Cloning of the cta Operon from Alkaliphilic Bacillus firmus OF4 and Characterization of the pH-regulated Cytochrome caa₃ Oxidase It Encodes*

Philip G. Quirk, David B. Hicks, and Terry A. Krulwich†

From the Department of Biochemistry, Mount Sinai School of Medicine of CUNY, New York, New York 10029-6574

We have cloned and sequenced the DNA of alkaliphilic Bacillus firmus OF4 encompassing the cta operon that encodes a pH-regulated cytochrome caa₃ oxidase. The gene organization is identical with that of the homologous Bacillus subtilis caa₃ oxidase locus (van der Oost, J., von Wachenfeld, C., Hederstedt, L. & Saraste, M. (1991) Mol. Microbiol. 5, 2063–2072). The deduced amino acid sequences of the four putative structural subunits (CtaC-F) indicate substantial similarity to caa₃-type oxidases from other Bacillus species and to other members of the family of mitochondrial-type aa₃ oxidases. A marked paucity of basic residues was noted in the cytochrome c-containing domain of CtaC, which faces the highly alkaline external milieu. We have also purified the enzyme as a three-subunit complex, with possible trace amounts of a fourth subunit. N-terminal sequence analysis of the two largest subunits confirmed them to be encoded by the cloned cta genes. An additional, minor caa₃ component with distinctive chromatographic properties was noted during purification. Analysis of mRNA with a ctaD probe revealed an abundant 4-kilobase message of the right size to encode CtaC-F. The cellular content of this message varied with growth pH. Cells grown at pH 10.5 contained 2 to 2.5 times more message than those grown at pH 7.5, in good correspondence with the relative amounts of caa₃ oxidase found in the cells. The ctaB gene, immediately upstream from the ctaC-F genes, was found to be transcribed onto a low abundance 5-kilobase message, which is likely also to encode CtaC-F. Levels of this message were not affected by growth pH.

The facultative alkaliphile, Bacillus firmus OF4, grows well over a pH range extending from 7.5 to above 10.5 (1). The organism possesses a branched respiratory chain with at least three terminal oxidases, cytochrome o, cytochrome d, and cytochrome caa₃, which have been studied by difference spectroscopy and partially purified (2). B. firmus OF4 contains very high concentrations of cytochromes throughout the pH range of growth. The caa₃ oxidase, in particular, is present in a 2–3-fold higher concentration in the membranes of cells grown at pH 10.5 versus pH 7.5, suggesting some mechanism of pH-dependent regulation (3). Increases in cytochrome o, albeit more modest on a percentage basis of this more abundant species, are also observed during growth at high pH. Elevated levels of caa₃ oxidase were additionally found in cells grown at pH 7.5 in the presence of sublethal amounts of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) (3). Thus, increased amounts of cytochrome caa₃ are associated with two distinct growth conditions where ΔpH is low: pH 10.5 or pH 7.5 in the presence of CCCP. In addition, a mutant, CCCP-resistant strain was found to contain constitutively elevated levels of cytochrome caa₃ when grown at pH 7.5 (3).

ATP synthesis by alkaliphilic bacilli such as B. firmus OF4 poses a bioenergetic problem. Oxidative phosphorylation at alkaline pH has been shown to deviate from the chemiosmotic model of Mitchell (4) in two respects (5, 6). Firstly, the protonmotive driving force (ΔpH) is very low at pH 10.5 and above and is insufficient to account for the observed phosphorylation potential unless a high and variable H⁺/ATP stoichiometry is posited. Secondly, artificially imposed diffusion potentials fail to energize ATP synthesis at pH values above 9.5. To explain these discrepancies, it has been hypothesized that oxidative phosphorylation at very alkaline pH values may involve the direct, intramembranous transfer of protons from respiratory chain complexes to the F₀ sector of the F₀F₁ ATP-synthase (5). In addition, one or more of the respiratory chain complexes whose concentration increases during growth at very alkaline pH is presumably responsible for the increased transmembrane electrical potential observed in cells grown at such pH values (1). By virtue of its elevated expression at high pH, the cytochrome caa₃ oxidase is an attractive candidate for mediating one of these roles. The involvement of a pH-regulated complex in the putative direct proton pathway is specifically supported by physiological data. For example, when cells grown at pH 7.5 were starved and then re-energized at pH 10.5, they synthesized much less ATP than similarly treated cells that had been grown at pH 10.5 (6), although the ΔpH generated was the same.

In order to better understand oxidative phosphorylation at the molecular level, we recently cloned and sequenced the ATP genes encoding the F₀F₁ ATP-synthase (7). Several nonconservative substitutions at otherwise highly conserved residues were noted in the predicted sequences of the a- and e-subunits of the F₀ moiety, and equivalent substitutions were found in the genes of a second, distinct alkaliphilic Bacillus (8). To complement these efforts, a parallel project was initiated on the cytochrome caa₃ oxidase, and we will pursue a comparable

*This research was supported by Grant DE-FG02-86ER13559 from the Department of Energy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 17 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence and reprint requests should be addressed: Dept. of Biochemistry, Box 1020, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029-6574. Tel.: 212-241-7280; Fax: 212-996-7214.

The abbreviations used are: CCCP, carbonyl cyanide m-chlorophenylhydrazone; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; aa, amino acid; kb, kilobase(s).
study of cytochrome o. Here, we report on the cloning and sequencing of the cta genes encoding the caa3 oxidase and the further purification and characterization of the enzyme.

