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Synthesis of holo *Paracoccus denitrificans* cytochrome c_{550} requires targeting to the periplasm whereas that of holo *Hydrogenobacter thermophilus* cytochrome c_{552} does not

Implications for *c*-type cytochrome biogenesis

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Abstract

Expression from a plasmid of the complete gene, including the codons for the N-terminal periplasmic targeting signal, for cytochrome c_{550} of *Paracoccus denitrificans* led to the formation of the holo protein in the periplasms of both *P. denitrificans* and *Escherichia coli*. Expression of the gene from which the region coding for the targeting signal had been specifically deleted resulted in formation of apo-protein in the cytoplasms of both organisms. These findings are consistent with haem attachment occurring in the periplasm. In contrast, the formation of holo cytochrome c_{552} from *Hydrogenobacter thermophilus* following expression of the gene lacking the periplasmic targeting sequence in either *P. denitrificans* or *E. coli* is attributed to spontaneous cytoplasmic attachment of haem to the thermostable protein.

Key words: Bacterial *c*-type cytochrome biogenesis; Heme attachment; Periplasm; Signal sequence

1. Introduction

In bacteria water-soluble *c*-type cytochromes, which contain haem covalently attached via thioether linkages to two cysteine residues of the protein, are usually located in the periplasm [1,2]. In eukaryotic cells it is known that attachment of haem to apo-cytochrome *c* is catalysed by an enzyme cytochrome *c* haem lyase in mitochondria [3], but there is no direct evidence for such an enzyme in bacteria. Recently, ABC (ATP binding cassette) transporters which may function to translocate haem or, less likely apo-cytochrome *c*, into periplasm have been found in *Rhodobacter capsulatus* and *Bradyrhizobium japonicum* [4,5]. Also, Ccl and CycH proteins which play important roles in biogenesis of cytochrome *c* have been discovered in both organisms [4–6]. Their active domains appear to be located in the periplasm. Moreover, apo-cytochromes *c* with the periplasmic N-terminal targeting sequence removed by proteolytic processing, have been found in the periplasm of *Paracoccus denitrificans* mutants pleiotropically deficient in cytochrome *c* biogenesis [7]. These results have all suggested that haem attachment to apo forms of c-type cytochromes takes place in the periplasm. However, in an apparent contradiction, expression in *Escherichia coli* of the signal sequence deleted *Hydrogenobacter thermophilus* gene for cytochrome c_{552} leads to cytoplasmic formation of a holo protein [8]. We report here an investigation of whether haem attachment for *P. denitrificans* cytochrome c_{550} occurs in the cytoplasm or the periplasm.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this work are listed in Table 1. The *E. coli* K12 strain JCB387 [9] was used for the production of exogenous *c*-type cytochromes. *E. coli* JM107 [10] was used for maintenance and propagation of all pKK223-3 and pEG400 plasmid derivatives. *E. coli* CRS603 [11], carrying plasmid pRK2013 [12], was used in triparental matings for mobilizing pEG400 plasmid derivatives into *P. denitrificans* mutant C010 which is deficient in the cytochrome c_{550} structural gene [13]. The transformed *E. coli* JCB387 cells were grown anaerobically in the minimal media in the presence of glycerol, nitrite and fumarate at 37°C [9] and other *E. coli* strains were routinely grown in LB broth at 37°C. *P. denitrificans* C010 and the transconjugants were grown anaerobically in minimal media containing succinate and nitrate at 37°C for the production of cytochromes *c* from plasmid-borne genes. Antibiotics were added to media as required at the following concentrations (μg/ml) for *E. coli*: ampicillin 50, kanamycin 50, spectinomycin 60; for *P. denitrificans*: kanamycin 50, streptomycin 50.

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Abbreviations: SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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2.2. Construction of gene cassette and plasmids

Plasmids used in this work are listed in Table 1. The pKK223-3 derivative, pKHC12 which carries *H. thermophilus* cytochrome c_{552} gene without signal sequence except the first methionine codon (*hcy* gene) was described previously [8].

