Microbiology (2003), **149**, 3543–3552 DOI 10.1099/mic.0.26566-0

Co-ordination of iron acquisition, iron porphyrin chelation and iron–protoporphyrin export via the cytochrome *c* biogenesis protein CcmC in *Pseudomonas fluorescens*

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Received 13 June 2003
Revised 22 August 2003
Accepted 1 September 2003

The cytoplasmic membrane protein CcmC is, together with other Ccm proteins, a component for the maturation of *c*-type cytochromes in Gram-negative bacteria. A *Pseudomonas fluorescens* ATCC 17400 *ccmC* mutant is cytochrome *c*-deficient and shows considerably reduced production of the two siderophores pyoverdine and quinolobactin, paralleled by a general inability to utilize various iron sources, with the exception of haem. The *ccmC* mutant accumulates in a 5-aminolevulinic acid-dependent synthesis a reddish, fluorescent pigment identified as protoporphyrin IX. As a consequence a *visA* phenotype similar to that of a ferrochelatase-deficient *hemH* mutant characterized by drastically reduced growth upon light exposure was observed for the *ccmC* mutant. The defect of iron–protoporphyrin formation was further demonstrated by the failure of *ccmC* cell-free proteinase K-treated extracts to stimulate the growth of a haem auxotrophic *hemH* indicator strain, compared to similarly prepared wild-type extracts. In addition, the *ccmC* mutant did not sustain *hemH* growth in cross-feeding experiments while the wild-type did. Significantly reduced resistance to oxidative stress mediated by haem-containing catalases was observed for the *ccmC* mutant. A double *hemH ccmC* mutant could not be obtained in the presence of external haem without the *hemH* gene *in trans*, indicating that the combination of the two mutations is lethal. It was concluded that CcmC, apart from its known function in cytochrome *c* biogenesis, plays a role in haem biosynthesis. A function in the regulatory co-ordination of iron acquisition via siderophores, iron insertion into porphyrin via ferrochelatase and iron–protoporphyrin export for cytochrome *c* formation is predicted.

INTRODUCTION

Free-living aerobes, such as the fluorescent pseudomonads, need to produce and excrete high-affinity Fe^{3+}-chelating siderophores to satisfy their need for iron (Braun & Killmann, 1999; Braun & Braun, 2002). Under conditions of iron limitation, fluorescent pseudomonads (among others, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens*) produce different fluorescent peptidic siderophores, named pyoverdines (PVDs) or pseudobactins (Cornelis & Matthijs, 2002; Ravel & Cornelis, 2003). *P. fluorescens* ATCC 17400 produces both a PVD and another, non-fluorescent, siderophore, quinolobactin (QB) (Mossialos *et al.*, 2000; Cornelis & Matthijs, 2002). PVDs are composed of a conserved dihydroxyquinoline chromophore, a variable peptide chain, comprising 6–12 amino acids, specific to a producing strain, and a side-chain, generally a dicarboxylic acid or an amide (Ravel & Cornelis, 2003). Both the chromophore and the peptide chain of PVDs are synthesized by non-ribosomal peptide synthetases (Merriman *et al.*, 1995; Lehoux *et al.*, 2000; Mossialos *et al.*, 2002). Ferribactin, the precursor of PVD, is probably exported to the periplasm where it is matured into PVD with a chromophore (Baysse *et al.*, 2002). Recently, we demonstrated that *de novo* haem biosynthesis is required for the production of PVD in *P. fluorescens* (Baysse *et al.*, 2001).

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Abbreviations: ALA, 5-aminolevulinic acid; CAS, chrome azurol S; PPIX, protoporphyrin IX; PVD, pyoverdine; QB, quinolobactin.

The porphyrin haem in pseudomonads is formed from glutamyl-tRNA via the general precursor molecule 5-aminolevulinic acid (ALA) (Hungerer *et al.*, 1995). Major genetic regulatory points for haem biosynthesis have been found at the initial step of ALA formation via control of *hemA* expression and the late step of coproporphyrinogen III decarboxylation via *hemN* and *hemF* regulation (Rompf *et al.*, 1998; Krieger *et al.*, 2002; Schobert & Jahn, 2002). The final step of haem biosynthesis, the insertion of iron into protoporphyrin IX (PPIX) catalysed by ferrochelatase encoded by *hemH*, was also proposed to be subject to regulation by iron availability (Qi & O'Brian, 2002). We isolated one mutant of *P. fluorescens* ATCC 17400 with a transposon insertion in the *ccmC* gene that showed greatly reduced production of PVD, an impaired maturation of the PVD chromophore, together with a decreased capacity to utilize this particular siderophore (Gaballa *et al.*, 1996, 1998; Baysse *et al.*, 2002). The product of this gene, the cytoplasmic membrane protein CcmC, is primarily involved in the biogenesis of *c*-type cytochromes (Thöny-Meyer, 1997; Kranz *et al.*, 1998). CcmC binds haem in the periplasm, probably via two conserved histidines in the first and the third periplasmic loops, the interaction being stabilized by hydrophobic amino acids in the second periplasmic loop, mostly composed of tryptophan residues (Goldman *et al.*, 1998; Schulz *et al.*, 2000). It has been established that CcmC further delivers haem to another membrane protein, CcmE, considered to be a haem chaperone that passes haem to a haem lyase complex (comprising the CcmF protein) before covalent binding of the haem prosthetic group to apocytochromes *c* (Schulz *et al.*, 1998, 1999, 2000). CcmC has been postulated to be a cytoplasm-to-periplasm haem exporter (Kranz *et al.*, 1998; Goldman *et al.*, 1998) and has been demonstrated to interact with the CcmAB ABC transporter (Goldman *et al.*, 1997). Some results, however, seem to indicate that CcmC can sustain cytochrome *c* biogenesis without CcmA or CcmB (Cook & Poole, 2000; Page & Ferguson, 1999; Schulz *et al.*, 2000). We here present evidence that, in the absence of CcmC, the production of siderophores and their utilization as iron source are impaired. We also demonstrate a reduced production of haem by the *ccmC* mutant, resulting in increased sensitivity to H_2O_2. Finally, a model is proposed where the reduced availability of haem is the source of the pleiotropic defects of *ccmC* mutants.

