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# Identification and Characterization of the *ccdA* Gene, Required for Cytochrome *c* Synthesis in *Bacillus subtilis*

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**The gram-positive, endospore-forming bacterium *Bacillus subtilis* contains several membrane-bound *c*-type cytochromes. We have isolated a mutant pleiotropically deficient in cytochromes *c*. The responsible mutation resides in a gene which we have named *ccdA* (cytochrome *c* defective). This gene is located at 173° on the *B. subtilis* chromosome. The *ccdA* gene was found to be specifically required for synthesis of cytochromes of the *c* type. CcdA is a predicted 26-kDa integral membrane protein with no clear similarity to any known cytochrome *c* biogenesis protein but seems to be related to a part of *Escherichia coli* DipZ/DsbD. The *ccdA* gene is cotranscribed with two other genes. These genes encode a putative 13.5-kDa single-domain response regulator, similar to *B. subtilis* CheY and Spo0F, and a predicted 18-kDa hydrophobic protein with no similarity to any protein in databases, respectively. Inactivation of the three genes showed that only *ccdA* is required for cytochrome *c* synthesis. The results also demonstrated that cytochromes of the *c* type are not needed for growth of *B. subtilis*.**

Cytochromes function in electron transfer reactions, e.g., in respiratory systems, and contain heme as a prosthetic group. Vegetative cells of the gram-positive, endospore-forming bacterium *Bacillus subtilis* can synthesize cytochromes of the *a*, *b*, *c*, and *d* types (70). The amounts of individual cytochromes in the cell vary with the growth conditions, growth phase, and strain. Cells grown under aerobic conditions contain three identified membrane-bound cytochromes *c*. These are the 36-kDa CtaC protein, which is a subunit of the terminal respiratory enzyme cytochrome $caa_3$ (cytochrome *c* oxidase) (68); the 29-kDa QcrC protein, which is a subunit of the menaquinol: cytochrome *c* oxidoreductase (*bc* complex) (73, 74); and the 16-kDa CccA protein (cytochrome $c_{550}$) (69). It has been demonstrated for each of the three identified cytochromes *c* that they are not required for growth of *B. subtilis* (73).

Cytochromes of the *c* type differ from other cytochromes in that the heme is covalently attached, via thioether linkages, to two cysteine residues (in some cases, only one) in a conserved sequence motif in the protein: Cys-Xaa-Yaa-Cys-His (42). The imidazole side group of the His in the motif functions as a fifth, axial ligand to the iron atom in the heme molecule. The functional localization of cytochromes *c* in bacteria is on the outside of the cytoplasmic membrane, either as a soluble protein or firmly attached to the cytoplasmic membrane (cf. reference 13).

The process of cytochrome *c* biogenesis is not fully understood. It is apparently a multistep process requiring many protein factors (66). Both apocytochrome polypeptide and heme are synthesized in the cytoplasm. Experimental data from different gram-negative bacteria strongly suggest that cytochrome *c* maturation, i.e., covalent attachment of heme to a polypeptide, takes place in the periplasm (40, 57, 58, 66). Thus, heme, apocytochrome, and periplasmic macromolecules required for cytochrome *c* maturation must all be transported across the cytoplasmic membrane. Nearly a dozen different genes required for cytochrome *c* biogenesis in bacteria have been identified (5, 6, 10, 19, 33, 44, 46, 50, 51, 65). Suggested functions for the gene products are involvement in heme transport, folding or unfolding of apocytochrome (chaperones), thioether bond formation (heme lyases), and reduction of heme-iron and cysteine residues in the apocytochrome (oxidoreductases).

Molecular studies on cytochrome *c* biogenesis in bacteria have thus far been restricted to gram-negative species, *Bradyrhizobium japonicum*, *Escherichia coli*, and *Rhodobacter capsulatus*, in particular. In this paper, we report the identification of a gene required for cytochrome *c* synthesis in the gram-positive bacterium *B. subtilis*.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** 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 LB medium or on LA plates (59). *B. subtilis* strains were grown on tryptose blood agar base (TBAB) plates (Difco). Liquid cultures of *B. subtilis* were grown in nutrient sporulation medium supplemented with phosphate (NSMP) (15) or in Spizizen minimal medium (62) supplemented with required growth factors (20 mg/liter) and with glucose (0.5%, wt/vol), glutamate (0.5%, wt/vol), lactate (0.5%, wt/vol), or succinate (0.5%, wt/vol) as the carbon source. To investigate growth on different nitrogen sources, *B. subtilis* strains were streaked on minimal medium plates (supplemented with 10 mg [each] of tryptophan, adenine, and methionine per liter) in which the ammonium sulfate was replaced by sodium sulfate (15 mM), together with $NH_4Cl$ (37 mM), $NaNO_3$ (24 mM), glutamate (12 mM), or glutamine (14 mM). The following antibiotics were used when appropriate: ampicillin at 75 mg/liter; tetracycline at 12.5 mg/liter; chloramphenicol at 12.5 (*E. coli*) or 5 (*B. subtilis*) mg/liter; and phleomycin at 1.5 mg/liter (*B. subtilis*).

**Specific radioactive labeling of cytochromes *c*.** *B. subtilis* cells were grown overnight at 37°C in 25 ml of NSMP supplemented with 12 μM 5-[4-$^{14}$C]aminolevulinic acid (biosynthetic precursor to heme). The cells were harvested and washed in 1 ml of 50 mM potassium phosphate buffer, pH 8.0. Membranes were isolated by incubating the cells in 2 ml of buffer with lysozyme (100 mg/liter), DNase (6 mg/liter), RNase A (6 mg/liter), and phenylmethylsulfonyl fluoride (1 mM) at 37°C for 1 h. After the incubation, 50 μl of 0.3 M Na-EDTA, pH 7.4, and 5 μl of 200 mM phenylmethylsulfonyl fluoride (in ethanol) were added. Two minutes later, 30 μl of 1 M $MgSO_4$ was added and the membranes were collected by centrifugation at 150,000 × *g* for 1 h at 4°C and washed once in 20 mM sodium MOPS (3-[*N*-morpholino]propanesulfonic acid)-HCl buffer, pH 7.4. Finally, the membranes were homogenized in 75 μl of MOPS-HCl buffer and stored frozen at −80°C until used. The membranes were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (36) after incubation of the sample in 100 mM dithiothreitol and 70 mM SDS for 30 min at 40°C. Gels were fixed in methanol-acetic acid to remove noncovalently bound heme, stained with Coomassie brilliant blue R250, and finally treated with salicylic acid (9). The dried gel was exposed to Fuji RX film at −80°C for up to 1 week.

