Roles of *c*-type cytochromes in respiration in *Neisseria meningitidis*

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Three *c*-type cytochromes were identified in *Neisseria meningitidis*, based on predictions from genome sequences, that were hypothesized to be involved in electron transport to terminal electron acceptor reductases for oxygen (the cytochrome *cbb*₃ oxidase) and nitrite (the nitrite reductase, AniA). Mutants were generated by allelic exchange with disrupted copies of the genes encoding these cytochromes and the phenotypes of the resultant mutants analysed. It was found that cytochrome *c*₅ is required for *in vivo* nitrite reductase activity, whereas cytochromes *c*_x and *c*₄ are both required for efficient growth using oxygen as an electron acceptor. Mutants in *c*_x, *c*₄, and *c*_x + *c*₄ have a decreased capacity to reduce oxygen, but there is a background oxygen-reduction activity, indicating that there may be other routes for electron transfer from the cytochrome *bc*₁ complex to the cytochrome *cbb*₃ oxidase, whereas cytochrome *c*₅ appears to be the sole route of electrons to the nitrite reductase in *N. meningitidis*. Interestingly, cytochrome *c*_x is highly similar to a domain of copper nitrite reductases from various proteobacteria, whereas cytochrome *c*₅ has high identity with a domain of the cytochrome *cbb*₃ oxidase of *Neisseria gonorrhoeae*, yet these two proteins function in oxygen respiration and nitrite respiration, respectively. This highlights a limitation of predicting protein function from similarity to known proteins, i.e. very closely related protein domains in different organisms can have different redox partners.

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INTRODUCTION

The β -proteobacterium *Neisseria meningitidis* is commonly identified as part of the commensal flora of the human nasopharyngeal mucosa. Very occasionally colonization is followed by invasion of the bloodstream by *N. meningitidis*, leading to life-threatening illness in the form of meningitis or septicaemia (van Deuren *et al.*, 2000). The nasopharyngeal mucosa is habitat to a wide variety of other bacteria, including both aerobes and anaerobes (Brook, 2003). In previous work, we have determined that under aerobic conditions *N. meningitidis* is able to support respiration by reducing oxygen to water via the enzyme cytochrome *cbb*₃ oxidase (which is the only oxygen reductase in *N. meningitidis*), and that when oxygen becomes limiting the bacterium expresses genes encoding a nitrite reductase (*aniA*) and a nitric oxide reductase (*norB*) that collectively catalyse the respiratory reduction of nitrite to nitrous oxide (Anjum *et al.*, 2002). *N. meningitidis* is able to employ this partial denitrification pathway to support growth under microaerobic conditions in the presence of nitrite (Rock *et al.*, 2005).

We have proposed an organization for the respiratory chain of *N. meningitidis*, based on genome sequence analysis and

experimental analysis with specific inhibitors (Deeudom *et al.*, 2006). The nitric oxide reductase of *N. meningitidis* appears more similar to the quinol-oxidizing nitric oxide reductase (qNOR) than the cytochrome *c*-oxidizing NOR (cNOR) (de Vries & Schröder, 2002), and hence it was proposed that the nitric oxide reductase receives its electrons directly from the ubiquinone pool. This was confirmed experimentally by showing that nitric oxide reduction is insensitive to the cytochrome *bc*₁ complex inhibitor myxothiazol. Contrastingly, the reduction of the other two electron acceptors (oxygen and nitrite) is very sensitive to myxothiazol. The enzymes responsible for these reductase reactions typically receive their electrons from *c*-type cytochromes in other micro-organisms, and hence it was proposed that these two enzymes terminate the electron-transport chain downstream of the cytochrome *bc*₁ complex and *c*-type cytochromes (Deeudom *et al.*, 2006). The genome of *N. meningitidis* MC58 reveals the presence of genes encoding three putative *c*-type cytochromes that might mediate the transfer of electrons between the cytochrome *bc*₁ complex and the reductases for oxygen and nitrite. These three *c*-type cytochromes are conserved within the genomes of other *N. meningitidis* strains and in *Neisseria gonorrhoeae*.

