Cytochrome c-based domain modularity governs genus-level diversification of electron transfer to dissimilatory nitrate reduction

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Summary

The genus Neisseria contains two pathogenic species (N. meningitidis and N. gonorrhoeae) in addition to a number of commensal species that primarily colonize mucosal surfaces in man. Within the genus, there is considerable diversity and apparent redundancy in the components involved in respiration. Here, we identify a unique c-type cytochrome (c₅) that is broadly distributed among commensal Neisseria, but absent in the pathogenic species. Specifically, c₅ supports nitrite reduction in N. gonorrhoeae strains lacking the cytochromes c₃ and CcoP established to be critical to NirK nitrite reductase activity. The c-type cytochrome domain of c₅ shares high sequence identity with those localized c-terminally in c₃ and CcoP and all three domains were shown to donate electrons directly to NirK. Thus, we identify three distinct but paralogous proteins that donate electrons to NirK. We also demonstrate functionality for a N. weaverii NirK variant with a C-terminal c-type heme extension. Taken together, modular domain distribution and gene rearrangement events related to these respiratory electron carriers within Neisseria are concordant with major transitions in the macroevolutionary history of the genus. This work emphasizes the importance of denitrification as a selectable trait that may influence speciation and adaptive diversification within this largely host-restricted bacterial genus.

Introduction

Bacterial respiratory chains and their associated redox proteins are essential for overall metabolism, to maintain proton motive force and enable the chemiosmotic coupling of electron transport to adenosine triphosphate (ATP) synthesis. The relatively high degrees of diversification manifest within bacterial systems undoubtedly reflect corresponding variability in the environments inhabited. As such, a better understanding of the underlying components involved in electron transfer, the pathways incorporated and the terminal electron acceptors utilized reveal a higher resolution picture of the environmental, ecological and evolutionary forces involved. Under conditions of low oxygen, many bacteria utilize alternate substrates as terminal electron acceptors with perhaps the best-studied systems involving denitrification initiating with the reduction of nitrate. Such studies and efforts to understand how microaerobic respiration and denitrification have co-evolved by integrating hybrid electron transport chains have focused primarily on free-living species of environmental importance (Chen and Strous, 2013).

The genus Neisseria is comprised of Gram-negative, oxidase-positive diplococci/rods primarily inhabiting mucosal surfaces of man. Most research has focused on the two species associated with disease, namely Neisseria gonorrhoeae and Neisseria meningitidis. The former is the cause of the sexually transmitted disease gonorrhea, whereas N. meningitidis is primarily a commensal of the human naso/oropharynx that, under some circumstances, causes invasive disease and meningitis (Stephens, 2009). In addition to these, a large number of commensal neisserial species have been identified that colonize the human oropharynx as part of the normal flora (Tønjum, 2005). Thus, although most human neisserial species appear to be evolving in related niches (Tønjum, 2005), they retain tightly restricted evolutionary trajectories. This is particularly interesting given the evidence for
significant levels of interspecies horizontal gene transfer (Marri et al., 2010; Higashi et al., 2011). Neisseria gonorrhoeae and N. meningitidis demonstrate the ability to combine an aerobic respiration pathway with that of a truncated denitrification pathway. In aerobic respiration, the reduction of dioxygen to water is solely carried out by their cytochrome cbb3 oxidase (Pitcher and Watmough, 2004). Canonical cytochrome cbb3 oxidases consist of four subunits in which CcoN is the catalytic subunit that carries a dinuclear centre formed by the iron of a high-spin heme and an associated copper ion (CuA) in which dioxygen is reduced (Rauhamaki et al., 2009). CcoO and CcoP act as membrane-bound c-type cytochromes that channel electrons to the dinuclear centre, whereas CcoQ oxides link oxygen reduction to proton translocation across the inner membrane such that metabolic energy is retained for ATP synthesis (Toledo-Cuevas et al., 1998).

The truncated denitrification pathway entails the copper-containing nitrite reductase (CuNIR) NirK-mediated one-electron reduction of nitrite (NO2−) to nitric oxide (NO) that is then in turn reduced to nitrous oxide (N2O) by NorB (Mellies et al., 1997; Householder et al., 2000; Anjum et al., 2002; Rock et al., 2005). The linked nirK and norB genes are controlled differentially by transcriptional regulators that are responsive to alterations in the levels of oxygen, NO2− and NO (Overton et al., 2006; Rock et al., 2007; Heurlier et al., 2008; Isabella et al., 2008; 2009).

Electron transport via NirK and NorB is associated with a lower theoretical generation of proton motive force (pmf) compared with electron transport to oxygen via cbb3. Therefore, the co-metabolism of oxygen and nitrite as electron acceptors under microaerobic conditions represents a trade off in which electron flux through the respiratory chain is maintained under reduced levels of O2 at the cost of reduced energy conservation.

Given the interconnected nature of the aerobic respiration and denitrification pathways, an important question relates to which redox partners carry out direct electron transfer to cbb3 and NirK. We as well as others have provided genetic evidence that the gonococcal CcoP is capable of electron transfer to NirK either directly or indirectly by virtue of a unique c-type cytochrome domain located at its C-terminus (Hopper et al., 2009; Aspholm et al., 2010). The extended, tri-heme c-type cytochrome architecture of this CcoP (as opposed to the di-heme form found in most other CcoP proteins) is prevalent among neisserial species. Genetic data show that Cc5, implicated in electron transfer to the cbb3 oxidase, can also facilitate electron transfer to NirK (Deedum et al., 2008). The latter activity is mediated by virtue of its C-terminally localized c-type cytochrome domain that shares 74% identity to the corresponding terminal domain of tri-heme CcoP (Aspholm et al., 2010). As such, both CcoP and Cc5 act as electron carriers ultimately targeting both aerobic respiration and denitrification pathways (Deedum et al., 2008; Hopper et al., 2009; Aspholm et al., 2010). These findings demonstrated in an unprecedented fashion how components of the aerobic respiratory complex can be linked to functionality of a denitrification pathway.

Two major evolutionary transitions relating to the interplay between aerobic respiration and denitrification are evident within the genus Neisseria (Aspholm et al., 2010). As previously shown, the tri-heme-encoding ccoP likely had its genesis via homologous recombination involving Cc5 and a primordial, di-heme-encoding ccoP allele. This event appears to have occurred once and subsequently become fixed within the genus by virtue of its adaptive value in coordinating aerobic respiratory processes with denitrification. The second major event relates to the single base change in extant ccoP genes of N. meningitidis, resulting in a reversal to the primordial allelic state (Aspholm et al., 2010). Therefore, changes in CcoP domain architecture and ensuing alterations in function are key traits in successive, adaptive radiations within the genus Neisseria.

Here, we extend studies of redox partners targeting NirK by examining the distribution of cytochrome c domain—containing proteins within commensal Neisseria genome draft assemblies. In particular, we report the identification of a distinctive mono-heme c-type cytochrome, widely distributed among commensal Neisseria species, which complements a defect in electron transfer to NirK in N. gonorrhoeae. Its cytochrome c domain shares high degrees of identity with those in CcoP and Cc5 involved in electron transfer to NirK. Moreover, we demonstrate biochemically that each of these three c-type cytochromes can directly deliver electrons to NirK. These results reveal novel insights into the interconnectivity and evolution of hybrid electron transport chains and the importance of domain-based modularity in the diversification of redox partners.

