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The Cytochrome *bc* Complex (Menaquinone:Cytochrome *c* Reductase) in *Bacillus subtilis* Has a Nontraditional Subunit Organization

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We have identified an operon in *Bacillus subtilis*, designated *qcr*, that is thought to encode a quinone: cytochrome *c* reductase. Northern (RNA blot) analysis suggests a tricistronic operon. The operon is located at about 200° on the *B. subtilis* map. Disruption of the operon leads to loss of a 22-kDa cytochrome *c* from membrane preparations. The structure of the putative protein products of the *qcr* operon suggests a protein complex that is closely related to but distinct from known cytochrome bc_1 and b_6f complexes, which catalyze electron transfer from a quinol to a *c*-type cytochrome or to plastocyanin. QcrA is similar to Rieske-type iron-sulfur proteins; QcrB is similar in size and sequence to *b*-type cytochromes from b_6f complexes; and QcrC has a novel structure that resembles a fusion of a subunit IV (found in b_6f complexes) to a cytochrome *c*. Transcription of the operon is induced at the end of exponential growth from a σ^A-like promoter. This transition state induction appears to be dependent on the downregulation of *abrB* expression, which is mediated by Spo0A activation. As bacteria move from the transition state into sporulation, transcription of the operon is reduced in a σ^F-dependent manner.

The cytochrome bc_1 complex (quinol:cytochrome *c* oxidoreductase; EC 1.10.2.2) is an integral membrane enzyme that catalyzes electron transfer from a quinol to a *c*-type cytochrome. Coupled to the electron transfer, protons are translocated across the membrane, generating a transmembrane electrochemical gradient. This enzyme has been isolated from mitochondria and from several bacterial species. A very similar complex, b_6f, is associated with photosynthesis in cyanobacteria and in chloroplasts of higher plants (for a review, see reference 32).

The number of polypeptides in isolated bc_1/b_6f complexes varies, but common to all is the presence of an iron-sulfur protein with a [2Fe-2S] cluster (the Rieske FeS protein), a cytochrome *b* with two low-spin hemes, and a cytochrome *c*. The cytochrome *b* polypeptide of bc_1 complexes has about 420 residues, with eight membrane-spanning segments, and the cytochrome c_1 has His/Met axial ligation of heme C. In b_6f complexes, the cytochrome *b* can be regarded as being split into two polypeptides, a polypeptide of about 220 residues that harbors the two hemes and has four membrane-spanning segments and a polypeptide of about 160 residues, subunit IV, which is similar to the C-terminal part of the cytochrome *b* polypeptide of bc_1 complexes (32). Cytochrome *f* of the b_6f complex is a *c*-type cytochrome that has Met/Tyr (α-amino group)-ligated heme C and is distinct in sequence from cytochromes c_1 (22).

Relatively little information is available on the quinol:cytochrome *c* reductases of gram-positive bacteria (11, 32). Only a quinol:cytochrome *c* reductase of the thermophilic bacterium *Bacillus* sp. strain PS3 has been purified (16). This enzyme shows features of a b_6f complex, with a 21-kDa cytochrome *b*. Sone et al. have recently identified separate genes for cytochrome *b* and subunit IV in a related species, *Bacillus stearothermophilus* (28). It is of considerable evolutionary and functional interest whether a group of nonphotosynthetic bacteria such as *Bacillus* species contain a complex of the b_6f type. *Bacillus subtilis* contains several *c*-type cytochromes and a cytochrome *c* oxidase (36). The presence of a menaquinone:cytochrome *c* reductase in *B. subtilis* is therefore predicted but has not yet been demonstrated. In the course of studies of σ^F-directed transcription during sporulation of *B. subtilis*, we have identified an operon which appears to encode core subunits of a quinol:cytochrome *c* reductase. The characterization of this locus is described here.

MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strain used was DH5α [F^- *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 ϕ80*dlacZ* Δ*M15*]. The *B. subtilis* strains used are listed in Table 1. Strain SL5651, which carries the *erm* (erythromycin resistance) gene replacing much of *amyE*, was kindly provided by Margaret Karow (Temple University School of Medicine). The *erm* gene of SL5651 was replaced by a *qcr-lacZ* fusion in strain SL6635. The substitutions of a *neo* (neomycin resistance) gene for part of *qcrA* and for part of *qcrA* and the *qcr* promoter region in strains SL6579 and SL6580, respectively, were confirmed by Southern blot analysis. When appropriate, *B. subtilis* strains were grown in the presence of antibiotics at the following concentrations: chloramphenicol, 5 μg/ml; neomycin, 5 μg/ml in agar and 0.25 μg/ml in broth; and erythromycin, 1 μg/ml.

Plasmids. All plasmids were maintained in *E. coli* DH5α. All constructions were checked by analyzing digestion patterns obtained with appropriate endonucleases and by sequencing the junction regions between insert and vector. The plasmids constructed for this study are listed in Table 1, and the *qcr* regions contained within the plasmids are illustrated in Fig. 1. Plasmid pPP207 is a derivative of the integrative *lacZ* transcriptional fusion vector pJM783 (5) containing a polylinker 5′ to *lacZ*. A clone bank of *B. subtilis* DNA inserted at the *Bam*HI site of the polylinker in pPP207 was constructed from a partial *Sau*3AI digest of DNA from strain BR151. Plasmid pPP433 was isolated from this clone bank as showing reduced β-galactosidase activity in strain SL4342 when σ^F was induced from the P_{spac} promoter by addition of isopropyl-β-D-thiogalactopyranoside (IPTG). Plasmid pPP433 contains a 1,135-bp *Sau*3AI fragment extend-

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TABLE 1. *B. subtilis* strains and plasmids used

Strain or plasmid	Relevant genotype or description[a]	Source or reference[b]
Strains		
1A1	*trpC2*	BGSC
BR151	*trpC2 lys3 metB10*	F. E. Young
JH642	*trpC2 phe1*	J. A. Hoch
LUH179	*trpC2* Δ*qcr1*::*neo*	SL6579→1A1
LUH180	*trpC2* Δ*qcr2*::*neo*	SL6580→1A1
LUH680	*trpC2 met ade* Δ*ctaCD*::*ble* Δ*cccA*::*cat* Δ*qcr*::*neo*	SL6580→LW5610
LW5610	*trpC2 met ade* Δ*ctaCD*::*ble* Δ*ccc*A::*cat*	33
SL1013	*trpC2 lys3 spoIIAA69*	17
SL3902	*spo0A12 abrB15 trpC2 phe1*	J. A. Hoch
SL4342	*spoIIIG*Δ*1 trpC2 phe1* P_{spac}-*spoIIAC*	RS217 (24)
SL4675	*spo0A*::*erm trpC2 phe1*	A. Grossman
SL5433	*trpC2 lys3 metB10* Δ*spoIIG*::*neo*	25
SL5651	Δ*amyE*::*erm trpC2 lys3 metB10*	M. L. Karow
SL6579	Δ*qcr1*::*neo trpC2 lys3 metB10*	pPP452→BR151
SL6580	Δ*qcr2*::*neo trpC2 lys3 metB10*	pPP447→BR151
SL6628	*trpC2 lys3 metB10 qcrA*::pPP435	pPP435→BR151
SL6629	*trpC2 lys3 metB10 qcrA*::pPP439	pPP439→BR151
SL6635	*trpC2 lys3 metB10 amyE*::*qcr-lacZ*	pPP449→SL5651
SL6637	*spoIIIG*Δ*1 trpC2 phe1* P_{spac}-*spoIIAC amyE*::*qcr-lacZ*	SL6635→SL4342
SL6640	*spo0A12 abrB15 trpC2 phe1 amyE*::*qcr-lacZ*	SL6635→SL3902
SL6642	*trpC2 lys-3 spoIIAA69 amyE*::*qcr-lacZ*	SL6635→SL1013
SL6643	*spo0A*::*erm trpC2 lys3 metB10 amyE*::*qcr-lacZ*	SL4675→SL6635
SL6649	*trpC2 lys3 metB10* Δ*spoIIIG*::*neo amyE*::*qcr-lacZ*	SL6635→SL5433
Plasmids		
pPP433	1,135-kb *Sau*3AI (−635) *Sau*3AI (+500)	pJM783
pPP435	498-bp *Xba*I (+2) *Sau*3AI (+500)	pJM783
pPP439	234-bp *Dra*I (+266) *Sau*3AI (+500)	pJM783
pPP440	1.5-kb *Hin*dIII (TBD) *Sau*3AI (+500) + 1,579-bp *Xba*I (+2) *Hin*dIII (+1581)	pJM783
pPP447	1.5-kb *neo* inserted between *Nde*I (−325) and *Xba*I (+266)	pJM783
pPP449	825-bp *Nde*I (−325) *Sau*3AI (+500)	pDH32
pPP452	1.5-kb *neo* inserted between *Xba*I (+2) and *Dra*I (+266)	pJM783
pPP453	3.1-kb *Dra*I (+266) *Cla*I (TBD)	pBR322