Gene NMB0717 (from *N. meningitidis* MC58) is predicted to encode a periplasmic mono-haem cytochrome with a molecular mass of 12.5 kDa. We call the putative product of this gene cytochrome c_x . A homologue of NMB0717, cytochrome c_{552} from *Thermus thermophilus*, has been proposed to transfer electrons between the cytochrome bc_1 complex and ba_3 oxidase in that organism (Muresanu *et al.*, 2006). BLAST searching reveals that the predicted protein sequences with closest similarity to NMB0717, outside of the *Neisseria* homologues, are predicted copper-type nitrite reductases in *Bdellovibrio bacteriovorus*, *Pseudoalteromonas haloplanktis* and several *Burkholderia* species. In these organisms, the gene for the copper nitrite reductase (homologous to the nitrite reductase gene, *aniA*, of *N. meningitidis*) is fused to a cytochrome domain homologous to NMB0717. This suggests a possible role for cytochrome c_x in transfer of electrons to nitrite reductase in *N. meningitidis*.

Gene NMB1805 is predicted to encode a periplasmic di-haem cytochrome with a molecular mass of 21.5 kDa. We call the putative product of this gene cytochrome c_4 , consistent with the nomenclature for the homologous gene (also known as *cycA*) from *N. gonorrhoeae* (Turner *et al.*, 2005). Cytochrome c_4 homologues are found in many other proteobacteria and a crystal structure has been found for the protein from *Pseudomonas stutzeri* (Kadziola & Larsen, 1997). Both haem groups are hexacoordinated, consistent with a role in electron transfer, rather than enzyme catalysis.

Gene NMB1677 is predicted to encode a membrane-associated di-haem cytochrome with a molecular mass of 30 kDa. We call the putative product of this gene cytochrome c_5 , consistent with the nomenclature for the homologous gene (also known as *cycB*) from *N. gonorrhoeae* (Turner *et al.*, 2005). The protein contains a predicted N-terminal membrane span, followed by two soluble domains containing two covalently bound haems located within the periplasm. The second haem-containing domain of cytochrome c_5 bears a striking similarity (74 % identity) to a region of the cytochrome *cbb3* oxidase subunit III (encoded by *ccoP*) from *N. gonorrhoeae*. CcoP from *N. gonorrhoeae* is predicted to contain three haem groups, whereas CcoPs from other organisms that have been characterized contain just two haem domains (Pitcher & Watmough, 2004). Indeed, the predicted CcoP from *N. meningitidis* contains two haems. The similarity of cytochrome c_5 to a domain of the oxidase immediately suggests a role for cytochrome c_5 in the electron transfer from cytochrome bc_1 to the cytochrome *cbb3* oxidase.

The possession of multiple *c*-type cytochromes as part of multiply branched respiratory chains is characteristic of many bacterial species. The unambiguous assignment of function to these cytochromes is often difficult due to their large number and apparent redundancy of function. The aim of the work presented in this paper was to investigate the roles of the three putative electron-carrier proteins of

N. meningitidis in respiration in this organism, which is an important pathogen but which may also be viewed as a good model for analysis of branched respiratory metabolism due to its relatively small number of respiratory electron-acceptor reductases.

METHODS

Bacterial strains, plasmids and growth conditions. All strains of *N. meningitidis* used in this work were derived from *N. meningitidis* MC58 (McGuinness *et al.*, 1991) (Table 1). *N. meningitidis* strains were routinely cultured at 37 °C, in air supplemented with 5 % CO₂ on Columbia agar plus 5 % (v/v) horse blood plates, or in liquid culture in Mueller–Hinton Broth (MHB) supplemented with 10 mM NaHCO₃. Aerobic culture was carried out in 5 ml of broth in 25 ml Sterilin McCartney bottles shaken at 200 r.p.m. Microaerobic culture was carried out using 20 ml of broth in a 25 ml Sterilin McCartney bottle, shaken at 90 r.p.m. and, where appropriate, supplemented with 6 mM NaNO₂. Growth was monitored by measuring OD₆₀₀ in a Jenway 6305 spectrophotometer. For whole-cell spectroscopy experiments cultures were supplemented with 5 mM glucose in order to provide a defined electron donor that could be included as physiological reductant in cuvettes. Antibiotics were used at the following concentrations: tetracycline 20 µg ml⁻¹, erythromycin 50 µg ml⁻¹, kanamycin 50 µg ml⁻¹ and spectinomycin 50 µg ml⁻¹.

