

Cytochrome *bd* Biosynthesis in *Bacillus subtilis*: Characterization of the *cydABCD* Operon

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Under aerobic conditions *Bacillus subtilis* utilizes a branched electron transport chain comprising various cytochromes and terminal oxidases. At present there is evidence for three types of terminal oxidases in *B. subtilis*: a *caa*₃-, an *aa*₃-, and a *bd*-type oxidase. We report here the cloning of the structural genes (*cydA* and *cydB*) encoding the cytochrome *bd* complex. Downstream of the structural genes, *cydC* and *cydD* are located. These genes encode proteins showing similarity to bacterial ATP-binding cassette (ABC)-type transporters. Analysis of isolated cell membranes showed that inactivation of *cydA* or deletion of *cydABCD* resulted in the loss of spectral features associated with cytochrome *bd*. Gene disruption experiments and complementation analysis showed that the *cydC* and *cydD* gene products are required for the expression of a functional cytochrome *bd* complex. Disruption of the *cyd* genes had no apparent effect on the growth of cells in broth or defined media. The expression of the *cydABCD* operon was investigated by Northern blot analysis and by transcriptional and translational *cyd-lacZ* fusions. Northern blot analysis confirmed that *cydABCD* is transcribed as a polycistronic message. The operon was found to be expressed maximally under conditions of low oxygen tension.

The gram-positive soil bacterium *Bacillus subtilis* is able to grow with various substrates as carbon sources, and it can use oxygen or nitrate as terminal electron acceptors. During aerobic respiration *B. subtilis* utilizes a branched electron transport chain comprising various cytochromes and terminal oxidases (52). At present there is biochemical and genetic evidence for three types of terminal oxidases in *B. subtilis*: a *caa*₃-, an *aa*₃-, and a *bd*-type oxidase (52). The first of these probably functions as a cytochrome *c* oxidase, whereas the latter two use menaquinol as a substrate (32). Both *a*-type oxidases are members of the well-characterized heme-copper superfamily of terminal oxidases (11). The cytochrome *bd* complex is unrelated to this superfamily (25). In addition, some *B. subtilis* strains seem to express a CO binding *b*-type cytochrome that may function as a terminal oxidase (50). The composition of the aerobic respiratory chain depends on the growth conditions (52). The flexibility of the energy-generating machinery may be one important factor that enables free-living bacteria such as *B. subtilis* to cope with the variation in oxygen and nutrient supply that is a common characteristic of their natural environment.

Cytochrome *bd* is a widely distributed prokaryotic terminal oxidase present in *Archaea* and *Bacteria* (25, 29). Most studies on this oxidase have been carried out on the enzymes from *Azotobacter vinelandii* and *Escherichia coli*. The *E. coli* cytochrome *bd*-type quinol oxidase comprises three spectroscopically distinct cytochromes (*b*₅₅₈, *b*₅₉₅, and *d*) and contains two subunits (28, 34). The *E. coli cydA* and *cydB* genes form one operon, which encodes the two polypeptide subunits of the cytochrome *bd* complex (16). Two additional genes, *cydC* and *cydD*, encoding a heterodimeric ATP-binding cassette (ABC)

transporter, are required for proper assembly of the *E. coli* cytochrome *bd*-type quinol oxidase (12, 39).

Relatively few data are available on cytochrome *bd* in *B. subtilis* or in gram-positive bacteria in general. However, cytochrome *bd* has recently been isolated from the facultative alkaliphile *Bacillus firmus* OF4 (13) and from the thermophile *Bacillus stearothermophilus* (41). In these bacteria, cytochrome *bd* has been detected only in mutant strains lacking the *caa*₃-type terminal oxidase.

To further characterize the terminal segment of the respiratory chain of *B. subtilis*, we have isolated and analyzed a gene cluster, designated *cyd* (43), that includes the structural genes, *cydA* and *cydB*, for the cytochrome *bd* terminal oxidase. Downstream of the structural genes, *cydC* and *cydD* are located. The latter genes encode proteins showing similarity to bacterial ABC-type transporters. Using gene disruption experiments, we have shown that the *cydC* and *cydD* gene products are required for the production of a functional cytochrome *bd* complex. We also show that the *cydABCD* genes form an operon transcribed as one polycistronic message and that expression of this operon is influenced by, e.g., oxygen tension.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this study are listed in Table 1. Plasmids used in this work are shown in Fig. 1 or are described in the text. *B. subtilis* strains were grown at 37°C in nutrient sporulation medium with phosphate (NSMP) (10), in NSMP supplemented with 0.5% glucose (NSMPG), in DSM (43), in DSM supplemented with 0.5% glucose, or in minimal glucose medium (23, 46). Tryptose blood agar base medium (TBAB) (Difco) was used for growth of bacteria on plates. Either L broth, L agar, or 2× YT (42) was used for growth of *E. coli* strains. The following antibiotics were used when required: chloramphenicol (5 µg/ml), kanamycin (5 µg/ml), tetracycline (15 µg/ml), and a combination of erythromycin (0.5 µg/ml) and lincomycin (12.5 µg/ml) for *B. subtilis* strains, and ampicillin (100 µg/ml) and chloramphenicol (12.5 µg/ml) for *E. coli* strains.

Batch cultures of *B. subtilis* LUW48 were grown in a bioreactor fitted with a 3-liter vessel and operated at a 2-liter working volume. The degree of air saturation was varied by manipulating the stirring and the flow of air.

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TABLE 1. Bacterial strains used

Strain	Relevant characteristics	Source or reference ^a
<i>E. coli</i>		
MM294	<i>endA1 hsdR17(r_K⁻ m_K⁺) supE44 thi-1</i>	Laboratory stock
XL1-Blue	<i>endA1 gyrA96 thi hsdR17(r_K⁻ m_K⁺) supE44 relA1 lac/F' proAB lac^f lacZΔM15 Tn10</i>	Stratagene, Inc.
C600	<i>supE44 hsdR thi-1 leuB6 lacY1 tonA21</i>	Stratagene, Inc.
<i>B. subtilis</i>		
1A1	<i>trpC2</i>	BGSC ^b
1A771	<i>trpC2 pheA1 amyE::ery</i>	BGSC
168A	<i>trpC2</i>	Laboratory stock ^c
JO1	<i>trpC2 ΔctaCD::ble</i>	49
Δqox	<i>trpC2 galT ΔqoxABCD::kan</i>	50
CydAd	<i>trpC2 cydA::pCYDAd (cydA'-lacZ)</i>	pCYDAd→1A1
CydBd	<i>trpC2 cydB::pCYDBd (cydAB'-lacZ)</i>	pCYDBd→1A1
CydCd	<i>trpC2 cydC::pCYDCd (cydABC'-lacZ)</i>	pCYDCd→1A1
CydDd	<i>trpC2 cydD::pCYDDd (cydABCD'-lacZ)</i>	pCYDDd→1A1
LUH15	<i>trpC2 ΔctaCD::ble</i>	JO1→168A
LUH17	<i>trpC2 galT ΔctaCD::ble ΔqoxABCD::kan</i>	Δqox→LUH15
LUW3	<i>trpC2 cydA::pCYDcat</i>	pCYDcat→168A
LUW9	<i>trpC2 cydD::pCYD12</i>	pCYD12→168A
LUW10	<i>trpC2 ΔcydABCD::cat</i>	pCYD13→168A
LUW20	<i>trpC2 ΔcydABCD::tet</i>	pCYD14→168A
LUW48	<i>trpC2 amyE::pCydLacZ1 (cydA'-lacZ)</i>	pCydLacZ1→1A1
LUW98	<i>trpC2 amyE::ery</i>	1A771→1A1

^a Arrows indicate transformation and point from donor DNA to recipient strain.

