Bacillus subtilis CcdA-defective mutants are blocked in a late step of cytochrome c biogenesis.

T Schiött, M Throne-Holst and L Hederstedt

Cytochromes of the c type contain covalently bound heme. In bacteria, they are located on the outside of the cytoplasmic membrane. Cytochrome c synthesis involves export of heme and apocytochrome across the cytoplasmic membrane followed by ligation of heme to the polypeptide. Using radioactive protoheme IX produced in *Escherichia coli*, we show that *Bacillus subtilis* can use heme from the growth medium for c c protein synthesis. The *B. subtilis* ccdA gene encodes a 26-kDa integral membrane protein which is required for cytochrome c synthesis (T. Schiött et al., J. Bacteriol. 179:1962–1973, 1997). In this work, we analyzed the stage at which cytochrome c synthesis is blocked in a ccdA deletion mutant. The following steps were found to be normal in the mutant: (i) transcription and translation of cytochrome c structural genes, (ii) translocation of apocytochrome across the cytoplasmic membrane, and (iii) heme transport from the cytoplasm to cytochrome polypeptide on the outer side of the cytoplasmic membrane. It is concluded that CcdA is required for a late step in the cytochrome c synthesis pathway.

**Materials and Methods**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1.

**Media and general growth of bacteria.** *E. coli* strains were grown in Luria broth (LB) medium and on Luria agar (LA) plates (36). *B. subtilis* strains were grown on tryptose blood agar base (TBAB) plates (Difco) and in nutrient broth (LB) medium and on Luria agar (LA) plates (36). Bacterial strains and plasmids used in this study are listed in Table 1.

**Production of 14C-heme.** A system for the production of labeled protoheme IX (14C-heme) was developed based on an *E. coli* strain, AN344, containing plasmid pTYR13. AN344 is blocked in ALA synthesis and requires ALA for growth. Anti-biotics at the following concentrations were used when appropriate: ampicillin, 35 to 100 mg/liter; chloramphenicol, 12.5 (E. coli) or 3.5 to 5 (B. subtilis) mg/liter; and phleomycin, 1.5 mg/liter (B. subtilis).

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Plasmids

E. coli

AN344
t

leu pro hem A

Strain or plasmid Description Source or reference

B. subtilis

168A trpC2

C. Anagnostopoulou

168A qoxB::Cm

37

G. Venema

3G18 ade met trpC2

41

JO4 ade met trpC2

46

J06050 ade met trpC2

39

J06068 ade met trpC2

168A qoxB::Cm

3G18

pPCpC20

pUC19 with cccA-phoA fusion encoding Cyt c'-ALP (Fig. 2); Ap R

48

This work

pPCpC32

pHP13 with 3.9-kb Prvl-HindIII fragment from pCPc20; Cm R

This work

pCPN21

pUC19 with cccA-phoA fusion encoding Cyt c'-ALP (Fig. 2); Ap R

48

This work

pCPN23

pHP13 with 3.9-kb Prvl-HindIII fragment from pCPN21; Cm R

This work

pLU130

Shuttle vector; Cm R Em R

13

This work

pLU1301

pHP13 with cccA-cybC fusion encoding Cyt c'-Cyt b on a 1.1-kb EcoRI-BamHI fragment; Cm R Em R

This work

pLU1303

pUC19 with part of cccA on a 0.6-kb EcoRI-KpnI fragment; Ap R

This work

pLU1305

pUC19 with cccA-cybC fusion encoding Cyt c'-Cyt b on a 1.1-kb EcoRI-BamHI fragment

