Site-directed modifications indicate differences in axial haem *c* iron ligation between the related NrfH and NapC families of multihem *c*-type cytochromes

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During the last decade, a number of related bacterial membrane-bound multihem *c*-type cytochromes, collectively referred to as the NapC/NirT family, were identified. These proteins are generally thought to catalyse electron transport between the quinone/quinol pool and periplasmic oxidoreductases. The best-characterized members, the tetrahem *c*-type cytochromes NrfH and NapC, mediate electron transport to NrfA and NapA respectively. Amino acid sequence alignments suggest that the nature and position of distal haem *c* iron ligands differs in NrfH and NapC proteins. Site-directed modification of potential haem *c* iron-ligating histidine, lysine and methionine residues in *Wolinella succinogenes* NrfH was performed to determine the implication in electron transport from formate to nitrite. Two histidine, one lysine and one methionine residues were found to be essential, whereas

the replacement of three other conserved histidine residues, one methionine and two lysines did not prevent growth by nitrite respiration. The results contrast those previously obtained for *Paracoccus pantotrophus* NapC, in which four essential histidine residues have been identified that are highly likely to serve as distal haem *c* iron ligands. The combined experimental evidence suggests different haem ligation patterns within NapC and NrfH proteins, which might reflect their different functions in the bacterial electron transfer.

Key words: axial haem *c* iron ligation, CymA, cytochrome *c* nitrite reductase (Nrf), multihem *c*-type cytochrome, NapC/NirT family, periplasmic nitrate reductase (Nap).

INTRODUCTION

Cytochromes *c* are key components of electron transport chains and are involved in bacterial respiration. The haem *c* group is bound covalently via two thioether bonds formed between two cysteine residues organized in a haem *c*-binding motif (in most cases CXXCH) and two haem vinyl side chains (see [1–3] for recent reviews). The histidine residue of the haem *c*-binding motif normally serves as proximal haem iron ligand, whereas, in the case of low-spin haem, the distal ligand is located elsewhere in the primary structure. This sixth haem *c* iron ligand is usually a histidine or a methionine residue (although lysine is regarded as a potential alternative) and has considerable influence on the midpoint potential of the haem *c* group.

The abundant multihem *c*-type cytochromes of the NapC/NirT family play an essential role in bacterial electron transport between the quinone/quinol pool and oxidoreductases located outside of the cytoplasmic membrane [4–6]. Members of this family were originally described as membrane-bound proteins donating electrons to the Nap complex (NapAB) or NirS in the course of anaerobic electron transport in various proteobacteria [4,7]. Later on, the NapC-related protein NrfH was shown to catalyse electron transport from menaquinol to NrfA in the ϵ -proteobacterium *Wolinella succinogenes* [5,8]. In contrast with NapC, tetrahem NrfH cytochromes have been reported to form a stable complex with their redox partner NrfA in the membrane [9]. The NapC, NirT and NrfH proteins consist of approx. 175

amino acid residues and can be divided into an N-terminal hydrophobic region that most likely forms a membrane-spanning helix as well as a hydrophilic, possibly globular domain harbouring four CXXCH motifs (Figure 1). A subgroup of the NapC/NirT family comprising the TorC, DorC and DmsC proteins contains an additional C-terminal monohaem *c* domain of approx. 200 residues that is involved in electron transfer from the tetrahem domain to molybdenum-containing TMAO and DMSO reductases [10] (Figure 1). It is notable that the classification of these cytochromes is exclusively based on the genomic context of the corresponding genes, e.g. every NapC-encoding gene is part of a *nap* gene cluster. To date, NapC and NrfH proteins appear not to be present in the same organism. This is illustrated by the intriguing fact that *nap* clusters lacking a *napC* gene are found in *nrfH*-containing organisms [11] and that the function of NrfH is apparently carried out by dissimilar proteins in organisms encoding a NapC protein [12].

