JOURNAL OF BACTERIOLOGY, Dec. 2000, p. 6557–6564 Vol. 182, No. 23
0021-9193/00/$04.00+0

Terminal Oxidases of *Bacillus subtilis* Strain 168: One Quinol Oxidase, Cytochrome aa_3 or Cytochrome *bd*, Is Required for Aerobic Growth

LENA WINSTEDT AND CLAES VON WACHENFELDT*

Department of Microbiology, Lund University, Lund, Sweden

Received 26 June 2000/Accepted 8 September 2000

The gram-positive endospore-forming bacterium *Bacillus subtilis* has, under aerobic conditions, a branched respiratory system comprising one quinol oxidase branch and one cytochrome oxidase branch. The system terminates in one of four alternative terminal oxidases. Cytochrome caa_3 is a cytochrome *c* oxidase, whereas cytochrome *bd* and cytochrome aa_3 are quinol oxidases. A fourth terminal oxidase, YthAB, is a putative quinol oxidase predicted from DNA sequence analysis. None of the terminal oxidases are, by themselves, essential for growth. However, one quinol oxidase (cytochrome aa_3 or cytochrome *bd*) is required for aerobic growth of *B. subtilis* strain 168. Data indicating that cytochrome aa_3 is the major oxidase used by exponentially growing cells in minimal and rich medium are presented. We show that one of the two heme-copper oxidases, cytochrome caa_3 or cytochrome aa_3, is required for efficient sporulation of *B. subtilis* strain 168 and that deletion of YthAB in a strain lacking cytochrome aa_3 makes the strain sporulation deficient.

Aerobic and facultative aerobic bacteria can respond to changes within the environment by using different types of respiratory pathways (3, 23). During aerobic growth, the final step in the pathway, the four-electron reduction of dioxygen to two water molecules, is catalyzed by a group of membrane-bound enzymes called terminal oxidases. Many bacteria use more than one terminal oxidase (1, 3, 29). For example in the gram-negative bacterium *Escherichia coli*, there are two types of terminal oxidases—cytochrome bo_3 and cytochrome *bd*. The former is used under aerobic growth conditions, whereas the latter is induced under microaerobic conditions (7, 30). In the soybean symbiont bacterium *Bradyrhizobium japonicum*, the main terminal oxidase under free-living conditions is an aa_3-type cytochrome *c* oxidase (20, 21). When *B. japonicum* lives endosymbiotically, it uses a cbb_3-type oxidase. This terminal oxidase has an extremely high affinity for oxygen, which allows it to operate under the low oxygen pressure of the root nodules (21). Another example is from the obligately aerobic, nitrogen-fixing bacterium *Azotobacter vinelandii*, which has two known terminal oxidases, a cytochrome bo_3 and a cytochrome *bd*. In *A. vinelandii*, cytochrome *bd* with its high oxygen affinity protects the oxygen-labile nitrogenase by keeping the oxygen levels sufficiently low (19). The gram-positive endospore-forming soil bacterium *Bacillus subtilis* synthesizes under aerobic growth conditions a branched electron transport chain comprising three or possibly four terminal oxidases (Fig. 1) (33, 34). The physiological role(s) of the specific terminal oxidases in *B. subtilis* is unknown. The long-term objective of our work is to define the physiological roles of the terminal oxidases in *B. subtilis*.

The electron transport chain in *B. subtilis* contains two major branches, one quinol oxidase branch and one cytochrome oxidase branch (Fig. 1). Three known terminal oxidases are present. Cytochrome caa_3 is a cytochrome *c* oxidase, whereas cytochrome aa_3 and cytochrome *bd* are quinol oxidases (16, 34). Both *a*-type oxidases belong to the well-characterized heme-copper oxidase superfamily of respiratory oxidases (4, 6, 34). Characteristic for the bacterial heme-copper oxidases is that they have a subunit homologous to subunit I of the mitochondrial cytochrome *c* oxidase, contain copper, and pump protons across the cytoplasmic membrane in response to electron transfer (4, 34).

Four structural genes, *qoxABCD*, are required for expression of *B. subtilis* cytochrome aa_3 (26). Cytochrome caa_3 is encoded by the *ctaCDEF* genes (27). Two additional genes, *ctaA* and *ctaB*, are also required for production of both cytochrome caa_3 and cytochrome aa_3 (28, 31). The *ctaA* and *ctaB* gene products are involved in the biosynthesis of the heme *a* prosthetic group (28). The *bd*-type of oxidases is a distinct group of terminal oxidases, not related to the heme-copper oxidases. They do not pump protons or contain copper (14). As there is no proton pumping, less energy is conserved by cytochrome *bd* compared to the heme-copper oxidases.

Expression of cytochrome *bd* requires *cydA* and *cydB*, which code for the two subunits of the enzyme as well as two additional genes, *cydC* and *cydD* (33). The latter two genes encode a putative ATP-binding-cassette (ABC) type of transporter. In *B. subtilis*, the presence of a fourth terminal oxidase can be predicted from the genome sequence (15, 33). A gene cluster containing three genes, *ythA*, *ythB*, and *ythC*, has been identified. The translated sequences of *ythA* and *ythB* are closely related to *Bacillus stearothermophilus* CbdA and CbdB, which constitute a terminal oxidase of *bd* type (24). No homologue of *ythC* has been found in *B. stearothermophilus*. The *ythA* and *ythB* genes might encode a terminal oxidase related to the *bd*-type oxidases. However, there is no direct experimental evidence for the presence of this terminal oxidase in *B. subtilis*. Throughout this article, the product of these genes is referred to as YthAB. In addition, there is spectroscopic evidence for a putative terminal oxidase of *bb′* type. The genes encoding this oxidase have not been identified, but it is not the product of *ythA* and *ythB* (2).

In this work, we show that, in *B. subtilis*, cytochrome aa_3 is the most important terminal oxidase during the exponential-growth phase. Moreover, we show that no single terminal ox-

* Corresponding author. Mailing address: Lund University, Department of Microbiology, Sölvegatan 12, SE-223 62 Lund, Sweden. Phone: 46 46 222 3456. Fax: 46 46 157839. E-mail: claes.von_wachenfeldt@mikrbiol.lu.se.

