Multiple phenotypic alterations caused by a *c*-type cytochrome maturation *ccmC* gene mutation in *Pseudomonas aeruginosa*

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In some *Proteobacteria* biogenesis of *c*-type cytochromes depends on the products of the *ccmABCDEFHG* genes, which encode inner-membrane proteins. Inactivation of some *ccm* genes, in particular *ccmC*, has an impact on other processes as well, including siderophore production and utilization. Non-polar insertions were generated in the *Pseudomonas aeruginosa* *ccmA*, *ccmC*, *ccmE*, *ccmF* and *ccmH* genes, and their impacts on different phenotypes were compared. Only in the case of the *ccmC* mutant was cytochrome *c* production totally abrogated. The *ccmC* mutant, and to a lesser extent the *ccmF* mutant, showed a range of other phenotypic changes. The production of the siderophore pyoverdine was very low and growth under the condition of iron limitation was severely restricted, but production of the second siderophore, pyochelin, was increased. Interestingly, other traits were also strongly affected by the *ccmC* mutation, including the production of pyocyanin, swarming and twitching motility, and rhamnolipid production. The production of *N*-acyl homoserine lactones or the *Pseudomonas* quinolone signal (PQS) was, however, not affected in the *ccmC* and *ccmF* mutants. The *ccmC* mutant was also found to accumulate porphyrins, and catalase production was undetectable, consistent with the increased sensitivity to hydrogen peroxide. Finally, reduction in the content of [Fe–S] clusters was evidenced in both *ccmC* and *ccmF* mutants. Wild-type phenotypes were restored by complementation with a *ccmC* gene from *Pseudomonas fluorescens* ATCC 17400. In conclusion, we have demonstrated that CcmC is a key determinant for cytochrome *c* biogenesis, pyoverdine maturation, and expression of some quorum sensing-regulated traits.

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INTRODUCTION

Type *c* cytochromes, involved in anaerobic and aerobic respiration, have their haem covalently linked to the apocytochrome (Thöny-Meyer, 1997). In α - and γ -proteobacteria, the biosynthesis of *c*-type cytochromes is mediated by cytoplasmic membrane proteins, called Ccm (cytochrome *c* maturation), which covalently attach haem to the CXXCH motif of apocytochromes in the periplasm (Thöny-Meyer, 1997, 2000; Cianciotto *et al.*, 2005). The most common arrangement found in γ -proteobacteria is for all *ccm* genes to be in one cluster and transcribed in the same orientation, with *Shewanella oneidensis* as an

exception (Cianciotto *et al.*, 2005). CcmA and CcmB presumably form an ABC transporter together, and it has been proposed that CcmAB transports a reductant in the periplasm (Stevens *et al.*, 2004). CcmC, an integral membrane protein (Ahuja & Thöny-Meyer, 2003; Gaballa *et al.*, 1998), binds haem *c* in the periplasm and transfers it to the membrane-anchored periplasmic haem chaperone CcmE (Thöny-Meyer, 2003), with CcmD stabilizing their interactions (Ahuja & Thöny-Meyer, 2005). From CcmE, haem is transferred to the haem lyase, CcmF, which is associated with CcmH (Ahuja & Thöny-Meyer, 2003). CcmH keeps the cysteines in the apocytochrome reduced, while CcmG (DsbE) is a protein thiol/disulfide oxidoreductase that passes its electrons to CcmH (Edeling *et al.*, 2004).

There are a growing number of reports that describe pleiotropic effects of *ccm* mutations that cannot be explained only in terms of the absence of synthesis of *c*-type cytochromes (reviewed by Cianciotto *et al.*, 2005; Yurgel *et al.*, 2007). Negative effects of mutations in some

Abbreviations: AHL, *N*-acylhomoserine lactone; CAA, casamino acids; CAS, chrome azurol; HSL, homoserine lactone; EDDHA, ethylenediamine-dihydroxyphenylacetic acid; HHQ, 2-heptyl-4-quinolone; PQS, *Pseudomonas* quinolone signal; TMBZ, 3,3',5,5'-tetramethylbenzidine.

A supplementary figure showing production of quorum-sensing signal molecules and two video clips showing motility of wild-type and *ccmC* mutant *P. aeruginosa* are available with the online version of this paper.

ccm genes on the production and/or uptake of siderophores in different bacteria have been described (Cianciotto *et al.*, 2005), including the pseudomonads *Pseudomonas fluorescens* (Gaballa *et al.*, 1996, 1998; Baysse *et al.*, 2002, 2003) and *Pseudomonas aeruginosa* (De Chial *et al.*, 2003). In *P. fluorescens*, inactivation of *ccmC* causes a drastic reduction in the production of the fluorescent siderophore pyoverdine (Gaballa *et al.*, 1996, 1998; Baysse *et al.*, 2002, 2003). The same *ccmC* mutant is also defective in the utilization of not only pyoverdine but also other heterologous siderophores (Baysse *et al.*, 2003). Ferribactin, the non-fluorescent precursor of pyoverdine (Mossialos *et al.*, 2002), is accumulated by the *ccmC* mutant, which fails to mature the pyoverdine chromophore (Baysse *et al.*, 2002). In *P. fluorescens* ATCC 17400, a double pyoverdine-deficient/*ccmC* mutant also fails to produce the secondary siderophore quinolobactin (Matthijs *et al.*, 2004; Baysse *et al.*, 2003). However, no reduction of pyoverdine production is observed for two different *ccmF* mutants of *Pseudomonas putida* GB-1 (de Vrind *et al.*, 1998). The *ccmC* mutation in *P. fluorescens* also causes the accumulation of the haem precursor protoporphyrin IX (PPIX) and haem depletion (Baysse *et al.*, 2003). A hypothesis has been formulated in which the combination of haem shortage and PPIX accumulation leads to oxidative stress, since catalases are haemoproteins and PPIX generates reactive oxygen species (ROS) (Baysse *et al.*, 2003; Cianciotto *et al.*, 2005). One possible consequence of this continuous oxidative stress is the destruction of iron-sulphur clusters in proteins that have them in their active sites (Imlay, 2006). Interestingly, Matzanke *et al.* (2004) have described an [Fe-S] protein, FhuF, as being a ferrisiderophore reductase. If some ferrisiderophore reductases are [Fe-S] oxidoreductases, it could explain why a *ccmC* mutant is unable to use ferrisiderophores as a source of iron. The effects of different *ccm* mutations have not, to our knowledge, been investigated in *P. aeruginosa*. We therefore decided to generate mutants in different *ccm* genes in *P. aeruginosa* in order to assess their impact on the physiology of this important opportunistic pathogen that is characterized by the production of a rich array of virulence factors, among which are siderophores (Lamont *et al.*, 2002). *P. aeruginosa* is a well-studied model for cell to cell communication, genomics and virulence. It also displays a range of signalling molecules and iron chelators that are not found in *P. fluorescens*; therefore, investigation of the impact of inactivation of *ccm* genes in this strain will yield significant new data to understand the pleiotropic role of some of these proteins in the physiology of the cell.