Primary structure analysis suggests that NrfH and NapC belong to distinct protein families that are restricted to individual phylogenetic groups. On the basis of unrooted scaled trees derived from CLUSTAL W alignments, it appears that the NapC and NirT sequences as well as the pentahem sequences form a monophyletic group present exclusively in α -, β - and γ -proteobacteria, whereas NrfH proteins form a distinct group whose sequences are not as closely related to each other. In line with this fact, *nrfH* genes are more widely distributed and found in δ - and ϵ -proteobacteria as well as in several Gram-positive

Abbreviations used: Nap, periplasmic nitrate reductase; NirS, cytochrome *cd*₁ nitrite reductase; NrfA, cytochrome *c* nitrite reductase.

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Figure 1 Topological model of the related tetrahaem (A), pentahaem (B) and monohaem (C) *c*-type cytochromes discussed in the present study

The numbers in diamonds denote the number of haem *c*-binding motifs in predicted protein domains. The location of the quinone (Q)/quinol (QH₂)-binding site is not known. *n* and *p* denote the negatively charged inside and positively charged outside of the bacterial membrane respectively. 'Mo oxidoreductase', molybdenum cofactor-containing oxidoreductase.

bacteria and species of the Planctomycetes and the Bacteroides group, suggesting horizontal transfer of *nrfH* genes between unrelated organisms [9].

Several members of the NapC/NirT family have been purified. These are NapC from *Paracoccus pantotrophus*, CymA from *Shewanella oneidensis* MR-1 (both produced as soluble forms lacking the N-terminal hydrophobic domain) [4,6,13], native CymA from *S. frigidimarina* NCIMB400 [14] and the pentahaem proteins DorC from *Rhodobacter capsulatus* [15] and TorC from *Escherichia coli* [10]. *S. oneidensis* CymA is encoded by an orphan gene and is required in electron transport chains terminating with either fumarate, nitrate, nitrite, Fe(III) or DMSO [13,16]. Spectroscopic analysis of *P. pantotrophus* NapC indicated the presence of four low-spin haems, each most likely with bis-histidine axial haem *c* iron ligation, although the presence of histidine/lysine-ligated haem(s) has not been excluded [4]. Furthermore, four essential histidine residues have been identified by site-directed modification in *P. pantotrophus* NapC that are conserved throughout the NapC primary structures [6]. These histidine residues are also present in CymA, in accordance with the spectroscopically demonstrated presence of four bis-histidine-ligated haem *c* groups [14]. In contrast with NapC and CymA, haem *c* iron-ligating residues are not predicted easily from sequence alignments of NrfH proteins (and some members of the pentahaem group) since two of the likely haem *c* iron-ligating histidine residues of NapC are only loosely conserved in NrfH proteins [9]. To date, no representative of the NrfH family has been purified in a native form impeded by the fact that NrfH proteins appear to form stable membrane-bound complexes with NrfA. This is the first report that describes variants of an NrfH protein constructed in order to identify catalytically essential residues that potentially serve as distal haem *c* iron ligands.

EXPERIMENTAL

Growth of *W. succinogenes* and cell fractionation

The wild-type strain *W. succinogenes* DSMZ1740 and derivative mutants were grown with formate and nitrate as described in [17], except that (NH₄)₂SO₄ (5 mM) was present in the medium

instead of K₂SO₄. The medium was supplemented with brain-heart infusion broth (0.5 %, w/v). The capability of cells to grow by nitrite respiration was estimated from the growth curve of a batch culture in a minimal medium containing 50 mM formate and 10 mM nitrate as well as 5 mM fumarate as the carbon source. Under these conditions, accumulation of nitrite is observed since nitrate consumption is four times faster than ammonium generation. After nitrate has been completely reduced to nitrite, growth is solely dependent on nitrite respiration. Cells that do not grow by nitrite respiration reach only half the cell density of wild-type cells.