[a] For the plasmids, the position corresponding to the transcription start site in the sequence (Fig. 1) is indicated in parentheses. TBD indicates that the position is still to be determined. The vector is listed in the final column.

[b] X→Y indicates the donor DNA (X) and the recipient strain (Y) used in the strain construction. BGSC, *Bacillus* Genetic Stock Center.

ing from −635 to +500 (relative to the *qcr* operon transcription start site) (Fig. 1). Plasmids pPP435 and pPP439 were derived from pPP433 via intermediate plasmids; pPP435 contains a 498-bp *Xba*I-*Sau*3AI fragment (+2 to +500) inserted 5′ to *lacZ* in pPP207, and plasmid pPP439 contains a 234-bp *Dra*I-*Sau*3AI fragment (+266 to +500) inserted 5′ to *lacZ* in pJM783.

Regions flanking the initial clone were obtained by chromosome walking, with DNA from a *B. subtilis* BR151 derivative that contains a copy of pPP435 integrated into the *qcr* region of the chromosome. The DNA was digested with *Hin*dIII (which did not cut pPP435), ligated in dilute solution to favor unimolecular reactions, and then transformed into *E. coli* DH5α, selecting for ampicillin resistance (50 μg/ml). Such a protocol favors the recovery of pPP435 together with the flanking chromosomal regions extending to the nearest *Hin*dIII sites. In this way, plasmid pPP440 was obtained (Fig. 1); the method of construction resulted in pPP440's containing a duplication of the *B. subtilis* DNA that was contained in pPP435. A second chromosome walk was taken with DNA from strain BR151::pPP439. In this case, the DNA was digested by *Cla*I and *Stu*I, and the ends were filled in by Klenow fragment before ligation. Because of the choice of enzymes, the walk was downstream only, and the resulting plasmid, pPP453 (Fig. 1), does not contain any duplicated DNA; pPP453 retains the *ori* and *amp* regions of pBR322 that were present in pJM783.

Plasmid pPP449 contains an 825-bp *Nde*I-*Sau*3AI fragment (from −325 to +500) cloned 5′ to *lacZ* in pDH32 (21, 26). Plasmids pPP447 and pPP452 are derivatives of pPP433 in which a 1.5-kb *Bam*HI-*Pvu*II *neo* cassette from pBEST501 (14) replaces the 591-bp *Nde*I-*Dra*I fragment (from −325 to +266) (pPP447) or the 264-bp *Xba*I-*Dra*I fragment (from +2 to +266) (pPP452) (Fig. 1).

Nucleic acid analysis. RNA preparation and primer extension analysis were performed as described previously (38). Northern and Southern blot analyses were performed as described by Sambrook et al. (23); an RNA ladder (G3151; Promega, Madison, Wis.) was used as size standards for Northern blots. DNA preparation and DNA sequencing were performed as described previously (38).

Heme-specific labeling. The strains were grown overnight at 37°C in NSMP supplemented with 2 μM 5-[4-^{14}C]-aminolevulinic acid (51 mCi/mmol). The cultures, 25 ml each, were harvested by centrifugation and washed twice in 50 mM potassium phosphate buffer, pH 8.0. The cell pellet was suspended in 1 ml of the phosphate buffer, and 10 μl of lysozyme (10 mg/ml), 10 μl of DNase (0.6 mg/ml), 10 μl of RNase (0.6 mg/ml), and 5 μl of phenylmethylsulfonyl fluoride (PMSF; 200 mM in ethanol) were added. After 1 h at 37°C, 50 μl of 0.3 M sodium EDTA, pH 7.4, and an additional 5 μl of PMSF were added. Two minutes later, 60 μl of 1 M $MgSO_4$ was added, and the sample was placed on ice. The particulate cell fraction was collected by centrifugation (1 h, 150,000 × *g*, 4°C). The supernatant was discarded, and the pellet was washed once in 1.5 ml of 20 mM sodium MOPS (morpholinepropanesulfonic acid)-Cl buffer, pH 7.4. Finally, the pellet was homogenized in 75 μl of the MOPS buffer and stored frozen at −80°C until used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The particulate material from the labeled bacteria was incubated at 40°C for 30 min or 100°C for 5 min in the presence of SDS and fractionated by SDS-PAGE. After electrophoresis, the gel was incubated in methanol-acetic acid to remove noncovalently bound heme. The gel was then stained for protein with Coomassie brilliant blue R250 and finally treated with salicyclic acid (as an enhancer of fluorescence), dried, and analyzed by autoradiography (35).

Cytochrome *c* absorption spectroscopy. Bacteria were grown in NSMP at 37°C (1 liter per 6-liter baffled E-flask) on a rotary incubator (200 rpm). The cultures were harvested approximately 1 h after the end of exponential growth. Membranes were isolated by a published procedure (8), suspended in 20 mM sodium MOPS-Cl buffer, pH 7.4, and stored at −80°C.

The light absorption difference (reduced minus oxidized) spectra of isolated membranes were recorded at room temperature with a Shimadzu UV3000 spectrophotometer (double-beam mode; slit, 1 nm; scan speed, 100 nm/min; cuvette light path, 10 mm). The spectra were recorded at a membrane protein concentration of 4 mg/ml, and 1 mM KCN was included in the MOPS buffer. Membranes were oxidized with 1 mM $K_3Fe(CN)_6$ and reduced with 8 mM sodium ascorbate, pH 6.5, or by the addition of solid dithionite.

Conditions for growth and sporulation. NSMP medium was as described by Fortnagel and Freese (6). Other media have been described previously (25). Growth was monitored by measuring the optical density at 600 nm (OD_{600}) and converting it to bacterial dry weight by using a standard calibration curve. Sixteen hours after the end of exponential growth, cultures were analyzed for sporulation with a phase-contrast microscope.

