Mutational and biochemical analysis of cytochrome *c'*, a nitric oxide-binding lipoprotein important for adaptation of *Neisseria gonorrhoeae* to oxygen-limited growth

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Neisseria gonorrhoeae is a prolific source of *c*-type cytochromes. Five of the constitutively expressed cytochromes are predicted, based on *in silico* analysis of the *N. gonorrhoeae* genome, to be components of the cytochrome *bc*₁ complex, cytochrome *c* oxidase *cbb*₃ or periplasmic cytochromes involved in electron transfer reactions typical of a bacterium with a microaerobic physiology. Cytochrome *c* peroxidase was previously shown to be a lipoprotein expressed only during oxygen-limited growth. The final *c*-type cytochrome, cytochrome *c'*, similar to cytochrome *c* peroxidase, includes a lipobox required for targeting to the outer membrane. Maturation of cytochrome *c'* was partially inhibited by globomycin, an antibiotic that specifically inhibits signal peptidase II, resulting in the accumulation of the prolipoprotein in the cytoplasmic membrane. Disruption of the gonococcal *cycP* gene resulted in an extended lag phase during microaerobic growth in the presence but not in the absence of nitrite, suggesting

that cytochrome *c'* protects the bacteria from NO generated by nitrite reduction during adaptation to oxygen-limited growth. The cytochrome *c'* gene was overexpressed in *Escherichia coli* and recombinant cytochrome *c'* was shown to be targeted to the outer membrane. Spectroscopic evidence is presented showing that gonococcal cytochrome *c'* is similar to previously characterized cytochrome *c'* proteins and that it binds NO *in vitro*. The demonstration that two of the seven gonococcal *c*-type cytochromes fulfil specialized functions and are outer membrane lipoproteins suggests that the localization of these lipoproteins close to the bacterial surface provides effective protection against external assaults from reactive oxygen and reactive nitrogen species.

Key words: cytochrome *c'*, cytochrome *c* peroxidase, gonococci, microaerobic growth, *Neisseria gonorrhoeae*, NO.

INTRODUCTION

Neisseria gonorrhoeae is an obligate human pathogen that must be capable of surviving under different conditions encountered in the body during the disease process and chemical assault from the immune system. Such conditions include changes in oxygen availability. Phagocytic cells are one of the first lines of defence encountered by an invading pathogen. Reactive oxygen species are produced by the respiratory burst and NO is generated by the inducible NO synthase [1]. In combination, superoxide and NO form peroxynitrite that is extremely toxic and damages lipids, DNA and proteins [2–4]. Early studies showed that some internalized gonococci could survive in human polymorphonuclear phagocytes [5,6].

Gonococci are a prolific source of *c*-type cytochromes [7], and multiple proteins with covalently attached haem have been detected on SDS/polyacrylamide gels [7–9]. They can grow either aerobically or anaerobically in the presence of nitrite, using glucose, pyruvate or lactate as the primary source of carbon and energy. Only two types of electron-transfer chains appear to be used. During aerobic growth, oxygen is reduced by a *cbb*₃-type cytochrome oxidase. During oxygen-limited or anaerobic growth, nitrite is reduced by NO to N₂O, catalysed by the copper-containing nitrite reductase, AniA and the NO reductase NorB [8,10–13]. Antibodies to AniA were detected in the serum of patients with complicated and uncomplicated gonorrhoea, indicating that oxygen-limited growth occurs during the disease process [14]. Furthermore, gonococcal pelvic inflammatory dis-

ease is often a mixed infection with obligate anaerobes [15,16]. Clearly, the gonococcus must be capable of adapting from aerobic to oxygen-limited growth conditions.

We recently reported the characterization of the 45 kDa CPP (cytochrome *c* peroxidase) lipoprotein that is induced during oxygen-limited growth and could be important in protecting the bacteria from reactive oxygen species [9]. Other defence mechanisms that protect gonococci against oxidative and nitrosative stress include catalase and the NO reductase NorB [17,18]. There is also a homologue of the yeast Sco protein, which is required for the synthesis of cytochrome oxidase, but the neisserial homologue provides protection against oxidative stress [19].

The expression of AniA is completely dependent on the FNR protein, the oxygen-sensing transcription regulator, and up-regulated by NarP [8]. However, the expression of the NO reductase NorB is independent of FNR and NarP, but is up-regulated in response to NO [18]. In the closely related bacterium *Neisseria meningitidis*, NorB and, to some extent, cytochrome *c'* are capable of reducing the toxic effect of NO [20]. In this paper, we present results of an *in silico* analysis of the *c*-type cytochromes predicted to be encoded in the gonococcal genome, which revealed that gonococcal cytochrome *c'* includes, at residues 16–19 in the predicted translation product of *cycP*, the LSAC motif typical of a lipoprotein. The effects of mutagenesis of the cytochrome *c'* gene on the ability of gonococci to adapt from aerobic to microaerobic growth and the biochemical characterization of recombinant cytochrome *c'* expressed in *Escherichia coli* are reported here.

Abbreviations used: CCP, cytochrome *c* peroxidase; IPTG, isopropyl β-D-thiogalactoside.

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Table 1 Bacteria strains used in the present study

Strain	Genotype	Source
<i>E. coli</i> JM109	<i>recA1, endA1, gyrA96, thi, hsdR17</i> (r_k^- , m_k^+), <i>relA1, supE44</i> , Δ (<i>lac-proAB</i>), [<i>F'</i> , <i>traD36, proAB, lacI^q ZΔM15</i>]	Promega (Chilworth, Southampton, U.K.)
<i>E. coli</i> BL21(λ DE3)	<i>F-ompT gal[dcm][lon]hsds_B</i> (r_b - m_b -; an <i>E. coli</i> B strain) with DE3, a λ prophage carrying the <i>T7</i> RNA polymerase gene	Novagen (Madison, WI, U.S.A.)
Epicurean coli TM XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [<i>F'</i> <i>proAB lacI^q ZΔM15 Tn10</i> (<i>Tet^r</i>)]	Stratagene
<i>N. gonorrhoeae</i> F62	Parental strain	Strain F62 transformed with linear <i>ermC</i> fragment from pST503
<i>N. gonorrhoeae</i> JCGC214	F62 <i>cycP::ermC</i>	

