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Bacillus subtilis Contains Two Small *c*-Type Cytochromes with Homologous Heme Domains but Different Types of Membrane Anchors*

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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 c_{551} lipoprotein described for the first time in *B. subtilis*. Apparently, CccB corresponds to cytochrome c_{551} of the thermophilic bacterium *Bacillus* PS3. The heme domain of *B. subtilis* cytochrome c_{551} is very similar to that of cytochrome c_{550}, 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 c_{551}, and its function remains unknown.

The cytoplasmic membrane of the Gram-positive bacterium *Bacillus subtilis* contains cytochromes of *a*-, *b*-, *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 caa_3 complex (encoded by the *ctaC* gene) (2), cytochrome *c* of the cytochrome *bc* complex (encoded by the *qcrC* gene) (3), and the monomeric cytochrome c_{550} (encoded by the *cccA* gene) (4). Cytochrome caa_3 is a cytochrome *c* oxidase. The cytochrome *bc* complex oxidizes menaquinol and transfers electrons to cytochrome *c*.

Cytochrome c_{550} 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 c_{550} 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 c_{551}. As compared with the other *c*-type cytochromes in wild type cells, CccB is present in very low amounts, *i.e.* less than 10^3 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)[1], 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 *Pwo* 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 TTG CTG CCA GTG 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

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

TABLE I
Bacterial strains and plasmids used in this work

Strains and plasmids	Genotype or relevant properties[a]	Origin or Ref.
E. coli SURE	e14$^-$ (*mcrA*) Δ(*mcrCD*$^-$ *hsdSMR-mrr*)171 *endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC:Tn5* (kan^r) *uvrC* [F′ *proAB lacI*^{q}ZΔM15 Tn:*10*(tet^r)]	Stratagene
B. subtilis 168	*trpC2*	Laboratory stock
B. subtilis L16205	*trpC2* Δ*cccB::tet*	This work
B. subtilis L16224	*trpC2* Δ*(yvjA-cccB)::tet*	This work
B. subtilis L16225	*trpC2* Δ*(yvjA-cccB)::tet* Δ*cccA::cat*	This work
B. subtilis L16238	*trpC2, amyE*::pCR977 (*yvjA-lacZ*)	This work
B. subtilis LUH20	*trpC2* Δ*ctaCD::ble* Δ*cccA::cat*	This work
B. subtilis LUH36	*trpC2* Δ*ctaCD::ble* Δ*cccA::cat* Δ*cccB::tet*	This work
pBluescript SK(−)	Ap^r	Stratagene
pUC18, pUC19	Ap^r	8
pHP13	Em^r Cm^r	9
pMY2	*B. subtilis sdh* promoter on a 792-bp fragment in pHP13	10
pLUT191	part of *cccA* on a 600-bp *Eco*RI-*Kpn*I fragment in pUC19	11
p4303	pMTL20EC with *cccB* on a 1671-bp *Pst*I fragment	12
pCR977	transcriptional fusion of *B. subtilis yvjA-cccB* promoter and *E. coli lacZ* in pDH32	This work (Fig. 1)
pCRΔ*cccB*	Δ*cccB*::Tet^r in pUC18	This work (Fig. 1)
pCRΔ972	Δ(*yvjA-cccB*)::Tet^r in pUC18	This work (Fig. 1)
pLUJ104	*cccB* on a 1010-bp *BamHI-HindIII* fragment in pMY2	This work
pLUJ105	*cccA-cccB* hybrid gene on a 915-bp *EcoRI-BamHI* fragment in pHP13	This work

[a] Ap^r, ampicillin resistance; Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance; and Tet^r, tetracycline resistance.

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 $MnCl_2$, 3.2 mM dNTP mixture, and 4 units of *Tth* DNA polymerase (Roche Molecular Biochemicals). Under these conditions and in the presence of Mn^{2+}, the *Tth* DNA polymerase can perform reverse transcription and thus catalyze the synthesis of the cDNA strand complementary to the *cccB* mRNA. Subsequently, 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 Mg^{2+} 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 pCRΔcccB 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. pCRΔ972 was obtained from pCRΔcccB by substituting the distal part of *yvjA* with a PCR-obtained fragment homologous to the chromosomal region located upstream of *yvjA* (Fig. 1).

