplasmic nitrate reductase NapA [7].

It is as yet unknown whether menaquinol oxidation by N_2O (or nitrate) is an electrogenic or electroneutral process. Electron movement from menaquinol to NosZ (or NapAB) and proton release upon menaquinol oxidation by the NosGH (or NapGH) complex are both most likely periplasmic events and thus these redox steps do not give rise to charge separations over the membrane (Fig. 1). A proton-pumping activity of a putative NosGH (or NapGH) complex, however, cannot be excluded. If N_2O (or nitrate) reduction by menaquinol is electroneutral, the generation of an electrochemical proton potential during N_2O respiration would be entirely due to H_2- or formate-dependent menaquinone reduction which are electrogenic processes in *W. succinogenes* [1,21].

5. Regulation of cytochrome *c* N_2O reductase synthesis

The *W. succinogenes* genome harbors three *dnr* paralogs whose deduced proteins belong to the Crp–Fnr superfamily of transcription regulators [26]. The gene encoding DnrD is located upstream of *nosZ* (Fig. 1), the two other Dnr factors (Ws1181 and Ws1193) are encoded by genes located in close vicinity to each other within the genome region encoding Nap proteins and factors for molybdopterin biosynthesis [7,8]. All three regulators belong to the phylogenetically defined Dnr group of the Crp–Fnr protein tree [27].

Dnr-type regulators have been found to be involved in *nos* gene regulation. In *P. stutzeri*, DnrD activates, in response to NO, the *nosR* gene, whose product is required for *nosZ* transcription [28]. In *Pseudomonas aeruginosa*, the *nos* genes are arranged as a *nosRZDFYL* operon which is under the control of Dnr [29]. Dnr-dependent regulation requires cognate recognition motifs in the promoter of target genes similar to the Fnr-type consensus, TTGAT-N_4-ATCAA [26]. Given these precedents from other bacteria, one can reasonably predict for *W. succinogenes* the following scenario. The *dnrD* gene located upstream of *nosZ* encodes the regulator for *nosZ* expression. The *nosZ* promoter does carry the putative DnrD recognition sequence, aTGAc-N_4-ATCAA. The *dnrD* promoter also carries a recognition sequence, TTGAT-N_4-gTCAA, indicating *dnrD* autoregulation as is often observed with Dnr factors. The distance of the motifs from the respective translational start codons is in each case about 70 nucleotides, which clearly allows for the frequently observed distance of 41–43 nucleotides from the transcriptional start site in so-called class II promoters. The putative regulatory circuit predicts induction of *nos* genes of *W. succinogenes* in response to NO or nitrate as shown for other organisms with Crp–Fnr factors [28,30–32].

A conspicuous feature of the *W. succinogenes nos* gene cluster is the absence of *nosR* which is otherwise a regular component of *nos* gene clusters in N_2O-respiring bacteria [9]. The *nosR* gene is nearly always located directly upstream or downstream of *nosZ*, only *Burkholderia* spp. terminate their *nos* clusters with *nosR*. NosR has been shown to be required for *nosZ* expression in *P. stutzeri* [33] but not in *P. aeruginosa* [29], whereas in *P. denitrificans* the NosR homolog NirI is required for transcription of *nirS* encoding cytochrome cd_1 nitrite reductase [34]. Given this evidence, it is likely that NosR proteins have more than one function. The deduced NosR protein has a multidomain structure extending at both sides of the cytoplasmic membrane. A transmembrane function is likely, though this function has not yet been clarified. It has been noted previously that NosR shares common structural motifs with NapH [35], a fact that also holds true for *W. succinogenes* NosH. This raises the possibility that NosH substitutes a particular NosR function residing in the C-terminal transmembrane part and/or in potentially metal-binding portions of the protein, e.g., a function in sensing the redox status of the cell. It will be interesting to examine whether future genomic data will show a correlation between the absence of NosR and the presence of NosH.

The *W. succinogenes nos* gene cluster harbors *nosD*, *-F*, *-Y* and *-L* genes. The *nosD*, *-F* and *-Y* gene products are essential for the assembly of active *P. stutzeri* NosZ carrying its Cu cofactors [20]. The *W. succinogenes nosL* gene located directly downstream of *nosY* is considered to be the genuine *nosL*, since the deduced protein has the highest similarity to NosL proteins of denitrifying bacteria; but we note that there are two more *nosL* paralogs in *W. succinogenes* (see above). The gene *nosX*, which is frequently found in *nos* gene clusters, is also absent from the *W. succinogenes* genome. This indicates a non-essential function for the NosX protein or its substitution by other proteins as discussed for *P. stutzeri* [20].

6. Conclusion

The *nos* gene cluster of *W. succinogenes* shows an unprecedented arrangement of open reading frames predicting an electron transport chain from menaquinol to cytochrome *c* N_2O reductase. It might turn out that *nos* clusters similar to that of *W. succinogenes* are present in other (and largely unrelated) bacteria underlining the implications suggested here. Re-examination of cytochrome *c* N_2O reductase as well as studying other predicted electron transfer components will lead to a better understanding of electron transport to the multinuclear copper centers of NosZ and of the catalytic mechanism of N_2O reduction. A *W. succinogenes* strain that overproduces full-length cytochrome *c* N_2O reductase was obtained recently and will be the starting point for investigations in our laboratories.

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