MATERIALS AND METHODS

**Bacterial Strains and Growth Conditions**—*B. firmus* OF4 and its derivative strain 811M (met+, str+) were grown as previously described (3). During the course of this work, it was noted that cells grew better at pH 10.5 when the ammonium sulfate was omitted from the growth medium, and this modification was then adopted routinely. *Escherichia coli* strains DH5αMCtr (Gibco BRL) and JM109, used as plasmid hosts, were grown in LB medium, with the addition of ampicillin (100 μg/ml) where appropriate.

**Cloning Procedures**—Standard protocols were employed for all cloning procedures (9, 10). Radioactive probes were prepared by random priming of appropriate DNA templates, using a commercial kit (New England Biolabs). Plasmid DNA for sequencing was prepared by large scale alkaline lysis and purified by centrifugation through a CsCl gradient. Both strands of clones were sequenced using an Applied Biosystems 373A Sequencer. Appropriate oligonucleotide primers were synthesized using an Applied Biosystems 380B DNA synthesizer or were obtained commercially (Cetus, Paris). Computer analysis of nucleotide sequences was done using the GCG suite of programs (11) running on a VAX 4000–5000 computer. A Perkin-Elmer Cetus Instruments thermal cycler was used to perform polymerase chain reactions (PCR). The entire DNA sequence has been deposited in the GenBank library; throughout this paper, the numbering of bases follows that of the deposited sequence.

Initial attempts to generate a probe for the *B. firmus* OF4 caa3 oxidase genes utilized a DNA fragment from the *Bacillus subtilis* cta operon, but this DNA failed to generate a signal in hybridization screens. Instead, PCR was performed on purified *B. firmus* OF4 chromosomal DNA using two oligonucleotides, D1 and D2, designed to hybridize to the DNA encoding two highly conserved regions of subunit I about 150 bases apart. The probe sequences were: D1, GGCACCGGAGTTTAC (1 denotes inosine); D2, GACCTGAGTTTACCAACCAT (Fig. 2D). After 25 cycles of amplification, the hybridized product at 50°C was fractionated through a 6% polyacrylamide gel and the base pair product was found and ligated into pGEMZf(+) (Promega) by the method of Marchuk et al. (12). The resulting construct was designated pCOX1101 (Fig. 1). Sequencing revealed the insert to be highly homologous to the corresponding regions of the cytochrome caa3 oxidase genes cloned from *Bacillus subtilis* (13) and *Bacillus species* PS3 (14). The PCR product was used to probe a library of *B. firmus* OF4 DNA [library 3 of Ref. 15]. Three overlapping clones were isolated (pCOX17, pCOX45, pCOX163), which encompassed most of the cta-cdA region (Fig. 1). To obtain the 5’ end of the ctaB gene and upstream sequence, a nested set of primers was designed on an already sequenced portion of the gene, was prepared by PCR (primers: B1, GATCTGAGCATGATCCTTTAG; B2, TAACTGGATACCGCTGAGCTC, complement of bases 2736–2759), and further sequencing of the library led to the isolation of pCOX1711 (Fig. 1). To obtain the 3’ end of the ctaD gene and downstream sequence, a further probe was prepared by PCR, using a forward primer (D3, CTTGCTTTATCTACAGTGTT, bases 5699–5718) based on a known subsequence from the pCOX43 insert, and a reverse primer (E1, CCAACACATCATATAAATGCCA) designed to recognize a portion of the ctaD gene, which was expected to lie immediately downstream from ctaD. Subsequent sequencing of the library allowed the isolation of the cta region (Fig. 1).

As a precaution against the possibility of deletions or rearrangements of DNA (13), PCR was performed on *B. firmus* OF4 chromosomal DNA and the pCOX clones. Surprisingly, several common sequences of sequencing primers. Products of the expected size were obtained in all cases, indicating that no rearrangements had occurred during the cloning procedures.

**Analysis of RNA**—RNA was prepared from logarithmic phase cells, electrophoresed, blotted, and probed as described. DNA and PCR product complementary to the cta and ctaD genes were also used as probes. The primer sequences for PCR reactions were: ctaA, A1 (CCTGTCAGAGCTCAGCTCTG, bases 1289–1309), and A2 (ACGATTGAAGATTTATAGTGTC, complement of bases 1990–2011); ctaB, B1 and B2 (see above); ctaD, D1 (CCTGTCAGAGCTCAGCTCTG, bases 1807–1827) and D2 (ATAATCCTGAGTTAGCTCG, bases 573–582). Equal amounts of RNA (typically 5, 10, and 20 μg, as measured by spectrophotometric absorbance at 260 nm) from each of several growth conditions were electrophoresed; the relative intensities of the RNA bands were visualized by ethidium bromide staining and were found to vary by less than 20% between preparations.

