We demonstrate that the cccB gene, identified in the Bacillus subtilis genome sequence project, is the structural gene for a 10-kDa membrane-bound cytochrome c551 lipoprotein described for the first time in B. subtilis. Apparently, CccB corresponds to cytochrome c551 of the thermophilic bacterium Bacillus PS3. The heme domain of B. subtilis cytochrome c551 is very similar to that of cytochrome c550, a protein encoded by the cccA gene and anchored to the membrane by a single transmembrane polypeptide segment. Thus, B. subtilis contains two small, very similar, c-type cytochromes with different types of membrane anchors. The cccB gene is cotranscribed with the yvjA gene, and transcription is repressed by glucose. Mutants deleted for cccB or yvjA-cccB show no apparent growth, sporulation, or germination defect. YvjA is not required for the synthesis of cytochrome c551, and its function remains unknown.

The cytoplasmic membrane of the Gram-positive bacterium Bacillus subtilis contains cytochromes of α-, β-, c-, and d-type (1). The c-type cytochromes differ from other cytochromes by having heme covalently bound to the polypeptide via cysteine residues in a consensus motif, Cys-Xaa-Xaa-Cys-His, in which the His residue functions as the fifth axial ligand to the heme iron. Three different membrane-bound c-type cytochromes have been described in B. subtilis. They are all dispensable for growth, repressed by glucose, and expressed in the early stationary phase (1). These cytochromes c are subunit II of the cytochrome caa3 complex (encoded by the ctaC gene) (2), cytochrome c of the cytochrome bc complex (encoded by the qcrC gene) (3), and the monomeric cytochrome c550 (encoded by the cccA gene) (4). Cytochrome caa3 is a cytochrome c oxidase. The cytochrome bc complex oxidizes menaquinol and transfers electrons to cytochrome c.

Cytochrome c550 is a 13-kDa protein with a membrane anchor domain consisting of a single α-helical transmembrane segment of about 30 residues and a heme domain of about 74 residues (4). The latter domain, like that of all bacterial c-type cytochromes, is located on the outer surface of the cytoplasmic membrane (5). At pH 7.0, cytochrome c550 has a midpoint redox potential of +178 mV (6). The function of this cytochrome is not known, and deletion or overexpression of the cccA gene does not affect the respiration activity of the cell (4).

Understanding the respiratory system and energy metabolism of B. subtilis requires detailed knowledge of the cytochromes and their specific biological roles. Sequence analysis of the entire B. subtilis genome revealed the cccB gene encoding a possible novel cytochrome c in B. subtilis. The deduced CccB sequence shows about 35% identity to CccA and has the cytochrome c consensus motif in the C-terminal part of the polypeptide. This was the only new c-type cytochrome found in the B. subtilis genome sequencing project. The cccB gene is located at 310° on the chromosome far away from the cccA gene at 222° (7). In this paper we demonstrate that cccB is the structural gene for a membrane-anchored cytochrome c551. As compared with the other c-type cytochromes in wild type cells, CccB is present in very low amounts, i.e. less than 105 molecules/cell. We have also analyzed the transcription of cccB and the properties of cccB null mutants. This new B. subtilis cytochrome has been purified and some of its characteristics are presented.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Bacterial strains and plasmids used in this work are presented in Table I.

Growth Media—Escherichia coli cells were grown on Luria agar plates or in LB (13). Unless otherwise stated, B. subtilis cells were grown on tryptose blood agar base (Difco) plates or in nutrient sporulation medium with phosphate (NSMP)2, pH 7.0 (14). The concentration of antibiotics used for B. subtilis was 4 μg/ml chloramphenicol and erythromycin, 15 μg/ml tetracycline, and the concentration used for E. coli was 100 μg/ml ampicillin, 12.5 μg/ml chloramphenicol, 15 μg/ml tetracycline.

Molecular Genetic Techniques—Plasmids were isolated using CsCl density gradient centrifugation (15) or by using the Quantum Prep® plasmid mini preparation kit (Bio-Rad). General DNA techniques were as described by Sambrook et al. (13).

The procedure for transformation of B. subtilis was based on a method described by Arwert and Venema (16) or according to Karamata and Gross (17). E. coli competent cells were prepared and transformed according to the calcium chloride method (13) or by electroporation as described in Ref. 18. PCR was done using the AmpliTaq polymerase (Perkin-Elmer) or Pfu DNA polymerase (Roche Molecular Biochemicals) according to the suppliers’ instructions.

