# A novel cytochrome *c* peroxidase from *Neisseria gonorrhoeae*: a lipoprotein from a Gram-negative bacterium

Susan TURNER, Eleanor REID, Harry SMITH and Jeffrey COLE[1]

School of Biosciences, University of Birmingham, Birmingham B15 2TT, U.K.

A cytochrome *c* peroxidase (CCP) produced by *Neisseria gonorrhoeae* has been shown to have novel characteristics by investigating its location, expression and role in *Neisseria gonorrhoeae* and by expression in *Escherichia coli*. Analysis of the N-terminus of CCP indicated that it is a lipoprotein with a signal peptide for cleavage by signal peptidase II. Expression of the gonococcal CCP in *E. coli* revealed that it is first synthesized as a pro-apo-cytochrome that is translocated across the cytoplasmic membrane. The signal peptide is cleaved and haem is attached in the periplasm. The gonococcal CCP was associated with the membrane of both *E. coli* and *N. gonorrhoeae*. The expression of a MalE–CCP fusion protein has allowed characterization of CCP *in vitro*. Evidence is presented that CCP protects gonococci from hydrogen peroxide, presumably in the periplasmic compartment of the cell. The expression of CCP is dependent on the transcription factor FNR, but is repressed by nitrite, indicating that it could be most important in the stationary-phase response. These data support the hypothesis that the gonococcal lipoprotein CCP is anchored to the membrane in the periplasm, where it might be responsible for the reduction of hydrogen peroxide. Other putative CCP lipoproteins have been identified, representing a new subclass of bacterial CCP proteins.

Key words: cytochrome *c* peroxidase, gonococcal anaerobic regulation, gonococci, lipoprotein, post-translational modification.

## INTRODUCTION

In their natural environments, most bacteria are exposed to alternating stresses of oxygen starvation and excess. This requires them to be able to avoid damage mediated by a variety of reactive oxygen species, including hydrogen peroxide, organic peroxides, superoxide ions, hydroxyl radicals, and compounds derived from reactive oxygen species such as peroxynitrite. Bacteria have evolved a variety of mechanisms to provide protection from such compounds generated either within their cytoplasm or cytoplasmic membrane or released into their environment by other organisms. In their mammalian host, pathogenic bacteria such as *Neisseria gonorrhoeae* must be able to survive exposure to reactive oxygen and nitrogen species produced as part of the immune response. Multiple defence mechanisms involving different types of enzyme provide multiple routes for inactivating the same toxic chemical. Interesting examples are the bacterial cytochrome *c* peroxidases (CCPs), which protect bacteria from damage by reducing hydrogen peroxide to water. Catalase also breaks down hydrogen peroxide by dismutation to oxygen and water.

Bacterial CCPs have been purified from *Pseudomonas fluorescens*, *P. aeruginosa*, *P. nautica*, *Paracoccus denitrificans*, *Rhodobacter capsulatus*, *Nitrosomonas europea* and *Methylococcus capsulatus* [1–7]. The crystal structures of CCPs from *P. aeruginosa* and *Nitrosomonas europea* have been determined [8,9]. *P. aeruginosa* CCP is a di-haem *c*-type cytochrome located in the periplasm. A periplasmic location has also been established for CCPs from *Paracoccus denitrificans*, *Rhodobacter capsulatus* and several other pseudomonas species, although some of the earlier studies concluded that CCP is associated with the cytoplasmic membrane [3,4,10,11]. The source of this controversy was traced to ineffective methods used to release periplasmic proteins. All of these enzymes catalyse the reduction of hydrogen peroxide to water, and in each case the physiological electron donor is another *c*-type cytochrome. An intriguing feature of CCP from *Paracoccus denitrificans* and several pseudomonad species is that, although its function appears to be to protect the bacteria from oxygen damage, it is synthesized predominantly, or only, during anaerobic or microaerobic growth [1,3,12]. The transcription factor FNR (for the regulator of *f*umarate and *n*itrate *r*eduction), has been shown to be essential for expression of CCP in *Paracoccus denitrificans* [12].

*N. gonorrhoeae* can adapt to oxygen starvation by synthesizing a copper-containing nitrite reductase, AniA, that reduces nitrite to nitric oxide [13–16]. Expression of the *aniA* gene is FNR-dependent: optimal expression requires a two-component regulatory system, NarQ/NarP, which responds to the presence of nitrite, but is insensitive to nitrate [15,17]. During both aerobic and anaerobic growth, gonococci synthesize moderately high levels of five *c*-type cytochromes, but during anaerobic growth a sixth cytochrome of 45 kDa is also detectable. The N-terminus of this anaerobically expressed cytochrome was blocked, but it was identified by MS as the product of a putative gonococcal *ccp* gene [15]. This is the only protein apart from AniA that has been shown to be expressed under the control of the gonococcal FNR.

The CCP designation for the anaerobically induced *c*-type cytochrome of *N. gonorrhoeae* rested solely on the similarity of its predicted sequence with those of better characterized CCPs from other bacteria. Although several of the gonococcal *c*-type cytochromes are predicted to be soluble, periplasmic proteins, all six major *c*-type cytochromes synthesized during anaerobic gonococcal growth were found to be associated with the membrane fraction [15]. However, methods for separating periplasmic proteins from neisseria are poorly developed, so it was possible that, as in some pseudomonads, the apparent membrane association of gonococcal CCP was due to the use of ineffective methods for the release of periplasmic proteins. The aim of this project

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

[1] To whom correspondence should be addressed (e-mail j.a.cole@bham.ac.uk).

**Table 1 Bacterial strains used in this work**

| Strain | Genotype | Source |
|---|---|---|
| JM109 | *F′ traD36 lacI$^q$ Δ(lacZ)MISproA$^+$ B$^+$/e14$^-$ (McrA$^-$) Δ(lac-proAB)thigyrA96 (Nar$^r$) endA1 hsdR17 ($r_k^-$ $m_k^+$) relA1 supE44 recA1* | Promega |
| JCB 7120 | *ΔnirnarL$^+$ nrf$^+$* | Laboratory stocks |
| JCB7124 | *ΔnirnarL$^+$ nrf::tet$^R$* | Laboratory stocks |
| BL21(λDE3) | F-*ompT gal[dcm][ion]hsds$_B$* ($r_B$-$m_B$-; an *E. coli* B strain); with DE3, a λ prophage carrying the T7 RNA polymerase gene | Novagen |
| F62 | Parental strain | |
| JCGC201 | F62 *ccp* promoter–*lacZ* fusion | [22] |
| JCGC211 | F62Δ*ccp*::*ermC* | Transformed with linear fragment from pST103 containing *ermC* |
| JCGC212 | F62 *kat*::*ermC* | Transformed with linear fragment from pST302 containing *ermC* |
| JCGC213 | F62 Δ*katccp*::*ermC* | Transformed with linear fragment from pST402 containing *ermC* |

**Table 2 Primers used in this work**

Engineered restriction enzyme cleavage sites are in bold.

| Primer | Sequence (5′–3′) |
|---|---|
| ST_CCPFP1 | GTC GAT GGA TGC GTT TGC CG |
| ST_CCPRP1 | CGC **GGA TCC** TAC CCT GCG TAT CTG ACG CG |
| ST_CCPFP3 | CCC **ACA TGT** CTT TCA AAC TCC GTT ACC TCG |
| ST_CCPRP3 | CCC C**GA ATT C**GC TTT GTT GTC CGG CTT AGA TTC CAT CGG |
| ST_CCPFP4 | GGT C**GG ATC C**GG ATG TCT TTC AAA CTC CGT TAC CTC G |
| ST_CCPFP5 | TAT **GGA TCC** GGC GGT CAG GAA AAG TCT GCG GCA GG |
| ST_KATFP3 | ACT CGC CGA CTT CGT GCG |
| ST_KATRP2 | GTC AAA CGA CGT TTC AGA CGG |
| ST_ERYFP1 | ATA CCC **AAG CTT** AGA GTG TGT TGA TAG TGC AG |
| ST_ERYRP1 | ATA CCC **AAG CTT** CGA TAC AAA TTC CCC GTA GGC G |
| ST_ERYFP2 | CCG **GAA TTC** AGA GTG TGT TGA TAG TGC AG |
| ST_ERYRP2 | CCG **GAA TTC** CGA TAC AAA TTC CCC GTA GG |
| DJE11 | CAG GGT TAT TGC GTG GG |
| DJE12 | TCC GGC ATA AAC TAA GCG C |

was to determine the subcellular location of the gonococcal CCP, and to investigate its biochemical function and physiological role.