Results
Identification of a novel c-type cytochrome in commensal Neisseria species

Access to genome-sequencing projects for a number of commensal neisserial species allowed us to explore the genetic potential for respiratory pathways at the genus level. BLAST searches were performed using the polypeptide sequence of the distal cytochrome c domain of Cc5 from N. gonorrhoeae strain MS11 as a query. As expected, strong hits were observed to the orthologous domains of Cc5 and the third heme domain of CcoP from various Neisseria species. All species carry di-heme Cc5.
and the tri-heme version of CcoP (with the exception of \textit{N. meningitidis} and some commensals – see below). Unexpectedly, the search also revealed the presence of a highly related open reading frame (ORF) corresponding to a mono-heme c-type cytochrome within the genomes of some commensal \textit{Neisseria} species. The structural relatedness of this ORF from the type strain of \textit{N. elongata} subspecies \textit{glycolytica} (hereon designated as \textit{Nel}) to the C-terminal heme domains of \textit{N. gonorrhoeae} \textit{c5} and CcoP was remarkable (Fig. 1A and B). Its predicted amino acid sequence also includes an N-terminal motif indicative of lipoprotein modification and processing followed by a short sequence stretch of reduced complexity (rich in serine, alanine and proline). The latter is associated with sites of glycan attachment in gonococcal lipoproteins targeted by the general O-linked protein glycosylation pathway (Vik \textit{et al.}, 2009). Interestingly, these motifs are also found at the N-terminus of a conserved neisserial glycolipoprotein termed Ngo1043 in gonococci. In fact, the first 58 residue stretches of the ORF show 70% identity to that of Ngo1043 (Fig. 1C). The potential roles of Ngo1043 and its orthologues widely distributed within the genus are yet unknown.

We sought next to determine if this ORF was expressed using \textit{Nel} strain ATCC29315 as it had been previously established that this strain was amenable to genetic manipulation (Higashi \textit{et al.}, 2011). We constructed accordingly a strain in which the ORF was tagged by virtue of a C-terminal six-histidine extension and employed Western blotting using anti-His antibodies and an antisera raised towards a recombinant form of the terminal heme domain of CcoP. As shown in Fig. 2, these antisera detected a common species with a mass of 25 kD that was greater than that of 17 kD predicted from the deduced amino acid sequence. Given the O-linked glycosylation established for \textit{c5}, CcoP and Ngo1043 in pathogenic \textit{Neisseria} (Vik \textit{et al.}, 2009) and the presence of a minimal, functional set of glycosylation genes \textit{pgl} identifiable in the \textit{Nel} genome sequences (Higashi \textit{et al.}, 2011), we asked if this discrepancy might be related to such post-translational modification. Therefore, we constructed a \textit{Nel} derivative carrying a null mutation in the \textit{pglC} gene whose product is required for formation of the

![Fig. 1. Domain structure and structural relatedness of neisserial NirK – targeting redox proteins and their paralogues.](image)

- **A.** Structural organization of \textit{c5}, CcoP, Ngo1043 and the ORF subsequently designated as \textit{cN}. Heme domains in grey, low complexity regions (serine, proline, alanine rich) in green and lipoprotein maturation signal peptides in blue.
- **B.** Amino acid alignments of the C-terminal heme domains of \textit{c5}, \textit{ccoP}, (both from \textit{N. gonorrhoeae}) and \textit{cN} from \textit{Nel}. Amino acids identical in all three proteins are shown in red, amino acids identical in two of the three proteins are shown in blue, whereas similar amino acids are shown in green. Numbers refer to residues in the respective proteins.
- **C.** Amino acid alignment of Ngo1043 from \textit{N. gonorrhoeae} and \textit{cN}. Amino acids identical in both proteins are shown in red, whereas similar amino acids are shown in green.

![Fig. 2. Identification of \textit{cN} in \textit{Nel} and its post-translationally modified form. Immunoblots of wild-type \textit{Nel} and a \textit{pglC} mutant expressing 6His-tagged \textit{cN} probed with a polyclonal antibody made against CcoP shows reactivity against heme domains similar to the third heme domain of CcoP, heme-domain (left panel) and penta-His (right panel).](image)
undecaprenyl pyrophosphate-linked glycan donor and thus protein glycosylation (Hartley et al., 2011). Both antisera detected a common species with a mass of 17 kDa in this background (concurrent with the absence of the 25 kDa species), suggesting that this protein is glycosylated in *Nel*. We designated this protein as c5 and the corresponding gene *cycC*.

*Neisseria elongata* subspecies *glycolytica* reduces nitrite under aerobic conditions

Given that the *Nel* genome carries homologues of *nirK* (encoding nitrite reductase), *cycB* (encoding c5) and triheme encoding *ccoP*, it seemed likely that it would be able to undergo nitrite-dependent, microaerobic growth. Surprisingly however, nitrite reduction was observed immediately following addition of NO\(_3^-\) under aerobic conditions. In accordance with this observation, NirK was expressed in large amounts under aerobic conditions and only a modest increase in NirK was seen during a switch to microaerobic growth conditions in the presence of nitrite (Finn Erik Aas, Marina Aspholm, unpubl. results).

When *Nel* was cultured in the presence of nitrite under aerobic conditions, a reduced growth rate was observed although nitrite was consumed within a few hours. In contrast, the *nirK* mutant grew better than the wild-type strain for about 2 h until it reached an OD\(_{600}\) of about 0.5 (Fig. 3). These growth phenotypes were unique to the presence of nitrite as no such differences were seen in its absence. A likely explanation for the poor growth of *Nel* in the presence of nitrite is that NirK activity is relatively high compared with NO reductase activity, leading to accumulation of toxic NO in the system. The enzymes involved in handling NO might, by analogy to those in *N. gonorrhoeae* and *N. meningitidis*, be positively upregulated by NO, via inhibition of the repressor NsrR (Overton et al., 2006; Rock et al., 2007; Heurlier et al., 2008). Thus upon sudden addition of high levels of nitrite, levels of NO might increase quicker than the rate at which NO reductase can be synthesized. To examine this situation in more detail, we preincubated cells with low levels of nitrite. Thus, *Nel* was cultured for 1 h without nitrite followed by 1 h incubation with 0.5 mM nitrite before diluting the cultures back to OD\(_{600}\) = 0.2 and adding 2.5 mM nitrite (Hopper et al., 2013). However, the preincubation with low levels of nitrite did not have any effect on the growth, implying that *Nel* has an inherently low ratio of NO reductase/nitrite reductase activity. However, it may be that the poor growth phenotype on nitrite is not related to the ratio of nitrite to NO reductase activity. Rather, the presence of nitrite might divert electrons away from oxygen respiration, which is more tightly coupled to pmf generation.