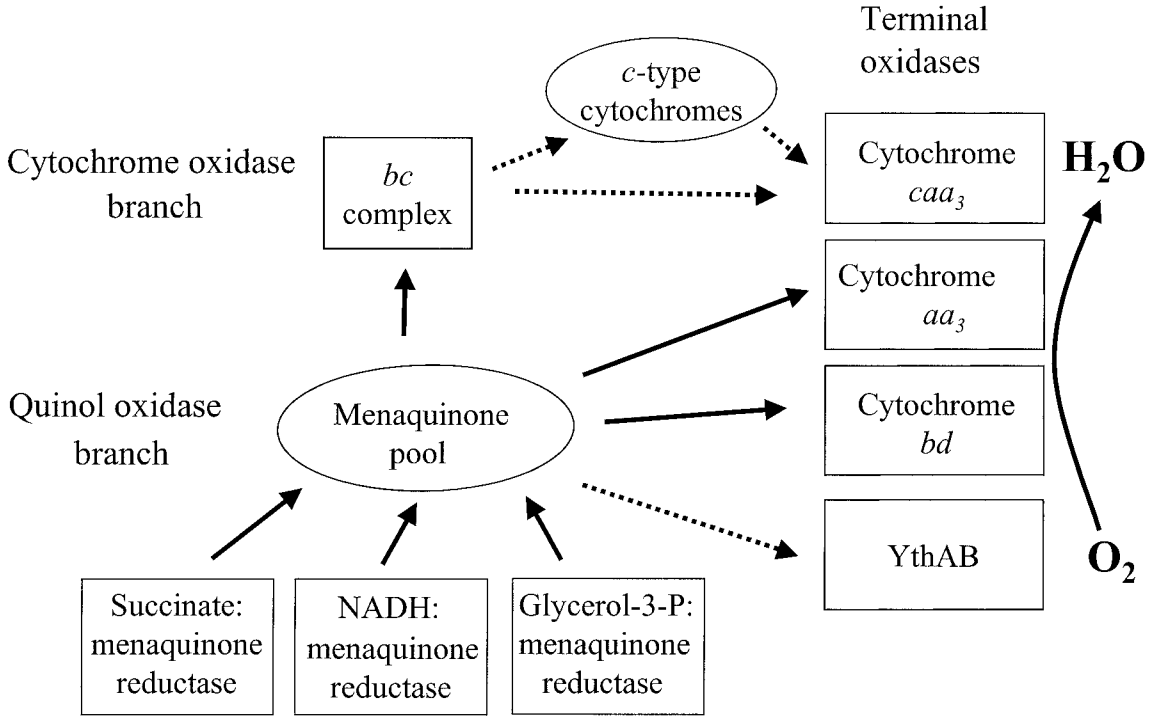

FIG. 1. Aerobic respiratory pathways in *B. subtilis* strain 168. Solid arrows, known electron pathways; dashed arrows, tentative pathways.

idase is essential for aerobic growth of *B. subtilis*. However, the presence of one of the quinol oxidases, cytochrome aa_3 or cytochrome *bd*, is essential for aerobic growth. In addition, we show that one of the heme-copper oxidases, cytochrome caa_3 or cytochrome aa_3, is required for normal sporulation of *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were kept on Luria agar (25). *B. subtilis* strains grown aerobically were kept on tryptose blood agar base (TBAB) (Difco) plates, which when indicated were supplemented with 1% (wt/vol) glucose. Liquid media were inoculated with *B. subtilis* cells grown on TBAB plates over night. The cultures were grown at 37°C in an orbital shaker at 200 rpm in nutrient sporulation medium phosphate (NSMP) (5) or in NSMP supplemented with 0.5% (wt/vol) glucose (NSMPG) or in minimal medium supplemented with 0.5% (wt/vol) glucose (MM) (36). The doubling times in the exponential-growth phase were calculated as follows: doubling time equals $(t_2 - t_1) \times \log 2]/[\log$ optical density at 600 nm (OD_{600}) at $t_2 - \log OD_{600}$ at $t_1]$, where t_1 and t_2 are the times of measurement. *B. subtilis* cells were also grown on minimal medium plates supplemented with 0.5% (wt/vol) of one of the following carbon sources: glucose, malate, glutamate, or succinate. For the sporulation frequency experiment, strains were grown in NSMP at 37°C for 30 h. The number of viable cells per milliliter of culture was determined as the total number of CFUs on TBAB plates. The number of spores per milliliter of culture was determined as the number of CFUs after heat treatment at 80°C for 10 min.

B. subtilis strains were grown anaerobically on TBAB plates, supplemented with 20 mM KNO_3 and 1% (wt/vol) glucose, at 37°C. The plates were incubated for 24 h in an anaerobic cabinet (Don Whitley Scientific). The gas composition in the anaerobic cabinet was 10% H_2–10% CO_2–80% N_2. For *B. subtilis*, the following concentrations of antibiotics were used: chloramphenicol, 5 g/liter; kanamycin, 5 g/liter; and tetracycline, 15 g/liter. For *E. coli*, ampicillin was used at 100 g/liter.

DNA techniques. *E. coli* cells were transformed using the electroporation method described by Hanahan et al. (9). Chromosomal DNA was isolated and competent *B. subtilis* cells prepared essentially as described by Hoch (12). General DNA techniques were performed as described by Sambrook et al. (25). PCR was performed essentially as described previously (35), using *Taq* DNA polymerase. The primers used to amplify a 269-bp fragment of *qoxA* were QoxA1 (5′-GCAAGCTTTTGAGGAAGTATGCACTTCAGA-3′) and QoxA2 (5′-GC TCTAGAGTCGCGGTATTTTACTAAAATAATGG-3′). Chromosomal DNA (0.1 ng) from *B. subtilis* 1A1 was used as a template. To construct double or triple mutants, *B. subtilis* strains were transformed with nonsaturating amounts of chromosomal DNA.

Spectral analysis on membranes. Membranes were prepared as described previously (10) and suspended in 20 mM sodium morpholinic propane sulphonic buffer (pH 7.4). Reduced minus oxidized difference light absorption spectra were recorded as described previously (33).

Construction of a *cydCD* expression plasmid. A plasmid containing the *cydC* and *cydD* genes under control of the *cyd* promoter was constructed by removing the 2-kb *Bgl*II and *Nde*I fragment containing *cydA* and *cydB* from plasmid pCYD23. The remaining part of pCYD23 was treated with the large (Klenow) fragment of *E. coli* DNA polymerase I, self-ligated, and used to transform *B. subtilis* 168A to chloramphenicol resistance. This resulted in plasmid pCYD25 containing *cydC* and *cydD* under the control of their native promoter (Fig. 2).

Construction of a *cydCD* null mutant. To make a *cydCD* deletion-insertion mutant, the 0.5-kbp *Eco*RI-*Hin*dIII fragment of pCYD13 was replaced by a 1-kb *Eco*RI-*Hin*dIII fragment of pCYD22 carrying a part of the *cydC* gene. The resulting plasmid, pCYD24 (Fig. 2), was used to transform strain 168A to chloramphenicol resistance. The deletion-insertion within the chromosomal *cydC* and *cydD* genes arising from a double-crossover recombination event was confirmed by Southern blot analysis (data not shown).