## MATERIALS AND METHODS

### Bacterial strains, plasmids and oligonucleotides

The *N. gonorrhoeae* and *Escherichia coli* K-12 strains used, their relevant genotypes and the method of construction or source are listed in Table 1. Oligonucleotides used in the construction of plasmids are listed in Table 2, and plasmids used in the study are listed in Table 3.

### Construction of gonococcal mutant strains

To obtain the *ccp* mutant, the gonococcal *ccp* gene was amplified by PCR from *N. gonorrhoeae* F62 chromosomal DNA using primers ST_CCPFP1 and ST_CCPRP1. Plasmid pST101 was constructed by ligating the PCR fragment into pGEM®-T Vector. Plasmid pST101 was digested with *Hin*dIII to remove 800 bp of the *ccp* coding region. To construct pST103, the *ermC* gene was amplified from pMGC20 with primers ST_ERYFP1 and ST_ERYRP1 and ligated into the *Hin*dIII sites of pST101.

The catalase gene (*kat*) was cloned by PCR using primers ST_KATFP3 and ST_KATRP2, and *N. gonorrhoeae* F62 chromosomal DNA as a template. Plasmid pST301 was constructed by ligating the *kat* PCR fragment into pGEM®-T Vector. To construct pST302, the *ermC* gene was amplified from pMGC20 using primers ST_ERYFP2 and ST_ERYRP2 and ligated into the *Eco*RI site of pST301.

To create the *kat ccp* double mutant, a single PCR fragment encoding the *kat* and *ccp* genes was amplified from the *N. gonorrhoeae* F62 chromosome by PCR using primers ST_KATFP3 and ST_CCPRP1. The *kat ccp* fragment was ligated into pGEM®-T Vector to construct pST401. pST401 was digested with *Hin*dIII and *Eco*RI to remove the 3′ end of *kat* and the 5′ end of *ccp*. The *ermC* gene was amplified from pMGC20 using ST_ERYFP1 and ST_ERYRP2. Plasmid pST402 was constructed by ligating the resultant PCR fragment into the *Eco*RI–*Hin*dIII sites of pST401.

Naturally competent, piliated *N. gonorrhoeae* F62 were then transformed with a linear DNA fragment from the relevant plasmid as described in Table 1. The DNA fragment contained the *ermC* gene flanked by DNA identical to the gene, or genes, to be disrupted and also the gonococcal uptake sequence.

### Construction of plasmids for the expression of CCP

To construct pST201, the *ccp* gene was amplified by PCR from the F62 gonococcal chromosome. The upstream primer ST_CCPFP3 introduced an *Afl*III site at the 5′ end of *ccp*. The downstream primer ST_CCPRP1 introduced a *Bam*HI site at the 3′ end of *ccp*. The 1.2 kb fragment, which included the ATG start codon for *ccp*, was ligated into *Nco*I–*Bam*HI-digested pGCFNR2. The *ccp* gene was fused in-frame to the *E. coli fnr* promoter.

Plasmid pST202 contains the 1.2 kb PCR fragment encoding the *ccp* gene from pST201 cloned into pBAD-Myc/His (Invitrogen). The upstream primer, ST_CCPFP3, introduced an *Afl*III site at the 5′ end of *ccp*, and the downstream primer, ST_CCPRP3, introduced an *Eco*RI site at the 3′ end of *ccp*. Similarly, plasmid pST203 contains the *ccp* gene, including the ATG start codon, amplified by PCR from pST201 and cloned into pET-11c (Novagen). The upstream primer, ST_CCPFP4, introduced a *Bam*HI site at the 5′ end of *ccp*. The downstream primer, ST_CCPRP1, introduced a *Bam*HI site at the 3′ end of *ccp*.

To construct pST204, the *ccp* gene was amplified by PCR from pST201. The upstream primer ST_CCPFP5 contained a *Bam*HI site engineered at the 5′ end. The downstream primer ST_CCPRP1 contained a *Bam*HI site engineered at the 3′ end. The 1.2 kb fragment encoding CCP from the glycine residue at +2 of the predicted mature CCP was cloned into the *Bam*HI site of the commercial vector pMALp2x (New England Biolabs). Plasmid pST204 expresses CCP fused to the C-terminus of MalE, the periplasmic maltose-binding protein, via a polylinker containing a Factor Xa cleavage site. The MalE signal sequence directs the fusion protein via the Sec pathway through the cytoplasmic membrane into the periplasm.

Plasmid pST2 was constructed to express the *E. coli* cytochrome *c* maturation (*ccm*) genes aerobically. The plasmid is functionally similar to pEC86 [19]. To construct pST2, the *ccm* genes were amplified by PCR from pJG70. The upstream primer DJE11 contained an *Nco*I site engineered at the 5′ end, and the downstream primer DJE12 contained a *Sal*I site engineered at the 3′ end. This 6.5 kb fragment was ligated into pACYC184 (New England Biolabs) cut with *Sal*1 and *Bsp*H1.

**Table 3 Plasmids used in this work**

| Plasmid | Description | Source |
|---|---|---|
| pGEM®-T Vector | Commercial vector for cloning PCR products | Promega |
| pACYC184 | Commercial cloning vector | New England Biolabs |
| pBAD-Myc/His | Commercial overexpression vector; expression is induced with arabinose | Invitrogen |
| pET-11c | Commercial overexpression vector; expression is induced by IPTG | Novagen |
| pMALp2x | Commercial overexpression vector; expression is induced by IPTG | New England Biolabs |
| pJG70 | *ccmA-H* cloned into pBR322 | Laboratory stock |
| pMGC20 | Gonococcal shuttle vector and the source of *ermC* | [18] |
| pGCFNR2 | *E. coli fnr* promoter fused in frame to the first codon of the gonococcal *fnr* in pBR322 | [15] |
| pST2 | *CcmA-H* *Nco*I–*Sal*I fragment cloned into pACYC184 | This work |
| pST101 | Gonococcal *ccp* gene cloned into pGEM®-T Vector | This work |
| pST103 | *ermC* gene ligated into *Hin*dIII site of pST101 | This work |
| pST201 | *E. coli fnr* promoter fused to first codon of *ccp* coding sequence. | This work |
| pST202 | *araBAD* promoter fused to the first codon of the gonococcal *ccp* in pBAD-MycHis-A | This work |
| pST203 | T7 promoter fused to the first codon of the gonococcal *ccp* in pET-11c | This work |
| pST204 | *E. coli* MalE fused to the +2 glycine of gonococcal CCP in pMalp2x | This work |
| pST301 | Gonococcal *kat* gene cloned into pGEM®-T Vector | This work |
| pST302 | *ermC* gene ligated into *Eco*RI site of *kat* in pST301 | This work |
| pST401 | Gonococcal *kat–ccp* cloned into pGEM® T Vector | This work |
| pST402 | *ermC* gene ligated into *Eco*RI site of *kat* and *Hin*dIII site of *ccp* in pST401 | This work |

### CCP expression

*E. coli* strains JCB7124 pST201, JCB7124 pST202 and JCB7124 pST204 were grown anaerobically in the presence of 5 mM sodium nitrate. Expression from pST202 was induced with arabinose. Expression from pST204 was induced with isopropyl $\beta$-D-thiogalactoside (IPTG). Strain BL21($\lambda$DE3) pST203 pST2 was grown aerobically in Lennox broth and expression of CCP was induced with IPTG. Globomycin (kindly donated by Sankyo Co. Ltd, Tokyo, Japan) was used at a concentration of 10 $\mu$g/ml. *N. gonorrhoeae* strains F62 and JCGC201 were grown in gonococcal (GC) broth under oxygen-limiting growth conditions [15]. Sodium nitrite was added at a concentration of 5 mM when required.