**Purification of the Cytochrome caa3 Complex**—The initial stages of purification followed precisely those employed in the F,F-,ATPase isolation procedure (17). Briefly, washed membranes were extracted with 50 mM octyl glucoside in a buffer containing 50 mM Tricine-KOH, pH 8, 5 mM MgCl2, 5 mM p-aminobenzoamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, and 3 mg/ml soybean aseoin. The cytochrome c oxidase-containing supernatant from high speed centrifugation was subjected to ammonium sulfate fractionation. The cytochrome remained soluble at 60% saturation; the salt concentration was lowered in this fraction by several steps of ultrafiltration and dialysis during which Triton X-100 was added to a concentration of 0.1%. The sample was then diluted with 2 volumes of Buffer A (10% glycerol, 25 mM Tris-SO4, pH 7, 1 mM EDTA, 0.1 mM PMSF, and 0.5% Triton X-100) and loaded onto a DEAE-Sepharose CL-6B column (2.5 × 18 cm) equilibrated with Buffer A. After washing the column with several bed volumes of Buffer A, a linear gradient between 0 and 0.6 M (NH4)2SO4 (800 ml) was applied to the column, and 5.2-ml fractions were collected. The major peak of cytochrome c oxidase activity, eluting as a broad peak between about 300 and 450 mM (NH4)2SO4, was concentrated by ultrafiltration using a PM-30 membrane and dialyzed overnight against 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, and 0.1 mM PMSF. The dialyzed preparation was split into 2 volumes, and each portion was applied to a 1.5 × 9.5-cm hydroxyapatite column. The column was washed successively with 2 bed volumes each of 10, 25, and 50 mM potassium phosphate, pH 7.5 (buffers also contained 1 mM EDTA, 0.1 mM PMSF, and 0.5% Triton X-100). Cytochrome c oxidase was eluted with 100 mM potassium phosphate. The enzyme eluted rather sharply off this column and was collected in a small volume which was further concentrated by ultrafiltration. The preparation was divided into aliquots, frozen in liquid N2, and stored at −70°C.

**SDS-Polyacrylamide Gel Electrophoresis and Related Procedures**—The polypeptide composition of cytochrome c oxidase was examined on SDS-10% polyacrylamide gels (12 × 15 × 0.15 cm, with 1.5% stacking gel) according to the procedure of Schagger and von Jagow (18). Samples were incubated in sample buffer for 15 min at room temperature prior to electrophoresis. The gels were run overnight, fixed for 1 h in 10% acetic acid, 45% methanol and stained with Coomassie G (19) or with silver (20). Heme staining was performed as described in Refs. 21 and 22. For N-terminal amino acid sequence analysis of subunits I and II, the purified complex was applied to a mini-gel of identical composition. Following electrophoresis, the gel was transferred, without pre-equilibration, to a polyvinylidene difluoride membrane (Applied Biosystems), using a buffer containing 20% methanol, 192 mM glycine, 25 mM Tris free base, pH 8.3. To identify the subunits, the membrane was briefly stained with Coomassie Brilliant Blue R (0.25% in 9% acetic acid, 45% methanol), destained, and equilibrated with 50% methanol (high performance liquid chromatography grade) before drying. Amino acid sequencing was performed on a Protein Instruments Model 2299E automated gas phase sequencer, using standard Edman degradative chemistry.

**Assays**—Cytochrome c oxidase activity was measured by the change in absorbance at 550 nm of reduced horse heart cytochrome c. The reaction mixture contained, in a 1-ml volume, 50 mM MES-NaOH, pH 6.0, 30 μM cytochrome c, 0.1% Triton X-100, and sample. The reaction was carried out at 20 °C in a Shimadzu UV-100 spectrophotometer. Protein content of samples was estimated by the method of Lowry et al. (23); Triton X-100-containing samples were compared to bovine serum albumin standards also containing Triton X-100 and were centrifuged in a table top model following color development.

**RESULTS**

**DNA Sequence Analysis**—Fig. 1 shows the organization of the *B. firmus* OF4 cta genes and flanking regions. One incomplete and nine complete open reading frames were identified.
**Alkaliphile cta Genes and caa₃ Oxidase**

**Fig. 1. Organization of the B. firmus OF4 cta genes.** Open reading frames are boxed, with the direction of transcription indicated by the arrows. The cloned fragments are shown below; the initial PCR product, cloned as pCOX1011, is indicated by the *.

**pta**—An incomplete open reading frame upstream of ctaA encoding 339 amino acids (aa) was found by the FASTA program to be 42% identical with the C-terminal 335 aa of E. coli deoxyribodipyrimidine photolyase (photoreactivating enzyme) (24). In contrast, the pycA pyruvate carboxylase gene is found in the equivalent position in B. subtilis (25). Interestingly, B. subtilis, along with other naturally competent eubacteria, is reported to lack a photoreactivation mechanism (26, 27); to date, we have been unable to induce a competent state in B. firmus OF4.

**ctaA**—The ctaA gene is predicted to encode a 297-aa protein with a pI of 10.3 and a molecular weight of 32,369. The most likely initiation codon is a GUG (complement of bases 2017–2019). As in B. subtilis, the gene is transcribed in the opposite direction to the rest of the cta genes (28). A region of dyad symmetry lies midway between the stop codons of the phr and ctaA genes and probably acts as a transcriptional terminator, perhaps for both genes. Overall, the alkaliphile ctaA gene product is 35% identical with that of B. subtilis, the only other species in which the sequence of this gene has been reported (25), with most identity in the N-terminal 200 aa (Fig. 2A).