W. succinogenes cells harvested in the exponential growth phase were suspended (10 g of cell protein/l) in an anoxic buffer (pH 8.0) containing 50 mM Tris/HCl and 1 mM dithiothreitol. The suspension was passed through a French press at 130 MPa and at a flow rate of 10 ml/min. 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 activities

All activities were determined at 37 °C. The activity of electron transport from formate to nitrite was determined using whole cells as described in [5]. The assay is dependent on the intact membrane-bound electron transport chain of nitrite respiration [9]. One unit of electron transport activity is equivalent to the consumption of 1 μmol of formate (two electrons) per min. Nitrite reduction by reduced benzyl viologen was measured photometrically [18]. This activity is dependent solely on the NrfA protein. Six molecules of reduced benzyl viologen are oxidized in order to reduce one molecule of nitrite to ammonium. In order to normalize both enzyme assays on the basis of the turnover of two electrons, 1 unit of enzyme activity is defined as the oxidation of 2 μmol of benzyl viologen/min. Protein was measured by the biuret method with KCN [19].

Construction of *W. succinogenes* *nrfH* mutant strains

All *nrfH* mutants were constructed as described previously [5]. The strains were obtained by integration of the modified plasmid

Table 1 Residues replaced in *W. succinogenes* NrfH and their degree of conservation in the NrfH and NapC families

Suitable alignments of NrfH and NapC proteins are presented in [4,6,9]. The pentahaem cytochromes (TorC, DorC and DmsC) as well as *Shewanella* spp. CymA are not included here since their residue conservation differs considerably in some cases. The primary structures of 20 NrfH and 37 NapC proteins have been taken into account.

Residue in <i>W. succinogenes</i> NrfH*	Degree of conservation in NrfH primary structures	Degree of conservation in the corresponding residues in NapC primary structures
<u>Met-48</u>	Completely conserved	Present in the vast majority of sequences
His-57	Conserved in nine out of 20 sequences; replaced by lysine in five sequences	Not present
His-60	Completely conserved	Corresponds to the completely conserved, proposed haem c iron ligand following the first CXXCH motif (His-81 in <i>P. pantotrophus</i> NapC)
Met-76	Not conserved	Not conserved
Lys-79	Conserved in 17 out of 20 sequences, part of Lys-X ₃ -Lys sequence in 16 cases	Completely conserved in Lys-X ₃ -Lys sequence
<u>Lys-83</u>	Conserved in 19 out of 20 sequences, part of Lys-X ₃ -Lys sequence in 16 cases	Completely conserved in Lys-X ₃ -Lys sequence
<u>His-90</u>	Conserved histidine (15 cases) or aspartate residue (five cases)	Not present
Lys-104	Present in six out of 20 sequences	Corresponds to Lys-128 of <i>P. pantotrophus</i> , which is completely conserved in NapC sequences
His-136	A histidine, methionine or lysine residue in an otherwise poorly conserved region of variable length between the third and fourth haem c-binding motifs	A completely conserved, proposed haem c iron ligand is located in that region (His-174 in <i>P. pantotrophus</i> NapC)
<u>His-158</u>	Completely conserved	Corresponds to the completely conserved, proposed haem c iron ligand following the fourth CXXCH motif (His-194 in <i>P. pantotrophus</i> NapC)

* The underlined residues have been found to be essential for electron transport from formate to nitrite (see Table 2).