β-Galactosidase assays. Samples were assayed with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate as described by Nicholson and Setlow (20). Specific β-galactosidase activity is expressed as nanomoles of ONPG hydrolyzed per minute per milligram (dry weight) of bacteria.

GenBank nucleotide sequence accession number and nomenclature. The DNA sequences reported here have been submitted to GenBank and given accession number U25535. Genes for polypeptides of cytochrome bc_1 or b_6f complexes are generally called *pet* (for petite, which refers to yeast colonies) or *fbc* (for iron, cytochrome *b*, cytochrome *c*). The *B. subtilis* complex is not a classical bc_1 or b_6f complex with respect to the subunit organization, and we have named the genes for the *B. subtilis* complex *qcr*, for quinone:cytochrome *c* reductase.

RESULTS

Cloning and sequencing of the *qcr* operon. From a clone bank of *B. subtilis* DNA, plasmid pPP433 was identified as containing a promoter that displayed reduced expression in *B. subtilis* SL4342 when expression of σ^F was artificially induced. The plasmid was found to contain the 5′ end of an open reading frame (ORF) for a protein showing similarity to genes for Rieske iron-sulfur proteins. Subclones from pPP433 were used to clone the flanking regions. Southern blot analysis indicated that no gross rearrangements had occurred during cloning (data not shown). A region of 2.4 kb was sequenced in both directions by the strategy shown in Fig. 1. The DNA sequence is shown in Fig. 2, together with the predicted amino

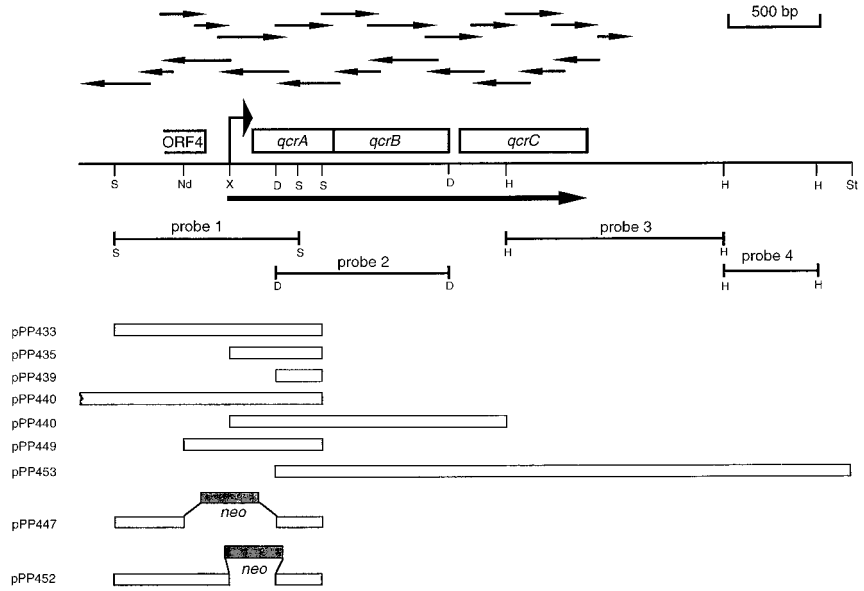

FIG. 1. Restriction map of the *qcr* region of the *B. subtilis* chromosome, showing the segments present in various plasmids. ORFs are shown as rectangles immediately above the restriction map. The sequencing strategy used is indicated at the top of the figure. The thick arrow below the restriction map indicates the likely extent of the 2.0-kb transcript. The bars below the restriction map show the probes used in Northern blot analysis and screening of the YAC library. Open rectangles at the bottom of the figure indicate the regions present in different plasmids. Restriction sites are indicated as follows: S, *Sau*3AI; Nd, *Nde*I; X, *Xba*I; D, *Dra*I; H, *Hin*dIII; St, *Stu*I.

acid sequences of four large ORFs that were identified in the region.

Three of the ORFs were found to be in an operon (see below). The first gene of the operon, designated *qcrA*, potentially encodes a 167-residue protein of 18.7 kDa. The initiating ATG codon is preceded by a potential ribosome-binding site site (RBS), with a ΔG of -16.4 kcal/mol, calculated by the rules of Tinoco et al. (31) and with the *B. subtilis* 16S rRNA sequence (19). The QcrA protein shows 21 to 34% identity and 43 to 55% similarity to Rieske iron-sulfur proteins in cytochrome bc_1/b_6f complexes. Furthermore, it contains the cysteines (Cys-100, Cys-105, and Cys-121) and histidines (His-102 and His-124) in an arrangement that is conserved in all such proteins (32). Residues Cys-105, Cys-121, His-102, and His-124 are the most likely ligands to the [2Fe-2S] cluster.

The second gene, designated *qcrB*, potentially encodes a product of 224 amino acid residues (25.4 kDa) and is preceded by a potential RBS (ΔG, -19.6 kcal/mol). The QcrB protein is similar in size to the cytochromes b_6 from cytochrome b_6f complexes (these vary in size from 215 to 232 residues) and shows 44 to 47% identity and 69 to 72% similarity to them. It also shows similarity to the N-terminal part of cytochromes *b* from cytochrome bc_1 complexes (33 to 40% identity and 59 to 68% similarity). This is illustrated as a dendrogram (Fig. 3). All invariant residues (4) are conserved in *B. subtilis* QcrB. QcrB is 92% identical to the 224-residue cytochrome *b* of *B. stearothermophilus* (28).

The third gene, designated *qcrC*, potentially encodes a protein whose structure is related to but distinct from that of known proteins from the cytochrome bc_1/b_6f family. The putative protein contains 255 residues and has a predicted size of 28 kDa; the *qcrC* ORF is preceded by a potential RBS with a ΔG of -23.8 kcal/mol. The QcrC N-terminal region of about 170 residues is hydrophobic and is similar to the 17-kDa subunit IV of known cytochrome b_6f complexes (21 to 33% identity and 51 to 57% similarity) and to the C terminus of cytochromes *b* from cytochrome bc_1 complexes (15 to 26% identity and 45 to 54% similarity). The C-terminal region (85 residues) of QcrC contains the CxyCH heme-binding amino acid motif that is found in all *c*-type cytochromes, including cytochrome *f* and cytochrome c_1 (18). It shows extensive similarity to cytochrome *c*-550, encoded by *cccA* of *B. subtilis* (35), and to other small *Bacillus* cytochromes *c* (Fig. 4 and Fig. 5A). There is a potential transcription termination structure (ΔG, -26.6 kcal/mol) located immediately downstream from *qcrC*, at positions 2418 to 2442 (Fig. 2).

There is a fourth ORF, located upstream of *qcrA*, which was not fully sequenced. It was encoded by a separate transcript and showed no similarity to proteins in the database.

Analysis of the RNA transcript of the *qcr* operon. RNA was extracted from *B. subtilis* BR151 at the end of exponential growth in modified Schaeffer's sporulation medium (MSSM) (25) and analyzed by Northern blotting with probes for four different parts of the *qcr* region (Fig. 1). Probes 1, 2, and 3 hybridized to a 2.0-kb transcript (Fig. 6). The size and the hybridization pattern obtained agree with the predicted size of a transcript encompassing *qcrA*, *qcrB*, and *qcrC*. Probes 2 and 3 also detected a band of about 750 bases; we think that it is a breakdown product of the 2.0-kb band but have not investigated it further. There was no indication of any larger transcript. The 2.0-kb band (and the 750-base band) was not detected with probe 4, which corresponds to a region downstream of *qcrC*. There were differences in the efficiencies of hybridization with the different probes, and so the failure to detect the 2.0-kb band was not necessarily significant in itself. However, probe 4 did detect a distinct 950-base band. Thus, there is a qualitative difference in the bands detected by probe 4 and by probes 1 through 3.