Construction of mutant strains. The gene encoding cytochrome c_x (NMB0717) and 500 bp flanking on each side was amplified with primers c552F and c552R (Table 1) using *Pfu* polymerase (Promega). The product was cloned into pCR-Blunt II TOPO (Invitrogen). The product was cleaved with *Bgl*I, which recognizes a site located centrally within the NMB0717 gene. Subsequently, the ends of the linearized plasmid were rendered blunt with Klenow fragment and the product was ligated with the spectinomycin resistance (*Spc*^R) gene derived from pHP45Ω (Prentki & Krisch, 1984) to yield pTOPO- c_x ::*Spc*^R. The gene encoding cytochrome c_4 (NMB1805) and 500 bp flanking on each side was amplified with primers c554F and c554R (Table 1) and disrupted by essentially the same method as described for cytochrome c_x except that the gene was disrupted by digestion with *Bsu*36I (which recognizes a site located centrally within the NMB1805 gene). Insertion of an erythromycin resistance (*Ery*^R) gene [derived by PCR from a strain of *N. meningitidis* in which the gene *fir* was disrupted by *Ery*^R (Rock *et al.*, 2005)] yielded pTOPO- c_4 ::*Ery*^R. The gene encoding cytochrome c_5 (NMB1677) and 500 bp flanking on each side was amplified with primers c555F and c555R (Table 1) and disrupted by essentially the same method as described for cytochrome c_x except that the gene was disrupted by digestion with *Xmn*I (which recognizes a site centrally within the NMB1677 gene) and insertion of a tetracycline resistance (*Tet*^R) gene derived from Tn916, yielding pTOPO- c_5 ::*Tet*^R.

The recombinant plasmids containing disrupted copies of the genes encoding cytochromes were transformed into *N. meningitidis* MC58 using the method of Bogdan *et al.* (2002), selecting for recombinant strains using the appropriate antibiotic selection on plates, and verifying the correct chromosomal rearrangement by PCR.

Gels and blotting. Whole-cell extracts of *N. meningitidis* were prepared by harvesting 1 ml samples of cultures in late exponential phase by centrifugation, resuspending the pellets in 500 µl 30 mM Tris/HCl (pH 8) + 1 % (w/v) n-dodecyl β-D-maltoside + 1 mg lysozyme ml⁻¹ + 1 mg DNase I ml⁻¹, and subjecting the suspension to 8–10 cycles of freezing and thawing. Samples were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were stained with Ponceau S to check for efficient protein transfer and

Table 1. Strains, plasmids and oligonucleotide primers used in this work

Strain, plasmid or primer	Description	Reference or source
<i>N. meningitidis</i> strains		
MC58	Wild-type serogroup B strain	McGuinness <i>et al.</i> (1991)
<i>fnr</i>	Derivative of MC58 with insertion of erythromycin cassette into <i>fnr</i>	Rock <i>et al.</i> (2005)
c_x^-	Derivative of MC58 with insertion in NMB0717 (Sp^R)	This work
c_4^-	Derivative of MC58 with insertion in NMB1805 (Ery^R)	This work
c_5^-	Derivative of MC58 with insertion in NMB1677 (Tet^R)	This work
$c_x^-.c_4^-$	Derivative of MC58 with insertion in NMB0717 and NMB1805 ($Sp^R Ery^R$)	This work
Plasmids		
pCR-Blunt II-TOPO	Vector for cloning blunt-ended PCR products; Kan^R	Invitrogen
pHP45 Ω	Source for Ω cassette (Sp^R)	Prentki & Krisch (1984)
pTOPO_ c_x :: Sp^R	NMB0717 disrupted with Sp^R cassette maintained in pCR-Blunt II-TOPO	This work
pTOPO_ c_4 :: Ery^R	NMB1805 disrupted with Ery^R cassette maintained in pCR-Blunt II-TOPO	This work
pTOPO_ c_5 :: Tet^R	NMB1677 disrupted with Tet^R cassette maintained in pCR-Blunt II-TOPO	This work
Primers		
c552F	5'-TTGGAAACCATGGAAGAAATC-3'	This work
c552R	5'-GACAGTACAAATAGTACGGAA-3'	This work
c554F	5'-GTTTTTGAACGATAGGCAAGA-3'	This work
c554R	5'-CCGAATTTGACCAAATAATCCG-3'	This work
c555F	5'-AATTGGCAAAGGTTATCTTGCG-3'	This work
c555R	5'-GGAAAATTATGAAGACTGACT-3'	This work

