Bacillus subtilis holo-cytochrome c-550 can be synthesised in aerobic Escherichia coli

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Bacillus subtilis membrane-bound holo-cytochrome c-550 was found to be expressed from the structural gene cloned on a plasmid vector in aerobically grown *Escherichia coli* and exhibited normal biochemical properties. This occurs despite the lack of endogenous cytochrome c and suggests that cytochrome c-heme lyase activity is also present in aerobic *E. coli*. The membrane topology of *B. subtilis* cytochrome c-550 was studied using fusions to alkaline phosphatase (PhoA). The results show that the heme domain (at least when fused to PhoA) can be translocated as apo-cytochrome and confirm that the N-terminal part of the cytochrome functions as both export signal and membrane anchor for the C-terminal heme domain. A model for the organisation of *B. subtilis* cytochrome c-550 in the cytoplasmic membrane is presented.

phoA; cccA; Hemoprotein; Cytochrome c biogenesis

1. INTRODUCTION

The heme group in c-type cytochromes is generally attached to the polypeptide via thioether linkage to two cysteine residues in a sequence -Cys-X-Y-Cys-His-. Specific enzymes, cytochrome c-heme lyases, are required for the formation of covalently bound heme as demonstrated for mitochondrial cytochrome c and c_1 [1,2]. Corresponding lyases have not been identified in bacteria but can be assumed to exist [3–5].

Cytochrome of c-type is found in *Escherichia coli* grown anaerobically with nitrate or trimethylamine N-oxide [6] and a *Rhodobacter sphaeroides* small soluble holo-cytochrome c can be synthesized in *E. coli* strain JM83 grown anaerobically on trimethylamine N-oxide [7]. Attempts to express different heterologous holo-cytochrome c, including the *Rh. sphaeroides* cytochrome, in aerobically grown *E. coli* have been reported unsuccessful [7,8] in correlation with the apparent lack of c-type cytochrome in aerobically grown *E. coli* [6]. In this communication we will demonstrate that *Bacillus subtilis* holo-cytochrome c-550 can be expressed in *E. coli* grown aerobically.

B. subtilis cytochrome c-550 is a tightly membrane-bound 15-kDa protein containing one heme group and with a yet unknown specific function [9]. The *cccA* gene encoding apo-cytochrome c-550 has been isolated and the primary structure of the 120 residue protein has

been deduced from the DNA sequence [9]. The C-terminal part of the cytochrome polypeptide is hydrophilic and constitutes the heme domain whereas the N-terminal part contains a stretch of 23 apolar residues and is proposed to function as an anchor for the cytochrome in the cytoplasmic membrane. *B. subtilis* cytochrome c-550 is found in the membrane fraction also when expressed in *E. coli*. To probe the topography of the cytochrome c-550 polypeptide in the *E. coli* membrane and also to identify polypeptide segments that can serve as a signal sequence for membrane translocation we have made use of the in vivo PhoA fusion system developed by Manoil and Beckwith [10,11]. The results obtained show that the 32 residues at the N-terminal end of the cytochrome polypeptide can function as a signal peptide, confirm the proposed topography and provide information on the binding of heme to the cytochrome polypeptide.

2. MATERIALS AND METHODS

2.1. Bacterial strains, phage and plasmids

E. coli strains used were CC118 (*araD*139 Δ(*ara leu*)7697 Δ*lacX*74 *phoA*Δ20 *galE galK thi rpsE rpoB argE*am *recA*1) [12]; JM83 (*ara* Δ(*lac-proAB*) *rpsL* Φ80 *lacZ*ΔM15) [13]; AN345 (*pro leu*) [14]; LE392 (*supE*44 *supF*58 *hsdR*514 *galK*2 *galT*22 *metB*1 *trpR*55 *lacY*1) [15] and 5K (*hsdR hsdM rpsL thiA thr*) [16]. Bacteriophage λ::Tn*phoA*-1 (*b*221 *cI*857 Pam3 with Tn*phoA* inserted in or near the *rex* gene) [17]. Phage λ::Tn*phoA* was propagated on *E. coli* LE392 as described in [18]. Plasmid pUC19 [13], pLUW1900 (pUC19 with the *B. subtilis cccA* gene on a 3.1 kb *Bam*HI fragment) and pLUW1954 pUC19 with *cccA* on a 0.9 kb *Dra*I-*Eco*RV fragment) have been described before [9].