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Description[a] | Source[b] or reference |
|---|---|---|
| *Bacillus subtilis* | | |
| W168 | Prototroph | J. A. Hoch |
| 1A1 | *trpC2* | Bacillus Genetic Stock Center |
| 3G18 | *ade met trpC2* | G. Venema |
| KA11 | *hemA20 met trpC2* | 25 |
| LU1801 | *ade met trpC2*::pLTS101 $Cm^r$ | This work (pLTS101→3G18) |
| LU1802 | *ade met trpC2*::pLTS102 $Cm^r$ | This work (pLTS102→3G18) |
| LU1803 | *ade met trpC2*::pLTS103 $Cm^r$ | This work (pLTS103→3G18) |
| LU1804 | *ade met trpC2*::pLTS104 $Cm^r$ | This work (pLTS104→3G18) |
| LU1807 | *ade met trpC2*::pLTS107 $Cm^r$ | This work (pLTS107→3G18) |
| LU1808 | *ade met trpC2*::pLTS108 $Cm^r$ | This work (pLTS108→3G18) |
| LU6010 | *ccdA115 trpC2* | This work (LU6020→LU8025) |
| LU6018 | *ade met trpC2* Δ*ccdA*::*ble* $Pm^r$ | This work (pLTS32 [*Sca*I]→3G18) |
| LU6020 | *ccdA115 hemA20 trpC2* | This work (OX115→KA11) |
| LU6101 | *ccdA115 trpC2*::pLTS101 $Cm^r$ | This work (pLTS101→LU6010) |
| LU8025 | *citB25 trpC2* | 39 |
| OX115 | *ccdA115* | This work (mutant of W168) |
| *Escherichia coli* | | |
| JM83 | *ara* Δ(*lac-proAB*) *strA thi-1* (Φ80 *lacZ*Δ*M15*) | 71 |
| MM294 | *supE44 hsdR endA1 thi* | 45 |
| XL1-Blue | *supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac* F′ [*proAB*$^+$ *lacI*$^q$ *lacZ*Δ*M15* Tn*10* $Tet^r$] | 8 |
| Plasmids | | |
| pBLE-1 | $Ap^r$ $Pm^r$ | 17 |
| pBluescript II KS(−) | $Ap^r$ | Stratagene |
| pBluescript II SK(−) | $Ap^r$ | Stratagene |
| pCCD1 | $Ap^r$ | This work |
| pCCD2 | $Cm^r$ $Em^r$ | This work |
| pHP13 | $Cm^r$ $Em^r$ | 20 |
| pHV32 | $Ap^r$ $Cm^r$ $Tc^r$ | 37 |
| pLTS1 | $Cm^r$ $Em^r$ | This work (Fig. 2) |
| pLTS10 | $Cm^r$ $Em^r$ | This work (Fig. 2) |
| pLTS11 | $Cm^r$ $Em^r$ | This work (Fig. 2) |
| pLTS12 | $Cm^r$ $Em^r$ | This work (Fig. 2) |
| pLTS13 | $Ap^r$ | This work |
| pLTS17 | $Ap^r$ | This work |
| pLTS31 | $Ap^r$ $Pm^r$ | This work |
| pLTS32 | $Ap^r$ $Pm^r$ | This work |
| pLTS44 | $Ap^r$ | This work |
| pLTS101 | $Ap^r$ $Cm^r$ | This work (Fig. 2) |
| pLTS102 | $Cm^r$ $Tc^r$ | This work (Fig. 2) |
| pLTS103 | $Cm^r$ $Tc^r$ | This work (Fig. 2) |
| pLTS104 | $Ap^r$ $Cm^r$ | This work (Fig. 2) |
| pLTS105 | $Ap^r$ $Cm^r$ | This work |
| pLTS107 | $Ap^r$ $Cm^r$ | This work (Fig. 2) |
| pLTS108 | $Ap^r$ $Cm^r$ $Tc^r$ | This work (Fig. 2) |
| pMR22 | $Ap^r$ $Tc^r$ | 54 |
| pMR26 | $Ap^r$ $Tc^r$ | 54 |
| pMR27 | $Ap^r$ $Cm^r$ $Tc^r$ | 54 |

[a] $Ap^r$, $Cm^r$, $Em^r$, $Pm^r$, and $Tc^r$ indicate resistance to ampicillin, chloramphenicol, erythromycin, phleomycin, and tetracycline, respectively.
[b] An arrow indicates transformation of the indicated strain with chromosomal or plasmid DNA.

**Light absorption spectroscopy of membranes.** *B. subtilis* strains were grown in NSMP at 37°C in 2-liter batches in 5-liter baffled E flasks on a rotary shaker (200 rpm). The cultures were harvested approximately 1 h after the end of the exponential growth phase. Membranes were isolated as described before (23) and stored at −80°C in 20 mM sodium MOPS-HCl buffer, pH 7.4. Protein concentrations were determined by using the bicinchoninic acid protein assay (Pierce Chemical Co.) with bovine serum albumin as the standard. Difference (reduced-minus-oxidized) absorption spectra of membranes were obtained by using a Shimadzu UV-3000 spectrophotometer in the double-beam mode at room temperature with the 1.0-nm slit, a scan speed of 100 nm/min, and cuvettes with a 10-mm light path. Membranes in 20 mM sodium MOPS-HCl buffer, pH 7.4, containing 2 mM KCN were reduced in the sample cuvette with either 8 mM sodium ascorbate, pH 6.3, or a few grains of sodium dithionite and oxidized in the reference cuvette with 1 mM potassium ferricyanide.

**DNA and RNA techniques.** The general DNA techniques used were those described by Sambrook et al. (59). *B. subtilis* chromosomal DNA was isolated as described by Marmur (32) or by using CsCl gradient centrifugation. Plasmid DNA was isolated as described by Ish-Horowicz and Burke (27) or by using a Jet Prep Plasmid Miniprep kit (Genomed). *E. coli* was transformed by electroporation by using 50-μl cell suspensions in cuvettes with a 2-mm gap length and a Bio-Rad Gene Pulser with settings of 2.0 kV, 200 Ω, and 25 μF. *B. subtilis* was grown to competence essentially as described before (2). Southern blots were performed by using a digoxigenin-based DNA labeling and detection kit (Boehringer Mannheim).

For Northern blotting and primer extension analysis, *B. subtilis* strains were grown in NSMP, pH 7.0, at 37°C on a rotary shaker (200 rpm). Total RNA was extracted essentially as described by Resnekov et al. (48), and the RNA was quantitated from the $A_{260}$.

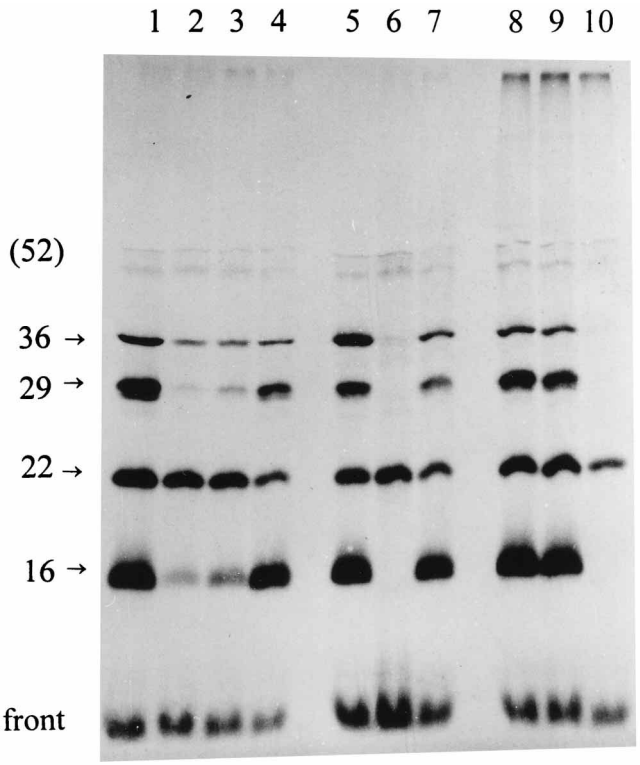



FIG. 1. Cytochrome *c* content in membranes from different *B. subtilis* strains analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography after heme-specific radioactive labeling. Lanes: 1, KA11 (wild-type control); 2, LU6020 (*ccdA115* mutant); 3, LU6020/pHP13; 4, LU6020/pLTS1; 5, 3G18 (wild-type control); 6, LU6018/pHP13 (Δ*ccdA* mutant); 7, LU6018/pLTS1; 8, LU1801(::pLTS101); 9, LU1802(::pLTS102); 10, LU1803(::pLTS103). About 40 μg of membrane protein (≈19 nCi of $^{14}C$) was loaded in each lane. The apparent masses in kilodaltons, of the different cytochrome polypeptides are indicated on the left. The small amount of radioactive polypeptide seen at 52 kDa is probably the 22-kDa polypeptide in a complex with another polypeptide(s). The radioactivity at the gel front is [$^{14}C$]heme.

For primer extension experiments, an oligonucleotide complementary to bp 1817 to 1800 in the *ccdA* gene was used (5′-ACA TCG TCC ATG CTG ACC-3′). The primer (0.2 pmol) 5′ end labeled with [γ-$^{32}P$]ATP (Amersham) was annealed to 36 μg of RNA and extended for 45 min at 37°C by using avian myeloblastosis virus reverse transcriptase (Pharmacia Biotech) in a total volume of 12 μl containing 37.5 mM Tris-HCl (pH 8.3), 45 mM NaCl, 5.6 mM dithiothreitol, 7.5 mM $MgCl_2$, and 0.5 mM deoxynucleoside triphosphate. The reaction was stopped by addition of 6 μl of a formamide-dye mixture (95% formamide, 20 mM Na-EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol), and the primer extension products were analyzed on a 6% polyacrylamide–7 M urea sequencing gel.

