

# Mutants of *Escherichia coli* lacking disulphide oxidoreductases DsbA and DsbB cannot synthesise an exogenous monohaem *c*-type cytochrome except in the presence of disulphide compounds

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**Abstract** Absence through mutation of two proteins involved in periplasmic disulphide bond formation, DsbA and DsbB, results in failure of anaerobically grown *Escherichia coli* to synthesise the holo forms of either its endogenous *c*-type cytochrome nitrite reductase or exogenous cytochrome *c*<sub>550</sub> from *Paracoccus denitrificans*. The synthesis of both cytochromes can be restored to the mutants by inclusion in the growth media of compounds containing disulphide bonds, e.g., the oxidised form of glutathione. The results suggest that the attachment of haem to the CXXCH motif of a periplasmic *c*-type cytochrome may be preceded by the formation of one or more intra- or inter-molecular disulphide bonds involving the cysteine residues of this motif.

**Key words:** Disulphide oxidoreductase; Bacterial *c*-type cytochrome biogenesis; Periplasm; *Escherichia coli*

## 1. Introduction

Proteins that function in the periplasm of the Gram-negative bacteria frequently undergo post-translational modifications. Prominent amongst these are the formation of disulphide bonds and the covalent attachment of haem to give *c*-type cytochromes. At first sight, these two types of process seem unrelated, but it has been shown that the gene *dsbD* [1–3] (initially called *dipZ* [1] and *cutA2* [3]), coding for a membrane-anchored protein with its catalytic site for disulphide isomerase activity located in the periplasm, is required in *Escherichia coli* for biogenesis of both endogenous and exogenous *c*-type cytochromes [1,4]. Deficiency in *c*-type cytochrome biogenesis could be corrected by inclusion in the growth medium of either L-cysteine or coenzyme M, 2-mercaptoethane sulphonic acid [4]. More recently a mutant of *E. coli* unable to synthesise endogenous multi haem *c*-type cytochromes has been shown to be defective in the *dsbA* gene which codes for an enzyme catalysing disulphide bond formation in the bacterial periplasm [5].

Not only DsbA and DsbD but also DsbC participate in the formation of disulphide bonds in the periplasm and each has sequence motifs CXXCZ, with Z notably being H in the case of DsbC [6]. The two cysteines of these motifs are known to undergo cycles of oxidation to a disulphide and re-reduction

to two cysteines as part of their catalytic activity. The sequence CXXCH provides, together with a methionine residue found many residues further towards the C-terminus of the polypeptide, the haem attachment site for a *c*-type cytochrome. Many lines of evidence (e.g., [7–9]) indicate that such haem attachment occurs in the periplasm (reviewed in [10,11]). Thus the CXXCH motifs in polypeptides destined to become *c*-type cytochromes have to be recognised differently from the similar motifs in the Dsb proteins. The requirement for *dsbD* in *c*-type cytochrome biogenesis [1,4] suggests that any differential recognition is not such that a cytochrome polypeptide is excluded from interaction with the Dsb proteins. In the present paper we report the consequences for *c*-type cytochrome biogenesis of deficiency in *dsbA* and *dsbB* genes and the extent to which these deficiencies can be overcome by inclusion of disulphide-containing compounds in the growth medium. Certain such compounds are already known to restore disulphide bond formation in the periplasm in *dsb* minus backgrounds [12,13].

## 2. Materials and methods

### 2.1. Chemical compounds

Disulphide compounds used in this study were L-cystine dihydrochloride, D-cystine, cystamine dihydrochloride and oxidized glutathione (GSSG) disodium salt, all of which were purchased from Sigma, St. Louis, MO.