Plasmid pCR977 carries a transcriptional fusion of the *yvjA-cccB* promoter region with the *lacZ* gene from *E. coli* (Fig. 1). It was obtained by cloning the PCR-derived DNA fragment used for the pCRΔ972 construction into pDH32. The latter plasmid allows the ectopical integration of the gene fusion into the *B. subtilis amyE* locus (20).

Plasmid pLUJ104, used for overproduction of CccB, was constructed as follows. Plasmid p4303 was cleaved by *Eco*RI and *Hin*dIII, and the obtained 990-bp fragment containing the *cccB* gene was ligated into pBluescript SK(-). From this plasmid, multiplied in *E. coli* SURE, a 1010-bp *Bam*HI-*Hin*dIII fragment containing the *cccB* gene was ligated into pMY2 downstream of the *B. subtilis sdh* promoter.

Plasmid pLUJ105 was constructed as follows. Plasmid pLUT191, which is a pUC19 derivative and contains 600 bp of the *B. subtilis cccA* gene region corresponding to the promoter and the part of the gene encoding amino acid residues 1–33, was cleaved by *Kpn*I and *Bam*HI and treated with alkaline phosphatase. The part of the *cccB* region that encodes residues 28–112 of CccB and contains the proposed transcription termination loop (Fig. 1.) was amplified by PCR using two primers, 03III, 5′-CG GGT ACC AAG ACA GAC ACT GGC AGC AAG (the *Kpn*I site is *underlined*), and 03IV, 5′-CG GGA TCC ATA TTG TCA AGG CAT AAA AAC ATC (the *Bam*HI site is *underlined*). Plasmid p4303 was used as the template. The PCR was performed using *Pwo* DNA polymerase and buffer from Roche Molecular Biochemicals containing 4 mM $MgSO_4$. The PCR product was cleaved with *Kpn*I and *Bam*HI, and the 315-bp fragment was ligated into pLUT191. The resulting pLUJ105 has the *cccA-cccB* hybrid gene under the native *B. subtilis cccA* promoter.

Construction of B. subtilis cccB Deletion Strains—The *cccB* gene was deleted by gene replacement consisting of the integration of linearized pCRΔcccB into the *B. subtilis* 168 chromosome via a double crossover event resulting in strain L16205. The deletion of the *yvjA-cccB* segment was performed in a similar way by using linearized pCRΔ972.

Strain LUH20 was obtained by the transformation of strain 168 to phleomycin resistance with chromosomal DNA containing a Δ*ctaCD::ble* gene replacement (21) and then to chloramphenicol resistance with DNA containing a Δ*cccA::cat* gene replacement (5). LUH36 was obtained by the transformation of LUH20 to tetracycline resistance with L16205 (Δ*cccB::tet*) chromosomal DNA.

Differential Solubilization of Membrane-bound c-type Cytochromes using Cholate and Triton X-100—Membranes isolated from LUH36/pLUJ104 and LUH36/pLUJ105 were diluted to 1.5 mg protein/ml in solubilization buffer (30 mM Tris/SO_4, pH 8, 0.5 M Na_2SO_4, and 1 mM Na-EDTA, 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* LUH20/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_r 20,000) to a final concentration of 8% (w/v) and centrifuged at 32,000 × *g* for 20 min at room temperature. To the supernatant, polyethylene glycol was added to a final concentration of 30% (w/v), and $MgSO_4$ 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 with a 3.5-kDa cut-off. The sample was applied on a QMA MemSep® 1010 Ion Exchange Membrane Chromatography Cartridge (Millipore) connected to an 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 c_{551} from *Bacillus* PS3.