Northern blot analysis was performed in accordance with protocols from Amersham. RNA was electrophoretically separated in 1% (wt/vol) multipurpose agarose gels (Boehringer Mannheim) in buffer (20 mM sodium MOPS-HCl, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) containing 6% formaldehyde and transferred to Hybond N filters (Amersham) by the capillary blotting procedure. The DNA probes employed in hybridizations, bp 1379 to 2473 ("*ccdA*"; *Sac*I-*Cla*I fragment) and bp 3067 to 3399 ("*orf160*"; *Rsa*I-*Pst*I fragment), were labeled with [α-$^{32}P$]dCTP and the Rediprime DNA labeling system (Amersham). RNA size markers from Gibco Bethesda Research Laboratories were used as size references in the blots.

Nested deletions in pLTS13, which consists of a 2.2-kb *Sal*I-*Kpn*I fragment from pLTS1 (the *Sal*I site in the cassette of pHP13) cloned in pBluescript II SK(−), were obtained by using exonuclease III and mung bean nuclease as described before (22). DNA sequence analysis was done by the dideoxynucleotide chain termination method (60) with universal and specific primers, Sequenase version 2.0, and Redivue [α-$^{35}S$]dATP (Amersham). Computer-assisted analysis of the sequence data was achieved with the University of Wisconsin GCG package (version 8.1) (11). Sequence alignments were obtained by using the PILEUP and BESTFIT programs and edited by using the GeneDoc program (see Fig. 4). Searches of gene data banks were done by using the BLAST algorithm (1).

**Construction of plasmid pCCD2.** Plasmid pCCD2, exploited for mRNA analysis and containing the *ccdA-orf120-orf160* gene cluster (bp 1379 to 3486), was constructed as follows. Plasmid pLTS17, corresponding to pBluescript II SK(−) with the *Sac*I-*Eco*RI fragment of pLTS1 (the *Eco*RI site in the cassette of pHP13), was cleaved with *Sac*I and *Eco*RV. The resulting fragment (bp 1379 to 2692) was ligated into pLTS44, which is pBluescript II SK(−) carrying bp 2473 to 3486 as a *Cla*I fragment. The resulting plasmid (pCCD1) was cleaved with *Bss*HII, and the *ccdA-orf120-orf160*-containing fragment was isolated. The ends of the fragment were made blunt by treatment with Klenow enzyme and deoxynucleoside triphosphates, and the fragment was then cleaved with *Sal*I and ligated to pHP13 cleaved with *Sma*I and *Sal*I, resulting in pCCD2.

**Mutant isolation.** Spores of *B. subtilis* W168 were mutagenized by treatment with ethyl methanesulfonate as described by Ito and Spizizen (28) and plated on TBAB plates. Colonies that appeared within 2 days at 37°C were tested for *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) oxidation activity essentially as described before (18). Cells from colonies that stained negative, i.e., remained "white" for 10 min, were picked to a fresh TBAB plate which was incubated overnight at 37°C. A single colony was restreaked, and the TMPD oxidation-negative phenotype was confirmed. Isolated mutants were tested for growth on minimal plates containing 0.5% (wt/vol) glucose, 0.5% (wt/vol) lactate, or 0.5% (wt/vol) malate as the carbon source. Mutants that grew on glucose, but not on lactate or malate, were kept and named OX followed by consecutive numbers. The frequency of TMPD oxidation-negative colonies was 1.8%; 4.6% of these mutants did not grow on lactate.

**Construction of a fractionated library of *B. subtilis* chromosomal DNA in pHP13 and cloning of *ccdA*.** Chromosomal DNA isolated from *B. subtilis* 1A1 was partially cleaved with *Sau*3A1 and size fractionated by centrifugation in a 10 to 40% (wt/vol) sucrose gradient. Fragments in the range of 1 to 5 kbp were pooled, ligated to pHP13 (vector-to-fragment ratio of about 1:1 [wt/wt]), and then used to transform *E. coli* MM294 to chloramphenicol resistance. Plasmid pHP13 is a low-copy-number plasmid (five to six copies per cell) in *B. subtilis* and a high-copy-number plasmid in *E. coli* (20). About 90% of the transformants obtained contained plasmids with an insert. The transformants on each plate (about 500 colonies per plate) were pooled by suspension in LB, and plasmid DNA was isolated. The plasmid pools were used to transform strain LU6020 to chloramphenicol resistance. One TMPD oxidation-positive clone was found after screening of 15 plasmid pools corresponding to about 50,000 LU6020 transformants. Plasmid DNA was isolated from this *B. subtilis* clone and used to transform *E. coli* MM294 to chloramphenicol resistance. Plasmid pLTS1 was isolated from one such *E. coli* transformant and used to transform *B. subtilis* LU6020 to chloramphenicol resistance. All of the transformants were TMPD oxidation positive.

**Insertion mutagenesis and construction of *ccdA* deletion strain LU6018.** Insertion mutants were obtained by transforming *B. subtilis* 3G18 with plasmid pHV32 containing various fragments from the *ccdA-orf120-orf160* region (see Fig. 2). pHV32 encodes chloramphenicol resistance but cannot replicate autonomously in *B. subtilis* (37). Transformants were selected on rich medium (TBAB plates) containing chloramphenicol; i.e., cells were selected in which the plasmid had integrated into the chromosome by a single recombination event between the homologous sequences in the chromosome and the plasmid.

Plasmid pLTS32, used to delete the *ccdA* gene (see Fig. 2), was constructed by first cloning the 1.5-kb *Hin*dIII-*Pst*I fragment of pLTS1 (bp 325 to 1841) upstream of the *ble* gene in pBLE-1, resulting in pLTS31. Subsequently, the 0.5-kb *Kpn*I-*Eco*RI fragment of pLTS1 (bp 2224 to the cloning cassette in pHP13) was inserted downstream of the *ble* gene in pLTS31, resulting in plasmid pLTS32. Plasmid pLTS32 was linearized with *Sca*I (unique cleavage site in the *bla* gene) and used to transform 3G18 to phleomycin resistance on TBAB plates. The deletion was confirmed both by close linkage between the phleomycin resistance marker and *citB25* and by Southern blot analysis (blot not shown).

**Cloning and sequence analysis of the *ccdA115* mutation.** To clone the *ccdA115* mutation, strain LU6010 was transformed with integrative plasmid pLTS101. Chromosomal DNA was extracted from one chloramphenicol-resistant and TMPD oxidation-positive transformant, LU6101. This DNA was digested with *Sph*I, ligated, and used to transform *E. coli* JM83 to ampicillin resistance. Plasmid pLTS105 was isolated from one *E. coli* transformant. It contains the same fragment as carried by pLTS101 but with the *ccdA115* mutation and an additional 1.4-kb fragment (bp 77 to 1500). Plasmid pLTS105, in contrast to pLTS101, could not transform LU6020 (*ccdA115*) to a TMPD oxidation-positive phenotype but transformed wild-type strain 3G18 to a TMPD oxidation-negative phenotype (28% of the chloramphenicol-resistant transformants were oxidation negative). The DNA sequence of pLTS105 corresponding to bp 1500 to 1841 of the mutant *ccd* region was determined.

**Nucleotide sequence accession number.** The DNA sequence reported here has been submitted to the GenBank-EMBL database and given accession no. X87845.

## RESULTS

**Isolation of a cytochrome *c*-deficient mutant.** The ability of *B. subtilis* to efficiently oxidize the dye TMPD is dependent on the terminal respiratory enzyme cytochrome $caa_3$ (68). This