A 1.0 kb *Sal*I-*Pst*I restriction fragment containing the *P. denitrificans* cytochrome c_{550} gene (*cycA* gene, [14]) was cut from the plasmid pUCC5502 kindly provided by Dr. R. van Spanning (Vrije Universiteit, Amsterdam). The fragment was then inserted into the *Sal*I and *Pst*I multiple cloning sites of pKK223-3 to generate a recombinant plasmid pKPD1 carrying the complete cytochrome c_{550} gene with its signal sequence. Four oligonucleotides (Fig. 1) were synthesised to amplify DNA fragments in pKPD1 by a PCR procedure to make a signal sequence deleted *P. denitrificans* cytochrome c_{550} gene cassette (*cycA2* gene). The primers XF01 and XF04 were annealed respectively with the 5′ side of *Sal*I site and 3′ side of *Pst*I site in the vector sequence. The primer XF02 was designed to anneal with the DNA sequence of the upstream region from the initiation codon ATG. Another primer XF03 was annealed with the region of the gene sequence corresponding to the beginning of mature protein N-terminal sequence. The primers XF02 and XF03 were 5′ phosphorylated and PCR amplifications were done between XF01 and XF02, and between XF03 and XF04 using Taq polymerase under the condition of 29 cycles of denaturation at 92°C (30 s), annealing at 58°C (30 s) and extension at 72°C (30 s). Each PCR fragment was treated with T4 DNA polymerase. After heat inactivation of the polymerase, the former fragment was cut by *Sal*I and the latter was cut by *Pst*I. The resulting DNA fragments were ligated with the pKK223-3 previously cut by *Sal*I and *Pst*I to make a new plasmid pKPD10 carrying the *P. denitrificans* cytochrome c_{550} gene with the signal sequence deleted (*cycA2* gene). PCR DNA products were sequenced on M13 phage single-stranded DNA templates by the dideoxy chain termination method with the Sequenase kit obtained from US Biochemical and [^{35}S]dATP obtained from Amersham.

A 1.2 kb *Bam*HI-*Sca*I restriction fragment containing the *tac* promoter, *hcy* gene and ribosomal terminator sequences from the plasmid pKHC12 was inserted into the broad host range vector pEG400 [15], cut by *Bam*HI and *Sma*I, to generate pEHC12. 0.8 kb *Sal*I-*Xba*I restriction fragments of pKPD1 and pKPD10 were ligated with pEG400 cut by *Sal*I and *Xba*I to make recombinant plasmids, pEPD1 and pEPD10, respectively.

Plasmid DNA isolations, transformations and other routine DNA manipulations were carried out by standard procedures [16]. Restriction enzymes and DNA modifying enzymes used here were purchased from Boehringer Mannheim.

2.3. Preparation of subcellular fractions

The periplasmic fractions of *E. coli* JCB387 cells carrying the expression plasmids were prepared by the cold osmotic shock procedure described previously [17]. The periplasmic proteins of the *P. denitrificans* C010 transconjugants were released using a procedure of Alefounder and Ferguson [18]. The spheroplasts obtained from both *E. coli* and *P. denitrificans* cells described above were resuspended in 10 mM Tris-HCl (pH 8.0) followed by sonication. The crude sonicates were centrifuged at low-speed to remove the cell debris and unbroken cells and then at $100.000 \times g$ for 1 h to pellet the membranes. The membrane fractions were solubilised with 1.5% Triton X-100 in 10 mM Tris-HCl (pH 8.0). The supernatant was the cytoplasmic fraction.

2.4. Detection of holo cytochrome c

Sample solutions were diluted twofold with 2 × SDS-PAGE sample buffer without β-mercaptoethanol, and subjected to SDS-PAGE (12.5% acrylamide). The cytochromes *c* were specifically detected on SDS-PAGE gels by a haem staining procedure [19].

2.5. Detection of immunoreactive proteins

Sample 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 proteins were then electrophoretically transferred from the SDS-PAGE gel to a nitrocellulose filter. *P. denitrificans* cytochrome c_{550} polypeptides were detected by the anti-cytochrome c_{550} antibody with a goat anti-rabbit IgG alkaline phosphatase conjugate. The immunoblots were developed with Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl and 50 mM $MgCl_2$.