Bacteria were harvested and washed, and the periplasm was prepared [20]. In some experiments, minor modifications to the standard procedure were introduced [4,21]. 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 (5000 ***g*** for 5 min). Membranes and soluble fractions were separated by centrifugation at 150000 ***g*** for 30 min at 4 °C. Inner membrane proteins were solubilized in sarkosyl solution and the outer membranes removed by centrifugation at 150000 ***g*** for 1 h at 4 °C. Inner membranes were then precipitated with ethanol and harvested by centrifugation at 150000 ***g*** for 1 h at 4 °C.

The MalE–CCP fusion protein was purified by amylose affinity chromatography from the periplasm of *E. coli* JCB7124 pST204 using a commercial protocol (New England Biolabs). The conditions required for cleavage of MalE from CCP by Factor Xa were investigated at a range of enzyme concentrations, temperatures and time points. Periplasmic MalE–CCP was not cleaved by Factor Xa. Purified MalE–CCP (55 $\mu$g) was cleaved by 1 $\mu$g of Factor Xa after 2 h at room temperature or 16 h at 4 °C.

### Biochemical assays

The protein concentration of cell fractions was determined using the Folin method [22]. Proteins were resolved by Tris/Tricene 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 [23].

Enzyme activity was measured using the procedure described by Gilmour et al. [24]. Horse heart ferrocytochrome *c* (25 $\mu$M) in 5 mM Hepes supplemented with 1 mM $CaCl_2$ was reduced with ascorbate or dithionite. Cleaved or uncleaved MalE–CCP was added and incubated for 20 min. The assay was initiated by the addition of a freshly prepared solution of hydrogen peroxide up to a final concentration of 80 $\mu$M. Activity was assayed with up to 1 mg of concentrated periplasmic proteins containing MalE–CCP.

Periplasm containing MalE–CCP was concentrated to 75 mg/ml protein. Absorption spectra were measured between 500 and 580 nm. Periplasm was reduced by the addition of ascorbic acid or sodium dithionite and oxidized by the addition of 50 $\mu$M hydrogen peroxide or potassium ferricyanide.

The susceptibilities of strains to hydrogen peroxide were compared in a disk diffusion assay adapted from King et al. [25]. A lawn of bacteria was spread on to a fresh GC agar plate supplemented with 2 mM sodium nitrite. An 8 mm-diam. filter paper disc was placed on the centre of the plate and wetted with 5 $\mu$l of 0.88 M hydrogen peroxide. The plate was incubated for 4 days at 37 °C in an anaerobic jar before the area of growth inhibition was calculated.

The $\beta$-galactosidase activities of JCGC201 cell lysates were measured as described by Lissenden et al. [15] to determine *ccp* promoter activity. The units of $\beta$-galactosidase activity are nmol of *o*-nitrophenol $\beta$-D-galactoside hydrolysed $\cdot$ $min^{-1}$ $\cdot$ (mg of dry mass)$^{-1}$.

## RESULTS

### Subcellular location of CCP in the gonococcus

Initial experiments were designed to determine whether our original designation of CCP as a peripheral membrane protein was an error due to the use of suboptimal conditions to release periplasmic proteins. *N. gonorrhoeae* strain F62 was grown under oxygen-limiting conditions in the presence of 5 mM nitrite, harvested and incubated under various conditions that are effective for releasing periplasmic proteins from other bacteria [4,15,20,21]. The treated bacteria were then broken by passage

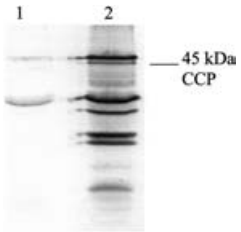


**Figure 1 Location of CCP in the gonococcus**

Oxygen-limited cultures of *N. gonorrhoeae* strain F62 grown in the presence of 5 mM nitrite were harvested and lysed by passage through a French press. Membranes and soluble fractions were separated by high-speed centrifugation and the inner membranes were dissolved in 0.25 % sarkosyl. Proteins (300 $\mu$g) in the inner and outer membrane fractions were separated by SDS/PAGE and stained for haem-dependent peroxidase activity. Lane 1, sarkosyl-insoluble outer membranes; lane 2, sarkosyl-soluble inner membranes.

through a French press, and soluble, cytoplasmic proteins were separated from the membrane fraction by centrifugation. The localization of *c*-type cytochromes in the various fractions was determined by separating proteins by SDS/PAGE and staining gels for covalently bound haem.

At least six *c*-type cytochromes, including the putative CCP, were readily detected in unbroken bacteria, but in none of these preliminary experiments were *c*-type cytochromes released into the soluble, periplasmic fraction. All six cytochromes always remained associated with the membrane fraction and, as expected, none of them were found in the soluble, cytoplasmic fraction. However, up to 50 % of the total membrane-associated cytochrome was released by washing membranes with 0.5 % sodium deoxycholate, including nearly all of the CCP. Neither CCP nor other *c*-type cytochromes were released by washing the membranes with 2 M NaCl. All six cytochromes were solubilized with 0.25 % sarkosyl, a treatment that separates integral membrane proteins associated with the inner and outer membranes of Gram-negative bacteria (Figure 1). Gels stained with Coomassie Blue showed that known gonococcal outer membrane proteins, such as porin, were enriched and abundant in the sarkosyl-insoluble, outer membrane fraction (results not shown).

These experiments confirmed that CCP, as well as other *c*-type cytochromes, are associated with the gonococcal membrane fraction and are sarkosyl-soluble. Possible explanations for this, and for earlier controversies concerning the location of CCP in different pseudomonads, were therefore sought.

### Predicted N-terminal sequences of CCPs from various bacteria

DNA sequence databases for complete and incomplete genomes were searched for putative open reading frames similar to that of *N. gonorrhoeae* CCP using a PSI-BLAST search on Virulogenome (http://www.vge.ac.uk/). More than 20 potential CCP proteins were identified, including known CCP proteins from *Paracoccus denitrificans*, *Methylococcus capsulatus*, *Mesorhizobium loti*, *Pseudomonas aeruginosa* and *Nitrosomonas europaea*. Candidates were also found in the genomes of other bacteria, including *E. coli*, *Helicobacter pylori*, *Bacteroides fragilis*, *Aquifex aeolicus* and *Salmonella typhimurium*. Inspection of the N-terminal amino acid sequences revealed two subgroups (Figure 2). The N-terminal amino acid sequences of all of the previously characterized CCPs include typical signal peptides that, after secretion by the Sec general secretion pathway, would be cleaved by signal peptidase I to generate soluble, periplasmic proteins. In contrast, the putative CCPs from *E. coli*, *Salmonella typhimurium*, *H. pylori* and *N. gonorrhoeae* contained possible targets for signal peptidase II (Figure 2), which cleaves the leader sequence immediately before an essential, conserved cysteine residue. Signal sequences cleaved by peptidase II are usually shorter than those cleaved by peptidase I, and the consensus sequence of the cleavage site is Leu-Ala/Ser-Ala/Gly-Cys [26,27]. The prediction that gonococcal CCP is a lipoprotein is consistent with its previously reported blocked N-terminus and its association with the membrane rather than with the periplasmic fraction [15]. The presence of a potential lipoprotein signal sequence at the N-terminus of CCP from *P. fluorescens* would also explain its peripheral association with the membrane [11]. In contrast, CCP from *P. aeruginosa* is synthesized with a signal peptide that is cleaved by signal peptidase I, consistent with its targeting to the periplasm [28]. Note, however, that although the gonococcal signal sequence includes a perfect match to the Leu-Ala/Ser-Ala/Gly-Cys consensus, there is considerable divergence from the consensus in the six other cysteine-containing sequences. Direct experimental proof that any or all of these CCPs are lipoproteins is therefore required.

