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A mutation blocking the formation of membrane or periplasmic endogenous and exogenous *c*-type cytochromes in *Escherichia coli* permits the cytoplasmic formation of *Hydrogenobacter thermophilus* holo cytochrome c_{552}

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Abstract

A mutant of *Escherichia coli*, JCB606, shown to be pleiotropically deficient in the formation of endogenous membrane and periplasmic *c*-type cytochromes, synthesised the apo form of the exogenous cytochrome c_{550} from *Paracoccus denitrificans*, but not the holo form. In contrast, a cytoplasmically located holo form of *Hydrogenobacter thermophilus* cytochrome c_{552} was found in *E. coli* JCB606. These findings support the proposition that the formation of the cytoplasmic *H. thermophilus* cytochrome c_{552} in *E. coli* does not involve the physiological pathway of *c*-type cytochrome biosynthesis in *E. coli* and that the haem insertion may be uncatalysed.

Key words: Bacterial *c*-type cytochrome biogenesis; *Escherichia coli*; Exogenous *c*-type cytochrome expression

1. Introduction

The haem groups of *c*-type cytochromes are covalently attached to polypeptide chains via two covalent bonds formed between thiol groups of the polypeptide and the vinyl groups of protohaem IX. In mitochondria this bond formation is catalysed by enzymes known as haem lyases [1–2]. In bacteria such enzymes are, by analogy, widely assumed to be present but to date none has been identified. On the other hand, several genes in different bacteria have been implicated in playing as yet undefined roles in *c*-type cytochrome biogenesis [3–6]. Such genetic analysis, plus various biochemical observations [7], lead to the current hypothesis that haem attachment occurs in the bacterial periplasm, or at least as polypeptides destined for the periplasm pass across the cytoplasmic membrane. Most recently this has been supported by the finding that the holo form of *P. denitrificans* cytochrome c_{550} is only found in either *P. denitrificans* or *E. coli* when the polypeptide, expressed from a plasmid, is targeted to the periplasm by its signal sequence [8]. Removal of the latter sequence resulted in the appearance of an apo form of the protein in the cytoplasm of both organisms. This contrasts with the cytoplasmic appearance for both organisms of a holo form of a cytochrome c_{552} from *H. thermophilus* [8], suggesting that the synthesis of this protein may be atypical and possibly independent of the cellular mechanism for bacterial *c*-type cytochrome assembly. This proposition suggests that uncatalysed insertion of haem into the apo form of the *H. thermophilus* cytochrome c_{552} may be possible. The present paper supports this view with the results of testing for the synthesis of this holo protein in the cytoplasm of a mutant of *E. coli* that is pleiotropically defective in *c*-type cytochrome synthesis.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The *E. coli* K-12 strain JCB387 is the parent strain of JCB606, which has a kanamycin resistance (Kan^R) insertion mutation [9]. The plasmid pKPD1 carries the complete gene for *P. denitrificans* cytochrome c_{550} with its signal sequence [8]. The plasmid pKHC12 carries *H. thermophilus* cytochrome c_{552} gene with signal sequence deleted [8]. Both *E. coli* strains were transformed with the plasmids by standard methods and cells were grown at 37°C anaerobically in minimal medium in the presence of glycerol, fumarate and nitrite supplemented with 50 μg/ml ampicillin and 25 μg/ml kanamycin when required [9].

2.2. Preparation of subcellular fractions

Fractionation of periplasmic, membrane and cytoplasmic proteins was performed by the cold osmotic shock procedure described previously [10]. The cytoplasmically-located glutamate dehydrogenase enzyme activity was determined according to the method of Windass et al. [11]. The activity of a periplasmic enzyme, β-lactamase, was assayed by an optimized direct spectrophotometric method described by Jansson [12]. Briefly, hydrolysis of 100 μg/ml of ampicillin by the fractionated sample in 0.1 M potassium phosphate buffer (pH 7.5) was measured by following the decrease in the absorbance at 240 nm.

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2.3. Analysis of expressed c-type cytochromes

The haem staining method was used to detect holo cytochromes *c* after SDS-PAGE (15% (Fig. 1), 12.5% (Fig. 2) acrylamide) as described previously [9,13]. For detection of cytochrome c_{550} polypeptide, immunoblotting with anti-cytochrome c_{550} antibody was used. Protein solutions were diluted twofold with 2 × SDS-PAGE sample buffer with β-mercaptoethanol, boiled for 2 min and subjected to SDS-PAGE (12.5% acrylamide). The methods for electroblotting and detection by the antibody were the same as previously [8].