In this work, we show that a single non-polar mutation in *ccmC* and, to a lesser extent, in *ccmF* causes multiple phenotypic changes in *P. aeruginosa*, including reduced pyoverdine production, reduced motility and decreased production of the phenazine pigment pyocyanin. Furthermore, we could confirm the destructive effect of the mutation on [Fe-S] clusters and the absence of catalase activity, a hypothesis formulated in a previous publication (Baysse *et al.*, 2003).

METHODS

Growth of micro-organisms. *P. aeruginosa* PAO1 and its mutants (listed in Table 1) were grown at 37 °C in LB or in casamino acids (CAA) medium in the absence or presence of 50 µM FeCl₃ (Cornelis *et al.*, 1992). To further increase iron limitation, the strong iron (III) chelator ethylenediaminedihydroxyphenylacetic acid (EDDHA; 1 mg ml⁻¹) was added to the CAA medium. Antibiotics were added to *P. aeruginosa* strains at the following concentrations: 300 µg chloramphenicol (Cm) ml⁻¹, 100 µg tetracycline (Tc) ml⁻¹, and 100 µg gentamicin (Gm) ml⁻¹. *Escherichia coli* strains were grown at 37 °C in LB with the appropriate antibiotics: 100 µg ampicillin (Amp) ml⁻¹, 50 µg Cm ml⁻¹, 25 µg spectinomycin (Sp) ml⁻¹ and 15 µg Tc ml⁻¹.

Growth parameters were measured using a Bioscreen apparatus (Life Technologies), with the following parameters: shaking for 20 s every 1 min; reading every 30 min; temperature 37 °C; volume of culture, 300 µl. As the inoculum, an overnight culture of PAO1 in medium was diluted to achieve a final OD₆₀₀ of 0.01. Each culture was grown in triplicate and each experiment was repeated three times. Cells were grown anaerobically (Pettigrew & Brown, 1988) by growing the cultures aerobically to a cell density of ~30 % of that of the stationary phase, at which point NaNO₃ (5 g l⁻¹) was added and aeration was stopped by addition of a layer of mineral oil (Gaballa *et al.*, 1996).

Construction of mutants. Non-polar mutations in *ccmA*, *ccmC*, *ccmE*, *ccmF* and *ccmH* were generated using the pKnockout system described by Windgassen *et al.* (2000). Primers used for the amplification of gene fragments are listed in Table 2. At the 5' end of all forward primers there was an *EcoRI* site, and a *Sall* site was present at the 5' end of all reverse primers, except in the case of the *ccmH* primers (see Table 2). The amplified fragments were cloned between the *EcoRI* and *Sall* sites of the pKnockout-G vector. After conjugation and transfer of the recombinant suicide vector to *P. aeruginosa*, correct insertion and gene inactivation were checked by amplification using the forward primers and the Z2 primer corresponding to the *lacZ* gene in the pKnockout vector. Further confirmation was obtained by PCR using primers designed to amplify the full-length genes. As a consequence of the insertion of the complete plasmid following the single recombination, amplification should be negative for the mutants because of the length of the insert. The *ccmC* gene was also inactivated by insertion of a gentamicin cassette, as described previously (Baysse *et al.*, 2003). A list of strains and plasmids used in this study is shown in Table 1 and a list of primers is shown in Table 2.

Cytochrome oxidase activity and haem staining. Cytochrome oxidase activity was determined using the Fluka kit. In the presence of cytochrome oxidase, *N,N*-dimethyl-*p*-phenylenediamine oxalate and α -naphthol react to form indophenol blue. Protein fractions for *c*-type cytochrome analysis were prepared as described by Feissner *et al.* (2003). Cells from an overnight culture were harvested by centrifugation at 15 000 *g* for 15 min and resuspended in 1/100 of the original culture volume in 10 mM Tris-HCl (pH 8.0). The cell suspension was then sonicated three times for 4 min each at 4 °C. Unbroken cells were removed by centrifugation at 15 000 *g* for 15 min at 4 °C and discarded. Protein concentrations were determined by bicinchoninic acid (BCA) assay using BSA as a standard. Protein fractions were prepared for SDS-PAGE by mixing them with DTT-free SDS loading buffer. Samples were separated by 15 % SDS-PAGE and transferred to nitrocellulose for 60 min at 200 mA using a Bio-Rad Mini Trans-Blot cell. A 6.3 mM 3,3',5,5'-tetramethylbenzidine (TMBZ) stock solution was made in methanol immediately prior to staining, and three parts TMBZ stock solution were mixed with seven parts 0.25 M sodium acetate (pH 5.0) to produce the TMBZ staining solution. Electroblooded membranes were incubated in TMBZ staining solution for 30 min at room temperature, followed by the addition of