Most of the bacterial CCP proteins studied so far are di-haem *c*-type cytochromes. The amino acid sequence of the gonococcal CCP includes only five cysteine residues, including the predicted N-terminal cysteine that would be the site of lipid modification in the mature protein. The other four cysteines occur in typical Cys-Xaa-Xaa-Cys-His motifs required for the covalent attachment of haem in the bacterial periplasm. Gonococcal CCP is therefore also predicted to be a di-haem *c*-type cytochrome. In contrast, CCPs from *E. coli*, *Bacillus fragilis* and *Haemophilus ducreyi* contain N-terminal extensions of 70–80 amino acids that, at least in the case of the first two bacteria, include a third potential haem-binding motif. Note that there is no correlation between the presence of this third putative haem-binding site and the presence of the possible cleavage site for signal peptidase II (Figure 2).

### Cloning and overexpression of the gonococcal *ccp* gene in *E. coli*

The gonococcal *ccp* gene was cloned into a series of expression vectors so that both the predicted processing events and the cellular location could be confirmed. The *ccp* gene was amplified by PCR using chromosomal DNA from *N. gonorrhoeae* strain F62 as the template, and cloned into three expression vectors. First CCP was expressed from the pBR322 derivative, plasmid pST201, under the control of the promoter of the *E. coli fnr* gene. After transformation into *E. coli* strain JCB7124, transformants were grown anaerobically in the presence of nitrate; protein expression was then analysed by SDS/PAGE and gels were stained for covalently bound haem. A clear band of a cytochrome of approx. 45 kDa that corresponded to CCP was detected in transformed bacteria (results not shown). This cytochrome was absent from the untransformed host. This confirmed that gonococcal CCP could be expressed and was not toxic to the heterologous host.

To increase the level of CCP produced by *E. coli*, the gonococcal *ccp* gene was cloned into two overexpression vectors, pET-11c and pBAD-Myc/His. The pBAD-Myc/His derivative pST202 was transformed into JCB7124. Transformants were grown anaerobically in the presence of nitrate and the expression of CCP was induced with arabinose. Bacteria were disrupted in the French press, and membrane and soluble fractions were separated by centrifugation. Proteins in each fraction were separated by

| Bacteria | N-terminal sequence: 30 amino acids | Compared with Ng_CCP | |
|---|---|---|---|
| | | Identity | Similarity |
| *Neisseria gonorrhoeae* | MSFKLRYLASVLALSSL**LAAC**GGQEKSAAG | 100 | 100 |
| *Silibacter pomeroyi* | MSPKSLFSFSLT**ALSC**TAGLAAANPLREQA | 45 | 61 |
| *Escherichia coli* K-12 | MKMVSRITAIGLAG**VAIC**YLGLSGYVWYHD | 45 | 64 |
| *Salmonella typhimurium* | MKKITLYATTVITV**GLLC**YLGLSGYVWYYD | 40 | 59 |
| *Helicobacter pylori* | MKKSILLGVCL**AFSC**AHALNDLELIKKARE | 36 | 54 |
| *Pseudomonas fluorescens* | MRKVRATTSAKPCRWTP**LPDC**WRPLSLVRG | 45 | 61 |
| *Pseudomonas putida* | MFAQTTCRFRSVLHPQR**LRRC**LSLWVLLVL | 43 | 59 |
| *Aquifex aeolicus* | MRKGLLLMAVFAGFVVAKEKIDDKELLKMA | 53 | 69 |
| *Pseudomonas aeruginosa* | MQSSQLLPLGSLLLSFATPLAQADALHDQA | 47 | 63 |
| *Mesorhizobium loti* | MASVQNAGRSLVKILFTTVAVALPAIAFAA | 44 | 63 |
| *Geobacter sulfurreducens* | MKTRNLAWGMALTLVAGVAWGKEDVMKRAQ | 45 | 61 |
| *Magnetococcus* MC1 | MDHPFNAIWRTAVKKSIALFAVLALAPLSG | 42 | 62 |
| *Rhodobacter sphaeriodes* | MRLTLTVLIATTALAGAAQADALRDKALGY | 43 | 60 |
| *Methylococcus capsulatus* | MFTLRLLTGALALAGVSAAVADWQALPAKA | 45 | 59 |
| *Ralstonia metallidurans* | MKSSRSRVLSTFSTATFITVLFAATVSSAH | 45 | 62 |
| *Nitrosomonas europea* | MIKRTLTVSLLSLSLGAMFASAGVMAANEP | 44 | 63 |
| *Shewanella putrefaciens* | MTKLTAIAITLTAIFASSYAVAAEPIEVIT | 46 | 65 |
| *Vibrio cholerae* | MKVVLTSLSLAISLSLAGYAFAASPRNEPV | 42 | 59 |
| *Campylobacter jejuni* | MKVKSLLIASLVAFSSLNAASLIDEAKNSG | 41 | 57 |
| *Yersinia pestis* | MIRRGLIGGGILVIAGYLGIAGYLYMTDNT | 37 | 55 |
| *Haemophilus ducreyi* | MKKYLLSALAVGGIGYFSLVGYAYWFDTEQ | 39 | 58 |
| *Bacteroides fragilis* | MKKSTKFIIALLVTVGALAITYRVVNQAPS | 43 | 60 |
| *Paracoccus denitrificans* | MLRLACLAPLAILIPAAGTAEQARPADDAL | 43 | 34 |

**Figure 2 Predicted N-terminal peptide sequences of known and putative CCP proteins**

Shown are the results of a PSI-BLAST search using virulogenome and the predicted amino acid sequence of CCP from *N. gonorrhoeae* (Ng_CCP). The N-terminal sequences revealed two subgroups with potential cleavage sites for signal peptidase I or II. Possible cleavage sites for signal peptidase II are highlighted.

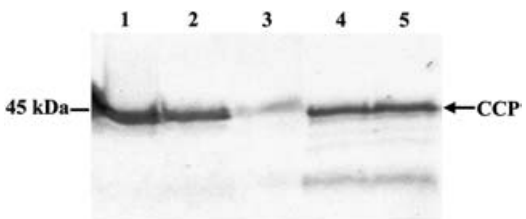


**Figure 3 Location of CCP in *E. coli***

*E. coli* strain JCB7124 transformed with plasmid pST202 was grown anaerobically in minimal medium supplemented with 10 % (v/v) Lennox broth, 0.4 % (v/v) glycerol, 40 mM fumarate and 5 mM nitrate to $A_{650} = 0.5$. Expression of CCP from plasmid pST202 was induced with 0.02 % (w/v) arabinose for 6 h. Bacteria were harvested and fractionated, and 300 $\mu$g portions of protein were separated by SDS/PAGE. The gel was stained for haem-dependent peroxidase activity. Lane 1, bacterial suspension; lane 2, unbroken bacteria and inclusion bodies; lane 3, soluble fraction; lanes 4 and 5, membrane fraction.