Reverse Transcription PCR—PCR was used to investigate the presence of mRNA molecules carrying the sequence corresponding to the yvjA-cccB intergenic region. For this purpose, the following oligonucleotides were prepared: CR108, 5'-GTC CGA TTT TAA TGT GCG TGG TTG-3', whose sequence is identical to the distal part of the yvjA-coding DNA strand; and CR109, 5'-GCT TCC GTC TGT CCG CCA GGG TCT-3', complementary to the mRNA encoding a proximal part of cccB. 32 μg of total RNA were extracted from 22 ml of a late exponential phase LB culture of B. subtilis 168 by using the RNeasy Mini Kit (Qiagen). The extract was incubated for 60 min with 5 units of DNase I at 37 °C. After

2 The abbreviations used are: NSMP, nutrient sporulation medium with phosphate; PCR, polymerase chain reaction; ALA, 5-amino-levulinic acid; bp, base pair(s).

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heat inactivation of the DNase (65 °C for 20 min), 5 ng of the RNA preparation were incubated for 20 min at 60 °C in reverse transcription buffer containing primer CR109 (2 μM), 0.9 mM MnCl₂, 3.2 mM dNTP mixture, and 4 units of Tth DNA polymerase (Roche Molecular Biochemicals). Under these conditions and in the presence of Mn²⁺, the Tth DNA polymerase can perform reverse transcription and thus catalyze the synthesis of the cDNA strand complementary to the DNA polymerase can perform reverse transcription and thus catalyze the synthesis of the cDNA strand complementary to the DNA fragment in pMY2. The mixture was supplemented with primer CR108 (2 μM), 0.75 mM EGTA, and PCR buffer according to the manufacturer’s instructions. The PCR was performed in the same tube, because the Tth enzyme can act as a thermostable DNA polymerase in the presence of the Mg²⁺ present in the PCR buffer. To confirm that the resulting product originated from template mRNA and not from eventual chromosomal DNA contamination, a negative control was performed by running in parallel the same RNA preparation previously incubated for 120 min at 37 °C with 5 mg/ml DNase-free RNase A.

Construction of Plasmids—Plasmid pCRαccB was constructed in several steps. Basically it is a derivative of pUC18 into which the two DNA fragments from the B. subtilis chromosome (Fig. 1), obtained by using PCR, and the tetracycline resistance gene from the plasmid pBEST307 (19) were introduced. pCRαccB was obtained from pCRαccB by substituting the distal part of yajA with a PCR-obtained fragment homologous to the chromosomal region located upstream of yajA (Fig. 1).

Plasmid pCRαccB carries a transcriptional fusion of the yajA-ccdB promoter region with the lasZ gene from E. coli. It was obtained by cloning the PCR-derived DNA fragment used for the pCRαccB construction into pHC3. The latter plasmid allows the ectopic introduction of the gene fusion into the B. subtilis amyE locus (20).

Plasmid pLJU104, used for overproduction of CccB, was constructed as follows. Plasmid p4303 was cleaved by EcoRI and HindIII, and the 990-bp fragment containing the cccB gene was ligated into pUC18 by p Bluescript SK(−). From this plasmid, multiplied in E. coli strains (21), the corresponding DNA fragment was inserted after the EcoRI site of pUC18 to obtain pUC18. The resulting pUC18 was linearized in 5 volumes of 0.1 M Tris/HCl, pH 8, 0.1 mM NaCl, respectively, the CccB cytochrome was eluted with 10 mM Tris/HCl, pH 8, 0.5 mM NaCl, and 1 mM Na₂EDTA, pH 8) containing 2% (w/v) cholate, Triton X-100, or no detergent. Phenylmethylsulfonyl fluoride was added to 0.5 mM, and the samples were sonicated and then centrifuged for 40 min at 140,000 × g at 4 °C. The supernatants and the pellets, homogenized in 2 ml of buffer without detergent, were analyzed by light absorption spectroscopy.

Purification of CccB—Membranes isolated from B. subtilis L16205 were suspended in 10 mM Tris/HCl, pH 8, containing 1% (w/v) Thesit and treated with alkaline phosphatase. The part of the cccB gene was ligated into the B. subtilis 168 chromosome via a double crossover event resulting in strain L16205. The deletion of the yajA-ccdB segment was performed in a similar way by using linearized pCRαccB.

