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Enterococcus faecalis V583 Contains a Cytochrome *bd*-Type Respiratory Oxidase

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We have cloned an *Enterococcus faecalis* gene cluster, *cydABCD*, which when expressed in *Bacillus subtilis* results in a functional cytochrome *bd* terminal oxidase. Our results indicate that *E. faecalis* V583 cells have the capacity of aerobic respiration when grown in the presence of heme.

Enterococcus faecalis, a gram-positive bacterium of low G+C content, is normally found in the human intestine. It is an opportunistic pathogen which can cause severe nosocomial infections (11, 16). Enterococci are generally considered to be facultative anaerobes that mainly use a homolactic fermentative pathway for energy production (3). However, the presence of cytochromes and a capacity for oxidative phosphorylation in *E. faecalis* strains have been reported in earlier studies (20, 22). The identity and function of these membrane-bound cytochromes have remained unknown. In this study, we demonstrate that *E. faecalis* contains a cytochrome *bd*-type oxidase which is expressed under some growth conditions.

Cytochrome *bd* terminal oxidase complexes are widely distributed in prokaryotes (13, 17). They are membrane-bound enzymes that comprise two subunits and three heme prosthetic groups. Cytochrome *bd* catalyzes the two-electron oxidation of quinol and the four-electron reduction of dioxygen to make water. The protons produced upon quinol oxidation are released on the outside of the cytoplasmic membrane, and the protons consumed in water production are taken up from the inside of the cell. This results in the production of an electrochemical gradient across the membrane (21). The cytochrome *bd* structural genes, *cydA* and *cydB*, have been cloned from different bacteria (6, 10, 14, 24, 28). In *Escherichia coli* and in *Bacillus subtilis*, two additional genes, *cydC* and *cydD*, have been shown to be required for expression of cytochrome *bd* (5, 18, 28). The *cydC* and *cydD* genes encode a putative heterodimeric ATP binding cassette (ABC) type of transporter (18). The cytochrome *bd* quinol oxidases contain two *b*-type cytochromes (a low-spin and a high-spin heme *b*) and one cytochrome *d* (15). The dioxygen reduction site of the enzyme is probably formed by the high-spin heme *b* together with heme *d* (8). Here we report the cloning of an *E. faecalis* gene cluster which encodes a cytochrome *bd* terminal oxidase.

***E. faecalis* can express a cytochrome of the *bd* type.** For membrane preparation, *E. faecalis* V583 was grown in indented flasks on a rotary shaker (200 rpm) at 37°C in a medium containing tryptone (15 g/liter), soy peptone (5 g/liter) (both from Lab M, Bury, England), NaCl (5 g/liter), and 1% (wt/vol) glucose. The medium was buffered with 30 mM sodium morpholinic propane sulfonic acid buffer (MOPS), pH 7.4, and 5 mM potassium phosphate buffer, pH 7.0. When indicated, 8 μM hemin (Sigma) was added to the medium. Twelve hours after inoculation, cells were harvested by centrifugation and washed in 20 mM sodium MOPS buffer, pH 7.4. All subsequent steps were done at 4°C or on ice. Cells were suspended in MOPS buffer containing DNase (0.1 mg/ml) (bovine pancreas, type 1; Sigma), 0.5 mM phenylmethylsulfonylfluoride and 5 mM $MgSO_4$ and broken using a French pressure cell. After a centrifugation at 5,000 × *g*, for 15 min, membranes were harvested from the supernatant by centrifugation at 200,000 × *g*, for 90 min, washed once, and then suspended in MOPS buffer. Protein concentrations were determined using the bicinchoninic acid assay (Pierce) with bovine serum albumin as the standard. Light absorption spectra were recorded as described previously (28).

Isolated membranes from *E. faecalis* cells grown aerobically in the presence of hemin demonstrated light absorption difference spectra with features characteristic for cytochrome *bd*, with absorption peaks at 561 nm (cytochrome *b*) and 626 nm (cytochrome *d*) (Fig. 1, spectrum A). The trough at about 650 nm indicates the presence of a stable oxygenated cytochrome *d* species [Fe(II)-O_2] (19). Membranes from cells grown without hemin lacked spectroscopically detectable cytochromes (Fig. 1, spectrum B).