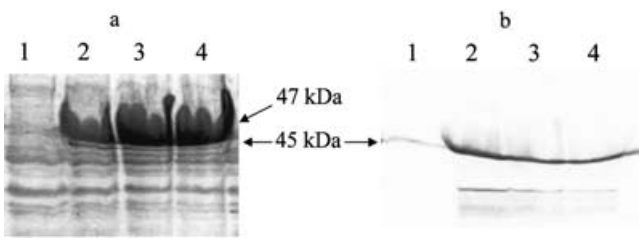


**Figure 4 Expression of two proteins from pST203 after induction with IPTG**

*E. coli* strain BL21(λDE3) doubly transformed with the *ccp* plasmid pST203 and the *ccm* plasmid pST2 was grown aerobically in Lennox Broth and the expression of CCP was induced at $A_{650} = 0.5$ with 0.5 mM IPTG. Proteins (300 $\mu$g) from lysed bacteria were separated by SDS/PAGE and stained (**a**) with Coomassie Blue or (**b**) for haem-dependent peroxidase activity. Lanes 1, uninduced bacteria; lanes 2–4, induced bacteria after 1, 2 and 3 h respectively.

SDS/PAGE and stained for covalently bound haem (Figure 3). A clear band of a 45 kDa cytochrome was detected in unbroken bacteria and in the membrane fraction of induced, transformed bacteria (Figure 3, lanes 1, 2, 4 and 5). This band was faint in uninduced fractions and almost absent from the soluble fraction of induced bacteria (Figure 3, lane 3). Using low concentrations of detergent (e.g. 0.1 % sodium deoxycholate), the 45 kDa *c*-type cytochrome could be solubilized, indicating that it was only loosely associated with the membrane. The membrane association of gonococcal CCP in *E. coli* therefore correlates with its location in the gonococcus.

Very high levels of an approx. 47 kDa protein accumulated after induction of CCP expression from the pET-11c derivative pST203; a faint 45 kDa band was also just visible in Coomassie-stained gels (Figure 4a). The expression of two different sized proteins suggested that CCP is synthesized initially with a signal peptide that is cleaved. Only the 45 kDa band stained for covalently bound haem (Figure 4b), and this cytochrome was associated exclusively with the membrane fraction. In contrast, after breaking bacteria in the French press, the 47 kDa component sedimented with unbroken bacteria during low-speed centrifugation, showing that it was unprocessed pre-apo-CCP that had accumulated in inclusion bodies in the cytoplasm. These experiments clearly demonstrated that the rate of secretion into the periplasm was rate-limiting for the formation of mature CCP. Furthermore, irrespective of the expression vector used, increased production of mature CCP was subsequently achieved by growing

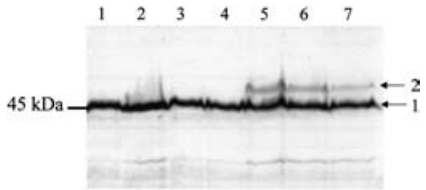


**Figure 5 Effect of globomycin on the production of mature CCP**

Whole-cell proteins (300 $\mu$g) from BL21(λDE3) pST2 pST203 were separated by SDS/PAGE and stained for haem-dependent peroxidase activity. Transformed bacteria were grown aerobically in Lennox Broth to $A_{650} = 0.5$ and expression of CCP was induced with 0.5 mM IPTG for 1–3 h. Globomycin at a final concentration of 10 $\mu$g/ml was added at induction. Lane 1, CCP marker; lanes 2–4, induced bacteria (no globomycin) after 1, 2 and 3 h respectively; lanes 5–7, induced bacteria in the presence of globomycin after 1, 2 and 3 h respectively. The 45 kDa band (labelled 1 on the right of the Figure) corresponds to mature CCP from which the signal peptide had been cleaved; the approx. 47 kDa band (2) corresponds to CCP with its signal peptide still attached.

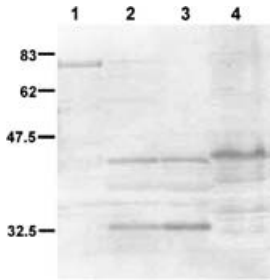


**Figure 6 Products of MalE–CCP cleaved with Factor Xa**

Purified MalE–CCP was cleaved with Factor Xa for 2 or 4 h at room temperature. Protein products were detected by SDS/PAGE and stained for haem-dependent peroxidase activity. Lane 1, uncleaved MalE–CCP; lane 2, MalE–CCP cleaved with Factor Xa for 2 h; lane 3, MalE–CCP cleaved with Factor Xa for 4 h; lane 4, membrane-bound mature CCP. Positions of molecular mass markers (kDa) are shown on the left.

transformants into stationary phase without induction (results not shown). This resulted in far less accumulation of unprocessed CCP in the cytoplasm, and a 10-fold increase in the amount of membrane-associated mature 45 kDa CCP.

### Identification of gonococcal CCP as a lipoprotein

Expression of CCP from pST203 showed that a signal peptide is cleaved from CCP as part of the post-translational modifications, and it has been predicted that peptidase II cleaves this signal peptide. The peptide antibiotic, globomycin, inhibits signal peptidase II, resulting in the accumulation of pro-lipoproteins in the cytoplasmic membrane [29–31]. In the absence of globomycin, bacteria accumulated only the 45 kDa mature CCP (Figure 5, lanes 2–4). After induction of CCP expression from pST203 in the presence of 10 $\mu$g/ml globomycin, the bacteria accumulated two proteins with covalently attached haem, of approx. 45 and 47 kDa (Figure 5, lanes 5–7). The 45 kDa band corresponds to mature CCP and the 47 kDa band to unprocessed CCP. The appearance of this new form of CCP is consistent with the transfer of pre-apo-lipoprotein to the periplasm, where haem was attached, but cleavage of the signal peptide was inhibited. It is also apparent that globomycin inhibited cleavage of the gonococcal CCP signal peptide. Furthermore, the accumulation of a haem-containing pre-lipoprotein suggests that, under these conditions, the rate of signal peptide cleavage, rather than the rates of secretion or haem attachment, limits the rate of formation of mature CCP.

### Expression and characterization of a soluble MalE–CCP fusion protein

Horse heart cytochrome *c* can be used as a surrogate reductant for CCP from many, but not all, bacteria, providing a convenient assay for CCP activity [24]. However, CCP is poorly expressed in gonococci [15], and in both gonococci and *E. coli* it is associated with membranes that rapidly oxidize horse heart cytochrome *c* even in the absence of hydrogen peroxide. Consequently, it was impossible to use the previously published methods to assay gonococcal CCP directly. Expression as a soluble, periplasmic MalE–CCP fusion protein provided two alternative approaches to demonstrating CCP activity.

First, periplasmic proteins released from *E. coli* strain JCB7124 transformed with pST204 were assayed for $H_2O_2$-dependent cytochrome *c* oxidation both before and after cleavage of the N-terminal MalE fusion partner with Factor Xa. Before cleavage, no activity was detected with up to 80 $\mu$M hydrogen peroxide, and higher concentration of peroxide bleached the haem of horse heart cytochrome *c*. After cleavage of the purified CCP with Factor Xa, a low rate of $H_2O_2$-dependent cytochrome *c* oxidation was detected. The rate was still extremely low, however, suggesting either that horse heart cytochrome *c* is a poor electron donor for gonococcal CCP or that the lipid modification is essential for peroxidase activity. Incubation with Factor Xa resulted in two major haem-containing cleavage products, indicating the presence of a secondary Factor Xa cleavage site within CCP (Figure 6). Significantly, however, the larger band was the size expected for the CCP cleavage product and, despite the presence of five extra N-terminal amino acids from the fusion linker, was slightly smaller than the membrane-associated gonococcal CCP expressed in *E. coli*. This result provided further evidence for post-translational modification of the gonococcal CCP.