^b BGSC, Bacillus Genetic Stock Center, Department of Biochemistry, Ohio State University, Columbus.

^c This isolate of strain 168 was found to be oligosporogenic.

DNA manipulations. Procedures for plasmid isolation, agarose gel electrophoresis, use of restriction and DNA modification enzymes, DNA ligation, Southern blot analysis, and PCR were performed according to standard protocols (42). *B. subtilis* chromosomal DNA was isolated by a published procedure (23). Preparation of electroporation-competent *E. coli* cells and plasmid transformation of *E. coli* strains with a GenePulser apparatus (Bio-Rad Laboratories) were performed as described elsewhere (20). Transformation of *B. subtilis* by chromosomal or plasmid DNA was performed as described by Hoch (23). DNA probes were radiolabeled with [α -³²P]dCTP by using the Rediprime DNA labeling system (Amersham) according to the manufacturer's instructions. Kyt-Doolittle (31) profiles were obtained with the software package pSAAM, written by A. R. Crofts (University of Illinois).

RNA techniques. To isolate RNA, an overnight culture of *B. subtilis* 1A1 was inoculated into NSMP or NSMPG to an optical density at 600 nm (OD₆₀₀) of about 0.05. The cultures were grown at 37°C with shaking. Cells (80 ml) were harvested at 2, 4, and 6 h after inoculation, corresponding to the exponential, deceleration, and stationary phases, respectively. RNA was isolated by using a modification of an established procedure (24). A 0.2-ml portion of a cell suspension in 10 mM Tris-Cl (pH 7.4)–1 mM Na-EDTA–10 mM sodium iodoacetate was mixed with 0.5 ml of glass beads (diameter, 0.5 mm), 0.4 ml phenol, and 0.8 ml of a solution containing 0.6% cetyltrimethylammonium bromide, 50 mM sodium acetate, and 1 mM dithiothreitol. The cells were lysed by shaking with a Mini-Beadbeater apparatus (Biospec Products) for 1.5 min at 65°C. Following reextraction of the aqueous phase with phenol-chloroform-isoamyl alcohol (125:24:1 by volume), RNA was recovered by precipitation with an equal volume of isopropanol in the presence of 0.3 M sodium acetate at 4°C. The RNA was precipitated a second time and then dissolved in 50 μ l of water containing 10 U of RNase inhibitor (GIBCO BRL).

To identify the transcriptional initiation site of the *cyd* operon, 50 μ g of each RNA was annealed to a primer (5'-TAGAACCAGACTTTGATCAG-3') that had been labeled at the 5' end with T4 polynucleotide kinase (Megalabel; Takara Shuzo Co., Ltd., Kyoto, Japan) and [γ -³²P]ATP (Amersham). Primer extension reactions were performed as described previously (55).

For Northern blot analysis, RNA was electrophoresed in glyoxal gels and transferred to a Hybond-N membrane (Amersham) (42). The PCR product used in construction of strain CydAd was labeled with [α -³²P]dCTP and used as a probe. Hybridization was carried out as described previously (42).

Cloning of the *B. subtilis* *cyd* locus. The following procedure was used to clone the *cyd* region. From the sequences of subunit I of the cytochrome *bd* terminal oxidase from *A. vinelandii* (CydA), *E. coli* (CydA and AppC [also referred to as CbdA]), and *Haemophilus influenzae* (CydA), the consensus sequences FWGKLFGINFA and WIL(V/N)ANGWMQ (corresponding to residues 54 to 64 and 143 to 152 of the *E. coli* CydA subunit [16]) were derived. These sequences were used to design the degenerate oligonucleotides 5'-TGGGG(A/

T/G/C)AA(A/G)(T/C)T(A/T/G/C)TT(T/C)GG(A/T/G/C)AT(A/T/C)AA(T/C)TT(T/C)GC-3', used as the sense primer, and 5'-TGCATCCA(A/T/G/C)CC(A/G)TT(A/T/G/C)GC(A/T/G/C)(A/T)(T/C)(A/T/G/C)A(A/G)(A/G/C)ATCC A-3', used as the antisense primer, for amplification of a *B. subtilis* gene fragment by PCR. The obtained PCR product was cloned in plasmid pT7Blue(R) (Novagen). The DNA sequence of the cloned 290-bp fragment was determined. It was found to encode part of a reading frame that showed significant similarity to the product of the *E. coli* *cydA* gene, which encodes subunit I of the cytochrome *bd* quinol oxidase. DNA from this plasmid, called pCYD, was radiolabeled with [α -³²P]dCTP. Southern blot analysis showed that the labeled plasmid DNA hybridized to a 2,800-bp fragment of *B. subtilis* 1A1 DNA digested with *Hind*III. The labeled pCYD plasmid DNA was used as a probe to screen a library of *B. subtilis* 1A1 chromosomal DNA in *E. coli* XL1-Blue. The library contained *Hind*III chromosomal DNA fragments, of approximately 3,000 bp, inserted in the unique *Hind*III site of pBluescript II KS(-). Of approximately 1,500 clones, 2 gave positive signals. Plasmid DNA isolated from one positive clone was found to contain a 2,800-bp *Hind*III insert. The plasmid was designated pCYD1. *E. coli* XL1-Blue cells harboring this plasmid grew poorly, and only limited amounts of plasmid DNA could be recovered from the cells. By using a similar strategy, an additional region of the *cyd* locus was cloned in pCYD9 (Fig. 1). DNA sequencing showed that the cloned region corresponds to the previously reported nucleotide sequence of the *B. subtilis* *cyd* region (54).

Disruption of the *cydA* and *cydD* genes. The *cat* gene from pHV32 (35) was isolated as a 2,000-bp *Hind*III-*Sal*I fragment and cloned in pCYD, resulting in pCYDcat. The plasmids pCYDcat and pCYD12 (Fig. 1), carrying internal fragments of the *cydA* and *cydD* genes, respectively, were integrated into the chromosome of strain 168A via a single-crossover event disrupting the open reading frame of *cydA* or *cydD*. The insertion of the respective plasmid within the chromosomal *cydA* or *cydD* gene was confirmed by Southern blot analysis (data not shown).

Construction of a *cydABCD* null mutant. To make a deletion-insertion mutant, a 500-bp *Eco*RI-*Hind*III fragment of pCYD2 corresponding to an internal part of *cydA* was ligated to pCYD12 that had been cut with the same enzymes. The resulting plasmid, pCYD13 (Fig. 1), was used to transform strain 168A to chloramphenicol resistance. The deletion-insertion within the chromosomal *cydABCD* genes arising from a double-crossover recombination event was confirmed by Southern blot analysis (data not shown). A *cydABCD* deletion-insertion strain with a tetracycline instead of a chloramphenicol resistance cassette was constructed by the same procedure, by replacing plasmid pCYD13 with pCYD14 (Fig. 1).