A *cyd* gene cluster is present in *E. faecalis*. The amino acid sequence of *B. subtilis* CydA was used to search for related sequences in the preliminary release of the *E. faecalis* genomic data obtained from The Institute for Genomic Research (TIGR). The BLAST search (1) resulted in the identification of a contig containing four putative genes, similar to *B. subtilis cydA*, *cydB*, *cydC*, and *cydD*. Alignments of the *B. subtilis* and *E. faecalis* amino acid sequences showed a sequence identity of 56% for CydA, 46% for CydB, 51% for CydC, and 49% for CydD.

To clone the *E. faecalis cydABCD* genes, the DNA sequence obtained from the TIGR *E. faecalis* database was used to design two primers, ECYD1 (5′ GGAGATCTAATGGAAAT GAACAATTCAGGTAAG-3′) (*Bgl*II restriction site underlined) and ECYD2 (5′-GGTCTAGACTATCATGGCGTTAC AGAAGCAC-3′) (*Xba*I restriction site underlined). The *Bgl*II restriction site is located 59 nucleotides upstream of the putative translational initiation site of *cydA*. These primers were used in a long-range PCR (Expand High Fidelity PCR system; Roche) with 500 ng of *E. faecalis* chromosomal DNA (prepared essentially as described by Hoch [9]) as the template. The amplified 6.2-kb fragment was cut with restriction enzymes *Bgl*II and *Xba*I and ligated into plasmid pCYD26, cut with *Bam*HI and *Xba*I. Plasmid pCYD26 contains the *B. subtilis cyd* promoter region (nucleotides −192 to +199 with respect to the transcription start site) (28) in the low-copy-number vector

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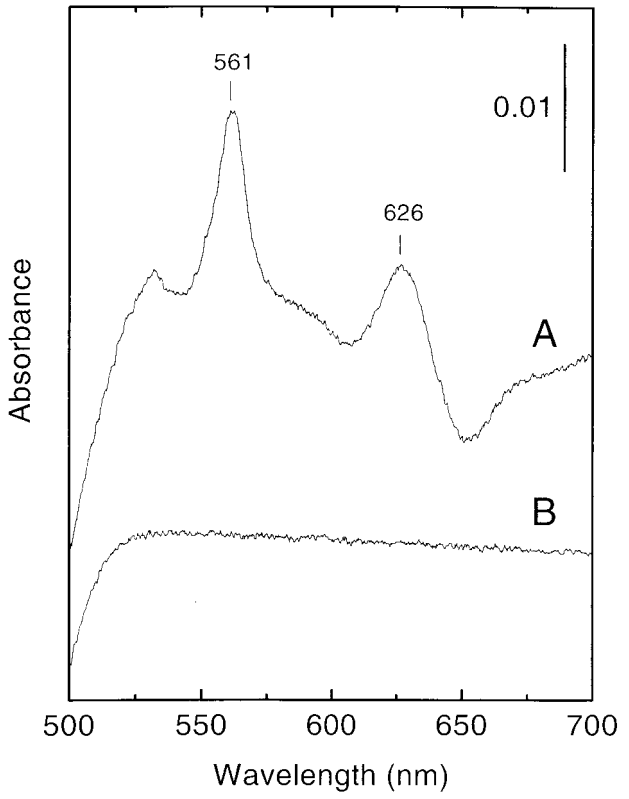

FIG. 1. Light absorption difference (dithionite-reduced minus air-oxidized) spectra of *E. faecalis* membranes (3 mg of protein per ml). (A) Membranes from cells grown in the presence of 8 μM hemin; (B) membranes from cells grown in the absence of added hemin. The vertical bar indicates the absorption scale. Difference spectra obtained after oxidation by potassium ferricyanide were identical to those obtained by air oxidation.

pHPSK. The ligate was used to transform *B. subtilis* 168A to chloramphenicol resistance, resulting in plasmid pLUF04 (Fig. 2). Restriction site mapping and partial DNA sequence analysis confirmed the identity of the cloned fragment.