pBR-N2 into the genome of *W. succinogenes* $\Delta nrfH$, thereby restoring the wild-type *nrfHAIJ* locus. Site-directed mutagenesis of *nrfH* was performed using the QuikChange site-directed mutagenesis kit (Stratagene, Cambridge, U.K.) and pBR-N2 as template. A pair of complementary primers was used for each modification, of which the forward primer sequence (5' → 3') is as follows. M48I, CTGTCATGTCATCAATCCGCAATACGC-1262; H57L, CGCTACATGGCAGCTCAGCTCCACGC-1286; H60L, GGCAGCACAGCTCCCTCGCAGAGAGAGCC-1296; M76I, CCCACGGGTAACATCGTGCAGAAATATATCTCC-1350, K79I, GGTAACATGGTGCAGATCTATATCTCCAAGGC-1355; K83I, GCAGAAGTATATCTCCATCGCAAGGGATGGATGG-1368; H90L, GGGATGGATGGAATCTCTCCGTCGCCTTTACGC-1390; K104I, CCTATGATCAGCATGATCATCAGCGAAGATGG-1430; H136L, GCTGATAGGAATCTCCAATTCAACGATCC-1526; H158L, CCACAAAAGTGTTCCACTCGGAAAAGTCCG-1589. The altered nucleotides are printed in boldface and the modified codons are underlined. The numbers drawn at the 3'-ends of the sequences correspond to the nucleotide positions given in the EMBL, GenBank® and DDBJ databases with accession number AJ245540. Transformation of *W. succinogenes* $\Delta nrfH$ cells with plasmid derivatives was performed by electroporation [20]. Transformants were selected in a medium containing formate and nitrate, kanamycin (25 mg/l) and chloramphenicol (12.5 mg/l). The desired integration of the plasmid into the genome was examined by PCR using suitable primer pairs. Each mutation was confirmed by sequencing the entire *nrfH* gene from a PCR fragment.

RESULTS AND DISCUSSION

The primary structure of *W. succinogenes* NrfH contains several histidine (His-57, His-60, His-90, His-136 and His-158), methionine (Met-48 and Met-76) and lysine (Lys-79, Lys-83 and Lys-104) residues that are potential distal haem c iron ligands.

The degree of conservation of these residues within the NrfH and NapC cytochromes varies and is presented in Table 1. The aforementioned ten residues were individually replaced by hydrophobic residues of similar size and the resulting *W. succinogenes* strains were characterized (Table 2). All strains contained wild-type amounts of NrfH in the membrane fraction as detected by staining cell homogenates applied to SDS/polyacrylamide gels for haem c-dependent peroxidase activity (results not shown). Each mutant had NrfA-dependent specific activity of nitrite reduction by reduced benzyl viologen that resembled that of the wild-type (Table 2). Most of the activity was retained in the membrane fraction, indicating the formation of the membrane-bound NrfHA complex in each mutant strain. In the absence of NrfH, NrfA was previously found to be located exclusively in the soluble fraction [5]. Four mutants (M48I, K83I, H90L and H158L) did not grow by nitrite respiration and their specific electron transport activity was 4 % (M48I) or less than 1 % of that of the wild-type. Cells of mutant M48I completely reduced nitrite to ammonium after 60 h of incubation, whereas cells of the strains K83I, H90L and H158L did not reduce nitrite within a week. For the latter three mutants, approx. 35 mM formate remained in the growth medium when the culture reached the stationary phase. The other six mutants (H57L, H60L, M76I, K79I, K104I and H136L) grew by nitrite respiration, although their respective specific activity of electron transport from formate to nitrite was reduced and amounted to between 32 % (H136L) and 74 % (M76I) of that of the wild-type (Table 2).

The results demonstrate that four potentially haem c iron-ligating residues are essential for electron transport to NrfA. With the exception of His-90, these four residues are completely conserved in NrfH sequences and are therefore legitimate candidates for distal haem c iron ligands (Table 1). On the basis of our results, however, it cannot be ruled out that other residues, e.g. His-57 or His-60, are indeed axial haem c iron ligands. It is conceivable that their replacement results in decreased

Table 2 Properties of *W. succinogenes* strains

The cells were grown in a medium containing formate (50 mM) and nitrate (10 mM) as energy substrates in the presence of 10 mM ammonium. The growth of cells incapable of nitrite respiration ceased after complete reduction of nitrate to nitrite. The membrane fraction was washed using an anoxic buffer (pH 8.0) containing 50 mM Tris and 0.5 M mannitol. Strain N2 contains the restored wild-type *nrfHAIJ* operon, whereas the *nrfH* gene is inactivated in the strain stopH by the introduction of consecutive stop codons [5]. Representative values derived from at least three different cultures of each strain are presented.

