Role of individual *nap* gene cluster products in NapC-independent nitrate respiration of *Wolinella succinogenes*

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Bacterial *nap* gene clusters, encoding periplasmic nitrate reductase (NapA), are complex and diverse, and the composition of the electron transport chain donating electrons to NapA is poorly characterized in most organisms. Exceptionally, *Wolinella succinogenes* transfers electrons from formate via the menaquinone pool to NapA independently of a membrane-bound *c*-type cytochrome of the NapC family. The role of individual ORFs of the *W. succinogenes* *napAGHBFLD* gene cluster is assessed here by characterizing in-frame gene inactivation mutants. The ability of the mutants to grow by nitrate respiration was tested and their NapA content and specific nitrate reductase activity were determined. The *napB* and *napD* gene products proved to be essential for nitrate respiration, with NapD being required for the production of mature NapA. Inactivation of either subunit of the putative membrane-bound menaquinol dehydrogenase complex NapGH almost abolished growth by nitrate respiration. Substitution of the twin-arginine sequence of NapG had the same effect as absence of NapG. Phenotypes of mutants lacking either NapF or NapL suggest that both proteins function in NapA assembly and/or export. The data substantiate the current model of the composition of the NapC-independent electron transport chain as well as of NapA maturation, and indicate the presence of an alternative electron transport pathway to NapA.

Received 18 May 2007

Revised 22 June 2007

Accepted 28 June 2007

INTRODUCTION

Prokaryotes are able to synthesize three distinct types of nitrate reductase that all reduce nitrate to nitrite but serve different physiological functions (for reviews see Lin & Stewart, 1998; Potter *et al.*, 2001; Richardson *et al.*, 2001; González *et al.*, 2006). Two nitrate reductases (Nar and Nap) terminate respiratory electron transport chains, while the cytoplasmic Nas enzyme is involved in assimilatory nitrate reduction. The Nar enzyme is bound to the membrane with the active site of nitrate reduction facing the cytoplasm, whereas Nap is located in the periplasmic space of Gram-negative bacteria. Electrons transported to Nar or Nap derive from the membranous quinol pool. The catalytic subunit of the periplasmic nitrate reductase (NapA) usually receives electrons from the dihaem *c*-type cytochrome NapB. Depending on the stability of interaction between NapA and NapB, heterodimeric nitrate reductases (NapAB) and monomeric nitrate reductases (NapA) have been reported (Jepson *et al.*, 2006, 2007; Arnoux *et al.*, 2003). In most cases NapB is thought to be reduced by NapC, a membrane-bound tetrahaem cytochrome *c* that is thought to catalyse quinol oxidation. NapC is prototypic for members of the NapC/NirT/NrfH

family, which mediate electron transfer in various anaerobic respiratory chains (Roldán *et al.*, 1998; Simon *et al.*, 2000, 2001; Gross *et al.*, 2005; Rodrigues *et al.*, 2006). More recently, a putative membrane-bound complex consisting of the two predicted iron–sulfur proteins, NapG and NapH, was proposed to be functionally equivalent to NapC (Richardson *et al.*, 2001; Simon *et al.*, 2003, 2004). NapG and NapH are likely to make up a quinol dehydrogenase that transports electrons from the quinone pool to a periplasmic *c*-type cytochrome, i.e. NapB or NapM (see below) (Brondijk *et al.*, 2002, 2004; Jepson *et al.*, 2006). The putative NapGH complex has been shown in *Escherichia coli* to function as a ubiquinol dehydrogenase that transfers electrons from ubiquinol via NapC to NapAB, while NapC catalyses menaquinol oxidation independently of NapGH (Brondijk *et al.*, 2004). Apparently, *napG* and *napH* genes are present when *napC* is lacking. The composition of bacterial *nap* operons is diverse (Marietou *et al.*, 2005; González *et al.*, 2006). While the *napA* and *napD* genes seem to be present throughout, NapF, NapL and NapM are encoded less frequently. NapF and NapD are predicted to be cytoplasmic proteins that function in NapA maturation (Berks *et al.*, 1995; Olmo-Mira *et al.*, 2004; Nilavongse *et al.*, 2006). To date, NapL has been found only in *nap* operons of ϵ -proteobacteria, while NapM is a multihaem cytochrome *c*

Abbreviations: BV, benzyl viologen; Tat, twin-arginine translocase.

that is encoded in some *nap* operons lacking *napB* (Jepson *et al.*, 2006). The functional role of both NapL and NapM is unclear.