This work

pLUW1300

pHP13 with cccA on a 3.1-kb BamHI fragment; Cm R Em R

46

This work

pLUW1335

pHP13 with cccA on a 0.9-kb DraI-EcoRV fragment; Cm R Em R

47

This work

pNS204

pUC18 with E. coli cybC on a 3.1-kb SauI fragment; Ap R

27

This work

pTyr13

pBR322 with mutant B. subtilis sdiC; Ap R

12

This work

pUC1815

Ap R

50

This work

Strain AN344/pTyr13 was grown at 37°C in LB, supplemented with 12 μM ferric citrate, 35 mg of ampicillin per liter, and 31 μM (50:8 C/mol) [14C]-ALA (14C-ALA; New England Nuclear) or 31 μM ALA, for the production of 14C-heme or nonlabeled heme, respectively. After overnight growth, the culture was harvested by centrifugation and washed twice in 30 mM Tris-HCl, pH 8.0. The subsequent steps were performed in dim light at 4°C. The cell pellet (from 60 ml of culture) was suspended in 8 ml of ice-cold acetone and transferred to a stoppered glass tube containing glass beads. The tube was shaken to homogenize the suspension and then subjected to centrifugation at 3,400 rpm for 20 min in a tabletop centrifuge. The pellet was extracted with 8 ml of chloroform-methanol (2:1, vol/vol) and then with 8 ml of ice-cold acetone. Finally, heme was extracted from the delipidated cells three times with 4 ml of ice-cold acetone containing 24 mM HCl. The acid acetone extracts were pooled and concentrated to 0.5 to 1 ml by evaporation. The yield of 14C-heme and nonlabeled heme with this procedure was typically about 20 nmol per 60-ml culture. About 28% of the 14C-ALA added to the medium was consumed during growth of the cells. The yield of 14C-heme was 10 to 15%; i.e., about 50% of the consumed 14C-ALA was recovered in the final 14C-heme preparation. Eight ALA molecules are used to build up the heme molecule. The obtained radioactive heme has 14C at positions 21, 3, 5, 6, 8, 11, 13, 17, and 19 of the tetrapyrole molecule. Analysis of 14C-heme preparations by reversed-phase high-pressure liquid chromatography (40) and thin-layer chromatography (9) showed only protoporph IX; no heme O or other porphyrin compounds were detected. The protoporph IX concentration determined by pyridine hemochromogen analysis (7) and that calculated on the basis of 14C radioactivity agreed to >95%, demonstrating that essentially all radioactivity of the preparations was in the heme.

Radioactive labeling of cytochromes in B. subtilis. B. subtilis strains were grown overnight at 37°C in 25 ml of NSMP supplemented with 12 μM (50:8 C/mol) 14C-ALA or 0.32 μM (405 C/mol) 14C-heme or with 12 μM (50:8 C/mol) 14C-ALA and 0.32 μM nonlabeled heme. Isolation of the membrane fraction, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of membrane proteins, and analysis for tightly bound radioactivity in polypeptides were performed as described previously (39).

Northern blot analysis. Total RNA was extracted from cultures in the exponential growth phase (t1), the end of the exponential growth phase (t2), and the beginning of the stationary growth phase (t3) and analyzed by Northern blotting as described before (39). A DNA fragment covering bp 1571 to 1880 of the qcrC gene (accession no. U25533) (51) was used as a probe.
with results for Bacillus residues) subunit.

The pattern of radioactive polypeptides obtained after SDS-PAGE of extracts from strain 3G18Δ8 grown in the presence of 14C-heme. Strain 3G18Δ8 has a deletion of the hemH gene, which encodes ferrochelatase, catalyzing the final step in protoheme IX synthesis. The mutant is completely blocked in heme synthesis and requires heme for growth (15). 14C-heme was produced in E. coli as described in Materials and Methods.

The 16- and 36-kDa polypeptides are cytochrome c550 (CcCA) and subunit II (CtaC) of the cytochrome c550, terminal oxidase, respectively. The 22- and 29-kDa polypeptides are most likely the cytochrome b (QcrB) and the cytochrome c (QcrC) subunits, respectively, of the bc complex. The radioactivity at 52 kDa is probably due to aggregation, including one of the labeled cytochromes (39). The radioactivity at the electrophoresis front is 14C-heme.

Lysozyme per liter or (for strains 168A and LU6068) in 10 ml of 50 mM sodium morphinolpropanesulfonic acid (MOPS)-HCl (pH 8.0) containing 0.5 M sucrose, 20 mM MgCl2, and 4 g of lysozyme per liter. After 10 min at 42°C with slow mixing, more than 95% of the cells had been converted to protoplasts as judged by phase-contrast microscopy. The protoplasts were pelleted by centrifugation at 5,000 × g for 10 min at 10°C. The uppermost part (~5 ml) of the protoplast supernatant was collected. Membranes were isolated from the protoplasts and suspended in 20 mM sodium MOPS-HCl, pH 7.4 (3G18 and LU6018) or 7.0 (168A and LU6068).