### 2.2. Bacterial strains, plasmid and growth conditions

The *E. coli* K12 strains used were JCB570 (parent strain) and its derivatives, JCB571 carrying the *dsbA::kanI* mutation and JCB789 carrying the *dsbB::kanI* mutation [12,14]. These *E. coli* strains were transformed by the plasmid pKPD1 [9] which carries the wild-type *P. denitrificans* cytochrome *c*<sub>550</sub> gene under the control of *tac* promoter of the original vector pKK223-3 (ampicillin resistance, Pharmacia). The transformed cells were grown in 2 ml of LB media overnight, and then 50 µl of fresh cells were inoculated into 15 ml conical tube filled with anaerobic minimal media [15] supplemented with 2.5 mM nitrite and 40 mM fumarate as electron acceptors, 0.4% (w/v) glycerol as a carbon source, and thiamine (1 µg/ml). To demonstrate the effects of disulphide compounds, freshly made stocks of these compounds in the same liquid media were diluted into the tubes. The antibiotics ampicillin (50 µg/ml) and kanamycin (25 µg/ml) were added to the media when required. The tubes were tightly screw capped and the cultures were incubated for 17 h at 37°C without shaking.

### 2.3. General methods

Molecular cloning methods were according to the procedures described by Sambrook et al. [16]. Fractionation of periplasmic protein from *E. coli* cells was performed by the cold osmotic shock procedure described previously [17,18]. SDS-PAGE was performed according to Laemmli [19] using 12.5% (v/v) acrylamide gels.

### 2.4. Quantitative determination of expressed holo-cytochrome *c*<sub>550</sub>

The periplasmic protein fractions were subjected to SDS-PAGE. The amount of exogenously expressed *P. denitrificans* holo-cyto-

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Abbreviations: AP, alkaline phosphatase; GSSG, oxidised glutathione

chrome  $c_{550}$  in the periplasmic fractions was evaluated by densitometric scans of haem-stained SDS gels [20]; a series of known amounts of purified cytochrome  $c_{550}$  was electrophoresed simultaneously as standards for the quantitative analysis. Gel scans were performed with a Bio-Rad Automatic DNA Sequence Reader and band intensities were analyzed by using IMAGE QUANT (Molecular Dynamics). The intensity of haem stain for a given amount of  $c$ -type cytochrome varies between individual gels. Consequently, for quantitation the following procedure was adopted. Each gel was loaded with several different amounts of cytochrome  $c_{550}$  in a range that had previously been established to give a linear response between the logarithm of the intensity of haem staining and the amount of protein loaded. The test material, periplasmic fraction from a *dsbA* or *dsbB* grown with a given amount of oxidised glutathione present, was loaded at several different protein concentrations onto a gel which, in each case, also had lanes containing at least three different amounts of the standard protein.

### 2.5. Alkaline phosphatase activity

Endogenous alkaline phosphatase (AP) activities from the periplasmic protein fractions were measured essentially according to Brickman and Beckwith [21] with some modifications. One millilitre of the suitably diluted periplasmic protein solutions in 50 mM Tris-HCl (pH 8.0) were added to 0.1 ml of phosphatase substrate Sigma 104 (0.4% w/v) in 50 mM Tris-HCl (pH 8.0) and incubated at 37°C. The reactions were stopped by adding 0.1 ml of 1 M  $\text{KH}_2\text{PO}_4$ . The increase in absorbance at 420 nm was measured.

## 3. Results

### 3.1. Chemical rescue of *dsbA* and *dsbB* mutations

Both the *dsbA* and *dsbB* mutations resulted in a complete loss of  $c$ -type cytochromes in the periplasm of *E. coli*, exemplified by either endogenous *E. coli* tetrahaem cytochrome  $c_{552}$ , otherwise known as nitrite reductase, or exogenous *P. denitrificans* cytochrome  $c_{550}$ . This result can be seen by contrasting the extreme left hand lane in panel A of Fig. 1 with the corresponding lanes in panels B and C.  $c$ -Type cytochromes were also completely absent from spheroplast fractions prepared from *dsbA* or *dsbB* mutants (data not shown). Addition of the disulphide compounds, L-cystine, D-cystine, cystamine (all at 1 mM) or GSSG (5 mM) to the growth media restored  $c$ -type cytochrome formation in both mutants. Of the compounds restoring the cytochrome synthesis, GSSG was studied in more detail.