Strain LUH20 was obtained by the transformation of strain L168 to phleomycin resistance with chromosomal DNA containing a Δcto/C::ble gene replacement (21) and then to chloramphenicol resistance with DNA containing a ΔccdB::cat gene replacement (5). LUH36 was obtained by the transformation of LUH20 to tetracycline resistance with L16205 (ΔccB::tet) chromosomal DNA.

**Differential Solubilization of Membrane-bound c-type Cytochromes using Cholate and Triton X-100—** Membranes isolated from strains L1628/ pLUJ104 and LUH36/pLUJ105 were diluted to 1.5 mg protein/ml in solubilization buffer (30 mM Tris/HCl, pH 8, 0.5 mM NaCl, and 1 mM Na₂EDTA, pH 8) containing 2% (w/v) cholate, Triton X-100, or no detergent. Phenylmethylsulfonyl fluoride was added to 0.5 mM, and the samples were sonicated and then centrifuged for 40 min at 140,000 × g at 4 °C. The supernatants and the pellets, homogenized in 2 ml of buffer without detergent, were analyzed by light absorption spectroscopy.

Purification of CccB—Membranes isolated from B. subtilis L16205/ pLUJ104 were diluted to 5 mg protein/ml in solubilization buffer containing 2% (w/v) cholate. Phenylmethylsulfonyl fluoride was added and the samples were incubated and centrifuged as for the differential solubilization described above. The supernatant was supplemented with polyethylene glycol (M, 20,000) to a final concentration of 8% (w/v) and centrifuged at 22,000 × g for 20 min at room temperature. To the supernatant, polyethylene glycol was added to a final concentration of 30% (w/v), and MgSO₄ was added to 5 mM. After mixing, the sample was centrifuged at 43,700 × g for 20 min at room temperature. The pellet was suspended in 10 mM Tris/HCl, pH 8, containing 1% (w/v) Thesit and then dialyzed at 4 °C against the same buffer using Spectrapor® tubing connected with a FPLC® system (flow rate 5 ml/min). After two washing steps with 10 mM Tris/HCl, pH 8, 0.1% Thesit, containing 5 and 20 mM NaCl, respectively, the CccB cytochrome was eluted with 10 mM Tris/HCl, pH 8, containing 0.1% Thesit and 100 mM NaCl. The 5-ml eluate was dialyzed as above against 10 mM Tris/HCl, pH 8, 0.1% Thesit. The purification procedure up to this point was based on a method described by Sone et al. (22) to purify cytochrome c₅₅₁ from B. subtilis strain PS3.

The cytochrome c₅₅₁ was further purified using isoelectric focusing with the Rotorfö® System (Bio-Rad) in the presence of 0.1% Thesit. Twenty fractions were collected, and the absorption at 414 nm was determined. The fractions with high absorption at 414 nm (pH 3.7–4.0) were diluted in 5 volumes of 0.1 M Tris/HCl, pH 8, containing 0.1% Thesit, pooled, and concentrated using Microcon 10-kDa cut-off concentrators.

Miscellaneous Methods—Light absorption spectroscopy at room temperature, in vivo labeling of heme using 2 μM and 0.1 μCi/ml of 5-14Caminolevulinic acid (14CALA) and SDS-polyacrylamide gel electrophoresis were performed as described in Ref. 23 except that the Schagger von Jagow gel system (24) was used. B. subtilis membranes were isolated according to Ref. 25. Low temperature (77 K) light absorption spectroscopy was done as described in Ref. 26. Protein concentration...
titations were determined using the BCA protein assay reagent (Pierce) with bovine serum albumin as standard. β-galactosidase assays were performed according to Ref. 26. Heme C was determined from the pyridine hemochromogen difference (reduced minus oxidized) spectrum in alkaline solution using the absorption coefficient 23.97 mM$^{-1}$ cm$^{-1}$ (550 nm minus 535 nm) (27).