3. Results

3.1. Expression of exogenous cytochromes c *in E. coli*

Fig. 2A shows haem stains following separation by SDS-PAGE of periplasmic, membrane and cytoplasmic proteins from the transformed *E. coli* cells. The *H. thermophilus hcy* gene was expressed as a holo protein in the cytoplasm of JCB387 cells (Fig. 2A, lane 3) as observed previously in a different strain of *E. coli* [8]. The expressed *H. thermophilus* cytochrome c_{552} also co-frac-

Table 1
Bacterial strains and plasmids

Strain/Plasmid	Relevant characteristics	Ref./Source
E. coli		
JM107	host of pKK223-3 and pEG400 derivatives	10
CRS603	host of pRK2013	11
JCB387	host for production of exogenous cytochrome *c*	9
P. denitrificans		
C010	Kan^R; *cycA* gene is non-functional	13
Plasmids		
PKK223-3	Amp^R; *E. coli* expression vector	Pharmacia
pEG400	Spc^R, Sm^R; broad host range vector	15
pRK2013	Kan^R *tra*$^+$; helper plasmid for mating	12
pUCC5502	pUC19 derivative carrying 1.0 kb *Sal*I-*Pst*I *P. denitrificans* gene containing *cycA*	14
pKHC12	pKK223-3 derivative carrying *hcy*	8
pKPD1	pKK223-3 derivative carrying *cycA*	This work
pKPD10	pKK223-3 derivative carrying *cycA2*	This work
pEHC12	pEG400 derivative carrying *hcy*	This work
pEPD1	pEG400 derivative carrying *cycA*	This work
pEPD10	pEG400 derivative carrying *cycA2*	This work

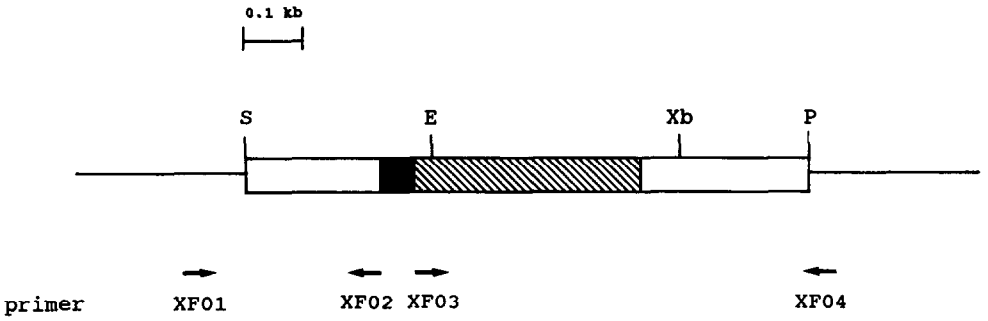

Fig. 1. PCR primers for construction of the signal sequence deleted *P. denitrificans* cytochrome c_{550} gene (*cycA2* gene). The relevant part of the plasmid pKPD1 is shown. A 1.0 kb *SalI-PstI P. denitrificans* chromosomal DNA fragment containing *cyc*A gene is flanked by the vector sequence (straight line). The coding regions for the signal sequence and mature protein are shown by the solid black box and the striped box, respectively. PCR primers are shown by the arrows with the direction from 5′ to 3′. The sequences were: XFO1 (5′-TGTGTGGAATTGTGAGCGGA-3′), XFO2 (5′-CATCGCGTTTCCTCTTGGGTAT-3′), XFO3 (5′-CAGGATGGCGACGCCGCCAAA-3′), XFO4 (5′-CTTCTCTCATCCGCCAAAAC-3′). Restriction sites: E, *Eco*RI; P, *Pst*I; S, *Sal*I; *Xb*, *Xba*I.