Like many other bacteria, the ϵ -proteobacterium *Wolinella succinogenes* grows by anaerobic respiratory nitrate ammonification, using formate or H_2 as electron donor substrates (Bokranz *et al.*, 1983; Lorenzen *et al.*, 1993; for reviews see Kröger *et al.*, 2002; Simon, 2002). This mode of respiration is dependent on two terminal reductases, periplasmic nitrate reductase (NapA) and cytochrome *c* nitrite reductase (NrfA). Mutants deficient in *napA* or in *nrfA* do not grow by nitrate and nitrite respiration, respectively (Simon *et al.*, 2000, 2003). The *W. succinogenes* genome sequence does not predict any other obvious nitrate- or nitrite-reducing enzyme (Baar *et al.*, 2003). It has been demonstrated that *W. succinogenes* nitrate respiration is independent of a NapC-like protein, since a mutant lacking both NapC homologues encoded in the genome (NrfH and FccC) grows by nitrate respiration and exhibits wild-type growth parameters (Simon *et al.*, 2003). Together with NrfA, the NrfH protein forms a membrane-bound complex that catalyses nitrite reduction using menaquinone as electron donor (Simon *et al.*, 2000).

The *W. succinogenes* genome contains a rather unusual *napAGHBFLD* gene cluster (Simon *et al.*, 2003). A model of the electron transport chain of *W. succinogenes* nitrate respiration has been proposed in which one or more of the *napF*, *G*, *H* and *L* gene products mediate(s) electron transport from menaquinol to the periplasmic NapAB complex, hence substituting for the function conventionally carried out by NapC (Simon *et al.*, 2003). The function of each *nap* gene product is tested here by characterizing a set of in-frame deletion mutants each defective in a single *nap* gene situated downstream of *napA*.

METHODS

Growth of *W. succinogenes*. *W. succinogenes* cells were grown by nitrate respiration in medium (pH 7.5) containing Tris (50 mM), sodium formate (80 mM), potassium nitrate (50 mM), K_2HPO_4 (1 mM), fumaric acid (5 mM), ammonium sulfate (5 mM), $MgCl_2$ (1 mM), $CaCl_2$ (0.2 mM), glutamic acid (0.68 mM), cysteine (0.57 mM) and trace element solution (0.2 ml l⁻¹), as used by Pfennig & Trüper (1981). Brain–heart infusion broth (0.25 or 0.5%, w/v) was added where appropriate. Growth by fumarate respiration was performed in the medium described by Kröger *et al.* (1994). Where indicated, 20 mM potassium nitrate was added.

Cell fractionation. *W. succinogenes* cells harvested in the exponential growth phase were suspended (10 g cell protein l⁻¹) in an anoxic buffer (pH 8.0) containing 50 mM Tris/HCl. The suspension was passed through a French press at 130 MPa and 10 ml min⁻¹ flow rate. The resulting cell homogenate was centrifuged for 45 min at 100 000 g to yield the membrane fraction (sediment) and the soluble fraction.

Determination of specific enzyme activities, and analytical procedures. Nitrate reductase activity was measured under anoxic conditions at 37 °C by photometrically recording the oxidation of

reduced benzyl viologen (BV) by nitrate at 546 nm. The test solution contained 50 mM potassium phosphate (pH 7.0), 1 mM BV and 10 mM sodium nitrate. BV was reduced by sodium dithionite and the reaction was started by addition of nitrate. Specific activities were calculated using an extinction coefficient (ϵ) of 19.5 mM⁻¹ cm⁻¹. For each micromole nitrate reduced to nitrite, 2 μ mol BV were oxidized. Protein was measured using the Biuret method with KCN (Bode *et al.*, 1968). Nitrate, nitrite and formate (using NAD⁺ and formate dehydrogenase) were determined as described elsewhere (Hartley & Asai, 1963; Rider & Mellon, 1946; Bergmeyer, 1974).