Role of related c-type cytochrome domains of c\(_N\), c\(_S\) and CcoP on NirK-related phenotypes in *N. elongata* subspecies *glycolytica*

The high degree of identity shown here between the c-type heme domain of c\(_N\) and the terminal heme domains of c\(_S\) and *ccoP* suggests a possible role for c\(_N\) in electron donation to NirK and nitrite reduction (Aspholm et al., 2010). To investigate this putative role of c\(_N\) in *Nel*, we created a *cycC* null allele. The absence of expression of c\(_N\) in the resulting null mutant was verified by immunoblotting using CcoPB antibodies (Fig. 4A) and heme staining (Fig. 4B). The null mutant did not demonstrate any significant difference in growth or nitrite reduction under either aerobic or microaerobic conditions, compared with the wild-type strain, suggesting that the presence of c\(_N\) is not critical for growth or nitrite reduction under the conditions used (Fig. 4C and D and data not shown). Because both c\(_S\) and CcoP are likely to function as electron donors to NirK (Aspholm et al., 2010), we next constructed a *cycC* null mutant in *Nel* expressing a monoheme version of c\(_N\) (c\(_N1\), encoded by *cycB*_1) and a di-heme version of CcoP (CcoP\(_{52}\)) from the native loci. The expression of the mono-heme version of c\(_N\) and the...
The di-heme version of CcoP was verified by immunoblotting using CcoPB antibodies (Fig. 4A) and heme staining (Fig. 4B). Analysis of growth and nitrite reduction revealed that the triple mutant lacking all three putative electron donors to NirK still reduced nitrite, although at a slightly slower rate compared with the wild-type and the cycB\textsuperscript{1X} ccoP\textsuperscript{2x} double mutant (Fig. 4C and D). Notably, however, both the double and triple mutant strains grew better than the wild-type strain and the cycC (single) mutant, and at a rate similar to the nirK mutant (Fig. 4C and D). In contrast, under aerobic conditions without nitrite, the double (cycB\textsuperscript{1x} ccoP\textsuperscript{2x}), triple (cycC, cycB\textsuperscript{1x} ccoP\textsuperscript{2x}) and nirK mutant strains grew at a similar rate as the wild-type strain (data not shown). As noted above, the toxicity of nitrite suggests a low NO reductase activity in Nel. The relatively minor change in nitrite disappearance in double/triple mutants may reflect NO reduction being the rate-limiting step in denitrification here. It is also worth noting that the colorimetric nitrite assay used here actually measures total NO\textsuperscript{2−} and at least some of the NO present, which is converted to nitrite under assay conditions. This could obscure potentially larger differences in denitrification rates between the mutants. Interestingly, the present data clearly indicate that there must exist a yet undefined route for electrons to be delivered to NirK in Nel, in addition to those involving CcoP, α\textsubscript{5} and potentially α\textsubscript{1}.

**Expression of α\textsubscript{5} in N. gonorrhoeae supports NirK-dependent nitrite reduction**

From a basic overview of gene content, one would expect Nel and N. gonorrhoeae to have similar electron transport pathways to NirK. However, our results suggest significant redundancy in electron transport pathways to NirK in Nel or that the electron transport pathways operating in this species are distinctly different from those in N. gonorrhoeae. To test the function of α\textsubscript{5} as a putative electron donor to NirK, we expressed it in a gonococcal background. Here, Nel cycC was expressed ectopically in an N. gonorrhoeae strain carrying the truncated forms of

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**Fig. 4.** Nel c-type cytochrome mutant characterization and nitrite turnover during aerobic conditions.
A. Western blot using a polyclonal antibody made against CcoP shows reactivity against heme domains similar to the third heme domain of CcoP.
B. Heme staining of whole-cell extracts from Nel wild-type and mutant strains.
C. Growth of the wild type and mutants supplemented with 2.5 mM nitrite.
D. Concentration of nitrite remaining in the growth medium from cultures in C. The results shown are representative of three independent experiments.
c5 (c51x) and CcoP (CcoP2x). The latter background thus precluded endogenous NirK targeting electron transport pathways. Expression of c5 was verified by immunoblotting using CcoPB antibodies (Fig. 5A). Analysis of growth and nitrite reduction revealed that ectopic expression of c5 complements the defect in microaerobic growth and nitrite reduction in the gonococcal cycB1x ccoP2x double mutant strain (Fig. 5B and C). These results strongly suggest that c5 represents yet another direct pathway for electron donation to NirK.

Expression of a Ngo1043-c5 composite protein complements a defect in nitrite-dependent, microaerobic growth in N. gonorrhoeae

Given the activity demonstrated for c5 and its modular structural similarities to both Ngo1043 and the C-terminal heme domains of c5 and CcoP, we wished to assess the potential functional relatedness and constraints of the domains involved vis-à-vis electron transport to NirK. In particular, it was of interest to determine if a functional equivalent to c5 could be reconstituted by creating a hybrid protein consisting of the N-terminal domain of Ngo1043 fused to the C-terminal heme domain of c5. We constructed a translational fusion consisting of the first 93 amino acids of Ngo1043 plus 300 base pairs upstream promoter sequence and the conserved C-terminal heme domain of c5 (both template sequences were derived from our N. gonorrhoeae N400 background) (Fig. 6A). The ngo1043-cycB hybrid allele was then expressed ectopically in the c51x ccoP2x mutant background and expression of the fusion protein was confirmed by immunoblotting and heme staining (Fig. 5A lane 4 and Fig. 6B lane 4). Analysis of growth and nitrite reduction under microaerobic conditions showed that expression of the Ngo1043-c5 hybrid protein restored growth and nitrite reduction in the N. gonorrhoeae cycB1x ccoP2x background to a level comparable with that of the wild-type strain (Fig. 6C and D). This finding shows that the terminal heme domain of c5 is functionally equivalent to that found in c5 when expressed in the context of the amino-terminal lipoprotein domain of Ngo1043.

Purified c-type cytochrome heme domains from CcoP, c5 and c5 transfer electrons directly to NirK in vitro

Given the significant experimental evidence that the third heme domain of CcoP, and cytochromes c5 and c5 are part of electron transport pathways to NirK in Neisseria, we sought to test whether this electron transfer was direct. We purified cytochrome c5, each of its two soluble heme-containing domains [c51x and c52x], the soluble third heme domain of CcoP [CcoP2x] and the soluble heme domain of Nel c5. As controls, we expressed cytochromes c5, c5 and the soluble domain of cupredoxin lipid-modified azurin (Laz) from N. meningitidis. The proteins were expressed and purified, made reduced by treatment with sodium dithionite, and their oxidation by purified soluble NirK was followed by stopped flow kinetics, using the change in
The rate of electron transfer from holo-cytochrome \( c_5 \) to NirK was determined under different salt concentrations and across a range of pH, and two rates were calculated from the raw data by fitting the data to two exponentials (Fig. 8A–D). The two rates are presumed to arise from the intermolecular electron transfer from \( c_5^{(2)} \) to NirK and the intramolecular electron flow from \( c_5^{(1)} \) to \( c_5^{(2)} \) (with the measured quantity (increase in \( A_{402} \)) being the subsequent (much faster) transfer of electrons from \( c_5^{(2)} \) to NirK). This reasoning is based on the fast (intermolecular) rate being dependent on NirK, whereas the slow (intramolecular) rate is independent of \([\text{NirK}]\) (Fig. 8B). Interestingly, the two rates vary differently with pH (Fig. 8D), suggesting that the two domains of \( c_5 \) do not operate as a single solid body, but as two loosely associated domains. This, and the relatively low value of the second-order intermolecular rate constant for holo-\( c_5 \) compared with the
Fig. 7. Direct electron transfer between cytochrome c domains and NirK subunits in vitro. The change in absorbance of CcoP$_{(3)}$ was monitored for 1 s in a stopped flow experiment in the presence of NirK subunits. For each data point, data were fitted to exponentials from which the observed rate ($k_{obs}$) was determined. In all cases, the concentration of NirK subunits was kept in large excess over cytochrome in order to maintain pseudo-first-order conditions. (A) $k_{obs}$ was plotted against NirK, and the second-order rate constant ($k$) was calculated from the slopes of the resultant best fit lines. These plots and rate constants are shown for (B) CcoP$_{(3)}$, (C) c$_{5(1)}$, and (D) c$_{5(2)}$. (E) The impact of increasing salt on the second-order rate constant with c$_{5(2)}$ (filled triangles), CcoP$_{(3)}$ (open triangles), c$_{(2)}$ (open circles) and c$_{(4)}$ (filled circles). Laz showed no activity with NirK.