Table 1. Strains and plasmids used in this study

Strains	Genotype	Reference
P. aeruginosa		
<i>P. aeruginosa</i> PAO	Wild-type	ATCC 15692
<i>P. aeruginosa</i> ccmA	pKnockout mutant in the <i>ccmA</i> gene, Gm ^R	This work
<i>P. aeruginosa</i> ccmC	pKnockout mutant in the <i>ccmC</i> gene, Gm ^R	This work
<i>P. aeruginosa</i> ccmC1	Mutant with a Gm cassette in the <i>ccmC</i> gene	This work
<i>P. aeruginosa</i> ccmE	pKnockout mutant in the <i>ccmE</i> gene, Gm ^R	This work
<i>P. aeruginosa</i> ccmF	pKnockout mutant in the <i>ccmF</i> gene, Gm ^R	This work
<i>P. aeruginosa</i> ccmH	pKnockout mutant in the <i>ccmH</i> gene, Gm ^R	This work
<i>P. aeruginosa</i> pvdD	Pyoverdine-negative mutant	Ghysels <i>et al.</i> (2004)
<i>P. aeruginosa</i> pchEF	Pyochelin-negative mutant	Ghysels <i>et al.</i> (2004)
<i>P. aeruginosa</i> pvdD pchEF	Pyoverdine- and pyochelin-negative mutant	Ghysels <i>et al.</i> (2004)
<i>P. aeruginosa</i> pqsA::lux	Reporter strain for PQS detection	Diggie <i>et al.</i> (2006a)
E. coli		
S17-1	<i>thi pro hsdR recA</i> ; chromosomal RP4 (Tra ⁺ Tc ^s Km ^s Ap ^s)	Simon <i>et al.</i> (1983)
DH5 α	<i>recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1 Δ(lacZYA-argF) U169 ϕ80lacZ ΔM15</i>	Hanahan (1983)
Other bacteria		
<i>C. violaceum</i> CV026	Mini-Tn5 mutant of ATCC 31532 deficient in AHL and violacein production	McClean <i>et al.</i> (1997)
<i>A. tumefaciens</i> pZLR4	Carries plasmid pZLR4 containing <i>traR</i> and <i>traG::lacZ</i> reporter for AHL detection in pBBR1MCS (Ap ^R , Gm ^R)	Cha <i>et al.</i> (1998)
Plasmids		
pKnockout-G	Vector for gene inactivation in <i>Pseudomonas</i> , Gm ^R	Windgassen <i>et al.</i> (2000)
pBR325	ColE1 vector; Ap ^R , Cm ^R , Tc ^R	Bolivar (1978)
pBBR-1MCS	Wide-host-range cloning vector, Cm ^R	Kovach <i>et al.</i> (1994)
pPyov35	0.8 kb <i>HindIII</i> – <i>SphI</i> fragment containing the <i>P. fluorescens</i> ATCC 17400 <i>ccmC</i> gene in pBBR-1MCS	Bayse <i>et al.</i> (2003)

30 % H₂O₂ to a final concentration of 0.3 % (v/v), and incubated for another 30 min (Goodhew *et al.*, 1990).

Detection of porphyrins. A 3 ml overnight culture was centrifuged at 5000 g for 5 min. After discarding the supernatants, the pellet was dissolved in one-tenth of the volume (300 μ l) of a mixture of acetone/0.1 M NH₄OH (9:1, v/v). Samples were again centrifuged and the supernatant was collected. These were analysed with a Shimadzu fluorimeter (excitation, 405 nm; emission, 630 nm).

Motility studies. Twitching motility was assayed as described by McMichael (1992). LB agar medium (1.3 %) was stab-inoculated with 3 μ l culture of the test organism so that the tip came into contact with the bottom of the plate. After 48 h of incubation at 37 °C, the agar was removed and the bottom of the Petri dish was stained with crystal violet (Merck). The swimming and swarming activities were tested in 0.4 and 1 % agar, respectively. A culture was grown to the optimal density (OD₆₀₀=0.5) and 3 μ l was spotted on the surface. The plates were incubated at 37 °C for 2 days.

Detection of rhamnolipids. Rhamnolipids were detected by the drop-collapsing method to detect the presence of wetting agents (Déziel *et al.*, 2003) and by the more specific blue plate assay of Siegmund & Wagner (1991). This assay is based on the formation of an insoluble ion pair between cetyl-trimethylammonium bromide and methylene blue. Positive cultures are surrounded by a dark blue halo. Wells were inoculated with decreasing numbers of cells and the plates were incubated at 28 °C for 48 h, and at room temperature for another 48 h.

Detection of signal molecules. Supernatants from overnight cultures in LB at 37 °C were extracted with dichloromethane, as

described previously (Diggie *et al.*, 2002; Yates *et al.*, 2002) and analysed by TLC. Samples (10 μ l) were spotted onto reverse-phase silica RP-18 F₂₅₄ S plates (Merck) and separated using a methanol:water (60:40, v/v) system. C4-homoserine lactone (C4-HSL; *N*-butanoyl-L-homoserine lactone) was assayed using *Chromobacter violaceum* CV026 as the indicator (McClean *et al.*, 1997). For the detection of 3-oxo-C12 HSL [*N*-(3-oxododecanoyl)-L-homoserine lactone], samples were spotted onto RP-2 plates (Macherey-Nagel) and the TLC was resolved using a mixture of methanol and water, 45:55 (v/v). The *Agrobacterium tumefaciens* pZLR4 strain, which carries a *lacZ* transcriptional fusion under the control of an *N*-acylhomoserine lactone (AHL)-inducible promoter for longer-chain AHLs, was used to detect 3-oxo-C12-HSL (Shaw *et al.*, 1997; Cha *et al.*, 1998).

