# Overproduction of CcmABCDEFGH restores cytochrome *c* maturation in a DsbD deletion strain of *E. coli*: another route for reductant?

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Received 9 July 2004; revised 24 August 2004; accepted 30 August 2004

Available online 11 September 2004

Edited by Peter Brzezinski

**Abstract The multidomain transmembrane protein DsbD is essential for cytochrome *c* maturation (Ccm) in *Escherichia coli* and transports reductant to the otherwise oxidising environment of the bacterial periplasm. The Ccm proteins ABCDEFGH are also essential and we show that the overproduction of these proteins can unexpectedly complement for the absence of DsbD in a deletion strain by partially restoring the production of an exogenous *c*-type cytochrome under aerobic and anaerobic conditions. This suggests that one or more of the Ccm proteins can provide reductant to the periplasm. The Ccm proteins do not, however, restore the normal disulfide mis-isomerisation phenotype of the deletion strain, as shown by assay of the multidisulfide-bonded enzyme urokinase.**


*Keywords:* DsbD; DipZ; Cytochrome *c* maturation; Disulfide bond isomerization

## 1. Introduction

*C*-type cytochromes are essential proteins found in virtually all organisms. Their biosynthesis in some Gram-negative bacteria and in plant mitochondria is dependent on the eight so-called cytochrome *c* maturation (Ccm) proteins [1], all of which are periplasmic and anchored to the cytoplasmic membrane or integral to it. This group of proteins is known as System I; there are two other distinct cytochrome *c* biogenesis systems that occur in other organisms and organelles [2]. In addition to the Ccm proteins, the disulfide bond (Dsb) protein system is also involved, and includes thioredoxin, DsbA, B and C as well as the membrane-bound, three-domain protein DsbD (also known as DipZ), which has one transmembrane and two periplasmic domains [3,4]. The Dsb system is also involved in the reduction of incorrectly formed disulfides in other periplasmic proteins, in a disulfide isomerase system (as reviewed elsewhere, [5]). It is thought that disulfide bonds first form between the two cysteines of the CXXCH motif in apocytochromes, as has been demonstrated in vitro [6], and are later reductively cleaved. The cysteine thiols in this motif form the covalent bonds to the vinyl groups of heme that are characteristic of *c*-type cytochromes. The formation of the disulfide bonds between the cysteines is believed to be catalysed by the periplasmic protein DsbA which has been shown, in at least some circumstances, to be required for cytochrome *c* maturation [7,8], though DsbA does not appear to be essential for this process in all organisms and under all conditions [9,10]. The function of DsbD has been partially elucidated by studies of its individual domains and of its three pairs of conserved cysteine residues [11,12], each of which occurs in one of the three domains. Electrons are transported from thioredoxin in the bacterial cytoplasm to the transmembrane domain of DsbD (DsbD($\beta$)), to the periplasmic C-terminal domain DsbD($\gamma$), then to the N-terminal domain DsbD($\alpha$) [13], a process involving a disulfide cascade that has been studied in some detail [14]. DsbD($\alpha$) next reduces the Ccm proteins G and H [15], which then presumably reduce the apocytochrome disulfides via a mechanism that has not yet been elucidated. The structures of the two soluble domains of DsbD have been determined. DsbD($\alpha$) has an immunoglobulin-like structure [16], whereas DsbD($\gamma$) has a thioredoxin fold [17].

The *Escherichia coli c*-type cytochrome biogenesis system can be used to produce heterologously cytochromes *c*, from a variety of organisms [18,19], including the cytochrome *c* domain of the nitrite reductase cytochrome $cd_1$ from *Paracoccus pantotrophus*, which is a soluble globular domain of the enzyme that contains a covalently attached heme and has been characterised [20,21]. This protein was selected as the cytochrome *c* marker in this study because of its distinctive spectroscopic properties, its relatively small size, because it contains a single heme, and has been produced previously in its matured form in *E. coli*.

