ELSEVIER

FEMS Microbiology Letters 161 (1998) 1–6

FEMS MICROBIOLOGY LETTERS

An *Escherichia coli ccm* (cytochrome *c* maturation) deletion strain substantially expresses *Hydrogenobacter thermophilus* cytochrome c_{552} in the cytoplasm: availability of haem influences cytochrome c_{552} maturation

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Received 22 January 1998; accepted 24 January 1998

Abstract

The maturation of *Hydrogenobacter thermophilus* cytochrome c_{552} in the cytoplasm of *Escherichia coli* is unique among bacterial *c*-type cytochromes. It is now shown to be matured in a strain lacking the whole set of *ccm* (cytochrome *c* maturation) genes that are normally required for *c*-type cytochrome biogenesis in *E. coli*. As this cytochrome is thermostable we propose that the apocytochrome c_{552} has sufficient tertiary structure to allow the haem to slot into its binding pocket, which in turn triggers the spontaneous covalent attachment between apocytochrome c_{552} and haem. The *ccm* deletion strain of *E. coli*, derived from a strain that synthesizes elevated levels of endogenous *c*-type cytochromes, also produces larger amounts of cytoplasmic *H. thermophilus* cytochrome c_{552} than a reference strain. This implies that elevated production of *c*-type cytochromes is not a consequence of high activity of *ccm* genes but rather an enhanced ability to supply haem, a view that is supported by the increase in thermophilic cytochrome c_{552} biogenesis that occurs in a reference strain following supplementation of growth media with δ-aminolevulinic acid.

Keywords: *Hydrogenobacter thermophilus*; Cytochrome *c* biogenesis; Haem; δ-Aminolevulinic acid

1. Introduction

C-type cytochrome biogenesis in bacteria involves a post translational pathway for the conversion of pre-apocytochrome *c* into the mature holocytochrome *c*. *C*-type cytochromes differ from other classes of cytochromes on account of the mechanism of haem attachment to the cytochrome *c* polypeptide. The haem moiety is attached to the polypeptide by thioether bonds between the two haem vinyl groups and the thiol groups of two cysteine residues of the conserved motif Cys-X-Y-Cys-His. Several lines of evidence suggest that this covalent attachment takes place in the periplasm and a relatively detailed model has been proposed [1] for the biogenesis of *c*-type cytochromes in bacteria. This model is in agreement with the results of studies on *Paracoc-*

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0378-1097/98/$19.00
PII S0378-1097(98)00037-8

cus denitrificans cytochrome *c* biogenesis indicating that the maturation of *c*-type cytochrome in bacteria takes place in the periplasm [2]. Genes directly involved in the biogenesis of *c*-type cytochromes have been found in many Gram-negative organisms such as *Rhodobacter casulatus* [3,4], *Bradyrhizobium japonicum* [5,6], *Escherichia coli* [7] and *P. denitrificans* [8–10]. Some of the components involved in the biogenesis of *c*-type cytochromes are thought to be periplasmic or attached to the cytoplasmic membrane with their functional domain facing towards the periplasm [11–13]. It has been shown that the holoform 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 [14]. Removal of the latter sequence results in the appearance of an apoform of the protein in the cytoplasm of both the organisms [14]. Cytochrome c_{552} from a thermophilic bacterium, *Hydrogenobacter thermophilus*, is the only cytochrome *c* which has been shown to be matured in the cytoplasm of *E. coli*; this occurs when it is expressed from a construct which lacks the coding region for its signal sequence [14]. Furthermore, it was shown that this cytochrome c_{552} was also expressed in an *E. coli* mutant strain lacking a gene, *dipZ*, coding for a disulfide isomerase or thioredoxin like protein [15], essential for other normal *c*-type cytochrome maturation [16]. Thus, it was suggested that the cytoplasmic maturation of this thermophilic cytochrome c_{552} does not need any enzymatic assistance [14,15]. However, this suggestion, with its very significant implication that covalent attachment of haem to the polypeptide of apocytochromes *c* can be uncatalysed, relies on the supposition that none of the other genes required for *c*-type cytochrome biogenesis in *E. coli* (*ccm* (cytochrome *c* maturation) genes) have any unsuspected role in this cytoplasmic synthesis of *H. thermophilus* cytochrome c_{552}, perhaps through currently unrecognized activities of cytoplasmic facing regions of some of the gene products. The present paper, therefore, addresses the important question as to whether the expression of the cytoplasmic cytochrome c_{552} continues in a strain of *E. coli* from which the *ccm* genes are deleted. The *ccm* deletion strain used is derived from a parent strain, JCB712, which produces, for unknown reasons, elevated levels of endogenous, periplasmic facing, *c*-type cytochromes [17]. Thus it was of considerable interest to determine whether this strain would also produce elevated levels of cytoplasmic cytochrome c_{552}. The outcome of this pair of experiments was thus expected to establish whether the activity of the *ccm* gene products might be responsible for the relatively high extent of endogenous cytochrome *c* biogenesis in *E. coli* JCB712, and if not, whether another factor, for example supply of haem, might be important.