Construction of *cyd-lacZ* translational and transcriptional fusions. A fragment containing part of *cydA*, bases -116 to +199 (relative to the *cyd* operon transcription start site), was amplified by PCR with oligonucleotides 5'-CCGG ATCCCTAGCAGCGACATAAATAAG-3' (*Bam*HI site underlined) and 5'-C

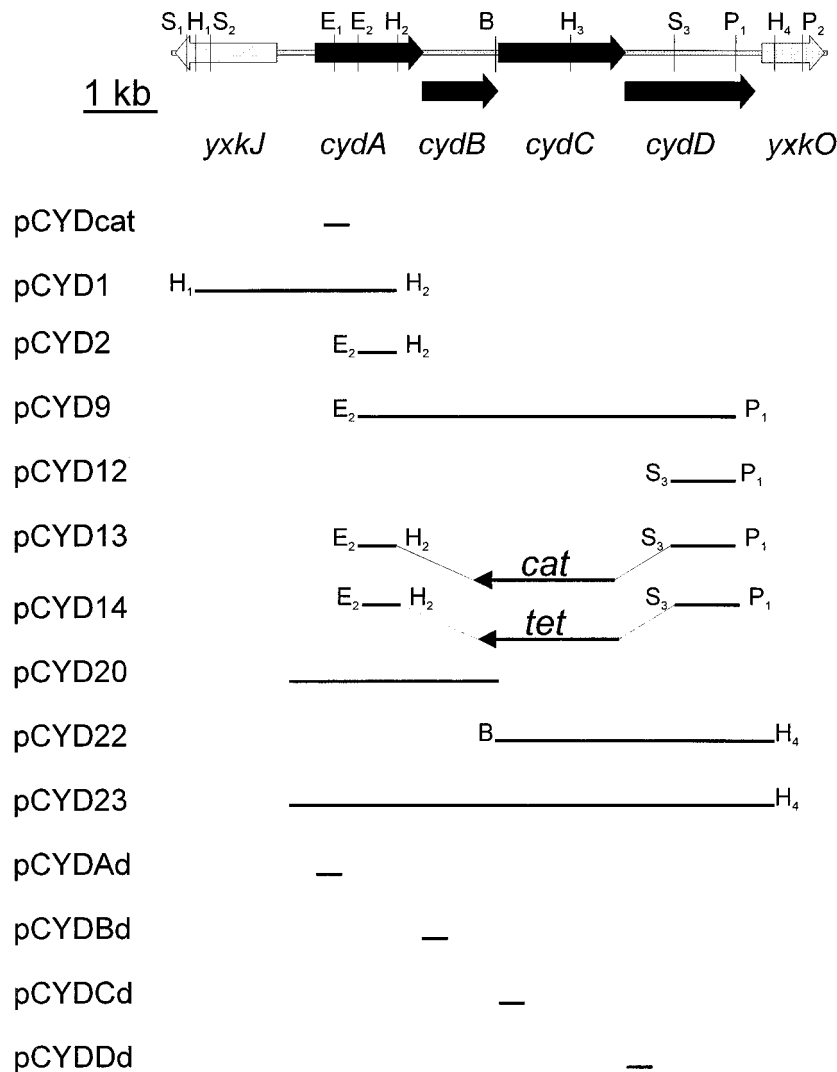


FIG. 1. Restriction map of the *B. subtilis* *cyd* region and plasmids carrying different parts of this region. The sequence of this region was determined previously (54). The *cyd* genes are oriented in the same direction as replication of the *B. subtilis* chromosome. The insert carried by each plasmid is shown as a solid line. Restriction sites used for subcloning fragments of the *cyd* region are abbreviated as follows: B, *Bsp*EI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sph*I. Plasmids pCYD1 and pCYD2 are derivatives of pBluescript II KS(-) (Stratagene, Inc.); plasmid pCYDcat is a derivative of pT7Blue(R) (Novagen) that contains the chloramphenicol acetyltransferase gene of pHV32 (35) on a 2,000-bp *Hind*III-*Sal*I fragment; plasmid pCYD9 is a derivative of pUC18 (53); plasmids pCYD12 and pCYD13 are derivatives of pHV32; pCYD20, pCYD22, and pCYD23 are derivatives of the *E. coli*/*B. subtilis* shuttle vector pHP13 (19); pCYD14 is a derivative of pCYD13 in which the *cat* gene (*Hind*III-*Bam*HI) has been replaced with the *tet* gene of plasmid pDG1515 (carried on a 2,142-bp *Hind*III-*Bam*HI fragment) (18); pCyAd, pCyBd, pCyCd, and pCyDd are derivatives of pMutin2 (*lacZ lacI amp ery*) (48).

CGGATCCCACTCATGCTTTCTCCTCCATTTC-3' (*Bam*HI site underlined) and was cloned into pBluescript II KS(-). The identity of the DNA fragment was verified by DNA sequencing. The fragment was then cloned into pMD431 (5), resulting in plasmid pCydLacZ1, which was subsequently integrated by double-crossover recombination at the *amyE* locus of the *B. subtilis* 1A1 chromosome by transformation of strain LUW98.

B. subtilis strains CydAd, CydBd, CydCd, and CydDd carrying transcriptional fusions of *cydA*, *cydAB*, *cydABC*, and *cydABCD* to *lacZ*, respectively, were constructed as follows. DNA fragments (approximately 350 bp) corresponding to internal parts of each of the *cyd* genes were amplified by PCR using specific primer pairs and chromosomal DNA of *B. subtilis* 1A1 as a template. The specific primers used for the constructions were as follows (restriction sites are underlined): for CydAd, 5'-GCCGAAGCTTTTCACTTCTTGTGGTCCG-3' (*Hind*III) and 5'-GCGCAGATCTTCGGTCCGAATGATACGAGC-3' (*Bgl*II); for CydBd, 5'-GCCGAAGCTTTTGGAAGGCTTTGATTTCCGG-3' (*Hind*III) and 5'-GCGCAGATCTCACAAACGGAGGAATTAGAC-3' (*Bgl*II); for CydCd, 5'-GCCGAAGCTTTGGGAATGAAGCGGATTTCCAC-3' (*Hind*III) and 5'-GCGCAGATCTACTGGCTGATGCCTTCCATC-3' (*Bgl*II); and for CydDd, 5'-GCCGAAGCTTTGCGCTGTTCGTTCTGGTTATC-3' (*Hind*III) and 5'-GCGCAGATCTCCTGCAAATGCTCAATATCC-3' (*Bgl*II). Each of the PCR

products was cleaved with *Hind*III and *Bgl*II and was then ligated with pMutin2 previously digested with *Hind*III and *Bam*HI. Plasmid pMutin2 (*lacZ lacI amp ery*) replicates in *E. coli* but not in *B. subtilis* and carries an erythromycin resistance gene that is active in *B. subtilis* (48). In addition, pMutin2 carries a promoterless *lacZ* gene derived from *E. coli* that can be used as a reporter gene (48). The ligated DNAs were introduced into *E. coli* C600 by transformation. The identity of each of the PCR products cloned into pMutin2 was verified by DNA sequencing. The resulting plasmids, pCYDAd, pCYdBd, pCYDCd, and pCYDDd (Fig. 1), were used to transform *B. subtilis* 1A1 to erythromycin resistance. Correct integration of a single copy of each plasmid into the respective *cyd* gene through a single-crossover event was confirmed by Southern blot analysis. In these disruptants, the *lacZ* gene of pMutin2 is placed under the regulation of all upstream sequences, including the *cyd* promoter.