Construction of *W. succinogenes* mutants. Standard genetic procedures were used (Sambrook *et al.*, 1989). DNA was isolated from *W. succinogenes* using the DNeasy Tissue kit (Qiagen). PCR was carried out using BIOTAQ DNA polymerase (Bioline) or the Expand High Fidelity PCR System (Roche) with standard amplification protocols. *W. succinogenes* $\Delta napG-D$ was constructed using a deletion plasmid in order to replace the genomic *napAGHBFLD* region by a chloramphenicol resistance gene (*cat*) cartridge according to a strategy outlined previously (Simon *et al.*, 2000). The deletion plasmid p $\Delta napG-D$ contained the *cat* gene flanked by two PCR fragments identical to regions of the *W. succinogenes* genome. The two PCR fragments were obtained using the following primer pairs: 5'-CGGAATTCACCTGAGATGCCTAGTAGCG-2911 and 5'-CGGATCCCCGCTCCTAAAGCCCTTAGGC-3262 for amplifying the upstream fragment; and 5'-CGGGATCCGAGGAGCTAGAGCTAGAGCGCC-7164 and 5'-ACGCGTTCGACGGGGCTCATTCTCGCGTCTCC-7658 for the downstream fragment. The primers carried an *EcoRI*, *BamHI* or *Sall* restriction site (underlined) for cloning. The numbers at the 3' ends of the primer sequences correspond to the nucleotide positions in the EMBL, GenBank and DDBJ database (accession number AJ512686). Transformation of *W. succinogenes* wild-type cells (DSMZ 1740) with p $\Delta napG-D$ was carried out by electroporation, as described previously (Simon *et al.*, 2000), except that transformants were selected on agar plates containing formate/fumarate medium and 12.5 mg chloramphenicol l⁻¹. The desired substitution in the transformant genome was confirmed by PCR and Southern blot analysis employing suitable *nap* and *cat* probes (not shown). The deleted region comprises the entire *napG*, *H*, *B*, *F* and *L* genes as well as 225 bp of the 5' end of *napD* (64% of the gene).

In order to restore the complete *nap* gene cluster on the genome of *W. succinogenes* $\Delta napG-D$, plasmid pnapG-Dkan was constructed, which contained 36% of *napA* (3' end) as well as the complete *napG*, *H*, *B*, *F*, *L* and *D* genes (Fig. 1). The *nap* region was present on a PCR fragment amplified with the primer pair 5'-ACGCGTTCGACCCCGTAGGAAATGCGATGCC-2297 and 5'-TCCCCGCGCGGGGGC-TATTCTCGGTCTCC-7658; see above for numbering. The primers carried a *Sall* or *XmaIII* restriction site (underlined) for cloning. The resulting 5.4 kb fragment was incorporated into a derivative of pBR322 containing a *cat* gene inserted into its unique *BamHI* site. Sequencing of the entire *nap* insert revealed two single-nucleotide mutations in *napH* (positions 4818 and 4826) that were corrected by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) and an appropriate primer pair (sequences not shown). The single *BamHI* site of the *nap* insert (positions 2886–2891) was silently mutated to GGACCC to allow the subsequent replacement of *cat* by a kanamycin resistance gene cartridge (*kan*; obtained by *BamHI* excision from pUC4K). PCR analysis showed that plasmid pnapG-Dkan contained the *kan* gene in the same orientation as the *nap* cluster (Fig. 1). After electroporation of *W. succinogenes* $\Delta napG-D$ cells with pnapG-Dkan, transformants were selected in formate/fumarate medium containing 50 mM nitrate, 25 mg kanamycin l⁻¹ and 12.5 mg chloramphenicol l⁻¹. The desired integration of the plasmid into the genome was confirmed by PCR and Southern blot analysis (not shown). The correctness of the 991 bp *napA* sequence involved in plasmid

Fig. 1. Restoration of the complete *nap* operon on the genome of *W. succinogenes* Δ *napG-D* through homologous recombination using plasmid pnapG-Dkan. See Methods for details.

integration was proven by amplification and sequencing of an appropriate PCR product.

Site-directed mutagenesis of *nap* genes was performed using the QuikChange site-directed mutagenesis kit and pnapG-Dkan (or a derivative) as a template. A pair of complementary primers was used for each modification, for which the forward primer sequences (5'→3') were as follows; stop-napG-1, GGATGATGAAAGAGCT-TTAATGAGACGAACGAGG-3318; stop-napG-2, GGAGCACCCC-TTGATAAATGTGCGAGGATATTCC-3596; stop-napH-1, GAAAT-TTTTAAACACTAATGATTCCTTACTCCTTCGG-4123; stop-napH-2, GGGGCTTCCCGCTTAATAGTTTATCAGCCC-4533; stop-napB-1, GGTTCATCCACGTGATAGGCTTTAGCCTTC-4970; stop-napF-1, GGTTTTTCACCTCTCTTTAGTGATCCAAAAGGG-5513; stop-napF-2, GGGGATGCACCTTTTGATAAGCATGTGCGTTGC-C-5707; stop-napL-1, CCCTCTATCTCATCTAATGAGTCACTCTC-TTGG-6010; stop-napL-2, CCCCTAAAATCTAATGAATCGATAC-ACTCG-6232; stop-napD-1, GAATATTTCTAGTGTGTTTGATAG-GTCAAGCCCGAGG-6957; RRnapG, CAATCAGACGAACGGGGG-GGGGTTATCTAGG-3329 (see above for numbering). The altered nucleotides are indicated in bold type and the modified codons are underlined (see Table 1 for details of modified codons and encoded residues). Transformation of *W. succinogenes* Δ *napG-D* cells with plasmid derivatives and genetic characterization of the transformants were performed as described above. In addition, each mutation was confirmed by sequencing of an appropriate PCR fragment. The preservation of desired stop codons was routinely confirmed after growth experiments.