2.2. Media and reagents

E. coli strains were kept on LA-plates. Liquid cultures (0.5 liter) were grown in LB-broth (10 g Bacto-tryptone (Difco), 5 g yeast ex-

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Abbreviations: SDS, sodium dodecyl sulfate; kb, kilobase(s)

tract (Difco), 5 g NaCl, in 1 liter water, pH 7.4) [18] supplemented with 0.5% (w/v) glucose at 37°C in 2.8 liters indentated Fernbach flasks in a rotary (200 rpm) incubator. Antibiotics were used at the following concentrations: ampicillin, 50 mg/l and kanamycin 30 or 300 mg/l. The higher kanamycin concentration was used in the initial screening of phoA gene fusions.

2.3. DNA manipulations

Agarose gel electrophoresis of nucleic acids was performed as described by Sambrook et al. [18]. Plasmid DNA was prepared according to Ish-Horowicz and Burke [19]. DNA was sequenced by the dideoxy nucleotide termination method [20] using modified T7 DNA polymerase (Sequenase), with double-stranded DNA templates [21] and pUC19 specific primers. E. coli cells were made competent by the CaCl$_2$ method [22].

2.4. Generation and analysis of PhoA fusions

Random cccA-phoA fusions on plasmids were generated by infecting E. coli CC118(pLUW1954) with λ::TnphoA-1 and selection on LA-plates supplemented with antibiotics and the indicator dye 5-bromo-4-chloro-3-indolylphosphate-p-toluidine (40 mg/l) essentially according to the protocol of Manoil and Beckwith [12]. Plasmids in PhoA$^+$ clones were first categorized by their restriction enzyme digestion patterns and the fusion point in two extreme variants was then determined by nucleotide sequence analysis of fragments subcloned in pUC19. Plasmids coding for the two fusion protein variants were stabilized by deletion of the transposase and kanamycin resistance genes using restriction endonuclease HindIII. The resulting plasmids were named pCPC20 (encoding the larger fusion protein) and pCPN21 (encoding the smaller fusion protein). Alkaline phosphatase enzyme activity was determined on samples suspended in 1 M Tris-HCl, pH 8.0 at 30°C using a double-beam spectrophotometer and 1-ml cuvettes. Permeabilized cells were prepared according to Gutierrez et al. [17]. Spheroplasts were prepared as described by Kaback [23]. The same amount of sample was added to both cuvettes and the enzyme reaction was started by the addition of 0.1 ml (0.4% (w/v) p-nitrophenyl phosphate (Sigma Chemical Co.) to one cuvette, a corresponding volume of water was added to the other cuvette and the increase in absorbance at 420 nm as a function of time was recorded. Enzymatic activity is given as μmol substrate hydrolysed per minute. The molar extinction coefficient for p-nitrophenol used is 13.1 mM^{-1}·cm^{-1} at 420 nm and pH 8.0 in 1 M Tris-HCl. This value was calculated from the extinction coefficient 18.3 mM^{-1}·cm^{-1} at 400 nm and pH 9-10 [24].

2.5. SDS-polyacrylamide gel electrophoresis, antisera and immunoblotting

Preparation of cytochrome c-550 antiserum has been described before [9]. Antiserum against E. coli alkaline phosphatase (Pharmacia LKB Biotechnology Inc.) was prepared from rabbits. The immunoglobulins were purified and stored as described by Harboe and Ingild [25] except that the ion-exchange step was omitted.

SDS-polyacrylamide gel electrophoresis was done according to Neville [26]. Molecular weight markers used were the same as described before [9]. For immunoblotting, proteins were separated in a gradient gel (10-16% (w/v) acrylamide, 0.26-0.43% (w/v) bisacrylamide) and electrotransferred to a nitrocellulose filter by the method of Towbin et al. [27]. The filter was blocked, probed with primary antibody that had been preadsorbed with an E. coli CC118 lysate [28] and developed according to Blake et al. [29]. Goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio Zac AB) was used as the secondary antibody.