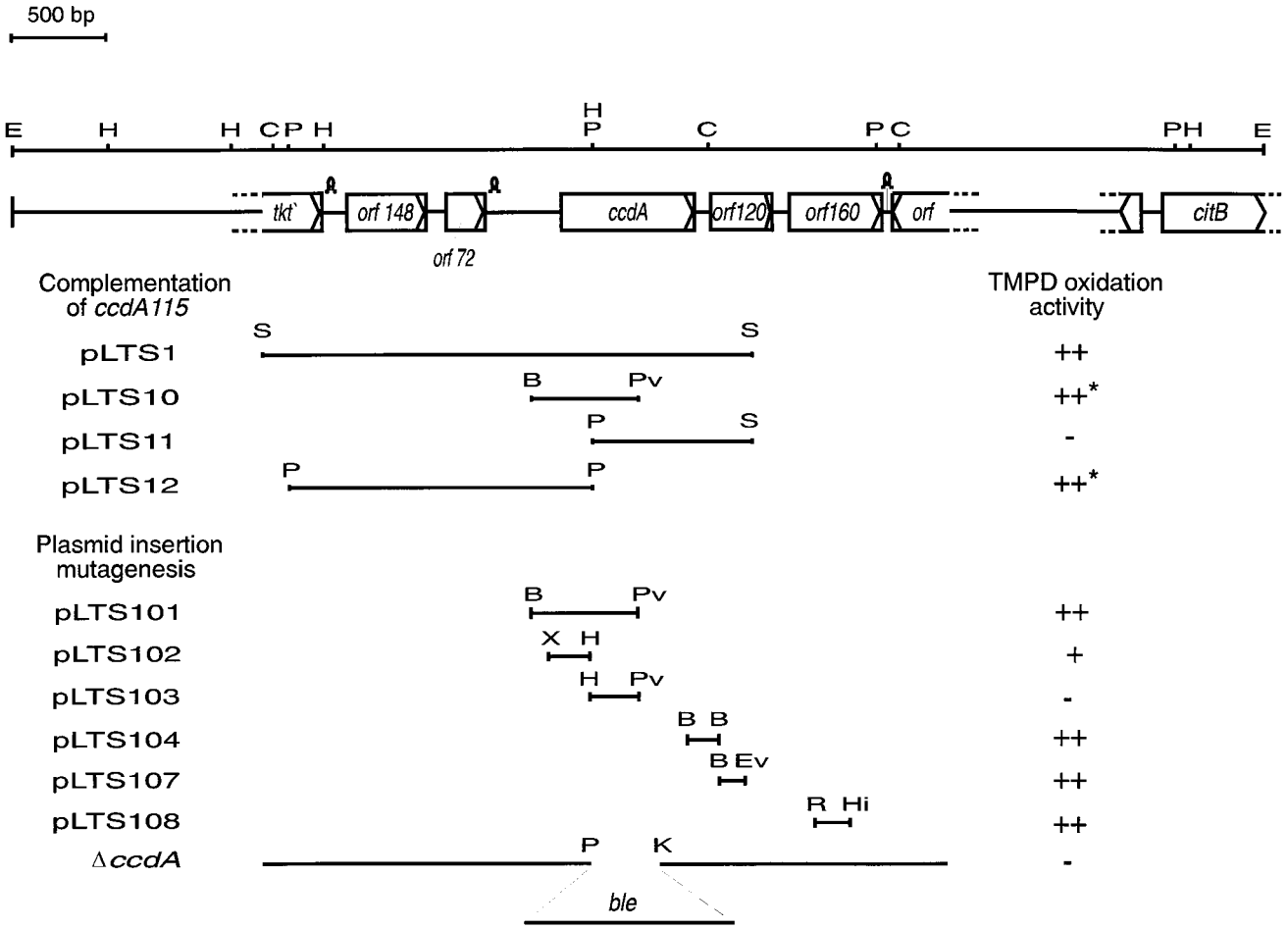



FIG. 2. Map of the *ccdA* region at about 173° on the *B. subtilis* chromosome. The map is derived from Southern blot analysis data and the DNA sequence reported here and that previously reported for *citB* by Dingman and Sonenshein (12). The lower part of the figure shows cloned DNA fragments present in the different plasmids used for complementation analysis and insertion mutagenesis. The strategy used for the deletion of *ccdA* and its replacement with the *ble* gene is also shown. Strain LU6020 was used for the complementation experiments, and strain 3G18 was used for mutagenesis. The TMPD oxidation activity of the transformants is indicated as follows: −, no activity; +, low activity; ++, wild-type activity. An asterisk indicates that a mixture of TMPD oxidation-positive and -negative transformants was obtained. Restriction enzyme cleavage sites: B, *Bgl*II; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Hi, *Hin*fI; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; R, *Rsa*I; S, *Sau*3AI; X, *Xmn*I.

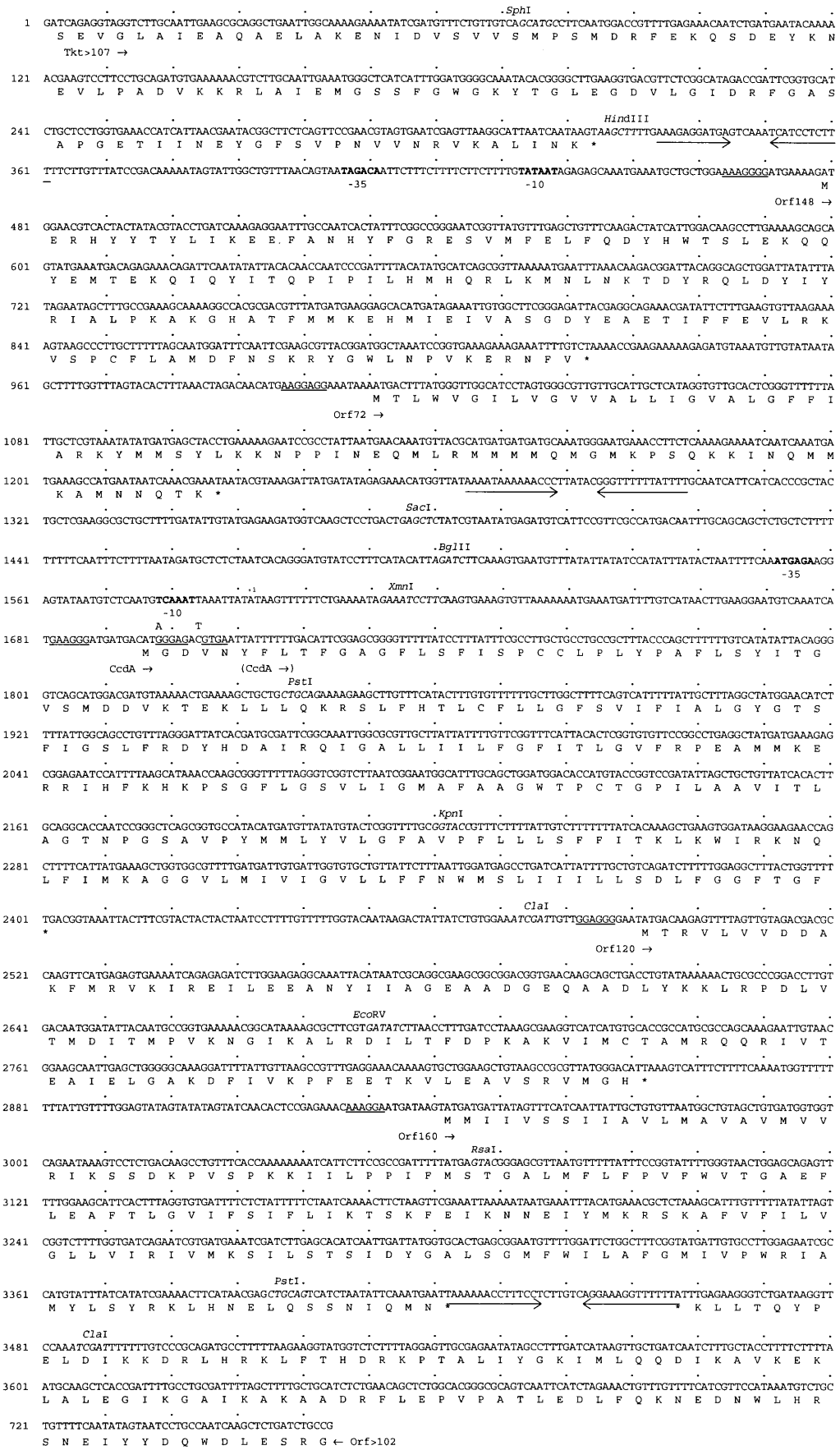
fact can be exploited to isolate mutants with a general block in cytochrome *c* synthesis. Such mutants cannot oxidize TMPD because the 36-kDa cytochrome *c*-containing subunit (CtaC) of cytochrome $caa_3$ is defective. A functional cytochrome $aa_3$, which is a quinol oxidase, is expected to be present in cytochrome *c* biogenesis mutants.

TMPD oxidation-negative mutants that grow on minimal medium with glucose, but not on lactate or malate, were isolated as described in Materials and Methods. The cytochrome composition of membranes from 14 mutants was analyzed by light absorption spectroscopy. One mutant, named OX115, contained cytochrome *a*, as judged from the absorption peak at about 600 nm seen with dithionite-reduced membranes, but had only low amounts of cytochrome *c* (little ascorbate-reducible cytochrome absorbing in the 550-nm region) compared to wild-type cells grown under the same conditions (spectra not shown). These properties of the mutant indicated a pleiotropic deficiency in cytochromes *c*.