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c5(2) domain, indicates that the first domain of c5 and NirK may interact with an identical or overlapping surface of the second domain of c5. The loose association between the two heme domains of c5 [limited by the length of the low complexity region (LCR) connecting the two heme domains] may be important for electron transfer from the inner membrane across the periplasm to the outer membrane location of NirK.

**Distribution of cycC within the genus Neisseria**

To better understand the forces shaping the pathways for electron delivery to NirK, we examined the occurrences of cycB, cycC and ccoP in a broader sampling within the genus Neisseria. As shown in Fig. 9, cycB was found in all strains as was ccoP (in its di- or tri-heme encoding forms), whereas cycC was limited in its distribution to four species groups including the *N. cinerea*, *N. mucosa*, *N. polysaccharea* and *N. subflava* species groupings. The results for cycC distribution were in line with the phylogenetic clusters established using whole-genome sequence data. Moreover, cycC was variably detected in strains within the *N. polysaccharea* group. This may be consistent with other data supporting the idea that the *N. polysaccharea* group is in fact polyphyletic and probably includes more than one taxonomically distinct organism (Bennett et al., 2012). Together, the data indicate that cycC was likely lost in conjunction with the divergence of pathogenic *Neisseria* from commensals occurring over time.

**Evolutionary relationships among neisserial NirK-targeting c-type heme domains**

The gain and loss of c-type heme domains targeting NirK within the genus *Neisseria* provides a striking example of the modular evolution of proteins. To better understand the molecular processes behind these expansions and

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**Fig. 8.** Intramolecular and intermolecular electron transfer involving cytochrome c5. Oxidation of cytochrome c5 was monitored at 402 nm, and the rate fitted to a two exponential equation (y = y0 + ae^bt + ce^dt) in Sigmaplot. (A) Shows typical data. The impact on the fast rate (filled circles) and slow rate (open circles) is shown for changes in (B) NirK concentration, (C) [NaCl] ([NirK] = 192 μM) and (D) pH ([NirK] = 40 μM).
contractions in the redox chain, the structural relation-
ships between the relevant domains of \( \alpha_c \), \( \alpha_n \) and CcoP within single strains from a broad array of species was assessed (Table S1). For strains within the *N. gonorrhoeae*, *N. meningitidis* and *N. lactamica* species groups (all lacking \( \alpha_n \)), identities between the domains of \( \alpha_c \) and CcoP were comparable with those reported previ-
ously (averaging 74–75% identity) (Aspholm et al., 2010). Among the other species groups, three basic patterns were discernible. Strains within the *N. polysaccharea* and *N. cinerea* species groups formed one group in which the overall values were most comparable and in which values were highest for the \( \alpha_c \) versus \( \alpha_p \) percent identities (85%) and similarities (92%). A second cohesive group was comprised of strains within the *N. subflava* and *N. mucosa* species groups with the highest values seen for the CcoP versus \( \alpha_p \) percent identities (92%) and similarities (96%). An outlier to these two groupings was the *Nel* strain with highest values seen for the \( \alpha_c \) versus CcoP percent identities (93%) and similarities (98%). These three unique patterns of heme domain relatedness dove-
tail well with the phylogenetic relationships established previously (Fig. S1) (Marri et al., 2010; Bennett et al., 2012).

We previously proposed a model for the genesis of the unique neisserial tri-heme CcoP form entailing a non-
reciprocal recombination event involving the 3' ends of the \( \alpha_p \)-encoding (*cycB*) gene and a primordial *ccoP* gene.
encoding a di-heme CcoP (Aspholm et al., 2010). This model was supported by the relatively high levels of identity and similarity between the C-terminal heme domains of CcoP and c5 and the presence of the AlaSerPro-rich region between the second and third CcoP heme domains. Given the new information relating to c5 including the high degree of relatedness between its heme domain and that of CcoP (as seen particularly in the N. subflava and N. mucosa species groups) and its AlaSerPro-rich region, one cannot formally rule out a role for cycC in the primordial neofunctionalization of CcoP.

NirK from Neisseria weaveri with a natural C-terminal heme domain extension is sufficient to sustain nitrite reduction in N. gonorrhoeae

Many Betaproteobacteria including N. weaveri express NirK homologues with a covalent C-terminal c-type heme domain with a high degree of similarity to neisserial cytochrome c5/c6 (Fig. 10A and B). We speculate that NirK in these organisms might be able to receive electrons and reduce nitrite in the absence of the c5 and/or CcoP-assisted routes observed in N. gonorrhoeae. To test this hypothesis, we cloned N. weaveri nirK including upstream promoter sequences and introduced it into an ectopic site in an N. gonorrhoeae c5(1x) ccoP2x nirK triple mutant (Fig. 10C). Here, ectopic expression of NirK from N. weaveri in this background supported nitrite reduction during growth under microaerobic conditions (Fig. 10D and E).

Discussion

Here, we identified c5 as a novel lipoprotein c-type cytochrome, supporting NirK function that is disseminated among three species groups in the genus Neisseria (Fig. 9 and Fig. S1). As isolates within these three commensal species groups also carry the genes for c5 and CcoP, they possess three distinct cytochromes supporting NirK function. Therefore, within the genus, one sees species-related differences where NirK function is supported by either one c-type cytochrome in N. meningitidis, two in N. gonorrhoeae and related species groups or three in the species expressing c6 (Figs 9 and 11). In addition, it is clear that Nel must have at least one further source for electron transfer to NirK in addition to c5, CcoP and c6.