Expression of *E. faecalis cydABCD* in *B. subtilis*. To facilitate characterization of *E. faecalis* cytochrome *bd*, we aimed to find a method for overproduction of the enzyme complex. Since cytochrome *bd* from *B. subtilis* and *E. faecalis* appeared to be closely related, we choose to use *B. subtilis* as our expression host. Strains and plasmids used in this study are listed in Table 1. *B. subtilis* strain LUW20 lacks cytochrome *bd*, and hence membranes from this strain lack the spectroscopic features of cytochrome *bd* (28). LUW20/pLUF04 (*E. faecalis cydABCD*), LUW20/pCYD23 (*B. subtilis cydABCD*) (28), and LUW20/pCYD26 (vector only) were grown at 37°C in nutrient sporulation medium with phosphate (4) supplemented with 0.5% glucose (NSMPG) and chloramphenicol (5 mg/liter). The cultures were harvested in the stationary phase. Membranes were prepared as described previously (7) and suspended in 20 mM sodium MOPS buffer, pH 7.4. Light absorption difference spectra of membranes from LUW20/pLUF04 showed an increased absorption at 561 nm and a peak at 626 nm, due to expression of a cytochrome *bd* (Fig. 3, spectrum B). Membranes from LUW20/pCYD23 showed a spectrum with an increased absorption at 563 nm and a peak at about 627 nm (Fig. 3, spectrum C), whereas membranes from the control, LUW20/pCYD26, lacked the peaks characteristic for cytochrome *bd* (Fig. 3, spectrum A), as expected. The absorption

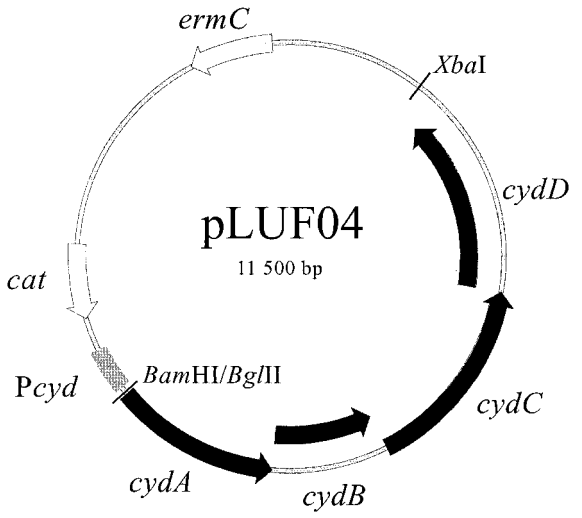

FIG. 2. Map of plasmid pLUF04, carrying the *E. faecalis cydABCD* genes. P*cyd* indicates the *B. subtilis cyd* promoter region. *Bam*HI/*Bgl*II shows where the PCR fragment (cut with *Bgl*II) was ligated to pCYD26 (cut with *Bam*HI). The chloramphenicol and erythromycin resistance genes are indicated by *cat* and *ermC*, respectively.

peak at about 600 nm in the spectra is mainly due to cytochrome *a* of the cytochrome aa_3 oxidase (27). These results show that the *cydABCD* genes of *E. faecalis* V583 can be expressed in *B. subtilis*, resulting in the formation of a spectroscopically detectable cytochrome *bd*.

The *E. faecalis cydABCD* genes can complement a *B. subtilis* cytochrome *bd*-deficient mutant. *B. subtilis* 168A cannot grow aerobically if both its quinol oxidases, cytochrome *bd* and cytochrome aa_3, are absent (L. Winstedt and C. von Wachenfeldt, unpublished data). The cytochrome aa_3 is encoded by the *qoxABCD* operon (27). To determine if *E. faecalis* cytochrome *bd* functions as a terminal oxidase, we examined whether a *B. subtilis* strain devoid of both cytochrome *bd* and cytochrome

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. faecalis V583		23
B. subtilis		
168A	*trpC2*	Laboratory stock
LUW20	*trpC2* Δ*cydABCD::tet*	28
LUH14	*trpC2* Δ*qoxABCD::kan*	Δqox→168A[b]
Δqox	*trpC2* Δ*qoxABCD::kan*	26
LUW173	*trpC2* Δ*qoxABCD::kan* Δ*cydABCD::tet* pCYD23	This work
LUW174	*trpC2* Δ*qoxABCD::kan* Δ*cydABCD::tet* pLUF04	This work
Plasmids		
pHPSK	Cm^r Em^r	12
pCYD23	*B. subtilis cydABCD* in pHP13	28
pCYD26	*B. subtilis cyd* promoter in pHPSK	This work
pLUF04	*E. faecalis cydABCD* in pCYD26	This work
pDG148	Km^r Em^r	25

[a] Cm^r, Em^r, and Km^r indicates resistance to chloramphenicol, erythromycin, and kanamycin, respectively.
[b] An arrow indicates transformation and point from donor to recipient.