W. succinogenes Δ *nosG-H kan* was constructed using a deletion plasmid in order to replace the genomic *nosGC1C2H* region by a kanamycin resistance gene cartridge (*kan*) by a similar strategy to that described above. The deletion plasmid p Δ *nosG-H* contained the *kan* gene flanked by two PCR fragments identical to regions in the *W. succinogenes* genome. The two PCR fragments were obtained using the following primer pairs: 5'-CGGAATTCCTATTTGAGCGCAACT-CTGTGGGG-181303 and 5'-CGGGATCCCAAAATTCGCCTCCA-TTTTGGGC-181855 for amplifying the upstream fragment; and 5'-CGGGATCCAAGAGCCAAAAGGAGAACTATTGATTG-184541 and 5'-ACGCGTTCGACCATGGCAAACTCTCTCAAAAGCGC-185042 for the downstream fragment. The primers carried an *Eco*RI, *Bam*HI or *Sal*I restriction site (underlined) for cloning. The numbers at the 3' ends of the primer sequences correspond to the nucleotide positions given in the EMBL, GenBank and DDBJ database (accession number BX571659). The desired substitution in *W. succinogenes* transformants (selected on formate/nitrate medium with 25 mg kanamycin l⁻¹) was confirmed by PCR analysis employing

suitable *nos* and *kan* primers (not shown). The deleted region starts with the *nosG* start codon and comprises each *nosG* and *nosH* codon up to and including the *nosH* stop codon.

RESULTS AND DISCUSSION

In-frame inactivation of *W. succinogenes* *nap* genes

A set of *nap* mutants was generated as described in Methods and illustrated in Fig. 1. First, *W. succinogenes* Δ *napG-D* was constructed, which lacks the *napG*, *H*, *B*, *F*, *L* and part of the *napD* genes. In a second step the entire *nap* operon was restored on the genome of this mutant upon integration of pnapG-Dkan, resulting in the presence of one copy of the *nap* operon located at its genuine genomic position under the control of the wild-type *nap* promoter (Fig. 1). The mutant containing the restored wild-type *nap* operon (*W. succinogenes* N5) showed wild-type properties with respect to growth by nitrate respiration, specific nitrate reductase activity and NapA content (Tables 1 and 2, Fig. 2). In contrast, the Δ *napG-D* mutant did not grow by nitrate respiration and lacked NapA-dependent nitrate reductase activity when grown under fumarate-respiring conditions. The phenotype of the latter mutant resembled that of the NapA-deficient strain *W. succinogenes* *napA::kan* (Simon *et al.*, 2003). Interestingly, the Δ *napG-D* mutant did not synthesize an immunodetectable form of NapA, demonstrating that at least one of the other *nap* gene products is essential for the synthesis of folded and functional NapA (Fig. 2). During nitrate respiration, wild-type cells produced nitrite at a concentration that almost corresponded to the concentration of nitrate degraded (Fig. 3a). Only about 5 mM nitrite was converted to ammonium before formate was exhausted, indicating that there was only a minor contribution of nitrite respiration to total cell growth under these growth conditions. Like *W. succinogenes* *napA::kan*, the Δ *napG-D* mutant did not reduce nitrate and no nitrite was produced (Fig. 3b).

Table 1. Growth parameters of *W. succinogenes* *nap* gene cluster mutants

Representative data from at least two independent cultures are shown.