**ctaB**—The ctaB gene is predicted to encode a 312-aa protein with a pI of 9.8 and a molecular weight of 34,325. B. firmus OF4 CtaB is 52% identical with the B. subtilis protein and 61% identical with the Bacillus PS3 partial sequence deduced from the published DNA sequence (14) (Fig. 2B). All of the 25 residues noted as invariant in B. subtilis CtaB, E. coli CyoE, Paracoccus denitrificans ORP1, and yeast COX10 (13) are present in the predicted alkaliphile CtaB. A hydropathy plot (not shown) suggests a topology similar to those suggested for the Paracoccus and E. coli homologues (29, 30), with seven transmembrane segments. A conserved DNA sequence exists upstream of the B. subtilis qoxA, ctaB, and men genes (31), but no equivalent sequence is apparent in B. firmus OF4.

**ctaC**—The ctaC gene for subunit II of the cytochrome oxidase complex is predicted to encode a 342-aa protein with a pI of 4.3 and a molecular weight of 38,141. However, N-terminal amino acid sequencing yielded the sequence (X)-L-G-E-E-N-L-T-A-L-D-P-K-G-P-Q-A-Q-(X)-I-Y-D-N-M-I-L (X denotes unidentifiable residue), corresponding to residues 22–48 and suggested that the mature protein begins with Cys-22; its molecular weight would be 35,588. A similar cleavage of a signal sequence has been demonstrated in Bacillus PS3 (14) and postulated in B. subtilis, where Cys residues are predicted in the corresponding regions of both the ctaC and caaA gene products (13, 31). Subunits II of these oxidases may be lipoproteins, as the sequences surrounding the cleavage site cysteines match the consensus sequence of Gram-negative lipoprotein precursors (31); however, that of B. firmus OF4, L-T-G-C-L-G, fits the consensus before the cysteine, but not after.

**Fig. 2. Deduced amino acid sequence alignments of cta gene products.** Residues identical in B. firmus OF4 and any of the other sequences are boxed. A, B. firmus OF4 ctaA (OF4cta) and B. subtilis ctaA (Bsucta); B, B. firmus OF4 ctaB (OF4cta), B. subtilis ctaB (Bsucta), and Bacillus PS3 (PS3cta) partial sequence of presumed caaB, deduced from the published DNA sequence (14). For PS3, one frameshift was assumed in the vicinity of residue 66. C, cytochrome oxidase subunits II from B. firmus OF4 ctaC (OF4cta), B. subtilis ctaC (Bsucta), Bacillus PS3 (PS3cta) partial sequence of presumed caaC, Bacillus PS3 partial sequence of presumed caaC (PS3cba), and B. subtilis caaC (Bsucaa). Resides obtained from N-terminal amino acid sequencing are underlined. D, cytochrome oxidase subunits I from B. firmus OF4 ctaD (OF4cta), B. subtilis ctaD (Bsucta), Bacillus PS3 ctaD (PS3cta), and B. subtilis caaD (BsucaD). Resides obtained from N-terminal amino acid sequencing are underlined. The regions to which PCR primers D1 and D2 were directed are indicated (###). E, cytochrome oxidase subunits III from B. firmus OF4 ctaE (OF4cta), B. subtilis ctaE (Bsucta), Bacillus PS3 ctaE (PS3cta), and B. subtilis caaE (BsucaE). The region to which PCR primer E1 was directed is indicated (####). F, cytochrome oxidase subunits IV from B. firmus OF4 ctaF (OF4cta), B. subtilis ctaF (Bsucta), Bacillus PS3 ctaF (PS3cta), and B. subtilis caaF (BsucaF).
Alkaliphile cta Genes and caa₃ Oxidase

The alkaliphile CtaC protein exhibits 39% sequence identity with B. subtilis CtaC, 43% identity with PS3 CaaC, and 22% identity with B. subtilis QoxA (Fig. 2C). All of the residues implicated in the formation of the Cu₃ and heme c binding sites are conserved in the B. firmus OF4 subunit II (13, 14, 32). One change of note concerns the conserved aromatic sequence F-W-W-Q-F-D-Y-E (residues 139-146), where Glu-146 replaces the Pro-146 of PS3 and both B. subtilis subunits II. In addition, the large C-terminal region contain-

ing the cytochrome c domain, which is probably external to the cytoplasmic membrane, is extremely acidic in the alkaliphile. This is due in part to the deletion of otherwise conserved basic residues, or by their replacement with neutral or acidic residues, e.g. Val-125, Leu-174, Pro-203, Glu-238, Asn-262, Val-289, Glu-302.

ctaD—The alkaliphile ctaD gene for subunit I of the oxidase is predicted to encode a 625-aa protein with a pl of 7.1 and a molecular weight of 69,885. N-terminal amino acid sequencing gave A-T-Q-K-Q-E-K-S-V-I-(X)-D, corresponding to residues 2-13, and indicating cleavage of the N-terminal methionine, as in PS3 (14). Interestingly, the start codon is UUG (confirmed by sequencing in both pCOX163 and pCOX17), which allows efficient translation initiation in B. subtilis (33). The predicted amino acid sequence is 64% identical with that of B. subtilis CtaD, 63% identical with PS3 CaaD, and 47% identical with B. subtilis QoxB (Fig. 2D). Subunit I contains the binding sites for heme a, and Cu₃ (the binuclear center) and heme a. All the residues likely to be involved in these centers (32) are conserved in the alkaliphile protein. A hydropathy plot (data not shown) suggests the presence of 12 transmembrane segments, as postulated for the B. subtilis CtaD and PS3 CaaD proteins (13, 14). By contrast, subunits I of the B. subtilis a₃ type (QoxB) and E. coli o-type oxidases (CyoB) have extended C termini, probably with two extra transmembrane segments (31, 34).