tionated with the membrane fraction, suggesting electrostatic adsoption to the cytoplasmic side of the membrane, probably because of the highly basic character of the cytochrome [20]. The *P. denitrificans cycA* gene was expressed as a processed holo protein in the periplasm (Fig. 2A, lane 4). The mobility of the expressed *P. denitrificans* cytochrome c_{550} in *E. coli* cells on an SDS-PAGE gel was the same as for authentic cytochrome c_{550} purified from *P. denitrificans* (cf. Fig. 2A, lane 10). The periplasmic haem stained band with approximate molecular weight 50 kDa corresponds to the *E. coli* endogenous tetrahaem nitrite reductase known as cytochrome c_{552} (Fig. 2A, lanes 1, 4 and 7) whilst the haem staining seen for a periplasmic species of approximate molecular weight 20 kDa (Fig. 2A, lanes 1 and 7) corresponds to a poorly defined *c*-type cytochrome of *E. coli* [21]. No haem stained band corresponding to cytochrome c_{550} was observed in any fraction from *E. coli* cells carrying the *cycA2* gene in which the N-terminal periplasmic targeting sequence is deleted (Fig. 2A, lanes 7–9). The absence of a haem stain in these fractions was confirmed by running the samples at a three-fold higher loading (not shown). The holo cytochrome c_{550} located in the periplasm of *E. coli* cells carrying the *cycA* gene was detected by immunoblotting using anti-cytochrome c_{550} antibody (Fig. 2B, lane 1). At lower loadings (not shown) this was clearly a single band, consistent with the observation of one band with this mobility in Coomassie blue stained gels (not shown). A higher molecular weight form, assumed to be the cytochrome c_{550} precursor, was reproducibly detected in the cytoplasm (Fig. 2B, lane 3). This putative precursor did not have covalently attached haem (cf. absence of staining in Fig. 2A, lane 6). The *cycA2* gene product was detected by the antibody in the cytoplasm of *E. coli* carrying pKPD10 (Fig. 2B, lane 6). This cytoplasmic polypeptide had the same apparent molecular weight as both the periplasmic holo protein (Fig. 2B, lane 1) and the authentic purified protein (Fig. 2B, lane 7) but a lower value than the putative precursor form (compare lanes 3 and 6 in Fig. 2B). The small amount of immunoreactive protein in the periplasm (Fig. 2B, lane 4) derived from the transformant carrying pKPD10 is likely due to leakage from the cytoplasmic fraction during the subcellular fractionation. The same results as reported here were also obtained when using *E. coli* laboratory strain JM107 as a host (data not shown).

3.2. Expression of plasmid-borne cytochrome genes in *P. denitrificans*

Fig. 3A shows the results of haem-staining subcellular fractions, following separation by SDS-PAGE, from *P. denitrificans* C010 expressing various *c*-type cytochromes. Lanes 1 to 3 (Fig. 3A) show the staining pattern observed in the periplasm, membrane and cytoplasm from various endogenous haem proteins; the absence of periplasmic cytochrome c_{550} from lane 1 is clear. The cells carrying pEHC12 in which the *hcy* gene is under the control of the one or both of the *lac* and *tac* promoters, which are in tandem within the construct, produced holo *H. thermophilus* cytochrome c_{552} in the cytoplasm (Fig. 3A, lane 6). The deficiency of cytochrome c_{550} in the C010 strain was complemented by the introduction of pEPD1 (Fig. 3A, lane 7). The expressed holo cytochrome c_{550} was located in the periplasm. The processed cytochrome c_{550} was also detected in the periplasm by immunoblotting (Fig. 3B, lane 1), although unlike the situation in *E. coli* no precursor form was detected in the cytoplasm (Fig. 3B, lane 3). However, a weak immunoreactive band corresponding to the mature protein was detected in the cytoplasm (Fig. 3B, lane 3); this is attributed to a contaminant from periplasm because haem staining corresponding to the periplasmic cd_1 nitrite reductase (approx. 60 kDa) is seen in the cytoplasmic fraction. A weak haem stain at the mobility for contaminating cytochrome c550 could reproducibly be seen (Fig. 3A, lane 9). The *cycA2* gene product was detected by immunoblotting (Fig. 3B, lane 6) in the cyto-