Construction of conditional *qoxABCD* mutant strains (P_{spac}-*qoxABCD*). A 269-base-pair fragment (*qoxA′*) of the 5′ region (−48 to +221 relative to the putative *qox* translational start site) of *qoxA* was amplified by PCR. The resulting fragment contains a part of *qoxA* and includes a putative ribosome-binding site but lacks the promoter region. Plasmid pDH88 contains the artificial hybrid promoter *spac*, which can be induced by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG) to the growth medium. The amplified *qoxA′* fragment was cleaved with restriction enzymes *Hin*dIII and *Xba*I and inserted into plasmid pDH88, cleaved with the same enzymes. The resulting plasmid was used to transform *E. coli* XL1-Blue to ampicillin resistance, creating plasmid pSPOX, containing the *spac* promoter followed by the *qoxA′* fragment (Fig. 3). When pSPOX was used to transform *B. subtilis* strains to chloramphenicol resistance, the plasmid was integrated into the chromosome by a single homologous recombination event in front of the *qoxABCD* genes. This resulted in strains in which expression of the *qoxABCD* operon could be controlled by IPTG (Fig. 3).

RESULTS AND DISCUSSION

Growth properties of single oxidase mutants. Doubling times and growth yields of *B. subtilis* strains lacking one of the terminal oxidases, cytochrome caa_3, cytochrome aa_3, cyto-

TABLE 1. List of strains and plasmids used in this work

Strain or plasmid	Relevant characteristics[a]	Source or reference[b]
Strains[c]		
Escherichia coli XL1-Blue	*endA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *supE44 relA1 lac*/F′ *proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10*	Stratagene, Inc.
B. subtilis JH642	*pheA1 trpC2*	BGSC[d]
Δqox	*trpC2* Δ*qoxABCD*::*kan*	32
Δspo0A	*pheA1 trpC2* Δ*spo0A*::*erm*	13
168A	*trpC2*	Laboratory stock
Strains derived from 168A		
LUH14	Δ*qoxABCD*::*kan*	Δqox→168A
LUH15	Δ*ctaCD*::*ble*	33
LUH17	Δ*qoxABCD*::*kan* Δ*ctaCD*::*ble*	33
LUW10	Δ*cydABCD*::*cat*	33
LUW20	Δ*cydABCD*::*tet*	33
LUW22	Δ*cydABCD*::*tet qox*::pSPOX	pSPOX→LUW20
LUW23	Δ*ctaCD*::*ble* Δ*cydABCD*::*tet*	LUW20→LUH15
LUW24	Δ*ctaCD*::*ble* Δ*cydABCD*::*tet*, *qox*::pSPOX	pSPOX→LUW23
LUW29	Δ*cydABCD*::*cat* Δ*qoxABCD*::*kan*	LUW10→LUH14
LUW32	*qox*::pSPOX	pSPOX→168A
LUW33	Δ*ctaCD*::*ble* Δ*cydABCD*::*tet* Δ*qoxABCD*::*kan*	LUH14→LUW23
LUW42	Δ*ctaCD*::*ble qox*::pSPOX	pSPOX→LUH15
LUW122	Δ*ythAB*::*tet*	2
LUW128	Δ*cydCD*::*cat*	pCYD24→168A
1A1	*trpC2*	BGSC[d]
Strains derived from 1A1		
LUW34	Δ*cydABCD*::*cat*	LUW10→1A1
LUW46	Δ*qoxABCD*::*kan*	LUH14→1A1
LUW112	Δ*spo0A*::*erm*	Δspo0A→1A1
LUW137	Δ*ythAB*::*tet*	LUW122→1A1
LUW138	Δ*qoxABCD*::*kan* Δ*ythAB*::*tet*	LUW122→LUW46
LUW142	Δ*ctaCD*::*ble*	LUH15→1A1
LUW143	Δ*ctaCD*::*ble* Δ*qoxABCD*::*kan*	LUW46→LUW142
LUW145	Δ*ctaCD*::*ble* Δ*cydABCD*::*cat*	LUW34→LUW142
LUW147	Δ*ctaCD*::*ble* Δ*ythAB*::*tet*	LUW122→LUW142
LUW148	Δ*ctaCD*::*ble* Δ*qoxABCD*::*kan* Δ*ythAB*::*tet*	LUW46→LUW147
LUW196	Δ*ctaCD*::*ble* Δ*cydABCD*::*cat* Δ*ythAB*::*tet*	LUW10→LUW147
LUW198	Δ*cydABCD*::*cat* Δ*ythAB*::*tet*	LUW10→LUW137
Plasmids:		
pDH88	Cm^r Am^r	11
pSPOX	*qoxA′* in pDH88	This work
pHP13	Cm^r Em^r	8
pCYD13	*cydA′* and *cydD′* in pHV32	33
pCYD22	*cydCD* in pHP13	33
pCYD23	*cydABCD* and the *cyd* promoter in pHP13	33
pCYD24	*cydC′* and *cydD′* in pHV32	This work
pCYD25	*cydCD* and the *cyd* promoter in pHP13	This work

[a] Am^r, Cm^r, and Em^r indicates resistance to ampicillin, chloramphenicol, and erythromycin, respectively.
[b] Arrows indicate transformation and point from donor to recipient.
[c] All *B. subtilis* strains derived from the parental strains 168A and 1A1 contain the *trpC2* mutation. During this work, strain 168A was found to be oligosporogenic, which affects growth in liquid media. Therefore, doubling times of oxidase mutants were calculated for strains derived from the parental strain 1A1.
[d] Bacillus Genetic Stock Center. Department of Biochemistry, Ohio State University, Columbus.

chrome *bd*, or YthAB, were compared with the doubling time and growth yield of the wild-type strain in different media (Table 2). In a broth medium (NSMP) or in MM, the doubling times and growth yields of the strains lacking cytochrome caa_3, cytochrome *bd*, or YthAB did not differ from those of the wild type. However, the strain lacking cytochrome aa_3 grew significantly more slowly and reached the stationary phase at a lower cell density compared to the wild type in both media (Table 2). When grown in NSMP supplemented with 0.5% glucose, all four mutant strains showed a doubling time and growth yield similar to those of the wild-type strain (Table 2). We concluded that none of the terminal oxidases are, by themselves, essential for aerobic growth. The significantly reduced growth rate of the cytochrome aa_3 mutant in NSMP and MM media suggests that cytochrome aa_3 in these growth media is the most important oxidase in exponentially growing *B. subtilis* cells.