3. Results

3.1. An E. coli mutant pleiotropically defective in c-type cytochromes

The mutant JCB606 has been described as defective in the periplasmic nitrite reductase, cytochrome c_{552} [9,14]. The mutated gene is located in the 94 minute region of the *E. coli* chromosome close to, but separate from, the *nrf* operon which includes the structural gene for cytochrome c_{552} [14–15]. Plasmids which complement the *nrf* deletion strain FM932 were unable to restore cytochrome synthesis to this mutant [9]. The mutation carried by JCB606 was transferred by P1 transduction to the parent JCB387. The transductant was then grown under conditions known to be optimal for the synthesis of *c*-type cytochromes by *E. coli* [16–17]. Synthesis, detected by staining for covalently bound haem, of the 50 kDa cytochrome c_{552} as well as traces of several low molecular weight proteins with masses in the range 16 to 24 kDa, with the latter being visible only at higher loading than in Fig. 1, (see Iobbi et al. [16]) was induced by nitrite and repressed by nitrate (Fig. 1, lanes 1 and 2). Synthesis of a 43 kDa membrane *c*-type cytochrome [17] was strongly stimulated by the presence of trimethylamine N-oxide (TMAO) (Fig. 1, lane 3). No holo *c*-type cytochromes were detected in corresponding cultures of a strain carrying the JCB606 mutation (Fig. 1, lanes 5 and 6). These results, which were also obtained for JCB606 itself (cf. Fig. 2), confirm that the insertion mutation in JCB606 pleiotropically affects a locus which is essential for the biogenesis of *c*-type cytochromes.

Table 1
Analysis, using enzyme markers, of subcellular fractionation of *E. coli* JCB387 and JCB606 expressing *P. denitrificans* cytochrome c_{550}

Enzyme	Fraction	Activity	
		JCB387	JCB606
β-lactamase	periplasm	100	100
	membrane	0	0
	cytoplasm	0	0
Glutamate dehydrogenase	periplasm	9	12
	membrane	0	0
	cytoplasm	91	88

Enzyme activities are expressed as the percentage in each fraction of the totals. The amounts of activity for both enzymes in each fraction were very similar for equivalent amounts of material from the two strains.

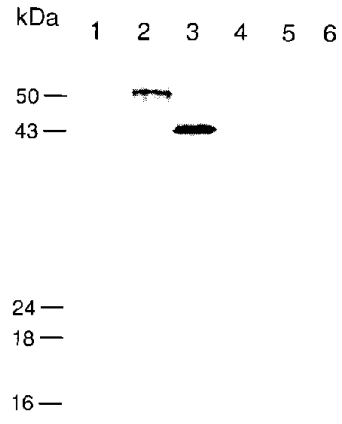

Fig. 1. The mutation in *E. coli* JCB606 results in the pleiotropic loss of all *c*-type cytochromes. Equal amounts (120 μg dry weight) of total cell mass [9] synthesised anaerobically by the parental and mutant strains with glycerol, fumarate and the supplements noted below were separated by SDS-PAGE and the gel stained for covalently bound haem [9]. Lanes 1–3: parental strain, JCB387, supplemented with 20 mM nitrate (lane 1), 2.5 mM nitrite (lane 2), or 20 mM TMAO (lane 3) during anaerobic growth. Lanes 4–6: the JCB387 Kan^R transductant carrying the mutation from JCB606 supplemented with 20 mM nitrate (lane 4), 2.5 mM nitrite (lane 5) or 20 mM TMAO (lane 6).

3.2. Analysis of exogenous holo c-type cytochrome formation

Fig. 2 shows haem stains following separation by SDS-PAGE of periplasmic, membrane and cytoplasmic proteins from the transformed *E. coli* cells. As observed previously [8], the mature holo form of cytochrome c_{550} was detected in the periplasm of JCB387 cells carrying pKPD1 which contains the complete *P. denitrificans* cytochrome c_{550} gene with its signal sequence (Fig. 2, lane 1). The endogenous *E. coli* cytochrome c_{552}, nitrite reductase (50 kDa), was also detected in the periplasm of this transformant. Haem staining of the membrane fraction (Fig. 2, lane 2) was negligible whilst contamination of the cytoplasm, though slight (Table 1), was sufficient to give some haem stain originating from the cytochrome c_{552} nitrite reductase (Fig. 2, lane 3) whilst cross-contamination of cytochrome c_{550} was less significant. In contrast, no haem-stained band corresponding to either the exogenous cytochrome c_{550} or the endogenous cytochrome c_{552} was observed in any fraction from JCB606 cells carrying pKPD1 (Fig. 2, lanes 4–6). However, a cytoplasmically-located holo form of the exogenous *H. thermophilus* cytochrome c_{552} was observed in the JCB606 strain carrying pKHC12 which contains *H. thermophilus* cytochrome c_{552} gene with its signal sequence removed (Fig. 2, lane 9). The heterologously-expressed *H. thermophilus* cytochrome c_{552} was also detected in the membrane (Fig. 2, lane 8) as discussed previously [8].