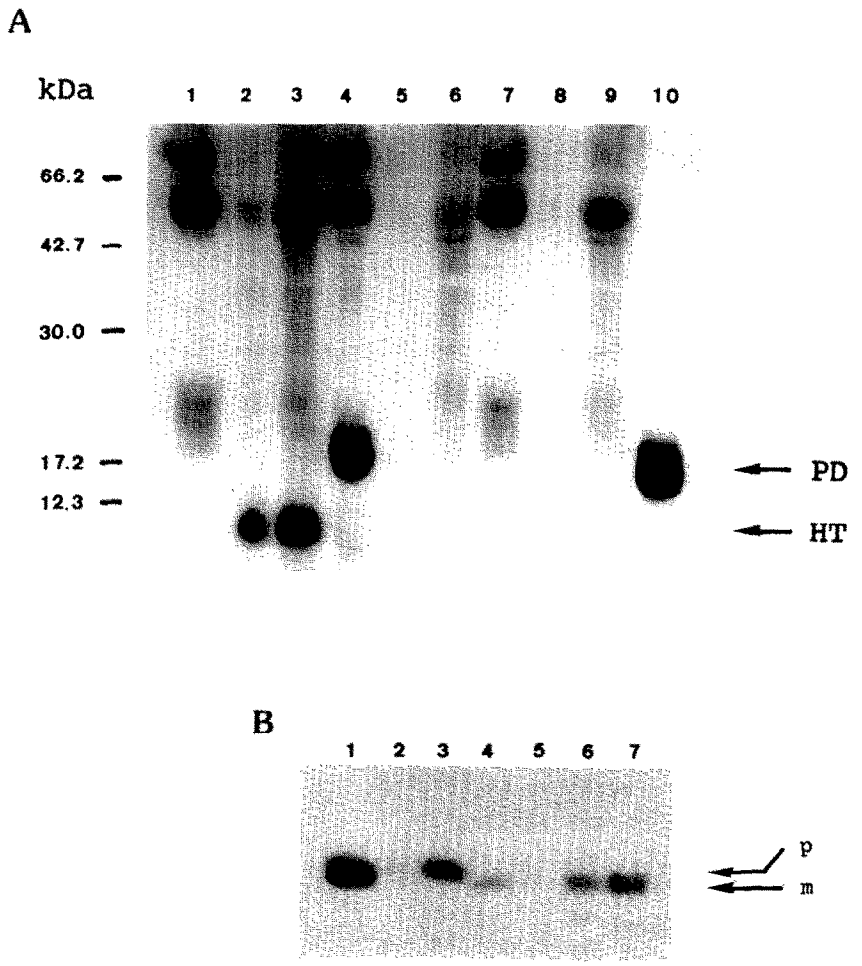

Fig. 2. Expression of exogenous cytochromes *c* in *E. coli* analysed by (A) haem staining and (B) immunoblotting using anti-*P. denitrificans* cytochrome c_{550} antibody. (A) cells of JCB387 carrying pKHC12 (lanes 1–3), pKPD1 (lanes 4–6) and pKPD10 (lanes 7–9) were fractionated into periplasm (lanes 1, 4 and 7), membrane (lanes 2, 5 and 8) and cytoplasm (lanes 3, 6 and 9) as described in section 2. Material from the different fractions derived in each case from approximately 10^9 cells was subjected to SDS-PAGE. Lane 10: purified cytochrome c_{550} from *P. denitrificans*. Arrows indicate the positions of *P. denitrificans* cytochrome c_{550} (PD) and *H. thermophilus* cytochrome c_{552} (HT). (B) cells of JCB387 carrying pKPD1 (lanes 1–3), pKPD10 (4–6) were fractionated into periplasm (lanes 1 and 4), membrane (lanes 2 and 5) and cytoplasm (lanes 3 and 6). Each fraction contained material from approximately 10^8 cells. Lane 7: purified cytochrome c_{550} from *P. denitrificans*. The positions of the putative precursor (p) and the mature (m) cytochrome c_{550} are shown.