Western blot analysis. Proteins separated by SDS-PAGE were electroblotted (KemEnTek Semidry Blotter II) to a polyvinylidene difluoride membrane. Rabbit antiserum against E. coli Alkaline phosphatase (48) was used as the primary antibody (diluted 1/5,000). Immunoreactive polypeptides were visualized using a chemiluminescence detection system (Clonetech).

Other methods. E. coli was transformed by electroporation and B. subtilis was grown to competence as previously described (1, 39). SDS-PAGE was carried out by using the Schägger and von Jagow system (38). Malate dehydrogenase and alkaline phosphatase enzyme activities were determined as previously described (48). Membranes from B. subtilis strains were isolated essentially as described by Hederstedt (16) and stored at ~80°C. Light absorption spectroscopy was carried out as described before (39). The protein concentration of membrane fractions was determined by using the bicinchoninic acid protein assay (Pierce Chemical Co.), with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Labeling of B. subtilis c-type cytochromes with 14C-heme. ALA is a biosynthetic precursor to heme. Four major radioactive polypeptides of 16, 22, 29, and 36 kDa are found if total cell extracts or isolated membranes from B. subtilis, grown in the presence of 14C-ALA, are subjected to SDS-PAGE analysis (39, 47, 51) (Fig. 1). The radioactivity remains associated with the polypeptides after electrophoresis and extensive washing of the resulting gel in acid methanol (51). The 16- and 36-kDa proteins have been identified as the cytochrome c550 (CcCA) and the subunit II (CtaC) of the cytochrome c550, terminal oxidase, respectively (46, 47). The 22- and 29-kDa proteins are both missing in mutants in which the ctaABC operon is inactivated (51). This operon encodes menaquinol:cytochrome c oxidoreductase (the bc complex), which contains a diheme cytochrome b (QcrB; 224 residues) and a monoheme cytochrome c (QcrC; 255 residues) subunit. B. subtilis Qcr mutant data (51, 52) combined with results for Bacillus strain PS3 (18, 43) and Bacillus stearo-thermophilus (41) suggest that the labeled 22- and 29-kDa polypeptides correspond to QcrB and QcrC, respectively.

All of the radioactive polypeptides obtained by growing cells in the presence of 14C-ALA do not necessarily contain heme. For example, the protein might contain covalently bound pyrro-del as in the case of porphobilinogen deaminase (20). For this reason and to determine if heme added to the bacteria can be incorporated into cytochromes of the c type in vivo, we analyzed membranes from B. subtilis 3G18Δ8 grown in the presence of 14C-heme. Strain 3G18Δ8 has a deletion of the hemH gene, which encodes ferrochelatase, catalyzing the final step in protoheme IX synthesis. The mutant is completely blocked in heme synthesis and requires heme for growth (15). 14C-heme was produced in E. coli as described in Materials and Methods.

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FIG. 1. B. subtilis proteins labeled in vivo with 14C-ALA or 14C-heme. An autoradiograph of an SDS-polyacrylamide gel is shown. Lane A, membranes of strain 3G18 (wild type) grown in the presence of 14C-ALA; lane B, membranes of strain 3G18Δ8 (blocked in heme synthesis) grown in the presence of 14C-heme; lane C, membranes of 3G18Δ8 grown in the presence of 14C-ALA and nonlabeled heme. The apparent masses of labeled polypeptides are indicated.
ALP has to be transported to the periplasm to become enzymatically active (2, 6). It has previously been demonstrated in E. coli that the Cyt c'-ALP and Cyt c'+ALP proteins are membrane bound, with ALP facing the periplasm (48).