3.3. Detection of the exogenous cytochrome c_{550} by immunoblotting

Fig. 3 shows the results of immunoblotting with anti-

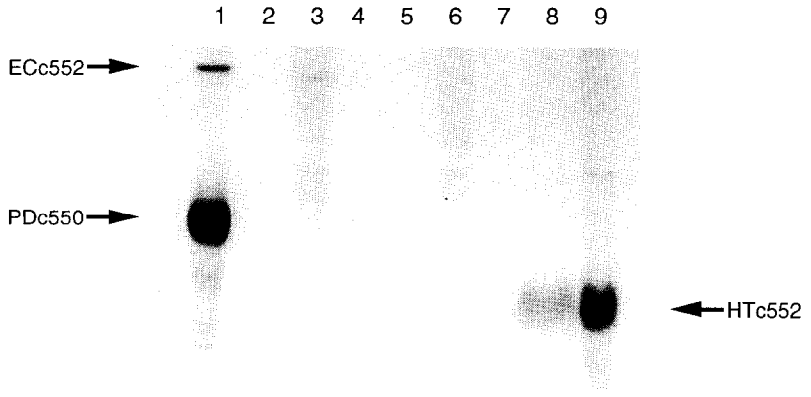

Fig. 2. Determination by haem staining of expression of holo forms of *P. denitrificans* cytochrome c_{550} and *H. thermophilus* cytochrome c_{552} in *E. coli* JCB387 and 606. Lanes 1–3 show, respectively, periplasmic, membrane and cytoplasmic fractions from JCB387 expressing *P. denitrificans* cytochrome c_{550}. Lanes 4–6 corresponding fractions from JCB606 cells expressing *P. denitrificans* cytochrome c_{550}. Lanes 7–9 corresponding fractions from JCB606 expressing the gene, minus the periplasmic targeting sequence, for *H. thermophilus* cytochrome c_{552}. Each fraction contained material from approximately 5×10^8 cells. The positions of the *E. coli* endogenous cytochrome c_{552} (ECc552), *P. denitrificans* cytochrome c_{550} (PDc550) and *H. thermophilus* cytochrome c_{552} (HTc552) are shown.

body against cytochrome c_{550} following separation by SDS-PAGE of subcellular fractions from *E. coli* JCB387 and JCB606 strains expressing the complete gene for *P. denitrificans* cytochrome c_{550}. In the total cellular fraction from the JCB387, two immunoreactive bands were detected close together (Fig. 3, lane 1). As suggested previously [8], the lower-molecular weight band corresponds to the mature holo protein derived from periplasmic fraction while the higher-molecular weight band is a putative, cytoplasmically-located, apo precursor protein of cytochrome c_{550} (Fig. 3, compare lanes 2 and 4 with lane 9 which contained the purified cytochrome c_{550}). In contrast, for the JCB606 cells, the expressed cytochrome c_{550} polypeptide was mainly detected by immunoblotting as an apo precursor form in the cytoplasm (Fig. 3, lane 8). It is clear that almost all the immunoreactive material in the total cell lysate was in the cytoplasm and that the molecular weight of this cytoplasmic material was greater than that of the authentic purified holo protein (compare lanes 8 and 9 in Fig. 3). The same higher molecular weight band weakly detected in the periplasm of the JCB606 is likely (probably) due to a minor leakage from the cytoplasmic fraction during the subcellular fractionation. The data for recovery of the marker enzymes shows that no uncontrolled lysis of cells occurred during the fractionation, but there was some cross-contamination between periplasm and cytoplasm (Table 1). The lower-molecular weight band, which corresponds in mobility to the mature holo-cytochrome c_{550}, was also weakly detected in the periplasm of JCB606 together with the higher-molecular weight band (Fig. 3, lane 6).

4. Discussion

The mutation in JCB606 is the first to be described for *E. coli* that affects a locus which is essential for biogenesis of not only endogenous *c*-type cytochromes (Fig. 1) but also the exogenous *P. denitrificans* cytochrome c_{550} (Fig. 2). The latter is made as an apo form in JCB606 in contrast to the parent strain JCB387 which makes the holo form [8]. This establishes that the heterologous formation of the holo cytochrome c_{550} depends on the cellular apparatus for the *E. coli* *c*-type cytochrome biogenesis, at least one component of which is absent in the JCB606.