From the sequence information and Northern blot analysis, it seemed likely that the 5′ end of the *qcrABC* transcript would be located near the 5′ end of *qcrA*. Primer extension analysis was used to test this (Fig. 7). The size of the reverse transcript obtained with a 19-mer primer (5′ CCTCCTACGCCTGTG AGCG 3′) which corresponded to bases 458 to 440 indicated

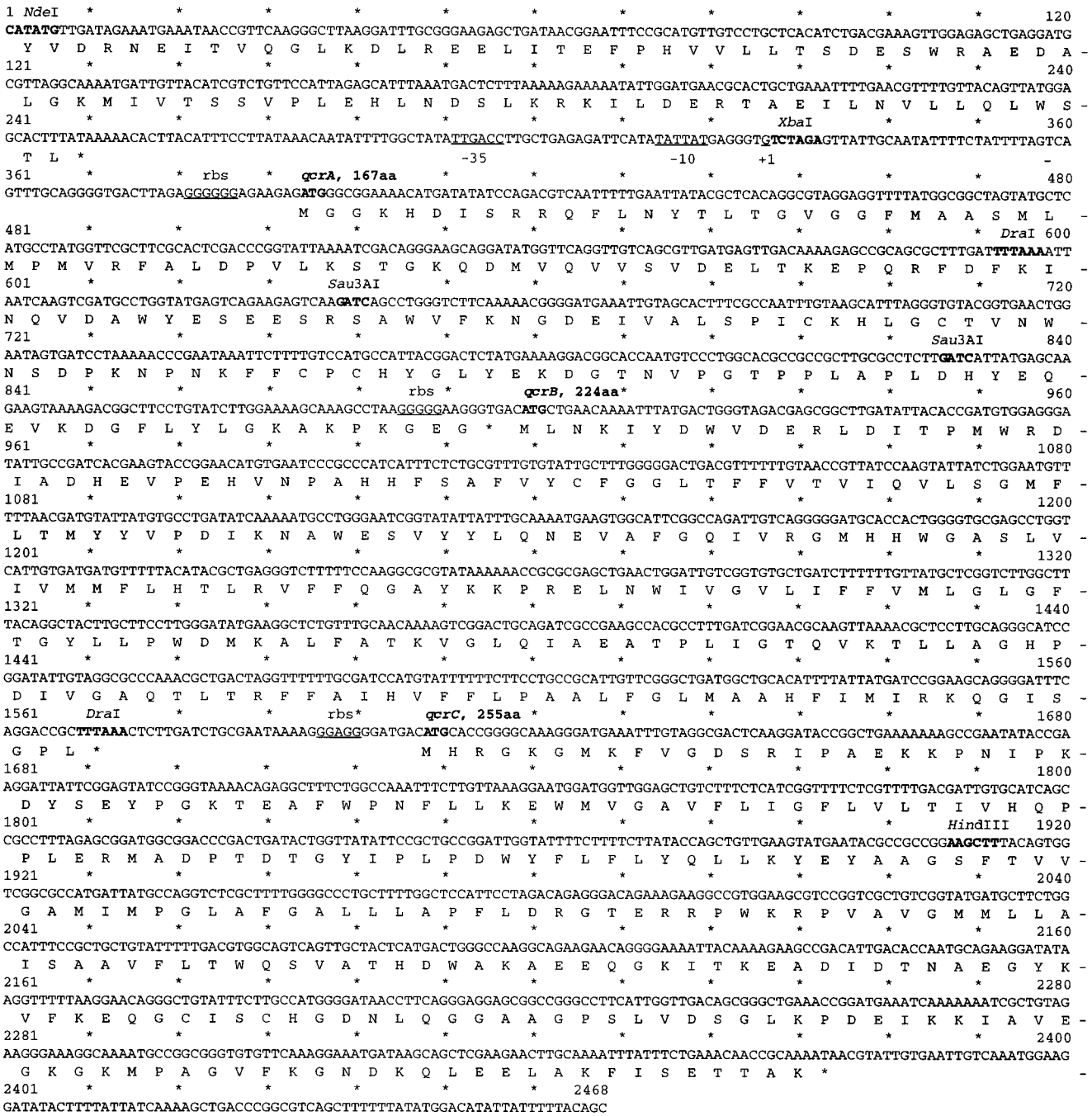

FIG. 2. Nucleotide sequence of the *qcrABC* region. The amino acid (aa) sequences of long ORFs are shown below the nucleotide sequence; the single-letter aa code is used. The probable transcription start site is underlined and is marked, below the sequence, as +1. Putative −35 and −10 regions are also underlined. Possible RBSs for *qcrA*, *B*, and *C* are underlined. Boldface ATG codons indicate the likely start codon for each ORF. Asterisks following the long ORFs indicate stop codons.

that the 5′ end of the message was the G at position 326 of the sequence (Fig. 2). The same 5′ end was identified with a second 23-mer primer (data not shown), which corresponded to bases 406 to 384 in the sequence. Assuming that there was no message processing or degradation, we deduce that the transcription start site is the G at position 326 in the nontranscribed DNA strand.

Phenotype of *qcr* mutants. In order to test for the role of the *qcrABC* operon in *B. subtilis*, two mutants were constructed. Strains SL6579 and SL6580 have part of *qcrA* and part of *qcrA* plus the *qcr* promoter region, respectively, replaced by a *neo* gene. Both of these deletion-replacements are expected to totally block *qcr* gene expression. The two mutants and the parental strain BR151 showed no significant differences in growth rate and reached the same final cell density upon batch cultivation in Luria broth, NSMP, and MSSM.

QcrB and QcrC are predicted from the sequence to be membrane-bound cytochromes of the *b* and *c* types, respectively. Membranes were isolated from the two mutant strains and BR151 grown in NSMP and analyzed for cytochromes by

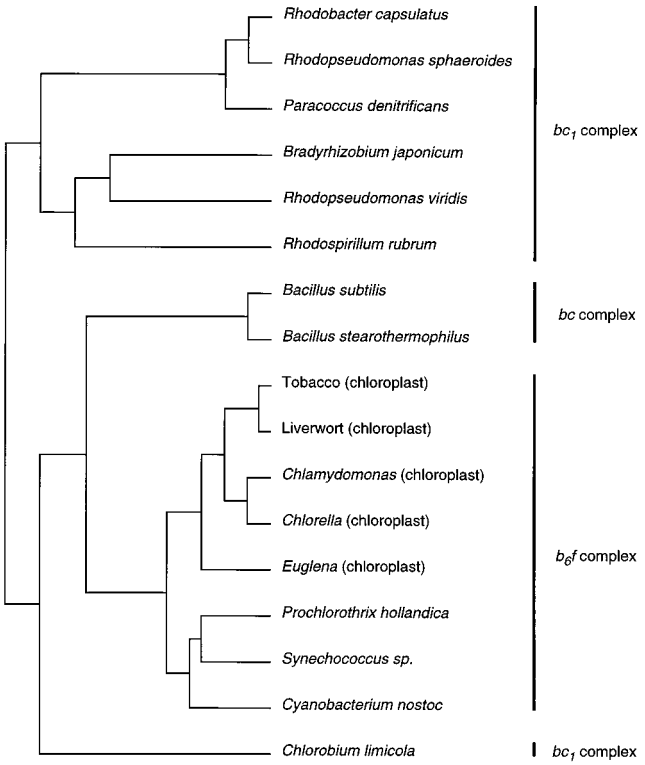

FIG. 3. Dendrogram based on sequence comparisons of QcrB, cytochromes b_6 of b_6f complexes, and the N-terminal part, encompassing the first four transmembrane segments, of cytochromes b in bc_1 complexes.