Table 2 Oligonucleotides used in the present study

Primer	Sequence (5'-3')
ST_CycPFP3	GGTGGTCGGATCCGGATGAAACCCAAATCAGCCTTGCCG
ST_CycP RP1	CTGCCCTACGGTTTGAACAAATCG
ST_CycPRP2	GGTGGTCGGATCCGGATGTTATTCGGCGCGCGG
ST_ERYFP2	CCGGAATTCAGAGTGTGTGATAGTGCAG
ST_ERYRP2	CCGGAATTCGATACAAATCCCGTAGG
ST_QC3	GCCTACGGCGAATTCGGCGCAAGCTG
ST_QC4	CAGCTTGCGCCGAATTCGCCGTAGGC

Table 3 Plasmids used in the present study

Plasmid	Genotype	Source
pGEM [®] T-vector	Commercial vector for cloning PCR products	Promega
pET-11c	Commercial overexpression vector, expression is induced by IPTG	Novagen
pMGC20	Gonococcal shuttle vector and the source of <i>ermC</i>	[52]
pST2	<i>CcmA-H</i> NcoI-Sall fragment cloned into pACYC184	[9]
pST205	<i>T7</i> promoter and <i>T7</i> tag (Novagen) fused to the first codon of gonococcal <i>cycP</i> in pET-11c	This work
pST501	<i>cycP</i> coding region and 1 kb of 3'-DNA cloned into pGEM [®] T vector	This work
pST502	pST501 with an EcoRI site engineered at the 3'-end of <i>cycP</i>	This work
pST503	<i>ermC</i> ligated into EcoRI site of pST502	This work

MATERIALS AND METHODS

Bacterial strains, plasmids and oligonucleotides

The *N. gonorrhoeae* and *E. coli* and their relevant genotypes and method of construction or source are listed in Table 1. Oligonucleotides used in the construction of plasmids are listed in Table 2. Plasmids used in the present study are listed in Table 3.

Construction of an *N. gonorrhoeae* cytochrome *c'* mutant strain

To obtain the cytochrome *c'* mutant, the gonococcal *cycP* coding region and 1 kb of downstream DNA were amplified by PCR from *N. gonorrhoeae* strain F62 chromosomal DNA using the primers ST_CycPFP3 and ST_CycPRP1. The plasmid pST501 was constructed by ligating the PCR fragment into pGEM[®] T-vector. An EcoRI site was introduced just upstream of the 3'-haem attachment site using a Quik Change[®] site-directed mutagenesis kit (Stratagene, Cambridge, U.K.), ST_QC3 and ST_QC4 primers and pST501 DNA as the template. This construct was named pST502. The *ermC* gene was amplified from pMGC20 with ST_ERYFP2 and ST_ERYRP2, which introduced EcoRI sites at each end. The *ermC* gene was ligated into the EcoRI site of

pST502 to create the construct pST503. Naturally competent, pilated *N. gonorrhoeae* F62 were then transformed with a linear DNA fragment amplified from pST503 using primers ST_CycPFP3 and ST_CycPRP1. The DNA fragment contained the *ermC* gene flanked by DNA homologous with the coding region of the gene, downstream non-coding DNA and also the gonococcal uptake sequence. Erythromycin-resistant colonies were screened for the disruption of the *cycP* gene by PCR and sequencing.

Construction of the cytochrome *c'* overexpression plasmid

To construct pST205, the *cycP* gene was amplified by PCR from the F62 gonococcal chromosome. The upstream primer ST_CycPFP3 introduced a BamHI site at the 5'-end of *cycP*. The downstream primer ST_CycPRP2 introduced a BamHI site at the 3'-end of *cycP*. The 500 bp fragment, which included the ATG start codon for *cycP*, was ligated into BamHI-digested pET-11c. The construct was checked by sequencing using primers that annealed to pET-11c.

Cytochrome *c'* expression and location

The *E. coli* strain BL21(λ DE3) pST205 pST2 was grown aerobically in Lennox broth and expression of cytochrome *c'* was induced with 0.5 mM IPTG (isopropyl β -D-thiogalactoside). Ampicillin and chloramphenicol were used at concentrations of 100 and 50 μ g/ml respectively. Globomycin (a gift from Sankyo Labo Services, Tokyo, Japan) is highly toxic, but preliminary experiments established that it could be used at concentrations of 10 and 20 μ g/ml in cultures of *N. gonorrhoeae* and *E. coli* respectively. For optimal expression of cytochrome *c'*, the strain BL21(λ DE3) pST205 pST2 was grown to stationary phase in Lennox broth without the addition of IPTG. *N. gonorrhoeae* strains F62 and JCGC214 were grown in GC (gonococcal) broth under the oxygen-limiting growth conditions described previously [8]. Sodium nitrite was added at a concentration of up to 3 mM.

Bacteria were harvested and washed and periplasm was prepared [21]. Whole cells or sphaeroplasts were broken by passage through a French pressure cell at 70 MPa. Unbroken cells and inclusion bodies were removed by low-speed centrifugation. Membranes and soluble fractions were separated by centrifugation at 150 000 *g* and 4 °C. Sucrose density gradients were used to separate inner and outer membranes using an adaptation of previously published procedures [22,23]. A step gradient was prepared in a 12 ml tube consisting of 54, 51, 45 and 36 % (w/v) sucrose solutions. Each layer was frozen in liquid nitrogen before the next layer was added. The gradient was allowed to thaw slowly. Concentrated membranes were prepared from *E. coli* or *N. gonorrhoeae* and treated with 10 μ g/ml RNase, 10 μ g/ml DNase and 5 mM MgCl₂ for 1 h at 37 °C before loading on to the top of the sucrose gradient. Membranes were separated by centrifugation for 48 h at 100 000 *g* at 4 °C.