For both biosensors, AHLs were visualized as bright spots on a dark background when viewed with a Luminograph LB 980 (Berthold) photon video camera. For the extraction of extracellular *Pseudomonas* quinolone signal (PQS), cell-free supernatants were prepared from overnight cultures (10 ml) and extracted with 10 ml acidified ethyl acetate. The organic phase was dried and resuspended in 50 μ l methanol. A 10 μ l sample was spotted onto normal-phase silica 60 F₂₅₄ (Merck) TLC plates, pretreated by soaking in 5 % K₂HPO₄ for 30 min and activated at 100 °C for 1 h. Extracts were separated using a dichloromethane: methanol mix, 95:5 (v/v). PQS was visualized under UV light and identified by comparison with a synthetic standard (5 μ l of a 10 mM stock). PQS and 2-heptyl-4-quinolone (HHQ) could also be visualized by using the indicator strain *pqsA::luxCDABE* (Diggie *et al.*, 2006a). Standards of C4-HSL, 3-oxo-C12-HSL and PQS were obtained from P. Williams (University of Nottingham).

Determination of iron–sulphur clusters. Cells from an LB or CAA culture (OD₆₀₀ 0.4–0.6) were collected by centrifugation (7500 r.p.m.

Table 2. Primers used in this study

EcoRI and *Sall* sites are underlined. For *ccmH*, the primers contain *Bam*HI (forward) and *EcoRI* (reverse) sites.

Primer designation	Sequence	Use
ccmAF	5' ATGCGAATTCATGTCCGATCCTGCTGAAGT 3'	<i>ccmA</i> gene inactivation
ccmAR	5' TGGCGTCGACCACCAGGTGCGTTTCCAGTTG 3'	
ccmAC-F	5' GTGGTGTGTGCTGGTGGCGCTGAC 3'	<i>ccmA</i> gene amplification
ccmAC-R	5' GTGCAGGATCAGCAGCGCCAGCA 3'	<i>ccmA</i> gene amplification
ccmCF	5' ATGCGAATTCAGCTTGGGTGCGCCAAGT 3'	<i>ccmC</i> gene inactivation
ccmCR	5' TGGCGTCGACCTAGCCGAGGACCATGAT 3'	
ccmCC-F	5' GTGATGATGAATTGGACATGG 3'	<i>ccmC</i> gene amplification
ccmCC-R	5' GTGAGCTCATCGTGCCTTCTCCAC 3'	<i>ccmC</i> gene amplification
ccmEF	5' ATGCGAATTCCTGCAACAGAACATCAACC 3'	<i>ccmE</i> gene inactivation
ccmER	5' TGGCGTCGACGTTCTCGTAGTGCTTCA 3'	
ccmEC-F	5' GTGAATCCGGTCCGCAAGAAA 3'	<i>ccmE</i> gene amplification
ccmEC-R	5' GTGCTACTTGCCCTCCTGGTT 3'	<i>ccmE</i> gene amplification
ccmFF	5' ATGCGAATTCCTGGGATCCGGTGGAAAAC 3'	<i>ccmF</i> gene inactivation
ccmFR	5' TGGCGTCGACGGTAAAGCCGGCATCGA 3'	
ccmFC-F	5' GTGCTAGATGATCCCGAACT 3'	<i>ccmF</i> gene amplification
ccmFC-R	5' TGGCGCGACGATCAGGAAGAT 3'	<i>ccmF</i> gene amplification
ccmHFF	5' ATGCGGATCCATCGACACCTACGAGTTC 3'	<i>ccmH</i> gene inactivation
ccmHFR	5' TGGCGAATTCCTTCAACAGGTTGGCGAG 3'	
ccmHC-F	5' GTGGGAGGCCCGATGAAGCGTTTC 3'	<i>ccmH</i> gene amplification
ccmHC-R	5' GTGCCACGCAGCAGCGGAATCAGC 3'	<i>ccmH</i> gene amplification
GmF	5' TACAGTCTATGCCTCGGG 3'	Gm cassette amplification
GmR	5' ACTACGCGGCTGCTCAA 3'	
Z2	5' CTGCAAGGCGATTAAGTTGG 3'	pKnockout <i>lacZ</i> gene

for 10 min at 4 °C) and washed twice with 0.85 % (w/v) NaCl and once with cell storage buffer (100 mM Tris/HCl, pH 7.3, 1 mM DTT, 1 mM PMSF). Cells were ultrasonically disrupted in the cell storage buffer at a power level of 25 % (15 min at 2 s intervals). The supernatant fraction was collected and centrifuged at 180 000 *g* for 60 min at 4 °C to yield membrane and soluble components. The soluble fraction was further centrifuged for 2 h at the same speed to yield a membrane-free system. Protein concentration was measured and a spectrum of the cell extracts between 350 and 550 nm was taken (Middaugh *et al.*, 2005).

Chrome azurol (CAS) assay for the detection of siderophores.

The presence of siderophores was determined by a universal chemical method using the high affinity of CAS for iron (III) (Schwyn & Neilands, 1987). Agar plates were made containing CAA with the ternary complex chrome azurol S/iron (III)/hexadecyl trimethylammonium bromide as the indicator. A 5 µl sample of a CAA culture (OD₆₀₀ 0.5), was spotted onto CAS plates and incubated overnight. The diameter of the orange halo was measured the next day.

Detection of pyoverdine by IEF and purification of pyoverdine.

Pyoverdine production was assessed by measuring the OD₄₀₀ of 24 h CAA cultures normalized to the OD₆₀₀ (Höfte *et al.*, 1993). Pyoverdine isoforms were detected by IEF of the supernatants of the same CAA liquid cultures, followed by CAS overlay, as described previously (Koedam *et al.*, 1994).