The alternative approach to demonstrating CCP activity was to demonstrate rapid oxidation of reduced CCP by hydrogen peroxide. The MalE–CCP in freshly isolated periplasmic proteins was oxidized, with no absorption maxima in the visible region of the spectrum (Figure 7A). Absorption maxima at 525 and 554 nm were observed with ascorbate-reduced CCP, but the absorbance increased on addition of the more powerful reductant dithionite, and the absorption maximum decreased from 553.7 to 551.4 nm. Addition of 50 $\mu$M hydrogen peroxide instantly restored the ascorbate-reduced spectrum, consistent with the expectation that only one of the two haem groups is oxidized as the substrate binds to the active site (Figure 7B) (see also [1,32,33]). An interesting feature of the hydrogen peroxide re-oxidized CCP is that ferricyanide failed to regenerate the fully oxidized cytochrome, even after prolonged incubation.

### Construction and phenotype of a gonococcal CCP mutant

The translation start codon and 800 bp of the *ccp* gene were deleted from pST101 and replaced by a 1.2 kb erythromycin-resistance cassette. The resulting plasmid, pST103, was used as the template to amplify a linear fragment that included the erythromycin-resistance cassette flanked by 550 bp of gonococcal chromosomal DNA from upstream of the *ccp* gene and 650 bp of downstream DNA. This linear fragment was transformed into naturally competent piliated strain F62, and erythromycin-resistant recombinants were selected, purified and screened by

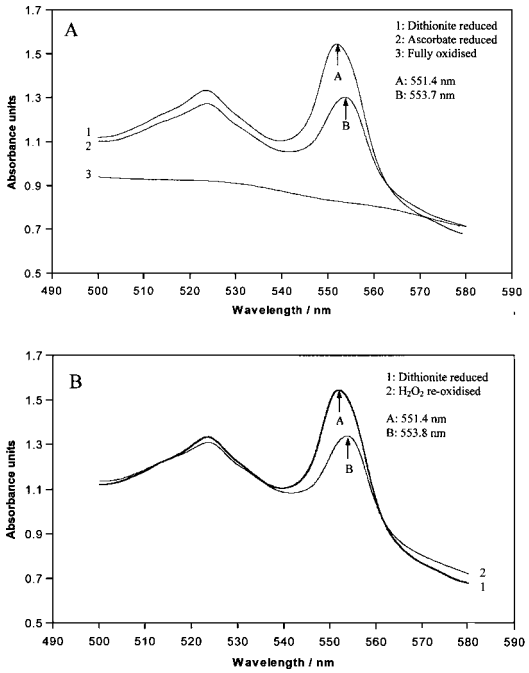


**Figure 7 Spectral analysis of periplasmic MalE–CCP**

(**A**) Oxidized, ascorbate-reduced and dithionite-reduced spectra of the periplasmic fraction of JCB7124 pST204 containing MalE–CCP. (**B**) Dithionite-reduced and 50 $\mu$M hydrogen peroxide re-oxidized spectra of the periplasmic fraction of JCB7124 pST204 containing MalE–CCP.

PCR for replacement of the parental *ccp* gene by the interrupted gene.

In contrast with strain F62, which accumulated six *c*-type cytochromes including the 45 kDa CCP, only five bands were found with the mutant bacteria. The ability of the *ccp* mutant (JCGC211) and the parental strain (F62) to survive exposure to hydrogen peroxide was then compared. Each strain was spread as a lawn on to a fresh GC agar plate supplemented with 2 mM sodium nitrite and seeded with a central filter paper disc that had been impregnated with 5 $\mu$l of 0.88 M hydrogen peroxide. After 4 days' growth in an anaerobic jar, the area of growth inhibition was calculated. The area was slightly greater around the disc on plates seeded with the mutant compared with the parental strain, suggesting the CCP had been expressed in the parental strain and was partially able to protect the gonococci (Figure 8). However, this effect was too small to be considered significant.

### Construction and phenotype of a gonococcal catalase mutant and a *kat ccp* double mutant

Catalase protects many bacteria from damage caused by hydrogen peroxide production. Gonococci are catalase-positive, and after exposure to hydrogen peroxide a catalase mutant of *N. gonorrhoeae* showed extensive single-strand breakages in chromosomal and plasmid DNA [34]. Any protective effect against hydrogen peroxide due to CCP might have been masked by the presence of catalase in the *ccp* mutant. A mutation of the *kat* gene was therefore introduced into the parental strain, F62, and into the *ccp* mutant. Being adjacent on the chromosome, *kat* and *ccp* were both deleted by insertion of a single fragment of DNA into F62. All of the expected cytochromes with covalently bound haem were clearly detectable in the *kat* single mutant, but the 45 kDa CCP band was missing from the *kat ccp* double

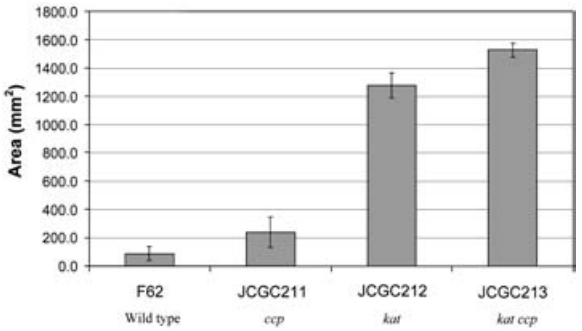


**Figure 8 Growth inhibition in response to hydrogen peroxide of the gonococcal parental strain F62 and mutants defective in catalase and/or CCP**

A lawn of bacteria was spread on to a fresh GC agar plate supplemented with 2 mM nitrite. An 8 mm filter paper disc was inoculated with 5 $\mu$l of 0.88 M hydrogen peroxide. The plates were incubated in an anaerobic jar at 37 °C for 4 days. The area of growth inhibition for each strain was then calculated. Error bars are the S.D.s of triplicates. Strain JCGC211 is defective in *ccp*, strain JCGC212 is defective in catalase and strain JCGC213 is a *kat ccp* double mutant.

mutant. The effects of hydrogen peroxide on the resulting strains, JCGC212 and JCGC213, were compared (Figure 8). Both of the catalase-defective strains were far more sensitive to hydrogen peroxide than the *kat*$^+$ parental strains, but the *ccp* deletion in a *kat* background again slightly increased the zone of growth inhibition. These data suggest that hydrogen peroxide might be less damaging in the periplasm, where it is removed by CCP, than in the cytoplasm, where it is removed by catalase. If so, the physiological role of CCP might be to protect gonococci from peroxides generated during their life cycle and as part of the immune response in the human body.

### Effect of nitrite on expression of the gonococcal *ccp* gene

Preliminary experiments reported by Lissenden et al. [15] indicated that expression of CCP is unaffected by the presence of nitrite during oxygen-limited growth. In *N. gonorrhoeae* strain JCGC201, a translational fusion of the *ccp* promoter to a *lacZ* reporter gene has been integrated by homologous recombination into the *proAB* region of the chromosome. This strain was grown in oxygen-limited GC broth in the presence or absence of 5 mM sodium nitrite, and expression from the *ccp* promoter was monitored throughout growth. For both strains, expression increased with increasing absorbance of the culture, presumably because of increased activation of the gonococcal FNR with increasing oxygen starvation. However, in contrast with the indications from preliminary experiments, the activities of cultures supplemented with nitrite were only about half those of the unsupplemented cultures (Figure 9). The gonococcal NarP might therefore be a repressor rather than a transcription activator at the *ccp* promoter.