Table 4 The *c*-type cytochromes of *N. gonorrhoeae*

Gene	Cytochrome	Predicted mass (kDa)	Putative function	N-terminal amino acid sequence
<i>ccoP</i>	CcoP	48	Subunit III cytochrome <i>c</i> oxidase (<i>cbb</i> ₃ type)	MNATSQFTSNFVNIYIAIVLLSFIALAWL
<i>ccp</i>	CCP	42	CCP	MSFKRLRYLASVLALSSLL LAACGG QKEKSAAG
<i>petC</i>	Cytochrome <i>c</i> ₁	30	Cytochrome <i>c</i> of <i>bc</i> ₁ complex	MRVEMKQAMKNWFAALLSAVPMMSAFAASG
<i>cycB</i>	Cytochrome <i>c</i> ₅	29	Putative electron transport	MKQLRDNKAQGSALFTLVSGIVIVIAVLYF
<i>ccoO</i>	CcoO	23	Subunit II cytochrome <i>c</i> oxidase (<i>cbb</i> ₃ type)	MKLQQLAEKIGVLIVFTLLVSVGLLIEV
<i>cycA</i>	Cytochrome <i>c</i> ₄	22	Putative electron transport	MRRLTLAFVLAAGAVSASPADVEKGKQV
<i>cycP</i>	Cytochrome <i>c'</i>	16	Binds NO	MKTQISLAATALALF LSACG NGGAPAKPGK

Analytical techniques

The protein concentration of cell fractions was determined using the Folin method [24]. Proteins were resolved by Tris/Tricine SDS/PAGE using a 15 % (w/v) polyacrylamide gel. Total protein was detected with 0.02 % (w/v) Coomassie Blue. Proteins containing covalently attached haem were detected using haem-dependent peroxidase activity [25].

E. coli membranes containing gonococcal cytochrome *c'* were diluted to a concentration of 1.8 mg/ml in Tris/HCl (pH 8.0). UV-visible spectra were measured between 350 and 700 nm in a Jasco V-550 spectrophotometer fitted with a Jasco ISV-469 reflective sphere to allow the capture of scattered light. Ammonium persulphate was used as an oxidizing agent and sodium dithionite as a reducing agent. To generate anaerobic conditions, the cuvette was sealed with a septum and sparged with nitrogen gas. The sample was then sparged with NO for up to 5 min.

The presence of nitrite in the growth medium was measured qualitatively by mixing approx. 50 µl each of culture, 1 % sulphanimide and 0.02 % *N*-naphthylethylenediamine dihydrochloride. A pink colour indicated the presence of nitrite in the culture.

Sequence data for *N. gonorrhoeae* were obtained from the University of Oklahoma genome sequencing project (<http://dna1.chem.ou.edu/gono.html>). A VGE-PSI BLAST search on the ViruloGenome site of the University of Birmingham (<http://www.vge.ac.uk/>) was used to identify putative protein homologues in the *N. gonorrhoeae* genome. The GenBank® accession number for the completed *N. gonorrhoeae* genome is AE004969.

RESULTS

In silico analysis of gonococcal *c*-type cytochromes

Six confirmed *c*-type cytochromes have been identified in the genomes of *N. meningitidis* serogroups A and B [26,27]. The amino acid sequences of these proteins were used to search the gonococcal genome to identify the *c*-type cytochromes. The gonococcal genome encodes open reading frames for all the confirmed meningococcal cytochromes and, in addition, also encodes a seventh cytochrome corresponding to the microaerobically induced CCP [8]. Table 4 shows the N-terminal amino acid sequence, gene and predicted homologue of the seven gonococcal *c*-type cytochromes. The meningococcus does not encode a CCP protein. Identification of the seventh cytochrome is consistent with experimental results from gels of proteins expressed by oxygen-limited gonococci that have been separated by SDS/PAGE and stained for covalently bound haem.

We previously reported that gonococcal CCP is a lipoprotein that was predicted, but not shown, to be anchored in the outer membrane [9]. The N-terminal sequence of CCP includes a consensus lipoprotein signal peptide for cleavage by signal peptidase II. Analysis of the other *c*-type cytochromes in the gonococcus

Figure 1 Presence or absence of cytochrome *c'* from gonococcal strains F62 and JCGC214

Total proteins from samples of aerobically grown bacteria were harvested in the middle of exponential growth, lysed in sample buffer and separated by SDS/PAGE. The gel was then stained for peroxidase activity associated with covalently bound haem. Lane 1, the cytochrome *c'* mutant strain JCGC214; lane 2, the parental strain F62. The densitometer trace on the left shows the prominent top band of CcoO (a component of the only cytochrome oxidase in the gonococcus) and cytochrome *c*₄; the less intense lowest band in track 2, absent from the mutant, is the 16 kDa cytochrome *c'*.

coccus showed that the putative cytochrome *c'*, encoded by *cycP*, also has a signal peptide with a lipobox, namely LSAC, from residues 16–19, that matches the consensus sequence (Table 4). On this basis, it was predicted that cytochrome *c'* too is a lipoprotein. Two preliminary observations were consistent with this proposal. First, cytochrome *c'*, similar to CCP, is membrane-associated [9]. Secondly, both CCP and cytochrome *c'* were solubilized on washing fractionated gonococcal membranes with 1 % sarkosyl, but were not solubilized by 2 M KCl or 0.1 % deoxycholate. This is typical of lipoproteins that associate with membranes through non-covalent attachment of palmitoyl residues linked to the N-terminal residue of the mature proteins.

Construction and phenotype of a gonococcal cytochrome *c'* mutant

A cytochrome *c'* mutant strain of *N. gonorrhoeae* was constructed by disruption of the chromosomal gene with an antibiotic selection marker. An EcoRI site was introduced into the *cycP* gene by site-directed mutagenesis of the plasmid pST501 and an erythromycin resistance cassette was inserted at this site. The resulting plasmid, pST503, was used as the template to amplify a linear fragment that included the erythromycin resistance cassette flanked by the *cycP* coding region and downstream DNA. This linear fragment was transformed into naturally competent pilated strain F62, and erythromycin-resistant recombinants were selected, purified and screened by PCR for replacement of the parental *cycP* gene by the interrupted gene. In contrast with strain F62, which accumulated six *c*-type cytochromes during aerobic growth and seven others during microaerobic growth, the 14 kDa cytochrome *c'* was absent from the mutant strain JCGC214 (Figure 1).