RESULTS AND DISCUSSION

Genetic Context and Transcription of cccB—Inspection of the B. subtilis genome sequence reveals that the cccB gene is flanked by the genes yvjA and ftsE (Fig. 1). Like cccB, these flanking genes are transcribed in the direction of DNA replication. The ftsE gene encodes a 25.5-kDa protein with sequence similarities to FtsE from E. coli, which is an ATP-binding protein involved in cell division. The putative 29.8-kDa polypeptide encoded by the yvjA gene shows about 30% sequence identity to several proteins of unknown function in B. subtilis, e.g. YgfU, YxkD, and YpJ. Judging from the sequence, there is no obvious promoter located immediately upstream of the cccB gene and no transcription terminator between yvjA and cccB. Downstream of cccB there is an inverted repeat followed by a run of Ts that probably functions as a rho-independent transcription terminator. The DNA sequence upstream of yvjA shows the features of a transcription terminator followed by a promoter region. Together, these observations suggest that yvjA and cccB are co-transcribed as an approximately 1.55-kilobase mRNA. Northern blot analysis of total B. subtilis RNA, using yvjA as the probe, has also shown a 1.6-kilobase transcript. The presence of such a di-cistronic mRNA was confirmed by reverse transcription PCR on total RNA extracted from strain 168 (Fig. 2). To study the expression pattern of yvjA-cccB during growth, a transcriptional yvjA-lacZ fusion was constructed (Fig. 1) and inserted into the chromosome at the amyE locus in strain 168 resulting in strain L16238. β-galactosidase activity values obtained with strain L16238 (squares) carrying the yvjA-lacZ fusion inserted into the amyE locus and those of the parent strain 168 corresponding to the background level of activity (circles). Closed and open symbols refer to cultures grown in NSMP and in NSMP supplemented with 0.5% glucose, respectively. Time zero corresponds to the beginning of the stationary phase.

FIG. 1. Map of the yvjA-cccB region in the B. subtilis chromosome. Genes are indicated by open arrows and putative transcription terminators by stem loop symbols. Short arrows indicate positions of putative promoters. Thick bars (lower part of the figure) show fragments cloned into the indicated plasmids. The zigzag line indicates the position of a reversed transcription (RT) PCR product obtained with primers CR108 and CR109 and total RNA isolated from strain 168 (Fig. 2). BglII (B), EcoRI (E), HindIII (H), and PstI (P) restriction sites are indicated.


FIG. 3. Effect of glucose on yvjA-cccB expression. β-galactosidase activity values obtained with strain L16238 (squares) carrying the yvjA-lacZ fusion inserted into the amyE locus and those of the parent strain 168 corresponding to the background level of activity (circles). Closed and open symbols refer to cultures grown in NSMP and in NSMP supplemented with 0.5% glucose, respectively. Time zero corresponds to the beginning of the stationary phase.

![Diagram](https://example.com/diagram.png)
PS3 cytochrome B. subtilis upstream of conclusion is supported by the fact that cccA CccB is therefore most likely a lipoprotein anchored to the modified Cys at the N-terminal end of the protein (28). Subsequently is cleaved by type II signal peptidase resulting in the Cys residue by the addition of a diacylglycerol moiety and sequence, Leu-Ala-Ala-Cys. This suggests that it is modified at signal peptide but contains the bacterial lipoprotein consensus (5). The N-terminal part of CccB also has the features of a function as a noncleaved signal sequence for membrane inser-
tion and peptide membrane anchor for the cytochrome domain (29). This cytochrome shows an absorbance maximum of two palmitic acid (C16:0) residues/molecule of cyto-

The predicted mass of the mature CccB lipoprotein with covalently bound heme is about 10 kDa. CccA protein in membranes of the parental strain 168 labeled with [14C]ALA gives rise to a diffuse but rather strong, radioactive cytochrome c band in the 15-kDa region of the gel. This band can hide other small cytochrome polypeptides. Therefore, to assess the presence of CccB, we constructed and analyzed strain LUH20, in which both cccA and ctcA are deleted. As expected, the QcrC and QcrB polypeptides were present in this strain, whereas a very faint, diffuse, radioactive polypeptide was found in the 14-kDa region of the gel. This polypeptide is most likely CccB because it was not present in labeled membranes from strain LUH36, which in addition to cccA and ctcCD has been deleted for the cccB gene (Fig. 5).

Overproduction of CccB—To facilitate the detection of 14C-heme labeled CccB as well as the isolation of the protein for biochemical characterization we have constructed pLUJ104. This plasmid is a derivative of pH13, an E. coli. subtilis shuttle vector with a copy number of about 5 in B. subtilis (9), containing the cccB gene cloned downstream of the sdh promoter. [14C]ALA labeled membranes obtained from B. subtilis strain LUH36 containing pLUJ104 presented a strong diffuse band migrating faster than CccA but at the same position as the weak band observed with LUH20 (Fig. 5, lane 5). The results show that CccB contains covalently bound heme, i.e. is a cytochrome c. The diffuse polypeptide bands observed with CccA and CccB are because of inherent properties of these cytochromes (not to the electrophoresis system as previously shown for CccA (6)).