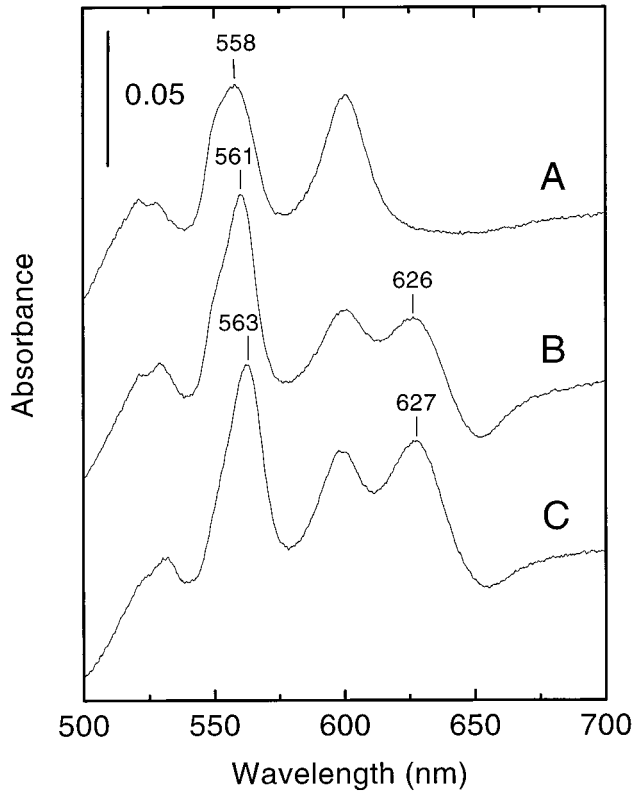

FIG. 3. Light absorption difference (dithionite-reduced minus ferricyanide-oxidized) spectra of *B. subtilis* membranes (2.5 mg of protein per ml). (A) LUW20/pCYD26; (B) LUW20/pLUF04 (*E. faecalis cydABCD*); (C) LUW20/pCYD23 (*B. subtilis cydABCD*). The vertical bar indicates the absorption scale.

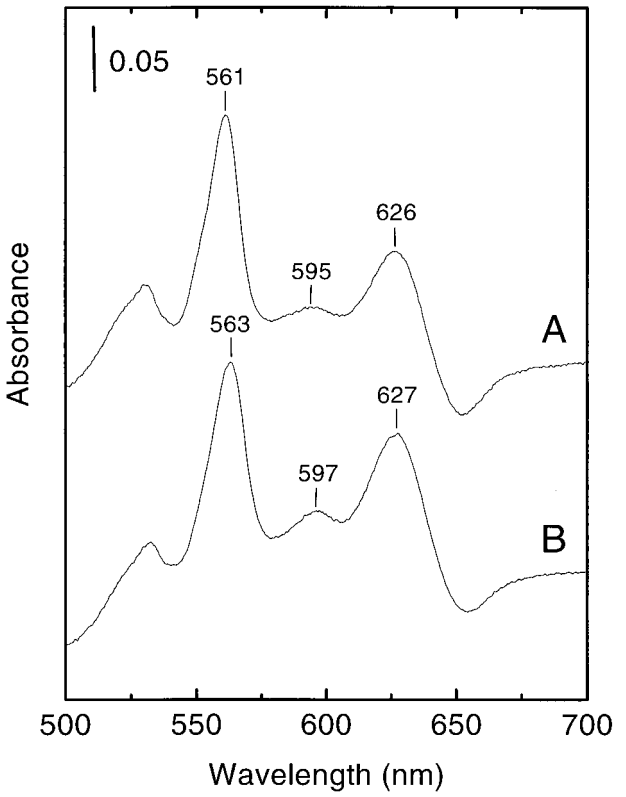

FIG. 4. Light absorption difference (dithionite-reduced minus ferricyanide-oxidized) spectra of *B. subtilis* membranes (2.5 mg of protein per ml). (A) LUW174 (Δ*qoxABCD::kan* Δ*cydABCD::tet* pLUF04), (B) LUW173 (Δ*qoxABCD::kan* Δ*cydABCD::tet* pCYD23). The vertical bar indicates the absorption scale.