These findings raise obvious questions as to what factors have shaped the differential distribution of NirK-targeting cytochromes. In N. meningitidis, there is clear evidence that strains are under relaxed selective constraints with regard to NirK-mediated nitrite reduction, as many strains lack nirK altogether or carry nirK pseudogenes (Stefanelli et al., 2008). The reduction in cytochromes targeting NirK from two to one seen there (due to the single nucleotide polymorphism-associated truncation in CcoP) may simply be a part of relaxed selection for NirK function (Aspholm et al., 2010). The situation in species additionally carrying c5 may relate to differences in the denitrification pathway itself as many of the strains carry genes (nosR2DFYFLX encoding a functional nitrous oxide reductase (Nos) complex (Barth et al., 2009). Although the nosR2DFYFLX genes are present in N. meningitidis and N. gonorrhoeae, most of the genes are non-functional because of small deletions and other ORF-terminating point mutations (Barth et al., 2009). The retention of functional nos genes in most Neisseria species suggests that there is a selection pressure to maximize the size of the O2-independent electron sink in these species. This may reflect limited nitrite availability in their habitats. This assertion would both explain the requirement for more pathways (including cytochrome c5) for electron transport to nitrite reductase (to optimize the scavenging of a scarce, but important respiratory electron sink) and explain the poor growth in laboratory culture of Nel in the presence of high nitrite concentrations, as observed here. The loss of a functional nos cluster in N. meningitidis and N. gonorrhoeae may reflect the adaptation of these species to thrive in environments with a higher concentration of nitrite and NO, suiting their lifestyle as pathogens, in which they must deal with NO and its congeners, generated by the nitrosative burst from an alert innate immune response (Poole, 2005). It is relevant to note, however, that strains of N. lactamica possess an active complement of nos genes, but lack c5, indicating that the selection landscape with respect to denitrification is complex and presumably reflects multiple different microniches occupied by the Neisseria species.

A major topic here relates to which redox partners carry out direct electron transfer to nitrite reductases. Biochemical studies have implicated both azurins (of the cupredoxin family) and c-type heme cytochromes as electron donors to the CuNIR family members (Murphy et al., 2002; Koteishi et al., 2009; Nojiri et al., 2009). Neisseria express a lipoprotein form of azurin termed Laz, but it has been reported that it is dispensable for growth under anaerobic conditions (in the presence of nitrite) (Cannon, 1989). A high-resolution crystal structure of a CuNIR together with a c-type cytochrome demonstrated the potential direct transfer of electrons between these partners as well as the nature of the contact (Nojiri et al., 2009). The biochemical data here clearly demonstrate that the implicated c-type cytochrome domains of c5, CcoP and c6 each are capable of direct electron donation to NirK. In this work, we have not addressed directly the redox properties of the different cytochromes. However, the kinetic analysis indicates that although the forward rates of electron donation from each cytochrome to NirK is
similar, the difference in intercept values on plots of $K_{\text{obs}}$ against NirK indicates a variation in redox potential between $c_5$, CcoP(3) and $c_N$.

A striking finding of this work is that despite the number of proteins transferring electrons to canonical neisserial NirK, they all consist of highly related modular domains. These include not only the relevant cytochrome c domains sharing high levels of identity, but also the related LCR domains that map N-terminal to cytochrome domain. Although it is difficult to assign values of identity/similarity between LCRs because of their repetitiveness and tendency to degeneration, their overall character and sequence composition support the idea that they share a common genetic ancestry. Furthermore, all LCRs involved

Fig. 10. Ectopic expression of $N. \text{weaveri}$ nirK complements a defect in nitrite-dependent, microaerobic growth in $N. \text{gonorrhoeae}$. A. Domain structure and structural relatedness of $N. \text{weaveri}$ NirK containing a C-terminally fused $c$-type heme domain with $NeI$ NirK. Cu-oxidase domain in grey, low complexity regions (serine, proline, alanine rich) in green, cytochrome c domain in blue, and lipoprotein maturation signal in red.

B. Amino acid alignments of the C-terminal heme-domain of $N. \text{weaveri}$ NirK with that of $c_{2}$ from $N. \text{gonorrhoeae}$. Amino acids identities are shown in red and conservative amino acid substitutions are shown in green. Numbers refer to amino acid residues in the respective proteins.

C. Western blot with anti-NirK antibodies of samples of total cell extracts, from $N. \text{gonorhoeae}$ wild type (lane 1), $c_5$ ccoP2x (lane 2), $c_5$ ccoP2x ΔnirK (lane 3) and $c_5$ ccoP2x ΔnirK expressing nirK from $N. \text{weaveri}$ (lane 4).

D. Growth of the wild type and mutants under microaerobic conditions in cultures supplemented with 2.5 mM nitrite. Strains: wild type (VD300), $c_5$, ccoP2x, $c_5$ nirK and $c_5$ ccoP2x, nirK (gA::nirKNW).

E. Concentration of nitrite remaining in the growth medium from cultures in D. The results shown are representative of three independent experiments.
here share the functional property of bearing serine occupancy sites for O-linked glycans (Vik et al., 2009). Thus, the data support a paralogous relationship for the three heteromeric LCR-cytochrome c configurations documented here. In that context, the relationships between \( \alpha \) and Ngo1043 orthologues and their distribution are noteworthy. Ngo1043 and its orthologues (found in all Neisseria except for Nel) are relatively abundant lipoproteins originally characterized in N. meningitidis as potential vaccine candidates (Chen et al., 2009). The primary protein structure shows reduced complexity composed of primarily Ala, Lys and Glu with variable numbers of tandem heptapeptide EAVTEAK repeats at the C-terminus. The observation that the first 58 residues of unprocessed \( \alpha \) show between 67% and 72% identity to the equivalent sequence from Ngo1043 and its orthologues clearly establish a paralogous relationship. Based on the current data, however, it remains unclear which gene might be the ancestral form (i.e. whether \( \alpha \) arose by Ngo1043, acquiring the cytochrome \( c \) domain, or whether Ngo1043 arose by \( \alpha \) domain loss).

Our findings here differ from those of Hopper and colleagues (2013) who reported that mutants expressing \( \alpha \) lacking its C-terminal heme domain and CcoP with a missense mutation disrupting the functionality of its third heme domain retained the ability to reduce nitrite albeit at reduced levels. It remains to be determined whether this disparity relates to differences in methodologies or the strain backgrounds employed. Hopper and colleagues (2013) argue for a role for cytochrome \( c \) in electron donation to NirK. Interestingly, sequences of \( c \) show very high identities for the first 132 amino acids between N. gonorrhoeae and N. meningitidis strains, but there are diverse sequences C-terminal to this (Finn Erik Aas, Marina Aspholm, unpubl. data). In N. gonorrhoeae, there are two variants among gonococcal strains with distinct amino acid sequences consisting of 20–30 amino acids, caused by a base insertion/deletion between strains. In N. meningitidis, cytochrome \( c \) (the meningococcal \( c \) homologue) terminates after 133 amino acids. The C-terminal sequence may be crucial for the role of \( c \) in electron donation to NirK in a subset of N. gonorrhoeae strains.