The importance of the protein DsbD in the biogenesis of *c*-type cytochromes in some bacteria has been known for some time. In a DsbD deletion strain of *E. coli*, *c*-type cytochromes are not produced [7,22,23] and the null mutation can be complemented in trans with DsbD on a plasmid [4,24], fully restoring *c*-type cytochrome biosynthesis, as well as by the addition of certain thiol compounds to the growth medium [25]. DsbD has also been shown to be involved in the biogenesis of *c*-type cytochromes in *Pseudomonas aeruginosa* [26]. In this work, we report that the absence of DsbD can be complemented by overproduction of the Ccm proteins

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*Abbreviations:* Dsb, disulfide bond; Ccm, cytochrome *c* maturation

0014-5793/$22.00 
doi:10.1016/j.febslet.2004.08.067

ABCDEFGH from a plasmid, suggesting that one or more of these proteins is able to supply some of the reductant necessary for *c*-type cytochrome biogenesis to the periplasm.

## 2. Materials and methods

### 2.1. Strains, plasmids and growth conditions

The strains JCB387 (*E. coli* RV Δ*nirB* [27]) and JCB606 (JCB387 Δ*dsbD* [23]) were kindly provided by Prof. J. Cole (Birmingham) and the strains RI89 (MC1000 *phoR* Δ*ara*714 *leu*$^+$) and RI242 (RI89 *dipZ*::mini-Tn*10*Cam1) [28] by Mary Berlyn (E. coli Genetic Stock Center). The plasmid pEG366a was used for the expression of the cytochrome *c* domain of NirS (cytochrome $cd_1$ nitrite reductase from *P. pantotrophus*) which together with its $d_1$ heme-containing domain is a nitrite reductase [20]. The Ccm proteins were expressed constitutively from the plasmid pEC86 [29], which was kindly provided by Prof. L Thöny-Meyer (ETH, Zurich) or from a form of this plasmid (pEG1026) that was modified to include a Gm cassette to allow selection in the strain RI242. The plasmid pRDB8b was used for the expression of mouse urokinase plasminogen activator constitutively in the periplasm; urokinase assays were performed as described [30] and zymogram images were quantified with Tnimage (http://brneurosci.org/tnimage.html). For aerobic cultures, bacteria were grown overnight on Luria–Bertani (LB) medium at 37 °C and shaken at 200 rpm. The volume of the growth media did not exceed 10% of the volume of the culture flask. For anaerobic cultures, a minimal medium described previously [8] was used to fill 50 ml tubes which were then inoculated with 0.5 ml of overnight cultures grown on LB medium, and were incubated without shaking at 37 °C for approximately 24 h. For cultures containing the plasmid pEG366a ampicillin was added to a concentration of 100 μg/ml, those with the plasmid pEC86 contained 33 μg/ml of chloramphenicol and those with the plasmid pEG1026 had 10 μg/ml gentamicin. The cells were harvested and resuspended in 50 mM Tris–HCl, pH 7.4, 250 mM NaCl. Polymyxin B sulfate was used to prepare the periplasmic fraction by adding it to cell suspensions to a concentration of 1 mg/ml, followed by incubation at 37 °C for 30 min and centrifugation at $10\,000 \times g$ for 20 min.

### 2.2. Protein characterisation

The periplasmic fractions were analysed for *c*-type cytochrome content by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 15% acrylamide) using the discontinuous buffer system (NuPAGE, Invitrogen), followed by staining for covalently attached heme according to the method published by Goodhew [31]. Visible absorption spectra of the periplasmic extracts were recorded on a Perkin–Elmer Lambda 2 spectrophotometer. Samples were reduced by the addition of a small amount of solid disodium dithionite where appropriate. The protein content of the samples was determined using the method of Bradford [32] and the results shown were normalised according to these values.

## 3. Results

### 3.1. Complementation of DsbD deficiency by the Ccm proteins

Fig. 1 shows an SDS–PAGE analysis of the periplasmic proteins from the parental strain JCB387 and the DsbD$^-$ strain JCB606 grown under aerobic conditions in panel A and anaerobic conditions in panel B. Equivalent amounts of protein were loaded onto the gels within each panel. Gels were treated according to a method that stains for covalently attached heme and therefore detects *c*-type cytochromes. Lanes 1 and 4 of Fig. 1A show periplasmic extracts of JCB387 and JCB606 containing only the plasmid pEC86 and, as expected, neither of these produced holocytochrome corresponding to the *c*-domain of nitrite reductase which was used (see Section 1) as the test for *c*-type cytochrome biosynthesis. Lanes 2 and 5 show that cultures of both strains did not produce holocytochrome in the presence of only the *c*-domain expressing plasmid pEG366a under aerobic conditions. This is expected as the endogenous Ccm proteins are expressed only under anaerobic conditions [33]. Lane 3 shows the aerobic expression of a holocytochrome of the expected mass of around 14.9 kDa (determined from comparison with molecular mass markers (not shown) and a sample of the purified protein) when both plasmids were present in JCB387. Lane 6 shows that there was also expression, albeit less than in JCB387 but nevertheless readily detectable, of the cytochrome *c*-domain in JCB606 under aerobic conditions when the two plasmids were present. Fig. 1B shows the results for the corresponding cultures grown under anaerobic conditions. Again, lanes 1 and 4 show that neither strain produces the holocytochrome when containing only pEC86. Lane 2 shows the effect of the anaerobic induction of expression of the Ccm proteins encoded by the chromosomal *ccm* operon of JCB387; a significant amount of the cytochrome was produced. That JCB606 did not produce any