Overproduction of cytochrome *bd* in *B. subtilis*. Plasmid pCYD20, containing the *cydA* and *cydB* genes under the control of their native promoter, was constructed as follows. The region of interest, -116 to +2601 (relative to the *cyd* operon transcription start site), was amplified by long-range PCR using DNA from *B. subtilis* 1A1 as a template and oligonucleotides 5'-CCGATCCTAGCAGCGGACATAAATAAG-3' (*Bam*HI site underlined) and 5'-CCCCTGCAGTTAATAAGTCATAGGCTCCTTATGG-3' (*Pst*I site underlined). Long-range

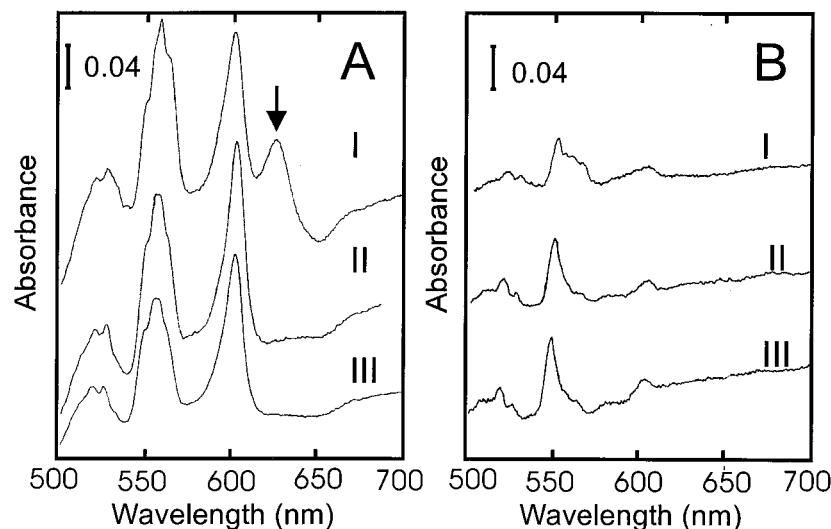


FIG. 2. Low-temperature (77 K) light absorption difference spectra of *B. subtilis* membranes. I, wild-type strain 168A; II, *cydD* mutant LUW9; III, *cydA* mutant LUW3. Membranes (10 mg of protein ml⁻¹) were reduced in the sample cuvette with sodium dithionite (A) or sodium ascorbate (B) and oxidized in the reference cuvette with potassium ferricyanide. The arrow indicates the 622-nm maximum of reduced cytochrome *d* of the cytochrome *bd* complex. The absorbance scales are indicated by the bars.

PCR was carried out by using the Expand High Fidelity PCR System (Boehringer Mannheim) according to the manufacturer's protocol. The PCR product was digested with *Bam*HI and *Pst*I and was then ligated with the shuttle vector pHP13 (19), which had previously been digested with *Bam*HI and *Pst*I. The ligate was used to transform *B. subtilis* 168A to chloramphenicol resistance. The plasmid isolated from these cells was called pCYD20. An intact pCYD20 cannot be maintained in *E. coli* XL1-Blue.

Plasmid pCYD23, containing the *cydABCD* operon under the control of the native promoter, was constructed as follows. The distal part of the *cydD* gene was amplified by PCR using chromosomal DNA from *B. subtilis* 1A1 as a template and oligonucleotides 5'-GAGCGCCAGCGGATCGCACTTGCG-3' and 5'-CGTCACGCCAATAGGTGCGCTCGG-3'. The sequences of these oligonucleotides were based on the previously determined sequence of the *cyd* region (54). The PCR product was cleaved with *Pst*I and *Hind*III and was then ligated with pBluescript II KS(-). The identity of the DNA fragment generated by PCR was verified by DNA sequencing. It was then cloned into pHP13, resulting in plasmid pCYD21. The 3,226-bp *Bgl*II-*Pst*I fragment of pCYD9 that carries *cydC* and part of *cydD* (Fig. 1) was ligated with pCYD21 cut with *Bam*HI and *Pst*I, resulting in pCYD22. This plasmid was cut with *Bsp*EI and *Nsi*I, and the *cydCD* fragment was isolated and ligated with pCYD20 cut with *Bsp*EI and *Nsi*I. The ligate was used to transform *B. subtilis* 1A1 to chloramphenicol resistance. The resulting plasmid, containing the *cydABCD* genes, was named pCYD23. As was noted for pCYD20, this plasmid, too, could not be transformed into *E. coli* XL1-Blue.

Biochemical analyses. For membrane preparation, *B. subtilis* strains were grown in NSMP or NSMPG. The bacteria were harvested when they had reached the stationary-growth phase. Membranes were prepared as described previously (21) and resuspended in 20 mM sodium morpholinic propane sulfonic buffer (MOPS), pH 7.4. Protein concentrations were determined by using the bicinchoninic acid assay (BCA; Pierce) with bovine serum albumin as the standard.

Difference (reduced minus oxidized) light absorption spectra were recorded as described previously (17). A few grains of sodium dithionite or 10 mM sodium ascorbate was used as the reducing agent, and 1.25 mM potassium ferricyanide was used as the oxidizing agent. When sodium ascorbate was used as the reductant, both cuvettes contained 5 mM potassium cyanide. CO spectra were obtained by bubbling the sample cuvette with carbon monoxide for 2 min.

B. subtilis cytochromes *c* were radiolabeled by growing cells in NSMP supplemented with 5-[4-¹⁴C]aminolevulinic acid (44).

An *N,N,N',N'*-tetramethyl-*p*-phenyleneamine (TMPD) oxidation assay was performed as described previously (17). β -Galactosidase activity was determined on cell extracts by using 2-nitrophenyl- β -D-galactopyranoside as a substrate, as described previously (1, 36). Alternatively, β -galactosidase activity was assayed with 4-methylumbelliferyl- β -D-galactoside as a substrate (56). At various intervals during growth, 0.5-ml samples were removed and immediately frozen in liquid nitrogen. Fluorometric readings of β -galactosidase assay samples were performed with a Shimadzu RF-5301PC spectrofluorophotometer.

RESULTS

Identification, organization, and mutagenesis of the *cyd* gene cluster. The *B. subtilis cydABCD* gene cluster (Fig. 1) is located at about 340° on the physical map of the *B. subtilis* chromosome (30). Analysis of the deduced sequences of *B. subtilis cydA* and *cydB* indicated that they are similar to the products of the *E. coli cydA* and *cydB* genes, respectively (41 and 36% identity, respectively), which encode the two subunits of the cytochrome *bd*-type quinol oxidase of *E. coli*. Studies using site-directed mutagenesis and various spectroscopic methods indicate that in *E. coli* CydA, His-19, His-186, and Met-393 provide three of the four axial ligands to the iron of the three hemes in the cytochrome *bd* complex (9, 27, 45, 47). These amino acid residues are preserved in *B. subtilis* CydA (His-18, His-183, and Met-334). In *E. coli* CydA, a large periplasmic loop, called the Q-loop, has been proposed to constitute a domain involved in quinol binding (7, 8). Inspection of the *B. subtilis* CydA amino acid sequence revealed that a large portion of this domain is absent. The *cydC* and *cydD* genes have the potential to code for the components of a heterodimeric ABC-type membrane transporter. CydC and CydD show 32 and 29% identity to *E. coli* CydD and CydC, respectively.

To study the roles of the different *B. subtilis cyd* genes, plasmid insertion mutagenesis was performed. Disruption of *cydA*, *cydD*, or *cydABCD* (see Materials and Methods) had no apparent effect on the growth of cells in broth or defined media. The total cytochrome contents of cytoplasmic membranes isolated from a wild-type strain and from the *CydA* and *CydD* mutants were analyzed by low-temperature (77 K) difference (reduced minus oxidized) spectroscopy (Fig. 2). Spectra of membranes from the wild-type strain demonstrated *c*-type (peaks in the 550-nm region), *b*-type (peaks in the 555- to 565-nm region), *a*-type (peak at 600 nm), and *d*-type (peak at 622 nm) cytochromes (Fig. 2A). The trough in the difference spectrum at 650 nm most likely originates from a stable oxygenated cytochrome *d* species (34, 40). The *CydA* and *CydD* mutants (Fig. 2A) both lack the peak at 622 nm as well as the

trough at 650 nm. This is a diagnostic feature of a strain lacking cytochrome *bd*. The mutants seem to express less of the *b*-type cytochromes relative to cytochrome *aa*₃ than does the wild type. By analogy with the *E. coli* system, it is likely that this is due to the lack of the low-spin cytochrome *b* component of the cytochrome *bd* complex in the mutants. The absence of this cytochrome gives a lower absorption in the 558- to 563-nm region.

