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Purification of a Cytochrome *bd* Terminal Oxidase Encoded by the *Escherichia coli app* Locus from a Δ*cyo* Δ*cyd* Strain Complemented by Genes from *Bacillus firmus* OF4

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***Escherichia coli* GK100, with deletions in the operons encoding its two terminal oxidases, cytochrome *bo* and cytochrome *bd*, was complemented for growth on succinate by a recombinant plasmid (pMS100) containing a 3.4-kb region of DNA from alkaliphilic *Bacillus firmus* OF4. The complementing DNA was predicted to encode five proteins, but neither sequence analysis nor complementation experiments with subclones provided insight into the basis for the complementation. Cytochrome difference spectra of everted membrane vesicles from the transformed strain had characteristics of a cytochrome *bd* spectrum but with features different from those observed for alkaliphile membranes. To determine the bacterial source and identity of the structural genes for the cytochrome *bd* in the transformed mutant, the complex was extracted and partially purified. On sodium dodecyl sulfate-polyacrylamide gels, two polypeptides were resolved from the preparation, 43 (subunit I) and 27 (subunit II) kDa. An internal peptide from subunit I was sequenced, and it yielded the same primary sequence as is found in positions 496 to 510 of *E. coli appC*. Consistent with the microsequencing results, pMS100 failed to complement a triple mutant of *E. coli* carrying a deletion in *appB* as well as in the *cyo* and *cyd* loci. The deduced sequence of AppBC had been predicted to be very similar to the sequence of CydAB (J. Dassa et al., Mol. Gen. Genet. 229:341–352, 1991), but this is the first demonstration that the former is indeed a cytochrome *bd* terminal oxidase. The enzyme catalyzed oxygen uptake coupled to quinol or *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine oxidation, and the activity was sensitive to cyanide. No cross-reactivity to subunit-specific polyclonal antibodies directed against the two individual subunits of *cyd*-encoded cytochrome *bd* was detected. Since this is the second cytochrome *bd* discovered in *E. coli*, it is proposed that the two complexes be designated cytochrome *bd*-I (*cydAB*-encoded enzyme) and cytochrome *bd*-II (*appBC*-encoded enzyme). In addition, *cbdAB* is suggested as a more appropriate gene designation for cytochrome *bd* than either *appBC* or *cyxAB*.**

The electron transport chains of aerobic bacteria typically contain multiple terminal oxidases, whose expression is regulated by developmental and/or environmental factors. The best-characterized system is in *Escherichia coli*, which has two terminal oxidases, cytochrome *bo*, encoded by the *cyo* operon, and cytochrome *bd*, encoded by the *cyd* operon (10, 43). Cytochrome *bd* has a high affinity for oxygen and is induced by microaerobiosis, while cytochrome *bo*, with a low affinity for oxygen, is highly expressed under oxygen-rich conditions (10). Genetic regulation of *cyo* and *cyd* expression occurs through the *arc* and *fnr* regulons and possibly a third, as yet unidentified, system (12, 15). The two terminal oxidases are structurally different, and only cytochrome *bo* functions as a proton pump. Both enzymes can nonetheless generate a proton motive force because of the liberation of scalar protons that occurs upon quinol oxidation (26, 27, 43). Cyd and Cyo are functionally equivalent insofar as mutants with alterations in either one of the enzymes grow as well as the wild type under standard laboratory conditions (10). In contrast, strains carrying point mutations and/or deletions in both *cyo* and *cyd*, e.g., strains RG129 and GK100, lose the ability to grow aerobically on nonfermentable substrates (2, 7, 30).

Our laboratory is engaged in an effort to enumerate and characterize the terminal oxidases of alkaliphilic *Bacillus firmus* OF4. The genes encoding one terminal oxidase complex, cytochrome caa_3, have thus far been cloned and sequenced (33). In the current study, a DNA library was screened for alkaliphile gene loci that were able to functionally complement mutants of *E. coli* that lack intact *cyo* and *cyd* and hence do not grow on succinate. It was anticipated that clones capable of complementing the *E. coli* mutants might include alkaliphile genetic regions that are responsible for the cytochrome *o*-like and cytochrome *bd*-like features that had been observed in membranes and partially purified extracts from the alkaliphile (14). Those proteins are still incompletely purified, and their genes have not been identified. As described here, however, the first complementing clone to be characterized does not itself encode a respiratory chain complex. Rather, it results in the expression of the *E. coli app* locus at a level sufficient to complement the terminal oxidase deficiency and to allow the first biochemical characterization of the *appBC* products. The similarity of the deduced *appBC* products to those of *cydAB* had been noted upon their discovery and the possibility of a role in resistance to oxygen toxicity had been advanced (5), but no biochemical information has since been reported in support of such proposals. The present characterization firmly establishes AppBC as a functional cytochrome *bd* complex.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* RG129, GK100, and SBS2019 were obtained

TABLE 1. *E. coli* strains used in this study

E. coli strain	Genotype	Reference or source
DH5αMCR	F⁻ *mcrA* (*mrr-hsdRMS-mcrBC*)	Gibco-BRL, Gaithersburg, Md.
RG129	*cydA2 cyo srl*::Tn*10recA*	2
GK100	Δ*cyo*::Kan^r Δ*cyd*::Cam^r	30
SBS2019	Δ*cyo*::Kan^r Δ*cyd*::Cam^r *appB*::Tn*phoA*	5
SBS2115	Δ*cyo*::Kan^r Δ*cyd*::Cam^r Δ*appBA*::Kan^r	P. L. Boquet

from R. Gennis. *E. coli* SBS2115 was provided by P. Boquet. All strains were maintained anaerobically on Luria broth (LB) containing 0.5% glucose. Anaerobiosis was achieved by using a BBL Gaspak 150 Chamber (Becton Dickinson, Cockeysville, Md.). A disposable anaerobic indicator supplied by the manufacturer was used to verify the establishment of anaerobic conditions. Chloramphenicol was added to the medium for strains GK100, SBS2019, and SBS2115. Ampicillin was added to all media for growth of transformants of all strains. Additional resistance markers were used to periodically check the identity and purity of the strains. The antibiotic concentrations used were as follows: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 12.5 μg/ml. For selection and small-scale growth of complemented strains, the medium used was usually M63 containing 0.5% succinate (37). Larger-scale cultures of complemented strains were grown in LB-succinate medium (succinate at 50 mM). Liquid cultures were shaken at 200 rpm to provide aeration. The incubation temperature for all experiments and culture maintenance was 37°C.