The hybrid genes for the two fusion proteins were cloned into the low-copy-number plasmid pHPl3 (13), giving pCPC23 and pCPN23. B. subtilis LU6018 and 3G18 containing pHPl3 formed white colonies after overnight growth on phosphate-supplemented TBAB plates containing X-phosphate, a zymogram substrate for ALP. Colonies of LU6018 and 3G18 containing pCPC23 or pCPN23 were blue on the X-phosphate plates. Membrane and protoplast supernatant cell fractions were isolated from the different strains, and analyzed for ALP by alkaline phosphatase activity measurements (Table 2) and by Western blotting (Fig. 3). No significant differences were observed between the mutant and the wild-type extracts. The membrane fractions of strains harboring plasmid pCPC23 or pCPN23 contained ALP antigen of >43 kDa, which is most clearly seen in the case of the longer fusion protein (Fig. 3A, lanes 3 and 5). Alkaline phosphatase activity was predominately found in the protoplast supernatant fraction (Table 2), where mainly 43-kDa ALP antigen, i.e., antigen of the same size as the ALP itself (Fig. 3B), was found.

The combined results show that the CccA polypeptide is translocated across the cytoplasmic membrane in both the wild type and the ccd4 mutant. To some extent, ALP is cleaved off the membrane-anchored fusion protein in vivo and/or during isolation of the protoplast supernatant fraction. The low activity of the ALP antigens found in the membrane fractions, especially in the case of the longer fusion construct, suggests that the CccA part interferes with proper folding and/or dimerization of ALP. Provided that enzymatically active ALP can form only on the outer side of the B. subtilis cytoplasmic membrane, we conclude that the active ALP protein found in the protoplast supernatant fraction must have been exported.

B. subtilis contains two a-type cytochromes, i.e., a quinol oxidase, aa₃ (encoded by the qoxABCD operon), and a cytochrome c oxidase, caa₃ (encoded by the ctaCDEF genes) (19). These cytochromes show an absorption peak at about 600 nm. The aa₃-type oxidase is dominating under most growth conditions, but the cytochrome caa₃ oxidase is predominantly reduced by ascorbate (46).

To analyze whether the ctaC and ctaD genes (encoding subunit II and subunit I of the caa₃ complex, respectively) are

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**TABLE 2. Alkaline phosphatase activities of subcellular fractions**

<table>
<thead>
<tr>
<th>B. subtilis strain</th>
<th>Membrane Activity (U)</th>
<th>Protoplast supernatant fraction Activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3G18/pHP13</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3G18/pCPC23</td>
<td>0.01</td>
<td>2.3</td>
</tr>
<tr>
<td>3G18/pCPN23</td>
<td>0.09</td>
<td>1.8</td>
</tr>
<tr>
<td>LU6018/pHP13</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LU6018/pCPC23</td>
<td>&lt;0.01</td>
<td>1.6</td>
</tr>
<tr>
<td>LU6018/pCPN23</td>
<td>0.11</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* One unit corresponds to 1 nmol of phosphoester bonds hydrolyzed per min and amount of cell fraction corresponding to that present in 1 ml of culture with an optical density at 600 nm of ~1.3.

**FIG. 2. Schematic illustration of B. subtilis cytochrome cᵅₛ (Cyt c) and the fusion proteins used in this work. The N-terminal membrane anchor domain of cytochrome cᵅₛ is indicated in dark grey. The location in the polypeptide of the -Cys-Xaa-Yaa-Cys-His- motif (heme C) and the sixth ligand to the heme iron (a methionine [M]) are indicated. The numbers refer to amino acid residues in cytochrome cᵅₛ (ccA gene product) and cytochrome bᵅ₃ (cybC gene product), respectively. When the cccA-cybC fusion gene was constructed, 6 bp encoding a glycine (G) and a threonine (T) residue were added at the fusion point (small black box).**

