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# Isolation and Sequence of *ctaA*, a Gene Required for Cytochrome $aa_3$ Biosynthesis and Sporulation in *Bacillus subtilis*

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**Cytochrome $aa_3$ is one of two terminal oxidase complexes in the *Bacillus subtilis* electron transport chain. A novel genetic strategy was devised which permitted the isolation of *B. subtilis* mutants lacking cytochrome $aa_3$ by selection for streptomycin-resistant clones which failed to oxidize the artificial electron donor *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Two mutations were studied intensively. Spectroscopic examination showed that each mutant lacked cytochrome $aa_3$; they were also asporogenous and unable to grow on lactate as the sole carbon and energy source. These mutations were mapped to a locus designated *ctaA*, located at 127 degrees between *pyrD* and *metC* on the *B. subtilis* chromosome. Both *ctaA* mutations were closely linked by transformation to the *pycA* locus. The *ctaA* locus and a portion of the *pycA* locus were cloned from a *B. subtilis* integration library constructed in *Escherichia coli*. A recombinant plasmid containing a 4.0-kilobase insert of *B. subtilis* DNA could transform both *ctaA* mutants to CtaA$^+$. Gene disruption and complementation experiments with subcloned fragments revealed that the *ctaA* locus consisted of a single transcriptional unit about 1.35 kilobase pairs in size. The nucleotide sequence of the *ctaA* transcriptional unit contains a single open reading frame capable of coding for a protein with a predicted molecular weight of 34,065. The predicted protein is extremely hydrophobic, with several probable membrane-spanning domains. No sequence similiarity was found between *ctaA* and the highly conserved procaryotic and mitochondrial oxidase polypeptides. Cloning and sequence analysis of two *ctaA* mutations revealed that one allele is a nonsense mutation in the carboxy terminus and the other is a missense mutation in the amino terminus; this indicates that the pleiotropic phenotype conferred by each mutation was caused by loss of CtaA or of its activity. Genetic evidence suggests that the *ctaA* gene product is required as an accessory protein in the genetic expression, posttranslational biogenesis, or both, of the cytochrome $aa_3$ complex and during an early stage of sporogenesis.**

Although most aerobic gram-positive bacteria are known to contain cytochromes *a*, *b*, and *c*, their integration into electron transfer pathways has not been well characterized (36). The membrane-bound respiratory chain of the gram-positive obligate aerobe *Bacillus subtilis* resembles closely the respiratory chain of mitochondria and contains as functional electron carriers cytochromes $aa_3$, *b*, $c_1$, *c*, and *o* (13). Cytochromes $aa_3$ and *o* function as terminal oxidases (13, 15), although the physiological roles played by the two oxidase-containing branches of the respiratory chain are not clear. In addition, the existence of a cytochrome *d* in *B. subtilis* has been suggested by detection of an absorbance maximum at 617 nm (28, 47). Cytochrome $aa_3$ catalyzes electron transfer from cytochrome *c* to dioxygen (13, 15) and has been purified to homogeneity and extensively characterized (10–12). The cytochrome $aa_3$ complex of *B. subtilis* is composed of three polypeptide subunits with hemes *a* and $a_3$ as the prosthetic groups (10). This complex is structurally and functionally similar to the three largest polypeptides of the mitochondrial oxidase (10–12, 26). Essentially nothing is known about the genetic regulation of this complex.

Although *Paracoccus denitrificans* has provided the paradigm for biochemical and biophysical analysis of bacterial cytochrome $aa_3$ complexes (26), the lack of a well-defined genetic system hinders analysis of cytochrome $aa_3$-deficient mutants and the cloned subunit genes of this bacterium (37, 45). However, the array of genetic manipulations available in *B. subtilis* makes it an especially attractive model system in which to undertake a detailed molecular analysis of cytochrome $aa_3$ gene expression, catalytic function, and biogenesis. The absence of cytochrome $aa_3$ in *Escherichia coli* precludes such studies in this bacterium.