*B. subtilis* KA11 was transformed to methionine prototrophy by using an excess of chromosomal DNA isolated from strain OX115. One transformant that stained TMPD oxidation negative was named LU6020. It grows on minimal medium with lactate as the carbon source, indicating that strain OX115 carries mutations at more than one locus. Analysis of the cytochrome *c* composition of strain LU6020 by specific radioactive labeling of heme (see Materials and Methods) showed a deficiency in cytochromes *c* (Fig. 1, lane 2). The mutant membranes contained reduced amounts of the 16-, 29-, and 36-kDa cytochromes *c*. A 22-kDa cytochrome with tightly bound heme remained at a normal level. The mutation causing this pleiotropic deficiency in cytochrome *c* was designated *ccdA115* (cytochrome *c* defective).

**Cloning and chromosomal localization of the wild-type *ccdA* locus.** A plasmid, pLTS1, complementing the TMPD oxidation-negative defect caused by the *ccdA115* mutation was isolated from a library containing *B. subtilis* wild-type (strain 1A1) DNA in pHP13 as described in Materials and Methods. The cytochrome *c* composition of LU6020/pLTS1, as analyzed by heme-specific radioactive labeling (Fig. 1, lane 4) and light absorption spectroscopy (spectra not shown), was found to be similar to that of the wild type. The relative amounts of the different cytochromes *c* in isolated LU6020/pLTS1 membranes were, however, not identical to those in membranes of parental strain KA11.

Southern blot hybridization analysis of *B. subtilis* 1A1 chromosomal DNA with the insert of pLTS1 as the probe con-


.SphI
Tkt>107 →
HindIII
-35
-10
Orf148 →
Orf72 →
SacI.
.BglII
-35
XmnI
-10
CcdA →
(CcdA →)
PstI
.KpnI
ClaI
Orf120 →
EcoRV
Orf160 →
RsaI.
PstI.
ClaI
Orf>102 ←

firmed that the cloned 2.7-kb fragment corresponds to only one chromosomal region (blots not shown). An ordered library of the *B. subtilis* chromosome in yeast artificial chromosomes (YACs) (3) was probed with the 1.7-kb *Pst*I fragment of pLTS12 (Fig. 2). Two YAC clones, 12-5 and 15-37, hybridized with the probe. Both of these clones contain *citB*, which encodes aconitase and is located at 173° on the *B. subtilis* genetic map (3). Transformation crosses with strain LU8025 (*citB25* mutant) and chromosomal DNAs from strains containing the *ccdA115* mutation confirmed that *citB* and *ccdA* are closely located.

Strain LU6020 was transformed to chloramphenicol resistance with plasmids pLTS10, pLTS11, and pLTS12. These plasmids are pHP13 containing different overlapping segments of the cloned 2.7-kb fragment (Fig. 2). The transformants obtained with pLTS10 and pLTS12 were a mixture of TMPD oxidation-negative and -positive colonies. This indicated that these plasmids can rescue the *ccdA115* mutation as a result of recombination rather than complementation in *trans*, which seemed to be the case for pLTS1. pLTS11 did not rescue the mutation. The results from the hybridization and transformation experiments together demonstrated that the cloned DNA in pLTS1 contains the complete functional wild-type *ccdA* gene and that the *ccdA115* mutation is located on a 0.34-kb *Bgl*II-*Pst*I fragment (Fig. 2).

**DNA sequence analysis.** The nucleotide sequence of the cloned fragment in pLTS1 was determined on both strands and found to be fully consistent with the restriction enzyme cleavage map of pLTS1 (Fig. 2 and 3).

Comparisons of the restriction map of a previously cloned *B. subtilis* 7-kb *Eco*RI fragment which carries the start of *citB* (12, 54) with the 2.7-kb fragment of pLTS1 and the information obtained from Southern blot analysis of chromosomal DNA indicated that the cloned DNA in pLTS1 corresponds to a region in the middle of the 7-kb fragment. By using DNA from the 7-kb fragment, i.e., plasmids pMR22, pMR26, and pMR27 (Table 1), we extended our nucleotide sequence by 1.0 kb in the direction of *citB* (Fig. 2 and 3).

At least five complete and two partial open reading frames (ORFs) can be found within the continuous 3,764-bp sequence (Fig. 3). The first ORF, most distal from *citB* and only partially sequenced, was named *tkt* because it most likely encodes transketolase. The C-terminal 107 residues deduced from *tkt* show 46 and 38% overall sequence identity to the C-terminal domains of *E. coli* transketolases TktA (major enzyme) (63) and TktB (26), respectively. The sequence identity to *Saccharomyces cerevisiae* transketolase (TKL1) (64), for which the three-dimensional structure is known (38), is 40%.

The second ORF, *orf148*, encodes a putative 18-kDa hydrophilic polypeptide without apparent sequence similarity to any protein in databases.

The third ORF, *orf72*, encodes a predicted 8.3-kDa methionine-rich (18% of the residues) polypeptide. The N-terminal end of the Orf72 polypeptide contains a sequence of 23 nonpolar residues. This sequence does not have the characteristics of a signal peptide for secretion (29) and might function to anchor the Orf72 polypeptide to the cytoplasmic membrane. Orf72 shows sequence similarity (32% identity) to a putative gene product of *Mycoplasma genitalium* (accession no. U02190) (41). This gene was not identified as an ORF in the published sequence of the *M. genitalium* genome (16); it ends 11 bp upstream of the putative gene MG335 and is transcribed in the same direction. The deduced protein encoded by MG335 has an unknown function but is similar to that of a *B. subtilis* gene, *orfX* (accession no. X76424) (49), located at 245° on the chromosome.

The fourth ORF was identified as *ccdA* since the *ccdA115* mutation mapped to this region (Fig. 2) and affects the expression of this gene (see below). There are two possible translation initiation codons for *ccdA* (Fig. 3), AUG at position 1696 and UUG at position 1717. Both of these codons are preceded, at a distance of 9 bp, by potential Shine-Dalgarno sequences with calculated (67) $\Delta G^\circ$ values of interaction with the 3′ end of *B. subtilis* 16S rRNA (35) of −9.6 and −18 kcal/mol, respectively. The deduced CcdA polypeptide consists of 235 or 228 residues, depending on the initiation codon used, and is most likely a polytopic integral membrane protein (Fig. 4). Genes with unknown functions encoding proteins similar to CcdA in sequence and size are present in *Haemophilus influenzae*, *Mycobacterium leprae*, *Synechocystis* sp., *Porphyra purpurea* chloroplast DNA, and *Mycobacterium tuberculosis* (Fig. 4) (14, 30, 43, 47, 52). We also found sequence similarity between CcdA and the central part of the much larger *E. coli* DipZ/DsbD protein (10, 34) and a DipZ homolog of *H. influenzae* (14) and *M. tuberculosis* (43) (Fig. 4).

The fifth and sixth ORFs in the sequence, *orf120* and *orf160*, are arranged in an operon-like fashion together with *ccdA* (Fig. 3). Orf120 is similar to *B. subtilis* single-domain response regulator proteins CheY (52% identity) and Spo0F (33% identity), which function in chemotaxis and sporulation, respectively (7, 72). Orf160 shows no sequence similarity to any protein in databases but may be a transmembrane protein since it contains three hydrophobic segments, each more than 18 residues long.