**FIG. 3. Western blot of fusion proteins in B. subtilis.** The membrane (A) and the protoplast supernatant (B) fractions from B. subtilis wild-type strain 3G18 (lanes 2, 3, 6, and 7) and ccd4 deletion mutant strain LU6018 (lanes 4, 5, 8, and 9), containing pHPl3 (lanes 2, 4, 6, and 8), pCPC23 (Cyt c'-ALP; lanes 3 and 5), or pCPN23 (Cyt c'+ALP; lanes 7 and 9), were analyzed for ALP antigen. Purified alkaline phosphatase (43 kDa) was loaded in lane 1, and its position in each gel is indicated by an arrowhead. After SDS-PAGE, samples were electroblotted onto a polyvinylidene difluoride filter and probed with polyclonal antiserum against E. coli alkaline phosphatase. Five micrograms of membrane proteins, 1 μl of the protoplast supernatant fraction, and 5 ng of purified alkaline phosphatase were loaded.
and the \(D_{P. \text{denitrificans}}\) mutant. Subunit II of the 600-nm region from cytochrome \(a\) was reduced in membranes of the wild type, the \(B. \text{subtilis}\) strain \(qoxB\), and JO4 (\(qoxB\) deletion mutant). Ascorbate-reduced membranes of the wild type, the \(qoxB\) deletion mutant, and the \(qoxB\) double mutant showed a peak in the 600-nm region from cytochrome \(a\) of the cytochrome \(ca_{a_3}\) complex (Fig. 4). Strain JO4 (deleted for both \(a\)-type cytochromes) lacked this absorption peak, as expected. The presence of cytochrome \(a\) in strain LU6050 strongly suggests that the \(ctaC\) and \(ctaD\) genes are expressed also in a \(ccdA\) deletion mutant. Subunit II of \(P. \text{denitrificans}\) cytochrome \(c\) oxidase (\(aa_{a_3}\) type), and thus probably also subunit II of \(B. \text{subtilis}\) cytochrome \(ca_{a_3}\), is required for assembly of the heme \(A\) containing subunit I (42). Hence, it is likely that the apoform of subunit II is present and assembled together with subunit I in the membrane of a \(ccdA\) deletion mutant. Since ascorbate can reduce cytochrome \(a\) in the heme \(C\)-defective \(B. \text{subtilis}\) cytochrome \(ca_{a_3}\) complex, it seems as if the apoform of subunit II contains the dicopper-Cu\(_A\) center.

The presence of the 22-kDa putative OcrB polypeptide in membranes of \(ccdA\) deletion mutants (39) indicates that the \(qcrABC\) operon (encoding the \(bc\) complex) is expressed. This was confirmed by analyzing wild-type cells (3G18) and the \(ccdA\) deletion mutant (LU6018) for mRNA corresponding to the \(qcrABC\) operon. Northern blot analysis of total RNA extracted from cells in different growth stages (see Materials and Methods) showed that \(qcrABC\) mRNA was present also in the mutant (Fig. 5). The highest amount of hybridizing RNA was found in cells harvested at the end of the exponential growth phase, i.e., at \(t_{e}\). It is notable that the cellular concentration of mRNA (39) and that of \(qcrABC\) mRNA are similarly growth stage dependent.

The combined data obtained using the \(ccca-phoA\) reporter system, spectroscopic analysis, and Northern blot analysis show that \(B. \text{subtilis}\) cytochrome \(c\) polypeptides are synthesized in a \(ccdA\) deletion mutant. They also strongly suggest that the heme domain of apocytochromes \(c\) is exported across the cytoplasmic membrane in such a mutant.

**Heme export across the cytoplasmic membrane can occur without CcdA.** As an integral membrane protein (39), CcdA could play a role in the transport of heme. To investigate this possibility, we made use of a reporter system for transmembrane heme transport and noncovalent incorporation of heme into apocytochrome on the outside of the cytoplasmic membrane. Goldman et al. (10) recently reported principally the same type of experiments with \(E. \text{coli}\) cytochrome mutants.