METHODS

Bacterial strains, vectors and growth conditions. Strains and plasmids used in this study are listed in Table 1. *P. fluorescens* ATCC 17400 and the *ccmC* mutant were maintained in Casamino acids (CAA) medium (Cornelis *et al.*, 1992). Unless otherwise indicated, 50 ml cultures were inoculated from an overnight pre-culture and incubated at 28 °C at 200 r.p.m. (New-Brunswick Innova shaker). Growth was followed in a Bio-Screen apparatus (Life Technologies) using the following parameters: shaking for 10 s every minute, reading every 20 min, temperature 28 °C. When needed, CAA medium was supplemented with ethylenediaminedihydroxyphenylacetic acid [EDDHA (0·5 mg ml^{-1})], purified PVD (50 μM) or ALA. Antibiotics were added to *P. fluorescens* strains at the following concentrations: kanamycin, 200 μg ml^{-1}; chloramphenicol, 300 μg ml^{-1}; tetracycline, 100 μg ml^{-1}; gentamicin, 100 μg ml^{-1}. *Escherichia coli* strains were grown at 37 °C in Luria–Bertani broth (LB) with the appropriate antibiotics: kanamycin, 100 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; tetracycline, 15 μg ml^{-1}.

H_2O_2 resistance. The susceptibility of the wild-type *P. fluorescens* strain and the *ccmC* mutant to H_2O_2 was tested as described previously (Baysse *et al.*, 2000).

DNA methodology. DNA manipulations were done according to standard protocols (Sambrook *et al.*, 1989). Restriction endonucleases, T4 DNA ligase and Klenow fragment (Pharmacia) were used according to the manufacturer's instructions. Sequencing was done by Eurogentec; sequences were analysed using the GENE COMPAR software package (Applied Maths) and the BLAST server of the NCBI or the *Pseudomonas* genome project (http://www.pseudomonas.com).

Siderophores detection. PVD concentration was estimated by fluorescence spectroscopy (Höfte *et al.*, 1993) and normalized by a biomass unit expressed as OD_{600} of the culture. Siderophore production was measured using the universal chrome azurol S (CAS) detection assay of Schwyn & Neilands (1987).

Porphyrins detection. Porphyrins were detected by spectrofluorimetry (Miyamoto *et al.*, 1992). After overnight growth, the cells were recovered by centrifugation (5000 ***g*** for 5 min), resuspended in 1/10 volume of acetone/0·1 M NH_4OH (9 : 1, v/v) and mixed. Cell extracts were centrifuged and the supernatants collected. Fluorescence emission spectra were recorded in a Shimadzu fluorimeter using an excitation wavelength of 405 nm (emission at 630 nm).

Inactivation of *ccmC* by allelic exchange. The gentamicin cassette was amplified with *Taq* polymerase (Life Technologies) from the pBBR1MCS-5 vector (Kovach *et al.*, 1994) using primers gent1 (5′-ATAAGAATGCGGCCGCACACCGTGGAAACGGA-3′) and gent2 (5′-ATAAGAATGCGGCCGCGATCTCGGCTTGAACGA-3′). The 800 bp fragment was then end-blunted using the T4 DNA polymerase.

The cassette was introduced into the blunt end *Sph*I site of the 2 kbp *Hin*dIII–*Pst*I fragment containing *ccmC*, *ccmD* and *ccmE* from *P. fluorescens* and subcloned into the suicide vector pBR325 (Gaballa *et al.*, 1996). After mobilization of the construct into *P. fluorescens*, *ccmC* mutants were selected for gentamicin resistance and chloramphenicol sensitivity. The gene replacements were confirmed by PCR using primers ccmc1 (5′-CATCGTCGGCCTGGTATGGA-3′) and ccmc2 (5′-GAAAGAGGCGGCGAAACTCATCCA-3′).

Inactivation of the *hemH* gene in the *pvsA* mutant. Construction of a *hemH* mutant was done by transposon mutagenesis using pTnmodOtc (Dennis & Zylstra, 1998). Colonies were screened on LB medium containing 100 μg tetracycline ml^{-1} and 20 μM of haemin for red fluorescence under UV light after 72 h incubation at 28 °C. One colony showing red fluorescence was isolated. The auxotrophy of this mutant for haemin was confirmed by its inability to growth on CAA medium without externally added haemin. The regions flanking the transposon insertion were isolated as described previously (Baysse *et al.*, 2001) and sequenced. The position of the Tn*5* insertion in the *hemH* gene is described by Baysse *et al.* (2001). In the double *pvsA hemH* mutant the insertion occurred at 127 bases from the 5′ end of the *hemH* ORF.

The vector pPAhemH, containing the *hemH* gene from PAO1 cloned in the expression vector pMMB208 (Baysse *et al.*, 2001), was introduced into the double mutant by conjugation with the helper strain carrying pRK2013. The transconjugants were selected on CAA medium without haemin to confirm the functional complementation.