## DISCUSSION

It was reported previously that a membrane-associated *c*-type cytochrome is induced in the gonococcus during oxygen-limited growth [15]. Its N-terminus was blocked, but sequencing by MS revealed that it was the product of the putative CCP gene, *ccp*. The N-terminus of CCP consists of a classic lipoprotein signal peptide with the lipobox cleavage site Leu-Ala-Ala-Cys. To investigate the prediction from sequence analysis that CCP

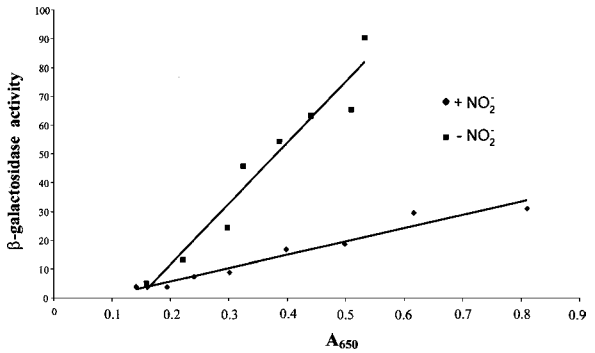


**Figure 9 Effect of nitrite during oxygen-limited growth on expression from the gonococcal *ccp* promoter**

Triplicate oxygen-limited liquid cultures of *N. gonorrhoeae* strain JCGC201 were grown in the presence or absence of 5 mM nitrite ($NO_2^-$). The activity of the *ccp* promoter throughout the growth cycle was determined by measuring $\beta$-galactosidase activity expressed from the *ccp* promoter of the *ccp*::*lacZ* fusion strain JCGC201.

is a lipoprotein, the coding sequence was cloned into three *E. coli* expression vectors. The 45 kDa *c*-type cytochrome was successfully expressed in *E. coli*, indicating that it was not toxic to the heterologous host. When CCP expression was induced to a higher level, two proteins were produced. The larger, approx. 47 kDa protein was located in inclusion bodies and corresponded to the pro-apo-cytochrome. It had not crossed the cytoplasmic membrane, no haem had been attached and the signal peptide had not been cleaved. However, in the presence of globomycin, a specific inhibitor of signal peptidase II, bacteria accumulated a 47 kDa protein with covalently attached haem. This corresponds to CCP without its signal peptide cleaved, confirming that in *E. coli* gonococcal CCP is processed as a pro-lipoprotein and that the signal peptide is cleaved by peptidase II. The smaller, 45 kDa protein was associated with the membrane with haem covalently bound. This corresponded to the mature CCP lipoprotein that had been exported across the cytoplasmic membrane by the Sec-dependent pathway, and its signal sequence had been cleaved by signal peptidase II. Haem is covalently attached to all *c*-type cytochromes in the periplasm of Gram-negative bacteria, indicating that gonococcal CCP must have been on the periplasmic side of the cytoplasmic membrane. The 45 kDa mature cytochrome *c* is only loosely associated with the *E. coli* membrane, presumably by the covalent lipid modification of the N-terminal cysteine residue. The identification of gonococcal CCP as a lipoprotein explains its membrane association and blocked N-terminus.

The cysteine of the lipobox of prolipoproteins is covalently modified post-translationally, resulting in an *N*-acyldiacylglycerylcysteine at the N-terminus [35,36]. Apolipoprotein diacylglyceryl transferase adds a diacylglyceryl to the cysteine to form diacylglycerylcysteine. This modification is an essential prerequisite for the cleavage of the signal peptide by peptidase II. The modified cysteine is then N-acylated by apolipoprotein N-acyltranferase; membrane phospholipids are the donors for this step [37]. All three processing enzymes, apolipoprotein diacylglyceryl transferase (Lgt), signal peptidase II (Lsp) and apolipoprotein N-acyltransferase (Lnt), appear to be essential for apolipoprotein processing in *E. coli* [35]. Analysis of the gonococcal genome revealed predicted open reading frames homologous to *E. coli* Lgt, Lsp and Lnt proteins. The predicted open reading frame for Lgt was 61 % similar and 50 % identical to *E. coli* Lgt, and the essential histidine at His-103 and important tyrosine at Tyr-235 are also conserved [38]. The predicted open reading frame for Lsp was 54 % similar and 43 % identical to *E. coli* Lsp, and the predicted open reading frame of Lnt was 44 % similar and 33 % identical to the corresponding *E. coli* protein. This suggests that the gonococcus processes pro-lipoproteins by a similar mechanism to *E. coli*.

Although the gonococcal CCP was expressed as a membrane-associated sarkosyl-soluble protein, it is premature to conclude that it is located in the gonococcal cytoplasmic membrane. From early lipoprotein studies, it was concluded that lipoproteins in which the +2 amino acid of the mature protein is aspartate are targeted to the cytoplasmic membrane, but that all other lipoproteins are localized in the outer membrane [39]. However, subsequent mutagenesis studies established that other residues at +2, including glycine, can result in retention in the cytoplasmic membrane [40]. Conversely, although integral outer membrane proteins are sarkosyl-insoluble, loosely attached lipoproteins are likely to be soluble. Consequently, whether the gonococcal CCP is attached to the inner or outer membrane remains uncertain.

Gonococcal CCP is the first documented example of a CCP lipoprotein in Gram-negative bacteria. Following the gonococcal work, we identified a number of putative CCP lipoproteins from other bacteria, including *E. coli*, *Salmonella enterica* and *H. pylori* (S. Turner, unpublished work). This defines a new subclass of bacterial CCPs, but raises the question of why lipid attachment is advantageous. Gram-positive bacteria, which do not have a true periplasm, synthesize many more lipoproteins than Gram-negative bacteria. The lipid modification anchors proteins to the membrane, allowing them to fulfil a variety of functions, such as substrate binding, antibiotic resistance and environmental sensing, that are fulfilled by periplasmic proteins in Gram-negative bacteria [41]. So what might be the advantage of lipid modification of CCP in a Gram-negative bacterium? First, it is possible that it serves a similar purpose as in Gram-positive bacteria. However, as the electron donors for CCPs are always reduced *c*-type cytochromes, which for gonococci are all located in, or associated with, the cytoplasmic membrane, the lipid modification might be required to maintain gonococcal CCP in close proximity to its electron donor. If the role of CCP is to protect the bacteria from hydrogen peroxide, it could be important in pathogenicity. The lipid modification could be essential to anchor the protein to the membrane and maintain it where its protective mechanism is essential.

This work was supported by a Research Studentship from the U.K. Biotechnology and Biological Sciences Research Council to S. T., and Project Grant 6/PRS12198 to J. C. The assistance of Deborah Eaves, Saahil Mehta and Elizabeth Cooper in the construction of the *ccp* and *ccm* expression plasmids, the help of Tim Overton with $\beta$-galactosidase assays, and helpful discussions of lipoprotein processing with Professor Colin Harwood, Professor Steve Busby and Professor Charles Penn are gratefully acknowledged.

## REFERENCES

1 Alves, T., Besson, S., Duarte, L. C., Pettigrew, G. W., Girio, F. M. F., Devreese, B., Vandenberghe, I., Van Beeumen, J., Fauque, G. and Moura, I. (1999) A cytochrome *c* peroxidase from *Pseudomonas nautica* 617 active at high ionic strength: expression, purification and characterization. Biochim. Biophys. Acta **1434**, 248–259

2 Arciero, D. M. and Hooper, A. B. (1994) A di-heme cytochrome *c* peroxidase from *Nitrosomonas europaea* catalytically active in both the oxidized and half reduced states. J. Biol. Chem. **269**, 11878–11886

3 Ellfolk, N. and Soininen, R. (1970) *Pseudomonas* cytochrome *c* peroxidase. Acta Chem. Scand. **24**, 2126–2136

4 Goodhew, C. F., Wilson, I. B. H., Hunter, D. J. B. and Pettigrew, G. W. (1990) The cellular location and specificity of bacterial cytochrome *c* peroxidases. Biochem. J. **271**, 707–712

5 Hu, W., De Smet, L., Van Driessche, G., Bartsch, R. G., Meyer, T. E., Cusanovich, M. A. and Van Beeumen, J. (1998) Characterization of cytochrome *c*-556 from the purple phototrophic bacterium *Rhodobacter capsulatus* as a cytochrome *c* peroxidase. Eur. J. Biochem. **258**, 29–36

6 Pettigrew, G. W. (1991) The cytochrome *c* peroxidase of *Paracoccus denitrificans*. Biochim. Biophys. Acta **1058**, 25–27