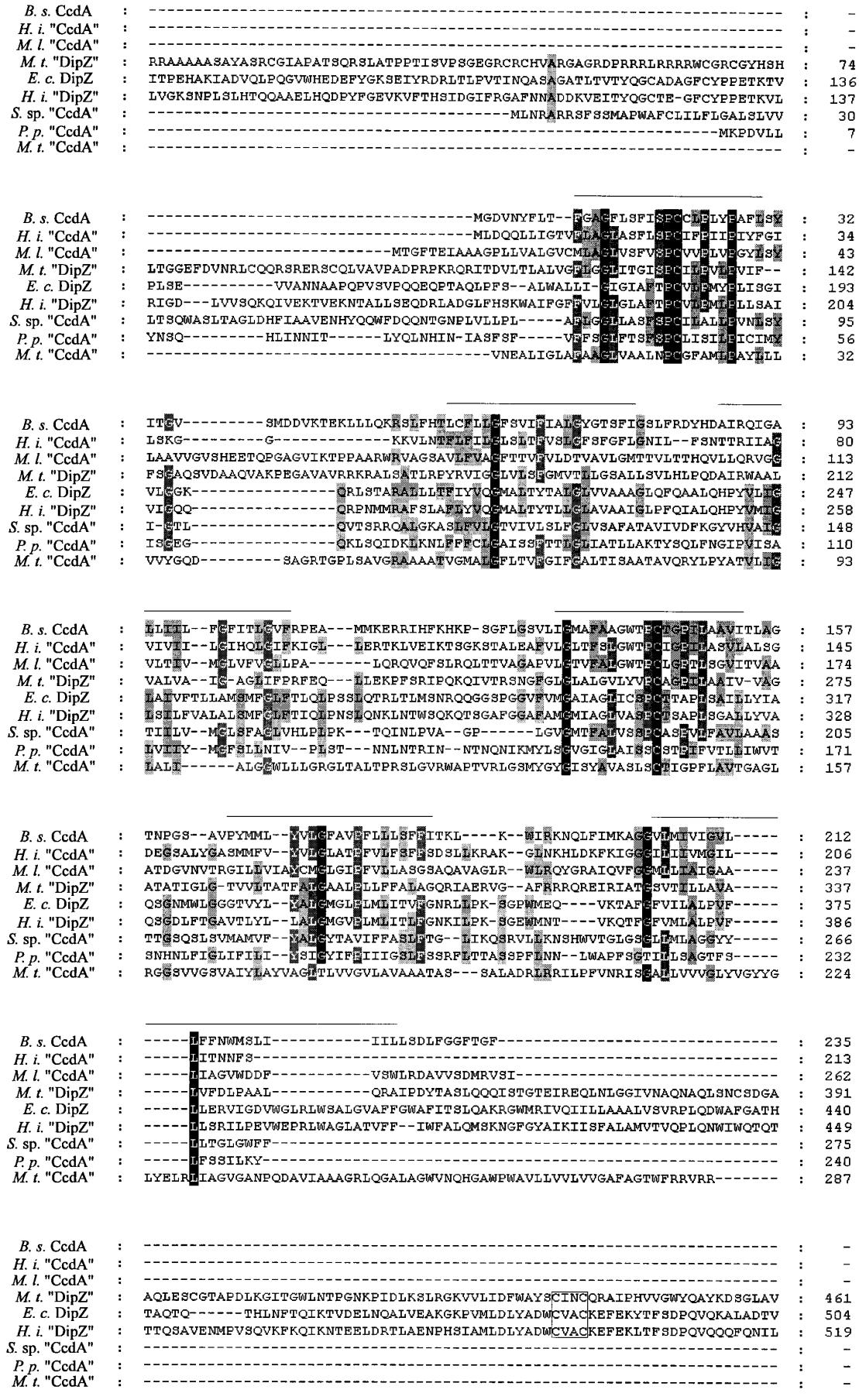
The *orf120* and *orf160* genes were found to be in the same reading frame and separated by 87 bp with no stop codon. Since two-component regulatory system response regulator proteins usually consist of a regulatory (N-terminal) domain and a catalytic (C-terminal) domain, it was important to rule out sequencing errors. Nucleotide sequence analysis of the *orf120-orf160* intergenic region in three independent original clones (pLTS1, pMR22, and a fragment cloned from strain 3G18 by insertion-excision of plasmid DNA) confirmed the TAA stop codon of *orf120* indicated in Fig. 3.

Only the terminal end of the ORF situated downstream of *orf160* was sequenced. Its orientation is opposite to that of all other ORFs in the sequence. The 102 C-terminal residues deduced from the partial sequence showed no similarity to any polypeptide in databases.

The DNA sequence was analyzed for perfect inverted repeats with $\Delta G^\circ$ values of $< -17$ kcal/mol that could function as Rho-independent transcription terminator signals. Such sequences were found downstream of *tkt*, *orf72*, and *orf160*, as indicated in Fig. 2 and 3.

After this work was completed and the sequence data had been submitted to the EMBL-GenBank database, a 26-kb se-

FIG. 3. Nucleotide sequence of the *ccdA* region in the *B. subtilis* chromosome. The noncoding strand is shown. Restriction enzyme cleavage sites mentioned in the text or referred to by position are indicated. The deduced sequence of the translation product of five complete and two partial ORFs and their putative Shine-Dalgarno sequences (underlined) are shown. Asterisks indicate translational stop codons. The two nucleotide substitutions (G-1698→A and C-1704→T) corresponding to the *ccdA115* mutation are indicated above the wild-type sequence. The base pair corresponding to the identified 5′ end of *ccdA* mRNA is designated +1. Possible −35 and −10 promoter sequences in front of *ccdA* and an almost perfect sigma-A consensus promoter sequence for *orf148* are in boldface letters. Three perfect inverted repeats (calculated $\Delta G^\circ$, $< -17$ kcal/mol) are indicated by arrows.


B. s. CcdA
H. i. "CcdA"
M. l. "CcdA"
M. t. "DipZ"
E. c. DipZ
H. i. "DipZ"
S. sp. "CcdA"
P. p. "CcdA"
M. t. "CcdA"

quence with accession no. Z73234 was deposited by the *B. subtilis* genome sequencing project (53). This sequence covers the 170° region and overlaps the sequence presented in this paper. Under accession no. Z73234, *orf120* and *orf160* are named *yneI* and *yneJ*, respectively, and it is reported that the region between *orf160* and *citB* contains two ORFs. This region, however, seemingly contains four ORFs, i.e., the ORF we have partially sequenced and the *cotKLM* gene cluster (accession no. U72073) (24).

**mRNA analysis.** Primer extension analysis of total mRNA extracted from *B. subtilis* 3G18 in the exponential growth phase ($T_{-1}$), at the end of exponential growth ($T_0$), and 1 h into the stationary phase ($T_1$) showed that most of the *ccdA* mRNA at the 5′ end contains the nucleotide corresponding to bp 1592, indicating that transcription initiates at this position (Fig. 5). Apparent −35 and −10 sigma-A promoter sequences (21) are present upstream of this putative initiation site (Fig. 3).

The same RNA preparations as used for primer extension and total RNAs from strains LU6018 and 3G18/pCCD2 (pCCD2 is pHP13 with the *ccdA-orf120-orf160* region) were analyzed by Northern blotting with probes for *ccdA* and *orf160*. A 1.8-kb *ccdA-orf120-orf160* transcript was detected with both probes in 3G18 and in larger amounts in 3G18/pCCD2 (Fig. 6). By using the "*orf160*" probe, we also detected 0.9-kb *orf120-orf160* and 0.5-kb *orf160* transcripts (Fig. 6). The *ccdA* deletion strain LU6018 (see below), as expected, lacked the 1.8-kb transcript. This finding is consistent with the fact that in LU6018 an internal part of the *ccdA* gene is deleted and replaced by the *ble* gene. Instead, it contained an about 0.6-kb mRNA that hybridized with the "*ccdA*" probe. The 0.6-kb mRNA showed the same induction pattern as the *ccdA-orf120-orf160* transcript. Strain 3G18/pCCD2 also contained a 1.1-kb mRNA that hybridized to the "*ccdA*" probe. This mRNA may be a *ccdA-orf120* transcript or a transcript initiated from a promoter within the vector DNA.

The concentration of *ccdA-orf120-orf160* mRNA varied, depending on the growth stage; it was most abundant in samples from bacteria harvested at the end of the exponential growth phase, i.e., at about $T_0$ (Fig. 6). This suggests that the activity of the *ccdA* promoter or the stability of the *ccdA-orf120-orf160* mRNA is modulated in the cell. It is notable that the genes for several cytochromes *c* in *B. subtilis* are induced at the end of the exponential growth phase (61, 73). The concentration of the *orf120-orf160* and *orf160* transcripts seemed less growth stage dependent, suggesting that *orf120* and *orf160* are constitutively expressed. The cellular concentration of *ccdA-orf120-orf160* mRNA in 3G18/pCCD2 was not in proportion to the copy number (five to six) of the plasmid (20) and could reflect loss of control of the *ccdA* promoter activity in this strain.