Strain	Growth by nitrite respiration	Electron transport activity (from formate to nitrite) (units/mg of dry cell weight)	Specific nitrite reductase activity (reduced benzyl viologen → nitrite) (units/mg of cell protein)		
			Cell homogenate	Membrane fraction	Soluble fraction
Wild-type	+	3.9	30	24	9.0
stopH	—	< 0.01	34	0.3	36
N2	+	3.8	36	23	7.0
M48I	—	0.15	29	22	6.5
H57L	+	1.7	28	22	5.5
H60L	+	1.9	25	22	10
M76I	+	2.9	29	24	7.5
K79I	+	1.3	29	22	5.5
K83I	—	0.04	28	23	6.0
H90L	—	< 0.01	34	20	16
K104I	+	1.9	27	21	5.5
H136L	+	1.3	27	23	5.5
H158L	—	0.02	25	20	6.5

W. succinogenes* NrfH** P. pantotrophus* NapC** **Figure 2** Arrangement of haem binding motifs and potential distal haem *c* iron ligands in NapC and NrfH primary structures

'Essential' and 'non-essential' residues in *W. succinogenes* NrfH refer to the capability of the corresponding *nrfH* mutants to grow by nitrite respiration. The position of the four haem *c*-binding motifs is depicted by diamonds and the numbers in the diamonds denote the distance between adjacent CXXCH motifs. The grey boxes indicate possible dihaem domains in NapC. Arrows indicate distal haem *c* iron ligation as proposed by Cartron et al. [6]. TMD, transmembrane domain.

electron transport activity without abolishing growth by nitrite respiration. Likewise, a decrease in electron transport activity might be caused by a modification of the active site for menaquinone/menaquinol or an impaired electron transfer rate between NrfH and NrfA. It is noteworthy that individual site-directed modification of every cysteine residue within the four haem *c*-binding motifs of *W. succinogenes* NrfH has demonstrated the functional importance of each haem *c* group in electron transport from formate to nitrite [21]. One of the mutants (C66S) still grew by nitrite respiration but had only 20 % of the specific electron-transport activity from formate to nitrite found in the wild-type [21].

The only other cytochrome of the NapC/NirT family for which potential distal haem *c* iron ligands were assessed by site-directed modification is NapC from *P. pantotrophus* (see the Introduction section) [6]. Figure 2 compares our findings for *W. succinogenes*

NrfH with those from *P. pantotrophus* NapC. Four histidine residues that are conserved throughout the NapC cytochromes have been modified in *P. pantotrophus* NapC and each of the resulting proteins has been shown to contain one high-spin haem. Since each of the four likely haem iron ligands in NapC follows one haem *c*-binding motif, Cartron et al. [6] proposed the existence of two dihaem domains structurally linked by the distal haem *c* iron ligands (Figure 2). A similar arrangement in NrfH proteins is questioned by the results presented here. Remarkably, the distances between adjacent haem *c*-binding motifs are not consistent in NapC and NrfH proteins (Figure 2), and only two of the four histidine residues proposed to be haem *c* iron ligands in NapC and CymA are conserved in NrfH sequences. These correspond to His-60 and His-158 in *W. succinogenes* NrfH following the first and fourth CXXCH motifs (Table 1 and Figure 2). The potentially haem *c* iron-ligating histidine residue that follows the second CXXCH motif in NapC and CymA has a corresponding histidine residue in 12 out of 20 NrfH sequences (excluding *W. succinogenes* NrfH), whereas the histidine following the third CXXCH motif in NapC and CymA is not conserved in NrfH sequences, although His-136 of *W. succinogenes* NrfH is located in that region. Another argument against four bis-histidine-ligated haem *c* irons in NrfH proteins derives from the primary structure of *Carboxydotherrus hydrogenoformans* NrfH that contains only three histidine residues outside of the four CXXCH motifs and that lacks any histidine residue between the second and the fourth CXXCH motifs [9].