The cytochrome *c* was further purified using isoelectric focusing with the Rotorfor® 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-[4-^{14}C]aminolevulinic acid ([^{14}C]ALA) and SDS-polyacrylamide gel electrophoresis were performed as described in Ref. 23 except that the Schägger/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. 21. Protein concen-

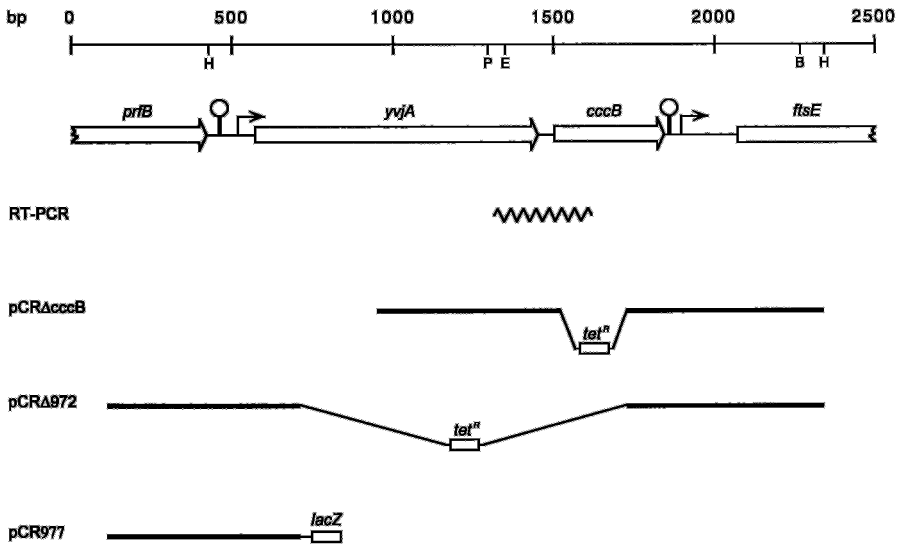

FIG. 1. **Map of the *yvjA-cccB* region in the *B. subtilis* chromosome.** Genes are indicated by *open arrows* and putative transcription terminators by *stem loop symbols*. *Short arrows* indicate positions of putative promoters. *Thick bars* (*lower part* of the figure) show fragments cloned into 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). *Bgl*II (*B*), *Eco*RI (*E*), *Hin*dIII (*H*), and *Pst*I (*P*) restriction sites are indicated.

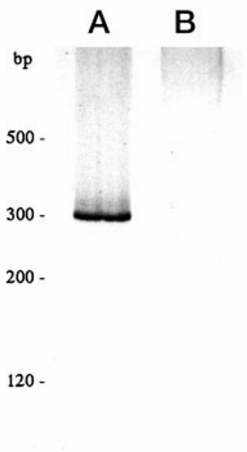

FIG. 2. **Reverse transcription PCR on the *yvjA-cccB* intergenic region.** *A,* reverse transcription PCR product obtained with primers CR108 and CR109 and total RNA isolated from *B. subtilis* 168 cells. *B,* negative control using a RNase-treated sample. See "Experimental Procedures" for details.

trations 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 *fstE* gene encodes a 25.5-kDa protein with sequence similarities to FstE 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 YpjC. 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

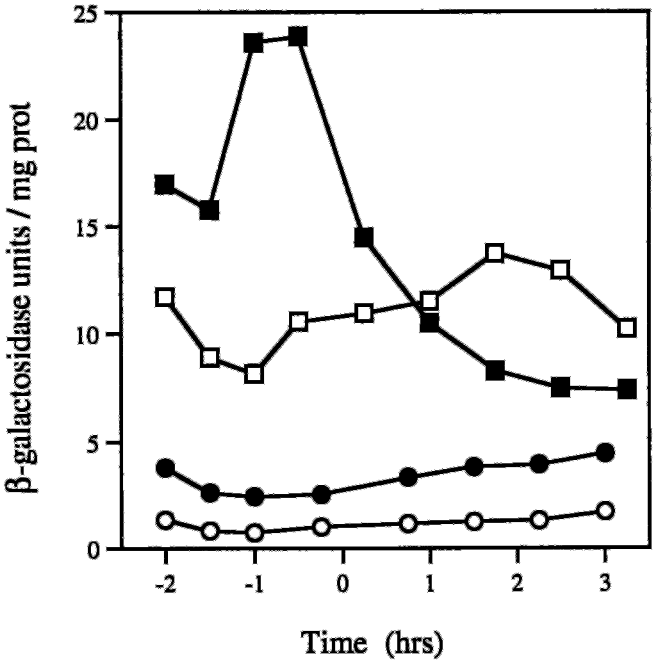

FIG. 3. **Effect of glucose on *yvjA-cccB* expression.** β-galactosidase activity values obtained with strain L16238 (*squares*) carrying the *yvjA-lacZ* fusion inserted into the *amyE* locus and those of the parent strain 168 corresponding to the background level of activity (*circles*). *Closed* and *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.