**Functional analysis of *ccdA-orf120-orf160*.** The presence of a *ccdA-orf120-orf160* transcript in wild-type cells indicates that the functions of the products of these three genes are related. Plasmid insertion mutagenesis was performed to determine if all three genes are required for cytochrome *c* synthesis (see Materials and Methods). Disruption of *ccdA*, i.e., integration of pLTS103, resulted in a TMPD oxidation-negative phenotype (Fig. 2). Inactivation of *orf120* (pLTS107) or *orf160* (pLTS108) or integration of plasmid DNA between *ccdA* and *orf120* (pLTS104) or integration of pLTS101 (which contains a relatively long fragment spanning the start of *ccdA*) did not affect the TMPD oxidation activity. Integration of pLTS102, which contains a short fragment spanning the start of *ccdA*, resulted in colonies with low TMPD oxidation activity, indicating that the inserted plasmid affected expression of *ccdA* (Fig. 3).

Light absorption spectra of isolated membranes from strains LU1801, LU1802, LU1803, LU1804, and LU1807, i.e., the strains with different pHV32-derived plasmids integrated into the chromosome, showed that the TMPD oxidation activity of colonies was correlated with cytochrome *c* content (spectra not shown). Radioactive heme-specific labeling demonstrated that disruption of *ccdA* (strain LU1803) causes total loss of all identified cytochromes *c* but does not affect the 22-kDa cytochrome (Fig. 1, lane 10).

A large internal part of *ccdA* (corresponding to amino acid residues 49 to 178) was deleted from the *B. subtilis* chromosome and replaced by the *ble* gene (see Materials and Methods) (Fig. 2). Light absorption spectra of membranes from deletion strain LU6018 showed a deficiency in cytochrome *c*, as judged from the low absorption at 550 nm in ascorbate-reduced minus ferricyanide-oxidized difference spectra (Fig. 7). Radioactive heme-specific labeling further showed that the deletion mutant lacks all cytochromes with tightly bound heme except for that of 22 kDa (Fig. 1, lane 6). Plasmid pLTS1 complemented the cytochrome *c* deficiency in LU6018; i.e., LU6018/pLTS1 colonies showed TMPD oxidation activity, and the cytochrome *c* composition and the cytochrome spectra were close to normal (Fig. 1, lane 7, and Fig. 7).

No apparent differences in growth properties were found between *ccdA* deletion mutant strain LU6018 and parental strain 3G18 in NSMP liquid medium, pH 7.0; minimal liquid medium supplemented with different carbon sources (glucose, succinate, and lactate); and modified minimal agar plates containing different nitrogen sources ($NH_4^+$, $NO_3^-$, glutamate, and glutamine). The deletion strain also grows similarly to the wild type on TBAB plates supplemented with nitrate under anaerobic conditions.

**The *ccdA115* mutation affects translation.** Plasmid pLTS102 (Fig. 2), which was used for insertion mutagenesis, rescued the *ccdA115* mutation in LU6010. From this observation and the transformation data summarized in Fig. 2, combined with the nucleotide sequence information, it was concluded that the *ccdA115* mutation is located between bp 1616 and 1841 (Fig. 3). The mutation was cloned and sequenced as described in Materials and Methods. Two mutations were detected, a G-to-A transition at bp 1698 and a C-to-T transition at bp 1704 (Fig. 3). The G-to-A mutation alters one of the two possible translation initiation codons (AUG) for *ccdA* and affects the tentative Shine-Dalgarno sequence for the second possible initiation codon (UUG). The C-to-T mutation probably gives no phenotype, since it does not change translation initiation signals or the amino acid sequence of CcdA.

FIG. 4. Sequence comparison between CcdA and DipZ homologs from different organisms. The *B. subtilis* (*B. s.*) CcdA polypeptide (assuming the AUG at bp 1696 to be the initiation codon) was aligned with "CcdA" proteins from *H. influenzae* (*H. i.*) (HI1454; accession no. U32823), *M. leprae* (*M. l.*) (accession no. U00018), *Synechocystis* sp. strain PCG803 (*S.* sp.) (accession no. D64001), *P. purpurea* (*P. p.*) (Orf240; accession no. U38804), and *M. tuberculosis* (*M. t.*) (MTCY274.08c; accession no. Z74024), and with the central part of *E. coli* (*E. c.*) DipZ/DsbD (accession no. X77707) and "DipZ" proteins from *M. tuberculosis* (MTCY274.05; accession no. Z74024 [referred to as a gene product with similarity to CcdA]) and *H. influenzae* (HI0885; accession no. U32770). Numbers to the right of the sequence indicate amino acid positions relative to the start of each polypeptide sequence. Note that the residues of *E. coli* DipZ are numbered on the basis of the notion that the ATG codon at bp 494 (10) is the translation start codon. The thioredoxin motif (Cys-Xaa-Yaa-Cys) in the C-terminal part of the DipZ proteins is boxed. Possible transmembrane segments in the *B. subtilis* protein, as suggested by the SWISS-PROT database (4), are indicated by horizontal bars.

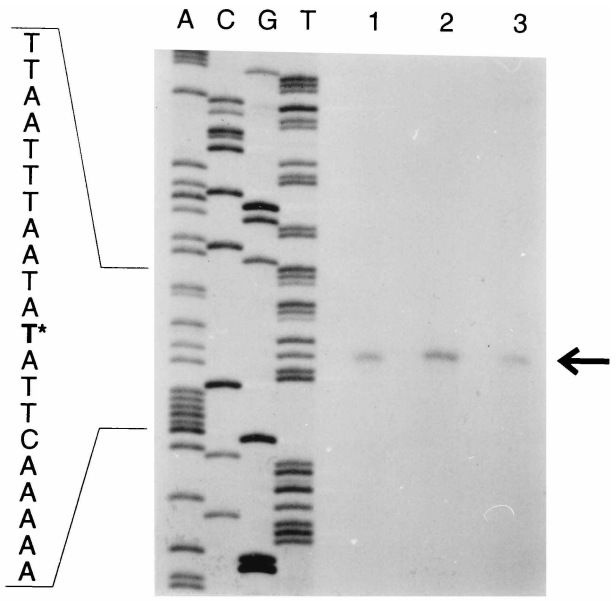



FIG. 5. Determination of the 5′ end of *ccdA* mRNA by primer extension analysis. A primer complementary to a sequence in the beginning of *ccdA* (see Materials and Methods) was end labeled with $^{32}P$, annealed to total *B. subtilis* RNA, and extended by using reverse transcriptase. Shown is an autoradiograph after separation of reaction products on an acrylamide sequencing gel. The RNA used was extracted from strain 3G18 cultured in NSMP, and samples were taken at different growth stages, i.e., at about $T_{-1}$ (lane 3), $T_0$ (lane 2), and $T_1$ (lane 1). Lanes A, C, G, and T contain dideoxynucleotide sequence ladders obtained by using the same primer and pLTS1 as the template. The asterisk in the sequence indicates the nucleotide corresponding to the 3′ end of the extension product (arrow), i.e., nucleotide 1592 (Fig. 3).

## DISCUSSION

In this paper, we have identified and characterized a gene, *ccdA*, which is required for the synthesis of *c*-type cytochromes in *B. subtilis*. In another paper (61), we will show that the CcdA protein is required for a step in cytochrome *c* synthesis occurring after heme and the apocytochrome polypeptide have been transported across the cytoplasmic membrane. Thus, CcdA is most probably required for a function on the outside of the cytoplasmic membrane.

The *ccdA* gene was identified by the isolation of a TMPD oxidation-negative mutant (carrying the *ccdA115* mutation) containing reduced amounts of holocytochrome *c*. *B. subtilis* contains four major membrane-bound polypeptides with tightly bound heme, three of which are known to be cytochromes of the *c* type. A *ccdA* insertion or deletion mutant lacks these three cytochromes *c*. The remaining 22-kDa cytochrome with tightly bound heme probably corresponds to QcrB, a *b*-type cytochrome of the menaquinol:cytochrome *c* oxidoreductase complex (*bc* complex) (61, 73, 74). We conclude that the CcdA protein is specifically required for the synthesis of cytochromes of the *c* type. The normal growth properties of a *ccdA* deletion mutant show that *c*-type cytochromes are not required in vegetative *B. subtilis* cells.

Strains carrying the *ccdA115* mutation are leaky; i.e., they contain the three cytochromes *c* but in reduced amounts. The explanation for this phenotype was provided by cloning and sequence analysis of the *ccdA115* mutation. The mutation alters one of the two possible translation initiation codons and affects a putative ribosome-binding site for *ccdA*. The effect of the *ccdA115* mutation is reduced, but not completely blocked, translation of *ccdA* mRNA.