Mutants defective in multiple terminal oxidases. Next, we attempted to make *B. subtilis* mutant strains lacking two, three, or four terminal oxidases. To make a strain lacking both cytochrome aa_3 and cytochrome *bd*, LUW20 (Δ*cydABCD::tet*) was transformed with nonsaturating amounts of chromosomal DNA (0.2 mg/liter) from LUH14 (Δ*qoxABCD::kan*), and transformants were selected on TBAB plates containing kanamycin, with and without glucose. However, no transformants were obtained. The reverse experiment, i.e., transformation of a strain lacking cytochrome aa_3 with chromosomal DNA from a strain lacking cytochrome *bd*, was performed with similar results. The experiment was also done with strains derived from

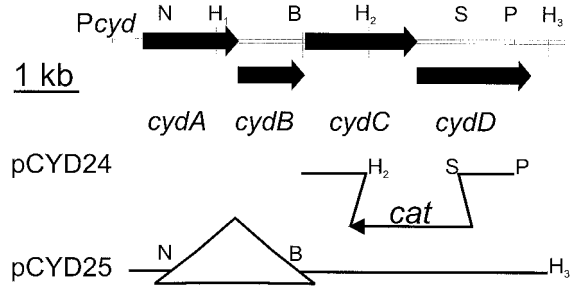

FIG. 2. Restriction map of the *cyd* region and plasmids carrying different parts of this region. At the top, the physical map of the *B. subtilis cyd* region is shown. The restriction sites are abbreviated as follows; B, *Bgl*II; N, *Nde*I; H, *Hin*dIII; P, *Pst*I; and S, *Sph*I. Plasmid pCYD24 is a derivative of pCYD13 (33), and plasmid pCYD25 is a derivative of pCYD23 (33). Construction of plasmids is described in Materials and Methods.

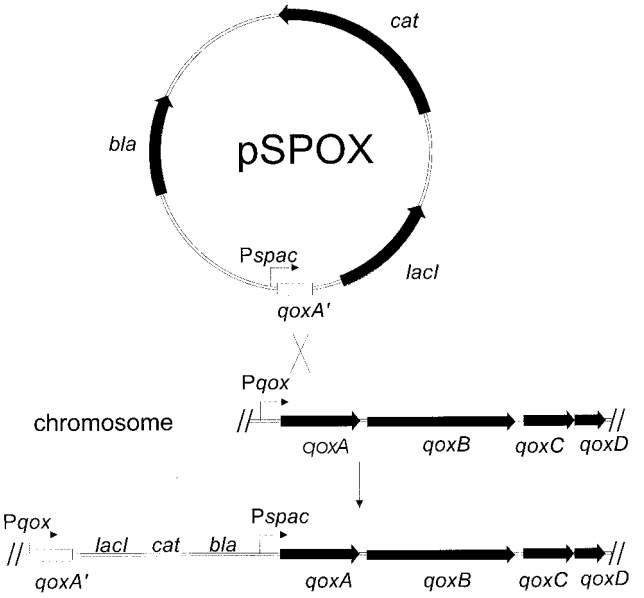

FIG. 3. Construction of *B. subtilis* strains with the *qoxABCD* operon under control of P_{spac}. The integrative plasmid pSPOX contains the inducible promoter *spac* and a 269-bp fragment of *qoxA* (*qoxA'*), including the ribosome-binding site but not the promoter region. Integration of pSPOX into the *B. subtilis* chromosome results in control of the *qoxABCD* genes by the *spac* promoter and control of the truncated *qoxA* by the native *qox* promoter. The genes for β-lactamase, chloramphenicol resistance, and the *lac* repressor are indicated as *bla*, *cat*, and *lacI*, respectively.

1A1 and JH642, with similar results. As a control, strain LUW20 (Δ*cydABCD::tet*) harboring plasmid pCYD23, which carries a functional set of the *cydABCD* genes, was transformed with LUH14 (Δ*qoxABCD::kan*) chromosomal DNA. Transformants (7.5×10^6/mg of DNA) were obtained, showing that the actual transformation event works in this strain. From this we concluded that a strain lacking both cytochrome aa_3 and cytochrome *bd* is not viable under the conditions employed.

Strains lacking two or three terminal oxidases were made by transformation of a recipient strain with donor chromosomal DNA as indicated in Table 1. All combinations that did not include deletion of both cytochrome *bd* and cytochrome aa_3 could be made. Mutants containing only one terminal oxidase, either cytochrome aa_3 or cytochrome *bd*, were further characterized. The strains were grown in liquid media and the doubling times calculated. In NSMPG, the doubling time of a strain containing only cytochrome *bd* did not differ from that of the wild type, but in NSMP and in MM, the doubling times were significantly longer, and the mutant strain reached the stationary phase at about half the cell density relative to that of the wild type (Table 2). In contrast, the doubling times and growth yields of a strain containing only cytochrome aa_3 did not differ from those of the wild type in either of the media (Table 2).

To further study the growth properties of oxidase mutants, strains were grown on plates containing minimal medium and one of the following carbon sources: glucose, malate, glutamate, or succinate. The strains lacking cytochrome caa_3 (LUH15), cytochrome *bd* (LUW10), or YthAB (LUW122) grew as well as the wild-type strain on all of the tested carbon sources. The same was observed for the strain containing only cytochrome aa_3 (LUW196). The strain lacking cytochrome aa_3 (LUH14) showed growth properties similar to those of the wild-type strain on glucose, but this strain grew more slowly and formed smaller colonies on the other carbon sources. The strain containing only cytochrome *bd* (LUW148) grew more slowly and formed colonies significantly smaller than those of the wild-type strain on glucose. This indicates that either cytochrome caa_3 or YthAB is required for optimal growth on glucose in a QoxABCD$^-$ mutant background. LUW148 did not grow on the nonfermentative substrates malate, glutamate, or succinate. Taken together, our data further indicate that cytochrome aa_3 is sufficient to support maximal growth rates in broth and defined media. It is likely that the other terminal oxidases play minor roles in exponentially growing, aerobic wild-type cells.

Anaerobic growth of double and triple mutants. Under anaerobic conditions, *B. subtilis* is able to utilize nitrate as a terminal electron acceptor (18). To find out if a strain lacking cytochrome aa_3 in combination with cytochrome *bd* is viable under anaerobic nitrate-respiratory conditions, the transformation experiments were carried out in an anaerobic atmosphere. LUH14 (Δ*qoxABCD::kan*) was transformed with chromosomal DNA from LUW10 (Δ*cydABCD::cat*). Transformants were selected on TBAB plates containing chloramphenicol, 20 mM KNO_3, and 1% (wt/vol) glucose, incubated at 37°C in an anaerobic cabinet for 24 h. Strain LUW29, lacking both cytochrome aa_3 and cytochrome *bd*, was obtained. A similar procedure was used to construct strain LUW33, lacking cytochrome caa_3, cytochrome aa_3, and cytochrome *bd*. The mutant strains LUW29 and LUW33 were streaked on two new plates of which one was incubated aerobically and the other one was incubated in the anaerobic cabinet. The strains grew well in the anaerobic cabinet but could not grow in an aerobic atmosphere. If the anaerobically incubated cells were exposed to oxygen, they could not resume growth in the anaerobic cabinet. The results showed that a strain lacking both cytochrome *bd* and cytochrome aa_3 grows under anaerobic, nitrate-respiratory conditions.