light absorption spectroscopy. The dithionite-reduced–minus–ferricyanide-oxidized spectrum gives the spectrum of all cytochromes. This spectrum of the mutant membranes did not display significant differences compared with that of BR151 except for a somewhat higher cytochrome *a* content (Fig. 8). Cytochromes of the *c* type constitute only a small amount of total cytochromes in *B. subtilis* grown in NSMP, and the 550- to 560-nm region is dominated by the cytochrome *b*-558 of succinate:menaquinone reductase (10). The ascorbate-reduced–minus–ferricyanide-oxidized spectrum shows, however, primarily high-potential cytochromes: cytochromes *c* at 550 to 560 nm and cytochromes *a* at 600 to 605 nm. This spectrum of membranes isolated from BR151 showed an absorption peak at 550 nm, with a shoulder on the "long-wavelength" side (indicated by an arrow in Fig. 8). The corresponding spectra of SL6579 and SL6580 were similar to the spectrum of BR151 except that the peak at 550 nm was more symmetrical. These results indicate that a minor high-potential cytochrome, probably of the *c* type and with an α-band absorption maximum at about 553 nm, is absent in the *qcr* mutants. From the spectral analysis, we cannot draw any conclusions regarding heme in QcrB. The spectrum of this presumptive cytochrome *b* would make only a small contribution to the peak at about 560 nm in dithionite-reduced membranes and would be masked by the absorption peak of cytochrome *b*-558.

Cytochrome *c* composition of *qcr* mutants. Cytochromes of the *c* type differ from other cytochromes in that they have covalently bound heme. Strains SL6579, SL6580, and BR151 were grown in the presence of 5-[4-^{14}C]-aminolevulinic acid to label heme specifically. Isolated membranes were subjected to SDS-PAGE. After electrophoresis, the gel was washed to remove noncovalently bound heme and then exposed to X-ray film. The resulting autoradiograph is shown in Fig. 9A. Strain BR151 contained three major cytochromes *c* of 16, 22, and 36 kDa that were described previously for *B. subtilis* (35). The 29-kDa cytochrome *c* that was present in *B. subtilis* 3G18 (33) was not apparent in extracts from strain BR151. The 16-kDa and 36-kDa cytochromes both have α-band absorption maxima of 550 nm and are cytochrome *c*-550 (CccA) and subunit II (CtaC) of the caa_3 oxidase, respectively (36). The 22-kDa cytochrome has not been identified.

The *qcr* mutant strains lacked the 22-kDa polypeptide that was present in BR151 (Fig. 9A); this band was not seen even when the autoradiographs were overexposed. The 16-kDa cytochrome *c*-550 band was of variable intensity in different preparations but was also present in the mutants. Cytochrome *c*-550 is known to be very prone to proteolytic degradation (37), and this is most probably the reason for the variation. In agreement with this explanation, a heme-containing fragment of about 10 kDa is seen when the intensity of the 16-kDa band is low, as in the case of the SL6580 sample.

Two other *B. subtilis* strains, 1A1 and LW5610, were also tested. The 1A1 strain gave a heme-labeling pattern similar to that of BR151 (Fig. 9B) except that a faint 29-kDa band was present. Disruption of *qcr* in strain 1A1 led to loss of the 22-kDa band (strains LUH179 and LUH180 [Fig. 9B]), as with strain BR151, and also to loss of the 29-kDa band. There was no indication of instability of the 16-kDa band in the 1A1 group of strains. Strain LW5610 is a derivative of 3G18 in which the structural genes for the 16- and 36-kDa cytochromes *c* have been deleted (33). Strain LW5610 produces the 29-kDa band as well as the 22-kDa band but not the other bands. Disruption of *qcr* in LW5610 (LUH680) led to the loss of the 22-kDa band and also to loss of the 29-kDa band (Fig. 9B). Unexpectedly, a weak, novel band moving slightly faster than the

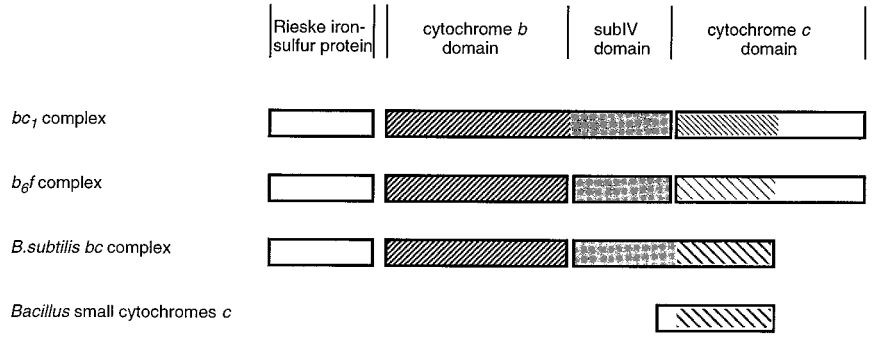

FIG. 4. Schematic illustration of domain organization in proteins of cytochrome bc_1, b_6f, and *bc* complexes and of *Bacillus* small cytochromes *c*.

A.