Complementation of mutant strains and isolation, sequence analysis, and subcloning of the complementing plasmid pMS100. Initial isolation of complementing plasmids was done with *E. coli* RG129. Cells were transformed by standard procedures with a DNA library from *B. firmus* OF4 that had been prepared with *Cla*I-digested DNA in pGEM3Zf(+) (Promega, Madison, Wis.) as described previously (16). Transformation protocols and other routine molecular biological procedures used in this study were taken from standard sources (34). The transformants were either plated directly on M63-succinate or first plated anaerobically on LB-glucose and then transferred to the selective plates. Cells transformed with the control vector, pGEM3Zf(+), did not grow on succinate-containing media but did form small colonies aerobically when glucose was provided as the carbon source. Formation of colonies by functionally complemented strains was observed on the selective plates within 48 h. The plasmid designated pMS100 was isolated from such a strain by the alkaline lysis method and purified by CsCl gradient centrifugation. The ability of pMS100 to complement was confirmed with *E. coli* RG129 and later with GK100 as well. The insert found in pMS100 and estimated to be 3.4 kb by restriction analysis was confirmed to be derived from *B. firmus* OF4 by Southern analysis; this analysis also indicated that there was no cross-hybridizing region in DNA from either *E. coli* or *Bacillus subtilis* (data not shown). The entire insert of pMS100 was sequenced on both strands by using synthetic oligonucleotide primers that were prepared commercially (Genset Corp., Paris, France). The sequencing was conducted by using an Applied Biosystems automated DNA sequencer at the Mount Sinai Brookdale Center for Molecular Biology. Sequence analysis was done with both FASTA and BLAST programs and utilized the Genetics Computer Group software package (6) run on a VAX 4000-300 computer. Estimations of molecular masses, isoelectric points, and hydropathy profiles were done with the MacVector software package.

Subclones of pMS100 (Fig. 1) were constructed in both pGEM and pSE420 (4), except for pMS104, which could only be prepared in pGEM3Zf(+). The subcloning was conducted in *E. coli* DH5αMCR. Digestion of pMS100 with *Xba*I, followed by religation of the recovered fragment containing the vector, generated the pMS102 subclone in pGEM3Zf(+). Insertion of the released *Xba*I fragment into *Xba*I-digested pGEM3Zf(+) resulted in the pMS101 subclone. The pMS103 and pMS104 constructs were prepared by blunt ligation of PCR products into *Sma*I-digested pGEM3Zf(+). The orientation of the inserts placed the genes under the control of the T7 promoter, except for the insert of pMS103, which had to be reversed by ligation into the similar vector pGEM4Z. The inserts from pMS101, -102, and -103 were recloned from pGEM plasmids into pSE420; all the inserts were present in this latter plasmid under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *trc* promoter.

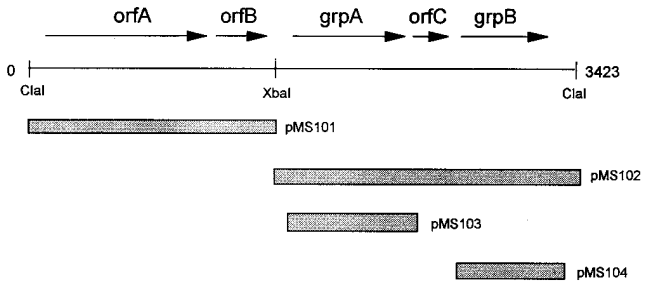

FIG. 1. Organization of ORFs present in the insert in pMS100. Arrows indicate the direction and length of the predicted gene transcripts. Construction of the depicted subclones is described in Materials and Methods.

Purification of cytochrome *bd* from the functionally complemented *E. coli* GK100 strain. Everted membrane vesicles were prepared from about 40 g (wet weight) of cells of *E. coli* GK100 transformed with pMS100 and grown in LB-succinate supplemented with ampicillin and chloramphenicol. The cell suspension was first washed with 50 mM potassium phosphate, pH 7.5–1 mM EDTA, pH 8.0 (buffer A) and suspended in the same buffer at approximately 5 ml/g (wet weight) of cells. The suspension was supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and a small amount of DNase and passed through a precooled French pressure cell at 18,000 lb/in^2. Unbroken cells and debris were removed by low-speed centrifugation (12,000 × *g*, 10 min), and the vesicles were collected by ultracentrifugation (200,000 × *g*, 60 min). The pellet was washed once with 5 mM potassium phosphate, pH 7.5–1 mM EDTA, pH 8.0–0.2 mM PMSF, centrifuged as described above, and suspended in buffer A at 5 to 10 mg of protein per ml. Sodium cholate was added to 2%, and the suspension was stirred for 2 h. Centrifugation as described above separated the yellow supernatant from the membrane pellet. The membranes were washed twice with buffer A plus 0.2 mM PMSF. The final pellet was suspended in buffer A plus 0.2 mM PMSF to 8 mg/ml. Lauryl maltoside (Anatrace, Maumee, Ohio) was added to 1.5%, and the suspension was incubated for 1 h at 4°C with stirring. The supernatant after ultracentrifugation (200,000 × *g*, 60 min) was brownish and contained over 90% of the cytochrome *bd* content of the membranes.