Strain	Modification*	Growth parameters on minimal medium [†]		Growth parameters on supplemented medium [‡]	
		Doubling time (h)	Final optical density (%)	Doubling time (h)	Final optical density (%)
Wild-type	None	2.5	100	2.0	100
$\Delta napG-D$	Deletion of <i>napGHBFLD</i> region	∞	0	∞	0
N5	Restored wild-type <i>nap</i> gene cluster	2.6	100	2.0	100
<i>stopnapG</i>	NapG: Q6stop, S7stop, C98stop, Y99stop	3.8	3	3.8	16
<i>RRnapG</i>	NapG: R11G, R12G	4.4	3	4.2	14
<i>stopnapH</i>	NapH: Y7stop, R8stop, F145stop, E146stop	2.7	8	2.5	23
<i>stopnapB</i>	NapB: G16stop, L17stop	∞	0	∞	0
<i>stopnapF</i>	NapF: L11stop, R12stop, C74stop, Q75stop	4.7	36	2.3	44
<i>stopnapL</i>	NapL: L11stop, G12stop, F85stop, G86stop	6.3	113	4.0	121
<i>stopnapD</i>	NapD: A8stop, K9stop	∞	0	∞	0
$\Delta nosG-H$	Deletion of <i>nosGC1C2H</i>	2.5	100	2.0	100

*See Methods for details of mutant construction.

[†]Cells were incubated at 37 °C in anoxic minimal medium containing formate (80 mM) and nitrate (50 mM) as energy substrates in the presence of 10 mM ammonium. Fumarate (5 mM), present as a carbon source, allowed limited growth by fumarate respiration during the initial stage (2–3 h) of growth. Cultures were inoculated using cells grown in the same medium supplemented with 0.25 % (w/v) brain–heart infusion broth. Doubling times were calculated from the exponential growth phase. The OD₅₇₈ of wild-type cells entering the stationary phase was typically about 0.55 (corresponding to 100 %), while that of mutant cells unable to grow by nitrate respiration was 0.08 (set as 0 %). All growing cultures became stationary after 7–9 h. ∞ , No growth.

[‡]Cells were grown as described above, except that the medium was supplemented with 0.25 % (w/v) brain–heart infusion broth. Cultures were inoculated with cells grown in formate/fumarate medium supplemented with 50 mM potassium nitrate and 0.5 % (w/v) brain–heart infusion broth. The OD₅₇₈ of wild-type cells entering the stationary growth phase was typically about 0.6 (corresponding to 100 %), while that of mutant cells unable to grow by nitrate respiration was 0.09 (set as 0 %).

Individual *nap* genes were inactivated on pnapG-Dkan by introducing consecutive stop codons into the predicted ORFs at their 5' ends and, in some cases, also in their central regions (Table 1). *W. succinogenes* mutants containing the respective modified plasmids integrated into their genomes were characterized (see Table 1 for mutant nomenclature).

Role of NapB

The mutant defective in *napB* neither grew by nitrate respiration nor did it reduce nitrate, indicating that NapB is an essential component in the respiratory chain of nitrate respiration (Table 1, Fig. 3c). The absence of NapB in cell extracts of the *stopnapB* mutant was confirmed by

Fig. 2. Immunodetection of NapA in cell homogenates of *W. succinogenes* wild-type (wt) and *nap* mutants. Cells were grown in the medium described in Table 2. SDS-PAGE was performed with 80 µg protein applied to each lane. For Western blot analysis, protein was transferred from the SDS gel to nitrocellulose by electroblotting in a discontinuous buffer system (Kyhse-Andersen, 1984). The NapA antigen was detected by indirect ELISA using an antiserum raised against NapA from *Paracoccus pantotrophus* (Berks *et al.*, 1994) and goat anti-rabbit IgG coupled to peroxidase (Cell Signaling Technology).

Fig. 3. Time-course of nitrate, nitrite and formate concentrations during incubation of different strains in minimal formate/nitrate medium (see Table 1). (●) Nitrate, (■) nitrite, (▲) formate.

peroxidase-linked haem staining of cell proteins after separation by denaturing PAGE (not shown). Compared with the wild-type, *stopnapB* contained an increased amount of NapA as detected by immunoblot analysis (Fig. 2) and had nearly double the specific nitrate reductase activity using reduced BV as electron donor, with excess NapA being located in the soluble cell fraction (Table 2). The reason for the apparent overproduction is not known. The results demonstrate that both the formation of NapA and its capacity for nitrate reduction by reduced BV are independent of the presence of its redox partner NapB. Notably, *W. succinogenes stopnapB* retained a considerable amount of nitrate reductase activity in the membrane fraction, indicating that membrane attachment of NapA occurred independently of NapB. Active NapA devoid of a NapB redox partner has been described in *Desulfovibrio desulfuricans*, and there are other known *nap* operons that lack *napB* (Dias *et al.*, 1999; Jepson *et al.*, 2006). It has been speculated that in such cases, NapG might functionally replace NapB (Jepson *et al.*, 2006). Assuming that the *nap* gene products other than NapB are present in wild-type

amounts in *stopnapB*, the results indicate that there is no physiologically relevant electron transport from NapG (or NapGH) to NapA in *W. succinogenes*. In *Rhodobacter sphaeroides* and *E. coli* the *napB* gene has also been found to be essential for nitrate reduction *in vivo* (Reyes *et al.*, 1998; Potter & Cole, 1999). The *napB*-deficient mutant of *R. sphaeroides* even lacks notable nitrate reductase activity using reduced methyl viologen as the electron donor (Reyes *et al.*, 1998).