destained with water. The expression of c-type cytochromes was assessed using a chemiluminescence method (Vargas *et al.*, 1993) to measure the peroxidase activity of haem groups covalently attached to protein. Five hundred microlitres of chemiluminescence detection reagents (SuperSignal West Dura substrate, Pierce) were mixed and pipetted onto the membrane, which was incubated, sandwiched between two acetate sheets, for 5 min. The membrane was subsequently exposed to X-ray film (for 5 s to 5 min) to achieve an optimum chemiluminescence signal. Bands due to c-type cytochromes were clearly visualized upon developing the film. Western blotting was used to detect expression of nitrite reductase AniA, as previously described (Rock *et al.*, 2007).

Activity assays. Oxygen and nitric oxide were measured using electrode-based methods and nitrite was assayed colorimetrically as described previously (Rock *et al.*, 2005). Nitrite reductase activity was assayed in whole-cell extracts using methyl viologen as electron donor as described previously (Moir *et al.*, 1993).

Spectroscopy. The spectra of cytochromes in intact cells were measured using a Jasco V550 UV-visible spectrophotometer fitted with Integrating Reflective Sphere (ISV-469) in order to compensate for the light scattering by very high-density bacterial suspensions. Bacterial cultures grown in MHB+5 mM glucose were harvested and resuspended in 25 mM HEPES+5 mM glucose (pH 7) to an OD₆₀₀ of 1.0–1.2 (in this spectrophotometer, equivalent to approx. 5–10 mg protein ml⁻¹). Spectral measurements were carried out in 1 ml cell suspensions in 1.4 ml quartz cuvettes with sealed caps (117.100F-QS, Hellma). Each spectral measurement was taken five times to allow averaging of multiple spectra to reduce noise. Reduction of respiratory components was achieved using the physiological electron donor glucose and leaving the cell suspension to exhaust the available oxygen over the course of 10 min. Oxygen-oxidized spectra were taken following vigorous shaking of the cell suspension. Oxidation of the suspension by nitrite was achieved by addition of 5 mM nitrite (in a total volume of 5 μ l to minimize dilution effect).

RESULTS

Construction and confirmation of mutants in cytochromes c_x , c_4 and c_5

Mutant strains deficient in c_x , c_4 , c_5 and c_x+c_4 were generated following transformation of the constructs described above into *N. meningitidis* MC58. In each case, the replacement of the wild-type copy of the gene with the copy containing an antibiotic resistance cassette was confirmed by PCR (data not shown). In order to obtain further biochemical confirmation, whole-cell extracts generated from each of the strains were run on SDS-PAGE, blotted onto nitrocellulose and stained for haem (Fig. 1a). The lane containing extract from strain *N. meningitidis* c_5^- reveals the absence of a cytochrome band with molecular mass of approximately 33 kDa, consistent with the absence of cytochrome c_5 (which has a predicted molecular mass of 30 kDa). The lanes containing extracts from strains *N. meningitidis* c_4^- and $c_x^-.c_4^-$ lacked an intense band with a molecular mass of approximately 23 kDa, consistent with the absence of cytochrome c_4 (predicted molecular mass 21.5 kDa) from these strains. There is no difference in the appearance of lanes between c_4^- and $c_x^-.c_4^-$ or between MC58 and c_x^- , indicating that a band due to cytochrome c_x cannot be observed by this method.