The supernatant was subjected to ammonium sulfate fractionation. Solid ammonium sulfate was added to the desired percent saturation, and the suspension was stirred for 15 min at 4°C before being subjected to a 15-min centrifugation at 27,000 × *g*. The suspension between 55 and 65% saturation was not completely clarified by this centrifugation, and so ultracentrifugation was carried out (twice at 90,000 × *g*, 15 min each time). The majority of the cytochrome *bd* was precipitated in this fraction. This material was brought up in a minimal volume of buffer A and loaded on a hydroxyapatite column (1.6 by 8.5 cm) equilibrated with 200 mM sodium phosphate, pH 7.5–1 mM EDTA, pH 8.0–0.02% lauryl maltoside. The hydroxyapatite chromatography was performed at room temperature, while all subsequent operations were performed at 4°C. In addition, all subsequent chromatography buffers also contained 1 mM EDTA and 0.02% lauryl maltoside. The column was washed with 2 bed volumes of 200 mM sodium phosphate and 2 bed volumes of 400 mM sodium phosphate. Most of the cytochrome *bd* could be eluted with 800 mM sodium phosphate. This fraction was diluted with 4 volumes of 0.02% lauryl maltoside so that it could be stored at 4°C. It was then concentrated by using a Centriprep-50 concentrator (Amicon, Beverly, Mass.), diluted, and reconcentrated to a minimum volume to lower the salt concentration to under 50 mM.

The cytochrome *bd* preparation was loaded on a DEAE-Sepharose CL-6B column (1.6 by 11.5 cm) equilibrated with 25 mM potassium phosphate, pH 7.5. A 200-ml 25 to 300 mM potassium phosphate gradient was applied to the column. Cytochrome *bd* eluted between 60 and 100 mM potassium phosphate. The tail of this peak overlapped with succinate dehydrogenase activity and was discarded. The peak fractions were concentrated to 1.0 ml and applied to a Sephacryl HR-300 gel filtration column (1.5 by 93 cm) equilibrated and run in 50 mM potassium phosphate, pH 7.5–100 mM NaCl–0.05% lauryl maltoside. Fractions exhibiting significant A_{412} were pooled and concentrated and then diluted to enable the preparation to bind to a Q Sepharose column (1.6 by 6 cm). The buffer was 25 mM potassium phosphate, pH 7.5, and the salt gradient consisted of 100 ml of 0 to 100 mM NaCl. The cytochrome *bd* did not elute, and a second gradient was applied to the column (100 ml of 100 to 250 mM NaCl). The complex eluted in this gradient between 130 and 170 mM NaCl. The peak fractions were concentrated on a Centriprep-50 concentrator, diluted, and reconcentrated to lower the salt concentration and then stored at −70°C.

SDS-PAGE, N-terminal amino acid sequence analysis, internal amino acid sequence analysis, and Western blots (immunoblots). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Schagger and von Jagow (35) in a mini gel apparatus. Samples were routinely denatured at room temperature in a buffer containing 50 mM Tris, 50 mM dithiothreitol, 4% SDS, 10% glycerol, and 0.01% bromophenol blue (pH adjusted to 6.8 with HCl). For amino acid sequencing, the gels were transferred following electrophoresis to polyvinylidene difluoride (PVDF) membranes in a buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid and 10% methanol (pH adjusted to 11.0 with NaOH). Following overnight transfer, the PVDF membranes were stained with 0.1% amido black–9% acetic acid–45% methanol for 5 min and then subjected to two 5-min periods of destaining with acetic acid-methanol without amido black. The membranes were washed briefly with deionized water and air dried. N-terminal amino acid sequence analysis was attempted by standard procedures with modified Edman chemistry. Internal amino acid sequence was obtained by Lys-C endoproteinase digestion of the PVDF-bound polypeptide, purification of the resulting peptides by microbore high-pressure liquid chromatography (HPLC), and amino acid sequencing of

selected purified peptides (8). Electrophoresis supplies and equipment were purchased from Bio-Rad, Hercules, Calif.

For Western blots, proteins were resolved on SDS–10% polyacrylamide gels as described above and transferred to nitrocellulose membranes in a standard Tris-glycine-methanol buffer by overnight electrophoresis. Western blotting was carried out according to the manufacturer's instructions (Bio-Rad) except that bovine serum albumin (BSA) was substituted wt/wt for gelatin; the second antibodies were coupled to alkaline phosphatase. The primary antibodies, generously provided by R. Gennis, were subunit-specific polyclonal antibodies directed against the previously purified *E. coli* cytochrome *bd* that is the *cydAB* product.

Spectral characterization. Absorption spectra were recorded at room temperature with a Perkin-Elmer 550 dual-beam spectrophotometer. Scans were performed at 2 nm/s with a slit width of 2 nm. The sample buffer was 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–NaOH (pH 7)–0.02% lauryl maltoside. For CO-reduced-minus-reduced difference spectra, the sample was reduced with dithionite and divided between two cuvettes. A baseline correction was obtained, and the sample cuvette was bubbled with CO for 2 min before the difference spectrum was recorded. Cytochrome *bd* content was estimated by using the extinction coefficient for the *cydAB*-encoded cytochrome *bd* of 7,400 M^{-1} cm^{-1} for $A_{628–605}$ (25). Pyridine hemochrome spectra were obtained in a solution of 0.1 N NaOH–20% pyridine. The reduced-minus-oxidized spectrum was measured by dithionite treatment of the sample and ferricyanide oxidation of the reference.