Table 1. Strains and plasmids used in this study

Bacterial strains	Genotype*	Source or reference†
***Pseudomonas* strains**		
P. aeruginosa PAO1 ATCC 15695	Wild-type; source of PVD_I	ATCC
P. aeruginosa ATCC 27853	Wild-type; source of PVD_{II}	ATCC
P. fluorescens ATCC 17400	Wild-type from which are derived all mutants described in this study; source of PVD_{Pf}	ATCC
P. fluorescens pvsA	Tn*5* mutant of ATCC 17400 wild-type, PVD-negative; Km^R, Tc^R	Mossialos *et al.* (2000)
P. fluorescens ccmC	Knock-out mutant of ATCC 17400 in the *ccmC* gene; Km^R	Gaballa *et al.* (1996)
P. fluorescens pvsA ccmC	Derivative of *pvsA* with *ccmC*::Gm	This work
P. fluorescens hemH	Tn*5* mutant of ATCC 17400 wild-type, ferrochelatase-negative; Tc^R	Baysse *et al.* (2001)
P. fluorescens pvsA hemH	Derivative of *pvsA* with Tn*5* insertion in *hemH*, ferrochelatase-negative; Km^R, Tc^R	This study
P. fluorescens hemH ccmC	Derivative of *hemH* with *ccmC*::Gm	This work
***E. coli* strains**		
DH5α	*supE44* Δ*lacU169* [ϕ80 *lacZ*(M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*]	Gibco-BRL
SM10	Mobilizer strain; Km^R	Gaballa *et al.* (1996)
Plasmids and vectors		
pBBR1MCS	Wide-host-range vector; Cm^R	Kovach *et al.* (1994)
pMMB208	Wide-host-range vector; Cm^R	Morales *et al.* (1991)
pBR325	Col E1 vector; Ap^R, Cm^R, Tc^R	Promega
pCR2.1	Vector for PCR fragment cloning; Ap^R, Km^R	Invitrogen
pPAhemH	1 kb *Eco*RI fragment containing *hemH* of *P. aeruginosa* PAO1, cloned in pMMB208	Baysse *et al.* (2001)
pPYOV35	0·8 kb *Hin*dIII–*Sph*I fragment containing *ccmC* cloned in pBBR1MCS	Gaballa *et al.* (1996)
pUT-mini-Tn*5lacZ*2	Suicide vector (Ap^R) with the mini-Tn*5lacZ*2 gene; Km^R	de Lorenzo *et al.* (1990)
pTnMod-OTc	Mini-transposon Tn*5* with an origin of replication (plasposon); Tc^R	Dennis & Zylstra (1998)

*Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Tc, tetracycline.
†ATCC, American Type Culture Collection, Manassas, VA, USA.

Cross-feeding of the *hemH* mutant. This experiment was performed on CAA medium plates containing 50 μg $FeCl_3$ ml^{-1} and 0, 20, 40 or 80 μg ALA ml^{-1}. Two vertical lines of the *hemH* mutant were streaked, followed by two perpendicular lines of the wild-type strain and the *ccmC* mutant. The plates were incubated at 28 °C for 72 h and the growth of the *hemH* mutant at the crossing zone was checked. Each experiment was repeated in duplicate.

Proteinase K-treated lysates. Cultures of wild-type *P. fluorescens* and *ccmC* mutant in CAA medium plus 50 μM $FeCl_3$ were harvested by centrifugation and resuspended in 0·1 vol. of 0·01 M Tris/HCl, pH 7·8, 0·01 M EDTA and 0·5 % (w/v) SDS. Cells were broken by sonication (6 × 30 s with 30 s pauses at 300 W). Cell debris was removed by 3 min centrifugation at 4000 ***g*** and the cell-free lysate was incubated for 2 h with 50 μg proteinase K ml^{-1} at 50 °C. Protease inhibitors (CompleteTM; Roche Diagnostics) were added to the samples before the cross-feeding assays.

Western blot with polyclonal antibodies against *P. fluorescens* HemH. Polyclonal antibodies against *P. fluorescens* HemH were generated as described previously (Baysse *et al.*, 2001). Western blot analysis was performed on cytoplasmic and inner membrane fractions of both wild-type and *ccmC* mutant using the BM Chromogenic Western Blotting Kit from Roche.

HPLC analysis of porphyrins in *P. fluorescens*. *P. fluorescens* was grown aerobically on M9 minimal medium (Sambrook *et al.*, 1989) containing 40 mM glucose as carbon source to the stationary phase in the presence or absence of 50 μg ALA ml^{-1}. Cells were harvested by centrifugation, resuspended in 100 μl H_2O and disrupted by brief sonication. Porphyrins were extracted by addition of 100 μl acetone/concentrated HCl (97·5/2·5, v/v). After centrifugation, 20 μl of the resulting supernatant was loaded directly onto a 4·6 × 250 mm ODS Hypersil C_{18} reversed-phase column (Techlab) with a pore width of 120 Å. Separation and identification of porphyrins were performed at a flow rate of 0·5 ml min^{-1} as described previously (Layer *et al.*, 2002). Coproporphyrin III (Porphyrin Products) and PPIX (Sigma) were used as porphyrin standards.