Role of NapD

A NapD homologue is encoded in every *nap* gene cluster currently known. Cytoplasmic NapD has been postulated to function as a NapA-specific chaperone probably involved in insertion of the molybdenum bis-molybdopterin guanine dinucleotide cofactor into NapA prior to twin-arginine translocase (Tat)-dependent export of folded NapA into the periplasmic space (Berks *et al.*, 1995; Palmer *et al.*, 2005). *W. succinogenes stopnapD* did not grow by nitrate respiration (Table 1) demonstrating that NapD is

Table 2. Specific nitrate reductase activity of *W. succinogenes* wild-type cells and *nap* gene cluster mutants

Representative values from at least two independent cultures are given.

Strain	Specific nitrate reductase activity BV _{red} →nitrate* [$\mu\text{mol nitrate reduced min}^{-1} (\text{mg cell protein})^{-1}$]			
	Intact cells	Cell homogenate	Membrane fraction	Soluble fraction
Wild-type†	1.8	1.8	0.9	1.1
$\Delta napG-D$	0.08	0.3‡	0.3‡	0.06
N5	1.6	1.7	0.9	1.2
stop $napG$	1.6	1.6	1.0	1.2
RR $napG$	1.9	1.9	0.7	1.0
stop $napH$	1.4	1.5	0.9	1.0
stop $napB$	3.6	3.7	1.3	2.6
stop $napF$	1.3	1.2	0.8	0.9
stop $napL$	0.4	0.2	0.3	0.3
stop $napD$	0.5	0.5	0.8	0.14
$\Delta nosG-H$	1.6	1.8	1.2	1.0

*Cells were grown in medium containing formate (100 mM), fumarate (90 mM) and nitrate (20 mM) as energy substrates, and supplemented with 0.5% (w/v) brain–heart infusion broth. Cells and membrane fractions were suspended in an anoxic buffer (pH 7.5) containing 50 mM Tris/HCl.

†Specific enzyme activities taken from Simon *et al.* (2003). Note that a considerable portion of activity is located in the membrane fraction (Simon *et al.*, 2003).

‡NapA-independent nitrate reduction activity. Such an activity has been reported previously for *W. succinogenes napA::kan* cells (Simon *et al.*, 2003).

an essential component of the *W. succinogenes* Nap system, as has been found in other organisms (Reyes *et al.*, 1998; Potter & Cole, 1999). NapA was not detectable in *W. succinogenes* stop $napD$ and the specific nitrate reductase activity of the mutant was similar to that of the *napA::kan* mutant (Fig. 2, Table 2). This activity is assigned to an as yet unknown second enzyme that reduces nitrate using BV but not formate as an electron donor (Simon *et al.*, 2003). The results can be explained by NapD serving in either NapA stabilization or folding and are in agreement with its proposed function as a twin-arginine signal chaperone for NapA.

Role of NapL

The NapL protein is predicted to be a soluble protein, but its precise localization and function are unknown. The *napL* gene seems to be restricted to *nap* operons of ϵ -proteobacterial genera, i.e. *Wolinella*, *Campylobacter*, *Helicobacter* and *Sulfurimonas* (Simon *et al.*, 2003; Pittman *et al.*, 2007). The stop $napL$ mutant of *W. succinogenes* exhibits a clearly reduced doubling time and nitrate consumption rate, although the cells eventually reached the optical density of wild-type cells in the stationary growth phase (Table 1, Fig. 3d). The specific nitrate reductase activity of the stop $napL$ mutant was significantly lower than that of the wild-type (Table 2), although the mutant cells contained only a slightly reduced

amount of NapA (Fig. 2). The results indicate that NapL plays a role in NapA maturation or function in an as yet unknown manner. Recently, a NapL-deficient mutant of *Campylobacter jejuni* was described (Pittman *et al.*, 2007). This mutant is not affected in growth with nitrate and has a normal nitrite accumulation rate, although it possesses only about half of the wild-type nitrate reductase activity using reduced BV as the electron donor. This points to a similar function of NapL in *W. succinogenes* and *C. jejuni*, and suggests a decreased stability or catalytic efficiency of NapA. Such a role might be taken over by other proteins in organisms that lack a *napL* gene. NapL is predicted to be synthesized with a putative Sec signal peptide, but its cellular location is unknown. An attempt to raise a peptide antiserum suitable for detecting *W. succinogenes* NapL was not successful.