Construction of strains with the *qoxABCD* genes under control of an IPTG-inducible promoter. To be able to study the growth properties of mutants lacking both cytochrome aa_3 and cytochrome *bd*, we constructed strains in which expression of the *qoxABCD* operon was controlled by the IPTG-inducible *spac* promoter. An integrative plasmid (pSPOX) carrying P_{spac} was constructed and used to transform different *B. subtilis* strains to chloramphenicol resistance as described in Materials and Methods and in Figure 3.

Plasmid pSPOX was used to transform *B. subtilis* 168A (wild type), LUW20 (Δ*cydABCD*), LUW23 (Δ*ctaCD* Δ*cydABCD*),

TABLE 2. Doubling times and relative yield of oxidase mutants grown in liquid media

Strain	Relevant genotype	Doubling time (min)[a]			Relative yield[b]		
		NSMP	NSMPG	MM	NSMP	NSMPG	MM
1A1	Wild type	41 ± 0.7	33 ± 1.7	49 ± 3.1	1.0	1.0	1.0
LUW34	Δ*cydABCD*	42 ± 0.9	34 ± 1.2	48 ± 1.1	0.97	0.95	1.0
LUW46	Δ*qoxABCD*	57 ± 0.9	32 ± 0.1	65 ± 2.0	0.57	0.95	0.78
LUW137	Δ*ythAB*	41 ± 0.9	31 ± 1.3	50 ± 2.1	1.0	0.96	0.98
LUW142	Δ*ctaCD*	39 ± 1.8	29 ± 1.9	50 ± 1.4	1.0	0.97	1.0
LUW148	Δ*qoxABCD* Δ*ctaCD* Δ*ythAB*	59 ± 2.3	32 ± 0.5	63 ± 5.8	0.48	0.76	0.53
LUW196	Δ*cydABCD* Δ*ctaCD* Δ*ythAB*	42 ± 1.5	32 ± 1.9	44 ± 0.8	1.0	1.0	0.99

[a] Doubling times were calculated for strains in the exponential growth phase, grown at 37°C in different media. Values are the mean of at least three experiments. The degree of spread is indicated as the standard error of the mean.

[b] Relative yield is defined as the highest optical density in early stationary phase in terminal oxidase mutant strain cultures relative to that in the wild-type strain cultures. Values are the means of at least three experiments. The variation is less than 6%.

and LUH15 (Δ*ctaCD*) to chloramphenicol resistance. Transformants were selected on TBAB plates with or without IPTG and incubated aerobically or anaerobically. Transformation of the wild-type strain and the strain lacking cytochrome caa_3 resulted in transformants under all growth conditions (Table 3). To confirm that cytochrome aa_3 was only synthesized in the presence of IPTG, the wild-type strain carrying pSPOX in its chromosome was grown in NSMP supplemented with 0.5% glucose, with or without IPTG. Spectral analysis of membranes from this strain showed that cytochrome aa_3 could be detected only in membranes from cells grown in the presence of IPTG (data not shown). When transforming the strain lacking cytochrome *bd* or the strain lacking both cytochrome *bd* and cytochrome caa_3, transformants were obtained only on plates incubated anaerobically or on plates incubated aerobically and supplemented with IPTG (Table 3). When colonies from plates supplemented with IPTG were streaked on new plates without IPTG and incubated aerobically, no growth was seen.

To study the growth properties in liquid cultures, LUW32 (P_{spac}-*qoxABCD*), LUW22 (P_{spac}-*qoxABCD* Δ*cydABCD*), LUW24 (P_{spac}-*qoxABCD* Δ*ctaCD* Δ*cydABCD*), and LUW42 (P_{spac}-*qoxABCD* Δ*ctaCD*) were grown in NSMPG in the presence of IPTG. After 2.25 h, the cells were harvested, washed, and resuspended in NSMPG, with or without IPTG. In the presence of IPTG, the growth rates of the strains did not differ from that of the wild-type (Fig. 4). When IPTG was removed, no effect was seen in LUW32 (Fig. 4A). Growth of LUW42 (lacking cytochrome caa_3) was slightly poorer than that of the wild type (Fig. 4B), whereas in LUW22 (lacking cytochrome *bd*) and LUW24 (lacking cytochrome *bd* and cytochrome caa_3), the growth rate was significantly decreased by about 1 h after removal of IPTG (Fig. 4C and D). Moreover, LUW22 and LUW24 failed to reach the final optical density exhibited by the LUW32 strain. The decrease in growth rate did not occur immediately after removal of IPTG, probably because cytochrome aa_3 was present in the cell membrane at the time of IPTG removal. The data further confirmed our results that a *B. subtilis* strain lacking both cytochrome aa_3 and cytochrome *bd* cannot grow vegetatively in an aerobic atmosphere.

Sporulation of oxidase mutants. Sporulation in *B. subtilis* is an energy-requiring process. To see whether the absence of any of the terminal oxidases affected sporulation, mutant strains were tested for sporulation efficiency. As shown in Table 4, the sporulation efficiency of the single-oxidase mutants did not differ from that of the wild-type strain. Mutants lacking both cytochrome caa_3 and cytochrome *bd* or both cytochrome caa_3 and YthAB or both cytochrome *bd* and YthAB showed normal sporulation (Table 4). The same was observed for the strain containing only cytochrome aa_3 (i.e., a strain lacking cytochrome caa_3, cytochrome *bd*, and YthAB). However, the strain containing only cytochrome *bd* showed an approximately 5,000-fold reduction of the sporulation frequency relative to that of the wild-type strain (Table 4). Sporulation was also inhibited in the strain lacking both cytochrome aa_3 and cytochrome caa_3. This is in line with previous data showing that a $CtaA^-$ mutant strain, which is unable to make the heme *a* prosthetic group, is sporulation deficient (17). The strain lacking cytochrome aa_3 and YthAB showed a 24-fold decrease in the level of sporulation (Table 4). These results showed that one of the heme copper terminal oxidases, cytochrome aa_3 or cytochrome caa_3, is required for efficient sporulation of *B. subtilis* strain 168, probably because at least one proton-pumping oxidase is required to conserve enough energy for sporulation. In addition, our results suggested that YthAB may have a role in sporulation and can compensate for the loss of cytochrome aa_3.

The role of the CydCD transporter. The *cydC* and *cydD* gene products are likely to encode a heterodimeric, membrane bound ABC type of transporter that is required for assembly of cytochrome *bd* (33). To analyze whether the CydCD ABC

TABLE 3. Relative frequency of transformants obtained in different *B. subtilis* strains transformed with plasmid pSPOX

Strain	Relative frequency of transformants under culture conditions[a]			
	$+O_2$		$-O_2$	
	+IPTG (+ cyt. aa_3)	−IPTG (− cyt. aa_3)	+IPTG (+ cyt. aa_3)	−IPTG (− cyt. aa_3)
168A (wild-type)	0.95	1.0	1.1	1.1
LUH15 (Δ*ctaCD*)	1.1	1.1	n.d.	n.d.
LUW20 (Δ*cydABCD*)	1.1	0	0.98	0.88
LUW23 (Δ*ctaCD* Δ*cydABCD*)	1.2	0	1.1	0.98

[a] Transformants were selected on plates containing chloramphenicol, with (+ cytochrome aa_3) or without (− cytochrome aa_3) 1 mM IPTG, incubated with ($+O_2$) or without ($-O_2$) oxygen. A value of 1.0 corresponds to 1,102 transformants. n.d., not done.