RESULTS

Defect in the haem exporter CcmC or the ferrochelatase HemH leads to PVD and QB deficiency

Previously, we demonstrated that a *P. fluorescens* ATCC 17400 *ccmC* mutant had a greatly reduced PVD production and was impaired in PVD utilization as iron source, but not

at the level of the uptake (Gaballa *et al.*, 1996, 1998; Baysse *et al.*, 2002). To investigate whether the absence of CcmC also affects other siderophore-mediated iron uptake systems, besides PVD, we decided to introduce the *ccmC* mutation into a mutant of *P. fluorescens* ATCC 17400 deficient for the non-ribosomal peptide synthetase PvsA, which is needed for the PVD chromophore synthesis (Mossialos *et al.*, 2002). The *pvsA* mutant still produces high levels of another siderophore, QB (Mossialos *et al.*, 2000). The *ccmC* gene in the *pvsA* mutant was disrupted by double recombination. The single and the double mutants grew similarly in liquid Casamino acid (CAA) medium (data not shown). The effects of the mutation on the production of QB were evaluated by examining the diameter of the halos of discoloration around colonies of the *pvsA* and *pvsA ccmC* mutants on a CAS agarose plate (Fig. 1a). A decrease in the diameter of the clear halos around the colony of the *pvsA ccmC* mutant was observed, indicating that QB production was strongly reduced.

Likewise, inactivation of *hemH* affects QB production since a double *pvsA hemH* mutant did not produce a detectable CAS discoloration. The production of the siderophore was restored, although weakly, by complementation of the mutant with the *hemH* gene from *P. aeruginosa* PAO1 (Fig. 1b), but not by externally added haemin present in the medium (data not shown). This observation might provide a first indication for the importance of a common factor that is limiting for the biosynthesis of both siderophores (PVD and QB) in the *ccmC* and the *hemH* mutants. One likely candidate is iron–PPIX, since *de novo* haem biosynthesis has been shown to be important for the biosynthesis of both siderophores (Baysse *et al.*, 2001; S. Matthijs, unpublished results, see further in Discussion). Recent data showed that overexpressing a heterologous ferrochelatase in *E. coli* did not lead to significant accumulation of haem, although the enzyme activity could be demonstrated *in vitro* (Kwon *et al.*, 2003). Indeed, the three last enzymes of the haem biosynthetic pathway are supposed to work in an interactive manner that requires a compatible set of enzymes. Using the *hemH* gene from *P. aeruginosa* PAO1 to complement a mutant of *P. fluorescens* may affect the *in vivo* activity due to insufficient enzyme compatibility.

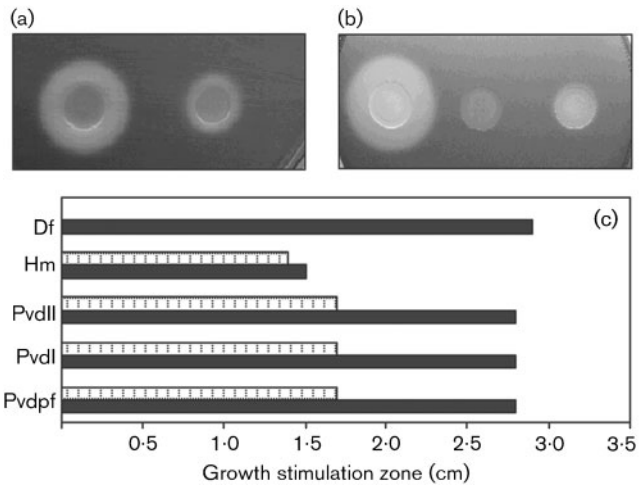

Fig. 1. (a) QB production by *P. fluorescens* mutant *pvsA* (left) and *pvsA ccmC* (right) as visualized by the CAS assay. (b) QB production by *P. fluorescens* mutant *pvsA* (left), *pvsA hemH* (centre) and *pvsA hemH* carrying pPAhemH (right) as visualized by the CAS assay. (c) Growth stimulation of *P. fluorescens* mutant *pvsA* (black bars) and *pvsA ccmC* (bars with squares) by desferrioxamine B (Df), haem (Hm), *P. aeruginosa* PVDs type II (PvdII) and type I (PvdI), and by cognate PVD (Pvdpf). The bars represent the diameter (in cm) of the growth stimulation zone around a disc impregnated with the iron source (see Methods for details). No value is shown for Df and *pvsA ccmC* because of the total lack of growth stimulation.

Haem-complexed iron, but not siderophore-bound iron, sustains growth of the *pvsA ccmC* double mutant

Since the *pvsA* mutant cannot grow in media supplemented with the strong iron(III) chelator ethylenediaminedi-hydroxyphenylacetic acid (EDDHA), it is possible to use this mutant as an indicator for the use of high-affinity heterologous siderophores as a source of iron. Fig. 1(c) shows that the growth of the single *pvsA* mutant was stimulated by both the cognate and two heterologous PVDs (from *P. aeruginosa*) and by desferrioxamine B. The *pvsA ccmC* double mutant on the other hand showed a reduced capacity to utilize the three PVDs and desferrioxamine B (Fig. 1c) could not stimulate its growth at all. Interestingly, the growth of this double mutant was still stimulated by haemin, to the same extent as the *pvsA* mutant, indicating that the *ccmC* mutation only affects siderophore-mediated iron acquisition but not haem-mediated iron uptake (Fig. 1c).