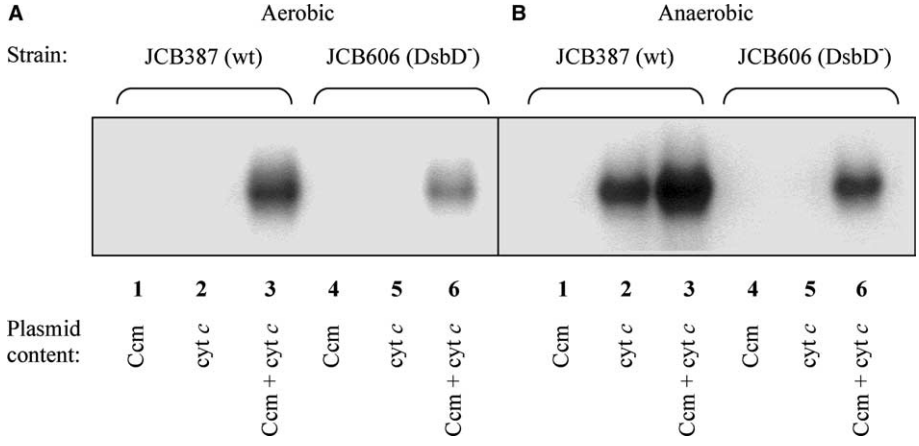


Fig. 1. SDS–PAGE analysis in conjunction with heme staining of periplasmic extracts of JCB387 (wild-type) and JCB606 (DsbD$^-$) cultures containing either or both of the plasmids pEC86 (encoding the Ccm proteins) or pEG366a (encoding the *c*-domain of cytochrome $cd_1$). Panel A shows the results for cultures grown under aerobic conditions and Panel B for anaerobic conditions. For each panel; lane 1: JCB387 containing pEC86; lane 2: JCB387 containing pEG366a; lane 3: JCB387 containing pEC86 and pEG366a; lane 4: JCB606 containing pEC86; lane 5: JCB606 containing pEG366a; lane 6: JCB606 containing pEC86 and pEG366a. The band observed is of the mass expected for the *c*-domain of cytochrome $cd_1$.

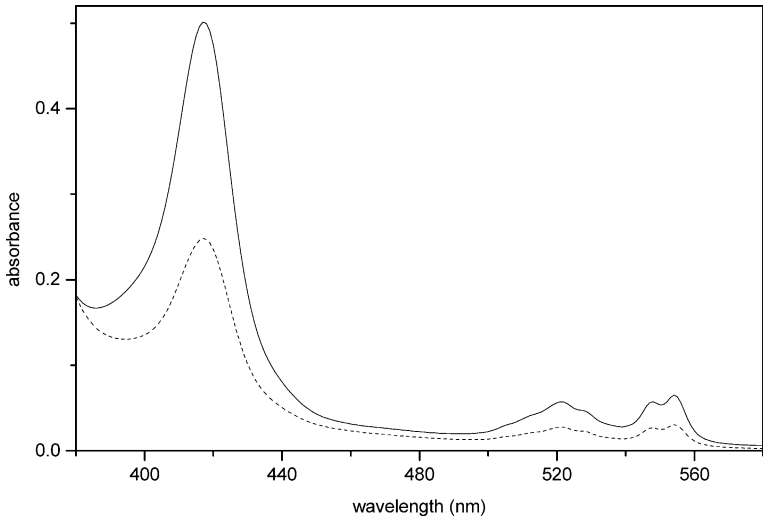


Fig. 2. Absorption spectra of reduced periplasmic extracts of wild-type JCB387 (—) and JCB606 (DsbD$^-$) (– – –) strains co-expressing the holocytochrome *c*-domain of cytochrome $cd_1$ nitrite reductase (from plasmid pEG366a) and the Ccm proteins ABCDEFGH (from plasmid pEC86) under aerobic conditions. The spectra were normalised according to total protein content and samples were reduced by the addition of a small amount of solid disodium dithionite.