Enzyme assays. Oxygen uptake was determined at 37°C by using a Clark type electrode. The oxygen concentration was assumed to be 230 μM. Quinol oxidase activity could be conveniently measured by the coupled reaction as described by Lemma et al. (22), in which the quinone is reduced in the reaction vessel by diaphorase, with NADH as the reductant. Reaction mixtures contained, in a volume of 3.0 ml, 50 mM potassium phosphate (pH 6.3), 1 mM EDTA, 0.02% lauryl maltoside, 0.6 mg of diaphorase per ml (Sigma, St. Louis, Mo.), 0.3 mM quinone, and 0.6 mM potassium NADH. After NADH addition, the rate of autoxidation of the reduced quinone was monitored and the sample was then added. The rate of oxygen uptake due to autoxidation was subtracted from the rate obtained with the enzyme to determine the cytochrome *bd*-dependent rate of oxygen uptake. The quinones were dissolved in methanol, and the solvent concentration in the reaction mixture was 0.5%. TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine) oxidase activity was measured in a buffer containing 50 mM potassium phosphate (pH 7.5), 1 mM EDTA, and 0.02% laurylmaltoside. Samples were preincubated for 10 min in the buffer in the absence or presence of inhibitors, and the reaction was initiated with 5 mM TMPD. Background rates were determined in the absence of sample and were subtracted from the enzymatic rates. Succinate dehydrogenase assays were carried out according to the method of Hatefi (13).

Protein determination. The protein content of samples was estimated by the Lowry procedure (24), with BSA (Boehringer Mannheim, Indianapolis, Ind.) as the standard and assuming that 0.1% BSA has an A_{279} of 0.667.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the GenBank Data Bank under the accession number U39410.

RESULTS

Characterization of the complementing plasmid pMS100. Both *E. coli* RG129 and GK100 were complemented for growth on succinate by pMS100. Spectral analysis of everted membrane vesicles obtained from succinate-grown *E. coli* transformants of both mutant strains indicated the presence of a cytochrome *bd* species strikingly similar to the *cydAB*-encoded cytochrome *bd* of *E. coli*. The reduced-minus-oxidized difference spectrum of membranes showed absorption peaks at 626 nm, typical of a cytochrome *d*, and at 589 nm, similar to the cytochrome b_{595} of the cytochrome *bd* complex (data not shown). The putative cytochrome *bd* complex that has been partially purified from membranes of *B. firmus* OF4 is distinct from the *cydAB*-encoded complex and that of the transformant membranes in having the former peak at about 622 nm rather than at 626 nm (14). The reduced-plus-carbon monoxide-minus-reduced difference spectrum of the transformant membranes showed that cytochrome *d* reacted with CO, resulting in a peak at 640 nm and a trough at 619 nm. The preliminary spectral evidence suggested that the membranes contained a cytochrome *bd*-type terminal oxidase that was unlikely to be the one previously detected in alkaliphile membranes. Regardless, however, of its origin and relationship to pMS100, this terminal oxidase probably accounted for the functional complementation.

The 3.4-kb insert in pMS100 was sequenced completely on both strands. The analysis of the nucleotide and deduced amino acid sequences (Fig. 2) suggested the presence of five open reading frames (ORFs) organized as shown schematically in Fig. 1. The deduced properties were as follows.

(i) *orfA*. The first ORF, designated *orfA*, is predicted to encode a 37,600-Da protein with an estimated pI of 8.7. Hydropathy analyses indicated three potential transmembrane segments. No significant homologies to proteins present in the databases were detected. The best matches were to several penicillin-binding proteins; for example, a stretch of 42 residues was 30% identical to *B. subtilis* penicillin-binding protein 4 (32) and a separate stretch of the same protein was 73% identical across 15 residues. The upstream region of *orfA* contained candidate promoter recognition sequences including the sequences AGGANNT (from nucleotides 48 to 54) and GAATT (nucleotides 108 to 112), which are exact matches of the σ^H stage 0 sporulation promoter consensus sequence from *B. subtilis* (41). The σ^H promoter sites would require that the ATG initiation codon at bases 121 to 123 be utilized rather than the one present at bases 106 to 108. A potential Shine-Dalgarno sequence was present at bases 87 to 91.

(ii) *orfB*. The next ORF, *orfB*, potentially encodes a 12,500-Da hydrophobic protein with a predicted pI of 8.81. Residues 30 to 49 were found to be 40% identical to an N-terminal region of PsbK of tobacco chloroplasts (36), and residues 78 to 100 were 34% identical to a separate region of PsbK. The function of the *psbK* gene product is unknown, but a role in stabilization of the photosystem II reaction complex in chloroplasts of *Chlamydomonas reinhardtii* (40) has been suggested. A separate weak homology to several regions of *ctaA* from *B. subtilis* was detected. *ctaA* was first identified as a gene required for assembly of cytochrome aa_3 (29) and more recently was shown to have a role in heme A synthesis (39) and to encode a cytochrome *b* (38).

(iii) *grpA*. The third ORF was designated *grpA*, for glutamate-rich protein, because of the presence of 33 glutamate residues in the predicted sequence of the gene product. Overall, GrpA contains 21% acidic residues (aspartate plus glutamate residues). The *grpA* initiation codon appears to be the atypical GTG, which is used more frequently in organisms with a high GC content such as *Micrococcus luteus* (31). An analysis of the predicted hydropathy profile indicated the potential presence of an N-terminal transmembrane span which could tether the acidic protein to the cell membrane. The predicted molecular mass and isoelectric point of the *grpA* gene product are 22,300 Da and 4.17, respectively. Sequence analysis indicated similarity to numerous glutamate-rich proteins, including many calcium-binding proteins. Several regions of DNA-binding proteins were also found to exhibit weak homology; for example, a 79-residue stretch was found to be 25.3% identical to RpoD, the RNA polymerase σ factor of *Streptomyces aureofaciens* (20). σ^H promoter recognition sequences were also present upstream of *grpA*, as shown in Fig. 2.

(iv) *orfC*. A short ORF, designated *orfC*, encodes a putative 9,000-Da highly hydrophobic protein; only three charged residues are present, all basic. No significant homologies to any proteins in the databases were detected.