UV-visible reduced-minus-oxidized spectroscopy of intact cells of *N. meningitidis* wild-type yielded spectra consistent with the presence of c-type (α band located near 550 nm) and b-type (α band shoulder near 560 nm) cytochromes in this organism (Fig. 2). Comparison of wild-type versus strains deficient in c_x , c_4 and c_5 indicates the absence of

Fig. 1. (a) Haem-stained blot of *N. meningitidis* extracts shows altered expression of cytochromes. Samples (13 µg) of total cell extracts from *N. meningitidis* wild-type MC58 and the mutants indicated (aerobically grown) were separated by 15% SDS-PAGE, blotted and stained for haem. (b) Western blot with anti-AniA antibodies. The *N. meningitidis* strains were grown under microaerobic conditions plus nitrite. Gels were run as for (a).

specific cytochromes in each of the constructs (Fig. 2). The overall intensities of the redox difference spectra were decreased for the cytochrome-deficient strains compared to the wild-type. Furthermore, the positions of the α bands in these difference spectra are shifted compared to the wild-type. For *N. meningitidis* MC58, the α band is positioned at 553 nm, whilst the c_x^- and c_5^- mutants have their α peak at 552 nm and the c_4^- mutant has an α band at 555 nm. The α

Fig. 2. UV-visible redox spectra of intact *N. meningitidis*. Spectra are endogenously reduced minus oxygen-oxidized spectra of *N. meningitidis* wild-type MC58 (black) and mutants c_x^- (red), c_4^- (green), c_5^- (blue), and $c_x^-c_4^-$ (purple). The positions of spectral peaks α , β and γ are marked. For each cell suspension, the OD₆₀₀ for the endogenously reduced sample was 1.0.

band of the $c_x^-c_4^-$ mutant is centred at 553 nm. It is notable that the mutation in c_x causes a shift in the α band as well as a decrease in spectral intensity (for MC58 versus c_x^- and for c_4^- versus $c_x^-c_4^-$), providing evidence that cytochrome c_x is expressed in wild-type *N. meningitidis* and absent from the c_x mutant strains (despite giving no band on haem-stain blots).

Growth properties of cytochrome-deficient mutants of *N. meningitidis*

N. meningitidis MC58, c_x^- , c_4^- , c_5^- and $c_x^-c_4^-$ were cultured under aerobic conditions and under microaerobic conditions in the presence and absence of 5 mM nitrite (Fig. 3). The growth rate of the c_5^- mutant was virtually the same as that of the wild-type during the exponential phase in aerobic culture (doubling time of 43 min), whereas the c_x^- , c_4^- and $c_x^-c_4^-$ mutants grew relatively poorly, with doubling times of 63 min, 130 min and 174 min, respectively (Fig. 3a). *N. meningitidis* MC58, c_x^- , c_4^- and c_5^- grew at similar rates to one another under microaerobic conditions in the absence but not the presence of nitrite (Fig. 3b, c). In the presence of nitrite, *N. meningitidis* MC58, c_x^- , c_4^- and $c_x^-c_4^-$ had an exponential phase of growth, as expected for cultures that have nutrient sufficiency when nitrite is present, whereas the c_5^- mutant had a linear growth curve, as would be expected for a strain that is limited by availability of a nutrient that is supplied at a constant rate, such as oxygen (Fig. 3c). This has been observed previously for strains of *N. meningitidis* that are unable to denitrify (Rock *et al.*, 2005), and suggests that under these growth conditions the cytochrome c_5 -deficient strain grows using the limiting nutrient oxygen and is unable to denitrify. Measurements of nitrite remaining in culture medium corroborated this proposition, since nitrite disappeared during the course of growth of all strains except *N. meningitidis* c_5^- (Fig. 3d). It is notable that *N. meningitidis* c_x^- , c_4^- and $c_x^-c_4^-$ grew to lower final cell densities than did *N. meningitidis* MC58, and yet utilized nitrite as quickly as or quicker than the wild-type. This is consistent with the relatively poor ability of these strains to grow aerobically (Fig. 3a), and indicates that under denitrifying conditions these strains use nitrite as the major electron sink for respiration whereas the wild-type combines nitrite and oxygen utilization simultaneously.