The reporter system is based on \(E. \text{coli}\) cytochrome \(b_{562}\), which is encoded by the \(cybC\) gene (27) and is a water-soluble, monomeric, periplasmic cytochrome of unknown specific function. It is a very stable 12-kDa four-helix bundle protein containing one non-covalently bound protoheme IX (14). The cytochrome is synthesized as a 128-residue-long polypeptide with a signal peptide of 22 residues at the N terminus which is cleaved off after export of the protein across the cytoplasmic membrane (27). A hybrid gene encoding a fusion protein, Cyt \(c^*\)-Cyt \(b\) (Fig. 2), corresponding to the membrane anchor domain of \(B. \text{subtilis}\) cytochrome \(c_{550}\) (CcdA), residues 1 to 32, and of the \(E. \text{coli}\) cytochrome \(b_{562}\) polypeptide (CybC), residues 23 to 128, was constructed in pHP13, resulting in pLUT130. The \(ccdA\) deletion mutant LU6068 and the corresponding wild-type strain 168A, containing pLUT130 or pHP13, were grown in NSMP. Isolated membranes were analyzed by light absorption spectroscopy. At neutral pH, cytochrome \(c_{550}\) and cytochrome \(b_{562}\) have relatively high midpoint redox potentials, +178 mV and +180 mV, respectively (26, 49). These cytochromes are therefore reducible by ascorbate.

\(B. \text{subtilis}\) 168A/pLUT130 and 168A/pHP13 contained cyto-
Membrane topology of the Cyt c'--Cyt b fusion protein. It is very unlikely that the fusion proteins used in this work have a topology in the B. subtilis cytoplasmic membrane which is different from that of cytochrome c$_{550}$P. However, since cytochrome b$_{562}$ can form in the cytoplasm when fused to a membrane-bound protein (30), we analyzed the transmembrane topology of the heme domain of the Cyt c'--Cyt b fusion protein. Cells of strain LU6068/pLUT130 were treated with lysisase in the presence of 0.5 M sucrose to avoid osmosis.

The resulting protoplasts were removed by centrifugation. The obtained supernatant (protoplast supernatant fraction) and membranes isolated from the protoplast pellet were analyzed for cytochrome b$_{562}$ by using absorbance spectroscopy. About 45% of the total cytochrome b$_{562}$ content was found in the protoplast supernatant fraction. The degree of protoplast lysis was only 8%, as determined from the malate dehydrogenase enzyme activity of the protoplast supernatant fraction compared to that of a total cell lysate. The effect of adding trypsin to protoplasts of LU6068/pLUT130 and 168A/pLUW1354 was also tested. After protease treatment, about 64% of the total cytochrome b$_{562}$ content and about 98% of the cytochrome c$_{550}$ content were removed from the LU6068/pLUT130 and the 168A/pLUW1354 protoplasts, respectively. These experimental results confirmed that the cytochrome b$_{562}$ domain of the Cyt c'--Cyt b fusion protein is located on the outside of the B. subtilis cytoplasmic membrane.

**What is the specific function of CcdA?** Cytochrome c synthesis in B. subtilis requires the CcdA protein (39). In this work, we have defined the level at which the cytochrome c biosynthetic pathway is blocked in a B. subtilis ccdA deletion mutant. It was demonstrated that the expression of cytochrome c genes and the export of cytochrome polypeptides are normal in the mutant. CcdA does not seem to play a role in either transport of heme across the membrane or delivery of heme to the apocytochrome after transport, as concluded from the observation that a periplasmic cytochrome b can be synthesized in the absence of CcdA. However, it cannot be ruled out completely that CcdA is involved in the delivery of heme to apocytochrome c specifically. We suggest that CcdA functions in one or more cytochrome c biosynthetic steps that occur outside the cytoplasmic membrane. Assuming that CcdA is directed involved in cytochrome c synthesis, it might have a role in maintaining apocytochrome c in the correct state for covalent heme attachment.

**ACKNOWLEDGMENTS**

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**REFERENCES**


**FIG. 6.** Reduced-minus-oxidized difference light absorption spectra of membranes from B. subtilis 168A/pHP13 (A), 168A/pLUT130 (B), LU6068/pHP13 (C), and LU6068/pLUT130 (D). Plasmid pLUT130 encodes the Cyt c fusion protein (Fig. 2), and pHP13 is the corresponding control. The absorbance scale is indicated by the bars. The cuvettes contained 4 mg of membrane protein per ml. Other conditions were as for Fig. 4. The sample was reduced first with 8 mM ascorbate (asc.) and then with dithionite (dit.) added as a solid. The empty and filled arrowheads indicate 550 and 562 nm, respectively.