2.6. Pyridine hemochromogen

Heme c and protoheme IX in E. coli membranes were quantitated as pyridine hemochromogens [30]. The amount of hemochrome b and hemochrome c was determined from the absorbance difference of a reduced minus oxidized spectrum using the wavelength pair 556-540 nm and 550-535 nm, respectively, and an extinction coefficient of 24 mM^{-1}·cm^{-1} [31].

2.7. Other procedures

E. coli membranes were prepared from 3-liter cell cultures as described before [32]. Visible-light absorption spectroscopy was done as previously described except that a 1-nm bandpass was used [33]. Protein was determined according to Lowry et al. [34] in the presence of 1.7% (w/v) SDS using bovine serum albumin as the standard.

3. RESULTS AND DISCUSSION

3.1. Synthesis of B. subtilis holo-cytochrome c-550 in E. coli K12

Plasmid pLUW1900 is pUC19 carrying the B. subtilis cccA gene with its native promoter region. E. coli JM83 containing plasmid pLUW1900 and pUC19, respectively, were grown aerobically in LB supplemented with 0.5% (w/v) glucose. Cells were harvested when the culture had reached early stationary growth phase. The presence of cytochrome c-550 polypeptide in different subcellular fractions was analyzed immunochemically using anti-cytochrome c-550 serum. The antigen was found in the membrane fraction of JM83(pLUW1900) and had an apparent molecular weight of 15 kDa. This size corresponds to that of holo-cytochrome c-550 in B. subtilis membranes [9].

The cytochrome content in isolated membranes of JM83(pLUW1900) and JM83(pUC19) was analyzed by light-absorption spectroscopy (Fig. 1). A difference spectrum at 77 K of ascorbate-reduced minus ferricyanide-oxidized E. coli JM83(pLUW1900) membranes is shown in Fig. 1A. The spectrum shows absorption maxima at 548 and 519 nm (550 and 520 nm at room temperature). Cytochrome c-550 in B. subtilis membranes is fully reducible by ascorbate and shows absorption maxima at the same wavelengths [9]. No ascorbate reducible cytochrome could be detected in membranes of JM83(pUC19) (Fig. 1B). Dithionite reduced JM83(pLUW1900) membranes showed absorption peaks at 548 and 519 nm which were not present in the control membranes. These results demonstrate the presence of cytochrome c-550 chromophore in aerobically grown JM83(pLUW1900). Cytochrome c-550 can be reduced both by NADH and by succinate in B. subtilis membranes ([9], unpublished results). Similarly the cytochrome c-550 in the heterologous membrane was found to be reduced by NADH (Fig. 1A) and succinate (not shown), indicating that it can functionally interact with the E. coli respiratory chain.

To verify that the heme group is covalently bound in B. subtilis cytochrome c-550 synthesized in E. coli and to quantitate the amount of holo-cytochrome c expressed we made use of differential heme extraction. Delipidated membranes (prepared as described in [30]) were first extracted with acid-acetone which removes non-covalently bound heme and c-type cytochrome in the residue was then quantitated as pyridine ferrohemochrome by difference spectroscopy. As a reference the amount of acid-acetone extractable pro-
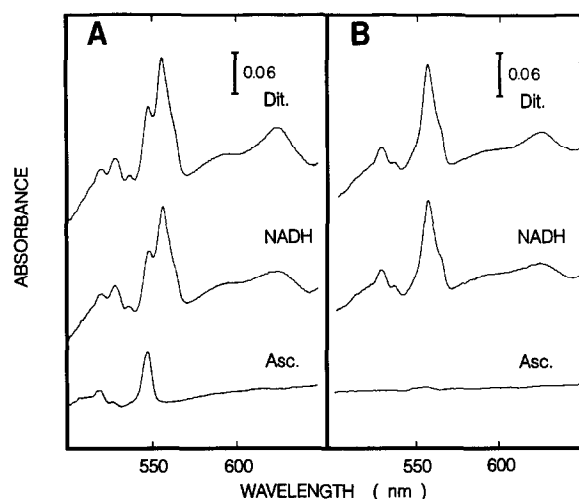
Fig. 1. Difference absorption spectra of *E. coli* membranes recorded at 77 K. (A) JM83(pLUW1900) (contains the *B. subtilis cccA* gene). (B) JM83(pUC19). Membranes, 15 mg protein/ml, were oxidized by 1 mM $K_3Fe(CN)_6$ in the reference cuvette and reduced by a few grains of sodium dithionite (Dit.), 2.5 mM NADH and 10 mM sodium ascorbate (Asc.), respectively, in the sample cuvette. When the sample was reduced by NADH or sodium-ascorbate 1 mM KCN was included in both cuvettes.