To test whether the impaired aerobic growth observed in mutants deficient in c_x , c_4 and $c_x + c_4$ was due to an inability to metabolize oxygen, the rate of oxygen uptake was measured in suspensions of *N. meningitidis* strains using a Clark-type oxygen electrode. The rate of oxygen uptake was measured for c_x^- and c_4^- strains relative to wild-type following aerobic growth. The rates of oxygen uptake were $83 \pm 5\%$ and $59 \pm 5\%$ of the wild-type rate in strains c_x^- and c_4^- respectively. The growth of *N. meningitidis* $c_x^-c_4^-$ was so poor aerobically that we cultured the wild-type and $c_x^-c_4^-$ strains under denitrifying conditions to analyse the relative oxygen respiration

Fig. 3. Effect of deficiency in cytochromes on growth of *N. meningitidis*. Cultures of *N. meningitidis* wild-type MC58 (■—■) and mutants c_5^- (□---□), c_4^- (△---△), $c_5^- c_4^-$ (●—●) and $c_5^- c_4^-$ (○---○) were grown under aerobic conditions (a), under microaerobic conditions without nitrite (b) and under microaerobic conditions plus 6 mM nitrite (c). In each case growth was monitored by measuring OD₆₀₀. Nitrite disappearance was monitored for microaerobic cultures supplemented with nitrite (d).

rate in this strain. The rate of oxygen uptake was $45 \pm 5\%$ of the wild-type rate in the $c_5^- c_4^-$ strain. Clearly both cytochromes c_5 and c_4 are involved in enabling *N. meningitidis* to utilize oxygen and to grow aerobically.

N. meningitidis c_5^- failed to utilize nitrite during incubation under microaerobic conditions with nitrite. To determine whether this was due to an inability to respire nitrite or nitric oxide we grew strains MC58 and c_5^- microaerobically in the absence of nitrite and then followed the accumulation of nitric oxide using an NO electrode after the addition of nitrite (Fig. 4). No nitric oxide accumulated from the c_5^- strain, whereas, as expected, it accumulated immediately on adding nitrite to the wild-type. This indicates that the lesion in the c_5 mutant relates to nitrite reduction, not nitric oxide reduction. To assess whether the defect in nitrite reduction in *N. meningitidis* c_5^- might be due to low nitrite reductase expression and/or activity rather than to a break in the electron-transport chain to the nitrite reductase we measured nitrite reductase expression and activity. Expression of nitrite reductase was assessed by Western blotting (Fig. 1b), which showed that AniA is expressed at similar levels in all strains. Using a methyl viologen-linked nitrite reductase assay in total cell extracts we found that *N. meningitidis* MC58 and c_5^- had similar overall rates of nitrite reductase activity (data not shown). In the absence of cytochrome c_5 the electron-transport chain to nitrite reductase is disabled, presumably because cytochrome c_5 is an electron carrier between the cytochrome bc_1 complex and AniA nitrite reductase.

Spectroscopic analysis of oxidation of *N. meningitidis* cytochromes by oxygen versus nitrite

Like oxygen, nitrite was found to be capable of oxidizing cytochromes in *N. meningitidis* MC58 (but only after

Fig. 4. Nitric oxide production in *N. meningitidis* wild-type MC58 but not the c_5^- mutant strain. *N. meningitidis* MC58 and c_5^- were grown under microaerobic conditions, and a 5 ml cell suspension was allowed to become anaerobic in a water-jacketed electrode chamber at 37 °C. Oxygen was monitored with a Clark-type electrode (dashed line). Nitric oxide was monitored with an NO electrode (solid line) before and after the addition of 5 mM nitrite.

growth under microaerobic conditions plus nitrite) (Fig. 5). The redox difference spectrum following oxidation by nitrite is less intense than that following oxidation by oxygen, consistent with nitrite being a less good oxidant than oxygen. Furthermore, the positions of the spectral maxima are different (the α band is located at 560 nm), indicating that oxidation by nitrite brings about predominantly the oxidation of *b*-type cytochromes. The redox difference spectra following oxidation by nitric oxide (provided by treating cells with 1 mM DEA-NONOate) gives similar spectra to those seen on oxidation by nitrite. Redox difference spectra of *N. meningitidis* c_5^- following treatment with nitrite showed no features, consistent with this strain possessing no significant electron-transport chain to nitrite reductase (Fig. 5).