In a further example of the diversity of electron donors to NirK within the genus Neisseria, N. weaveri possess a recently identified class of copper-containing NIRs that carry \( c \)-type mono-heme domains translationally fused to the carboxy-termini of the nitrite reductase domain. This domain organization is typical of many other soluble CuNIRs of the NirK family (Ellis et al., 2007). The fused heme domains there demonstrate strong identity to the soluble cytochrome \( c \) homologues present in most Neisseria species, suggesting that this protein has evolved by gene fission. In that respect, it is interesting to note that the cccA gene-encoding cytochrome \( c \) is located 200 base pairs downstream of the nirK ORF in Nel. It is also noteworthy that among neisserial species carrying such fused forms of NirK, (including N. wadsworthii, N. shayegani and N. weaveri), all express a di-heme form of CcoP. In contrast to the situation in N. meningitidis, these species carry an OmpA-like domain (Pfam PF00691) at their C-termini in place of the third heme domain. Thus, the extended heme domain configuration of these NirK forms may obviate or relax the phenotypic benefits to be had from direct electron donation from CcoP. Finally, the high-resolution structure solved for this class of NirK found in Ralstonia pickettii provided fine detail of the interface between nitrite reductase domain and the tethered cytochrome \( c \) domain allowing self-electron transfer (Antonyuk et al., 2013). To our knowledge, expression of N. weaveri NirK in N. gonorrhoeae is the first demonstration of in vivo functionality for this class of NIR.

In summary, this work reveals the remarkable potential of modular-based gene duplication and expansion in shaping the pathways of direct electron transfer to nitrite reductase. It also further emphasizes the importance of microaerobic denitrification to species within the genus Neisseria and how differential gene loss and retention can be connected to significant macroevolutionary shifts. Finally, it underscores the power of combining genetics, genus-wide comparative genomic and biochemistry to unravel the mechanisms shaping complex molecular metabolic networks.
Experimental procedures

Bacterial strains, plasmids and culture conditions

The gonococcal strains used in this study were derived from the VD300 background, and the strains of Neisseria elongata subspecies glycolytica (Nel) were derived from the ATCC29315 background. Strains used are listed in Table 1. Gene bank accession numbers refer to N. gonorrhoeae strain FA1090 and Nel strain ATCC2315 sequences where appropriate (Table S1). Neisseria meningitidis constructs were all derived from strain MC58, and N. weaveri nirK was derived from strain 00714. Strains were grown in conventional GC medium or on GC medium plates (Freitag et al., 1995), except that Thiotone E peptone was replaced by Proteose Peptone No. 3 (Difco). Aerobic cultures were carried out in 100 ml of broth, supplemented with 10 mM NaHCO₃, in 500 ml of nunc flasks with filter cap shaken at 190 r.p.m. (SARSTEDT, Nümbrecht, D). Microaerobic culture was 500 ml of nunc flasks with filter cap shaken at 190 r.p.m. (Rock et al., 2005). Where appropriate, cultures were supplemented with 2.5 mM or 5 mM NaNO₃. Growth was monitored by measuring the optical density at 600 nm (OD₆₀₀) in a WPA biowave CO 8000 Cell Density Meter. Nitrite concentrations in culture media were measured by a colorimetric assay as previously detailed (Deeudom et al., 2008).

Escherichia coli strains DH5α or Top10 (Invitrogen) were used for plasmid propagation and cloning experiments and were grown in Luria–Bertani media (LB). When needed, antibiotics were used in the following concentrations for Neisseria: kanamycin (50 μg ml⁻¹), chloramphenicol (30 μg ml⁻¹), tetracyclin (20 μg ml⁻¹) and erythromycin (8 μg ml⁻¹); for E. coli: kanamycin (50 μg ml⁻¹), chloramphenicol (30 μg ml⁻¹), tetracycline (15 μg ml⁻¹) and erythromycin (300 μg ml⁻¹). pP6 is a shuttle mutagenesis/integration vector that carries two gonococcal DNA uptake sequences (Wolfgang et al., 2000). p2/16/1 is a derivative of pUP6 carrying the ermC gene flanked by gonococcal iga gene sequences (Wolfgang et al., 2000), allowing integration of plasmid inserts into the iga locus of the N. gonorrhoeae chromosome. Isolation and purification of plasmid DNA was performed using QiAprep Spin Miniprep columns according to the manufacturer’s specifications (Qiagen, Chatsworth, CA, USA).

Oligonucleotides

The oligonucleotides used in his work are listed in Table S2.

Construction of an N. elongata subspecies glycolytica strain expressing C-terminally His-tagged cN

The 6His-tag was introduced into the C-terminus of cN from Nel by polymerase chain reaction (PCR)-based splicing-by-overlap extension (SOE) reactions. Each pair of PCR fragments containing the mutation was created using primers cycCF4 in combination with cycCR2 and cycCF2 in combination with cycCR3. The resulting overlapping PCR fragments were spliced together using primers cycCF4 and cycCR3, which inserted the domain-terminal 6His-tag and an EcoRV restriction site. The resulting PCR fragment was cloned into pTOPO (Invitrogen). Subsequently, the cat gene from plasmid pACYC184 (New England BioLabs) was released with the restriction enzymes BsrBI and Hincll and cloned into the EcoRV site, located after the stop codon in cycC, which generated the plasmid pTOPO cyc6His (cat). The cycC fragment linked to the cat cassette was amplified using primer pairs cycCF4 and cycCR3; the resulting PCR products were used to genetically transform Nel strains as previously described for N. gonorrhoeae (Aas et al., 2002), and the transformants were selected on plates containing chloramphenicol.

Table 1. Strains used in this study.

| Strain name | Parental strain | Relevant genotype | Reference |
|-------------|-----------------|-------------------|-----------|
| KS944* | N. elongata subsp. glycolytica 6171/75 | Type strain (wild type) | Bovre and Holten, 1970 |
| KS945 | KS944 | ΔpgIC::kan | This study |
| KS951 | KS944 | cycC-HIS::cat | This study |
| KS952 | KS950 | ΔpgIC::kan, cycC-HIS::cat | This study |
| KS953 | KS944 | ΔnirK::kan | This study |
| KS954 | KS944 | cycC::cat | This study |
| KS955 | KS944 | cycB::tet | This study |
| KS956 | KS944 | ccoP2::kan | This study |
| KS957 | KS955 | cycB::tet, ccoP2::kan | This study |
| KS958 | KS957 | cycB::tet, ccoP2::kan, cycC::cat | This study |
| N. gonorrhoeae VD300 strains | | | |
| VD300 | MS11 (N. gonorrhoeae) | opa derivative of MS11 | Koomey and Falkow, 1987 |
| KS337 | KS335 | cycB::tet, ccoP4 | Aspholm and colleagues, 2010 |
| KS959 | KS337 | cycB::tet, ccoP4, iga::cycC | This study |
| KS960 | KS337 | cycB::tet, ccoP4, iga::ngo1043-cycB | This study |
| KS961 | KS337 | cycB::tet, ccoP4, ΔnirK::kan | This study |
| KS962 | KS961 | cycB::tet, ccoP4, ΔnirK::kan, iga::nirKΔ | This study |

a. Wild-type isolate of Neisseria elongata subsp. glycolytica 6171/75 (= ATCC 29315 = CCUG 6508 A = CIP 82.85 = NCTC 11050).
b. A tetracycline resistance gene was introduced into the central LCR region of cycB such that a truncated mono-heme version of cN is expressed.
c. The nirK gene from Neisseria weaveri 3458 was introduced into the iga locus of N. gonorrhoeae strain KS961.