7 Zahn, J. A., Arciero, D. M., Hooper, A. B., Coats, J. R. and DiSpirito, A. A. (1997) Cytochrome *c* peroxidase from *Methylococcus capsulatus* Bath. Arch. Microbiol. **168**, 362–372

8 Fulop, V., Ridout, C. J., Greenwood, C. and Hajdu, J. (1995) Crystal structure of the di-haem cytochrome *c* from *Pseudomonas aeruginosa*. Curr. Biol. **3**, 1225–1233

9 Shimizu, H., Schuller, D. J., Lanzilotta, W. M., Sundaramoorthy, M., Arciero, D. M., Hooper, A. B. and Poulos, T. L. (2001) Crystal structure of *Nitrosomonas europaea* cytochrome *c* peroxidase and the structural basis for ligand switching in bacterial di-heme peroxidases. Biochemistry **40**, 13483–13490

10 Hanlon, S. P., Holt, R. A. and McEwan, A. G. (1992) The 44-kDa *c*-type cytochrome induced in *Rhodobacter capsulatus* during growth with dimethylsulphoxide as an electron acceptor is a cytochrome *c* peroxidase. FEMS Microbiol. Lett. **97**, 283–288

11 Soininen, R., Sojonen, H. and Ellfolk, N. (1970) Pseudomonas cytochrome *c* peroxidase. Acta Chem. Scand. **24**, 2314–2320

12 Van Spanning, R. J. M., De Boer, A. P. N., Reijnders, W. N. M., Westerhoff, H. V., Stouthamer, A. H. and Van Der Oost, J. (1997) FnrP and NNR of *Paracoccus denitrificans* are both members of the FNR family of transcriptional activators but have distinct roles in respiratory adaption in response to oxygen limitation. Mol. Microbiol. **23**, 893–907

13 Cardinale, J. A. and Clark, V. L. (1998) Expression of the *Neisseria gonorrhoeae* outer membrane protein AniA in *Escherichia coli* JCB7121 (*nirB Δnrf* ) results in nitrite reductase activity. Proceedings of the 11th International Pathogenic Neisseria Conference, p. 309, EDK Editions Médicales et Scientifiques, Paris

14 Knapp, J. S. and Clark, V. (1984) Anaerobic growth of *Neisseria gonorrhoeae* coupled to nitrite reduction. Infect. Immun. **46**, 176–181

15 Lissenden, S., Mohan, S., Overton, T., Regan, T., Crooke, H., Cardinale, J. A., Householder, T. C., Adams, P., O'Connor, C. D., Clark, V. et al. (2000) Identification of transcription activators which regulate gonococcal adaption from aerobic to anaerobic or oxygen-limited growth. Mol. Microbiol. **37**, 839–855

16 Mellies, J., Jose, J. and Meyer, T. F. (1997) The *Neisseria gonorrhoeae* gene *aniA* encodes an inducible nitrite reductase. Mol. Gen. Genet. **256**, 525–532

17 Householder, T. C., Belli, W. A., Lissenden, S., Cole, J. A. and Clark, V. (1999) *cis*- and *trans*-Acting elements involved in regulation of *aniA*, the gene encoding the major anaerobically induced outer membrane protein in *Neisseria gonorrhoeae*. J. Bacteriol. **181**, 541–551

18 Nassif, X., Puaoi, D. and So, M. (1991) Transposition of Tn*1545-Δ3* in the pathogenic Neisseriae: a genetic tool for mutagenesis. J. Bacteriol. **173**, 2147–2154

19 Arslan, E., Schulz, H., Zuffrey, R., Kunzler, P. and Thony-Meyer, L. (1998) Over-production of the *Bradyrhizobium japonicum c*-type cytochrome subunits of the $cbb_3$ oxidase in *Escherichia coli*. Biochem. Biophys. Res. Commun. **251**, 744–747

20 McEwan, A. G., Jackson, J. B. and Ferguson, S. J. (1984) Rationalization of properties of nitrate reductases in *Rhodopseudomonas capsulata*. Arch. Microbiol. **137**, 344–349

21 Hunter, D. J., Brown, K. R. and Pettigrew, G. W. (1989) The role of cytochrome c4 in bacterial respiration. Cellular location and selective removal from membranes. Biochem. J. **262**, 233–240

22 Lowry, O. H., Rosenberg, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275

23 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

24 Gilmour, R., Goodhew, C. F., Pettigrew, G. W., Prazeres, S., Moura, J. J. G. and Moura, I. (1994) The kinetics of the oxidation of cytochrome *c* by *Paracoccus* cytochrome *c* peroxidase. Biochem. J. **300**, 907–914

25 King, K. Y., Horenstein, J. A. and Caparon, M. G. (2000) Aerotolerance and peroxide resistance in peroxidase and PerR mutants of *Streptococcus pyogenes*. J. Bacteriol. **182**, 5290–5299

26 Braun, V. and Wu, H. C. (1993) Lipoproteins: structure, function, biosynthesis and model for protein export. In New Comprehensive Biochemistry, vol. 27: Bacterial Cell Wall (Ghuysen, J.-M. and Hachenbecks, R., eds.), pp. 319–341, Elsevier, Amsterdam

27 Hayashi, S. and Wu, H. C. (1990) Lipoproteins in bacteria. J. Bioenerg. Biomembr. **22**, 451–471

28 Ridout, C. J., James, R. and Greenwood, C. (1995) Nucleotide sequence encoding the di-haem cytochrome $c_{551}$ peroxidase from *Pseudomonas aeruginosa.* FEBS Lett. **365**, 152–154

29 Dev, I. K., Harvey, R. J. and Ray, P. H. (1985) Inhibition of prolipoprotein signal peptidase by globomycin. J. Biol. Chem. **260**, 5891–5894

30 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

31 Inukai, M., Enookita, 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

32 Foote, N., Peterson, J., Gadsby, P. M. A., Greenwood, C. and Thomson, A. J. (1985) Redox-linked spin-state changes in the di-haem cytochrome *c*-551 peroxidase from *Pseudomonas aeruginosa*. Biochem. J. **230**, 227–237

33 Ellfolk, N., Ronnberg, M. and Osterlund, K. (1991) Structural and functional features of *Pseudomonas* cytochrome *c* peroxidase. Biochim. Biophys. Acta **1080**, 68–77

34 Johnson, S. R., Steiner, B. M., Cruce, D. D., Perkins, G. H. and Arko, R. J. (1993) Charaterisation of a catalase-deficient strain of *Neisseria gonorrhoeae*: evidence for the significance of catalase in the biology of *N. gonorrhorae*. Infect. Immun. **61**, 1232–1238

35 Sankaran, K. and Wu, H. C. (1994) Lipid modification of bacterial prolipoprotein. J. Biol. Chem. **269**, 19701–19706

36 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

37 Hankte, K. and Braun, V. (1973) Covalent binding of lipid to protein. Eur. J. Biochem. **34**, 284–296

38 Sankaran, K., Gan, K., Rash, B., Qi, H.-Y., Wu, H. C. and Rick, P. D. (1997) Roles of histidine-103 and tyrosine-235 in the function of the prolipoprotein diacylglyceryl transferase of *Escherichia coli*. J. Bacteriol. **179**, 2944–2948

39 Pugsley, A. P. (1993) The complete general secretory pathway in Gram-negative bacteria. Microbiol. Rev. **57**, 50–108

40 Seydel, A., Gounon, P. and Pugsely, 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

41 Sutcliffe, I. C. and Russell, R. R. B. (1995) Lipoproteins of gram-positive bacteria. J. Bacteriol. **177**, 1123–1128


Received 8 January 2003/16 April 2003; accepted 29 April 2003
Published as BJ Immediate Publication 29 April 2003, DOI 10.1042/BJ20030088