2. Materials and methods

2.1. Bacterial strains and plasmids

The strains and plasmids used are listed in Table 1.

2.2. Preparation and analysis of normalized cell extracts for comparison of expression of cytochrome c_{552}

The normalized crude extracts were prepared by growing the cells in LB, aerobically, at 37°C until a culture had reached an OD of 1.5 at 580 nm. The same OD was obtained for each cell culture so that comparison of the expression in different strains of cytochrome c_{552} could be made. The cell pellet, collected by centrifugation at 12 000 rpm at 4°C, was resuspended in GTE (50 mM glucose, 25 mM Tris-HCl pH 7.5, 10 mM EDTA) and the cell walls were broken by freezing and thawing. The plasma membrane was removed by sonication and centrifugation. This resulting supernatant was then used as the crude extract. The amount of total cell protein in the crude extract was measured, using a protein assay kit from Biorad, and equal amounts of protein were loaded onto 15% SDS-PAGE gels for the comparisons of expression. Changes in the expression of cytochrome c_{552} were also determined for each sample by spectrophotometry. The crude extracts were suitably diluted, reduced with dithionite and the absorbance at 552 nm was measured on a Perkin Elmer UV/Vis spectrophotometer. SDS-PAGE and other molecular biology techniques were followed as described by Sambrook et al. [18]. Staining for the detection of covalently attached haem was done as

described by Goodhew et al. [19]. In some experiments the haem precursor, δ-aminolevulinic acid, was added to the growth medium, usually at a final concentration of 0.1 mM. Recombinant *H. thermophilus* cytochrome c_{552} was partially purified, so as to provide a standard for SDS-PAGE, by CM-cellulose chromatography of an extract from *E. coli* containing the construct pKHC12. This cytochrome c_{552} is the only cytochrome in such an extract that binds to the cation exchanger and thus sufficient purification for the present purpose was achieved.

3. Results

3.1. Expression of H. thermophilus cytochrome c_{552} in E. coli ccm deletion strain, JCB71202

Expression of *H. thermophilus* cytochrome c_{552} in *E. coli* strain JCB71202 was readily observed from the red colour of the cells, haem staining following SDS-PAGE (Fig. 1) and by spectrophotometry (Fig. 2A). Thus none of the *ccm* genes that are deleted in this strain are required for cytoplasmic synthesis of this cytochrome. JCB712, the parent strain of JCB71202, for unknown reasons synthesizes higher level of endogenous periplasmic or periplasmic facing *c*-type cytochromes than other strains [17,20]. The ready detection of *H. thermophilus* cytochrome c_{552} in the JCB71202 strain suggested that this higher level of expression may also apply to this cytoplasmically expressed protein. To test this proposal the expression of this cytochrome in strains JCB712 and JCB387, a commonly used strain in the studies of cytochrome *c* biogenesis and expressing lower levels

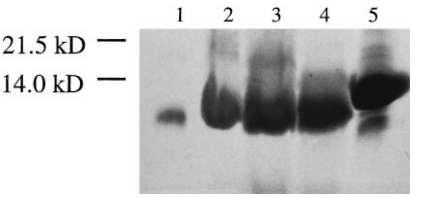

Fig. 1. Haem stained SDS-PAGE gel of the extracts of three strains of *E. coli* expressing *H. thermophilus* cytochrome c_{552} from the construct pKHC12. The normalized crude extracts, ~80 μg protein in each case, were loaded on an SDS-PAGE gel as described in Section 2. Lane 1: Partially purified recombinant cytochrome c_{552} (1 μg protein). Lane 2: Extract of JCB387 expressing cytochrome c_{552}. Lane 3: Extract of JCB712 expressing cytochrome c_{552}. Lane 4: Extract of JCB71202 (*ccm* deleted) expressing cytochrome c_{552}. Lane 5: Horse heart mitochondrial cytochrome *c* (10 μg protein) supplied by Sigma. The molecular masses on the left-hand side correspond to the electrophoresis of two colored marker proteins (rainbow markers supplied by Amersham Life Science), in one lane of the gel but which do not reproduce photographically. Mitochondrial cytochrome *c* in lane 5 serves as an additional molecular mass marker.

of cytochrome *c* [17,20–22], was examined. Fig. 1 shows that whereas the extent of expression of thermophilic cytochrome c_{552} in JCB712 was comparable with that in JCB71202, a significantly lower level was observed for strain JCB387. Although a normalized amount of crude extract was loaded on SDS-PAGE in each case, the higher level of expression of *H. thermophilus* holocytochrome c_{552} in JCB712 or JCB71202 was also demonstrated by measuring the visible absorbance of this cytochrome (Fig. 2A). This established that the expression of *H. thermophilus* cytochrome c_{552} was many-fold (approximately 10 times) higher in JCB712 strains than in JCB387.