cytochrome when transformed with pEG366a and grown anaerobically (lane 5) shows that DsbD is essential for cytochrome *c* maturation, as has been shown previously [7,22,23]. Lanes 3 and 6 show the same result as was observed aerobically; JCB606 produces the holocytochrome *c* when the plasmid-encoded Ccm proteins are expressed anaerobically, though to a lesser extent than the DsbD$^+$ strain JCB387.

Fig. 2 shows the visible absorption spectrum of dithionite-reduced periplasmic extract of JCB606 (DsbD$^-$) containing both pEC86 and pEG366a and grown under aerobic conditions, compared with the spectrum of the periplasmic extract of JCB387 (DsbD$^+$) containing the same plasmids and grown under identical conditions. Averaged over a number of growth experiments, the level of holocytochrome production in JCB606 compared with JCB387 was about 40%. This was determined according to the relative absorbance intensities of the Soret bands. The spectra of the periplasmic extracts indicate the presence of the holo-form of the *c*-domain and shows wavelength maxima expected for this protein in its reduced form [20,21]. The spectrum shows the characteristic split α-band with maxima at 548 and 554 nm as well as the expected Soret band at 417 nm and β-band at 521 nm. The spectrum of reduced periplasm from anaerobically grown JCB387 containing pEC86 showed small peaks with absorption maxima at 418, 522 and 551 nm which could be indicative of the expression of endogenous *E. coli* *c*-type cytochromes that are produced under anaerobic conditions [34]. These were not present in sufficient quantities to be detected by SDS–PAGE and heme staining.

In order to confirm that the effects observed above were not strain-specific, the same experiments were performed on a separate set of *E. coli* strains from a different source (namely *E. coli* strain RI242, which has a *dipZ*::mini-Tn *10*Cam1 genotype and the parental strain RI89). SDS–PAGE analysis followed by heme-staining of the periplasmic extracts of these strains containing the required plasmids (as above) was found to give the same result (not shown) showing that the complementation for cytochrome *c* maturation by the Ccm proteins in a DsbD deletion strain is a general effect.

### 3.2. Urokinase expression

Urokinase is an enzyme that is only active when its multiple disulfide bonds are correctly formed. The mouse urokinase expressed from the plasmid pRDB8b contains 12 disulfide bonds in its folded form and can be expressed in the periplasm of *E. coli* in its active state [30]. It is known that the enzyme is inactive in the absence of DsbD in *E. coli* [28] and so we were prompted to determine whether the Ccm system could complement for the loss of disulfide isomerase activity in the same way that it can for cytochrome *c* maturation. Expression of urokinase in the strain JCB606 was found to produce inactive enzyme (approximately 8% of the activity of the wild-type strain JCB387); the activity did not increase when the Ccm proteins were coexpressed (not shown) indicating that this phenotype cannot be reversed by the Ccm system.

## 4. Discussion

The periplasmic space of Gram-negative bacteria is known to be an oxidising environment. Yet, paradoxically, the maturation of *c*-type cytochromes, a post-translational process that requires reducing conditions, occurs in the periplasm. It is believed that the reaction of ferric heme with apocytochromes produces incorrect products instead of properly matured *c*-type cytochromes [35] and this has been demonstrated in vitro as normal thioether bond formation occurs only with ferrous heme [6]. As it is not known how heme is transported into the periplasm, we are unable to determine its redox state when it arrives in the periplasm from its site of synthesis in the cytoplasm. The heme chaperone CcmE, which is an intermediate in the cytochrome *c* maturation process that binds heme covalently via a histidine residue (described below), appears to undergo heme attachment exclusively under reducing conditions [36,37].