suggest that *yvjA* and *cccB* are co-transcribed as an approximately 1.55-kilobase mRNA. Northern blot analysis of total *B. subtilis* RNA, using *cccB* as the probe, has also shown a 1.6-kilobase transcript.[2] The presence of such a di-cistronic mRNA was confirmed by reverse transcription PCR on total RNA extracted from strain 168 (Fig. 2). The obtained cDNA product showed that *yvjA* and *cccB* mRNA is contiguous (Fig. 1).

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 was analyzed in cells growing at 37 °C in NSMP with or without 0.5% glucose. Activities were low and decreased in the presence of

[2] T. Schiött, personal communication.

FIG. 4. **Amino acid sequence comparison between *B. subtilis* CccA, *B. subtilis* CccB, and *Bacillus* PS3 CccA.** Identity in two of the polypeptides is indicated in *gray*. Residues invariant in all three polypeptides are in *black*.

0.5% glucose (Fig. 3). In L16238 cells grown in unsupplemented NSMP, the β-galactosidase activity reached a maximum at the end of the exponential growth phase. The results indicate that the *cccB* gene is expressed under exponential growth but at a low level.

YvjA and CccB Are Not Required for Growth—To analyze the role of YvjA and CccB, deletion mutants L16224 (Δ*yvjA-cccB*) and L16205 (Δ*cccB*) were constructed. No apparent growth defect was detected, *i.e.* the mutants grew as wild type on solid and liquid media including minimal medium. It can be noted that mutants deficient in cytochrome *c* synthesis also do not show any growth defect (23). The *cccB* deletion mutant showed normal sporulation, spore outgrowth, and sensitivity to lysozyme (data not shown).

CccB Compared with CccA of B. subtilis and CccA of Bacillus PS3—The amino acid sequence of the C-terminal part of CccB is very similar to that of CccA, the *B. subtilis* cytochrome c_{550} polypeptide (Fig. 4.). This part constitutes the heme domain of cytochrome c_{550} (6). The N-terminal parts of the two proteins are clearly different. In CccA, the first 32 residues are known to function as a noncleaved signal sequence for membrane insertion and peptide membrane anchor for the cytochrome domain (5). The N-terminal part of CccB also has the features of a signal peptide but contains the bacterial lipoprotein consensus sequence, Leu-Ala-Ala-Cys. This suggests that it is modified at the Cys residue by the addition of a diacylglycerol moiety and subsequently is cleaved by type II signal peptidase resulting in the modified Cys at the N-terminal end of the protein (28). CccB is therefore most likely a lipoprotein anchored to the membrane by fatty acid residues.

The thermophilic bacterium *Bacillus* PS3 contains a 10-kDa cytochrome *c,* which has been shown to be a lipoprotein containing two palmitic acid (C16:0) residues/molecule of cytochrome (29). This cytochrome shows an absorbance maximum at 551 nm and has therefore been named cytochrome c_{551}. The structural gene for this cytochrome in *Bacillus* PS3 is called *cccA* (30). Sequence similarities strongly suggest that *B. subtilis* CccB corresponds to CccA of *Bacillus* PS3 (Fig. 4). This conclusion is supported by the fact that *B. subtilis* YvjA and the protein encoded by the open reading frame located immediately upstream of *cccA* in the chromosome of *Bacillus* PS3 (30) show 70% sequence identity. It has been demonstrated that *Bacillus* PS3 cytochrome c_{551} can be synthesized from the cloned gene in both *Bacillus stearothermophilus* K1041 (29) and *B. subtilis* (31).