With sodium ascorbate as the reducing agent, preferential high-potential *c*-type cytochromes are seen in the difference spectra. From the reduction with sodium ascorbate, it can be seen that the cytochrome composition in the mutants differs slightly from that in the wild type (Fig. 2B). The spectra indicate a slightly increased expression of a putative cytochrome *c* in the mutants. The cytochrome composition of membranes from a strain in which *cydABCD* was deleted (see Fig. 4, spectrum A) was similar to that of the membranes from the *CydA* mutant strain (Fig. 2A). These data demonstrate that the formation of spectrally detectable cytochrome *bd* in *B. subtilis* requires intact *cydA* and *cydD* genes. Most probably, *cydA* codes for a polypeptide of the cytochrome *bd* complex, whereas the *cydD* gene is required for assembly of a functional complex.

Overproduction of cytochrome *bd*. To better resolve the spectral features and provide a convenient source for isolation of cytochrome *bd*, the complex was overproduced in *B. subtilis*. Plasmid pCYD20 is a low-copy-number vector containing the *cydAB* genes and the *cyd* promoter region. This plasmid was introduced into strain LUH17. This strain lacks the terminal *a*-type oxidases, cytochrome *aa*₃ and cytochrome *caa*₃. As a consequence, membranes isolated from this strain show no interfering signals in the 600-nm region. Membranes were prepared from cells harboring pCYD20 (*cydAB*) or pHP13 (vector) and were analyzed for cytochromes by absorbance spectroscopy. At room temperature, the α -absorption maxima of the cytochrome *bd* oxidase were present at 626, 597, and about 563 nm (Fig. 3). The last absorption maximum was split in two peaks (558 and 563 nm) when the temperature was lowered to 77 K. At this temperature, peaks attributable to cytochrome *bd* are present at 622, 593, 563, and 558 nm (spectrum not shown). Cytochrome *bd* was found to be overproduced about fourfold in a strain carrying plasmid pCYD20, as estimated from the intensity of the 626-nm peak (cytochrome *d*) and to be reduced by NADH via the respiratory chain (data not shown). The CO difference spectrum in the 300- to 400-nm region of membranes of LUH17/pCYD20 revealed absorption troughs at 428 and 440 nm that were about fourfold more pronounced than those of LUH17/pHP13 membranes (data not shown). This indicates that the overproduced enzyme reacts with carbon monoxide like the native enzyme.

Overexpression of *cydAB* from pCYD20 in a strain in which *cydABCD* had been deleted did not reveal any signals attributable to the cytochrome *bd* oxidase (Fig. 4, spectrum B). However, when plasmid pCYD23, carrying the complete *cydABCD* operon, was introduced into strain LUW20 ($\Delta cydABCD$), cytochrome *bd* was detected, confirming the functionality of the cloned DNA (Fig. 4C). In a wild-type strain transformed with pCYD20 or pCYD23, cytochrome *bd* was found to be overproduced about fourfold (data not shown). Membranes isolated from strain LUW9 (*CydD*⁻) transformed with pCYD20 showed a slight but significant increase in absorbance in the 558-nm region. No cytochrome *d* signal was seen in these membranes (data not shown).

The *cydD* gene is not required for the synthesis of *c*-type cytochromes. In *E. coli* the *cydCD* homologs (*cydDC*) are required for the assembly of *c*-type cytochromes (38). To analyze

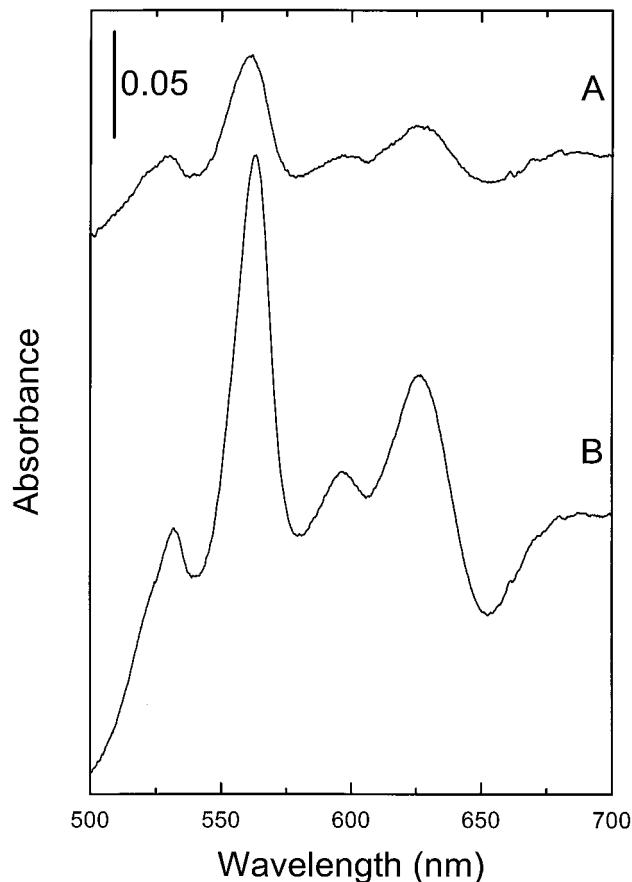


FIG. 3. Light absorption difference spectra of *B. subtilis* membranes recorded at room temperature. Membrane suspensions (4 mg of protein ml⁻¹) were reduced in the sample cuvette with sodium dithionite and oxidized in the reference cuvette with potassium ferricyanide. (A) LUH17/pHP13; (B) LUH17/pCYD20. Strain LUH17 has cytochromes *aa*₃ and *caa*₃ deleted. The vertical bar indicates the absorbance scale.

if mutations in *cydD* or *cydABCD* influence the synthesis of *c*-type cytochromes in *B. subtilis*, heme-specific radioactive labeling was performed. The pattern of *c*-type cytochromes in membranes isolated from the mutant strains was similar to that of the wild-type strain (data not shown).

B. subtilis strains that lack the structural genes for cytochrome *caa*₃ or are deficient in the synthesis of *c*-type cytochromes are TMPD oxidase negative (44, 49). Inactivation of *cydD* or deletion of *cydABCD* did not affect the ability to oxidize TMPD (data not shown).

We conclude that the *cyd* genes are not essential for synthesis of *B. subtilis* cytochromes *c*.