DISCUSSION

In this study we investigated the roles of the three putative electron-carrier *c*-type cytochromes of *N. meningitidis* in respiratory electron transport during oxygen respiration and denitrification of nitrite to nitrous oxide. The evidence establishes that cytochrome c_x and cytochrome c_4 are important for oxygen reduction and that cytochrome c_5 is necessary for the reduction of nitrite to nitric oxide. These findings run counter to the expectations based on similarity of the cytochromes to other proteins in sequence databases. Most notably, cytochrome c_x is highly homologous to a cytochrome-containing domain of copper nitrite reductases from various proteobacteria, which had indicated that cytochrome c_x might be involved in electron transfer to nitrite reductase in *N. meningitidis*, whereas the experimental finding was that mutation of cytochrome c_x has no deleterious effect on nitrite reduction. Similarly,

cytochrome c_5 (identified experimentally as required for nitrite reduction) is very similar to a domain of the cytochrome *cbb₃* oxidase of *N. gonorrhoeae*, which had suggested that this protein might be important for oxygen respiration. This is an important caveat to bear in mind when predicting function of electron-transport proteins, i.e. very closely related protein domains in different organisms can have different redox partners.

The simplest explanation of the data we have obtained is that cytochrome c_5 is a mediator that carries electrons from the cytochrome *bc₁* complex to the AniA nitrite reductase. Cytochromes c_x and c_4 are not required for the reduction or oxidation of c_5 . Is it feasible that cytochrome c_5 might be a direct electron donor to AniA nitrite reductase? AniA consists of a trimer of water-soluble subunits (Boulanger & Murphy, 2002) associated with the outer membrane via covalent attachment of N-terminal cysteine residues to fatty acid moieties (Clark *et al.*, 1987; Hoehn & Clark, 1992). It is presumed to be associated with the inner leaflet of the outer membrane, and hence located within the periplasmic compartment. To reduce AniA and drive nitrite reduction, electrons must be transported across the periplasm from the cytochrome *bc₁* complex (in the inner membrane) to AniA. Can cytochrome c_5 span this gap? Analysis of the sequence of cytochrome c_5 indicates that it arose by a duplication event from an ancestral gene encoding a monohaem protein. There is no structure available for a dihaem cytochrome c_5 , but structural information is available for a homologous monohaem cytochrome c_5 from *Shewanella putrefaciens* (Bartalesi *et al.*, 2002). This globular protein has a diameter of approximately 30 Å (3 nm), indicating that the dimeric cytochrome c_5 from *N. meningitidis* may span approximately 60 Å. *N. meningitidis* cytochrome c_5 is predicted to be attached to the membrane via a transmembrane helix, and thus it is anticipated that it can form a structure which protrudes up to approximately 60 Å into the periplasm from the inner membrane. The ingress of electrons to AniA occurs via a blue copper centre which is accessed from a site on the surface of AniA which is approximately 45 Å from the N-terminal face of the soluble protein structure (Boulanger & Murphy, 2002). Thus a complex of the globular domains of cytochrome c_5 and AniA could span some 105 Å across the periplasm. The width of the Gram-negative periplasm is approximately 170 Å, based on electron microscopy measurements (Matias *et al.*, 2003) and calculations based on the interaction of inner-membrane protein AcrB with outer-membrane protein TolC (Tamura *et al.*, 2005). Towards the N terminus of the mature AniA polypeptide there is a 35–40 residue region rich in alanine, proline and glutamate that may form an elongated unstructured linker that will allow AniA to get access to electrons from cytochrome c_5 . It will be of interest to shorten this 'linker region' to determine whether it is required for allowing AniA to obtain electrons.