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Subsection 1: 

**Construction of an N. elongata subspecies glycolytica strain expressing di-heme CcoP and mono-heme c5**

The premature stop codon was introduced into *Nel ccoP* by SOEing PCR. Each pair of PCR fragments containing the mutation was created using primers NelccoPF1 in combination with NelcoPLCR and, NelcoPLCRF in combination with NelccoPR2. The overlapping PCR fragments were spliced together using primers NelccoPF1 and NelccoPR2, which inserted a novel stop codon followed by an EcoRV restriction site into the site encoding the LCR located after the second heme domain. The resulting PCR fragments were digested using the unique, flanking BamH1 and EcoR1 sites and cloned into the polylinker of pUP6 cut with the same restriction enzymes. Direct DNA sequencing of plasmid DNA was done to verify the introduction of the stop codon and the absence of any other alterations. A kanamycin resistance gene cassette was cloned into the EcoRV site, which generated the plasmid pUP6 *Nel ccoP2x::kan*. The *ccoP* fragment containing the kanamycin resistance marker was amplified using primer pairs NelccoPF1 and NelccoPR2. The resulting PCR products were used to genetically transform *Nel* strains, and the transformants were selected on plates containing kanamycin.

The gene-encoding *Nel* cytochrome *c5* (*cycB*) was amplified with primers Nelc5F1 in combination with Nelc5R1. The resulting PCR fragments were digested using the unique, flanking BamH1 and EcoR1 sites and cloned into the polylinker of pUP6 cut with the same restriction enzymes. Direct DNA sequencing of plasmid DNA was done to verify the absence of any sequence alterations. The plasmid containing the *cycB* insertion was cleaved with EcoNi, which recognizes a unique site located centrally within the *cycB* gene. The ends of the linearized plasmid were rendered blunt with Klenow fragment, and the product was ligated with the tetraacyclin resistance gene derived from pTOPOc5::tet (Deedom et al., 2008). The *cycB* fragment containing the kanamycin resistance marker was amplified using primer pairs Nelc5F1 and Nelc5R1, and purified PCR products were used to genetically transform *Nel* strains. Correct transformants were screened by PCR, and expression of the di-heme form of *ccoP* and the mono-heme form of *c5* was verified by heme staining of SDS-PAGE separated whole-cell extracts.

Subsection 2: 

**Construction of null mutants in cycC and nirK in N. elongata subspecies glycolytica**

To completely inactivate *cycC* in *Nel*, we made a *cat* gene insertion mutant. PCR-based SOE-ing was used to introduce a unique EcoRV site 51 base pairs downstream of the start codon in *c5*. The primers FE2383 and FE2378 were used to amplify the N-terminal part of *cycC*, and about 500 base pairs of upstream sequence and the primers FE2384 and FE2377 were used to amplify an overlapping fragment encompassing about 150 base pairs of sequence downstream of *cycC*. The overlapping PCR fragments were spliced together using the primers FE2383 and FE2384 and the resulting PCR product was digested with SacI and cloned into a unique SacI site in the plasmid p2/16/1. Subsequently, the *cat* gene from plasmid pACYC184 was cloned into the unique EcoRV site in *cycC*. The resulting plasmid pEF596 was used as a PCR template to amplify *cycC* with the *cat* gene insertion and flanking sequences using the primers FE2383 and FE2384. The purified PCR product was used to transform *Nel*, and transformants were selected on chloramphenicol plates. Insertion of the *cat* gene was verified by PCR and immunoblotting of whole-cell extracts with the CcoPB antibodies confirmed the absence of the *c5* protein.

To obtain a complete deletion of the gene-encoding NirK in *Nel*, the primers ΔNelnirKR1 and ΔNelnirKF2 and ΔNelnirK2 were used to amplify a 561 base pairs fragment of genomic DNA upstream of the *nirK* gene, and the primers ΔNelnirKF1 and ΔNelnirKR2 were used to amplify a 535 base pairs fragment of genomic DNA downstream of the *nirK* gene. The resulting PCR products contained regions of homology at the 3’ end of the upstream fragment and at the 5’ end of the downstream fragment such that they could be spliced together by PCR-based SOEing using primers ΔNelnirKF1 and ΔNelnirKR2, leaving out the entire *nirK* gene. The resulting PCR fragments were digested using the flanking BamH1 and EcoR1 sites and cloned into the polylinker of the plasmid pUP6 (Wolfgang et al., 1999) cut with the same restriction enzymes. A kanamycin resistance cassette was cloned into the unique EcoRV site located centrally in the cloned fragment, which generated the plasmid pUP6 ΔNelnirK::kan. A purified PCR product, generated using primers ΔNelnirKF1 and ΔNelnirKR2 and pUP6 ΔNelnirK::kan as a template, was used to genetically transform *Nel*, and transformants were screened on plates containing kanamycin. The absence of *nirK* was verified by PCR and immunoblotting of whole-cell extracts using polyclonal anti-NirK antibodies.

Subsection 3: 

**Recombinant expression of c5, Ngo1043-c5, hybrid protein and N. weaveri NirK in N. gonorrhoeae**

The gene encoding a His-tagged form of *c5* and 500 base pair upstream sequence was constructed by SOEing PCR reactions. Each pair of PCR fragments containing the His-tag was created using primers NelcycCF1 in combination with NelcycCR1 and cycC6HisF in combination with cycC2R2. The resulting PCR products containing regions of homology at the 3’ end of the upstream fragment and at the 5’ end of the downstream fragment were spliced together by PCR-based SOEing reactions using primers cycCF1 and cycC2R2.

The ngo1043-cycB hybrid allele was constructed by PCR-based SOEing such that a region of ngo1043 encompassing about 300 base pairs of upstream promoter sequence and the first 279 base pairs of the coding region was fused to a 246 base pair region of cycB encoding the C-terminal heme domain of *c5*. The gene fusion was constructed so that the total length of the expressed hybrid protein was 175 amino acids. The ngo1043 part of the hybrid allele, including about 300 base pairs upstream sequence, was amplified by PCR using the flanking primer Ngo1043F1 in combination with the primer Ngo1043RcycB. The region encoding the second heme domain of *c5* was amplified by PCR using the flanking primer cycBF Ngo1043 in combination with the primer cycBR1. The PCR products containing regions of homology at the 3’ end of the upstream (ngo1043) fragment and at the
5’ end of the downstream (cycB) fragment were spliced together by SOEing PCR reactions using the primers Ngo1043F1 and cycBR1. For ectopic expression in N. gonorrhoeae the nirK gene from N. weaveri, including about 400 base pairs of upstream sequence, was PCR amplified from N. weaveri 3458 by using the primers FE2392 and E2394. Ectopic expression of the His-tagged c0, the ngo1043-c0 hybrid allele and the nirK from N. weaveri was performed by digesting the resulting PCR fragments with SacI and cloning them into a unique SacI restriction site in plasmid p2/16/1, allowing integration into the iga locus of the gonococcal chromosome. DNA sequencing of plasmid DNA was done to verify the absence of any unwanted sequence alterations. The plasmids containing the correct inserts were then used to transform gonococcal strains, and transformants were selected for growth on GC agar plates containing erythromycin. Insertion of constructs into the iga locus of N. gonorrhoeae was verified by PCR and immunoblotting of whole-cell extracts using anti-NirK antibodies for N. weaveri NirK, penta-HIS antibodies for His-tagged c0, and antibodies raised against recombinant gonococcal CcoP protein for the Ngo1043-c0 hybrid protein.