The *ccmC* mutant shows reduced haem production and accumulates PPIX

Colonies of the *ccmC* mutant showed a reddish fluorescence under UV, especially on iron-supplemented medium. Porphyrinogens and porphyrins accumulated by the *ccmC* mutant grown in the presence and absence of ALA were determined by HPLC analysis. ALA was added to growth media to overcome the initial rate-limiting step of tetrapyrrole biosynthesis and to allow significant detectable tetrapyrrole accumulation (Doss & Philipp-Dornston, 1971; Philipp-Dornston & Doss, 1973). The data presented in Fig. 2 indicate that there is a general decrease in the production of tetrapyrroles by the cells of the *ccmC* mutant in the absence of added ALA, an indication that the haem content might be decreased (Fig. 2c versus Fig. 2a). As expected, and in agreement with earlier observations for various other bacteria, the wild-type and mutant strains grown in the absence of ALA did not contain significantly

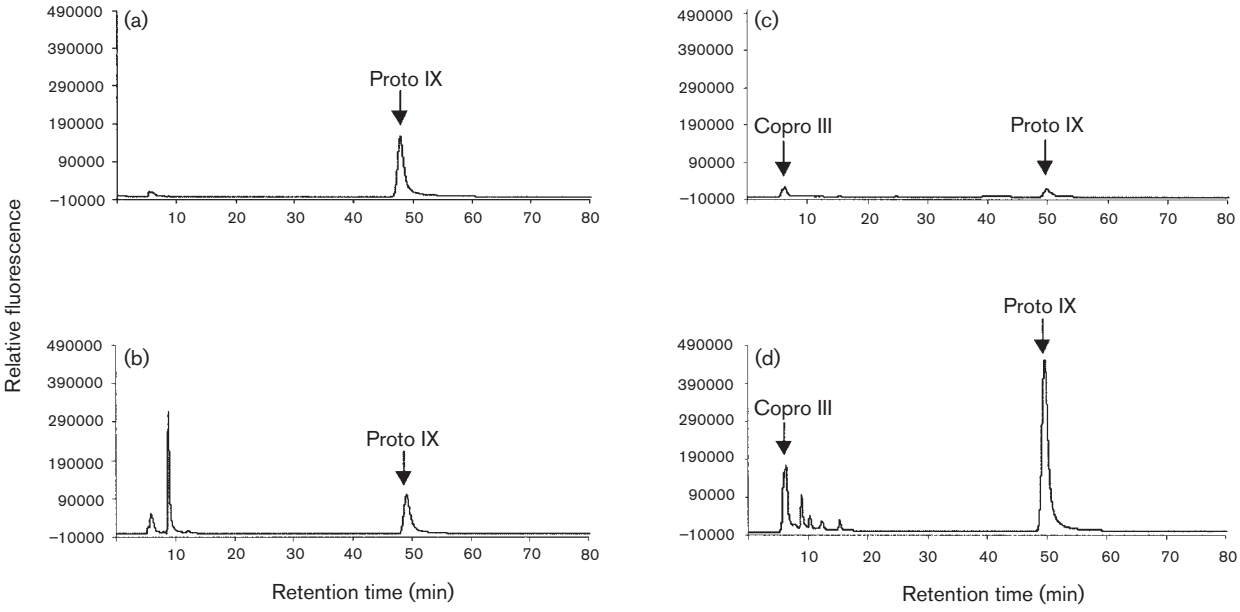

Fig. 2. Separation by HPLC of porphyrins from wild-type *P. fluorescens* (a and b) and *ccmC* mutant (c and d). In (a) and (c), the cultures were grown in the absence of ALA while, in (b) and (d), the cells were grown in the presence of ALA. See Methods for details. Copro III, coproporphyrinogen III; Proto IX, PPIX.

overproduced tetrapyrroles (Doss & Philipp-Dornston, 1971; Philipp-Dornston & Doss, 1973).

When grown in the presence of ALA, the mutant accumulated higher amounts of PPIX compared to the wild-type, indicative of a defective ferrochelatase activity (Fig. 2d versus Fig. 2b).

To distinguish between enzymically generated PPIX and oxidized protoporphyrinogen IX chemically formed during the isolation procedure, tetrapyrrole preparation was performed anaerobically. One half of the resulting preparation was analysed immediately, while the other half was completely oxidized using H_2O_2 treatment. No significant difference between the differentially treated tetrapyrrole preparations was observed. These experiments clearly indicate that PPIX accumulation was caused by the *ccmC* mutation. The parallel observed increase in the cellular coproporphyrinogen III concentration of the *ccmC* mutant (Fig. 2d) is in agreement with previous investigations of the physiological effects of an *E. coli hemH* mutant. Due to the rate-limiting step of coproporphyrinogen III oxidation on the late haem biosynthetic pathway, mutation of late genes always results in a minor coproporphyrinogen III accumulation (Schobert & Jahn, 2002).

Further *in vivo* evidence for PPIX accumulation is provided by the light-sensitive *vis* phenotype of the *ccmC* mutant. Similar to observations made for a *hemH* mutant of *E. coli*, the PPIX-accumulating mutant fails to grow in bright light due to the light-depending formation of detrimental radicals from the porphyrin ring system (Miyamoto *et al.*, 1991, 1992) (results not shown).