Expression analysis. To analyze the expression of the *cydABCD* genes, we constructed transcriptional and translational fusions with the *E. coli lacZ* reporter gene (see Materials and Methods). β -Galactosidase activities were measured in intact cells or in extracts of bacteria grown in different media. A transcriptional fusion of *cydA* to *lacZ* was constructed. This construct was integrated at the *cyd* locus to reconstruct the intact chromosomal DNA context upstream of the fusion. The *cydA-lacZ* fusion was not significantly expressed during growth in NSMP or in DSM (Fig. 5A). However, in NSMPG (broth medium supplemented with glucose), the fusion was activated at the transition between the exponential-growth and the stationary phase (Fig. 5A). Very low activities were detected also

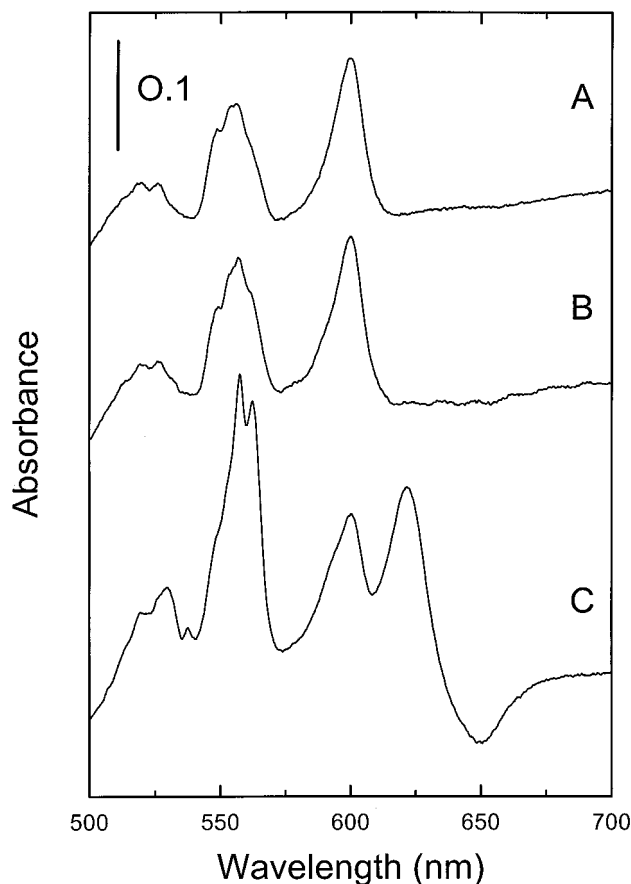


FIG. 4. Difference spectra of *B. subtilis* LUW20 membranes isolated from cells harboring plasmid pHP13 (vector alone) (A), pCYD20 (*cydAB*) (B), or pCYD23 (*cydABCD*) (C). Strain LUW20 has *cydABCD* deleted. The spectra were recorded at 77 K on membrane suspensions containing 10 mg of protein ml^{-1} . Membranes were reduced in the sample cuvette with sodium dithionite and oxidized in the reference cuvette with potassium ferricyanide. The vertical bar indicates the absorption scale.

when cells were grown in DSM supplemented with glucose. DSM is a nutrient broth-based growth medium that has a composition similar to that of NSMP but lacks the phosphate buffer. In this medium the bacteria grew to a density similar to that in NSMPG (Fig. 5A). To determine whether the levels of cytochrome *bd* in membranes reflect the patterns of *cydA-lacZ* expression, we analyzed the cytochrome composition of *B. subtilis* 1A1 grown in the media described above. In agreement with the *cydA-lacZ* expression, membranes from cells grown in NSMP or in DSM supplemented with glucose exhibited very small amounts of the cytochrome *bd* complex (data not shown).

The timing and amounts of β -galactosidase activity of the transcriptional fusions of *cydAB*, *cydABC*, and *cydABCD* to *lacZ* showed patterns of expression similar to that seen for the *cydA-lacZ* fusion (Fig. 5B). This result would be consistent with the promoter upstream of *cydA* determining the bulk of the expression of the *cydABCD* genes. The indicated coregulation suggests that the *cyd* genes are transcribed as an operon. The lack of an apparent transcription terminator in the region covering the *cydABCD* genes and the finding that the four *cyd* genes overlap each other further support the operon hypothesis.

A *cydA-lacZ* translational fusion, containing positions -307

to +6 relative to the initiation codon of *CydA*, was constructed and inserted by double-crossover recombination at the nonessential *amyE* locus. Expression in NSMP, NSMPG, DSM alone, and DSM supplemented with glucose followed the pattern described for the *cydA-lacZ* fusion above (data not shown). This shows that major sites for the regulation of the *cydA* promoter are located within the 312-bp fragment used for the *cydA-lacZ* translational fusion.

The expression pattern of the *cydA-lacZ* fusions suggested that the *cydA* promoter is activated in media that give a high cell density and that this activation may correlate with a decrease in oxygen tension in the culture medium. In support of this idea, we found that when the bacteria were cultivated at high aeration, the expression of the *cydA-lacZ* fusion was low, whereas the reverse was observed when bacteria were grown under oxygen-limiting conditions (Fig. 6).

Identification of the transcriptional start site for *cydA*. Primer extension analysis was used to identify the *cydA* promoter. Total RNA was isolated from cells grown in NSMP or NSMPG in the exponential-growth phase, at the time of tran-

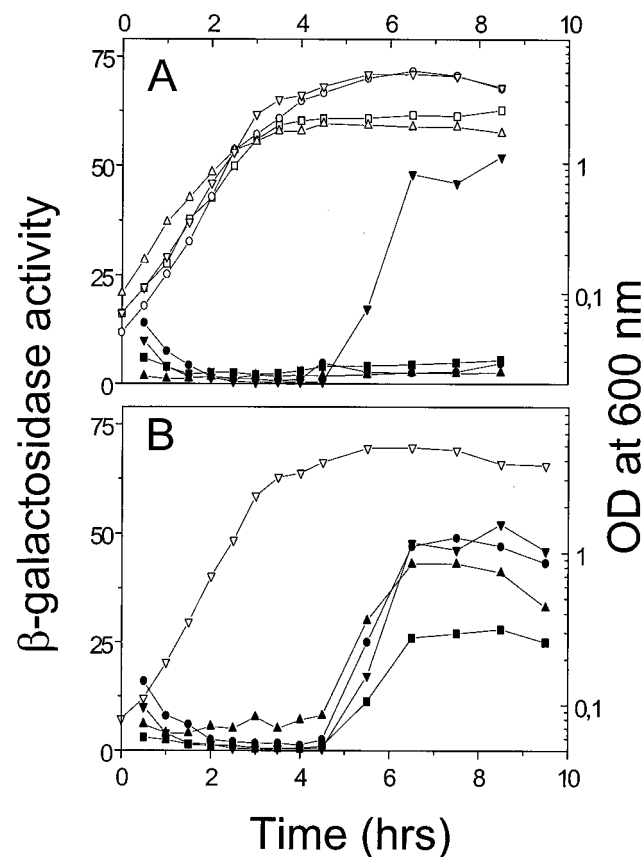


FIG. 5. β -Galactosidase expression from *cyd-lacZ* fusions as a function of growth. (A) Activities for the *cydA-lacZ* fusion are shown for cells grown in DSM (solid squares), NSMP (solid triangles), DSM supplemented with glucose (solid circles), and NSMPG (solid inverted triangles). The optical densities for cells grown in each medium are shown by the corresponding open symbol. (B) β -Galactosidase expression from transcriptional fusions to *lacZ* integrated by single-crossover recombination at the *cydA* (solid inverted triangles), *cydB* (solid circles), *cydC* (solid triangles), or *cydD* (solid squares) locus. β -Galactosidase activity is expressed as nanomoles of 2-nitrophenyl- β -D-galactopyranoside hydrolyzed per minute and milligram of protein. The growth (optical densities) of the *cydA-lacZ* fusion strain is shown (open inverted triangles). The four strains were grown in NSMPG and showed similar growth properties. Data from a single experiment are presented. Each experiment was repeated at least twice, with similar results.