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B.az c-552 (10) DPEGMVKASCASCHGQNLEGG.VGPALAD.............VGSRNAMPAGMVDPAK(53)
                        |  |||    |    |                      || |
B.az C-555 (1)     ALEASGCLNCHGTDLTGXPAXP.....................ATMPPGMFKGSN(34)
                        |  |||    |    |                      || |
PS3  c-551 (43) AEQIFKQN.CASCHGQDLSGG.VGPNLQKVGSKYSKDEIKNIIANGRGAMPAGIIKGED(97)
                        |  |||    |    | |  |       ||       |  || |
B.li c-552 (1)  GEEIYQQN.CTGCHGKDLAGG.SAPSLKEVGGKYKESEIKDIVVNGRGGMP.GNLVDEK(56)
                        |  |||    |    | |  |       ||       |  || |
B.su c-550 (52) PEEIVKAN.CIACHGENYEGV.SGPSLKGVGDKKDVAEIKTKIEKGGNGMPSGLVPADK(108)
                        |  |||    |    | |  |       ||       |  || |
B.st PetD  (183)GYKIAQANTCTSCHGENLSGG.AGPSLVGTGLT--AEEIAKIAKQGQGSMPGGIFKGTD(238)
                        |  |||    |    | |  |       ||       |  || |
B.su QcrC  (183)GYKVFKEQGCISCHGDNLQGGAAGPSLVDSGLK--PDEIKKIAVEGKGKMPAGVFKGND(239)
                        *  **                                  *
```

B.

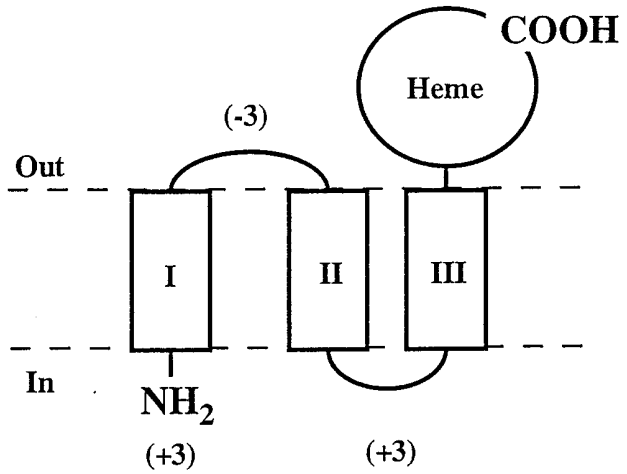

FIG. 5. (A) Alignment of *Bacillus* cytochrome *c* sequences. The positions of gaps were adjusted by using the Pileup and BestFit Program of the Wisconsin Genetics Computer Group package. Vertical bars show identical residues in all sequences. Heme ligands are indicated by stars. Extreme N-terminal and C-terminal parts are omitted. B.az, *Bacillus azotoformans* (13); PS3, *Bacillus* sp. strain PS3 (29); B.li, *Bacillus licheniformis* (13); B.st, *B. stearothermophilus* (27, 28); B.su, *B. subtilis* (35). (B) Predicted topology of QcrC in the *B. subtilis* membrane. The calculated net charge of each predicted hydrophilic loop is indicated.

16-kDa cytochrome *c* was detected with LW5610 and LU680. It may correspond to a hitherto undetected cytochrome *c*.

The results suggest that the 22-kDa polypeptide and/or the 29-kDa polypeptide corresponds to QcrC, since these were the (presumptive) cytochromes *c* that are affected by *qcr* mutations. That the heme-labeled bands survive boiling for 5 min (Fig. 9B) reinforces the conclusion that they are *c*-type cytochromes, with the heme covalently joined to the cytochrome. The relationship of the 29-kDa protein to the 22-kDa protein remains to be established. The 29-kDa protein was not detected in all Qcr^+ strains, but both proteins were always missing from Qcr^- strains. The C terminus of QcrC contains the heme-binding domain, and we suspect that the 29-kDa polypeptide is processed at its N terminus to yield the 22-kDa polypeptide. As the *neo* insertions at the 5′ end of *qcrA* (Fig. 1) prevent the formation of both polypeptides, it is unlikely that the 22-kDa polypeptide results from a distinct, truncated transcript; sequence analysis gave no indication of an RBS and start codon that might account for the 22-kDa polypeptide. The deduced mass of QcrC, 28 kDa without heme C, is similar to the size of the 29-kDa band, although the hydrophobic portion of QcrC could affect its mobility on SDS-PAGE.

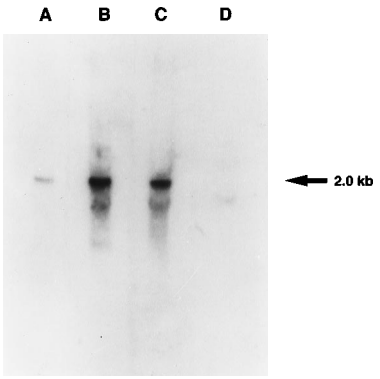

FIG. 6. Northern blot analysis of the *qcr* region of the chromosome. After denaturation, 20-μg samples of RNA extracted from *B. subtilis* BR151 were loaded into each of four wells on an agarose-formaldehyde gel; an additional well contained an RNA size ladder. The gel was subjected to electrophoresis (5 V/cm). After electrophoresis, the RNA was transferred to a nylon membrane. The nylon membrane with the RNA ladder was cut out and stained with methylene blue. The remaining nylon membrane was cut into four slices, with one RNA sample on each. Then these nylon membrane slices, labeled A, B, C, and D, were hybridized with four radiolabeled probes (probes 1, 2, 3, and 4, respectively, as indicated in Fig. 1; 1.2×10^6, 0.5×10^6, 1.8×10^6, and 1.6×10^6 cpm, respectively). The 950-base band detected with probe 4 was apparent as a faint band in lane D of the original autoradiograph but is difficult to see here.

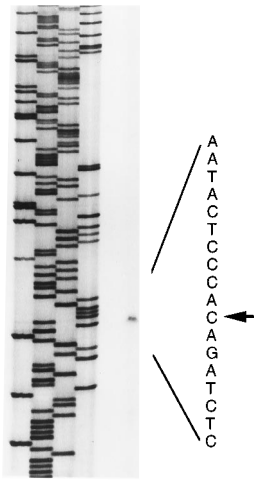

FIG. 7. Determination of the 5′ end of the *qcr* mRNA by primer extension analysis with a 19-mer primer. RNA extracted from *B. subtilis* BR151 at the end of exponential growth was used. A sequencing ladder with the same primer and pPP438 is also shown. The letters above the lanes indicate which dideoxynucleotide was used to terminate the sequencing reaction; lane P shows the primer extension product. The sequence indicated is of the nontranscribed strand and is the complement of the sequence that can be read from the sequencing ladder. The transcription start site is indicated by an arrow.

Mapping of the *qcr* operon. The *B. subtilis* YAC library of Azevedo et al. (3) and probe 2 (Fig. 1) were used to map the *qcr* operon on the genome. The library was arranged in pools so that each clone was uniquely present in two pools (15). Three different pools hybridized to the probe, and we identified two overlapping clones, 15-6B and 11-501 (3), as containing the *qcr* operon. This region is located at about 200° on the *B. subtilis* genetic map (2).