Haem staining was used successfully to identify cytochromes c_4 and c_5 in total extracts of *N. meningitidis*

Fig. 5. UV-visible redox spectra of intact *N. meningitidis* wild-type MC58 and mutant c_5^- . Spectra are endogenously reduced minus oxygen-oxidized spectra of strain MC58 (blue), reduced minus nitrite-oxidized spectra of strain MC58 (red), reduced minus nitric oxide-oxidized spectra of strain MC58 (green), and reduced minus nitrite-oxidized spectra of strain c_5^- (purple). For each cell suspension, the OD₆₀₀ for the endogenously reduced sample was equal to 1.0.

strains, but cytochrome c_x could not be seen by this method. Haem staining relies on the peroxidase activity of the haem group, an activity that is affected by the maturation state of the protein folding around the haem group (Diederix *et al.*, 2002). This activity varies between cytochromes and presumably is very low for cytochrome c_x . Spectroscopic measurements, however, showed a significant loss of c-type cytochrome from the c_x mutant (Fig. 2), confirming that this is a major cytochrome in *N. meningitidis*. The additive effects of mutations in c_x and c_4 on oxygen respiration indicate that these two proteins operate as independent parallel pathways of electrons from the cytochrome bc_1 complex to the cytochrome cbb_3 oxidase (Fig. 6). Additionally, there is residual oxidase activity in a $c_x^- .c_4^-$ mutant, indicating that there are alternative pathways to cytochrome cbb_3 . Cytochrome c_5 may act as an alternative electron carrier to the oxidase, and it is noticeable that growth of a c_5^- mutant aerobically is slower than that of the wild-type at high optical densities in late exponential phase (Fig. 4a). We were unable to construct a $c_x^- .c_4^- .c_5^-$ triple mutant, lending support to this proposition. An alternative route might involve the outer-membrane-associated cupredoxin Laz (lipid-modified azurin) (Gotschlich & Seiff, 1987; Kawula *et al.*, 1987). Mutants deficient in the gene encoding Laz grew well aerobically (Wu *et al.*, 2005; our unpublished data) but we were unable to generate a $c_x^- .c_4^- .laz$ triple mutant (data not shown). The proposed electron-transport chain of *N. meningitidis* is summarized in Fig. 6.

Spectroscopic analysis following oxidation of reduced suspensions of intact *N. meningitidis* with oxygen, nitrite and nitric oxide indicates that on oxidation of cells with nitrite the main chromophores that are oxidized are b-type cytochromes, which are also oxidized directly by nitric oxide [nitric oxide reductase itself is a b-type cytochrome (de Vries & Schröder, 2002)]. Whilst oxygen strongly oxidizes the cytochrome c pool in steady-state cell suspensions, nitrite does not. At steady state in the presence of nitrite, the cytochrome c pool remains largely reduced. Presumably this is related to the fact that nitrite is a poor oxidant relative to oxygen ($E'_{\text{NO}_2^-/\text{NO}} = 0.375$ V;

Fig. 6. Proposed electron-transport chain of *N. meningitidis*. Electron flow from cytoplasmic reductants (NADH, succinate, etc.: shown as [H]) to terminal electron acceptors O_2 , NO_2^- and NO is shown by solid arrows. A possible alternative electron transport pathway from cytochrome c_5 to the oxidase is shown by the dashed arrow. UQ, ubiquinone; NIR, nitrite reductase.

$E'_{\text{O}_2/\text{H}_2\text{O}} = 0.8$ V). It may also be related to the fact that nitrite reductase draws electrons from only cytochrome c_5 whereas oxygen oxidizes cytochromes cbb_3 , c_x , c_4 and possibly c_5 . Furthermore, this spectroscopic observation is consistent with the finding that oxygen almost completely inhibits nitrite reduction in intact cells, whereas the reverse is not the case (Rock *et al.*, 2005); i.e. oxygen is able to out-compete nitrite for electrons at the level of cytochrome c .

In summary, we have identified the roles of three c-type cytochromes in the respiratory chain of *N. meningitidis*. Respiration is crucial for survival of *N. meningitidis*, and these cytochromes are highly conserved and extracytoplasmic, making them potential targets for the development of novel therapeutics against pathogenic neisseriae.

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