Figure 2 Growth of the gonococcal strains F62 and its *cycP* mutant, JCGC214, in oxygen-limited cultures in the presence and absence of nitrite

Triplicate cultures of *N. gonorrhoeae* strains F62 and JCGC214 were grown under oxygen-limited conditions and the attenuation of the cultures was measured at regular intervals. (a) Attenuance of the cultures of F62 and JCGC214 grown in the presence of 3 mM nitrite. The graph is plotted on a semi-exponential scale and shows the means $\pm$ S.D. of absorbance readings for three experiments. The inset shows the same data plotted on a linear scale (OD = attenuation). (b) Strains F62 and JCGC214 were also grown without nitrite added to the growth medium. The graph shows the means $\pm$ S.D. for samples from triplicate cultures.

To compare the growth rate and nitrite consumption of the mutant with those of the parent, the strains F62 and JCGC214 were grown under oxygen-limited conditions in the presence of 3 mM nitrite. Growth was monitored at regular intervals and the amount of nitrite present in the medium was measured qualitatively. Both strains reduced nitrite during growth, indicating that cytochrome *c'* is not required for nitrite reduction, and both the mutant and parental strains showed a significant lag phase (Figure 2a). However, the lag phase before the exponential growth commenced was 2 h longer for the mutant. The exponential growth-rate constants of the parental and mutant strains were 0.167 and 0.206 h⁻¹, respectively, showing clearly that the mutation had no adverse effect during this growth phase. Both strains reached the stationary phase at a similar cell density.

To determine whether the production of NO, as the result of nitrite reduction, leads to the extended lag phase of the cytochrome *c'* mutant, the two strains were grown under oxygen-limiting conditions without nitrite and growth was monitored at regular intervals. In the absence of nitrite, the growth rate of both strains was linear rather than exponential (Figure 2b), and there was no significant difference between the growth rates of the two strains.

This indicated that the presence of nitrite in the growth medium or the production of NO by nitrite reduction was the cause of the extended lag phase observed for the mutant.

Post-translational modification of cytochrome *c'* in the gonococcus

Three enzymes are essential for the processing of lipoproteins in *E. coli*: apolipoprotein diacylglycerol transferase (Lgt), signal peptidase II (Lsp) and apolipoprotein *N*-acyltransferase (Lnt) [28]. The peptide antibiotic, globomycin, inhibits signal peptidase II, resulting in the accumulation of prolipoproteins in the cytoplasmic membrane [29–31]. To confirm that both cytochrome *c'* and CCP are processed as lipoproteins in *N. gonorrhoeae*, the gonococcal strain F62 was grown in oxygen-limiting conditions to mid-exponential phase in the presence of 2 mM nitrite. Cultures were then supplemented with globomycin at a concentration of 10 μ g/ml. Before the addition of globomycin, the gonococcus accumulated both cytochrome *c'* and CCP. After the culture had been supplemented with globomycin, additional bands of molecular mass approx. 2 kDa higher than CCP and cytochrome *c'* were also detected. These bands correspond to the proteins

Figure 3 Effect of globomycin on the production of cytochrome *c'*

Whole cell proteins (300 μ g) from BL21(λ DE3) pST2 pST205 were separated by SDS/PAGE and stained for haem-dependent peroxidase activity. Transformed bacteria were grown aerobically in Lennox broth to an A_{650} 0.5 and expression of cytochrome *c'* was induced with 0.5 mM IPTG for 2 h. Globomycin at a final concentration of 20 μ g/ml was added at the time of induction. Lanes 1 and 2, induced bacteria, without globomycin, after 2 h; lane 3, uninduced bacteria; lanes 4 and 5, induced bacteria in the presence of globomycin after 2 h. The approx. 14 kDa band (band 1) corresponds to mature cytochrome *c'* from which the signal peptide had been cleaved; the approx. 16 kDa band 2 corresponds to cytochrome *c'* with its signal peptide still attached.

with their signal peptides still attached, but were either too faint to document clearly (cytochrome *c'*) or were masked by the much more prominent band of CcoP, a component of the gonococcal cytochrome oxidase. This confirmed that, in the gonococcus, signal peptidase II is responsible for the cleavage of both CCP and cytochrome *c'* signal peptides.

Effect of globomycin on the cleavage of the signal peptide of the gonococcal cytochrome *c'* expressed in *E. coli*

To confirm that cytochrome *c'* is post-translationally modified as a lipoprotein, the *cycP* gene was cloned and the protein was expressed from pST205 in *E. coli*. The *cycP* gene was amplified by PCR using chromosomal DNA from *N. gonorrhoeae* strain F62 as the template and cloned into pET-11c. Bacteria were co-transformed with plasmid pST2, which expresses the cytochrome *c* maturation proteins (designated CcmA to CcmH), to allow the production of *c*-type cytochromes during aerobic growth. Cytochrome *c'* was expressed aerobically from pST205 under two conditions as described in the Materials and methods section. Significantly more cytochrome *c'* was accumulated when bacteria were grown to stationary phase without the addition of IPTG. This is consistent with results previously reported for the overexpression of gonococcal CCP in *E. coli*, suggesting that the post-translational modifications of lipoproteins are rate-limiting steps during overexpression [9].

In the absence of globomycin, bacteria accumulated only the 14 kDa mature cytochrome *c'* (Figure 3, lanes 1 and 2). After induction of cytochrome *c'* from pST205 in the presence of 20 μ g/ml globomycin, the bacteria accumulated two proteins with covalently attached haem of approx. 16 and 14 kDa (Figure 3, lanes 4 and 5). The 14 kDa band corresponds to mature cytochrome *c'* and the 16 kDa band corresponds to cytochrome *c'* with its signal peptide attached. The appearance of this new form of cytochrome *c'* is consistent with the transfer of preapolipoprotein to the periplasm where haem was attached, but cleavage of the signal peptide by signal peptidase II was inhibited.

The membrane location of cytochrome *c'*

In Gram-negative bacteria, lipoproteins can either be anchored to the inner or to the outer membrane, in each case facing the

Figure 4 The location of gonococcal cytochrome *c'* in *E. coli*

Cellular fractions (300 μ g of protein) from BL21(DE3) pST2 pST205 were separated by SDS/PAGE and stained for haem-dependent peroxidase activity. Transformed bacteria were grown to stationary phase, fractionated in a French press and soluble and membrane fractions were prepared by centrifugation. Lane 1, whole cells; lanes 2 and 3, soluble fraction; lanes 4 and 5, membrane fraction.