(v) *grpB*. The final ORF was predicted to encode a protein which, like GrpA, is very acidic. The isoelectric point and molecular mass of GrpB are predicted to be 5.3 and 20,300 Da, respectively. The best match found for GrpB was its 25% identity (residues 48 to 111) to a 63-amino-acid region of the

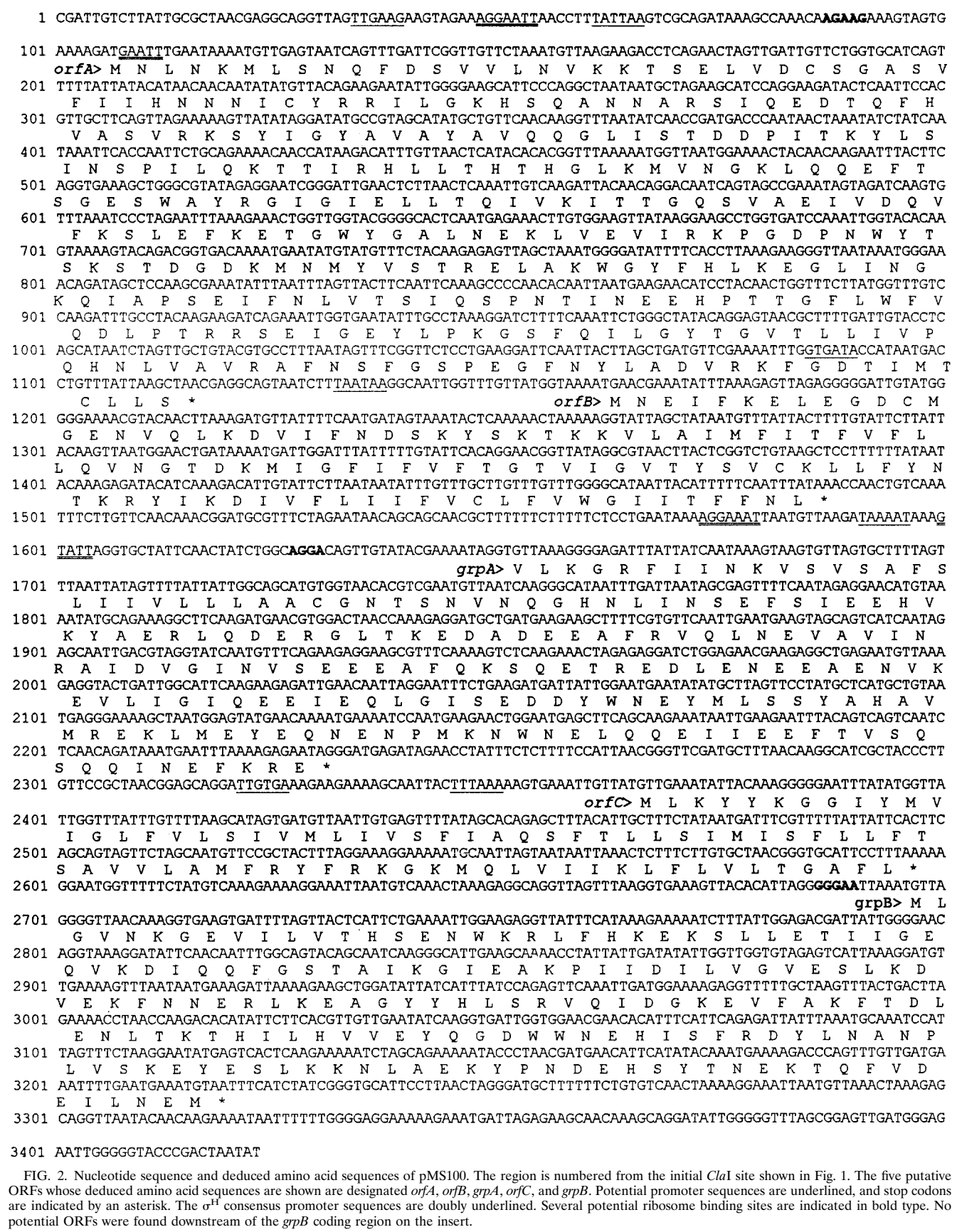

FIG. 2. Nucleotide sequence and deduced amino acid sequences of pMS100. The region is numbered from the initial *Cla*I site shown in Fig. 1. The five putative ORFs whose deduced amino acid sequences are shown are designated *orfA*, *orfB*, *grpA*, *orfC*, and *grpB*. Potential promoter sequences are underlined, and stop codons are indicated by an asterisk. The σ^{H} consensus promoter sequences are doubly underlined. Several potential ribosome binding sites are indicated in bold type. No potential ORFs were found downstream of the *grpB* coding region on the insert.

TABLE 2. Purification of cytochrome *bd*-II from *E. coli* GK100/pMS100

Step	Protein (mg)	Cytochrome *bd*		% Yield
		Amt (nmol/mg)	Total (nmol)	
Cholate-washed membranes	204.9	1.0	204.9	100
Lauryl maltoside extract	139.2	1.4	196.0	96
Ammonium sulfate fractionation	67.4	1.7	116.1	57
Hydroxyapatite chromatography	20.0	3.0	60.5	30
DEAE CL-6B chromatography	7.3	3.7	26.6	13
Gel filtration	4.6	4.2	18.9	9
Q Sepharose chromatography	2.55	5.5	14.1	7

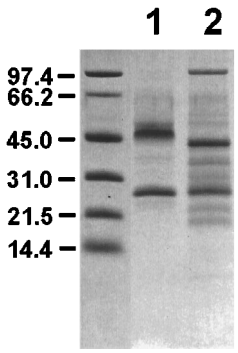

FIG. 3. SDS-PAGE of cytochrome *bd*-I and cytochrome *bd*-II. Lane 1, cytochrome *bd*-I, 7 μg; lane 2, cytochrome *bd*-II, 7 μg. Cytochrome *bd*-I was purified from *E. coli* essentially as described by others (26). Molecular mass standards, in kilodaltons, are indicated on the left.

activator gene of the phosphoglycerate transport system of *Salmonella typhimurium* (44).