tcaE—The tcaE gene for cytochrome oxidase subunit III is predicted to encode a 206-aa protein with a pl of 9.1 and a molecular weight of 22,856. In PS3, the N-terminal methionine is cleaved off (14). The predicted amino acid sequence is 59% identical with B. subtilis TcaE, 61% identical with PS3 CaaE, and 44% identical with B. subtilis QoxC (Fig. 2E). The alkaliphile protein contains the conserved, dicyclohexylcarbodiimide-binding, acidic residue (Glu-36). This residue was originally thought to play a role in proton translocation, but such a role appears doubtful, since the activity of the Paracoccus enzyme was apparently unimpaired when its equivalent glutamate residue was changed to glutamine or alanine (35), and disruption of the Paracoccus gene resulted instead in impaired assembly of the oxidase complex (36).

tcaF—The tcaF gene for oxidase subunit IV is predicted to encode a 114-aa protein with a pl of 10.0 and a molecular weight of 13,017. The alkaliphile sequence is 37% identical with B. subtilis TcaF, 31% identical with PS3 CaaF, and 25% identical with the first 100 residues of B. subtilis QoxD (Fig. 2F). Immediately downstream from the stop codon is a 30 base region of dyad symmetry, a likely transcriptional terminator. A similar structure has been identified in PS3 (14).

orfA-C—The remainder of the cloned DNA contained three potential open reading frames, designated orfA-C. The first, orfA, beginning at a GUG codon (bases 7460-7462), would encode a very hydrophobic 185-aa product with a pl of 10.8 and a molecular weight of 20,961. A 33-aa stretch of OrfA has 40% identity with part of several Ca²⁺-transporting ATPases (e.g. Ref. 37), but no obvious function has been ascribed to this region. Translation of orfB from a GUG codon (complement of bases 9238-9240) would yield a hydrophilic 385-aa protein with a pl of 10.6 and a molecular weight of 43,410. No significant homology to any sequence in the PIR database was found. Finally, orfC, reading from the AUG codon (bases 9381-9383) may encode a hydrophilic 153-aa protein with a pl of 4.5 and a molecular weight of 17,748. This product may also be lipid-anchored, as residues 18-23 (L-Å-A-C-G-S) match exactly the Gram-negative consensus sequence; its acidic pl is consistent with an exterior location. However, no significant homology to any deposited sequence was found. While this is the first description of the genes distal to a

FIG. 2.—continued.
**Bacillus** cytochrome oxidase operon, none of them seems likely to be involved with expression of the oxidase.

**mRNA Abundance and Size**

Results from a typical Northern blot are shown in Fig. 3. The *ctaD* probe hybridized to a message of approximately 4 kb. Such a transcript would be just the right length to encode the four structural subunits of the cytochrome oxidase (CtaC-F), but too small to encode CtaB in addition. Considerably more of this message was found in pH 10.5-grown cells than in pH 7.5-grown cells. Densitometric analysis of the autoradiographs gave a relative abundance of 2.4 ± 0.25:1 (n = 4), in good agreement with the relative abundance of cytochrome *aa3* oxidase in the cell membranes (3). Although data are not shown, the *ctaD* probe was also used to determine mRNA levels in cells grown at pH 7.5 in the presence of 1 μM CCCP. The levels of *cta* mRNA were generally higher in such cells than in the pH 7.5-grown controls, but were quite variable. One preparation contained as much message as pH 10.5-grown cells, while two others showed little or no increase over pH 7.5-grown controls. As cytochrome *aa3* levels were elevated in all three batches of cells, as observed previously (3), the timing of sampling for the mRNA determinations may be critical.

Although the coding region of the *ctaB* gene occupies only 1 kb, Northern analysis revealed a 5-kb low abundance message, with no sign of any shorter products (Fig. 4). As the *ctaA* gene, immediately upstream of *ctaB*, is transcribed in the opposite direction, it is likely that the *ctaB* message spans the *ctd* gene, immediately upstream of *ctaB*, is transcribed in the opposite direction. As the *ctd* gene contains a broad peak between 300 and 450 mM ammonium sulfate. A subunit I was better stained by silver than by Coomassie Blue staining (Fig. 6, lane 3) and silver staining of the complex (Fig. 6, lane 3) indicated that the preparation was quite pure. Subunit I was better stained by silver than by Coomassie Blue staining (Fig. 6, lane 3). The complex was resolved into three bands with approximate sizes of 44,000, 37,500, and 22,500 (Fig. 6). As observed for other very hydrophobic polypeptides, subunit I migrates anomalously. Both Coomassie staining (Fig. 6, lane 2) and silver staining of the complex (Fig. 6, lane 3) indicated that the preparation was quite pure. Subunit I was better stained by silver than by Coomassie Blue staining (Fig. 6, lane 3). The complex was resolved into three bands with approximate sizes of 44,000, 37,500, and 22,500 (Fig. 6). As observed for other very hydrophobic polypeptides, subunit I migrates anomalously. Both Coomassie staining (Fig. 6, lane 2) and silver staining of the complex (Fig. 6, lane 3) indicated that the preparation was quite pure. Subunit I was better stained by silver than by Coomassie Blue staining (Fig. 6, lane 3). Heme staining of the gel showed that the cytochrome *c* of the complex is in subunit II (Fig. 6, lane 4), consistent with the predicted amino acid sequence of subunit II. Importantly, N-terminal amino acid sequencing confirmed that subunits I and II were indeed the products of the cloned *ctaD* and *ctaC* genes.