toheme IX in the membrane was determined. The results (Table I) demonstrate presence of cytochrome *c* in JM83(pLUW1900) membranes whereas no heme *c* could be detected in membranes of JM83(pUC19). Membranes of JM83(pLUW1900) consistently contained more protoheme IX than those of JM83(pUC19). This difference can be accounted for by a slightly increased relative content of cytochrome *d* oxidase in cells expressing cytochrome *c*-550 (see Fig. 1).

3.2. Topology of cytochrome c-550 in the membrane

Cytochromes *c* in bacteria seem always to be located on the outside of the cytoplasmic membrane, either soluble in the periplasmic space or bound to the membrane [35]. Hence, biogenesis of a cytochrome *c* in a procaryot involves translocation of the polypeptide heme-domain across the cytoplasmic membrane and covalent attachment of heme. In bacteria, little is known about the mechanisms or temporal order of these events [36,37].

Table I

Heme content of *E. coli* membranes

Membranes	Heme c[a] (nmol/mg)	Heme b[b] (nmol/mg)	Heme c/heme b (ratio)
JM83(pLUW1900)	0.11	0.45	0.25
JM83(pUC19)	<0.005	0.34	<0.02

[a] Acid-acetone nonextractable heme
[b] Acid-acetone extractable protoheme IX

The topology of a membrane bound protein and amino acid sequences that function as an export signal can be probed simultaneously in vivo by using translational fusions to alkaline phosphatase [11]. The experimental system is based on the use of a modified *E. coli* alkaline phosphatase gene (*phoA*) lacking the first part of *phoA* which encodes the translational initiation site and the signal-peptide for export [11]. PhoA protein is expressed when the truncated *phoA* gene is fused in frame to another gene providing the translation initiation site. Furthermore, the alkaline phosphatase part of the hybrid protein is only exported if a signal sequence is provided in the N-terminal sequence and alkaline phosphatase has to be on the outside of the cytoplasmic membrane in order to be enzymatically active [11].

Cells expressing active alkaline phosphatase from different *cccA-phoA* gene fusions were selected on plates containing kanamycin and the indicator dye 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine after infection of *E. coli* CC118(pLUW1954) with λ::Tn*phoA*-1. Plasmid pLUW1954 is a pUC19 derivative with the *B. subtilis cccA* gene on a 0.9 kb *B. subtilis* DNA fragment. Two clones with plasmids encoding fusion proteins containing 104 and 32 amino acid residues, respectively, of the cytochrome polypeptide were studied in more detail (Fig. 2).