Role of NapF

The NapF proteins from *R. sphaeroides* and *E. coli* have been described recently as cytoplasmic iron–sulfur proteins that are possibly involved in the incorporation of the NapA [4Fe–4S] cluster (Olmo-Mira *et al.*, 2004; Nilavongse *et al.*, 2006). In *E. coli* the *napF* gene was found to be dispensable for nitrate reduction using either glycerol or formate as the growth substrate (Potter & Cole, 1999). *W. succinogenes* stop $napF$ grew by nitrate respiration, albeit with an increased doubling time as compared with wild-type cells

(Table 1). Furthermore, the mutant cells did not reach the final optical density of wild-type cells (Table 1). Western blot analysis revealed the presence of two distinct immunodetectable forms of NapA in stopnapF cells (Fig. 2). The larger form presumably represents the cytoplasmic NapA precursor awaiting export by the Tat system. The specific nitrate reductase activity was slightly lower than that of wild-type cells, indicating that functional NapA is still assembled even in the absence of NapF. Taken together, it seems that NapA maturation is retarded in the absence of NapF, supporting the hypothesis that NapF indeed plays a role in iron–sulfur cluster assembly, possibly supporting the capacity of the conventional iron–sulfur cluster maturation machinery (the NifSU system). Interestingly, many *Campylobacter* species lack a napF gene in their nap clusters, thus underlining the proposed accessory role for NapF.

Roles of NapG and NapH

The putative NapGH complex has been postulated to be a prime candidate for catalysing NapC-independent menaquinol oxidation and electron transfer to NapAB in *W. succinogenes* (Simon *et al.*, 2003). The *W. succinogenes* napG and napH genes were individually inactivated (Table 1). In addition, mutant RRnapG was constructed, whose NapG protein contains two glycine residues instead of the two conserved arginine residues of the putative Tat signal sequence. In supplemented medium all three mutants showed only residual growth by nitrate respiration. In minimal medium, growth of the stopnapG and RRnapG mutants was even more severely impaired. Under these conditions, the stopnapG and stopnapH mutants slowly consumed only about 6 and 11 mM nitrate, respectively, before both growth and nitrate reduction stopped (Fig. 3e, f). No nitrite accumulation occurred, indicating that the small amount of nitrite produced was instantly reduced to ammonium. The total amount of formate converted was in agreement with this assumption, as four molecules of formate are required to reduce one molecule of nitrate to ammonium. The results indicate the functional importance of NapG and NapH as well as that of the twin-arginine sequence of NapG for nitrate respiration, but also indicate the existence of a less efficient alternative electron transport route capable of electron transfer to NapA. Periplasmic localization of NapG is very likely, as has been indicated for *E. coli* NapG (Brondijk *et al.*, 2004). It is notable that some bacteria lack the twin-arginine residues in NapG, and it was hypothesized that in this case NapG is co-exported with NapA (Jepson *et al.*, 2006). The NapA protein and specific NapA-dependent nitrate reductase activity is retained in stopnapG, stopnapH and RRnapG mutants (Table 2 and Fig. 2), indicating that NapG and NapH are not involved in NapA maturation or function, in contrast to NapF, NapL and NapD. The similar phenotypes of the stopnapG and stopnapH mutants suggest that NapG and NapH are components of the same functional unit.

Alternative routes for menaquinol oxidation and electron transfer to NapA

There are several proteins encoded on the *W. succinogenes* genome that might participate in electron transport to NapA (via NapB) in the absence of either NapG or NapH. The most obvious candidates are NosG and NosH, which are homologous to their Nap counterparts (Simon *et al.*, 2004). The nosG and nosH genes are part of the nos locus, which encodes cytochrome *c* nitrous oxide reductase (NosZ). Both the nosGC1C2H and napGH transcripts were detected in RT-PCR experiments using wild-type cells grown with either nitrate or fumarate (results not shown). Quantitative real-time RT-PCR revealed that the transcript levels of nosG, nosC1, nosC2 and nosH were not significantly increased in stopnapG and stopnapH mutants when compared with wild-type cells (results not shown). This is not surprising, as nosZ is known to be disrupted by a copy of insertion element IS1302 (Simon *et al.*, 2004). Hence, constitutive transcription of the nosG, C1, C2 and H genes, situated downstream of nosZ, is likely to be initiated from a promoter located within IS1302, e.g. that of the transposase gene tnpA (Simon & Kröger, 1998). Accordingly, all four nos genes mentioned above were found not to be upregulated during transition from fumarate to nitrate respiration, in contrast to nap and nrf genes (A. M. Mager & J. Simon, unpublished data).