aa_3, but carrying pLUF04, could grow under aerobic conditions. Chromosomal DNA from LUH14 (Δ*qoxABCD::kan*), prepared as described by Hoch (9), was used to transform LUW20/pLUF04, LUW20/pCYD23, and LUW20/pCYD26 to kanamycin resistance. The same limiting amount of LUH14 DNA (0.2 mg/liter of competent cells) was used for all three strains. Transformants were selected on tryptose blood agar base plates supplemented with 1% (wt/vol) glucose and containing chloramphenicol (5 mg/liter) and kanamycin (5 mg/liter). To verify that the transformants obtained still lacked the chromosomal copy of the *B. subtilis cydABCD* operon, they were streaked on plates containing tetracycline (15 mg/liter). As shown in Table 2, kanamycin- and tetracycline-resistant transformants, i.e., transformants deleted for both the *qoxABCD* and the *cydABCD* operons in the *B. subtilis* chromosome, were obtained only with LUW20/pLUF04 and LUW20/pCYD23. One transformant from each strain was kept and designated LUW174 and LUW173, respectively. The few transformants obtained with LUW20/pCYD26 were all sensitive to tetracycline; i.e., the tetracycline resistance marker in LUW20 had been substituted with the *B. subtilis cydABCD* operon from the LUH14 chromosomal DNA.

TABLE 2. Functional complementation of *B. subtilis* cytochrome *bd*-deficient mutants

Recipient strain[a]	No. of transformants (Km^r)[b,c]	% of transformants which were also Tet^r[c]
LUW20/pCYD23	108	99
LUW20/pLUF04	130	98
LUW20/pCYD26	3	0

[a] *B. subtilis* LUW20, carrying a Δ*cydABCD::tet* mutation in the chromosome and one of three plasmids, was transformed with chromosomal DNA from LUH14, carrying a Δ*qoxABCD::kan* mutation. All strains showed a similar degree of competence as tested by transformation with plasmid pDG148.

[b] Transformants were selected on plates containing kanamycin and chloramphenicol and then restreaked on plates containing tetracycline.

[c] Km^r and Tet^r indicate resistance to kanamycin and tetracycline, respectively.

To further characterize LUW174 and LUW173 and to compare the spectroscopic features of *E. faecalis* V583 cytochrome *bd* and *B. subtilis* cytochrome *bd* in more detail, the two strains were grown in NSMPG and membranes were prepared as described above. The growth properties of LUW174 did not differ from those of LUW173. Light absorption difference spectra of membranes from LUW174 (containing *E. faecalis* cytochrome *bd*) showed peaks at about 561, 595, and 626 nm (Fig. 4, spectrum A), indicating the presence of three prosthetic groups (low-spin heme *b*, high-spin heme *b*, and heme *d*). Membranes from LUW173 (containing *B. subtilis* cytochrome *bd*) showed peaks at about 563, 597, and 627 nm (Fig. 4, spectrum B). The absence of a peak at about 600 nm confirmed that the strains lack cytochrome aa_3.

Conclusion. In this work, we show that *E. faecalis* V583 contains a *cydABCD* gene cluster and that membranes from this strain grown in the presence of heme contain a cytochrome *bd*. Under these growth conditions, cytochrome *bd* is the major (and possibly the only) membrane-bound cytochrome in *E. faecalis*. The cloned *E. faecalis cydABCD* gene cluster expressed in *B. subtilis* resulted in a cytochrome *bd* which showed a spectrum indistinguishable from that of the cytochrome *bd*

found in *E. faecalis*. The *E. faecalis* cytochrome *bd* can functionally complement a *B. subtilis* cytochrome *bd*-deficient mutant, indicating that the *E. faecalis* cytochrome *bd* is a menaquinol oxidase. *E. faecalis* and *B. subtilis* both contain naphthoquinones in the cytoplasmic membrane: demethylmenaquinone and menaquinone, respectively (2). Thus, a specific electron donor for cytochrome *bd* is present in *E. faecalis*. These results indicate that *E. faecalis* is capable of aerobic respiration. The physiological importance of the identified respiratory system and its role in pathogenesis remain to be determined.

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