Northern blot analysis revealed that the *ccdA* gene is transcribed together with two downstream genes: *orf120* and *orf160*. Insertion mutagenesis demonstrated that these two genes are not required for cytochromes *c* synthesis. Analysis of the 5′ end of *ccdA* mRNA and inspection of the DNA sequence strongly suggest that *ccdA* is transcribed mainly from a sigma-A-type promoter, giving an untranslated leader sequence of about 100 nucleotides. A mutant, LU1802, with plasmid pHV32 integrated into the leader sequence was found to express *ccdA* at a low level compared with the wild type. This finding confirms the assigned promoter region and indi-

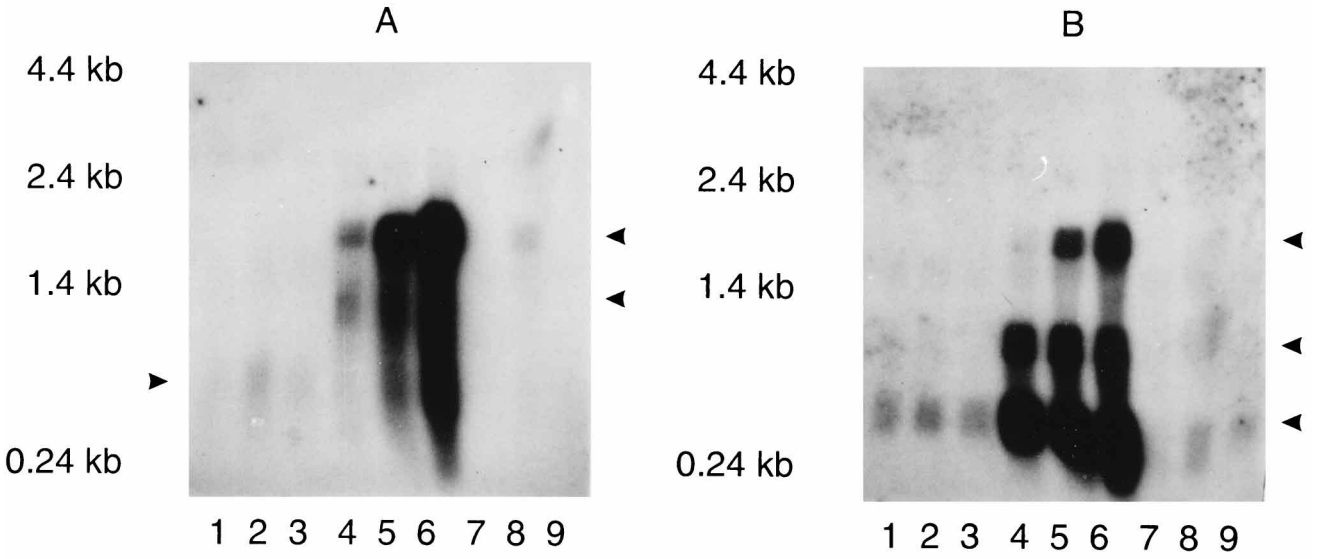



FIG. 6. Northern blot analysis of *B. subtilis* RNA using a "*ccdA*" probe (A) and an "*orf160*" probe (B) labeled with $^{32}P$. Autoradiographs are shown. Total RNA was extracted from strains LU6018 (lanes 1 to 3), 3G18/pCCD2 (lanes 4 to 6), and 3G18 (lanes 7 to 9) grown in NSMP. Samples were taken at the growth stages $T_{-1}$ (lanes 1, 4, and 7), $T_0$ (lanes 2, 5, and 8), and $T_{+1}$ (lanes 3, 6, and 9). About 15 μg of total RNA was loaded in each lane of a denaturing agarose gel and transferred to a filter after electrophoretic separation. The positions of RNA size standards are indicated on the left of each blot. Hybridizing mRNA fragments mentioned in the text are indicated by arrowheads. The largest mRNA detected with the "*ccdA*" probe is about 1.8 kb. The "*orf160*" probe hybridized with RNAs of about 1.8, 0.9, and 0.5 kb.

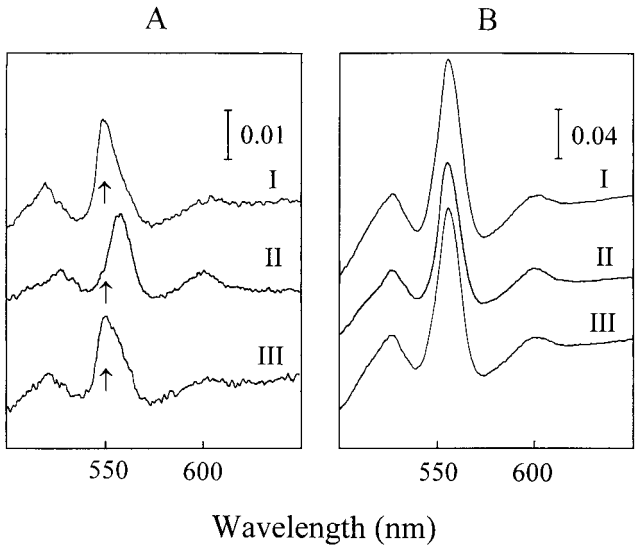



FIG. 7. Reduced-minus-oxidized difference spectra of membranes from different *B. subtilis* strains. Membranes were reduced in the sample cuvette with ascorbate (A) or dithionite (B) and oxidized in the reference cuvette with potassium ferricyanide. I, 3G18 (wild type); II, LU6018/pHP13 (Δ*ccdA*); III, LU6018/pLTS1. Cytochrome *c* absorption is apparent mainly at 550 nm (arrows) and can be seen more clearly in the ascorbate-reduced samples, where it is not obscured by overlapping cytochrome *b* absorption. The absorbance scales are indicated by the bars. The protein concentration was 3 mg/ml.

cates weak promoter activity from integrated pHV32 or from the region between the integration site and the translation initiation sequence.

CcdA is a 26-kDa integral membrane protein as deduced from the DNA sequence. Genes encoding proteins similar to CcdA in size and sequence are found in a variety of organisms (Fig. 4). The "*ccdA*" gene in these organisms has been found in the course of sequencing projects, and functional studies of the gene product have not been reported. The "CcdA" proteins show weak but significant similarity to the central region of the much larger "DipZ" proteins. The similarity is both in sequence and in the pattern of stretches of hydrophobic residues, which could correspond to transmembrane α-helical segments. Interestingly, *E. coli* DipZ/DsbD is required for the synthesis of *c*-type cytochromes in *E. coli* (10, 55). This protein has a redox function in the periplasm (56). The C-terminal region of DipZ-DsbD contains a motif, Cys-Xaa-Yaa-Cys, which is found in thioredoxins and protein disulfide isomerases (31). This motif and region is not present in CcdA. This fact and the presence of both a "DipZ" homolog and a "CcdA" homolog in *H. influenzae* suggest that CcdA is a novel cytochrome *c* biogenesis protein. The sequence similarity between "CcdA" proteins and the central part of "DipZ" proteins, however, indicates related functions or origins of these protein domains. Two invariant cysteine residues in the "CcdA" proteins are present also in the "DipZ" proteins (Fig. 4). These two cysteines are not in a thioredoxin type of sequence motif but might still be functionally redox active or serve as ligands to a prosthetic group. It is notable that a gene encoding a small thioredoxin-like protein is located adjacent to the "*ccdA*" gene in the bacteria listed in Fig. 4 (except *B. subtilis*). "CcdA" and the thioredoxin-like protein in those bacteria could, together, perform a function similar to that of DipZ/DsbD in *E. coli.*

The possibility that CcdA is only indirectly involved in cytochrome *c* synthesis cannot be excluded. For example, it might be required for the synthesis or activity of a factor which is directly involved in cytochrome *c* synthesis. However, we have not found any phenotype of *B. subtilis ccdA* mutants other than cytochrome *c* deficiency. The *orf120* and *orf160* genes were found to be cotranscribed with *ccdA* but also to be expressed separately from *ccdA*. Orf120 is, as judged from the strong similarity to the Spo0F and CheY proteins, most likely a single-domain response regulator. Like Spo0F and CheY, Orf120 probably interacts with other proteins rather than with DNA. Response regulator genes often lie adjacent to the gene for the cognate histidine kinase. The Orf160 protein does not, however, have the features of a typical kinase. It remains to be determined whether there is any functional relationship between Orf120-Orf160 and CcdA.

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