The two gene fusions were subcloned into pUC19 to give plasmid pCPC20, encoding the larger fusion protein, and pCPN21, encoding the smaller fusion protein. Both plasmids lack the major part of Tn5 encoding kanamycin resistance and transposase functions. *E. coli* CC118 cells containing plasmid pCPC20, pCPN21 and pLUW1954 (full length cytochrome *c*-550), respectively, were fractionated into cytoplasmic, periplasmic, and membrane subcellular fractions. Hybrid proteins were found predominantly in the membrane fraction as determined by immunoblotting using antibodies against alkaline phosphatase (Fig. 3). The apparent molecular weights of the respective hybrid proteins, 60 and 52 kDa, corresponded to those deduced from the DNA sequence. Degradation products with a size about the same as that of alkaline phosphatase (47 kDa) were found from both hybrid proteins. Similar proteolytic processing occurring in vivo or during the preparation of cell fractions has been reported for several other PhoA fusion proteins [10,38,39]. Fig. 3B shows that antibodies against cytochrome *c*-550 recognize the 60-kDa fusion protein but not the 52-kDa fusion protein. Both fusion proteins have high alkaline phosphatase activity as determined using permeabilized cells or purified membranes. The specific enzyme activity of the membrane fraction was 4–5 times higher than that of permeabilized cells (Fig. 2). The results with the permeabilized cells demonstrate that the alkaline phosphatase part of the fusion proteins is exposed on the outside of the cytoplasmic membrane.
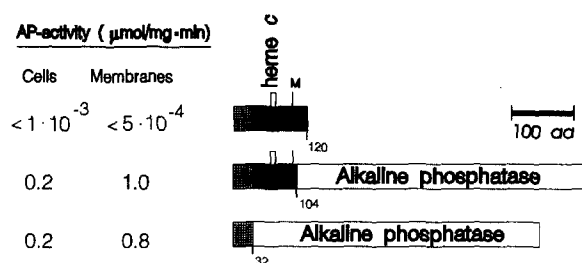
AP-activity (μmol/mg·min)

Cells	Membranes
$< 1 \cdot 10^{-3}$	$< 5 \cdot 10^{-4}$
0.2	1.0
0.2	0.8

Fig. 2. Primary structure of full-length cytochrome c-550 and of the two studied PhoA fusion proteins. The location of the heme binding site and a methionine proposed to be the sixth ligand to the heme iron atom in the cytochrome are indicated. The hydrophobic N-terminal segment (gray) and the hydrophilic heme domain (black) of the cytochrome polypeptide are indicated. The specific alkaline phosphatase (AP) activity of *E. coli* CC118 permeabilized cells and isolated membranes containing the different proteins are shown.

When spheroplasts were prepared from CC118(pCPC20) and CC118(pCPN21) less than 5 and 1%, respectively, of the total alkaline phosphatase activity was found in the supernatant after the spheroplasts had been removed by centrifugation. This activity remained in the supernatant after centrifugation for 30 min, at $200000 \times g$. The soluble alkaline phosphatase activity is probably a result of proteolytic processing of membrane bound hybrid proteins. The above results with the PhoA fusion proteins demonstrate that the N-terminal 32 residues of the cytochrome c-550 polypeptide can function as an export signal. They also strongly indicate that this N-terminal segment is sufficient for binding of cytochrome c-550 and hybrid proteins to the membrane. Finally, they suggest that the cytochrome c-550 antibodies are directed against the heme-domain (C-terminal part) of the cytochrome polypeptide.

3.3. Can heme be attached to hybrid cytochrome polypeptide?

The 60-kDa hybrid protein contains the major part of the cytochrome c-550 polypeptide including the heme binding site and a methionine proposed to be the sixth ligand to the heme iron atom (Fig. 2). To determine if heme is bound to the hybrid proteins, membranes of *E. coli* CC118(pCPC20), CC118(pCPN21) and CC118(pLUW1954) were prepared and analyzed by light absorption spectroscopy as before. Holocytochrome c-550 was present in CC118(pLUW1954) membranes but could not be found in membranes from CC118(pCPC20) and CC118(pCPN21). The lack of detectable cytochrome c-550 chromophore in membranes from these two strains cannot be explained by a low amount of membrane bound full-length hybrid protein. The amounts of the 52- and 60-kDa hybrid proteins were estimated from anti-alkaline phosphatase immunoblots by comparing the relative staining intensity of hybrid protein bands with that of a known
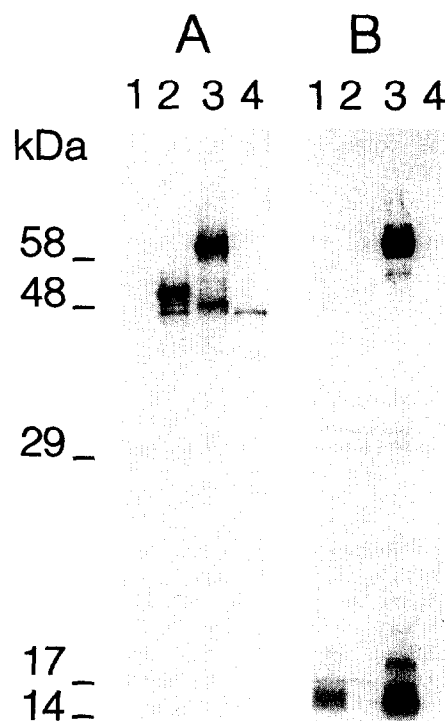