Detection of pyochelin. Pyochelin was extracted from overnight CAA culture supernatants and detected using the method of Farmer & Thomas (2004). Aliquots of culture (30 ml) were extracted with 12 ml acidified ethyl acetate. The organic phase was transferred to a fresh tube and dried to completion under a stream of helium gas. The

solute was resuspended in 50 µl methanol for further analysis. A spectrum was taken between 200 and 450 nm. A 10 µl sample was spotted onto a normal-phase silica 60F₂₅₄ (Merck) TLC plate. Extracts were separated using a chloroform/acetic acid/ethanol mix (90:5:2.5). The plate was visualized using a UV transilluminator and photographed. The pyochelin levels in culture supernatants were estimated by comparing them with ethyl acetate extracts of other strains with mutations in different pyochelin biosynthesis genes (Table 1). Stereoisomeric forms of pyochelin were detected under UV and by measuring the *R_F* value after spraying the plate with a solution of FeCl₃ (0.3 M in 0.1 M HCl). LC/MS analyses were performed on a Kontron 325 system, coupled to the mass spectrometer and equipped with a UV detector (model 322), an automatic injector (model 465) and LC-6A type pumps. The column used was a Vydac 218TP54 RP column (C18, 5 µm, diameter 0.46 cm, length 25 cm) and a flow rate of 1 ml min⁻¹ was maintained. MS data were recorded on a VG Quattro II spectrometer (electrospray ionization, cone voltage 70 V, capillary voltage 3.5 kV, source temperature 80 °C). Data collection was done using the Masslynx software.

Assay for pyocyanin production. Pyocyanin was visualized by growing bacteria for 48 h on P agar (Difco). Pyocyanin was extracted as described by Mavrodi *et al.* (2001). Briefly, the agar was collected in a Falcon tube and 10 ml chloroform was added per 12.5 g agar medium. The phenazine pigment was extracted for 2 h at 37 °C, after which 2 ml 0.5 M HCl was added and the mixture shaken vigorously. The pink top layer was removed and its A₅₂₀ was measured.

Catalase detection. Protein extraction was performed as described in Feissner *et al.* (2003), and the resulting protein extracts were analysed by 7.5 % SDS-PAGE with a DTT-free loading buffer. Catalase activity was detected as described by Wayne & Diaz (1986).

RESULTS

Construction of *P. aeruginosa* mutants in different *ccm* genes

Using the pKnockout vector delivery system for rapid gene inactivation (Windgassen *et al.*, 2000), we obtained mutants in the *ccmA*, *ccmC*, *ccmE*, *ccmF* and *ccmH* genes. No mutant could be obtained in *ccmG*. A mutant initially identified as being in *ccmB* had the vector inserted in an *xcp* gene and was therefore eliminated. All other mutants were verified by analysis of the sequence of the fragment amplified with the corresponding forward and Z2 primers (Table 2). Using primers corresponding to the 5' and 3' ends of the different genes, we could obtain amplification of *ccmA*, *ccmC*, *ccmE*, *ccmF* and *ccmH* for the wild-type, but not in the case of the corresponding mutants. As control for the quality of DNA we used primers for *oprL*, and this gene was amplified in the wild-type and the mutants (results not shown). This is due to the presence of the large pKnockout insert in the interrupted gene, giving a fragment that cannot be amplified by *Taq* polymerase (results not shown). In order to make comparisons with the previously described *ccmC* gene mutant in *P. fluorescens* ATCC 17400, which was obtained by the insertion of a gentamicin cassette, we also inactivated the *P. aeruginosa* *ccmC* gene via insertion of a gentamicin cassette, as previously described (Baysse *et al.*, 2003). This last mutant was termed *ccmC1* and had exactly the same phenotype as the pKnockout *ccmC* mutant (see below). Unless specifically mentioned, the results are given for the pKnockout *ccmC* mutant.

Growth characteristics

Delayed growth in LB medium was observed for the *ccmC* and *ccmF* mutants, while the other mutants grew like the wild-type (see Fig. 1a for wild-type, *ccmC* and *ccmF*). The wild-type strain reached stationary phase after 14 h, the *ccmF* mutant after 24 h, and the *ccmC* mutant after 48 h of growth. In the iron-limiting CAA medium and in the presence of the strong iron (III) chelator EDDHA, the growth of the *ccmF* mutant was further retarded, but the growth of the *ccmC* mutant was almost completely abolished (Fig. 1b). Addition of pyoverdine to the CAA plus EDDHA medium partially restored the growth of the *ccmF* mutant, but had only a small effect on the growth of the *ccmC* mutant (Fig. 1c). From these results we can say that the two *ccmC* mutants obtained via different gene inactivation procedures behave very similarly, and that the *ccmF* and *ccmC* mutations affect growth in all media tested, including CAA and CAA plus iron (results not shown). Furthermore, pyoverdine had only a small stimulatory effect on the growth of the *ccmC* mutant and only partially restored the growth of the *ccmF* mutant.

Cytochrome c and porphyrin production

All mutants, except *ccmC* and *ccmF*, were still oxidase positive, suggesting that they were still able to produce

Fig. 1. Growth of wild-type (●), *ccmC* (○) and *ccmF* (△) in LB medium (a), CAA plus 1 mg EDDHA ml⁻¹ (b), and CAA plus 1 mg EDDHA ml⁻¹ and 50 μM pyoverdine (c).

c-type cytochromes (results not shown). Accumulation of porphyrins was analysed by fluorimetry, and was most strongly evidenced for the *ccmC* and *ccmF* mutants (Fig. 2a). The *ccmC* and *ccmF* mutants accumulated a porphyrin with a peak of emission at 637 nm (Fig. 2a, results shown only for *ccmC*). When the *P. aeruginosa* *ccmC* mutant was complemented with the *P. fluorescens* ATCC 17400 *ccmC* gene (Baysse *et al.*, 2003), the porphyrin peak was strongly reduced (Fig. 2a). The combination of TMBZ and H₂O₂ is used as a stain for the peroxidase activity of cytochrome *c* in SDS polyacrylamide gels. This reagent can be used to detect very low levels of haem-associated

Fig. 2. (a) Detection of porphyrins in total cell extracts of wild-type (●), *ccmC* (○), and *ccmC* complemented by the *ccmC* gene from pPyov35 (+). (b) Haem staining of *c*-type cytochromes after SDS-PAGE. Lanes: 1, pre-stained molecular mass markers; 2, total extract from wild-type; 3, total extract from *ccmC* mutant; 4, total extract from *ccmF* mutant.

peroxidase activity (Goodhew *et al.*, 1990). Haem staining after SDS-PAGE confirmed the presence of *c*-type cytochromes in all mutants, except in the *ccmC* mutant, while the intensity of stained *c*-type cytochrome bands was strongly reduced for the *ccmF* mutant (Fig. 2b).