Cytochrome c Composition of cccB and yvjA-cccB Deletion Mutants—Membrane-bound cytochromes with covalently bound heme can be identified by a combination of *in vivo* radioactive labeling of heme using ALA, a precursor to heme, and SDS-polyacrylamide gel electrophoresis of isolated membranes followed by autoradiography, *cf.* Ref. 23. In wild type *B. subtilis* strains, four cytochromes are visualized by this method (Fig. 5, *lane 1*). These are the 39-kDa subunit II of cytochrome caa_3 (CtaC), the 28-kDa cytochrome *c* of the *bc* complex (QcrC), the 25-kDa cytochrome *b* subunit of the cytochrome *bc* complex (QcrB) (32), and the 13-kDa cytochrome c_{550} (CccA).

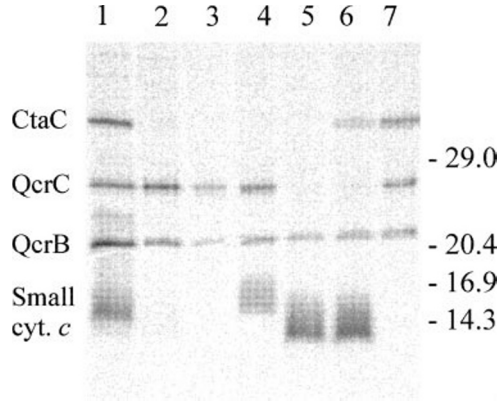

FIG. 5. **Autoradiogram showing membrane-bound proteins with tightly bound heme in different *B. subtilis* strains.** Isolated membranes from strains grown in the presence of [^{14}C]ALA were incubated at 40 °C for 30 min in buffer containing SDS and 2-mercaptoethanol and were analyzed on a 16.5% polyacrylamide gel. After electrophoresis, the gel was incubated in methanol/acetic acid, stained for protein, and dried. Approximately the same amount of protein was loaded in each lane as judged from the Coomassie Blue R250-stained gel. *Lane 1*, 168; *lane 2*, LUH20; *lane 3*, LUH36/pHP13; *lane 4*, LUH36/pLUJ105; *lane 5*, LUH36/pLUJ104; *lane 6*, L16225/pLUJ104; and *lane 7*, L16225/pHP13. The weak 28-kDa QcrC band seen in *lanes 5* and *6* is probably because of proteolytic activity as discussed before (3).

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 [^{14}C]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 *ctaC* 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 *ctaCD* has been deleted for the *cccB* gene (Fig. 5).

Overproduction of CccB—To facilitate the detection of ^{14}C-heme labeled CccB as well as the isolation of the protein for biochemical characterization we have constructed pLUJ104. This plasmid is a derivative of pHP13, an *E. coli/B. subtilis* shuttle vector with a copy number of about 5 in *B. subtilis* (9), containing the *cccB* gene cloned downstream of the *sdh* promoter. [^{14}C]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-

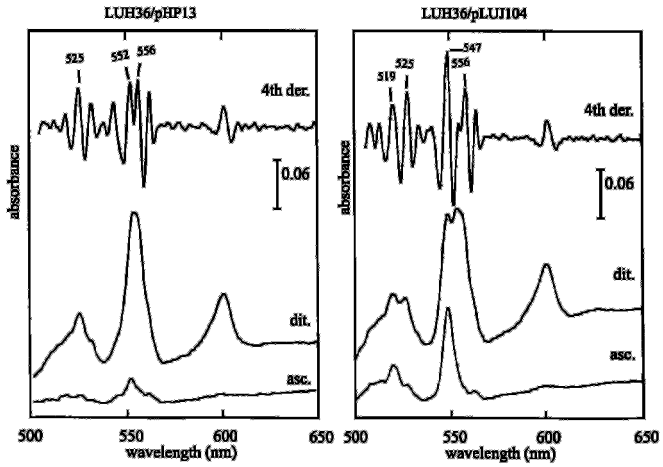

FIG. 6. **Reduced minus oxidized difference absorbance spectra of membranes recorded at 77 K.** *asc.* denotes ascorbate-reduced minus $K_3Fe(CN)_6$-oxidized spectra. *dit.* denotes dithionite-reduced minus $K_3Fe(CN)_6$-oxidized spectra. *4th der.* denotes the fourth derivative of the difference spectrum of dithionite-reduced membranes. The protein concentration was 10 mg/ml, and the cuvette path length was 4 mm.