Transcription regulation. A transcriptional fusion to *lacZ* of the −325 to +500 (relative to the transcription start point) region of the *qcr* operon was used to analyze the regulation of *qcr* transcription. The fusion displayed a strong postexponential induction in both sporulation (MSSM) (strain SL6635, Fig. 10A) and nonsporulation (Luria broth) media (Fig. 10B). There was a subsequent reduction in β-galactosidase activity in the sporulation medium but not in the nonsporulation medium. The postexponential induction of *qcr-lacZ* expression was blocked by mutation in the *spo0A* locus (strain SL6643 [Fig. 10]). This block was largely overcome by an *abrB* mutation (strain SL6640). A mutation in *spoIIAA*, which prevents σ^F activation (24), did not prevent the postexponential induction of *qcr-lacZ* but did prevent the subsequent decline in expression that ordinarily occurred in sporulation medium (strain SL6642 [Fig. 10A]); mutation in *spoIIIG*, which blocks sporulation after σ^F activation, had little effect on either phase of *qcr-lacZ* expression (strain SL6649 [Fig. 10A]).

The *qcr* locus was originally identified as a locus whose expression was reduced by artificially inducing σ^F expression. This is illustrated in Fig. 11 with strain SL6637, in which the *qcr-lacZ* fusion was inserted at *amyE* and the σ^F structural gene, *spoIIAC*, was placed under the control of the IPTG-inducible P_{spac} promoter (24, 39). Addition of IPTG at the end of exponential growth prevented the postexponential induction of *qcr-lacZ* expression that occurred in a control culture to which IPTG had not been added. This effect of IPTG addition was seen in both a sporulation (Fig. 11A) and a nonsporulation (Fig. 11B) medium.

DISCUSSION

The sequence of the *B. subtilis qcrABC* operon suggests that it codes for proteins of a respiratory complex which is similar to cytochrome bc_1 and b_6f complexes. These complexes catalyze the reduction of a cytochrome *c* or plastocyanin by quinol. QcrA, 18.7 kDa, is clearly similar to Rieske-type iron sulfur proteins. QcrB, 25.4 kDa, is similar to cytochromes *b* of bc_1 and b_6 of b_6f complexes, and it is similar in size to cytochromes b_6

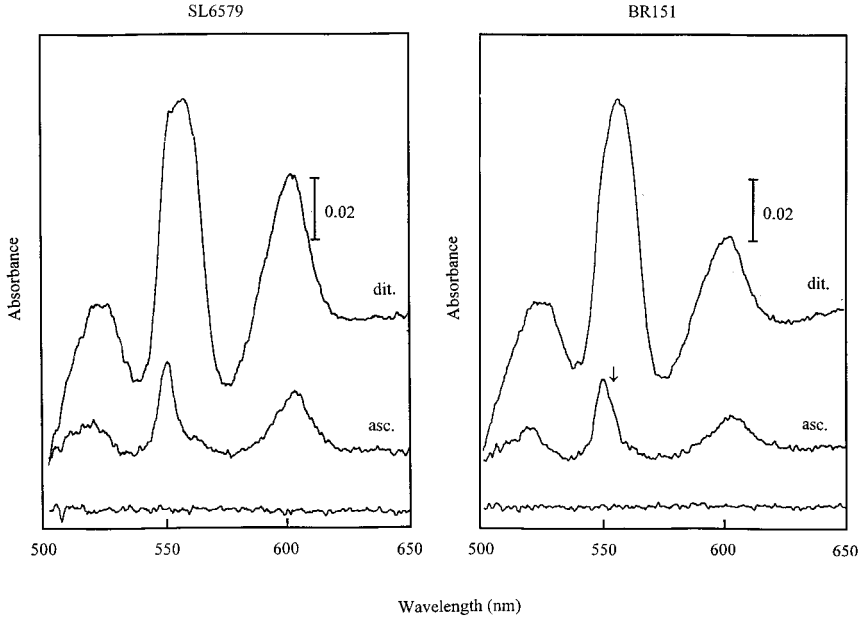

FIG. 8. Light absorption spectra of isolated membranes from *B. subtilis* BR151 and SL6579. The bottom trace shows the base line which was recorded first. asc., ascorbate-reduced–minus–ferricyanide-oxidized difference spectrum; dit., dithionite-reduced–minus–ferricyanide-oxidized spectrum. The spectra of membranes isolated from SL6580 were similar to those of membranes from SL6579.

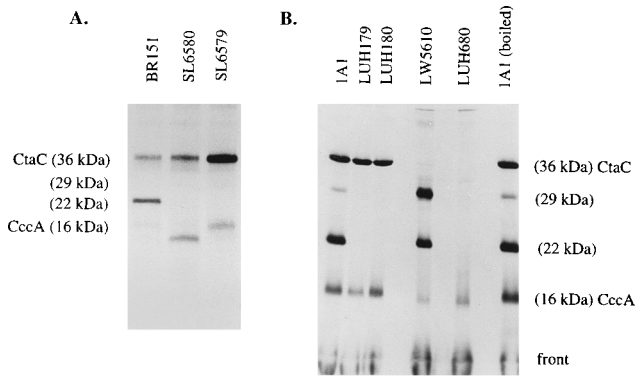

FIG. 9. Determination of cytochrome *c* composition by heme-specific labeling. *B. subtilis* strains were grown in NSMP medium supplemented with 5-[4-^{14}C]-aminlevulinic acid. Isolated membranes were analyzed by SDS–10% PAGE in the 10% Schägger-von Jagow buffer system (A) or on a 10 to 15% polyacrylamide gradient in the Neville buffer system (B). Thirty-six micrograms of protein was loaded in each lane. After electrophoresis, the gel was incubated with methanol-acetic acid and treated with salicylic acid. An autoradiograph of the dried gel is shown. (A) Membrane preparations from strains BR151, SL6579, and SL6580. (B) Membrane preparations from strains 1A1, LUH179, LUH180, LW6510, and LUH680.

of chloroplasts and cyanobacteria. Furthermore, it is almost identical to the cytochrome *b* of *B. stearothermophilus* menaquinol:cytochrome *c* reductase (28). QcrC, 28 kDa, is a novel protein composed of two distinct domains (Fig. 4). The N-terminal part of QcrC is similar in size and sequence to subunit IV of b_6f complexes. This sequence shows three putative transmembrane segments. The C-terminal part of QcrC has the CxyCH motif which is characteristic of cytochromes *c*; the heme is covalently bound via thioether linkages to the cysteines and with the histidine as an axial ligand to heme iron. The sequence of the cytochrome *c* domain in QcrC is distinct from that of both cytochromes c_1 and *f*. Sequence comparisons show extensive similarity of the C-terminal part of QcrC to small cytochromes *c* from *Bacillus* spp., including the *B. subtilis* 16-kDa cytochrome *c*-550, belonging to class I, group 6, as suggested by Sone and Toh (29). The sequence comparisons suggest that His and Met are the ligands axial to heme iron in QcrC. The likely membrane topology of the QcrC protein, based on hydropathy plots, the positive-inside rule (34), and the fact that cytochrome *c* domains are always on the outside of the cytoplasmic membrane in bacteria, is illustrated in Fig. 5B. This topology is distinct from that of cytochromes c_1 and *f*, which are anchored to the membrane by C-terminal transmembrane segments (32).