### 3.2. Effects of GSSG on the amount of cytochrome $c_{550}$ and alkaline phosphatase activity

The presence of up to 16.5 mM added GSSG had little effect on the expression of cytochromes  $c_{550}$  (from *P. denitrificans*) or  $c_{552}$  (endogenous) in wild-type cells (Fig. 1A). However, the synthesis of the holo forms of both *P. denitrificans* cytochrome  $c_{550}$  and *E. coli* cytochrome  $c_{552}$  (nitrite reductase) by the *dsbA* and *dsbB* mutants was enhanced by inclusion of increasing amounts of GSSG in the medium. Interestingly, judging from the intensities of the haem staining bands, the expression levels for both the endogenous and exogenous cytochromes  $c$  showed a similar dependence on the concentration of GSSG in the two mutant backgrounds (Fig. 1B,C).

The quantitative relationships between amount of holo-cytochrome  $c_{550}$  and GSSG concentration are shown in Fig. 2. AP, a periplasmic protein that requires disulfide bonds for function, activity was also restored by GSSG in the two mutants, essentially in parallel to the amount of cytochrome  $c_{550}$  formed in each case (Fig. 2B,C). However, the restoration pattern in the *dsbA* mutant did not exactly parallel that in

the *dsbB* mutant. In the former the restoration levels reached a plateau value gradually as the GSSG concentration was raised to 4.5 mM, while in the latter case a lower concentration (1 mM) was sufficient to attain the maximum level of formation of AP and holo-cytochrome  $c_{550}$ . These results are consistent with the observations of Bardwell et al. [12] and Dailey and Berg [13], who reported that higher concentrations of disulphide compounds, GSSG (> 10 mM) or cystine (8.6 mM) were required to restore the *dsbA* phenotype compared with restoration of *dsbB* mutation, indicating that these oxidants can substitute for the DsbB function, but not for DsbA itself. The higher concentrations of oxidants might permit direct oxidation of substrate proteins in the *dsbA* background without any involvement of DsbB. The extent to which alkaline phosphatase activity is lost in *dsbA* and *dsbB* backgrounds is reportedly variable [6,14,22,23], but a maximum activity loss of 60-fold [14] and restoration by cystine have been reported previously [23].