*B. firmus* OF4 cytochrome *aa3* oxidized cytochrome *c* from both horse heart and *Saccharomyces cerevisiae*, the latter being a slightly better substrate. The pH optimum of the reaction was acidic (pH 6). These results are in agreement with the those observed with the purified complex from alkaliphilic *B. firmus* RAB (38).

**DISCUSSION**

The organization of the cytochrome *aa3* oxidase genes in alkaliphilic *B. firmus* OF4 is identical with the corresponding loci in *B. subtilis* (13, 28) and *Bacillus* PS3 (14). Four genes, *ctaC-F*, correspond to the four putative structural subunits of the enzyme. Among these three species, however, a four-subunit complex has been isolated to date only from PS3 (39). The purified complex from the alkaliphile clearly has three subunits (Fig. 6). A faint silver-stained band at around 10 kDa (Fig. 6, lane 3) might correspond to subunit IV, although this would be present, at least in this preparation, in stoichiometric amounts. The presence of the putative fourth subunit has not yet been demonstrated in the *B. subtilis aa3* oxidase (40), although the qox operon has a homologue of *ctaF*.

The functions of the two other *cta* genes, *ctaA* and *ctaB*, remain to be elucidated. In view of the relatively low abundance of *ctaB* mRNA in *B. firmus* OF4, it would be surprising if the protein were present in stoichiometric amounts in the alkaliphile oxidase complex, although it could be one of the minor species. A nonsense mutation in *B. subtilis* *ctaA* and a spontaneous deletion encompassing the entire *ctaA* and *ctaB* genes both led to a total lack of detectable heme *a* (25, 28),...
**Alkaliphile cta Genes and caa₃ Oxidase**

**TABLE I**

| Step                        | Protein | Cytochrome oxidase activity | Specific activity | Yield | Enrichment |
|-----------------------------|---------|----------------------------|-------------------|-------|------------|
| 1. Octyl glucoside extract  | 149.8   | 28.08                      | 0.116             | (100%)| (1)        |
| 2. (NH₄)₂SO₄ fractionation  | 59.8    | 24.28                      | 0.406             | 86    | 3.5        |
| 3. DEAE-Sepharose CL-6B chromatography | 5.9 | 8.83                   | 1.50              | 31    | 12.9       |
| 4. Hydroxyapatite chromatography | 1.6    | 4.28                       | 2.66              | 15    | 22.9       |

* Membranes equivalent to about 400 mg of protein were extracted with octyl glucoside; measurement of the enzymatic activity of the membrane fraction underestimated the cytochrome caa₃ content.

**Fig. 5. Purification of cytochrome c oxidase activity on DEAE-Sepharose CL-6B.** As detailed under "Materials and Methods," the octyl glucoside extract of washed membranes was subjected to (NH₄)₂SO₄ fractionation, and the active, desalted fraction was loaded onto a DEAE-Sepharose CL-6B column. After washing the column with 2 bed volumes of starting buffer, a 0 to 0.6 M (NH₄)₂SO₄ gradient (in starting buffer) was applied to the column. Gradient fractions were assayed for cytochrome c oxidase activity (triangles). The units are ΔA₅₅₀/min × ml.

**Fig. 6. SDS-polyacrylamide gel electrophoresis of purified cytochrome c₃ oxidase.** The purified complex was electrophoresed on a 10% T polyacrylamide gel (18). Lane 1 contained molecular weight standards as labeled. Lane 2, which contained 8 μg of protein, was stained with Coomassie Brilliant Blue G, while lane 3, which contained 0.8 μg of protein, was stained with silver. Lane 4 (4 μg) was stained for 3,3',5,5'-tetramethylbenzidine oxidase activity (heme stain).

suggesting that neither caa₃ nor aa₃-type oxidase was present; there is no gene equivalent to ctaB upstream of the gox operon (31). Recent data of Svensson and Hederstedt (41) suggest that ctaA and ctaB are required, together, for the synthesis of heme a, and that one of them encodes a b-type cytochrome that functions in the synthetic reactions. The hydrophathy profiles of the two CtaA proteins whose deduced sequences are now available (Fig. 2A) are almost identical. Taking into consideration the fact that exterior connecting loops of bacterial plasma membrane proteins contain a paucity of positively charged residues relative to cytosolic loops (42), a speculative model would include eight transmembrane helices and would place the two pairs of conserved cysteines facing the exterior, where they could be involved in metal binding. No equivalent to CtaA has yet been identified in E. coli, which is unable to synthesize heme a.