tionally related proteins. To determine if the YvjA protein plays a role in the maturation of the CccB cytochrome a *yvjA-cccB*, *cccA* deletion strain L16225 was constructed. Membranes of L16225 containing pLUJ104 or the plasmid vector, pHP13, were analyzed for cytochrome *c* (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 Cytochrome c—To investigate the domain structure of CccB, a *cccA-cccB* in 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 [^{14}C]ALA contained a radioactive polypeptide corresponding to the CccA-CccB hybrid protein, which in the polyacrylamide gel migrated slightly slower than CccA (Fig. 5, *lane 4*). The results define the heme domain of CccB and demonstrate that the membrane anchor domain of CccA and CccB is functionally interchangeable.

CccB Is a Cytochrome c_{551}—Membranes from strain LUH36/pLUJ104 grown in NSMP were analyzed by light absorption spectroscopy. LUH36 lacks cytochrome c_{550} and cytochrome caa_3, which are the dominant high potential *B. subtilis* cytochromes absorbing in the 550-nm region of the spectrum (11) and is deleted for the *cccB* gene. Ascorbate-reduced minus ferricyanide-oxidized difference spectra at 77 K of membranes from LUH36/pLUJ104 showed an α-band absorption peak at 547 nm and a β-band peak at 519 nm (Fig. 6, *asc. spectrum*). These peaks are because of CccB, because they were not seen with membranes from LUH36/pHP13 (Fig. 6). Only cytochromes of high (>100 mV) midpoint redox potential are reduced by the ascorbate. Difference spectra of dithionite-reduced membranes, where all cytochromes are reduced, indicated that the CccB cytochrome is present in relatively large amounts in LUH36/pLUJ104 (Fig. 6, *dit. spectra*).

At room temperature, reduced CccB cytochrome showed absorption maxima at 551 ± 0.7 nm and 522 ± 0.7 nm (not shown). Because of its spectral properties and other similarities to the small cytochrome of *Bacillus* PS3, we name *B. subtilis* CccB cytochrome c_{551}. Membranes from strain LUH36 containing pLUJ105, which encodes the CccA-CccB hybrid protein, showed an 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 the unprocessed CccB) constitute the entire heme domain of cytochrome c_{551}.

TABLE II
Biochemical properties of cytochrome c_{551} of B. subtilis compared to that of Bacillus PS3

Properties	*B. subtilis*	*Bacillus* PS3
Number of amino acid residues[a]	92	93
Isoelectric point (pI)	3.8	4.0
Extinction coefficient (Reduced minus oxidized)	32 mM^{-1} cm^{-1} ($A_{551} - A_{535}$)	20.9 mM^{-1} cm^{-1} ($A_{551} - A_{535}$)
Midpoint redox potential	>100 mV	225 mV
Absorption maxima at room temperature		
Oxidized	409 nm	409 nm
Reduced	416, 522, 551 nm[b]	416, 522, 551 nm

[a] In the processed polypeptide.
[b] The numbers given are within an experimental error of ±0.7 nm.

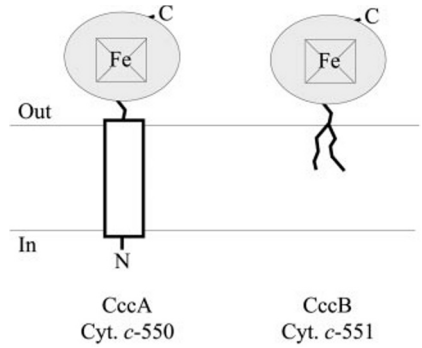

FIG. 7. **Schematic drawing of cytochrome c_{550} (CccA) and c_{551} (CccB) in the *B. subtilis* cytoplasmic membrane**. The homologous heme domains are indicated by a *circle*. The α-helical transmembrane polypeptide membrane anchor and the diacylglycerol membrane anchor, respectively, of the cytochromes 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.