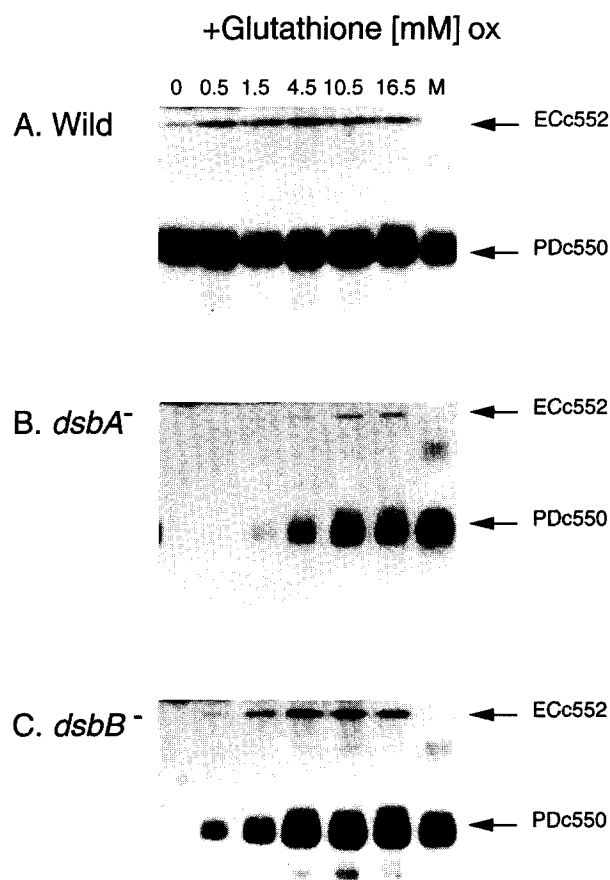


Fig. 1. The effect of inclusion of varying concentrations of oxidised glutathione (GSSG) in the growth medium of (A) wild type; (B) *dsbA*<sup>-</sup> and (C) *dsbB*<sup>-</sup> *E. coli* upon the synthesis of an endogenous and an exogenous  $c$ -type cytochrome. The synthesis of *E. coli* cytochrome  $c_{552}$ , nitrite reductase, (ECc552) and *P. denitrificans* cytochrome  $c_{550}$  (PDc550) was followed by staining SDS-PAGE for covalently attached haem. The concentrations of GSSG initially present in the growth media are indicated at the tops of the lanes. Periplasmic protein (7  $\mu\text{g}$ ) was loaded on to each lane. Lane M indicates loading of a sample (0.4  $\mu\text{g}$  protein) of pure *P. denitrificans* cytochrome  $c_{550}$ .

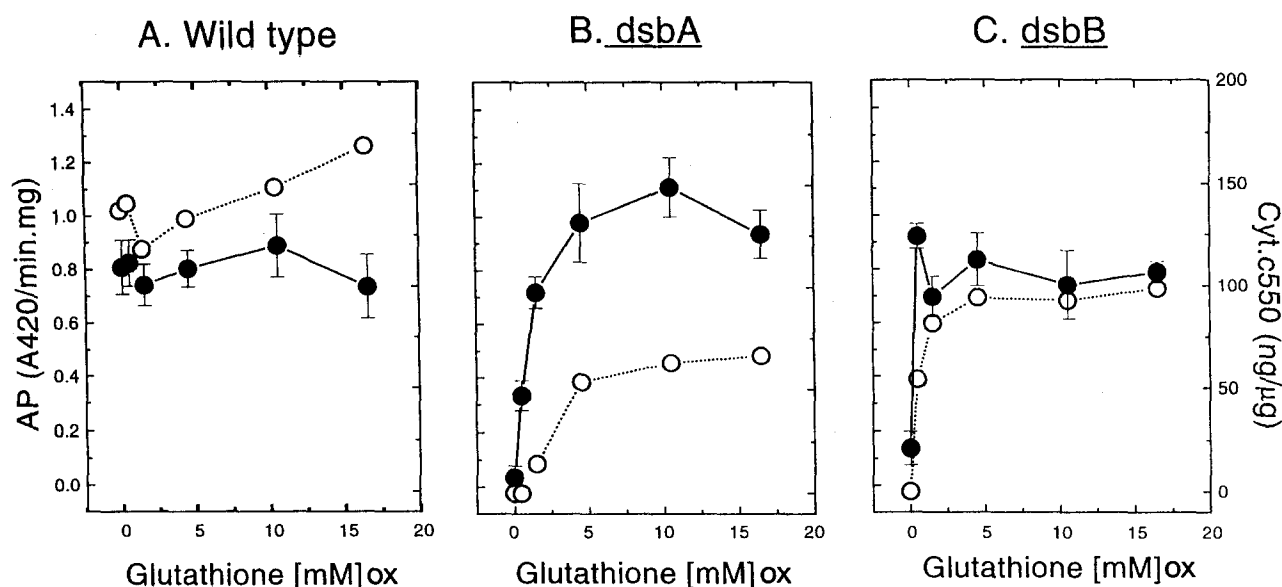


Fig. 2. Quantitation of the effect of GSSG in the growth medium upon the formation of alkaline phosphatase (AP) activity (●) and *P. denitrificans* cytochrome *c*<sub>550</sub> (○) in (A) wild type; (B) *dsbA*<sup>-</sup> and (C) *dsbB*<sup>-</sup> *E. coli*. Quantitation of haem staining was done as described in Section 2. The cytochrome *c*<sub>550</sub> contents (means of two independent assays) are expressed as ng/μg of total periplasmic protein. Error bars are shown for alkaline phosphatase activity (determined from two independent cultures, for which each set of measurements was done in duplicate).

#### 4. Discussion

The new findings presented in this paper are: (i) DsbA is required for synthesis of an exogenous mono haem *c*-type cytochrome in *E. coli*; (ii) DsbB is required for synthesis of both this exogenous *c*-type cytochrome and the endogenous *c*-type cytochromes; (iii) not only each of these types of cytochrome, but also alkaline phosphatase activity, are restored in parallel in cells with *dsbA* or *dsbB* mutations when cells are grown anaerobically in the presence of an oxidant carrying a disulphide bond.