To identify genes required for the functional formation of cytochrome $aa_3$ in *B. subtilis*, we undertook a genetic approach in which we isolated and characterized mutants defective in cytochrome $aa_3$ formation. In this report, we describe the isolation and preliminary characterization of mutant strains of *B. subtilis* lacking the cytochrome $aa_3$ terminal oxidase. We also describe the molecular cloning, genetic mapping, and nucleotide sequence of a new genetic locus, *ctaA*, affecting the synthesis of the oxidase. Mutations within this locus have pleiotropic effects, altering both cytochrome $aa_3$ formation and sporulation. Gene disruption and complementation experiments as well as nucleotide sequence analysis suggest that the *ctaA* locus is monocistronic and that the pleiotropic phenotype conferred by mutations at this locus is caused by loss of the *ctaA* gene product. Genetic evidence indicates that CtaA is required at a step in the biogenesis of the cytochrome $aa_3$ complex and that the role of CtaA in sporulation is independent of its function in the formation of cytochrome $aa_3$. A preliminary report on isolation of *cta* mutants and their genetic analysis has appeared (31).

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and bacteriophage.** Bacterial strains, plasmids, and phage used in this work are listed in Table 1.

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TABLE 1. Bacterial strains, plasmids, and bacteriophage

| Strain, plasmid, or phage | Relevant characteristics | Source or reference |
|---|---|---|
| Strains | | |
| *B. subtilis* | | |
| RB1 | *trpC2* | This laboratory |
| RB353 | *pyrD1 ilvA1 thyA1 thyB1 trpC2* | This laboratory |
| RB359 | *sacQ36*(Hy)[a] *metC3 trpC2* | This laboratory |
| RB521 | *thr-5 recE4 trpC2* | This laboratory |
| RB642 | *ctaA642 trpC2* | This work |
| RB645 | *trpC2* ϕ105J23 | J. Errington; 16 |
| RB648 | *zdg-648*::Tn*917*[b] *trpC2* | This work |
| RB676 | *ctaA676 scs-1 trpC2* | This work |
| RB771 | *citL22 trpC2* | BGSC[c] |
| RB772 | *pycA19 recA1 trpC2* | BGSC |
| RB773 | *spo0E11 pheA1 trpC2* | BGSC |
| RB774 | *spoVD156 rpoB2 trpC2* | BGSC |
| RB777 | *spoIIG279 cysC7 furA2* | BGSC |
| RB811 | RB1ΩpA1516 | This work |
| RB816 | RB642ΩpAI516 | This work |
| RB819 | RB676ΩpAI515 | This work |
| RB828 | Δ(*ctaA-pycA*)*1*::pAI522 *trpC2* | This work |
| RB829 | Δ(*ctaA-pycA*)*1 trpC2* | This work |
| RB840 | *thyA1 thyB1 dapE320 trpC2* | BGSC |
| RB874 | *spo0A12 pheA1 trpC2* | BGSC |
| RB888 | *pycA19 trpC2* | This work |
| RB907 | RB829 *recE4* | This work |
| RB923 | RB829 *sca-3* | This work |
| RB934 | *ctaA676 trpC2* | This work |
| RB944 | RB642 *scs-2* | This work |
| RB945 | RB934 *scs-4* | This work |
| RB971 | RB829 *scs-5* | This work |
| RB977 | ϕ105J23::pA1546 (ϕ105*ctaA*$^+$) *trpC2* | This work |
| RB995 | Δ*ctaA2*::pA1553 *trpC2* | This work |
| RB996 | Δ*ctaA2 trpC2* | This work |
| *E. coli* | | |
| JM107 | Δ(*lac-proAB*) *thi endA1 gyrA96 hsdR17 relA1* λ$^-$ *supE44* (F′ *traD36 proAB lacI*$^q$ZΔ*M15*) | This laboratory |
| JM109 | *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ$^-$ Δ(*lac-proAB*) (F′ *traD36 proAB lacI*$^q$ZΔ*M15*) | M. Belfort |
| Plasmids | | |
| pSGMU2 | *bla*$^+$ *cat*$^+$; integration vector 3.7 kb | J. Errington; 17 |
| pMK4 | *bla*$^+$ *cat*$^+$; shuttle vector 5.6 kb | BGSC; 46 |
| pBS+/− | *bla*$^+$; 3.2 kb | M. Belfort; 41 |
| Phage | | |
| ϕ105J23 | Cloning vector | J. Errington; 22 |

[a] (Hy), Hyperproducing.