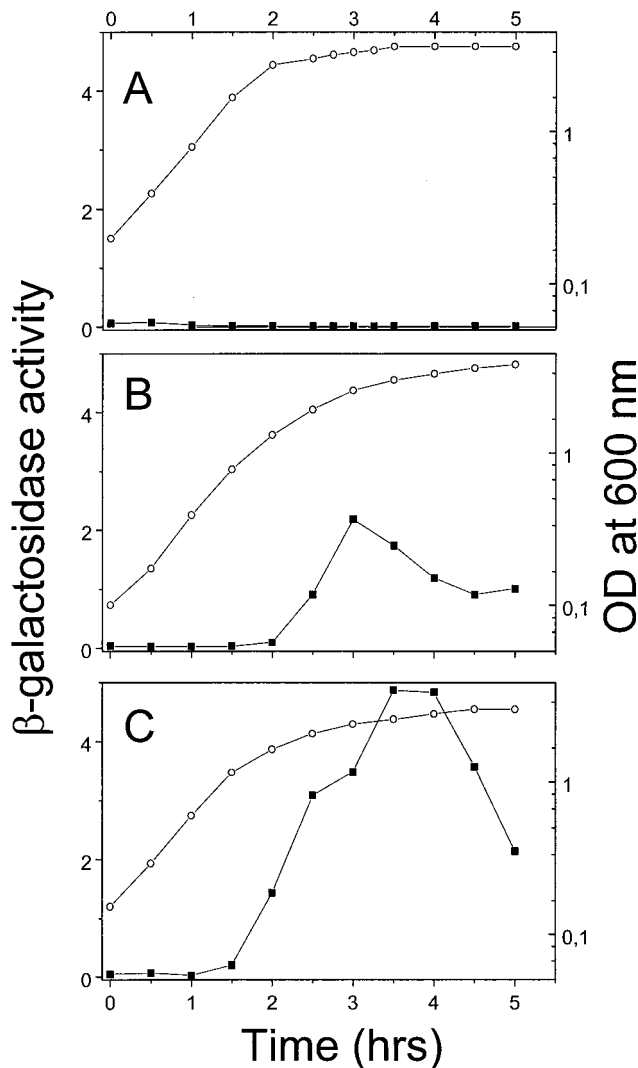


FIG. 6. Effect of aeration on *cydA-lacZ* expression. *B. subtilis* LUW48 was grown in NSMPG in a bioreactor. (A) High aeration, maintained during growth by manually increasing the stirring speed from 250 to 800 rpm. (B) Medium aeration, maintained by constant stirring at 250 rpm. (C) Low aeration, maintained by constant stirring at 250 rpm. The vessel was sparged with sterile air at a flow rate of 1 (A and B) or 0.5 (C) volume of air per volume of liquid per minute. β -Galactosidase activity (solid squares) is expressed as nanomoles of 4-methylumbelliferyl- β -D-galactoside hydrolyzed per minute per OD₆₀₀ unit. Optical densities are shown as open circles.

sition from the exponential-growth to the stationary phase, and approximately 1 h into the stationary phase. Consistent with the results of *lacZ* fusion studies, *cyd* mRNA was detected only in late-exponential-phase or stationary-phase cells and preferentially in cells grown in glucose-containing medium (Fig. 7A). The major extension product found indicated that the apparent 5' end of the *cydA* mRNA is located 193 bp upstream of the *cydA* translational start site and is initiated at an adenine nucleotide (Fig. 7A). The size of the major extension product was identical under both growth conditions. The intensity of the signal was highest with RNA isolated from cells grown in NSMPG (Fig. 7A, lanes 2 and 3). Upstream of the transcriptional start point, putative -35 and -10 sigma-A recognition sequences are located (Fig. 7B). The minor extension product seen in Fig. 7A probably originates from an incomplete reverse transcription. However, we cannot rule out the presence of a

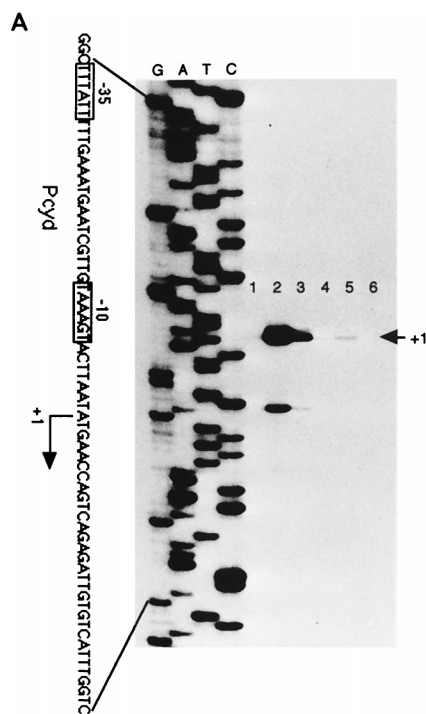
weaker promoter downstream of that indicated in Fig. 7. Inspection of the promoter region revealed a perfect, 16-bp palindromic sequence (Fig. 7B) 98 bp upstream of the AUG translation initiation sequence for *CydA*. This sequence may constitute the operator site for a putative regulatory protein.

Analysis of RNA transcripts from the *cyd* region. To confirm the organization of the *cyd* gene cluster, RNA was isolated from wild-type cells grown in NSMP and NSMPG media and was analyzed in Northern blot experiments. When a DNA fragment corresponding to a part of *cydA* was used as a probe, a transcript of approximately 6,000 nucleotides was detected with RNA extracted from cells grown in NSMPG (Fig. 8, lanes 1 to 3). This transcript has the length expected for a polycistronic *cydABCD* mRNA. We could not detect any full-length transcript in cells grown in NSMP (Fig. 8, lanes 4 to 6), which is in agreement with the much-lower expression of, e.g., the *cydA-lacZ* fusion, in NSMP than in NSMPG (Fig. 5A). Analysis of the signal intensities in this experiment are also in good agreement with that found in the primer extension analysis (Fig. 7A). Preliminary Northern blot experiments with RNA isolated from cells grown in NSMPG, using DNA fragments corresponding to *cydB*, *cydC*, and *cydD* as probes, also showed a transcript of approximately 6,000 nucleotides. The *cydABCD* transcript appears to be highly unstable (Fig. 8). To rule out any general problem with RNA degradation, the blots were reprobbed with DNA fragments corresponding to parts of *galE* and *yxjF*. These probes detected intact transcripts of the expected sizes and showed no hybridization smears (data not shown).

DISCUSSION

The respiratory chain of *B. subtilis* is branched, with at least three terminal oxidases, cytochrome *aa*₃, cytochrome *caa*₃, and cytochrome *bd*, being synthesized under different growth conditions (52). During vegetative growth, cytochrome *aa*₃ is likely to be the major terminal oxidase contributing to proton motive force generation. In this paper we have characterized four genes, *cydA*, *cydB*, *cydC*, and *cydD*, that are required for the expression of cytochrome *bd* in *B. subtilis*. Based on sequence comparisons, *cydA* and *cydB* are likely to code for the two subunits of a cytochrome *bd*-type quinol oxidase. The *cydC* and *cydD* gene products are most likely not part of the functional oxidase but are required for its assembly. The physiological roles of cytochrome *bd*-type quinol oxidases in bacteria are in general far from being fully understood. In *A. vinelandii* and *Klebsiella pneumoniae*, one important role of cytochrome *bd* is to scavenge oxygen that otherwise could inactivate the oxygen-sensitive nitrogenases of these bacteria (26, 37). In *E. coli*, cytochrome *bd* may perform two physiological roles: contributing to energy conservation under microaerobiosis and protecting the cell from oxidative stress (22). The physiological role of cytochrome *bd* in *B. subtilis* has not been established. Disruption of the *B. subtilis cyd* genes had no apparent effect on the growth of cells in broth or defined media. The presence of cytochrome *bd*-like quinol oxidases appears to be widespread among prokaryotes, indicating that this type of oxidase plays important functional roles. Sequences predicted to encode *bd*-type oxidases have been reported for *Archaea* and *Bacteria* but do not appear to be present among *Eukarya* (2, 4, 6, 25). Computer analysis of the deduced *B. subtilis* *CydA* sequence showed that it is most closely related to the corresponding protein of *Mycobacterium tuberculosis* (2).