Assuming the presence of four low-spin haem *c* groups in both the NrfH and NapC cytochromes, cumulative evidence suggests that the haem *c* iron ligation patterns are different in these two protein groups. Such a feature might reflect fine tuning of haem *c* midpoint potentials in order to optimize electron transfer to the respective redox partner proteins, the multihaem *c*-type cytochrome NrfA or NapB. Unfortunately, no structural or spectroscopic data on an isolated native NrfH protein are currently available. Various attempts to separate the NrfH and NrfA proteins from *W. succinogenes* under non-denaturing conditions have not been successful (J. Simon, unpublished work). The purification of *W. succinogenes* NrfH in the absence of NrfA will hopefully enable a direct investigation of the axial haem *c* iron ligands in the

future. A possible histidine/methionine-ligated haem *c* is expected to be detectable by redox titration and a combined spectroscopic approach using EPR and MCD (magnetic circular dichroism) spectroscopy.

Recent data bank searches using primary structures of the penta-haem members of the NapC/NirT family revealed a new type of monohaem *c*-type cytochrome that lacks the typical tetrahaem domain. Instead, the N-terminal hydrophobic part appears to be fused directly to the monohaem domain (Figure 1C). Such cytochromes are currently predicted from the genomes of the four ϵ -proteobacteria *W. succinogenes* (protein Ws1850), *Campylobacter jejuni* NCTC11168 (Cj0265c), *C. coli* RM2228 (CCO0283) and *C. lari* RM2100 (CLA0227). In each case, the corresponding gene is found adjacent to a gene encoding a typical molybdenum cofactor-containing oxidoreductase that is likely to catalyse DMSO and/or TMAO reduction. Provided these proteins were functional in quinone/quinol conversion, it is implied that the quinone/quinol-binding site in NapC or NrfH proteins might be predominantly composed of the hydrophobic N-terminal domain. Alternatively, the monohaem proteins might be functionally dependent on an 'adaptor' protein that takes over quinol oxidation. To date, none of the monohaem cytochromes has been characterized by biochemical or genetic experiments.