The *ccmC* mutant fails to rescue a haem-auxotrophic *hemH* mutant via cross-feeding

The product of the *hemH* gene, the enzyme ferrochelatase, is responsible for the insertion of Fe^{2+} into the protoporphyrin ring. Consequently, a mutant devoid of ferrochelatase activity accumulates large quantities of PPIX (Nakahigashi *et al.*, 1991; Nakayashiki & Inokuchi, 1997; Yang *et al.*, 1995). The accumulation of PPIX results in reddish-orange colonies that give a typical red fluorescence under UV and in light sensitivity (Nakahigashi *et al.*, 1991). As expected, the ferrochelatase-deficient mutant of *P. fluorescens* is auxotrophic for haem (Baysse *et al.*, 2001). Therefore, we decided to use this mutant as an indicator either for the presence of haem in cell extracts after proteinase K treatment or for the presence of free haem released by the cells. Fig. 3 shows that the wild-type *P. fluorescens* cells excrete enough haem to promote the growth of the *hemH* mutant in their vicinity. This growth promotion was dependent on the presence of both iron and ALA in the medium. Clearly this phenotype was not observed in the vicinity of the *ccmC* mutant (Fig. 3a). Growth promotion was again observed when the *ccmC* mutant was complemented with the plasmid pPYOV35 containing a wild-type copy of the *ccmC* gene (Gaballa *et al.*, 1996). Similar results were obtained when proteinase K-treated cell extracts were used. In this case, again, no stimulation of the growth of the *hemH* mutant was observed

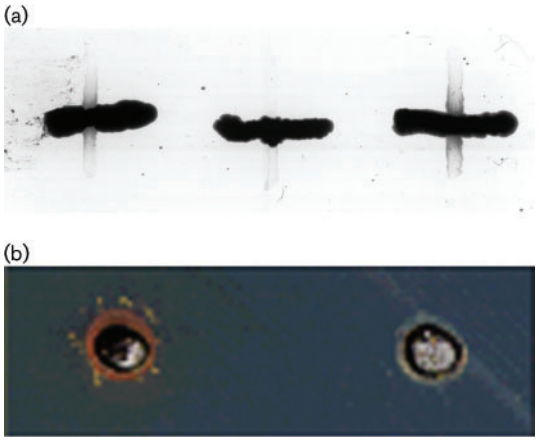

Fig. 3. (a) Cross-feeding of a *hemH* mutant (vertical streak) by wild-type *P. fluorescens* (left), *ccmC* (middle) and *ccmC* complemented with *ccmC in trans* (right). (b) Growth stimulation of the *hemH* mutant by proteinase K-treated cellular extracts from wild-type *P. fluorescens* (left) and *ccmC* mutant (right). The conditions are described in Methods. After overnight incubation, the plate was exposed to UV to detect the PPIX-accumulating *hemH* mutant.

when the extracts from the *ccmC* mutant were used, as shown in Fig. 3(b): under UV exposure, no growth of red-fluorescent bacteria, indicative of the PPIX-accumulating *hemH* mutant, is observed around the well containing cell-free extract from the *ccmC* mutant. These results confirm that *in vivo* the *ccmC* mutant produces and exports fewer tetrapyrroles, both functions being complemented by *ccmC in trans*.

The *ccmC* mutant shows reduced resistance to oxidative stress

Decreased haem content and increase in PPIX are likely to affect the response of the *ccmC* mutant to oxidative stress. Indeed, catalases are enzymes that employ haem as co-factor; therefore, a decrease in the supply of haem, as observed for the *ccmC* mutant, is expected to result in increased sensitivity to H_2O_2. However, PPIX is known to be toxic for the cells, especially because it is a photo-reactive molecule that can be easily degraded, a process resulting in the production of reactive oxygen species (Yang *et al.*, 1995).

The inhibition caused by H_2O_2 on the growth of the wild-type and the *ccmC* mutant was tested. The *ccmC* mutant displayed an increased sensitivity to H_2O_2 (zone of inhibition $5{\cdot}85 \pm 0{\cdot}15$ cm compared to $3{\cdot}9 \pm 0{\cdot}1$ cm for the wild-type strain; $n = 3$). Addition of external haem in the form of haemin (final concentration, 40 μM) restored an almost normal level of sensitivity to H_2O_2 to the *ccmC* mutant (zone of inhibition $4{\cdot}15 \pm 0{\cdot}05$ cm compared to $3{\cdot}9 \pm 0{\cdot}1$ cm for the wild-type strain), another confirmation of a haem shortage in the *ccmC* cells.

Lethality of a double *hemH ccmC* mutation

A double *hemH ccmC* mutant was obtained, but only when the *P. aeruginosa hemH* gene was present *in trans*. Furthermore, it was impossible to cure this *hemH ccmC* mutant from the plasmid bearing the *hemH* gene (results not shown). These results indicate that the combination of *hemH* and *ccmC* mutations is lethal.

Ferrochelatase expression is not influenced by the *ccmC* mutation

By performing a Western blot using antibodies against *P. fluorescens* HemH (Baysse *et al.*, 2001), we detected the protein in the membrane fractions of both the wild-type and the *ccmC* mutant. No significant difference in the HemH level was observed (data not shown).

DISCUSSION

The importance of the cytoplasmic membrane protein CcmC in the biogenesis of *c*-type cytochromes in Gram-negative bacteria has been clearly established, but its role is still controversial (Thöny-Meyer, 1997; Kranz *et al.*, 1998; Page & Ferguson, 1999; Cook & Poole, 2000; Schulz *et al.*, 2000). Obviously, absence of CcmC causes a range of defects which are not all due to the absence of *c*-type cytochromes (Gaballa *et al.*, 1998).