In addition to the requirement for reduced heme, it is also necessary for the cysteine residues of the apocytochrome to be reduced before the heme can be attached. It is known that a disulfide bond can form between the two cysteines of the

CXXCH motif in an apocytochrome in vitro [6] and evidence has been presented suggesting that such a disulfide bond forms in vivo in the case of an artificial cytochrome *c* [9]. It is generally accepted that as apocytochromes are transported into the bacterial periplasm by the Sec system [38], DsbA (known to be a strong oxidant [39]) oxidises the cysteines in the CXXCH. Reduction of the disulfide is then believed to be performed via a series of disulfide exchange reactions involving DsdD and the Ccm proteins CcmG and H.

In this study, we have shown that the absence of holocytochrome formation in a DsbD$^-$ strain of *E. coli* (JCB606) can be partially reversed, under both aerobic and anaerobic conditions, by overproduction of the Ccm proteins ABCDEFGH. A spectroscopic analysis of the periplasmic extracts of JCB606 and its parent strain JCB387, in the presence and absence of the Ccm-expressing plasmid and a plasmid expressing an exogenous cytochrome, showed that the Ccm system could partially complement for the deletion. The absorption spectra indicated that the holocytochrome was produced in its correctly matured form [20] and that, under aerobic conditions, about 40% of the holocytochrome production was restored. The same result was observed by SDS–PAGE analysis of the same extracts followed by heme staining; JCB606 with pEC86 produced the expected heme-staining band demonstrating the complementation effect by the Ccm system. Complementation to restore the production of active urokinase, the activity of which is dependent on the correct formation of multiple disulfide bonds, was not observed.

It seems possible, therefore, that the Ccm proteins (CcmABCDEFGH), all of which are anchored to or integral to the cytoplasmic membrane, contain a transporter of reductant to the periplasm. CcmA has features suggesting that it forms part of an ABC-transporter (ATP-binding cassette) on the cytoplasmic side of the membrane along with CcmB which is a membrane protein [40]. The substrate of this transporter has not been identified to date; there is conflicting evidence as to whether the substrate could be heme, as discussed elsewhere [2]. It is not clear whether the membrane protein CcmC functions with CcmAB [41,42], though a role for CcmC in the attachment of heme to the protein CcmE has been demonstrated [43]. CcmE is a membrane-anchored heme chaperone that has a periplasmic globular domain and has been shown to interact with both CcmC, CcmD [44]; CcmF [45]; it functions by binding heme covalently via a histidine residue before transferring the heme to apocytochromes [46]. CcmD is a small single transmembrane-helix protein that appears to stabilise the interactions of other Ccm proteins and enhances heme binding to CcmE [44]. CcmF is a large protein with at least 11 transmembrane helices and has been proposed to function as a transporter [47]; other studies propose that it functions as part of a heme lyase complex [45] but its function in vivo remains to be demonstrated. CcmG is a periplasmic thioredoxin-like protein [48] and functions along with CcmH in a presumed disulfide bond reduction pathway [49].

Of the Ccm proteins described above, there are several potential candidates (CcmAB and CcmF) for providing reductant to the periplasm and producing the complementation effect that we have observed. Such a reductant transport role for CcmAB has been suggested previously [42]. It is likely in fact that reductant is provided by one or more of the proteins CcmABCD, as these proteins are required for heme attachment to CcmE (known to require reduced heme [37]), which can occur in the absence of CcmFGH [46]. The elucidation of which of the Ccm proteins are involved is complicated by the fact that many, if not, all of the proteins appear to function in complexes with each other and so the consequence of absence of a single protein does not necessarily demonstrate the function of that protein and may relate only to the disruption of a complex in which that protein occurs. Co-immunoprecipitation experiments have provided some evidence of a Ccm supercomplex [50] which recently (in plant mitochondria) has been shown to be as large as 700 kDa [51]. A reductant transport function of the protein complex CydDC has been shown in *E. coli*; the complex appears to transport cysteine to the periplasm [52] and appears to be involved in cytochrome maturation. Further, detailed study of the Ccm proteins will reveal whether such a transporter is present or if increased production of the Ccm proteins attenuates the requirement for DsbD-dependent reductant in the periplasm by some other mechanism. It is possible that the effect we have observed reflects an increased rate of heme attachment to apocytochromes (due to elevated amounts of the Ccm proteins) competing kinetically with the process of disulfide bond formation, thereby by-passing the requirement for DsbD.

*Acknowledgements:* This work was supported by Grants C11888 and C20071 from the UK BBSRC to S.J.F. J.M.S. gratefully acknowledges funding from the BBSRC. The authors thank Michaël Cartron, Richard Zajicek and James Allen for assistance and advice.

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