In summary, the sequence of the *qcrABC* operon suggests that *B. subtilis* has a three-subunit menaquinol:cytochrome *c* reductase. The cytochrome *b* subunit is similar to that of b_6f complexes. However, the organization of QcrC and the sequence of its cytochrome *c* domain make us suggest that the *B. subtilis* complex encoded by the *qcr* operon be called a *bc* complex, with no subscript. The differences between the proteins of the various bc_1 and b_6f complexes and those of the *bc* complex (Fig. 4) appear to be an example of protein domaing swapping.

Sone et al. (28) have reported the sequence of a PCR fragment encoding a portion of the *bc* complex from *B. stearothermophilus* and showed that the gene for cytochrome *b* was separated from that for domain IV. They reported the sequence encoding the complete cytochrome *b* and the domain IV portions of the complex. This sequence is very similar to that reported here for *B. subtilis*. The published *B. stearothermophilus* sequence extends only a short distance 5′ and 3′ of the regions coding for cytochrome *b* and domain IV, respec-

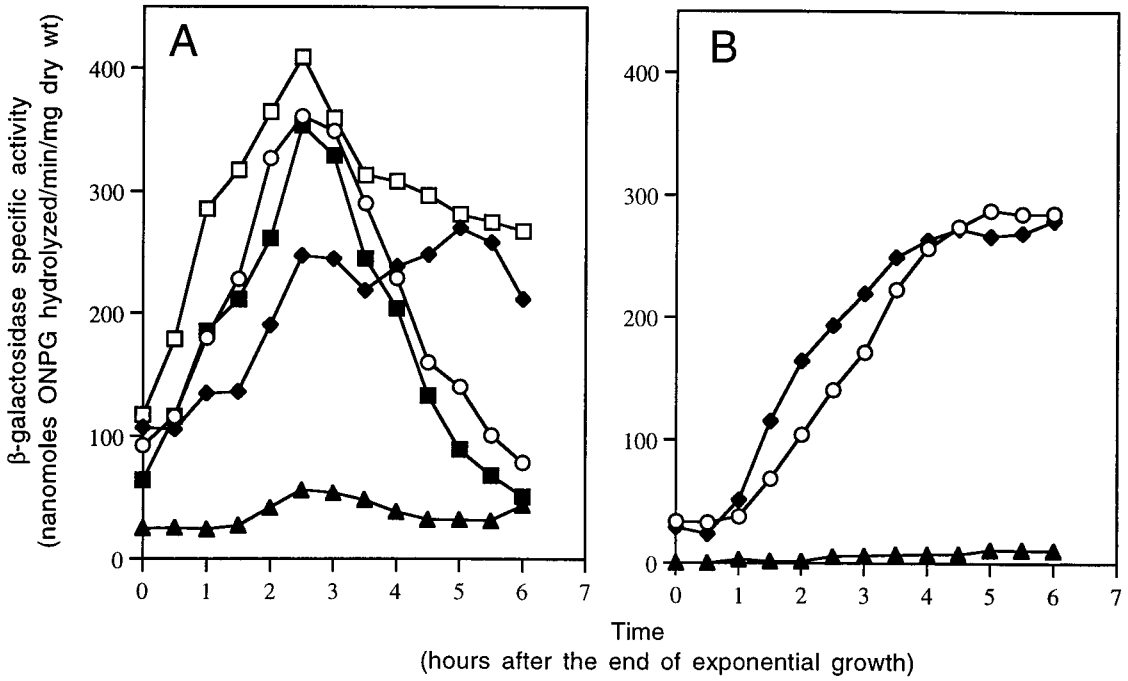

FIG. 10. Expression of a *qcr-lacZ* transcriptional fusion in wild-type and mutant backgrounds. Strains were cultured in (A) MSSM or (B) LB. Fifty microliters of sample was taken at the indicated time after the end of exponential growth for the determination of β-galactosidase activity. The *qcr-lacZ* fusion was contained in strains that were *spo*$^+$, SL6635 (○); *spo0A*::*erm*, SL6643 (▲); *spo0A12 abrB15*, SL6640 (◆); *spoIIAA69*, SL6642 (□); or *spoIIIG*Δ::*neo*, SL6649 (■). Strains were derivatives of BR151 except for SL6640, which was a derivative of JH642.

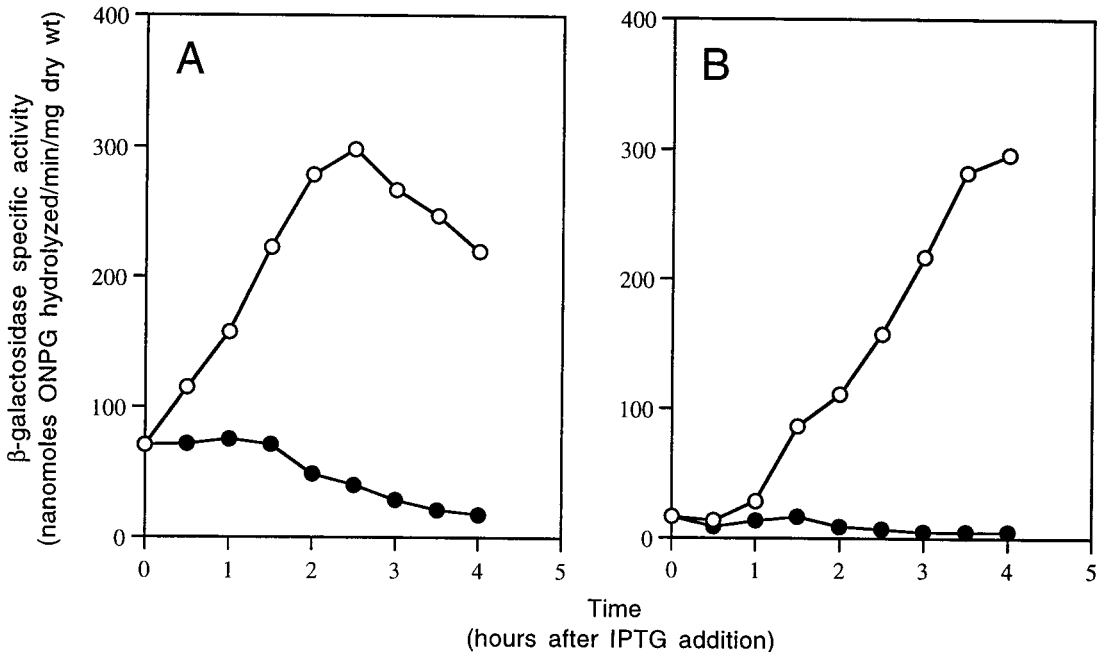

FIG. 11. Effect on *qcr-lacZ* expression of an IPTG-inducible σ^F in (A) MSSM and (B) LB medium. Values are shown for cultures with (●) and without (○) IPTG added. Strain SL6637 (*amyE*::*qcr-lacZ*) that carried a P_{spac}-*spoIIAC* fusion was used.

tively. Sone and coworkers (27) have recently cloned and sequenced the flanking regions and reexamined the published sequence. The gene organization and sequences of polypeptides encoded by these regions are very similar to those reported here for *B. subtilis*.

Analysis of membranes showed that a high-potential cytochrome with an α-band absorption maximum at about 553 nm and the presence of 29-kDa and 22-kDa cytochrome polypeptides are dependent on the intact *qcr* operon. We conclude that the 29-kDa polypeptide and/or the 22-kDa polypeptide is a cytochrome of the *bc* complex. The binding of heme to these polypeptide cytochromes resists boiling, and we conclude that the cytochromes are of the *c* type. We suggest that the 29-kDa polypeptide and/or the 22-kDa polypeptide is the product of *qcrC*. We have been unable to construct a strain with only *qcrC* disrupted in order to confirm this conclusion. It should be noted that Kutoh and Sone (16) purified a quinol:cytochrome *c* reductase from *Bacillus* sp. strain PS3 (which is similar to *B. stearothermophilus*) and reported that the cytochrome *c* from the complex migrated at 29 kDa on SDS-PAGE.

Northern blot analysis indicates that *qcrA*, *qcrB*, and *qcrC* are transcribed as an operon. Transcription of the operon is greatly increased as bacteria enter the postexponential transition state. The increase in transcription is blocked by a *spo0A* mutation, and this block is overcome by an *abrB* mutation. Thus, in common with other transition state events, the increase in transcription appears to be dependent on the downregulation of *abrB* expression that is mediated by Spo0A activation (12). It is of interest that other genes associated with respiration, such as those for menaquinone biosynthesis and for the citric acid cycle, are also induced postexponentially (9, 30).

As bacteria move from the transition state into sporulation proper, transcription of the *qcr* operon is reduced. This reduction did not occur in a medium that did not support sporulation. It appears to be dependent on the activity of σ^F, as it is blocked by a mutation that blocks σ^F activation. More strikingly, premature activation of σ^F prevents the postexponential induction of *qcr*. The mechanism by which σ^F curtails *qcr* transcription is not clear. However, there are interesting implications for subsequent sporulation events. σ^F is active in the prespore and not in the mother cell (7). Thus, it seems plausible that *qcr* transcription and therefore, speculatively, ATP synthesis might be downregulated in the prespore and not in the mother cells. As σ^F is activated by a reduction in the ATP/ADP ratio (1), the reduction in *qcr* transcription could help "fix" σ^F activation in the prespore. Disruption of the *qcr* operon reduced the frequency of sporulation to 50% of that of the parent strain (unpublished observations), suggesting that the Qcr complex might have some role in sporulation, although the nature of that role must remain speculative.

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