Figure 5 The separation of *E. coli* membranes containing gonococcal cytochrome *c'* by sucrose density-gradient centrifugation

E. coli membranes containing gonococcal cytochrome *c'* were loaded on to a sucrose step gradient and centrifuged at 100 000 *g* for 48 h. Fractions were extracted from the top of the gradient. Proteins were separated by SDS/PAGE and the gel was stained first for haem-dependent peroxidase activity to locate cytochrome *c'* (results not shown) and then restained for total protein. Cytochrome *c'* was detected in fractions 8–14 but was most abundant in fractions 10–14 (illustrated above). Note the significant changes in the protein profile down the gradient with the outer membrane marker OmpF accumulating in lower fractions.

periplasm. Attempts to separate gonococcal membranes by isopycnic centrifugation were unsuccessful, so the cellular location of gonococcal cytochrome *c'* was investigated in the heterologous host, *E. coli* strain BL21(DE3) co-transformed with plasmids pST2 and pST205. Bacteria were grown to stationary phase without induction, disrupted in a French press and the membrane and soluble fractions were separated by centrifugation. The proteins in each fraction were separated by SDS/PAGE and stained for covalently bound haem. As expected, a clear band of an approx. 14 kDa cytochrome was detected in unbroken bacteria (Figure 4, lane 1) and in the membrane fraction (Figure 4, lanes 4 and 5), but not in the soluble fraction (lanes 2 and 3). The membrane location of cytochrome *c'* in *E. coli* is consistent with its location in the gonococcus and the prediction that the N-terminal cysteine residue is lipid-modified during lipoprotein processing.

E. coli membranes containing gonococcal cytochrome *c'* were loaded on to a sucrose step gradient and separated by centrifugation at 100 000 *g* for 48 h. Fractions were extracted from the top of the gradient and the protein content of each fraction was analysed by spectroscopy and SDS/PAGE (Figure 5). Note that

Figure 6 Spectral analysis of oxidized and reduced *E. coli* membranes containing gonococcal cytochrome *c'*

Membranes extracted from aerobically grown BL21(λ DE3) pST205 pST2 and BL21(λ DE3) pST2 were diluted to a protein concentration of 1.8 mg/ml and analysed by spectroscopy. (a) Membranes from bacteria expressing cytochrome *c'* were oxidized with ammonium persulphate and the absorbance spectrum between 350 and 700 nm was recorded. The membranes were then reduced with dithionite and the spectrum was repeated. The wavelengths of absorbance maxima are shown for the oxidized and reduced spectra. (b) The ammonium persulphate oxidized spectrum was subtracted from the dithionite reduced spectrum to obtain the difference spectrum and compared with a difference spectrum for non-expressing cells. I, the Soret region; and II, the α and β regions for the membranes extracted from non-expressing and cytochrome *c'*-expressing bacteria.

this gel was first stained for covalently bound haem to reveal fractions enriched in cytochrome *c'* and then with Coomassie Blue to stain all proteins. Haem-dependent peroxidase activity due to cytochrome *c'* could be detected in fractions 8 and 9 and was enriched in fractions 10–14. A prominent 36 kDa band was excised from the gel, digested with trypsin and confirmed by MS to be the outer membrane protein OmpF. Conversely, no OmpF was detected in fraction 6, which was also devoid of cytochrome *c'* and gave a protein profile typical of inner membrane preparations. These results established that, in *E. coli*, gonococcal cytochrome *c'* is targeted to the outer membrane.

Spectroscopic evidence that gonococcal cytochrome *c'* binds NO

Soluble cytochrome *c'* proteins from both photosynthetic and denitrifying bacteria have been shown to bind NO, giving characteristic spectral changes [32–36]. Spectroscopy of *E. coli* membranes containing overexpressed gonococcal cytochrome *c'* was used to determine its absorbance spectra and ability to bind NO. Membranes extracted from expressing and non-expressing cells were diluted to a final protein concentration of 1.8 mg/ml in Tris/HCl (pH 8.0), oxidized with ammonium persulphate and the spectrum between 350 and 700 nm was recorded. The reducing agent sodium dithionite was then added and the spectrum was repeated (Figure 6a). Membranes from the cytochrome *c'*⁺ strain reveal features in the Soret region characteristic of cytochromes *c'*. In the oxidized state, there is an absorption maximum at 404 nm and a shoulder around 380 nm. In the dithionite reduced sample, the Soret band shifts to yield an asymmetric peak at 426 nm with a shoulder at 439 nm. The spectra, obtained from membranes containing cytochrome *c'* and

the control sample, were compared to verify that the characteristic properties detected were due to cytochrome *c'*. Difference (reduced spectra–oxidized spectra) spectra were obtained by subtracting the spectrum of ammonium persulphate-oxidized membranes from the dithionite-reduced membranes (Figure 6b). The characteristic asymmetrical Soret band was detected only in membranes containing cytochrome *c'* (Figure 6b, I). Both membrane samples gave absorption maxima at 560 nm, typical of a *b*-type cytochrome (Figure 6b, II) [37]. A negative feature at 649 nm was more clearly detected in cytochrome *c'*-containing membranes than in the control sample. This corresponds to the absorption maximum at 651 nm in the oxidized sample and might be due to cytochrome *c'*. The results show that the expression of gonococcal cytochrome *c'* causes the production of a membrane protein with the characteristic spectral features seen in previously characterized cytochrome *c'* proteins.

The NO-binding properties of gonococcal cytochrome *c'* were then determined. The sample was oxidized with ammonium persulphate and sparged with nitrogen gas to generate anaerobic conditions before the spectrum was recorded between 350 and 700 nm. It was then sparged with NO gas and the spectrum was repeated. The difference spectrum generated by subtracting the spectrum in the absence of NO from that in its presence revealed peaks at 564, 533 and 419 nm (Figure 7). This is typical of cytochrome *c'*, for example, *Paracoccus denitrificans* cytochrome *c'*, for which absorption maxima are at 562, 530 and 413 nm [35]. This spectrum was identical with that formed after reaction of ferrous cytochrome *c'* with NO. These results indicate that the cytochrome *c'* from the gonococcus is an NO-binding protein, as has been shown previously for other *c'*-type cytochromes [32].