[b] *zdg-648*::Tn*917* was isolated near the *ctaA* locus essentially as described by Youngman (54) by transformation of RB642 with a Tn*917* chromosomal library kindly provided by K. Sandman and P. Youngman.

[c] BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus, Ohio.

**Culture media and genetic techniques.** Luria-Bertani (LB) medium was used for routine culture of *B. subtilis* and *E. coli*. The minimal salts medium (M) for cultivation of *B. subtilis* was that of Anagnostopoulos and Spizizen (1). Tryptophan and glucose were added to final concentrations of 25 μg/ml and 0.5%, respectively, to give MGT medium. For strains containing additional auxotrophic markers, the appropriate amino acids were added from sterile stock solutions to give final concentrations as recommended by Dedonder et al. (9). Minimal lactate medium (MLT) was identical to MGT except glucose was replaced with 0.5% L-sodium lactate. DL-Sodium lactate (Sigma Chemical Co., St. Louis, Mo.) was neutralized with solid NaOH, diluted to 10.0% L-sodium lactate, and autoclaved. Purification agar medium was prepared and used as described by Carls and Hanson (6). Penassay (antibiotic medium 3; Difco Laboratories, Detroit, Mich.) broth was used for growth of phage PBS1 and recipient cultures in transduction experiments. Growth and sporulation in 2× nutrient sporulation (NS) medium were carried out as described previously (24, 30). Sporulation efficiency was estimated by determining the fraction of heat-resistant CFU (80°C, 15 min) from a 24-h culture grown in 2× NS broth. Competent *B. subtilis* cells were prepared and transformed as described by Piggot et al. (34). Transformations of *E. coli* strains were carried out as described by Hanahan (21). Preparation of phage PBS1-transducing lysates and transduction were as described previously (49). Techniques for the use of *B. subtilis* phage ϕ105 were as described by Errington (16). Chloramphenicol resistance (Cm$^r$) was induced by the addition of chloramphenicol to 0.1 μg/ml. Erythromycin (5 μg/ml) and lincomycin (25 μg/ml) were used to select for the presence of Tn*917* as described by Youngman (54).

**Plate assay for cytochrome $aa_3$ activity.** The TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) plate assay for the detection of cytochrome *d* oxidase activity in *E. coli* as described by Green and Gennis (19) was modified for detection of cytochrome $aa_3$ activity in *B. subtilis*. Cells were incubated on solid LB medium for 24 to 48 h at 37°C to allow mature colonies to form. Plates were partially frozen at −20°C for 30 to 45 min and were thawed at room temperature. Fixation of the partially permeabilized cells to the surface of the medium was achieved by spraying the plate with a light mist of Aqua Net (Clairol) hair spray. An overlay solution consisting of 2.0 ml of molten 1.5% Bacto-Agar (Difco), 1.0 ml of 10.0% Triton X-100 in 0.1 M $KPO_4$ (pH 7.0), 0.2 ml of 10.0% sodium deoxycholate, 1.0 ml of 95% ethanol, and 1.0 ml of 1.0% TMPD was prepared immediately before use in preheated tubes at 50°C and was poured over the fixed cells. TMPD oxidase activity could be detected in 1 to 5 min with the appearance of blue staining colonies. Cells which failed to oxidize TMPD (unstained) were extracted directly from the agar overlay, streaked for single colonies on a fresh LB plate, and rescreened prior to further analysis. When 1 mM KCN, which inhibits cytochrome $aa_3$ activity, was included during the staining procedure, the wild-type strain failed to stain blue. This demonstrated that it is the enzymatic activity of cytochrome $aa_3$ and not the mere presence of hemes *c* and $aa_3$ (or both) that is required for the TMPD staining reaction.

**Low-temperature spectroscopy of whole cells.** Cultures were grown for 48 h on solid media, and estimations of cytochrome levels were made at liquid nitrogen temperature with the use of a Hartree low-dispersion microspectroscope, as described by Taber (47).