plasm of *P. denitrificans* C010 cells carrying pEPD10. In common with expression in the *E. coli*, the *cycA2* gene product was not haem stained in the SDS-PAGE gel (Fig. 3A, lane 12) even when the protein was overloaded (data not shown), but had a similar mobility to the periplasmic holo protein (Fig. 3B, compare lane 6 with lane 7). This was not unexpected since *cycA2* lacks the removable periplasmic targeting N-terminal sequence and thus the cytoplasmically located apo-protein should have the same apparent molecular weight as the periplasmically located mature holo protein since the two forms of cytochrome c_{550} electrophorese indistinguishably on SDS-PAGE [7].

4. Discussion

Several c_2-type cytochrome genes, including that for *P. denitrificans* cytochrome c_{550} have been cloned and their periplasmic expression observed in *E. coli* [22–24]. The present study confirms, but at an improved expression level using a different host-vector system, the expression of the holo cytochrome c_{550} from *P. denitrificans* in the *E. coli* periplasm. Thus the *E. coli* protein transport system, signal peptidase and haem attaching machinery can recognise these precursor cytochromes *c* to form holo proteins in the periplasm. However, *Rhodopseudomonas viridis* cytochrome c_2 was synthesised only as an apo-precursor in the *E. coli* cytoplasmic membrane [25], but when expressed heterologously in *P. denitrificans*, this protein was detected in the periplasm as a holo protein [26], implying substrate and host specificities of bacterial cytochrome *c* biogenesis pathways.

It is unlikely that the present failure to form holo cytochrome c_{550} formation in the cytoplasms of either *E. coli* or *P. denitrificans* is a result of haem sequestration,