Table 1
E. coli strains and plasmids

Strain or plasmid	Relevant genotype/characteristics	Source [reference]
Strains		
JCB387	*E. coli* RV Δ*nirB*	Griffiths and Cole [21]
JCB712	*pro his trp*Δ*lac*	M. Jones-Mortimer
JCB71202	Δ*ccm(A'-'H)*::Ω	Grove et al. [17]
Plasmids		
pKHC12	Contains the coding region of cytochrome c_{552} mature protein, with the coding region for the signal sequence deleted.	Sanbongi et al. [27]
pKPHC12ΔSIG	Contains the first ten amino acids of *P. denitrificans* cytochrome c_{550} followed by the coding region for mature protein of cytochrome c_{552}.	Y. Sanbongi

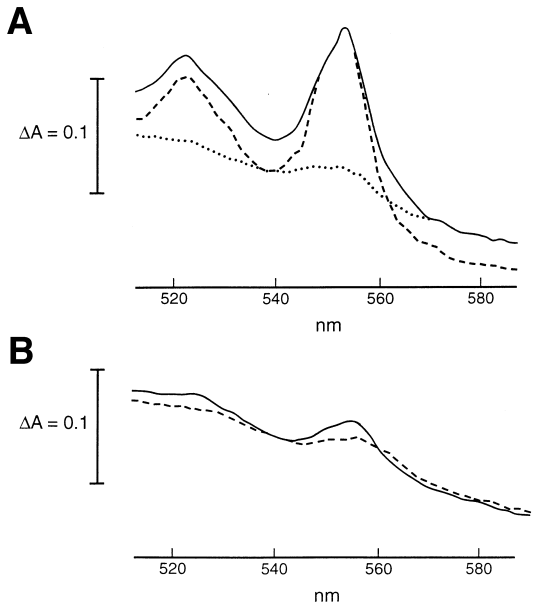

Fig. 2. A: Spectra of reduced *H. thermophilus* cytochrome c_{552} in crude extracts of *E. coli* strains JCB387 (dotted line), JCB712 (line) and JCB71202 (dashed line) expressing cytochrome c_{552} from the construct pKHC12. The crude extracts were prepared as described in Section 2. 0.8 mg protein/ml was used for the analysis after adding sufficient solid sodium dithionite to reduce the samples completely. The spectra were measured against a buffer reference. B: Spectra of reduced cytochrome c_{552} in crude extracts of *E. coli* strain JCB387 expressing cytochrome c_{552} from the construct pKHC12 either without (dashed line), or with (line) 0.1 mM δ-aminolevulinic acid added to the growth medium. Other conditions were as in A.

3.2. The effect of δ-aminolevulinic acid on the expression of cytochrome c_{552}

In principle, an explanation for the higher levels of expression of *c*-type cytochromes in *E. coli* JCB712 strain is that one or more genes (e.g. *ccm*, *dipZ*, *dsbB*) that are required for *c*-type cytochrome biogenesis are expressed to a higher level than in other strains. However, the fact that the cytoplasmically expressed cytochrome c_{552} from *H. thermophilus* is also expressed to a greater extent in both JCB712 and JCB71202 strains, despite its formation being independent of at least the *ccm* and *dipZ* genes, suggests that this cannot be the explanation. *C*-type cytochrome biogenesis requires an appropriate supply of a haem which prompted us to investigate whether the supply of haem precursor, δ-aminolevulinic acid, to the growth media influences the production of the holocytochrome c_{552}. In case of strain JCB387 the inclusion of 0.1 mM δ-aminolevulinic acid in the medium resulted in noticeably redder cells and an increase in the amount of thermophilic cytochrome c_{552} (Fig. 2B). A further increase in the concentration of δ-aminolevulinic acid had no effect (not shown). The dependence on the concentration of δ-aminolevulinic acid is similar to that reported for the expression of other haem proteins in *E. coli* [23]. In contrast, the addition of 0.1 mM δ-aminolevulinic acid to the growth medium for JCB712 strains was without effect on the synthesis of *H. thermophilus* holocytochrome c_{552}. The effect of supplementation of the growth medium with δ-aminolevulinic acid on the expression of cytochrome c_{552} in the strain JCB387 could also be clearly seen when the plasmid pKHC12ΔSig was used. This has the cytochrome c_{552} structural gene sequence preceded by the coding sequence for ten residues from the N-terminus of *P. denitrificans* cytochrome c_{550} protein. A periplasmic targeting sequence is absent, as in pKHC12. *H. thermophilus* holocytochrome c_{552}, extended by ten residues at N-terminus following expression from pKHC12ΔSig, was not readily detectable in the strain JCB387 unless the growth medium was supplemented with δ-aminolevulinic acid. In contrast, this form of *H. thermophilus* cytochrome c_{552} was expressed from the same plasmid at readily detectable levels in the strain JCB712 without supplementation of growth medium with δ-aminolevulinic acid, to an extent approximately equivalent to that found in JCB387 after supplementation with δ-aminolevulinic acid.