Sequence analysis thus indicated that the genes in pMS100 were not related to known cytochrome *bd* genes (11, 28). It was hypothesized that the region of alkaliphile DNA cloned in pMS100 is involved in sporulation or other stationary-phase functions with a probable relationship to Ca^{2+}. The most likely basis for the complementing activity appeared to be the two genes, *grpA* and *grpB*, that might well have regulatory functions. They might thus induce *E. coli* genes that encode a third terminal oxidase. One candidate for a third terminal oxidase is the *appBC* genes, which show strong sequence similarity to *cydAB* (5). The effect of *grpA* and/or *grpB* on such heterologous genes could be direct or through some regulatory network in *E. coli* which shares late-growth-stage functions with the alkaliphile *grp* genes. To test the hypothesis that *grpA* and/or *grpB* were the agents of the complementation, the subclones shown in Fig. 1 were transformed into *E. coli* GK100 in either pGEM vectors or pSE420; the latter complementations were assayed in both the presence and absence of IPTG. None of the subclones were able to functionally complement GK100. The negative results indicate either that the genes in the subclones were not expressed in the same way as they were in the parent clone, pMS100, or that genes from both halves of the insert are required for complementation. This remains unresolved.

Next, the involvement of the *appBC* genes in the complementation was examined by testing the ability of pMS100 to complement *E. coli* SBS2019 and SBS2115, which lack a functional *appB* gene in addition to carrying deletions in *cyo* and *cyd*. The sequence similarity of the *E. coli appBC* genes to the *cydAB* genes suggested to Dassa et al. (5) that they were likely to encode a cytochrome *bd*-type terminal oxidase complex, but this had not yet been confirmed. *E. coli* SBS2019 and SBS2115 were readily transformed to ampicillin resistance by pMS100, but the transformants did not grow aerobically on succinate. This was consistent with the hypothesis that the *appBC* genes were the structural genes whose enhanced activity directly resulted in growth of the transformant on succinate. Unambiguous demonstration of this connection, however, required the purification of the complex that is present in the transformant so that amino acid sequence information could be obtained.

Purification of cytochrome *bd* from the *E. coli* GK100/pMS100 transformant. Purification of the cytochrome, hereafter referred to as cytochrome *bd*-II, was carried out as described in Materials and Methods. The purification procedure is summarized in Table 2. Purification was monitored by SDS-PAGE, by the specific cytochrome *bd* content, and by the ratio of cytochrome *b* content relative to cytochrome *d* content. When analyzed by SDS-PAGE, the final preparation was found to contain two major bands, representing subunits I and II of the complex, as well as some contaminating bands migrating between the two subunits and below subunit II (Fig. 3). There was one prominent contaminant at approximately 100 kDa; this band may represent an aggregate of subunits I and II that has been observed with some preparations of the *cydAB*-encoded complex (19), hereafter referred to as cytochrome *bd*-I. The specific cytochrome *d* content was 5.5 nmol/mg in several different preparations, somewhat lower than the specific content observed with cytochrome *bd*-I preparations (e.g., about 10 nmol of cytochrome *d* per mg of protein [18]). The lower content of cytochrome *bd*-II probably reflects the fact that the preparation still contains impurities and may also result from the use of an extinction coefficient for cytochrome *bd*-I (25). As suggested by Junemann and Wrigglesworth (17), the ratio of $A_{559–580}$ to $A_{628–650}$ in the reduced-minus-oxidized difference spectrum can serve as an index of purity, with ratios of less than 1 expected in pure preparations. As shown in Fig. 6, this ratio is 0.8, suggesting minimal cytochrome *b* contamination.

Amino acid sequence of an internal peptide of subunit I. The N terminus of subunit I was blocked. The PVDF-bound subunit I was digested with endoproteinase Lys-C, the peptides were purified by microbore HPLC, and a peptide was chosen for sequence analysis (8). The peptide had the sequence YARLGPSAMQ(S)E(Q)P(T), the parentheses indicating tentative assignments. This corresponded exactly with amino acids at positions 496 to 510 predicted for the *appC* product (5). From this information, it was concluded that the subunits of cytochrome *bd*-II were encoded by *appC* (subunit I) and *appB* (subunit II).

Electrophoretic analysis of cytochrome *bd*-II. The complex contained two subunits that on SDS–10% polyacrylamide gels cast according to the method of Schagger and von Jagow (35) had estimated sizes of 43.0 ± 1.6 kDa (subunit I) and 27.0 ± 1.5 kDa (subunit II) ($n = 4$; to the nearest 0.5 kDa). The subunits of cytochrome *bd*-I were estimated to be 47.5 ± 1.7 kDa (I) and 26.5 ± 1.5 kDa (II) ($n = 4$). All the subunits migrated anomalously, i.e., they were calculated on the basis of their mobility to be smaller than their predicted sizes. Strikingly, however, the AppC gene product, which is predicted to be just 8 amino acids shorter than subunit I of cytochrome *bd*-I (514 amino acids compared with 522) and which has a very similar hydropathy profile (5), migrated on gels as if it were about 4.5 kDa smaller. There is no obvious explanation for this phenomenon, which was also observed on 10 to 20% gradient gels (data not shown). Subunit II migrated very similarly to subunit II of cytochrome *bd*-I, consistent with there being just

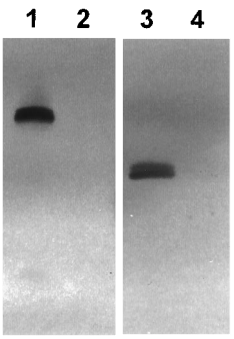

FIG. 4. Protein immunoblot of cytochromes *bd*-I and *bd*-II with antibodies against the subunits of cytochrome *bd*-I. Lanes 1 and 2, blots probed with anti-subunit I; lanes 3 and 4, blots probed with anti-subunit II. Lanes 1 and 3 contain cytochrome *bd*-I at 1 and 5 μg, respectively; lanes 2 and 4 contain cytochrome *bd*-II at 6 and 15 μg, respectively.

one amino acid difference between the two (378 amino acids compared with 379).