Properties of Cytochrome c_{551}—Cytochrome c_{551} 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 obtain pure *B. subtilis* cytochrome *c* as determined by SDS-polyacrylamide gel electrophoresis and staining for protein and covalently bound heme. The cytochrome polypeptide gave rise to a diffuse band in the gels (not shown) like that observed with ^{14}C-heme labeled cytochrome (Fig. 5).

The properties of isolated *B. subtilis* cytochrome c_{551} are very similar to those of cytochrome c_{551} from *Bacillus* PS3 (Table II). The latter cytochrome has been demonstrated to be a lipoprotein (29). That *B. subtilis* cytochrome c_{551} is a lipoprotein also, as suggested from the amino acid sequence, was confirmed by the finding that it could be efficiently extracted from LUH36/pLUJ104 membranes using cholate. In contrast, the CccA-CccB fusion protein, which contains a peptide membrane anchor, was poorly extractable from membranes of LUH36/pLUJ105 by cholate but, as expected, was solubilized by Triton X-100.

A small cytochrome *c* has recently been isolated and characterized from the Gram-positive photosynthetic bacterium *He-*

liobacterium gestii. This 18-kDa cytochrome c_{553} is a lipoprotein similar to CccB, the function of which, like that of CccB, remains unknown. It contains palmitate and stearate in the lipid moiety at the N terminus (33). Cytochrome c_{551} of *Bacillus* PS3 has been shown to contain two palmitate residues. We have not been able to detect radioactivity in CccB polypeptide after growth of LUH20/pLUJ104 in the presence of [^{3}H]palmitate followed by SDS-polyacrylamide gel electrophoresis of isolated membranes and autoradiography. This negative result can be explained by the low amount of CccB in the membrane and/or by the fact that the cytochrome contains fatty acid residues with an acyl chain shorter than that of palmitate.

What Is the Specific Function of Cytochrome c_{551}?—The heme domains of CccA and CccB seem from the amino acid sequence to belong to a family of small *c*-type cytochromes found in *Bacillus* species (34 and this work). *B. subtilis* cytochrome c_{550} and c_{551} differ essentially only in the way they are anchored to the membrane (Fig. 7). The very similar amino acid sequence and redox properties of the heme domain of these two cytochromes indicate that they might serve the same, yet unknown, function in electron transfer in the membrane. If so, *B. subtilis* would be endowed with two different membrane-anchoring systems for a conserved cytochrome *c* domain, each of which may offer a distinct advantage under specific growth conditions. Mutants, *e.g.* LUH36, lacking both these cytochromes grow as well as the parental strain, suggesting that the growth conditions used in the laboratory do not require any of the two cytochromes. It is possible that, under certain natural environmental conditions, *B. subtilis* may preferentially resort to lipid mediated anchoring to the cytoplasmic membrane, *i.e.* use CccB rather than CccA. To the best of our knowledge, this is the first example of two homologous membrane proteins with different types of membrane anchors that coexist in one organism.

The close similarity between cytochrome c_{551} of *B. subtilis* and *Bacillus* PS3 suggests that they fulfill the same function in their respective bacterium. In *Bacillus* PS3, c_{551} is a major cytochrome, whereas a cytochrome *c* corresponding to *B. subtilis* cytochrome c_{550} has not been found. The function of cytochrome c_{551} in *Bacillus* PS3 has been investigated by Sone *et al.* (35). This cytochrome is mainly synthesized under air-limited conditions and is efficiently oxidized by a novel cytochrome *c* oxidase, cytochrome ba_3/bo_3 (36). The structural genes, *cbaAB*, of this oxidase have recently been sequenced (37). If cytochrome c_{551} specifically interacts with cytochrome ba_3/bo_3 to donate electrons, *B. subtilis* would contain such an oxidase also. However, genes corresponding to *cbaA* or *cbaB* were not found in the genome of *B. subtilis* 168. This leaves open the question whether cytochrome c_{551} is required for the reduction of cytochrome ba_3/bo_3 only or whether it may have other functions.

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