The products of all four alkaliphile structural genes show substantial identity to their counterparts in other cytochrome oxidases from Bacillus species (Fig. 2). Subunit I in particular shows significant identity to subunits I from other prokaryotes and eukaryotes (32). Ultimately, we will focus on the possibility of specific sequence deviations that may relate to the bioenergetics of alkaliphiles (6); such an analysis will require a better understanding of the native structure, its subunits and their roles, and will entail the examination of mutants that are compromised in oxidative phosphorylation specifically at high pH, but generate the same Δp pattern as the wild-type. An observation in the current study that is relevant to alkaliphilicity, although not to the bioenergetics of the organism per se, is the striking deletion of basic residues in the external, hydrophilic cytochrome c₃-binding domain of CtaC, or their replacement by neutral or acidic residues; in Fig. 7, a model of the gene product is shown, with an x indicating residues at which such a replacement has occurred. In general, adaptation to growth at extremely alkaline pH features the avoidance of basic residues, even more than is usually found (42) in those enzymes (43), parts of enzymes or structural proteins (44) that must function in the highly alkaline exterior. With respect to polypeptide membrane proteins, the extreme avoidance of basic residues in the external hydrophilic loops of alkaliphile proteins may offer assistance in making topological predictions.

In addition to other, alternate terminal oxidases, B. subtilis contains two distinct cytochrome aa₃-type oxidases, the caa₃ counterpart to the complex studied here (13, 45) and an aa₃-
type quinol oxidase (40, 45) encoded by the gox genes (31). B. cereus also has both a caa3 and an aa3 oxidase (46). Might a similar situation exist in B. firmus OF4? To date, there is no evidence for more than one aa3/caa3-type oxidase in the alkaliphile, unless the minor component observed during the current purification work is such a species (Fig. 5). During extensive screening of the B. firmus OF4 DNA library, only clones of the cta region were obtained; however, Saraste et al. (13) also failed to detect hybridization of their B. subtilis cta probe to the chromosomal gox DNA, despite the similarity of the two sets of protein products. At the protein level, we have consistently observed only a single α-peak at 600 nm in reduced-minus-oxidized spectra. By contrast, the B. subtilis aa3 oxidase shows a peak at 600 nm, and the caa3 oxidase a peak at 605 nm. Furthermore, the B. subtilis aa3 enzyme, unlike the caa3, is not significantly reduced by ascorbate/TMPD (45). We have never seen any increased signal from dithionite-reduced versus ascorbate/TMPD-reduced samples, nor was any additional signal apparent when ascorbate/TMPD-reduced samples were further reduced with dithionite. No qualitative differences were observed in the 600 nm region when malate-grown and glucose-grown B. firmus OF4 were compared; by contrast, expression of the aa3 oxidase in B. subtilis was higher in rich or glucose minimal media than in succinate minimal media (31). The minor component eluted from the Sepharose column may indeed represent an additional aa3-type oxidase species in the alkaliphile, but is probably more likely to be a caa3 variant than a counterpart to the B. subtilis aa3. P. denitrificans possesses two very closely related genes for subunit I, with the protein products differing significantly only in the N-terminal 15 aa (47). The second gene was not detected until a deletion of the first gene was found to be without any phenotypic effect. It is notable that under conditions of slight oxygen limitation, Bacillus PS3 was found to be without any phenotypic effect. It is notable that significantly only in the N-terminal 15 aa related genes for subunit I, with the protein products differing more likely to be a caa3 variant than a counterpart to the B. subtilis ctaF) and may, instead, be part of a promoter region. It will

be of interest to further delineate the mechanism(s) of the pH-dependent increase in cta operon expression and to study transcription in the CCCP-resistant mutant strain, CC2, in which cytochrome oxidase levels are elevated in pH 7.5-grown cells (3).

Acknowledgments—We thank Dr. M. Saraste for providing the cta probe from B. subtilis, which was used in the initial cloning attempts, and for sharing sequence information with us prior to publication. Protein sequencing was performed by Dr. Ronald Kohanski, Protein Chemistry Core Laboratory, Mount Sinai School of Medicine, and DNA sequencing was conducted in the Brookdale Center DNA Core Facility, Mount Sinai School of Medicine.