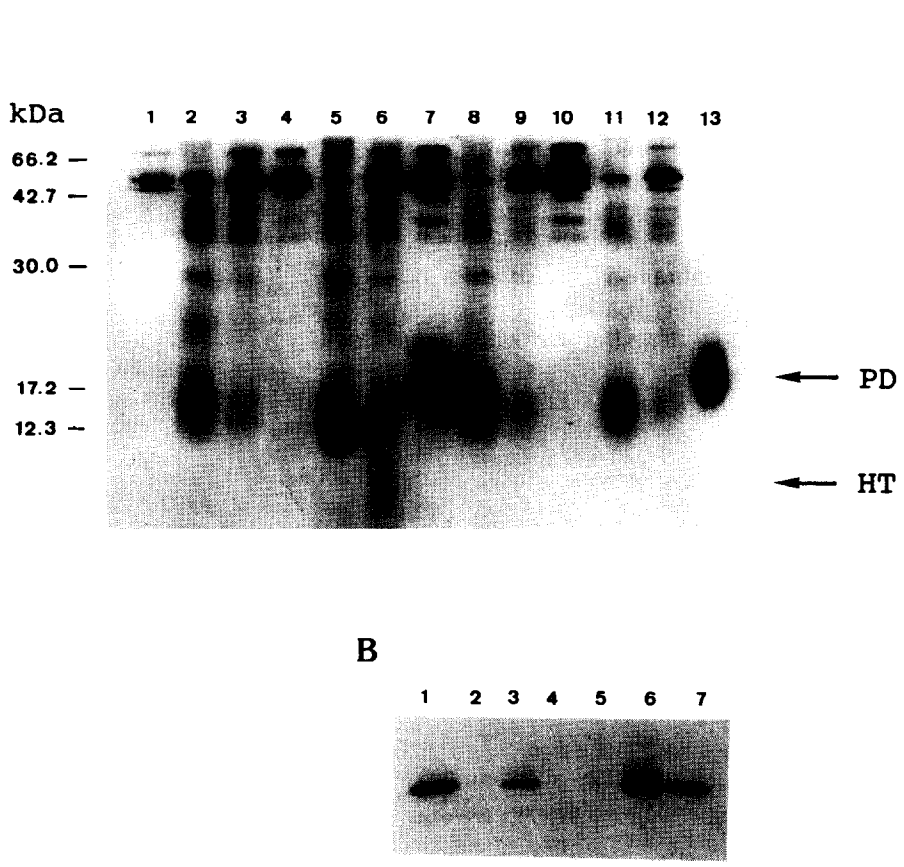

Fig. 3. Cytochrome *c* expression in *P. denitrificans* analysed by (A) haem staining and (B) immunoblotting of the cytochrome c_{550}. (A) Cells of strain C010 (lanes 1–3), C010 transconjugants carrying pEHC12 (lanes 4–6), pEPD1 (lanes 7–9) and pEPD10 (lanes 10–12) were fractionated into periplasm (lanes 1, 4, 7 and 10), membrane (lanes 2, 5, 8 and 11) and cytoplasm (3, 6, 9 and 12). Each fraction contains material derived from approximately 10^9 cells. Lane 13: purified cytochrome c_{550} from *P. denitrificans*. Arrows indicate the positions of the cytochromes c_{550} (PD) and c_{552} (HT). (B) cells of C010 transconjugants carrying pEPD1 (lanes 1–3) and pEPD10 (lanes 4–6) were fractionated into periplasm (lanes 1 and 4), membrane (lanes 2 and 5) and cytoplasm (lanes 3 and 6). Each fraction contains material derived from approximately 10^8 cells. Lane 7: purified cytochrome c_{550} from *P. denitrificans*.

since the *H. thermophilus* cytochrome c_{552} acquires haem cytoplasmically in both organisms. Apparently, export to the periplasm is necessary for the cytochrome c_{550} to be formed as a holo protein. This result implies that the haem attachment takes place in the periplasm as suggested previously [4–7] or possibly as the polypeptide crosses the membrane. Using immunoblotting to test for cytochrome c_{550} expression in *E. coli* cells carrying a *cycA* gene, both mature and a putative precursor forms of the cytochrome c_{550} were detected, with most of the latter soluble in the cytoplasm rather than membrane bound (Fig. 2B). The accumulation of precursor in the cytoplasm is likely due to overexpression of the cytochrome c_{550} gene.

All bacterial cytochromes *c* reported to date, with the possible exception of some species in sulphate-reducing bacteria [1], are periplasmic or face the periplasm if attached to the cytoplasmic membrane. However, haem can incorporate cytoplasmically into the heterologously expressed apo-cytochrome c_{552} from *H. thermophilus*. It seems unlikely that *E. coli* and, in particular, *P. denitrificans* have highly specific cytochrome *c* haem lyases involved in the addition of haem only to the apo-cytochrome c_{552}, and thus not to the *P. denitrificans* apo-cytochrome c_{550}, in the cytoplasm. *H. thermophilus* native holo cytochrome c_{552} is an extremely thermostable protein [27], suggesting that the apo-cytochrome c_{552} might form an ordered structure at the growth temperature of *E. coli* and *P. denitrificans*, whereas the *P. denitrificans* apo-cytochrome c_{550} does not in the same conditions. The haem might thus incorporate into such a structured apo-cytochrome c_{552} in the correct orientation noncovalently. In contrast to the oxidising environment of the periplasm where disulphide bond formation occurs, cysteine thiol groups remain reduced in the cytoplasmic environment [28, 29] which would allow spontaneous formation of the covalent thioether linkages between the presumably appropriately located cysteine thiol groups of the apo-cytochrome c_{552} and haem vinyl groups. This proposal is supported by recent observations with bovine liver cytochrome b_5. The apo-cytochrome b_5 has tertiary structure similar to that of the holo protein and haem

incorporates into the protein noncovalently without enzymatic assistance [30]. Interestingly, a mutant cytochrome b_5 in which Asn-57 is replaced by cysteine has a covalent thioether linkage, believed to be formed spontaneously, between the introduced Cys-57 and haem when the mutant protein is expressed in *E. coli* [31]. In view of these considerations it can be postulated that bacterial cytochrome *c* haem lyase might function in the periplasm or at the periplasmic side of the membrane as an enzyme to promote the formation of the proper folding of apocytochrome *c* with cysteines reduced and/or to assist haem to access the cysteines.

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