Figure 7 Spectral analysis of *E. coli* membranes containing gonococcal cytochrome *c'* in the presence of nitric oxide

The absorbance of membranes containing cytochrome *c'* oxidized with ammonium persulphate (ferric) was recorded between 350 and 700 nm. The sample was sealed with a septum and sparged with N_2 gas to generate anaerobic conditions. The sample was then sparged with NO for 5 min and the new spectrum was recorded. The spectrum of membranes containing gonococcal cytochrome *c'* in the absence of NO was subtracted from the spectrum obtained after exposure to NO to determine the difference spectrum.

DISCUSSION

Targeting of lipoproteins to the cytoplasmic or outer membrane

Inspection of the N-terminal sequences of the seven *c*-type cytochromes revealed that they split into two groups. Five were typical of *c*-type cytochromes found in bacteria that inhabit microaerobic environments. They include the haem *c* components of the bc_1 complex and a cytochrome *cbb*₃ oxidase, which in other bacteria has a high affinity for oxygen [38], and two di-haem periplasmic cytochromes typical of those that transfer electrons between the bc_1 complex and the cytochrome oxidase. It is also possible that one or more of these cytochromes transfer electrons from the quinol pool in the cytoplasmic membrane to outer membrane components (see below). The remaining two *c*-type cytochromes are predicted to be lipoproteins: one of them is the previously characterized CCP [8,9] and the other is clearly a member of the well-characterized cytochrome *c'* family of cytochromes.

Lipoproteins are translocated across the *E. coli* cytoplasmic membrane by the Sec general secretion pathway. The N-terminal cysteine residue is then lipid-modified before cleavage of the signal peptide by signal peptidase II [28,30,39,40]. After the initial post-translational modifications during secretion, five proteins (LolA, LolB, LolC, LolD and LolE) are involved in the targeting of lipoproteins to the *E. coli* outer membrane [41–43]. Retention in the cytoplasmic membrane depends on the ability of the lipoprotein to avoid the Lol system, which targets other lipoproteins to the outer membrane. The Lol avoidance signal is encoded by the combination of residues at +2 and +3 of the mature protein [44–47]. The strongest Lol avoidance signals are aspartic residue at position 2 followed by aspartic, glutamic, glutamine and asparagine residues at position 3 [44,44a,46]. It is still not known whether the rules for lipoprotein sorting vary among different bacteria; however, the Lol system has been identified in a number of Gram-negative bacteria [43]. Analysis of the genome sequence of *N. meningitidis* serogroup A has identified homologues of LolA, LolB, LolC and LolD [26].

Apart from our previous report that maturation of CCP is sensitive to inhibition by globomycin, a specific inhibitor of signal peptidase II [9], nothing is known about the processing of lipoproteins in the gonococcus. A PSI-BLAST search on

ViruloGenome shows that the gonococcal genome has predicted open reading frames with high levels of identity with LolA, LolB, LolC and LolD from *N. meningitidis* and a high degree of similarity to the *E. coli* proteins (results not shown). The +2 +3 amino acid sequence of the gonococcal cytochrome *c'*, Gly-Asn, suggests that it will be targeted to the outer membrane. No *lolE* gene is present in the gonococcus and LolE is essential in *E. coli*. However, in *E. coli*, the amino acid sequence of LolC is very similar to that of LolE and therefore it is possible that in the gonococcus the ATPase is made up of (LolC)₂ LolD [48].

We have confirmed unpublished observations that gonococcal inner and outer membranes are poorly separated during sucrose density-gradient centrifugation. Consequently, an indirect approach was required to demonstrate the subcellular localization of these two gonococcal lipoproteins. On the basis of analysis of both CCP and cytochrome *c'* in *E. coli* (Figure 7) and the amino acid sequences of the lipoboxes (Table 4), both proteins are predicted to be targeted to the outer membrane. If this is correct, there is a division in the location of the cytochromes with respect to function: those associated with the outer membrane are involved in combating oxidative and nitrosative stress; the others are predicted to be involved in respiration. In other bacteria, these cytochromes are soluble but it is possible that lipid modification is required to anchor the protein to its site of function and, for cytochrome *c'*, this might be in close proximity to another outer membrane lipoprotein, AniA [13,14,49].

Implications of the outer membrane localization of CCP and cytochrome *c'* for pathogenicity

Recent work has shown that cytochrome *c'* can protect a number of bacterial species, including *N. meningitidis*, from NO [20,50]. Cytochrome *c'* mutants of *N. meningitidis* and *Rhodobacter capsulatus* are more susceptible to exogenously supplied NO than the respective parental strains [20,50,51]. We have detected the expression of the gonococcal cytochrome *c'* homologue during both aerobic and oxygen-limited growth, so it could protect the bacteria from NO generated endogenously during respiration or from exogenous NO produced by the human host.

Since antibodies to AniA are detected in infected individuals, oxygen-limited growth of gonococci clearly occurs during the disease process. During adaptation, the FNR protein senses the decreased oxygen level and induces the synthesis of AniA, which can inhibit complement-mediated killing of gonococci [13]. Although it also reduces nitrite to potentially toxic NO, the constitutive synthesis of cytochrome *c'* provides an instant defence mechanism against NO toxicity until sufficient NorB has accumulated to reduce further NO to N_2O . By binding NO, cytochrome *c'* might also prevent the formation of peroxynitrite, thus helping the bacteria to survive in a macrophage or in abscesses where the oxygen tension is low. Furthermore, the induced synthesis of CCP provides an additional defence mechanism against oxidative stress.

The presence of novel lipoproteins in the gonococcus raises the fascinating possibility that many other types of Gram-negative bacteria will target lipoproteins to the outer membrane to provide protection against attack from reactive oxygen and nitrogen compounds. While the relevance to pathogenicity is obvious, it is also important for commensal bacteria to be capable of surviving host defence mechanisms such as the phagocytic burst and generation of NO.