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REFERENCES

- Ferguson, S. J. (2001) Keilin's cytochromes: how bacteria use them, vary them and make them. *Biochem. Soc. Trans.* **29**, 629–640.
- Allen, J. W. A., Daltrop, O., Stevens, J. M. and Ferguson, S. J. (2003) *C*-type cytochromes: diverse structures and biogenesis systems pose evolutionary problems. *Philos. Trans. R. Soc. Lond. B* **358**, 255–266.
- Stevens, J. M., Daltrop, O., Allen, J. W. A. and Ferguson, S. J. (2004) *C*-type cytochrome formation: chemical and biological enigmas. *Acc. Chem. Res.* **37**, 999–1007.
- Roldan, M. D., Sears, H. J., Cheesman, M. R., Ferguson, S. J., Thomson, A. J., Berks, B. C. and Richardson, D. J. (1998) Spectroscopic characterization of a novel multihaem *c*-type cytochrome widely implicated in bacterial electron transport. *J. Biol. Chem.* **273**, 28785–28790.
- Simon, J., Pisa, R., Stein, T., Eichler, R., Klimmek, O. and Gross, R. (2001) The tetraheme cytochrome *c* NrfH is required to anchor the cytochrome *c* nitrite reductase in the membrane of *Wolinella succinogenes*. *Eur. J. Biochem.* **268**, 5776–5782.
- Cartron, M. L., Roldan, M. D., Ferguson, S. J., Berks, B. C. and Richardson, D. J. (2002) Identification of two domains and distal histidine ligands to the four haems in the bacterial *c*-type cytochrome NapC; the prototype connector between quinol/quinone and periplasmic oxido-reductases. *Biochem. J.* **368**, 425–432.
- Jungst, A., Wakabayashi, S., Matsubara, H. and Zumbt, W. G. (1991) The *nirSTBM* region coding for cytochrome *cd*₁-dependent nitrite respiration of *Pseudomonas stutzeri* consists of a cluster of mono-, di-, and tetraheme proteins. *FEBS Lett.* **279**, 205–209.
- Simon, J., Gross, R., Einsle, O., Kroneck, P. M. H., Kroger, A. and Klimmek, O. (2000) A NapC/NirT-type cytochrome *c* (NrfH) is the mediator between the quinone pool and the cytochrome *c* nitrite reductase of *Wolinella succinogenes*. *Mol. Microbiol.* **35**, 686–696.
- Simon, J. (2002) Enzymology and bioenergetics of respiratory nitrite ammonification. *FEMS Microbiol. Rev.* **26**, 285–309.
- Gon, S., Guidici-Ortoni, M.-T., Mejean, V. and Iobbi-Nivol, C. (2001) Electron transfer and binding of the *c*-type cytochrome TorC to the trimethylamine *N*-oxide reductase in *Escherichia coli*. *J. Biol. Chem.* **276**, 11545–11551.
- Simon, J., Sanger, M., Schuster, S. C. and Gross, R. (2003) Electron transport to periplasmic nitrate reductase (NapA) of *Wolinella succinogenes* is independent of a NapC protein. *Mol. Microbiol.* **49**, 69–79.
- Clarke, T. A., Dennison, V., Seward, H. E., Burlat, B., Cole, J. A., Hemmings, A. and Richardson, D. J. (2004) Purification and spectropotentiometric characterization of *Escherichia coli* NrfB, a decaheme homodimer that transfers electrons to the decaheme periplasmic nitrite reductase complex. *J. Biol. Chem.* **279**, 41333–41339.
- Schwalb, C., Chapman, S. K. and Reid, G. A. (2003) The tetraheme cytochrome CymA is required for anaerobic respiration with dimethyl sulfoxide and nitrite in *Shewanella oneidensis*. *Biochemistry* **42**, 9491–9497.
- Field, S. J., Dobbin, P. S., Cheesman, M. R., Watmough, N. J., Thomson, A. J. and Richardson, D. J. (2000) Purification and magneto-optical spectroscopic characterization of cytoplasmic membrane and outer membrane multihaem *c*-type cytochromes from *Shewanella frigidimarina* NCIMB400. *J. Biol. Chem.* **275**, 8515–8522.
- Shaw, A. L., Hochkoeppler, A., Bonora, P., Zannoni, D., Hanson, G. R. and McEwan, A. G. (1999) Characterization of DorC from *Rhodobacter capsulatus*, a *c*-type cytochrome involved in electron transfer to dimethyl sulfoxide reductase. *J. Biol. Chem.* **274**, 9911–9914.
- Myers, C. R. and Myers, J. M. (1997) Cloning and sequence of *cymA*, a gene encoding a tetraheme cytochrome *c* required for reduction of iron(III), fumarate, and nitrate by *Shewanella putrefaciens* MR-1. *J. Bacteriol.* **179**, 1143–1152.
- Lorenzen, J. P., Kroger, A. and Unden, G. (1993) Regulation of anaerobic respiratory pathways in *Wolinella succinogenes* by the presence of electron acceptors. *Arch. Microbiol.* **159**, 477–483.
- Bokranz, M., Katz, J., Schroder, I., Robertson, A. M. and Kroger, A. (1983) Energy metabolism and biosynthesis of *Vibrio succinogenes* growing with nitrate or nitrite as terminal electron acceptor. *Arch. Microbiol.* **135**, 36–41.
- Bode, C., Goebell, H. and Stahler, E. (1968) Zur Eliminierung von Trubungsfehlern bei der Eiweissbestimmung mit der Biuret-methode. *Z. Klin. Chem. Klin. Biochem.* **6**, 418–422.
- Simon, J., Gross, R., Ringel, M., Schmidt, E. and Kroger, A. (1998) Deletion and site-directed mutagenesis of the *Wolinella succinogenes* fumarate reductase operon. *Eur. J. Biochem.* **251**, 418–426.
- Simon, J., Eichler, R., Pisa, R., Biel, S. and Gross, R. (2002) Modification of haem *c* binding motifs in the small subunit (NrfH) of the *Wolinella succinogenes* cytochrome *c* nitrite reductase complex. *FEBS Lett.* **522**, 83–87.

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