Production of siderophores

All mutants produced pyoverdine to the same extent as the wild-type, except for the two *ccmC* mutants, for which pyoverdine production was found to be drastically reduced, as evidenced by the colour of the CAA culture supernatant and the absence of fluorescence under UV (Fig. 3a, results shown only for the wild-type and the *ccmC* mutant). Quantitative pyoverdine measurements gave an OD₄₀₀ between 3 and 3.5 for stationary-phase cultures for all tested strains, except in the case of the two *ccmC* mutants, for which this value never exceeded 0.05. On CAS plates, however, high levels of production of siderophores by the *ccmC* mutant were evident (Fig. 3b), and the halo produced around the inoculated bacteria was similar in size and colour to the one produced by a *pvdD* pyoverdine-negative mutant which still produces the second siderophore pyochelin. Detection of siderophore activity by IEF followed by CAS overlay showed that the *ccmC* and

ccmF mutant siderophore patterns were different from those of the other strains and the wild-type (Fig. 3c). To investigate whether the *ccmC* mutant siderophore activity seen on CAS agar plates is due to pyochelin, this siderophore was detected by LC/MS. Two peaks were detected by HPLC, one eluting at 19.8 min and the other at 21.1 min, for both wild-type and the *ccmC* mutant (results not shown). The compound eluting at 19.8 min had a mass of 325 Da and the 21.1 min compound had a mass of 326 Da, close to the expected mass for the two interconvertible pyochelin isomers (324.1 Da) which account for the two different peaks in HPLC (Schlegel *et al.*, 2004). The *ccmC* mutant produced about twice the amount of pyochelin compared to the wild-type (results not shown).

Production of pyocyanin

During the course of our experiments we quickly realized that both *ccmC* mutants also showed a strongly reduced production of the blue-green phenazine pigment pyocyanin on LB or on P-agar medium (Fig. 4). Complementation of the *ccmC* mutant by the *P. fluorescens* ATCC 17400 *ccmC* gene *in trans* restored the capacity to produce pyocyanin (Fig. 4). Quantification of pyocyanin in P-agar medium showed that the production of this pigment is

Fig. 3. Production of siderophores. (a) Culture supernatants of wild-type (left) and *ccmC* mutant (right) grown in CAA for 24 h. The picture on the right shows the fluorescence of the same tubes when exposed to UV light. (b) Comparison of the discoloration haloes around inoculated wild-type (top), *pvdD* pyoverdine-negative mutant (middle right), a pyochelin-negative *pchEF* mutant (centre), a double *pchEF pvdD* mutant (bottom), and the *ccmC* mutant (middle left). The plate was incubated for 48 h. (c) IEF separation of semi-purified and concentrated pyoverdines from *ccmC* (lane 1), wild-type (wt; lane 2) and *ccmF* (lane 3). IEF was done on Ampholine PAG plates with a pH 3–10 gradient, and the bands were visualized after CAS overlay (see text for details).

significantly reduced in the *ccmC* mutants and the *ccmF* mutant compared to wild-type (10–12 % and 30–40 % of wild-type levels, respectively; results not shown). The *ccmC* mutant produced colonies of the same size as the wild-type on P agar (Fig. 4), and even prolonged incubation times (more than 3 days) did not induce pyocyanin production in the *ccmC* mutant.

Swarming, swimming and twitching motilities

Since pyocyanin production is a trait known to be controlled by quorum sensing in *P. aeruginosa* (see Discussion), we decided to look at another quorum sensing-controlled phenotype, motility. As shown in Fig. 5(a), the *ccmC* mutant, and to a lesser extent the *ccmF* mutant (results not shown), was much less motile in LB medium (swarming, swimming and twitching). Supplementary Fig. S2a, b shows the motility of wild-type and *ccmC* mutant cells observed under the phase-contrast microscope.

Detection of rhamnolipids

Rhamnolipids are glycolipids known to be involved in the swarming motility of *P. aeruginosa* (Caiazza *et al.*, 2005).

The drop-collapsing assay was used to measure the wetting capacity of a *P. aeruginosa* culture supernatant (Déziel *et al.*, 2003). The *ccmC* mutants produced less rhamnolipid than the other mutants, as judged by the absence of drop-collapsing activity, a phenotype also observed for the *ccmF* mutant (results not shown). In order to measure more accurately the production of rhamnolipids, we used the blue plate detection method of Siegmund & Wagner (1991). As can be seen in Fig. 5(b), there was a clear reduction of the diameter of the halo around *ccmC* and *ccmF* inocula compared to the wild-type. At a cell density of 1.25×10^8 per well there was no detectable production of rhamnolipids by the *ccmC* mutant.

Production of signal molecules

Since the phenotypes described above are all quorum sensing-dependent, we also looked at the production of the three signal molecules known to be produced by *P. aeruginosa*, 3-oxo-C12-HSL, C4-HSL, and PQS. For the detection of C4-HSL, the *Chromobacter violaceum* CV026 strain was used (McClean *et al.*, 1997), while the *Agrobacterium tumefaciens* pZLR4 strain, which carries a *lacZ* transcriptional fusion under the control of an

Fig. 4. Production of pyocyanin on P-agar plates incubated for 2 days by wild-type (1), *ccmC* (2), and *ccmC* complemented by the *ccmC* gene from pPyov35 (from *P. fluorescens* ATCC 17400; 3).