**Preparation and spectrophotometry of solubilized membrane vesicles.** *B. subtilis* membrane vesicles were prepared as described by Bisschop and Konings (4). Membrane vesicles were suspended at a concentration of 2 mg/ml in 50 mM Tris chloride (pH 7.4)–1.0 mM EDTA and were stored at −70°C. Cytochromes were solubilized by incubating membrane vesicles with 1.0% (wt/vol) dodecyl-β-D-maltoside (laurylmaltoside; Calbiochem-Behring, La Jolla, Calif.) in 50 mM Tris chloride–1.0 mM EDTA (pH 7.4) for 30 min at room temperature with gentle agitation (2 mg of membrane protein per mg of laurylmaltoside) (10). The mixture was centrifuged

at 48,000 × *g* for 30 min at 4°C. The supernatant was rapidly frozen in liquid nitrogen and stored at −70°C. Difference absorption spectra (dithionite reduced minus oxidized) of laurylmaltoside cytoplasmic membrane extracts were recorded at room temperature with a Perkin-Elmer Lambda 3B spectrophotometer at a scan speed of 2 nm/s. The membrane extracts were adjusted to 1.0 mg of protein per ml with 50 mM Tris chloride–1.0 mM EDTA (pH 7.4), and 1.0-ml volumes were placed in sample and reference cuvettes. Reduction of cytochromes in the sample cuvette was achieved by addition of a few crystals of solid sodium dithionite, while cytochromes in the reference cuvette were oxidized by addition of 1.5 μl of 10 mM potassium ferricyanide, to give a final concentration of 0.015 mM. Protein was determined by the method of Lowry et al. (25), using bovine serum albumin as a standard.

**Assays for extracellular products.** Antibiotic production was scored by inoculating colonies into 2× NS solid medium overlaid with soft agar containing a *B. subtilis spo0A* mutant (strain RB874), as described by Trowsdale et al. (53). Amylase production was scored by growth on 2× NS solid medium supplemented with 1% soluble starch (Sigma) followed by flooding of the plates with 0.08 N $I_2$–0.32 N KI in 1% (wt/vol) sodium tetraborate. Protease production was scored after growth on 2× NS solid medium supplemented with 1.5% Carnation instant milk powder followed by flooding with 25% trichloroacetic acid. Accumulation of organic acids was scored on purification agar medium plates as described by Carls and Hanson (6).

**In vitro manipulation of DNA.** All DNA manipulations were carried out as described by Maniatis et al. (27). Chromosomal DNA was isolated from *B. subtilis* strains as described by Saunders et al. (40). Preparative isolation of plasmid DNA from both *E. coli* and *B. subtilis* was carried out by the alkaline lysis procedure of Birnboim and Doly (3). Restriction enzymes and DNA modification enzymes were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and were used as recommended by the supplier.

**Construction of a *B. subtilis* genomic library.** Four hundred μg of high-molecular-weight chromosomal DNA from *B. subtilis* strain RB1 was partially digested with *Mbo*I and was fractionated in a 5 to 20% neutral sucrose gradient (27). Fractions of 0.5 ml were collected, and 10-μl samples were assayed for fragment size by agarose gel electrophoresis. The fractions containing fragments in the 2- to 5-kilobase (kb) range were pooled and dialyzed against 1,000 volumes of 1× TE (10 mM Tris chloride [pH 7.5]–1 mM EDTA) buffer at 4°C with two buffer changes over a 16-h period. The cloning vector was the integration plasmid pSGMU2, generously provided by J. Errington. This plasmid contains a chloramphenicol acetyltransferase gene from plasmid pC194 cloned into the *Nar*I site of pUC13 (17). Chromosomal fragments and *Bam*HI-digested pSGMU2 were ligated at a vector-to-insert molar ratio of 2 in a 50-μl reaction volume. A portion of the ligation products was used to transform *E. coli* strain JM109. Recombinants were identified as ampicillin-resistant ($Ap^r$) transformants that formed white colonies on LB agar plates containing 50 μg of ampicillin per ml, 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. A plasmid pool containing plasmids from 8,000 independent clones was used as a transformation stock.