Construction of a nirK null mutation in N. gonorrhoeae
The primers NgonirK1F and NgonirK1R1 were used to amplify a 500 base pair fragment of genomic DNA upstream of nirK; and the primers NgonirK3 and NgonirK3R were used to amplify a 450 base pair fragment of genomic DNA downstream of the nirK gene. The PCR products containing regions of homology at the 3’ end of the upstream fragment and at the 5’ end of the downstream fragment were spliced together using primers ΔNgonirK1F and NgonirK3R. The resulting PCR fragments were digested using the flanking BamHI and EcoRI sites and cloned into the polylinker of the plasmid pUP6 cut with the same restriction enzymes. Subsequently, a kanamycin resistance cassette was cloned into the unique EcoRV site, which generated the plasmid pUP6 Ngo nirK::kan. The plasmid was then used to transform gonococcal strains, and transformants were selected for growth on GC agar plates containing kanamycin; mutants containing the correct insert were verified by PCR. Immunoblotting of whole-cell extracts using the polyclonal anti-NirK antibody verified ablation of NirK expression.

Cloning of redox proteins from N. meningitidis and N. elongata subspecies glycolytica for expression in E. coli
Genes encoding c-type cytochrome proteins and their individual domains and copper-containing redox proteins Laz and NirK were expressed heterologously in E. coli using pET vectors. The genes encoding cytochromes c5, c2 and c3 were amplified and cloned into pETYSBLIC3C by ligation-independent cloning according to the published protocol (Bonsor et al., 2006). The primers c5F and c5R were used to amplify c5, the primers c2F and c2R were used to amplify c2, and the primers c4F and c4R were used to amplify c4. Hexa-His tags were introduced to the C-terminal ends of the protein-coding regions of cytochromes c5 and c2 by inverse PCR using primers c5HisF and c5HisR for c5 and c2HisF and c2HisR for c2. The DNA sequences encoding the third heme domain of CcoP [CcoP(3)] from N. gonorrhoeae, the first heme domain of c0 [c0(1)], the second heme domain of c0 [c0(2)], the heme domain from Nel cytochrome c0, the soluble domain of Laz and the soluble domain of NirK were cloned into pET22b downstream of the pelB leader sequence to allow their expression as soluble proteins and targeted to the periplasm. Restriction enzyme sites were incorporated into primers for amplification of the regions concerned, and these were used for conventional cloning into the pET vector. The region encoding CcoP(3) was amplified with ccoPFor and ccoPRev. The region encoding c0(1) was amplified with 1°c5For and 1°c5Rev and the region encoding c0(2) was amplified with 2°c5For and 2°c5Rev. cycC was amplified with NEFor and NERev. The gene-encoding Laz was amplified with LazFor and LazRev. NirK was amplified with AniAFor and AniARev.

Protein expression and purification
pET vectors containing the genes of interest were transformed into E. coli BL21(T7DE3). For expression of c-type cytochromes, plasmid pST2 [containing the E. coli cytochrome c maturation genes, under a constitutive promoter (Turner et al., 2003)] was also transformed into the BL21(T7DE3) expression strain. Protein expression was induced by culturing cells in autoinduction medium (containing 0.5 mM CuCl2 for expression of Laz and NirK) (Studier, 2005). For protein purification, 1 l of cultures was harvested by centrifugation at 5000 g for 10 min at 4°C. Pellets were resuspended in 50 mM Tris (pH 7.5) and sonicated with 6 × 10 s bursts using a Misonix 3000 sonicator. The resultant suspension was centrifuged at 20 000 g for 40 min and the supernatant taken for further purification. His-tagged recombinant proteins [CcoP(3), c5, c2, c0(1), c0(2), an, and c2] were purified using pre-packed HiTrap HP columns (GE Healthcare, UK). Columns were equilibrated with five column volumes of 40 mM imidazole, 50 mM Tris/HCl (pH 7.5) (buffer A). Cell-free extract was applied to the column, that was then washed with buffer A. His-tagged proteins were eluted with 500 mM imidazole, 50 mM Tris/HCl (pH 7.5) by a step gradient. Laz and NirK were purified by anion exchange chromatography using a column containing 40 ml of bed volume Diethylaminoethyl (DEAE) sepharose-CL6B (Amersham Biosciences, Sweden) equilibrated with 50 mM Tris/HCl (pH 7.5). Proteins were eluted with a gradient from 0 to 500 mM NaCl in 100 ml of 50 mM Tris/HCl (pH 7.5). Cytochrome c5 was purified by cation exchange chromatography using a carboxymethyl (CM) sepharose fast flow resin, under identical conditions as described above for anion exchange. Further purification of NirK, Laz and c5 was achieved by gel filtration using a column containing 150 ml of bed volume of Sephacryl S100 (Sigma Aldrich, UK) equilibrated with 50 mM Tris/HCl (pH 7.5). Protein purity was checked by SDS-PAGE. Purified proteins were exchanged into required buffers using pre-packed PD-10 desalting columns (GE healthcare, UK) according to the manufacturer’s instructions. Protein concentration was assessed using the Bradford assay (Biorad) for 16
Measuring kinetics of electron transfer

The kinetics of electron transfer between redox proteins was followed by pre-steady-state kinetics using an SX20 stopped-flow spectrometer (Applied Photophysics, UK). Electron donor proteins were reduced with dithionite and excess dithionite removed on a desalting column. Oxidized electron acceptors were oxidized as prepared. Experiments were carried out in 50 mM Tris/HCl (pH 7.5) with the cytochrome (or Laz) at a concentration of 7.5 μM except where otherwise stated. Potential redox partners were mixed rapidly (dead-time c. 3 ms), monitored at an appropriate wavelength for measuring a change in cytochrome/cupredoxin spectrum, and 1000 data points were collected in a 1 s experiment. Data for each experimental condition were collected in triplicate. Rates of electron transfer were calculated by fitting data to single or double exponential functions using the SIGMAPLOT software.

SDS-PAGE and immunoblotting and the detection of c-type cytochromes

Whole-cell lysates for immunoblotting were made from equal numbers of cells grown over night on GC plates. Procedures for SDS-PAGE and immunoblotting are described in Freitag et al. (2007). All primary antibodies used to detect cccO, cccP and cα is a polyclonal rabbit antibody made against recombinant gonococcal Ccp protein (lacking the first 88 N-terminal aminoacids) overexpressed in E. coli and purified by standard biochemical methods (GenScript). C-terminally His-tagged proteins were detected using penta-histidine (His) antibodies (Qiagen) and NirK was detected using polyclonal rabbit antibodies (Rock et al., 2007). All primary antibodies were used at 1:1000 dilutions. The heme-staining procedure for detecting c-type cytochromes in SDS-PAGE separated whole-cell extracts was done as described previously (Aspholm et al., 2010).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Evolutionary relationships among Neisseria based on concatenated sequences from 246 genes. The evolutionary history was inferred using the neighbour-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. ‘T’ denotes type strain. This figure serves as the template for the data shown in Fig. 9 [used with permission (Bennett et al., 2012)].

Table S1. Comparison of the C-terminal heme domains of $\delta_h$, CcoP and $\delta_n$.

Table S2. List of primers used in this work.