Absence of CcmC affects haem biosynthesis at the level of iron chelation

In this study, using both direct (detection of tetrapyrroles by HPLC) and indirect methods, we came to the conclusion that in a *ccmC* mutant haem production is reduced, while PPIX accumulates when haem biosynthesis is boosted by ALA. Wild-type *P. fluorescens*, but not a *ccmC* mutant, was able to cross-feed the *hemH* mutant on solid medium, in an iron- and ALA-dependent fashion. This result strongly suggests that wild-type cells can excrete free haem when its synthesis is boosted. We therefore propose a model (Fig. 4) where CcmC (probably in association with the other Ccm proteins) is a transporter for haem, and is associated with other haem biosynthesis proteins, including ferrochelatase, to form a metabolon. Inactivation of *ccmC* would directly or indirectly down-regulate the haem biosynthetic pathway to prevent formation of toxic free haem. It is interesting to mention that metabolite channelling has already been proposed for several steps in the pathway of haem biosynthesis (Moser *et al.*, 2001; Olsson *et al.*, 2002). Channelling is observed when precursors in a metabolic pathway are known to be potentially harmful for the cell (Massant *et al.*, 2002). This model also explains why different mutations in periplasmic loops of *P. fluorescens* CcmC affect differently the ability to produce and use PVD, on the one hand, and cytochrome *c* biogenesis, on the other (Gaballa *et al.*, 1998). Mutations affecting the binding of haem by CcmC in the periplasm preferentially affect cytochrome *c* biogenesis since only the transfer of haem from CcmC to the haem chaperone CcmE would be impaired (Schulz *et al.*, 2000).

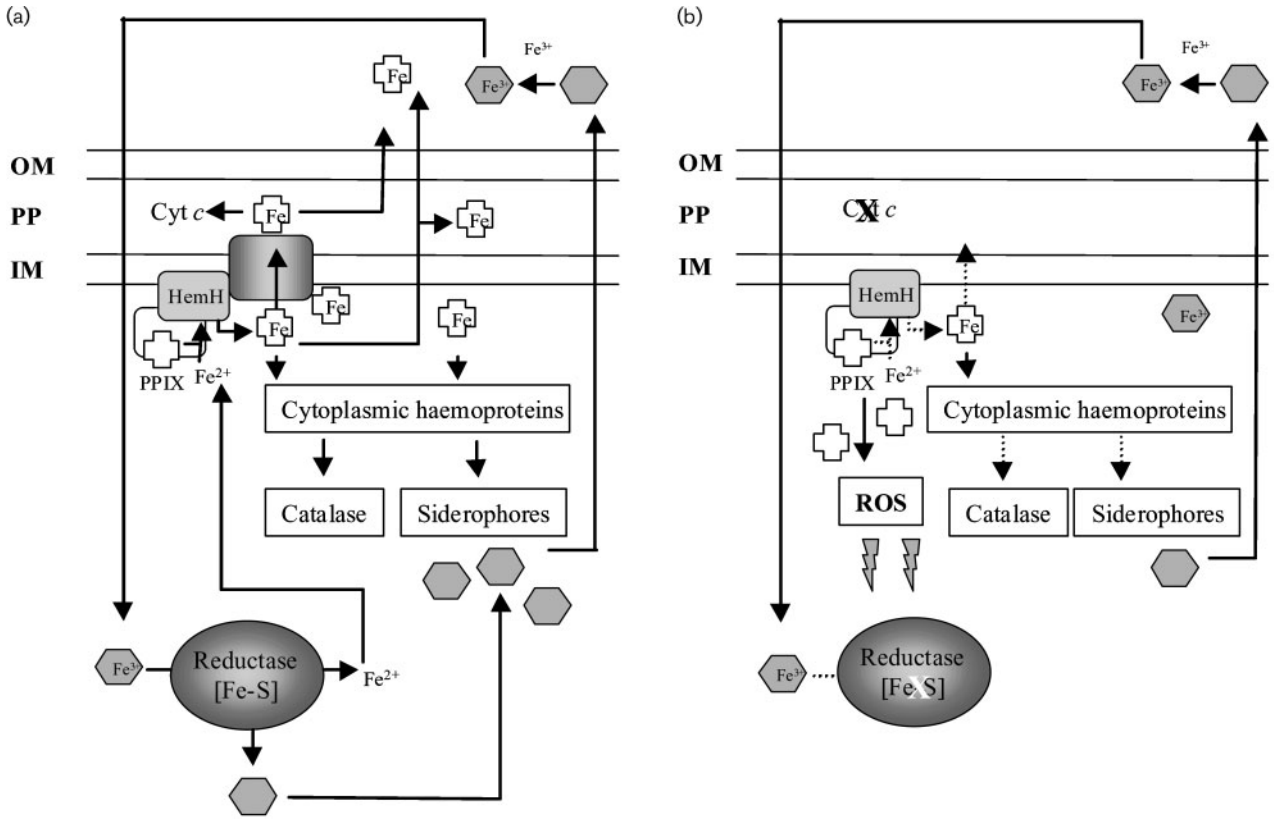

Fig. 4. Model explaining the different phenotypes observed in the *ccmC* mutant. (a) In the wild-type, the complex of Ccm proteins in the inner membrane (shown as a grey box) is associated with haem biosynthesis enzymes, including ferrochelatase (HemH). Haem (represented as a white cross with Fe^{2+}) is either channelled via CcmC to the periplasm (where it is transferred to CcmE and CcmF for the biogenesis of *c*-type cytochromes) or incorporated into cytoplasmic haemoproteins including some siderophore biosynthetic enzymes. Haem can also be transported or diffuse to the periplasm where it can be incorporated into cytochromes other than the *c*-type. Our results also indicate that haem can be exported. Iron(III) is released from ferrisiderophores by a [Fe–S] reductase. (b) In the absence of CcmC, the haem metabolon is destabilized, resulting in decreased haem production (shown in dotted lines) and accumulation of PPIX (white crosses), which can result in the accumulation of reactive oxygen species (ROS). As a consequence of the oxidative stress, the [Fe–S] centre(s) of the ferrisiderophore reductase(s) are destroyed (shown by the cross), resulting in an inability to release iron from them. The transport of ferrisiderophores is, however, unaffected. Lack of haem results in decreased siderophore production and reduced synthesis of catalase. Haem transport to the periplasm is decreased (dotted line). In the absence of the Ccm complex, no cytochrome *c* is produced.