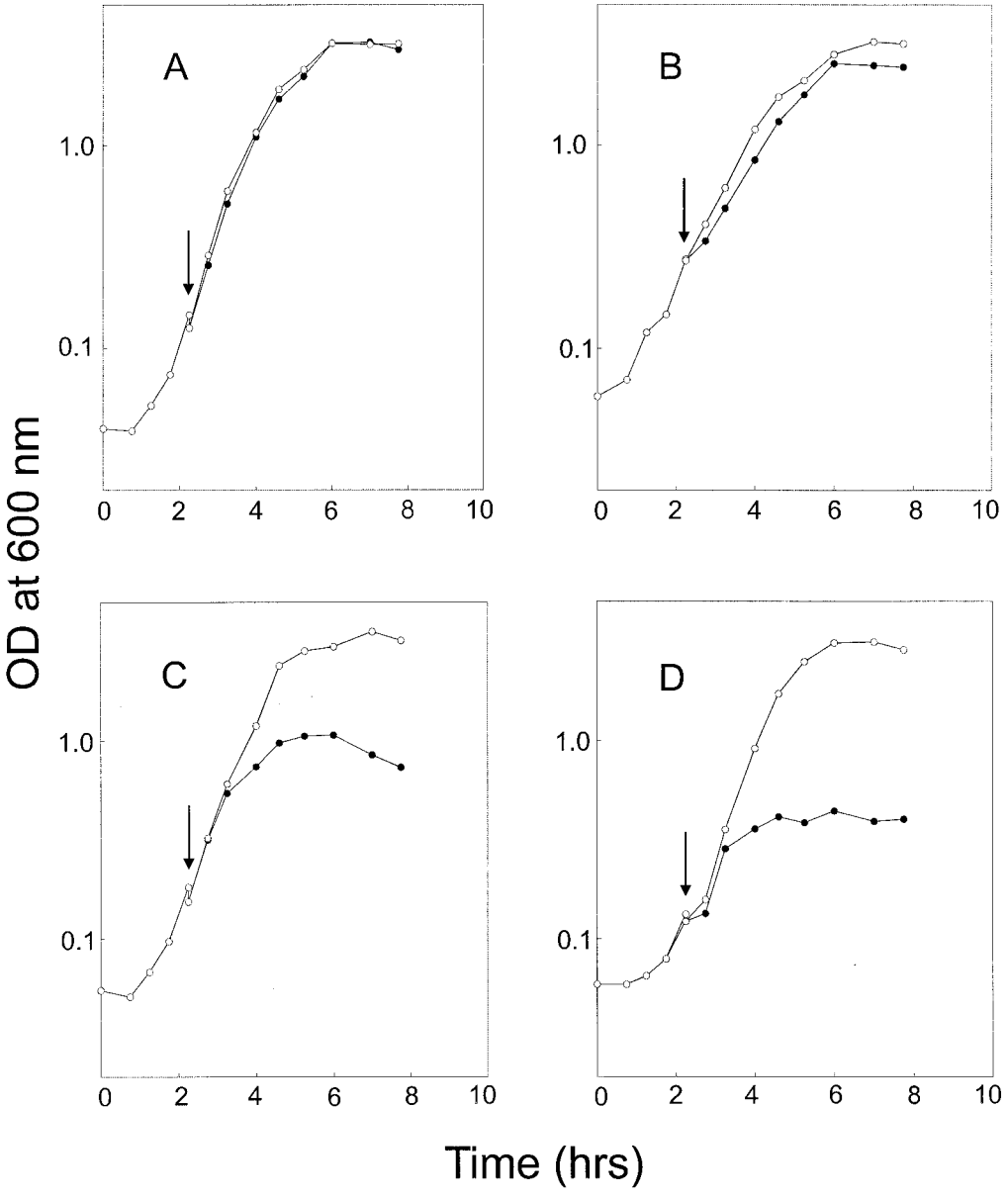

FIG. 4. *B. subtilis* strains carrying pSPOX grown in NSMPG with and without IPTG. Cells were grown in NSMPG supplemented with IPTG. After 2.25 h (OD_{600} between 0.15 and 0.24, indicated by arrows), cells were harvested, washed, and resuspended in fresh media. Open circles show cells grown in NSMPG containing 1 mM IPTG. Solid circles show cells grown in NSMPG without IPTG. (A) LUW32 (P_{spac}-*qoxABCD*); (B) LUW42 (P_{spac}-*qoxABCD* Δ*ctaCD*); (C) LUW22 (P_{spac}-*qoxABCD* Δ*cydABCD*); (D) LUW24 (P_{spac}-*qoxABCD* Δ*ctaCD* Δ*cydABCD*).

transporter is also required for the assembly of an additional terminal oxidase in *B. subtilis*, we constructed a strain containing the CydCD transporter but lacking cytochrome *bd*. A plasmid, pCYD25, carrying *cydCD* under control of the *cyd* promoter, was introduced into LUW20, thus creating a strain lacking the chromosomal *cydABCD* operon but carrying *cydCD* on a plasmid. To confirm that pCYD25 contained a functional set of *cydCD*, strain LUW128 lacking *cydCD* was constructed, and pCYD25 was introduced into this strain. LUW128 carrying pCYD25 and LUW128 carrying pHP13 were grown in NSMPG, and membranes were studied by light absorption difference (reduced minus oxidized) spectroscopy. Membranes from strain 1A1 (wild type) grown in the same way were used as a control. No cytochrome *bd* was detected in membranes from LUW128(pHP13). Membranes from LUW128(pCYD25) showed a spectrum similar to that of the wild-type strain, showing that pCYD25 expressed a functional *cydCD* and that overexpression of *cydCD* did not result in an increased production of cytochrome *bd* (Fig. 5).