REFERENCES
1. Guffanti, A. A. & Hicks, D. B. (1991) J. Gen. Microbiol. 137, 2375-3379
2. Hicks, D. B., Plass, R. J. & Quirk, P. G. (1991) J. Bacteriol. 173, 5010-5016
3. Quirk, P. G., Guffanti, A. A., Plass, R. J., Clejan, S. & Kruilikh, T. A. (1991) Biochim. Biophys. Acta 1058, 131-140
4. Mitchell, P. (1961) Nature 191, 144-146
5. Guffanti, A. A. & Kruilikh, T. A. (1992) J. Biol. Chem. 267, 9580-9588
6. Kruilikh, T. A. & Guffanti, A. A. (1989) Ann. Rev. Microbiol. 43, 453-463
7. Ivey, D. M. & Kruilikh, T. A. (1991) Mol. Gen. Genet. 229, 292-300
8. Ivey, D. M. & Kruilikh, T. A. (1992) Gene 115, 463-470
9. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
10. Ausubel, F. M., Brent, R., Kinston, R. E., Moore, D. D., Smith, J. A., Seidman, J. C. & Struhl, K. (eds) (1987) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York, NY
11. Deveraens, J., Haebeli, P. & Smithies, O. (1984) Nucleic Acids Res. 12, 287-296
12. Marchuk, D., Drumm, M., Saulino, A. & Collins, F. S. (1991) Nucleic Acids Res. 19, 1154
13. Saraste, M., Mett, T., Nakari, T., Jalli, T. & Laursen, M. & van der Oost, J. (1991) Eur. J. Biochem. 205, 575-579
14. Ishizuka, M., Machida, K., Shimada, S., Mogi, A., Tsuchiya, T., Ohmori, T., Suam, Y., Gonda, M. & Sone, N. (1993) J. Biochem. (Tokyo) 114, 866-875
15. Ivey, D. M., Guffanti, A. A., Bossewitch, J., Guffanti, A. A., Plass, R. J., Clejan, S. & Kruilikh, T. A. (1991) J. Biol. Chem. 266, 12483-12486
16. Beechhofer, D. H. & Duhau, D. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8522-8526
17. Hicks, D. B. & Kruilikh, T. A. (1990) J. Biol. Chem. 265, 20547-20554
18. Schagger, H. & von Jagow, G. (1987) Anal. Biochem. 166, 368-379
19. Wilson, C. M. (1983) Methods Enzymol. 91, 236-247
20. Anzorge, W. (1982) in Electrophoresis '82 (Stathakos, D., ed.) pp. 235-242, Walter de Gruyter, Inc., New York, NY
21. Thomas, P. R., Ryan, D. & Levin, W. (1976) Anal. Biochem. 75, 168-176
22. Gilkes, J. A. & Sherman, L. A. (1980) Biochim. Biophys. Acta 637, 189-201
23. Lowry, O. H., Rosebrown, N. J., Farr, A. L. & Randall, R. J. (1961) J. Biol. Chem. 193, 265-270
24. Saraste, M., Smith, F. W., Lorence, M. C., Rupert, C. & van der Oost, J. (1991) Molecular Biology of the Bacilli, Duhau, D. A., ed. Vol. 2, pp. 33-52, Academic Press, Orlando, FL
25. van der Oost, J., van Wachenfeld, C., Hederstedt, L. & Saraste, M. (1991) Mol. Microbiol. 5, 2063-2072

Fig. 8. Models of possible RNA structures between the ctaB/caaB and ctaC/caaC genes of B. firmus OF4 (A), Bacillus PS3 (B), and B. subtilis (C). Computed folding energies (53) are -9.5, -9.8, and -17.5 kcal/mol, respectively.
Alkaliphile cta Genes and caa3 Oxidase

29. van der Oost, J., Haltia, T., Raitio, M. & Saraste, M. (1991) J. Bioenerg. Biomembr. 23, 257–267
30. Chepuri, V. & Gennis, R. B. (1990) J. Biol. Chem. 265, 12978–12986
31. Santana, M., Kunst, F., Hullo, M. F., Rapoport, G., Danchin, A. & Glaser, P. (1992) J. Biol. Chem. 267, 10225–10231
32. Saraste, M. (1990) Q. Rev. Biophys. 23, 331–366
33. Vellanoweth, R. L. & Rabinowitz, J. C. (1992) Mol. Microbiol. 6, 1105–1114
34. Chepuri, V., Lemieux, L., Au, D. C.-T. & Gennis, R. B. (1990) J. Biol. Chem. 265, 12978–12986
35. Haltia, T., Saraste, M. & Wikstrom, M. (1991) EMBO J. 10, 2015–2021
36. Haltia, T., Finel, M., Harms, N., Nakari, T., Raitio, M., Wikstrom, M. & Saraste, M. (1989) EMBO J. 8, 3571–3579
37. Magyar, A. & Varadi, A. (1990) Biochem. Biophys. Res. Commun. 173, 872–877
38. Kitada, M. & Krulwich, T. A. (1984) J. Bacteriol. 158, 963–966
39. Sone, N., Shimada, S., Ohmori, T., Soume, Y., Gonda, M. & Ishizuka, M. (1990) FEBS Lett. 262, 249–252
40. de Vrij, W., Azzi, A. & Koning, W. N. (1983) Eur. J. Biochem. 131, 97–103
41. Svensson, B. & Hederstedt, L. (1992) EBEC Short Reports, Vol. 7, Report II-42, Elsevier Science Publishers B. V., Amsterdam
42. von Heijne, G. (1988) EMBO J. 7, 3021–3027
43. Durham, D. R., Stewart, D. B. & Stellwag, E. J. (1987) J. Bacteriol. 169, 2762–2768
44. Guffanti, A. A. & Eisenstein, H. C. (1983) J. Gen. Microbiol. 129, 3329–3342
45. Leurusea, M., Haltia, T., Saraste, M. & Wikstrom, M. (1991) Eur. J. Biochem. 197, 699–705
46. Garcia-Horsman, J. A., Barquera, B. & Escamilla, J. E. (1991) Eur. J. Biochem. 199, 761–768
47. Raitio, M., Fupa, J. M., Metso, T. & Saraste, M. (1990) FEBS Lett. 261, 431–435
48. Puustinen, A. & Wikstrom, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6122–6126
49. Sone, N. & Fujiiwara, Y. (1991) FEBS Lett. 288, 154–158
50. Qureshi, M., Yuzuto, I., Fujiiwara, T., Fukumori, Y. & Yamanaka, T. (1980) J. Biochem. (Tokyo) 107, 499–506
51. Tsuchikubo, K. (1971) J. Bacteriol. 106, 652–661
52. James, W. S., Gibson, F., Taroni, P. & Poole, R. K. (1989) FEMS Microbiol. Lett. 58, 277–282
53. Zaker, M. & Stiegler, P. (1981) Nucleic Acids Res. 9, 133–148