On the other hand, mutations affecting to a different extent the transport function of CcmC would only partially affect cytochrome *c* biogenesis if sufficient amounts of haem still reach the periplasmic space. These mutations seem to have more negative consequences at the level of haem biosynthesis, more probably by inactivating the last step of haem biosynthesis, resulting in accumulation of PPIX. We have evidence that this regulation is not at the level of the ferrochelatase expression (results not shown). However, absence of CcmC may affect the activity of the enzyme.

Absence of CcmC induces a state of oxidative stress

Accumulation of porphyrins is known to cause an oxidative stress, as demonstrated for ferrochelatase-deficient *E. coli* mutants (Yang *et al.*, 1995). PPIX is known to be toxic for the cells, especially because it is a photo-reactive molecule that can be easily degraded, a process resulting in the production of reactive oxygen species (Yang *et al.*, 1995; Maciver & Hansen, 1996). Indeed, we demonstrated that the growth of the *ccmC* mutant that accumulates PPIX is totally impaired when the cells are exposed to light (results not shown). In a double *hemH ccmC* mutant, the high levels of PPIX could result in cell death due to oxidative damage, explaining why such a double mutant could not be obtained in the absence of complementation by *hemH in trans* (Yang *et al.*, 1995). Similarly, catalase, which is a haemoprotein, shows a reduced activity in the *ccmC* mutant, as judged by the increased sensitivity of this mutant to H_2O_2. Interestingly, externally added haemin restores almost

wild-type levels of resistance to H_2O_2, but does not increase the production of siderophores. Haemin is probably taken up via a TonB-dependent receptor by *P. fluorescens*, as is the case in *P. aeruginosa* (Létoffé *et al.*, 2000; Ochsner *et al.*, 2000; Wandersman & Stojilkovic, 2000). It therefore seems that haemin taken up via TonB-dependent receptors can restore catalase activity.

Absence of CcmC affects the production of siderophores

One obvious phenotype of mutants affected in the biogenesis of *c*-type cytochromes is their reduced capacity to grow under conditions of iron limitation (Gaballa *et al.*, 1996, 1998; Yeoman *et al.*, 1997; Pearce *et al.*, 1998; Polesky *et al.*, 2001; Baysse *et al.*, 2002, Viswanathan *et al.*, 2002). The decrease in haem production can explain some of the observed phenotypes of the *ccmC* mutant, such as reduced production of siderophores PVD and QB. We have already demonstrated that *de novo* haem biosynthesis was needed for the production of PVD (Baysse *et al.*, 2001). Haem is probably a necessary component for the biosynthesis of QB as well, since the enzyme tryptophan-2,3-dioxygenase, a haemoprotein, is needed for its production (S. Matthijs, unpublished results). This is supported by the fact that a *hemH* mutant does not produce QB.

Absence of CcmC affects the utilization of ferrisiderophores as iron source

The cells of the *ccmC* mutant are also characterized by a general inability to use different ferrisiderophores as iron source. In the case of PVD, we have already demonstrated that the defect was not due to an impaired uptake or absence of production of siderophore receptors (Gaballa *et al.*, 1996). Haemin utilization, on the other hand, was not affected. For the release of iron from ferrisiderophores in the cytoplasm, a reduction mechanism is probably needed. The *E. coli* FhuF protein is likely to be the reductase for the release of iron from desferrioxamine B (Patzer & Hantke, 1999). The production of FhuF, a [2Fe–2S] protein, is repressed by iron and the stability of its iron–sulphur centre is maintained by different enzymes encoded by the *sufABCDSE* operon (Patzer & Hantke, 1999). In a recent article, Nachin *et al.* (2003) showed that SufC from *Erwinia chrysanthemi* (also part of the same iron-regulated operon) is needed for the biogenesis of [Fe–S] centres under conditions of oxidative stress (Nachin *et al.*, 2001, 2003). Interestingly, the same authors observed that a *sufC* mutant is unable to use the siderophore chrysobactin as iron source, even though the uptake of the ferrisiderophore was not impaired. This phenotype is very similar to the one we describe here. In fact, we can predict that the *hemH* mutation should give the same phenotype, but this is difficult to demonstrate experimentally since it needs haemin (itself a source of iron) to grow.

A model explaining the pleiotropic phenotype of the *ccmC* mutant

We propose a model (Fig. 4) where in the absence of a functional CcmC protein (and/or other Ccm proteins) the biosynthesis of haem is impaired, leading to a reduction in haem content and in an accumulation of PPIX. Since some haemoproteins are probably involved in the biosynthesis of PVD (Baysse *et al.*, 2001) and QB (S. Matthijs, unpublished results), this would explain why fewer siderophores are produced by the *ccmC* mutant. As mentioned above, PPIX can induce the production of active oxygen radicals, which in turn can destroy the [Fe–S] centre(s) of ferrisiderophore reductase(s), resulting in a general incapacity to use ferrisiderophores as iron source without affecting the capacity to utilize haemin.

Future research should help to get a better insight into the function of CcmC and other proteins of the Ccm complex, by using a proteomic approach.

ACKNOWLEDGEMENTS

We wish to thank the Flemish Fund for Scientific Research (FWO), and the Alphonse & Jean Forton fund against Cystic Fibrosis for their financial support. S. Matthijs was a recipient of a FWO fellowship. Many thanks to Willy Verheulpen for computer assistance.

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