Analysis of the genome sequence of *B. subtilis* revealed one *cydA* paralogue, *ythA*, which is the first gene of a putative *ythABC* operon (30). Sequence comparisons show that *YthA* is



B

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-65 TAGTGCGGGACTTTTTAGGTATTTTAGGCTTTTATTTTGAATGAATCGTTGTAAGTAC
+1
-5 TTAATATGAACCAGTCAGAGATTGTGTGCATTTGGTCAGTCTGGCAATCTTGATCATAT
+56 GGATGACTTTTTGACACATTTGTGAATATTGAGCAATATTTTTTCGCTATTTTGTGA

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FIG. 7. Primer extension mapping of the *cydA* promoter. (A) Identification of the transcriptional initiation site of the *cydA* promoter (*Pcyd*). A known nucleotide sequence ladder was used to estimate the size of the extended product, which is indicated by an arrow. The end-labeled primer was annealed to total RNA isolated from *B. subtilis* 1A1 cultured in NSMPG (lanes 1 to 3) or in NSMP (lanes 4 to 6). Samples were taken in the exponential-growth phase (lanes 1 and 4), at the time of transition from the exponential-growth to the stationary phase (lanes 2 and 5), and in the stationary phase (lanes 3 and 6). Boxes, -10 and -35 promoter regions of *cydA*. This promoter is likely to be recognized by RNA polymerase containing the vegetative sigma factor, σ^A . (B) Nucleotide sequence of the *yxkJ-cydA* intergenic region. The apparent transcription start site is indicated as +1. The -10 and -35 promoter regions of the *cydA* gene are indicated. Also shown is a putative operator sequence, the 16-bp palindrome starting at +80 (underlined). The coordinates are given with respect to the *cydA* transcription start point. A likely initiation codon (ATG) of *cydA* is at position +194.

distantly related to the known CydA sequences. YthB encodes a protein similar to CydB in size but does not reveal any convincing sequence similarity to the known CydB sequences. Nevertheless, the YthB and CydB sequences show striking similarities in their hydrophathy profiles, indicating that YthAB may be part of a cytochrome *bd*-like enzyme (data not shown).

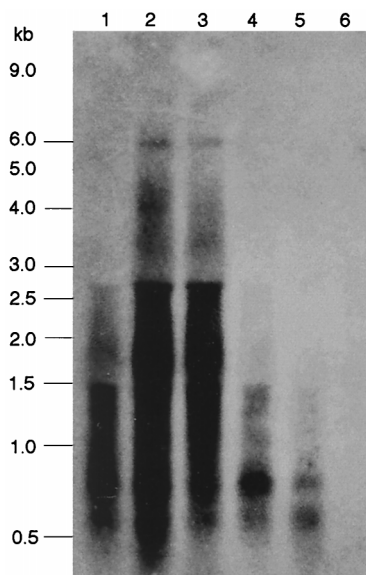


FIG. 8. Northern blot analysis of *B. subtilis* 1A1 total RNA using a *cydA* probe labeled with ^{32}P . RNA was isolated from cells grown in NSMPG (lanes 1 to 3) or in NSMP (lanes 4 to 6). Samples were taken in the exponential-growth phase (lanes 1 and 4), at the time of transition from the exponential-growth to the stationary phase (lanes 2 and 5), and in the stationary phase (lanes 3 and 6). The positions of RNA size standards are indicated on the left. In addition to *cydA*-specific transcripts, the probes hybridized nonspecifically to 16S (1,553 bases) and 23S (2,928 bases) rRNA.

The third gene, *ythC*, encodes a 55-residue amphiphilic and basic protein which does not show any striking similarity to sequences in databases. Analysis of *B. subtilis* membranes showed that inactivation of *cydA* or deletion of *cydABCD* resulted in the loss of spectral features associated with cytochrome *bd*, suggesting that YthAB has distinct spectral characteristics or that the *yth* genes are poorly expressed under the conditions used.

As reported herein, the *B. subtilis* *cydA*, *cydB*, *cydC*, and *cydD* genes are transcribed as a polycistronic message. A corresponding gene organization may also be present in *M. tuberculosis* (2). In *E. coli* and in other bacteria where *cydC* and *cydD* have been clearly identified, these genes are adjacent to each other but separate from *cydAB* (3). The sequences of *CydC* and *CydD* suggest that they comprise an ATP-dependent membrane transporter of the ABC superfamily (33, 39). Mutations in the *E. coli* *cydC* or *cydD* gene affect the assembly of cytochrome *bd* (12, 39). The apoproteins are made and inserted into the membrane, but the mutant complexes are missing heme *d* and presumably hemes b_{595} and b_{558} (38). However, cytochrome *bd* can be detected in a *cydC* mutant if the *cydAB* genes are overexpressed from a multicopy plasmid (14). In addition, *CydC* and *CydD* are required for synthesis of all *c*-type cytochromes and of cytochrome b_{562} (15, 38). In *B. subtilis*, mutations in *cydCD* or *cydD* result in the loss of all spectral features attributable to the cytochrome *bd* complex but do not affect the synthesis of *c*-type cytochromes or presumably of any other cytochrome complex. This suggests that *B. subtilis* *cydCD* genes are required only for the assembly of a functional cytochrome *bd*, whereas in *E. coli* these genes have additional roles. In a *B. subtilis* strain with *cydABCD* deleted, synthesis of cytochrome *bd* can be restored when the four genes are supplied on a plasmid. Overexpression of *cydAB* alone does not allow cytochrome *bd* to be produced even in a *CydD* mutant strain. The substrate of the *CydCD* transporter is not known. It has been suggested that the transporter is involved in the export of heme to the periplasm of *E. coli* (39). Goldman et al. (15) proposed, based on experiments with periplasmic heme reporters, that the *CydCD* transporter is not a heme exporter but is required for the control of the reducing environment within the periplasmic space. In *B. subtilis*, all cytochromes *c* have their heme domain exposed to the outer face of the cytoplasmic membrane (51). The presence of holo-cytochromes *c* in membranes from *B. subtilis* *CydABCD* or *CydD* mutant strains suggests that mutations in *cydCD* do not

affect the transport of heme across the membrane or its subsequent attachment to the apocytochromes *c*.

The regulation of the amount of cytochrome *bd* in *B. subtilis* under various growth conditions appears to be primarily at the level of transcription of the *cydABCD* operon. When cells are grown with high aeration, the expression of cytochrome *bd* is repressed. When the oxygen tension of the growth medium is decreased, expression of the *cyd* operon is induced and reaches its maximum during the transition from the exponential- to the stationary-growth phase. The *cydABCD* operon appears to be highly regulated in response to oxygen. However, in a nonbuffered, low-phosphate medium (DSM supplemented with glucose), expression of the *cydABCD* operon was significantly reduced from that in a buffered, high-phosphate medium (NSMPG). Thus, for maximal expression of the *cyd* genes, specific medium compositions as well as conditions of low oxygen tension are required.

It is likely that *B. subtilis* makes regulatory proteins that function either to activate *cyd* expression as oxygen becomes limiting or, alternatively, to repress *cyd* expression under conditions of high oxygen tension. The *lacZ* reporter constructs described in this paper should be helpful in identifying such regulatory proteins.

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