The first of these findings complements the observations of Metheringham et al. [5] where it was shown that endogenous multihaem *c*-type cytochromes of *E. coli* are formed only in the presence of active DsbA. In discussing the latter observations it was noted that the requirement for DsbA might be because a component of the *c*-type cytochrome biogenesis machinery may require a disulphide bond, formed by the action of DsbA, for function [5]. The CaaaAC motif in the CcmH (also known as Ccl2) protein [24,25] could, for example, present such a requirement. An alternative suggestion [5] for the role of DsbA was that it would correctly lock pairs of cysteines within each CXXCH motif of the polypeptide chain of the multihaem cytochromes before subsequent attachment of the haem. Alternatively, either the weak disulphide isomerase activity of DsbA alone [26], or DsbA in combination with the isomerase DsbC [26], may be required to ensure that unwanted cross disulphide bonds between different CXXCH motifs do not form. Yet another possibility is that without this type of activity the haem might be attached to two cysteines of different CXXCH motifs. The present finding that a mono-haem *c*-type cytochrome requires DsbA for its expression in *E. coli* argues against these last three possibilities, but is consistent with proposals that disulphide bond formation within the CXXCH motif of a *c*-type cytochrome, or between this motif and thiol groups on other proteins, is an obligatory step on the route leading to covalent attachment of haem to

the two cysteines [4,5]. This would be consistent with our recent finding [27] that replacement of either of the cysteines in the CXXCH motif of cytochrome *c*<sub>550</sub> results in the expression in the *E. coli* periplasm of apo-proteins, whereas in eukaryotic systems it is known that haem is covalently attached to a single cysteine in an AXXCH motif of a mitochondrial cytochrome *c* [28].

The present finding that both endogenous and exogenous holo *c*-type were not formed in the *dsbA* and *dsbB* mutants shows that, as in other processes, DsbB probably serves as a partner to DsbA. The finding that the phenotypes of the *dsbA* and *dsbB* mutants related to *c*-type cytochrome and alkaline phosphatase formation were rescued in parallel by the additions of disulphide compounds into the *E. coli* growth media strongly suggests that the DsbA–DsbB system functions as a provider of oxidising power during the formation of holo-cytochromes *c*.

In previous work we have shown that specific thiol compounds, L-cysteine and coenzyme M could complement the *dsbD* mutation with respect to cytochrome *c* biosynthesis [4]. The interpretation was that the DsbD is usually a provider of reductant in the periplasm, a view consistent with the proposals of others [1,2,5]. Thus the pathway of *c*-type cytochrome biogenesis after polypeptide translocation into the periplasm requires both thiol-disulphide reducing and oxidising powers generated by the DsbD and DsbA–DsbB system, respectively. This synergism means *c*-type cytochromes are processed under a subtle balance of thiol-disulphide interconversion to become holo proteins, probably sequentially undergoing oxidation and then reduction, rather than being protected from the oxidising power of the DsbA/B system as has been suggested [29]. Such thiol-disulphide redox balancing mechanisms in the periplasm could also in part explain the identification of periplasmic and thioredoxin-like proteins that are required for *c*-type cytochrome biogenesis in other bacteria [30,31]. It is notable that evidence in favour of CcmG, an example of such a thioredoxin-type protein, acting as a peri-

plasmic disulphide reductase in *P. denitrificans* has recently been obtained (M.D. Page and S.J. Ferguson, unpublished results). The latter protein, also known in *E. coli* as DsbE [2], might transfer reducing power from DsbD to periplasmic targets in similar fashion to the transfer to targets by DsbA of oxidising power provided by DsbB. This provision of reducing power is apparently not compromised with respect to *c*-type cytochrome biogenesis at the concentrations of GSSG used here.

It is clearly important that experimental methods now be developed to address the technically difficult question as to whether CXXC motifs in apocytochromes, or in components required for their synthesis, do form disulphide bridged structures.

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