Genes that are organized in one operon often encode func-
structure of CccB, a cccA-cccB hybrid gene is transcribed from the native promoter and is constitutively expressed, resulting in pLUJ105. The latter cytochrome has been demonstrated to be a lipoprotein also, as determined by SDS-polyacrylamide gel electrophoresis and staining for protein and lipids. The numbers given are within an experimental error of ±0.7 nm.

CccA-CccB Hybrid Cytochrome c—To investigate the domain structure of CccB, a cccA-cccB frame gene fusion was constructed and cloned into pHP13 resulting in pLUJ105. The hybrid gene is transcribed from the native cccA promoter and is expected to encode a protein with the CccA membrane anchor domain (residues 1–33) fused to the predicted heme domain of CccB (residues 28–112). Membranes from strain LUH36 containing pLUJ105 and grown in the presence of [14C]ALA showed the absorbance maximum at 551 nm at room temperature after reduction with ascorbate. This confirmed that residues 28–112 of CccB (amino acid numbering according to Bacillus PS3) constitute the entire heme domain of cytochrome c551.

Properties of Cytochrome c551—Cytochrome c551 was overproduced to about 0.36 nmol/mg membrane protein in strain LUH20/pLUJ104. The cytochrome was extracted from these membranes using cholate and purified according to steps 1 and 2 of a method described by Noguchi et al. (29), except that we used 1% (w/v) Thesit instead of Triton X-100. A final isoelectric focusing step in the presence of 0.1% Thesit was performed to completely resolve cytochrome c551 from cytochrome c550 (Fig. 5, lanes 6 and 7). The results showed that YvjA is not required for the synthesis of the membrane-bound CccB cytochrome or any other cytochrome with covalently bound heme.

CccA-CccB hybrid protein, which in the polyacrylamide gel contained a radioactive polypeptide corresponding to the processed polypeptide. The heme domains are located on the outer side of the cytoplasmic membrane.

**Table II**

<table>
<thead>
<tr>
<th>Properties</th>
<th>B. subtilis</th>
<th>Bacillus PS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of amino acid residues</td>
<td>92</td>
<td>93</td>
</tr>
<tr>
<td>Isoelectric point (pI)</td>
<td>3.8</td>
<td>4.0</td>
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<tr>
<td>Extinction coefficient</td>
<td>32 mm⁻¹ cm⁻¹</td>
<td>20.9 mm⁻¹ cm⁻¹</td>
</tr>
<tr>
<td>(Reduced minus oxidized)</td>
<td>(A&lt;sub&gt;551&lt;/sub&gt; - A&lt;sub&gt;550&lt;/sub&gt;)</td>
<td>(A&lt;sub&gt;551&lt;/sub&gt; - A&lt;sub&gt;550&lt;/sub&gt;)</td>
</tr>
<tr>
<td>Midpoint redox potential</td>
<td>&gt;100 mV</td>
<td>225 mV</td>
</tr>
<tr>
<td>Absorption maxima at room temperature</td>
<td>416, 522, 551 nm</td>
<td>416, 522, 551 nm</td>
</tr>
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Fig. 6. Reduced minus oxidized difference absorbance spectra of membranes recorded at 77 K. asc. denotes ascorbate-reduced minus K₃Fe(CN)₆-oxidized spectra. dit. denotes dithionite-reduced minus K₃Fe(CN)₆-oxidized spectra. 4th der. denotes the fourth derivative of the difference spectrum of dithionite-reduced membranes. The protein concentration was 10 mg/ml, and the cuvette path length was 4 mm.

Fig. 7. Schematic drawing of cytochrome c<sub>550</sub> (CccA) and c<sub>551</sub> (CccB) in the B. subtilis cytoplasmic membrane. The homologous heme domains are indicated by a circle. The α-helical transmembrane polypeptide membrane anchor and the diacylglycerol membrane anchor, respectively, of the cytochromes are indicated. N and C indicate the N- and C-terminal ends of the polypeptides. The heme domains are located on the outer side of the cytoplasmic membrane.
B. subtilis Cytochrome c₅₅₁


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Bacillus subtilis Contains Two Smallc-Type Cytochromes with Homologous Heme Domains but Different Types of Membrane Anchors
Jenny Bengtsson, Carlo Rivolta, Lars Hederstedt and Dimitri Karamata

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