Fig. 5. (a) Top pictures: swarming motility of wild-type and *ccmC* mutant strains grown on 0.4% agar LB medium; middle pictures: swimming motility of wild-type and *ccmC* mutant grown on 1% agar LB medium; bottom pictures: twitching motility of wild-type and *ccmC* mutant. The strains were grown for 24 h. In all three pictures wild-type and *ccmC* cultures are on the left- and right-hand plates, respectively. (b) Detection of rhamnolipid production. Plates were incubated at 28 °C for 48 h and at room temperature for another 48 h. Wells were inoculated with the number of cells indicated at the top of the figure. Top, wild-type (WT); middle, *ccmC*; bottom, *ccmF*. See text for details.

AHL-inducible promoter for longer-chain AHLs, was used to detect 3-oxo-C12-HSL (Shaw *et al.*, 1997; Cha *et al.*, 1998). The results show that the two *ccmC* mutants produce wild-type levels of the two AHLs. The levels of these two AHLs were also unchanged in all the other strains tested (Supplementary Fig. S1).

For the detection of PQS, extracts from spent medium were separated by TLC and fluorescent spots were detected by UV. PQS could be detected in all extracts. Overlay of the TLC plate by the *P. aeruginosa* *pqsA::lux* indicator strain confirmed that PQS was produced by all strains (results not shown).

Resistance to oxidative stress and [Fe-S] content

Both *ccmC* and *ccmF* mutants were also found to be more sensitive to H_2O_2 when grown in LB or in CAA medium with or without iron. The diameter of growth inhibition in LB was 3.57 cm ($SD \pm 0.06$ cm) for the wild-type and 4.07 cm ($SD \pm 0.15$ cm) and 6.17 cm ($SD \pm 0.06$ cm) for the *ccmC* and *ccmF* mutants, respectively. In CAA medium, the effect was even more pronounced since the following growth inhibition diameters were observed: 3.17 cm ($SD \pm 0.15$ cm) for the wild-type, 7.77 cm ($SD \pm 0.15$ cm)

for *ccmC*, and 8.5 cm ($SD \pm 0.06$ cm) for *ccmF*. After SDS-PAGE, no catalase activity could be detected for the *ccmC* mutant, although a clear band was observed for the cellular and extracellular extracts of the wild-type and the other mutants; in the case of the *ccmF* mutant the catalase band was present, but very faint (Fig. 6).

Spectrophotometric measurement revealed that the peak corresponding to [Fe-S] clusters was strongly reduced in *ccmC* and *ccmF* (Fig. 7).

DISCUSSION

Our results confirm the pleiotropic nature of the *ccmC* mutation (and to a lesser extent of the *ccmF* mutation) in *P. aeruginosa*, a feature already observed in other bacteria, in which it has been found that CcmC in particular is important for the utilization of ferrisiderophores as a source of iron (reviewed by Cianciotto *et al.*, 2005). However, to our knowledge, no systematic comparisons of the phenotypic effects of mutations in different *ccm* genes have been performed to date. Also, it was interesting to find out the effects of these mutations on the opportunistic pathogen *P. aeruginosa*. We successfully obtained knockouts in the *ccmA*,

Fig. 6. Detection of catalase activity after SDS-PAGE. Lanes: 1, molecular markers; 2, total cellular extract of wild-type; 3, total cellular extract of *ccmC*; 4, total cellular extract of *ccmC1*; 5, total cellular extract of *ccmF*.

ccmC, *ccmE*, *ccmF* and *ccmH* genes of *P. aeruginosa* PAO1. Unexpectedly, cytochrome *c* production was not affected in the *ccmA*, *ccmE* and *ccmH* mutants, while it was strongly reduced in the *ccmF* mutant and completely abolished in the *ccmC* mutants. This is maybe due to the method of inactivation that we chose. Indeed, the pKnockout system is designed to generate non-polar insertions of the recombinant plasmid after a single recombination event. This means that the genes downstream are transcribed, but also that the 5' end of the gene is transcribed, explaining why the effects are less drastic compared to what has been reported for other mutants (produced by transposon or antibiotic cassette insertion) described in the literature. In agreement with our previous results in *P. fluorescens* ATCC 17400, the *P. aeruginosa ccmC* mutant was defective for the production of pyoverdine (Gaballa *et al.*, 1996, 1998; Baysse *et al.*, 2002, 2003). Production of pyoverdine by the *ccmC* mutant of *P. aeruginosa* could be restored by complementation with the *P. fluorescens* ATCC 17400 *ccmC* gene *in trans* (Baysse *et al.*, 2003; and results not shown). Interestingly, production of pyochelin was not affected by the *ccmC* mutation and was even increased. In stark contrast, production of the secondary siderophore quinolobactin