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REFERENCES

- Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H. and Fang, F. C. (2000) Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages *in vitro*. *J. Exp. Med.* **192**, 227–236.
- Ischiropoulos, H., Zhu, L. and Beckman, J. S. (1992) Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.* **298**, 446–451.
- Zhu, L., Gunn, C. and Beckman, J. S. (1992) Bactericidal activity of peroxynitrite. *Arch. Biochem. Biophys.* **298**, 452–457.
- Brunelli, L., Crow, J. P. and Beckman, J. S. (1995) The comparative toxicity of nitric oxide and peroxynitrite to *Escherichia coli*. *Arch. Biochem. Biophys.* **316**, 327–334.
- Veale, D. R., Finch, H. and Smith, H. (1976) Penetration of penicillin into human phagocytes containing *Neisseria gonorrhoeae*: intracellular survival and growth at optimum concentrations of antibiotic. *J. Gen. Microbiol.* **95**, 353–363.
- Witt, K., Veale, D. R., Finch, H., Penn, C. W., Sen, D. and Smith, H. (1976) Resistance of *Neisseria gonorrhoeae* grown *in vivo* to ingestion and digestion by phagocytes of human blood. *J. Gen. Microbiol.* **96**, 341–350.
- Strange, J. C. and Judd, R. C. (1994) Identification of heme-containing proteins of *Neisseria gonorrhoeae*. In *Pathobiology and Immunobiology of Neisseriaceae* (Condeelis, C. J., Morse, S., Rice, P., Sparling, F. and Calderon, E., eds.), pp. 417–423. Proceedings of the Eighth Pathogenic *Neisseria* Conference held at Mexico City, Instituto Nacional De Salud Publica, Mexico City.
- Lissenden, S., Mohan, S., Overton, T., Regan, T., Crooke, H., Cardinale, J. A., Householder, T. C., Adams, P., O'Connor, C. D., Clark, V. L. et al. (2000) Identification of transcription activators which regulate gonococcal adaption from aerobic to anaerobic or oxygen-limited growth. *Mol. Microbiol.* **37**, 839–855.
- Turner, S., Reid, E., Smith, H. and Cole, J. (2003) A novel cytochrome *c* peroxidase from *Neisseria gonorrhoeae*: a lipoprotein from a Gram-negative bacterium. *Biochem. J.* **373**, 865–873.
- Morse, S. A. and Bartenstein, L. (1974) Factors affecting autolysis of *Neisseria gonorrhoeae*. *Proc. Soc. Exp. Biol. Med.* **145**, 1418–1421.
- Knapp, J. S. and Clark, V. (1984) Anaerobic growth of *Neisseria gonorrhoeae* coupled to nitrite reduction. *Infect. Immun.* **46**, 176–181.
- Mellies, J., Jose, J. and Meyer, T. F. (1997) The *Neisseria gonorrhoeae* gene *aniA* encodes an inducible nitrite reductase. *Mol. Gen. Genet.* **256**, 532–537.
- Cardinale, J. A. and Clark, V. L. (2000) Expression of *AniA*, the major anaerobically induced outer membrane protein of *Neisseria gonorrhoeae*, provides protection against killing by normal human sera. *Infect. Immun.* **68**, 4368–4369.
- Clark, V., Knapp, J. S., Thompson, S. and Klimpel, K. W. (1988) Presence of antibodies to the major anaerobically induced gonococcal outer membrane protein in sera from patients with gonococcal infections. *Microb. Pathog.* **5**, 381–390.
- Smith, L. D. S. (1975) *The Pathogenic Anaerobic Bacteria*. Charles C. Thomas, Springfield, IL.
- Burnakis, T. G. and Hildebrandt, N. B. (1986) Pelvic inflammatory disease: a review with emphasis on antimicrobial therapy. *Rev. Infect. Dis.* **8**, 86–116.
- Zheng, H.-Y., Hassett, D. J., Bean, K. and Cohen, M. S. (1992) Regulation of catalase in *Neisseria gonorrhoeae*. *J. Clin. Invest.* **90**, 1000–1006.
- Householder, T. C., Fozo, E. M., Cardinale, J. A. and Clark, V. (2000) Gonococcal nitric oxide reductase is encoded by a single gene, *norB*, which is required for anaerobic growth and is induced by nitric oxide. *Infect. Immun.* **68**, 5241–5246.
- Seib, K. L., Jennings, M. P. and McEwan, A. G. (2003) A Sco homologue plays a role in defence against oxidative stress in pathogenic *Neisseria*. *FEBS Lett.* **546**, 411–415.
- Anjum, M. F., Stevanin, T. M., Read, R. C. and Moir, J. W. (2002) Nitric oxide metabolism in *Neisseria meningitidis*. *J. Bacteriol.* **184**, 2987–2993.
- McEwan, A. G., Jackson, J. B. and Ferguson, S. J. (1984) Rationalization of properties of nitrate reductases in *Rhodospseudomonas capsulata*. *Arch. Microbiol.* **137**, 344–349.
- Witholt, B., Boekhout, M., Brock, M., Kingma, J., Heerikhuizen, H. V. and Leij, L. D. (1976) An efficient and reproducible procedure for the formation of spheroplasts from variously grown *Escherichia coli*. *Anal. Biochem.* **74**, 160–170.
- Doherty, H., Yamada, H., Caffrey, P. and Owen, P. (1986) Identification, immunochemical characterization, and purification of a major lipoprotein antigen associated with the inner (cytoplasmic) membrane of *Escherichia coli*. *J. Bacteriol.* **166**, 1072–1082.
- Lowry, O. H., Roseberg, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
- Thomas, P. E., Ryan, D. and Ledwin, W. (1976) An improved staining procedure for the detection of peroxidase activity of cytochrome P-450 on sodium dodecyl sulphate polyacrylamide gels. *Anal. Biochem.* **75**, 168–176.
- Parkhill, J., Achtman, M., James, K. D., Bentley, S. D., Churcher, C., Klee, S. R., Morelli, G., Basham, D., Brown, D., Chillingworth, T. et al. (2000) Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. *Nature (London)* **404**, 502–506.
- Tettelin, H., Saunders, N. J., Heidelberg, J., Jeffries, A. C., Nelson, K. E., Eisen, J. A., Ketchum, K. A., Hood, D. W., Peden, J. F., Dodson, R. J. et al. (2000) Complete genome sequence of *Neisseria meningitidis*. *Science* **287**, 1809–1815.