To find out whether there is an additional terminal oxidase present, which requires CydCD and can compensate for the loss of both quinol oxidases in *B. subtilis*, the following experiment was performed. Chromosomal DNA from LUH14 (Δ*qoxABCD::kan*) was used to transform LUW20(pCYD25), LUW128(pCYD25), and LUW128. Transformants were selected on TBAB plates containing kanamycin with and without glucose. A few transformants were obtained with LUW20(pCYD25) and LUW128, but these had all became

TABLE 4. Sporulation frequencies of oxidase mutants

Strain	Relevant genotype	Viable count (cells/ml)	Spore count (cells/ml)	Sporulation frequency (%)[a]
1A1	Wild-type	4.6×10^8	4.2×10^8	91
Single mutants				
LUW142	Δ*ctaCD*	2.9×10^8	2.6×10^8	90
LUW34	Δ*cydABCD*	4.5×10^8	4.4×10^8	97
LUW137	Δ*ythAB*	2.8×10^8	2.7×10^8	98
LUW46	Δ*qoxABCD*	4.8×10^8	4.5×10^8	93
Double mutants				
LUW145	Δ*ctaCD* Δ*cydABCD*	2.9×10^8	2.8×10^8	97
LUW147	Δ*ctaCD* Δ*ythAB*	4.0×10^8	3.6×10^8	90
LUW143	Δ*ctaCD* Δ*qoxABCD*	8.7×10^8	2.0×10^5	0.023
LUW198	Δ*cydABCD* Δ*ythAB*	4.0×10^8	3.7×10^8	92
LUW138	Δ*qoxABCD* Δ*ythAB*	8.4×10^8	3.3×10^7	3.8
Triple mutants				
LUW148	Δ*ctaCD* Δ*ythAB* Δ*qoxABCD*	9.8×10^7	1.8×10^4	0.018
LUW196	Δ*ctaCD* Δ*cydABCD* Δ*ythAB*	2.7×10^8	2.6×10^8	97

[a] Sporulation frequencies were calculated as spore count divided by viable count. The sporulation mutant strain LUW112 (Δ*spo0A*) was found to have a sporulation frequency of $>3.1 \times 10^{-6}$%. Each experiment was repeated at least twice; the variation was less than 5%. In each case, data from a single experiment are presented.

wild type with respect to cytochrome *bd*; i.e., the antibiotic resistance marker in the *cyd* locus had been substituted with the *cydABCD* or the *cydCD* genes from the LUH14 chromosomal DNA. Several transformants were obtained with LUW128(pCYD25) (data not shown). Our results indicate that there is no additional terminal oxidase in *B. subtilis*, requiring the CydCD ABC transporter, that could compensate for the loss of cytochrome *bd* and cytochrome aa_3. The results also confirm that no functional cytochrome *bd* is made if CydCD is not present, and they suggest that none of the other about 80 ABC transporters in *B. subtilis* (22) can compensate for the loss of CydCD.

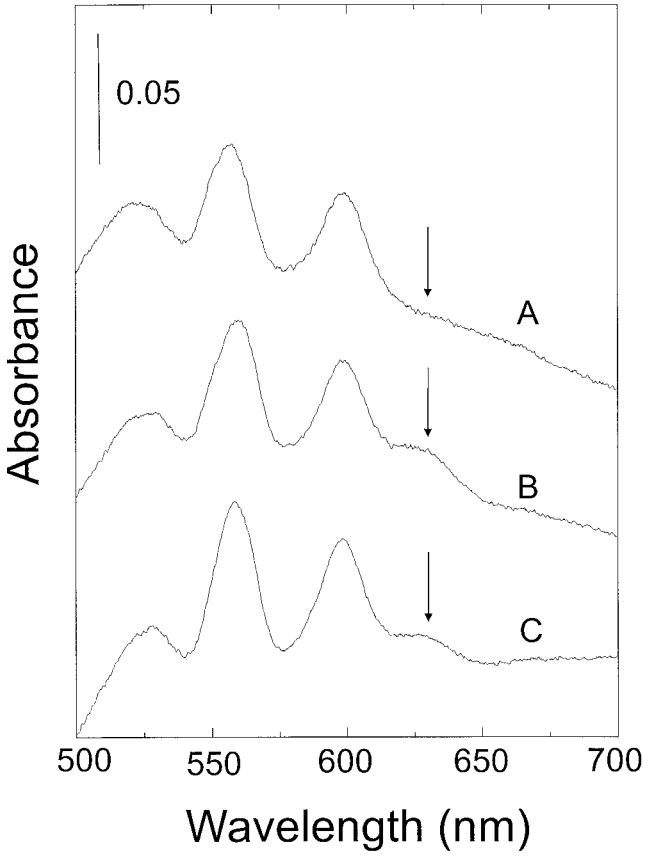

FIG. 5. Light absorption difference (dithionite-reduced minus ferricyanide-oxidized) spectra of membranes (3 mg of protein per ml) from strains 1A1 and LUW128 carrying different plasmids. *B. subtilis* strains were grown in NSMPG and harvested in the stationary-growth phase. Line A, LUW128(pHP13); line B, LUW128(pCYD25); line C, 1A1.

Conclusion. The aerobic respiratory pathways in *B. subtilis* terminate with one of three or possibly four alternative terminal oxidases, as indicated in Fig. 1. Taken together, our data strongly indicate that one of the quinol oxidases, cytochrome aa_3 or cytochrome *bd*, is essential for aerobic growth of *B. subtilis* strain 168. The reason that the cytochrome oxidase branch cannot compensate for the loss of the quinol oxidase branch is most probably that the cytochrome oxidase branch is not expressed until the cells enter the stationary phase. This hypothesis is supported by observations that the genes encoding the *bc* complex and probably also cytochrome caa_3 are repressed by the transition state regulator AbrB in the exponential growth phase (37; L. Winstedt and C. von Wachenfeldt, unpublished data). We do not know under which conditions the *ythAB* genes are expressed. However, it seems likely that they are not expressed in exponentially growing cells. Deletion of *ythAB* in a strain lacking cytochrome aa_3 makes the strain sporulation deficient, indicating a physiological role for YthAB in *B. subtilis*.

The combined results of this work show that cytochrome aa_3 is the most important terminal oxidase contributing to proton motive force generation in exponentially growing cells. The results also demonstrate that one of the proton-pumping heme-copper oxidases, cytochrome caa_3 or cytochrome aa_3, is required for efficient sporulation. It is likely that *B. subtilis* cannot conserve enough energy for initiation or completion of the sporulation cycle by using only the nonproton-pumping terminal oxidase, cytochrome *bd*.

ACKNOWLEDGMENTS

We thank L. Rutberg for valuable comments on the manuscript, E. Holst for help with anaerobic growth of bacteria, and P. A. Levin for the kind gift of the Δ*spo0A* strain.

This work was supported by grants from Crafoordska Stiftelsen and Emil och Wera Cornells Stiftelse.