could not be detected in the *P. fluorescens* ATCC17400 *ccmC* mutant (Baysse *et al.*, 2003). It has been demonstrated previously that haem is necessary for the production of both pyoverdine and quinolobactin (Baysse *et al.*, 2001, 2003). In line with this, the first enzyme in the pathway for quinolobactin synthesis, tryptophan 2,3-dioxygenase, is a haemoprotein, explaining why the *ccmC* mutation can affect the biosynthesis of this siderophore since it results in a haem shortage (Baysse *et al.*, 2003; Matthijs *et al.*, 2004, 2007). Recently, Matthijs *et al.* (2007) showed that synthesis of quinolobactin and its precursor thioquinolobactin is strongly impaired in a *hemA* haem biosynthesis mutant. By IEF and CAS overlay we also detected non-fluorescent isoforms of pyoverdine in our *ccmC* mutants similar to what was observed by Baysse *et al.* (2002) in the *P. fluorescens* ATCC 17400 *ccmC* knockout strain. This result supports the hypothesis that the maturation of the pyoverdine chromophore from the precursor ferriobactin, which takes place in the periplasm, does not occur in the *ccmC* mutant, because of a lack of oxidative power (Baysse *et al.*, 2002). Recently, it has been shown that at least one pyoverdine biosynthesis enzyme, PvdN, is periplasmic and is exported via the Tat secretion pathway (Voulhoux *et al.*, 2006). In their model, Baysse *et al.* (2003) also predicted that the observed accumulation of the direct haem precursor, protoporphyrin IX, could generate an oxidative stress, which in turn could result in the destruction of [Fe-S] clusters in proteins having them in their catalytic centres (Imlay, 2006). In this context it is interesting to mention that one siderophore reductase, FhuF, is a [Fe-S] protein (Matzanke *et al.*, 2004). Here we could demonstrate that indeed the *ccmC* and *ccmF* mutations cause an accumulation of porphyrins and a drastic reduction of the amount of [Fe-S] clusters. The previously described haem depletion in the *P. fluorescens* ATCC 17400 *ccmC* mutant (Baysse *et al.*, 2003) is also confirmed in our research by the absence of the haemoprotein catalase activity (Fig. 6).

To our surprise, the *ccmC* mutant was also deeply affected in some quorum-sensing-dependent phenotypes since it failed to produce pyocyanin and was non-motile (swarm-

Fig. 7. Detection of [Fe-S] clusters in cell extracts (LB) from wild-type (●), *ccmC* (○) and *ccmF* (△). The spectrum was taken between 350 and 550 nm.

ing, swimming and twitching). Interestingly, a recent report described that some mutations in some *c*-type cytochrome biogenesis genes, including *ccmC*, in *Sinorhizobium meliloti* also affect motility (Yurgel *et al.*, 2007). In *P. aeruginosa*, most virulence factors are regulated via cell-to-cell communication or 'quorum sensing' (Swift *et al.*, 2001; Withers *et al.*, 2001; Bassler, 2002; Cámara *et al.*, 2002), and in a growth phase-dependent manner (Diggle *et al.* 2002, 2003). *P. aeruginosa* has two AHL-dependent quorum-sensing systems, the *las* and *rhl* systems (Winzer & Williams, 2001; Lazdunski *et al.*, 2004). LasI is the 3-oxo-C12-HSL synthase, and LasR is its cognate response regulator, while the *rhl* system involves the RhlI C4-HSL synthase and the response regulator RhlR. Separately or together, the *las* and *rhl* systems regulate the production of extracellular virulence determinants such as elastase, the LasA protease, alkaline protease and exotoxin A, and the production of rhamnolipids, hydrogen cyanide, pyocyanin, siderophores and the cytotoxic lectins PA-IL and PA-III (Diggle *et al.*, 2002; Winson *et al.*, 1995; Lazdunski *et al.*, 2004). The *las* system exerts transcriptional control over the *rhl* system (Lazdunski *et al.*, 2004), but the *rhl* system can also function independently of the *las* system (Diggle *et al.*, 2003). A third signal molecule is characterized as 2-heptyl-3-hydroxy-4-quinolone and termed PQS (reviewed by Diggle *et al.*, 2006b). Synthesis of PQS depends on the *pqsABCDE* locus, which is responsible for generating multiple 4-hydroxyquinolones, including HHQ, the immediate PQS precursor, which is converted into PQS by PqsH, a putative periplasmic monooxygenase (Déziel *et al.*, 2004). Transcription of the *pqsH* gene is under the control of the *las* system (Gallagher *et al.*, 2002), which suggests that PQS synthesis depends on the production of 3-oxo-C12-HSL. However, LasR-independent production of PQS has also been demonstrated (Diggle *et al.*, 2003). On the other hand, McGrath *et al.* (2004) have demonstrated that the *rhl* system represses PQS production. It therefore seems that the PQS regulon is intertwined in the AHL quorum-sensing systems of *P. aeruginosa*. PQS is required for the production of pyocyanin in *P. aeruginosa* (Gallagher *et al.*, 2002; Diggle *et al.*, 2002, 2003; Dietrich *et al.*, 2006; Price-Whelan *et al.*, 2006).

Nevertheless, in our experiments the production of signal molecules was not really affected by any of the mutations. The *ccm* genes are not themselves subject to control by the quorum-sensing signal molecules AHL and PQS (Wagner *et al.*, 2003; Schuster *et al.*, 2003; Hentzer *et al.*, 2003; Bredenbruch *et al.*, 2006). The question remains why CcmC, and to a lesser extent CcmF, are important for functions other than *c*-type cytochrome biogenesis, and why phenotypes such as motility and pyocyanin production are affected. One possible explanation is that pyoverdine, which is a signal molecule as well (Lamont *et al.*, 2002; Visca *et al.*, 2002, 2007), also controls traits such as motility and phenazine pigment production. Others have made the interesting observation that pyoverdine-negative mutants are unable to form mature

biofilms, suggesting that pyoverdine acts not only as a siderophore but also as an important signal (Banin *et al.*, 2005). Accordingly, a very recent report from Matilla *et al.* (2007) has described the importance of pyoverdine for the motility of *P. putida* KT2440. Another hypothesis, not excluding the first one, is that CcmC is required for the oxidative power of the periplasm, as suggested by Baysse *et al.* (2002). Pyocyanin and 4-quinolones are also redox-active molecules which could play a role in the electron transport chain under aerobic conditions (reviewed by Price-Whelan *et al.*, 2006). It seems likely therefore that CcmC is a key component for the normal functioning of aerobic respiration and that its absence negatively influences many pathways in *P. aeruginosa*, which makes it an interesting target for future drug development.

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