In a different approach, the nosGC1C2H region was deleted from the genome of *W. succinogenes* wild-type cells. The nosC1 and nosC2 genes encode monohaem *c*-type cytochromes of unknown function (Simon *et al.*, 2004). Compared with wild-type cells, the resulting mutant *W. succinogenes* ΔnosG–H did not show any notable difference during growth with nitrate, nitrite or fumarate as the electron acceptor with respect to growth parameters and substrate turnover, and no difference in nitrate reductase activity or NapA content was observed (Tables 1 and 2, data not shown).

Taken together, it cannot be excluded that nos gene products participate in electron transport to NapA. In principle, however, any periplasmic iron–sulfur protein or *c*-type cytochrome might slowly reduce NapB (and hence NapA) in the absence of NapGH. When present, the latter clearly works as the physiological electron donor within the Nap system. Due to limitations of functional marker genes, it was not possible to delete nos genes in *W. succinogenes* stopnapG or stopnapH.

A napG mutant of *C. jejuni* has been described as having a phenotype similar to that of *W. succinogenes* stopnapG (Pittman *et al.*, 2007). In the case of *C. jejuni*, residual growth by nitrate respiration is abolished upon additional inactivation of nrfH, the gene encoding the quinol-oxidizing subunit of the cytochrome *c* nitrite reductase complex (NrfHA), a member of the NapC/NirT family (Pittman *et al.*, 2007; Gross *et al.*, 2005). In *W. succinogenes*, NrfH is known to form a tight and stable complex with NrfA, the pentahaem cytochrome *c* nitrite reductase

(Simon *et al.*, 2000; Simon, 2002), a feature that is also supported by the crystal structure of the *Desulfovibrio vulgaris* NrfHA₂ complex (Rodrigues *et al.*, 2006). As NrfA is formed in excess over NrfH in nitrate-grown *W. succinogenes* cells, it is unlikely that free NrfH donates electrons to a Nap component (Simon *et al.*, 2000). It cannot be excluded, however, that the NrfHA complex slowly feeds some electrons into the Nap system, possibly via NapB, thus bypassing NapG or NapGH. A *W. succinogenes* mutant lacking *nrfHA* has been described as not being affected in nitrate respiration (Simon *et al.*, 2003). For the reasons stated above, deletion of *nrfH* in *stopnapG* or *stopnapH* is not feasible at present.

Conclusions

The results presented here underpin the existing model of NapA-dependent bacterial nitrate respiration of *W. succinogenes* (see Fig. 3 in Simon *et al.*, 2003) and demonstrate that each *nap* gene product has a functional role in this metabolic pathway. The work further supports the view that various electron transfer pathways exist that form a network in the periplasmic space, thus making it difficult to determine the contribution of individual components. Despite numerous efforts, we did not succeed in setting up a biochemical assay to determine the specific electron transport activity from formate or a water-soluble menaquinone analogue to nitrate using intact cells or membranes of *W. succinogenes*. Any measured activity was much lower than the physiological minimal nitrate reduction rate calculated from growth rate and molar cell yield. We believe, however, that such an activity is accurately reflected here by the presented growth properties and changes in substrate concentrations. Future experiments will aim at the overproduction of tagged NapG and NapH proteins in order to directly demonstrate protein–protein and protein–quinol interactions.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Roland Gross for helpful discussions and Monica Sanger for excellent technical assistance. Professor David J. Richardson is acknowledged for generously hosting J.S. at the University of East Anglia, Norwich, UK, and for providing anti-*Paracoccus pantotrophus* NapA serum. This work was supported by the SFB 472 ‘Molecular Bioenergetics’ (projects P5 and P33) of the Deutsche Forschungsgemeinschaft (DFG) and by the Center for Membrane Proteomics (CMP) at the University of Frankfurt am Main. J. S. holds a DFG Heisenberg Fellowship.

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Edited by: J. Moir