4. Discussion

The biogenesis of *H. thermophilus* cytochrome c_{552} in the *ccm* minus background is striking and strengthens our previous hypothesis that its maturation is independent of enzymatic assistance [14]. The observations that δ-aminolevulinic acid increases the expression of cytochrome c_{552} from the strain JCB387 but that there is no effect of δ-aminolevulinic acid on the expression of cytochrome c_{552} from the strain JCB712, lead us to assume that *E. coli* JCB712, and strains derived from it are, at least to

some extent, producing more haem relative to the other strains e.g. JCB387. The observation of the synthesis of *H. thermophilus* holocytochrome c_{552} in the absence of *ccm* genes, and its dependence on a haem precursor in *E. coli* JCB387, together imply that the only identified factor contributing to the formation of this cytochrome is availability of haem. This may be because this cytochrome c_{552} is highly thermostable [24] with the consequence that its apocytochrome has some tertiary structure, including a binding pocket into which haem inserts. The covalent attachment between apocytochrome and haem would then take place spontaneously. Furthermore, the insertion of haem may enhance the folding of apocytochrome c_{552} and thus can retard its degradation. This proposal can explain why the expression of holocytochrome c_{552} from the construct pKHC12ΔSig in the strain JCB387 was negligible unless the growth medium was supplemented with δ-aminolevulinic acid while the expression was normal from the same construct in the strain JCB712 without the supplementation with δ-aminolevulinic acid. The extra ten N-terminal amino acid residues might retard folding of apocytochrome c_{552} and thus the availability of haem may be crucial for displacing an equilibrium to a state with tertiary structure.

It may be that in the case of normal periplasmic cytochrome *c* assembly part of the biogenesis machinery is involved in holding the apocytochrome *c* and haem in the appropriate conformation, rather than in catalyzing the chemical reaction of thiol addition to vinyl groups of haem. Analysis of the recently released genome of *Helicobacter pylori* [25], which has both membrane bound and periplasmic cytochromes *c*, shows that it lacks nearly all the homologous genes for *c*-type cytochrome biogenesis identified in other studied Gram-negative organisms [26]. It has been suggested that since proteins responsible for disulfide bond formation are absent from this bacterium the problem of inevitable disulfide bond formation once an apocytochrome *c* carrying cysteines enters the periplasm is avoided [26]. Thus much of the *c*-type cytochrome biogenesis machinery, present in the periplasm, for reduction of disulfide is dispensable in *H. pylori*. This hypothesis can be compared with the maturation of cytochrome c_{552} in the cytoplasm of *E. coli*. Thermostable apocytochrome c_{552}, having some tertiary structure in the reducing environment of cytoplasm, does not need any other enzymatic assistance for the covalent attachment. The same may be true in the non-oxidising environment of the *H. pylori* periplasm. In vitro studies will eventually demonstrate the exact requirements for the maturation of this cytochrome c_{552}. Such studies are in progress. The biogenesis of thermophilic cytochrome c_{552} has not, however, been studied in *H. thermophilus*. We assume that the apocytochrome c_{552} is first translocated to the periplasm, the normal site of cytochrome c_{552} maturation in *H. thermophilus* [28], where the covalent attachment between apocytochrome c_{552} and haem takes place. There is no information available concerning cytochrome *c* biogenesis genes in *H. thermophilus* but we expect their presence following the pattern seen in either typical Gram-negative organisms such as *E. coli* or that in *H. pylori* [26]. At the elevated growth temperature of *H. thermophilus* we suspect that the periplasmic insertion of haem into its cytochrome c_{552} will need protein-mediated assistance. This is because the apo-protein will probably not spontaneously take up an appropriate three-dimensional structure for haem to attach covalently without assistance.

Acknowledgments

We thank Professor J. Cole (Birmingham University) for supplying the strain JCB71202 and Dr. Y. Sanbongi for providing the cytochrome c_{552} gene. This work was financially supported by a Felix scholarship to N.S. and BBSRC grant C08007 to S.J.F.

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