Immunological reactivity of cytochrome *bd*-II to antibodies against cytochrome *bd*-I. The close similarity in deduced amino acid sequence between the AppBC and CydAB operons raised the issue of whether there might be cross-reactivity with antibodies against cytochrome *bd*-I. As shown in Fig. 4, subunit-specific polyclonal antibodies reacted very strongly and specifically with the appropriate subunits of cytochrome *bd*-I, as visualized by Western blot experiments. In contrast, no reaction could be detected with the subunits of cytochrome *bd*-II, despite loading of at least three times more II than of I, on a protein basis.

Spectral analysis of cytochrome *bd*-II. The absolute spectra of cytochrome *bd*-II are shown in Fig. 5. The air-oxidized spectrum had major peaks at 648 and 411 nm (Fig. 5A), while the reduced enzyme exhibited maxima at 628, approximately 589, 559, 530, and 428 nm (Fig. 5B). When the reduced enzyme was treated with carbon monoxide, peaks were observed at 632, 558, 530, and 425 nm, with a shoulder at about 588 nm (Fig. 5C). The reduced-minus-air-oxidized spectrum is shown in Fig. 6A; peaks were exhibited at 626, 589, 559, 529, and 433 nm. The difference spectrum indicates the presence of cytochrome *d* (626 nm), the high-spin cytochrome b_{595} (589 nm), and cytochrome *b* (559 nm). Most of the evidence supports a model for cytochrome *bd*-I of one cytochrome *d*, one cytochrome b_{595}, and one cytochrome *b* (low spin) per complex (43). The reduced-plus-CO-minus-reduced difference spectrum (Fig. 6B) gave maxima at 640, 432, and 416 nm and minima at 619, 440, 428, and 408 nm. This spectrum more closely resembled that of cytochrome *bd*-I from *E. coli* (26) than that of the cytochrome *bd* from *Azotobacter vinelandii* (19). The pyridine hemochrome difference spectrum showed peaks attributable to D heme (607 nm) and B heme (556 nm), indicating that only those hemes are present (data not shown).

Enzyme activity. To assign a terminal oxidase function to cytochrome *bd*-II, it was necessary to determine whether it had oxygen uptake activity. The preferred substrate in vitro for cytochrome *bd*-I, ubiquinone-1 (18, 26), was not available, and so other reduced quinones were tested as possible electron donors for cytochrome *bd*-II. Because of the susceptibility of quinols to autoxidation, it was desirable to use an assay system that allowed for reduction of the quinone in the reaction vessel (22), which minimized the rate of autoxidation. As shown in Table 3, cytochrome *bd*-II utilized a number of quinols as substrates; menadiol was the preferred quinol, with a specific

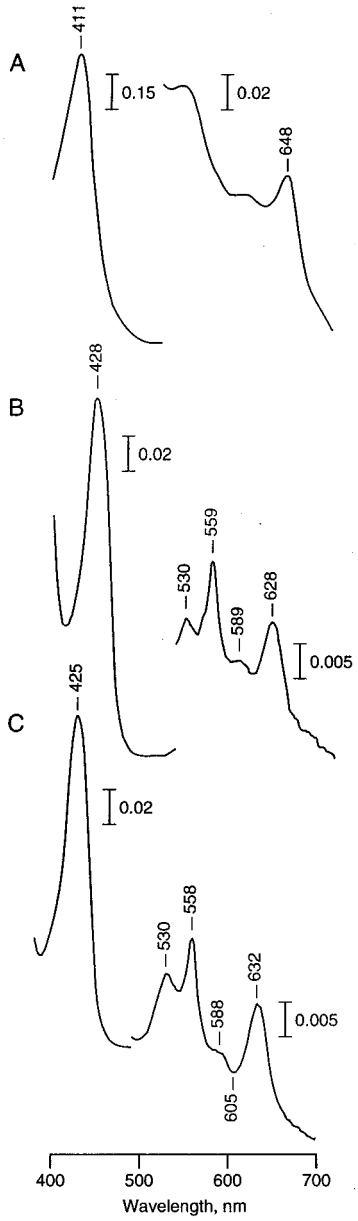

FIG. 5. Absolute absorption spectra of cytochrome *bd*-II. (A) Air oxidized; (B) dithionite reduced; (C) dithionite reduced plus CO. Protein concentrations were 1.5 (A) and 0.2 (B and C) mg/ml.

activity of 15.3 μmol of O_2 consumed min^{-1} mg of $protein^{-1}$. The effect of monovalent cations on enzyme activity was studied in light of the findings reported by Avetisyan et al. (3) that *E. coli* strains lacking cytochrome *bo* were able to grow at low proton motive force by induction of an Na^+-motive cytochrome *bd*. Neither NaCl nor KCl had a significant effect on enzyme activity. This correlated with an absence of any demonstrable stimulation by or dependence on Na^+ for growth of the complemented *E. coli* strain on succinate (data not shown). Cytochrome *bd*-II also catalyzed TMPD oxidation (Table 3). The effect of cyanide was measured by this assay because the longer persistence of a linear rate of reaction permitted more accurate calculation of the percent inhibition. As shown in Table 3, 1 mM KCN inhibited 89% of the activity and 10 mM caused essentially complete inhibition. Compared

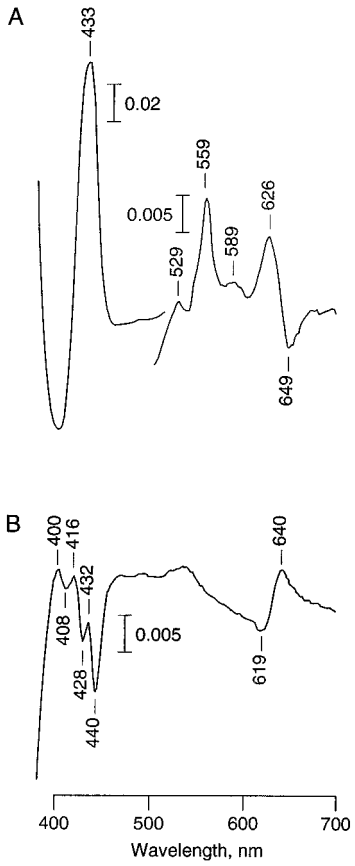

FIG. 6. Difference absorption spectra of cytochrome *bd*-II. (A) Dithionite reduced minus air oxidized; (B) dithionite reduced plus CO minus dithionite reduced. The protein concentration for both panels was 0.2 mg/ml.

with the relatively cyanide-resistant ubiquinol-1 oxidase activity of cytochrome *bd*-I (18), which was only about 20% inhibited by 1 mM KCN, the TMPD oxidase activity of cytochrome *bd*-II was much more sensitive to cyanide.