Fig. 3. Immunoblot of cccA-phoA hybrid proteins. Membrane proteins were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with antibodies against *E. coli* alkaline phosphatase (A) and *B. subtilis* cytochrome c-550 (B). Lanes 1, 2 and 3: membranes from strain CC118 harboring plasmid pLUW1954 (1); pCPN21 (2); pCPC20 (3). Lane 4: *E. coli* wild type alkaline phosphatase. In A, 1 μg of membrane protein and 5 ng purified alkaline phosphatase was loaded on the gel. The amount of protein loaded on gel B was 10 times higher than that in A.

amount of purified *E. coli* alkaline phosphatase (Fig. 3A). The content of hybrid protein in membranes was at least 0.1 nmol/mg. We conclude that both hybrid proteins lack covalently bound heme.

3.4. Conclusion

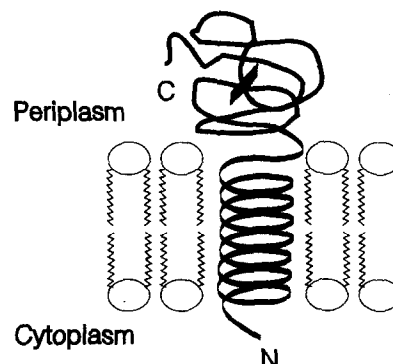The data obtained with the cytochrome c-550-PhoA fusion proteins show that the N-terminal 32 residues of

Fig. 4. Model of *B. subtilis* cytochrome c-550 in the cytoplasmic membrane based on sequence [9] and *phoA* gene fusion analyses.

the cytochrome polypeptide can function both as a signal sequence for membrane translocation and as a membrane anchor. They also suggest that the heme domain and the C-terminus are located on the extracytoplasmic side of the membrane as schematically drawn in Fig. 4.

B. subtilis holo-cytochrome *c*-550 was found to be expressed from the cloned *cccA* gene in aerobically grown *E. coli* and this seems not restricted to specific strains, e.g. holo-cytochrome was expressed in 5K and AN345 (data not shown) as well as in JM83 and CC118. Holo-cytochrome *c*-550 was also made in *E. coli* JM83(pLUW1900) grown aerobically in minimal glucose medium and anaerobically with trimethylamine *N*-oxide (data not shown). We conclude that cytochrome *c*-heme lyase activity is present in aerobic *E. coli* and can be active on a heterologous membrane-bound cytochrome polypeptide. The 0.9 kb fragment of *B. subtilis* DNA in pLUW1954 does not contain any open reading frame except *cccA*. Thus it can be excluded that the tentative lyase is encoded by the cloned DNA. The 60 kDa (Fig. 2) cytochrome *c*-550-PhoA fusion protein contains the first 104 amino acid residues of the 120 amino acid residue cytochrome polypeptide. The fact that this fusion protein lacks heme indicates that incorporation of heme is not required for translocation of the cytochrome across the cytoplasmic membrane. The lack of covalently bound heme in the 60-kDa fusion protein suggests that either the 16 C-terminal amino acid residues of apo-cytochrome *c*-550 are needed for heme binding or that the apo-cytochrome part of the fusion protein cannot obtain a conformation required for heme binding.

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