Gene sequences very recently identified at the *aeg-46.5* locus in *E. coli* (46.5 minute region of the *E. coli* chromosome) [18] appear to code for proteins similar to the putative haem transporter and thioredoxin-like proteins found in *Bradyrhizobium japonicum* [4] and *Rhodobacter capsulatus* [3,5], which are implicated in bacterial *c*-type cytochrome biogenesis. Furthermore, homologues of the *ccl1* and *ccl2* genes previously identified in the latter two organisms have been discovered at the end of the *nrf* operon at 92 minute region of *E. coli* chromosome [15, 19]. The locus of mutation in JCB606 is independent of both *aeg-46.5* and *nrf* operons, suggesting that the mutated gene might code for a novel part of the apparatus for *c*-type cytochrome biogenesis.

A lower-molecular-weight band, attributed to a small amount of translocated *P. denitrificans* cytochrome c_{550} from which the targeting sequence has been removed, was weakly detected in the periplasm of JCB606 by immunoblotting (Fig. 3, lane 6). This protein did not stain for haem (Fig. 2, lane 4). There are three ways to explain this observation. One possibility is that the cytochrome c_{550} protein is functionally translocated into the periplasm as in the normal *E. coli* cells. Since the haem is not attached to the apo protein in the periplasm of JCB606 cells, this processed apo protein could be degraded rapidly, with the consequence that it is only weakly detectable by immunoblotting. Secondly, it can be envisaged that the mutation in the JCB606 specifically affects the

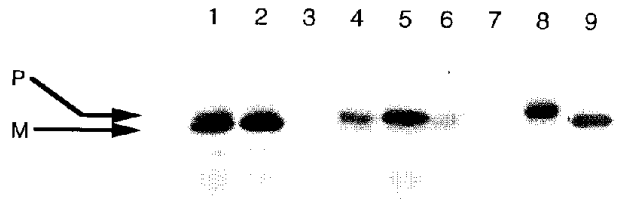

Fig. 3. Detection by immunoblotting of the expression of the *P. denitrificans* cytochrome c_{550} gene in *E. coli* strains JCB387 and 606. Lanes 1–4: show, respectively, total cell, periplasmic, membrane and cytoplasmic fractions from JCB387 expressing *P. denitrificans* cytochrome c_{550}. Lanes 5–8: corresponding fractions from JCB606 expressing *P. denitrificans* cytochrome c_{550}. Each fraction contained material from approximately 1×10^8 cells. Lane 9 shows purified cytochrome c_{550} isolated from *P. denitrificans*. The positions of the putative precursor (P) and the mature (M) cytochrome c_{550} are shown.

translocation of *c*-type cytochromes across the cytoplasmic membrane. Thus the cytochrome c_{550} might be translocated to a minor extent via an undefined and much less efficient alternative pathway. In this case, the haem would be postulated to attach to the apo cytochrome *c*, but the amount of translocated cytochrome *c* protein would be so small that it would escape detection by the haem staining method. It is unlikely that the mutation in JCB606 relates to general protein translocation, because the periplasmic β-lactamase activity is not affected in JCB606 (Table 1). Finally, one can consider a combination of the factors mentioned above in the sense that deficiency in haem attachment might cause the improper translocation of the cytochrome *c* polypeptide across the membrane.

The question as to why the apo form of cytochrome c_{550}, carrying the targeting sequence, accumulates in the cytoplasm of JCB606 (Fig. 3, lane 8), remains to be answered. If the gene product(s) absent in JCB606 normally assists in the handling of the apo protein on only one side of the membrane, the exact side depending upon the location of this gene product, then one might suggest that in JCB606 the precursor form might block the machinery for translocation across the cytoplasmic membrane. However, this proposition would have to be reconciled with the seemingly normal levels of periplasmic β-lactamase in JCB606.

The observation that the holo form of the signal sequence deleted *H. thermophilus* cytochrome c_{552} still accumulates in the cytoplasm of the mutant JCB606 (Fig. 2) clearly means that the formation of this protein occurs independently of a cellular component needed in *E. coli* for formation of periplasmic or membrane-bound *c*-type cytochromes. Whilst this may mean that there is a separate machinery for synthesising cytoplasmic *c*-type cytochromes in *E. coli*, it can equally be taken as evidence in favour of the suggestion [8] that haem can covalently insert into the apo-form of *H. thermophilus* cytochrome c_{552} without enzymic assistance.

Bacterial holo-cytochrome c_2 formation has recently been observed when the cytochrome c_2 gene deleted for the signal sequence was expressed in its natural host, *Rhodobacter sphaeroides* [20]. In contrast to the cytoplasmic accumulation of *H. thermophilus* cytochrome c_{552} in *E. coli*, the signal sequence deleted *R. sphaeroides* cytochrome c_2 gene product was found in the periplasm as a holo protein. The location of the haem attachment is of interest in the latter case. Apart from the major contrast between these observations with regard to the locations of the accumulated holo proteins, the two sets of observations contain the common feature that the signal sequence is not necessary for the covalent haem attachment to the apo-cytochromes *c*.

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