- Sankaran, K. and Wu, H. C. (1994) Lipid modification of bacterial prolipoprotein. *J. Biol. Chem.* **269**, 19701–19706.
- Inukai, M., Enokita, R., Torikata, A., Nakahara, M., Iwado, S. and Arai, M. (1978) Globomycin, a new peptide antibiotic with spheroplast-forming activity. I. Taxonomy of producing organisms and fermentation. *J. Antibiot.* **31**, 410–420.
- Hussain, M., Ichihara, S. and Mizushima, S. (1980) Accumulation of glyceride-containing precursor of the outer membrane lipoprotein in the cytoplasmic membrane of *Escherichia coli* treated with globomycin. *J. Biol. Chem.* **255**, 3707–3712.
- Dev, I. K., Harvey, R. J. and Ray, P. H. (1985) Inhibition of prolipoprotein signal peptidase by globomycin. *J. Biol. Chem.* **260**, 5891–5894.
- Iwasaki, H., Yoshimura, T., Suzuki, S. and Shidara, S. (1991) Spectral properties of *Achromobacter xylosoxidans* cytochromes *c'* and their NO complexes. *Biochim. Biophys. Acta* **1058**, 79–82.
- Yoshimura, T., Suzuki, S., Iwasaki, H. and Takakuwa, S. (1987) Spectral properties of nitric oxide complex of cytochrome *c'* from *Rhodospseudomonas capsulata* B100. *Biochem. Biophys. Res. Commun.* **145**, 868–875.
- Yoshimura, T., Fujii, S., Kamada, H., Yamaguchi, K., Suzuki, S., Shidara, S. and Takakuwa, S. (1996) Spectroscopic characterization of nitrosylheme in nitric oxide complexes of ferric and ferrous cytochrome *c'* from photosynthetic bacteria. *Biochim. Biophys. Acta* **1292**, 39–46.
- Moir, J. W. (1999) Cytochrome *c'* from *Paracoccus denitrificans*: spectroscopic studies consistent with a role for the protein in nitric oxide metabolism. *Biochim. Biophys. Acta* **1430**, 65–72.
- Lawson, D. M., Stevenson, C. E., Andrew, C. R. and Eady, R. R. (2000) Unprecedented proximal binding of nitric oxide to heme: implications for guanylate cyclase. *EMBO J.* **19**, 5661–5671.
- Kita, K., Konishi, K. and Anraku, Y. (1984) Terminal oxidases of *Escherichia coli* aerobic respiratory chain. *J. Biol. Chem.* **259**, 3375–3381.
- Preisig, O., Zufferey, R., Thony-Meyer, L., Appleby, C. A. and Hennecke, H. (1996) A high-affinity *cbb3*-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. *J. Bacteriol.* **178**, 1532–1538.
- Tokunaga, M., Tokunaga, H. and Wu, H. C. (1982) Post-translational modification and processing of *Escherichia coli* pro-lipoprotein *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2255–2259.
- Pugsley, A. P. (1993) The complete general secretory pathway in Gram-negative bacteria. *Microbiol. Rev.* **57**, 50–108.
- Matsuyama, S., Tajima, T. and Tokuda, H. (1995) A novel periplasmic carrier protein involved in the sorting and transport of *Escherichia coli* lipoproteins destined for the outer membrane. *EMBO J.* **14**, 3365–3372.
- Matsuyama, S., Yokota, N. and Tokuda, H. (1997) A novel outer membrane lipoprotein, LolB (HemM), involved in the LolA (p20)-dependent localization of lipoproteins to the outer membrane of *Escherichia coli*. *EMBO J.* **16**, 6947–6955.
- Yakushi, T., Masuda, K., Narita, S., Matsuyama, S. and Tokuda, H. (2000) A new ABC transporter mediating the detachment of lipid-modified proteins from membranes. *Nat. Cell Biol.* **2**, 212–218.
- Narita, S., Tanaka, K., Matsuyama, S. and Tokuda, H. (2002) Disruption of *lolCDE*, encoding an ATP-binding cassette transporter, is lethal for *Escherichia coli* and prevents release of lipoproteins from the inner membrane. *J. Bacteriol.* **184**, 1417–1422.
- Yamaguchi, K., Yu, F. and Inouye, M. (1988) A single amino acid determinant of the membrane localisation of lipoproteins in *E. coli*. *Cell (Cambridge, Mass.)* **53**, 423–432.
- Seydel, A., Gounon, P. and Pugsley, A. P. (1999) Testing the '+2 rule' for lipoprotein sorting in the *Escherichia coli* cell envelope with a new genetic selection. *Mol. Microbiol.* **34**, 810–821.
- Terada, M., Kuroda, T., Matsuyama, S. and Tokuda, H. (2001) Lipoprotein sorting signals evaluated as the LolA-dependent release of lipoproteins from the cytoplasmic membrane of *Escherichia coli*. *J. Biol. Chem.* **276**, 47690–47694.
- Hara, T., Matsuyama, S. and Tokuda, H. (2003) Mechanism underlying the inner membrane retention of *Escherichia coli* lipoproteins caused by Lol avoidance signals. *J. Biol. Chem.* **278**, 40408–40414.
- Narita, S., Tanaka, K., Matsuyama, S. and Tokuda, H. (2002) Disruption of *lolCDE*, encoding an ATP-binding cassette transporter, is lethal for *Escherichia coli* and prevents release of lipoproteins from the inner membrane. *J. Bacteriol.* **184**, 1417–1422.

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- 49 Clark, V., Campbell, L. A., Palermo, D. A., Evans, T. M. and Klimpel, K. W. (1987) Induction and repression of outer membrane proteins by anaerobic growth of *Neisseria gonorrhoeae*. *Infect. Immun.* **55**, 1359–1364
- 50 Cross, R., Aish, J., Paston, S. J., Poole, R. K. and Moir, J. W. B. (2000) Cytochrome *c'* from *Rhodobacter capsulatus* confers increased resistance to nitric oxide. *J. Bacteriol.* **182**, 1442–1447
- 51 Cross, R., Lloyd, D., Poole, R. K. and Moir, J. W. (2001) Enzymatic removal of nitric oxide catalyzed by cytochrome *c'* in *Rhodobacter capsulatus*. *J. Bacteriol.* **183**, 3050–3054
- 52 Nassif, X., Deidre, P. and So, M. (1991) Transposition of Tn1545- Δ 3 in the pathogenic neisseria: a genetic tool for mutagenesis. *J. Bacteriol.* **173**, 2147–2154

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