DISCUSSION

The major finding of this work is that the product of the *appBC* genes functions as a terminal oxidase complex allowing strain GK100/pMS100 to grow aerobically on succinate. The product is a cytochrome *bd*-type terminal oxidase that catalyzes quinol-dependent, Na^+-independent oxygen uptake. The *appBC* genes were discovered by Dassa et al. (5) during a study that had been undertaken to localize a promoter accounting for the induction properties of the downstream acid phosphatase gene, *appA*. Disruption of *appB* in the absence of disruptions of the loci for both of the other terminal oxidases resulted in no obvious growth phenotype under either aerobic or anaerobic conditions; the triple mutant, however, was more sensitive to aerobic conditions than was a strain disrupted only in *cyo* and *cyd* when glucose-containing medium was used to allow fermentative growth (5). This suggested a role for *appBC* in oxygen tolerance, and the sequence analysis suggested that a cytochrome *bd*-type activity was involved. On the presumption that the *appBC* products would prove to have such activity, Atlung and Brondsted (1) recently suggested that these genes be renamed *cyxAB*. Given the now clearly established cytochrome type and activity, and the need to differentiate between

TABLE 3. Oxidase activities of cytochrome *bd*-II

Activity	Substrate	Addition (concn [mM])	Sp act[a]	% of control
Quinol oxidase[b]	Menadiol		15.3	100
	Menadiol	NaCl (50)	17.1	112
	Menadiol	KCl (50)	17.3	113
	1,4-Napthoquinol		7.7	
	Duroquinol		6.2	
	2,3-Dimethyoxy-5-methyl-1,4-benzoquinol		1.0	
TMPD oxidase[c]			16.6	100
		KCN (0.1)	14.4	87
		KCN (0.5)	5.5	33
		KCN (1)	1.8	11
		KCN (10)	0.06	0.4

[a] Micromoles of O_2 consumed $minute^{-1}$ milligram of $protein^{-1}$.
[b] The assay pH was 6.3. See Materials and Methods for details.
[c] The assay pH was 7.5. See Materials and Methods for details.

genes encoding cytochromes *bd*-I and *bd*-II, we prefer and propose use of the gene designations *cbdAB* for the genes encoding the latter complex. The content of cytochrome *bd*-II in everted membrane vesicles from the transformant, 0.8 to 1.0 nmol of cytochrome *bd* per mg of membrane protein, was comparable to the membrane cytochrome *bd* content in a wild-type strain grown to stationary phase, consistent with its support of growth on succinate (data not shown).

Induction of *appA* is associated with (i) a shift to anaerobic growth, (ii) P_i starvation, or (iii) entrance into stationary phase (1). The operon is transcribed in the order *appC*, *appB*, *orfX*, and *appA* from a strong promoter upstream of *appC*. A weaker promoter that is utilized only during P_i starvation and stationary-phase expression and not, apparently, in response to anaerobiosis (1), is found in front of *appA*. *appY*, an *araC*-type regulatory gene (9) that is not linked to the *cbd-app* operon, is required for full induction of the operon by changes in growth conditions, e.g., anaerobiosis (1). In turn, the expression of *appY* is under complex global regulation that involves *rpoS* (which has also been studied under the designations *katF* and *appR*), *fnr*, and *arcA* (21, 23, 42). Clearly, the *cbd-app* locus is under complex regulation that reflects functions of the locus in stationary phase and in relation to oxygen tension. The mechanism whereby the alkaliphile genes in pMS100 lead to greatly enhanced levels of cytochrome *bd*-II in the membrane has not been established in this study. However, the mechanism probably involves cross talk between regulatory genes in the clone and the global networks regulating *cbd-app*. One possibility is that an *E. coli* global regulator up-regulates a gene or combination of genes in the complementing clone and those alkaliphile genes lead to strong positive regulation of *appY* or of the *cbd-app* operon itself. In this connection, it is notable that the proposed consensus sequence for the −35 region of the σ^S promoter is GTTAAGC (23) and residues 1585 to 1591, upstream of *grpA*, are GTTAAGA. The presence of σ^H recognition sites in the region cloned in pMS100 is consistent with a role of the cloned genes in physiological events that occur in late phases of growth, probably including sporulation and involving Ca^{2+}.

Finally, the finding of a condition, albeit one involving heterologous genes, under which high levels of cytochrome *bd*-II are found in the membrane raises the possibility that there are conditions under which *E. coli* normally produces high concentrations of this complex. There may be no single gene mutation

that leads to such expression, since strains lacking intact *cyo* and *cyd* do not produce cytochrome *bd*-containing revertants at high frequency when plated on succinate plates. However, appropriate signalling through the regulatory pathways, under specific conditions, may lead to levels of cytochrome *bd*-II that are a significant percentage of the total terminal oxidase content of the cells. Such a contribution of cytochrome *bd*-II could escape notice. The spectral properties of cytochrome *bd*-II closely resemble those of cytochrome *bd*-I, and it would be very difficult to distinguish one from the other. Secondly, cytochrome *bd*-II did not cross-react with antibodies to cytochrome *bd*-I. If a cross-reaction had been observed, the substantial difference in the apparent sizes of subunit I on SDS-PAGE would have been readily apparent. Antibodies raised against cytochrome *bd*-II will be very helpful in studying its expression and likely contribution under a variety of growth conditions.

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