REFERENCES

1. **Anraku, Y.** 1988. Bacterial electron transport chains. Annu. Rev. Biochem. **57:**101–132.
2. **Azarkina, N., S. Siletsky, V. Borisov, C. von Wachenfeldt, L. Hederstedt, and A. A. Konstantinov.** 1999. A cytochrome *bb′*-type quinol oxidase in *Bacillus subtilis* strain 168. J. Biol. Chem. **274:**32810–32817.
3. **Baker, S. C., S. J. Ferguson, B. Ludwig, M. D. Page, O. M. H. Richter, and R. J. M. van Spanning.** 1998. Molecular genetics of the genus *Paracoccus*: metabolically versatile bacteria with bioenergetic flexibility. Microbiol. Mol. Biol. Rev. **62:**1046–1078.
4. **Ferguson-Miller, S., and G. T. Babcock.** 1996. Heme/copper terminal oxidases. Chem. Rev. **96:**2889–2907.
5. **Fortnagel, P., and E. Freese.** 1968. Analysis of sporulation mutants. II. Mutants blocked in the citric acid cycle. J. Bacteriol. **95:**1431–1438.
6. **Garcia-Horsman, J. A., B. Barquera, J. Rumbley, J. Ma, and R. B. Gennis.** 1994. The superfamily of heme-copper respiratory oxidases. J. Bacteriol. **176:**5587–5600.
7. **Gennis, R. B., and V. Stewart.** 1996. Respiration, p. 217–261. *In* F. C. Neidhart (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
8. **Haima, P., S. Bron, and G. Venema.** 1987. The effect of restriction on shotgun cloning and plasmid stability in *Bacillus subtilis* Marburg. Mol. Gen. Genet. **209:**335–342.
9. **Hanahan, D., J. Jessee, and F. R. Bloom.** 1991. Plasmid transformation of *Escherichia coli* and other bacteria. Methods Enzymol. **204:**63–113.
10. **Hederstedt, L.** 1986. Molecular properties, genetics, and biosynthesis of *Bacillus subtilis* succinate dehydrogenase complex. Methods Enzymol. **126:** 399–414.
11. **Henner, D. J.** 1990. Inducible expression of regulatory genes in *Bacillus subtilis*. Methods Enzymol. **185:**223–228.
12. **Hoch, J. A.** 1991. Genetic analysis in *Bacillus subtilis*. Methods Enzymol. **204:**305–320.
13. **Ireton, K., D. Z. Rudner, K. J. Siranosian, and A. D. Grossman.** 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. Genes Dev. **7:**283–294.
14. **Jünemann, S.** 1997. Cytochrome *bd* terminal oxidase. Biochim. Biophys. Acta **1321:**107–127.
15. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
16. **Lauraeus, M., T. Haltia, M. Saraste, and M. Wikström.** 1991. *Bacillus subtilis* expresses two kinds of haem-A-containing terminal oxidases. Eur. J. Biochem. **197:**699–705.
17. **Mueller, J. P., and H. W. Taber.** 1989. Isolation and sequence of *ctaA*, a gene required for cytochrome aa_3 biosynthesis and sporulation in *Bacillus subtilis*. J. Bacteriol. **171:**4967–4978.
18. **Nakano, M. M., and F. M. Hulett.** 1997. Adaptation of *Bacillus subtilis* to oxygen limitation. FEMS Microbiol. Lett. **157:**1–7.
19. **Poole, R., and S. Hill.** 1997. Respiratory protection of nitrogenase activity in *Azotobacter vinelandii*—roles of the terminal oxidases. Biosci. Rep. **17:**303–317.
20. **Preisig, O., D. Anthamatten, and H. Hennecke.** 1993. Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen fixing endosymbiosis. Proc. Natl. Acad. Sci. USA **90:**3309–3313.
21. **Preisig, O., R. Zufferey, L. Thöny-Meyer, C. A. Appleby, and H. Hennecke.** 1996. A high-affinity cbb_3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. J. Bacteriol. **178:** 1532–1538.
22. **Quentin, Y., G. Fichant, and F. Denizot.** 1999. Inventory, assembly and analysis of *Bacillus subtilis* ABC transport systems. J. Mol. Biol. **287:**467–484.
23. **Richardson, D. J.** 2000. Bacterial respiration: a flexible process for a changing environment. Microbiology **146:**551–571.
24. **Sakamoto, J., E. Koga, T. Mizuta, C. Sato, S. Noguchi, and N. Sone.** 1999. Gene structure and quinol oxidase activity of a cytochrome *bd*-type oxidase from *Bacillus stearothermophilus*. Biochim. Biophys. Acta **1411:**147–158.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Santana, M., F. Kunst, M. F. Hullo, G. Rapoport, A. Danchin, and P. Glaser.** 1992. Molecular cloning, sequencing, and physiological characterization of the *qox* operon from *Bacillus subtilis* encoding the aa_3-600 quinol oxidase. J. Biol. Chem. **267:**10225–10231.
27. **Saraste, M., T. Metso, T. Nakari, T. Jalli, M. Lauraeus, and J. van der Oost.** 1991. The *Bacillus subtilis* cytochrome-*c* oxidase: variations on a conserved protein theme. Eur. J. Biochem. **195:**517–525.
28. **Svensson, B., M. Lübben, and L. Hederstedt.** 1993. *Bacillus subtilis* CtaA and CtaB function in haem A biosynthesis. Mol. Microbiol. **10:**193–201.
29. **Thöny-Meyer, L.** 1997. Biogenesis of respiratory cytochromes in bacteria. Microbiol. Mol. Biol. Rev. **61:**337–376.
30. **Unden, G., and J. Bongaerts.** 1997. Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors. Biochim. Biophys. Acta **1320:**217–234.
31. **van der Oost, J., C. von Wachenfeldt, L. Hederstedt, and M. Saraste.** 1991. *Bacillus subtilis* cytochrome oxidase mutants: biochemical analysis and genetic evidence for two aa_3-type oxidases. Mol. Microbiol. **5:**2063–2072.
32. **Villani, G., M. Tattoli, N. Capitanio, P. Glaser, S. Papa, and A. Danchin.** 1995. Functional analysis of subunits III and IV of *Bacillus subtilis* aa_3-600 quinol oxidase by in vitro mutagenesis and gene replacement. Biochim. Biophys. Acta **1232:**67–74.
33. **Winstedt, L., K. Yoshida, Y. Fujita, and C. von Wachenfeldt.** 1998. Cytochrome *bd* biosynthesis in *Bacillus subtilis*: characterization of the *cydABCD* operon. J. Bacteriol. **180:**6571–6580.
34. **von Wachenfeldt, C., and L. Hederstedt.** 1992. Molecular biology of *Bacillus subtilis* cytochromes. FEMS Microbiol. Lett. **100:**91–100.
35. **von Wachenfeldt, C., and L. Hederstedt.** 1993. Physico-chemical characterization of membrane-bound and water-soluble forms of *Bacillus subtilis* cytochrome *c-550*. Eur. J. Biochem. **212:**499–509.
36. **Yoshida, K., I. Ishio, E. Nagakawa, Y. Yamamoto, M. Yamamoto, and Y. Fujita.** 2000. Systematic study of gene expression and transcription organization in the *gntZ-ywaA* region of the *Bacillus subtilis* genome. Microbiology **146:**573–579.
37. **Yu, J., L. Hederstedt, and P. J. Piggot.** 1995. The cytochrome *bc* complex (menaquinone:cytochrome *c* reductase) in *Bacillus subtilis* has a nontraditional subunit organization. J. Bacteriol. **177:**6751–6760.