**Plasmid rescue of *ctaA* sequences.** Plasmid rescue (outcloning) from *B. subtilis* chromosomal DNA containing integrated structures was accomplished as follows. Approximately 5 μg of chromosomal DNA from a *B. subtilis* integrant strain was digested to completion with the restriction enzyme of choice. One μg of each sample was then ligated under dilute DNA concentrations to favor recircularization of linear plasmid molecules and then used to transform *E. coli* JM109.

**Gene disruption experiments.** Strain RB1 was transformed with 1 μg of DNA from the integrational plasmids, and $Cm^r$ transformants were selected on LB plates containing 5 μg of chloramphenicol per ml. Mature colonies were easily distinguished as $CtaA^+$ or $CtaA^-$, since the latter had a distinct small-colony phenotype on solid medium. Southern blot hybridization analysis was used to verify that the drug-resistant transformants had arisen by integration of the plasmid vector into the chromosome by single-crossover (Campbell-like) recombination between the *B. subtilis* DNA insert in the integration vector and the corresponding region of homology in the chromosome.

**Construction of *ctaA* deletion mutations.** To construct the Δ(*ctaA-pycA*)*1* deletion mutation, plasmid pAI503 was digested with *Bgl*II, ligated, and used to transform *E. coli* strain JM107. Plasmid DNA from one $Ap^r$ transformant shown by restriction analysis to lack the 2.2-kb *Bgl*II fragment was designated pAI522. The Δ(*ctaA-pycA*)*1* deletion was transferred to the *B. subtilis* chromosome by transforming competent cells of wild-type strain RB1 with pAI522 and selecting for $Cm^r$ transformants. Colonies were screened on MLT and MLT plus aspartate. Three types of transformants were noted: (i) 62% were phenotypically $CtaA^+$ $Pyc^+$, representing single crossovers in the *ctaA* portion of the construct; (ii) 31% were phenotypically $CtaA^+$ $Pyc^-$, representing gene disruptions caused by a single crossover in the *pycA* portion of pAI522; (iii) 7% were phenotypically $CtaA^-$ $Pyc^-$, representing a double crossover (gene replacement) of a linear plasmid concatemer (44). One of the $Cm^r$ $CtaA^-$ $Pyc^-$ colonies was purified and designated RB828. Growth of strain RB828 in the absence of chloramphenicol allowed isolation of chloramphenicol-sensitive ($Cm^s$) clones, arising from loss of the plasmid. Southern blot analysis verified the absence of plasmid sequences and the deletion of the expected *Bgl*II fragments (data not shown).

To construct the Δ*ctaA2* deletion, pAI549 was digested with *Acc*I and circularized in dilute solution, deleting sequences between the two *Acc*I sites and creating plasmid pAI553. This plasmid was introduced into competent *B. subtilis* RB1 cells by $Cm^r$ selection. As expected, both $CtaA^+$ and $CtaA^-$ transformants were identified after screening the clones on MLT plates. One $Cm^r$ $CtaA^-$ transformant, designated RB995, was purified, and $Cm^s$ segregants were isolated as described above. The internal deletion of the *ctaA* gene in strain RB996 was verified by Southern analysis.

**Construction of phage ϕ105*ctaA*$^+$.** Plasmid pAI546 was linearized at its unique *Bam*HI site and cloned into phage vector ϕ105J23 (22) by transfection of protoplasts of *B. subtilis* strain RB1 (7). The phage were pooled, and a crude lysate was prepared. This lysate was used to transduce strain RB907 to $Cm^r$ $CtaA^+$. One $CtaA^+$ lysogen was isolated, purified, and used to prepare phage, designated ϕ105*ctaA*$^+$. The ability of ϕ105*ctaA*$^+$ to complement various *cta* mutations was tested by the cross-streak method described by Jones and Errington (22). $Cm^r$ lysogens were scored for their CtaA phenotypes by the TMPD agar plate test.

**Nucleotide sequencing.** The *ctaA* gene was sequenced across both strands by the dideoxy-chain termination method of Sanger et al. (39), with modified T7 DNA poly-

merase (Sequenase; United States Biochemical Corp., Cleveland, Ohio; 51) and [$^{35}$S]thio-dATP (1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). Various subclones containing the *ctaA* coding sequence were transferred to the phagemids pBS+ and pBS− for single-stranded DNA isolation. Techniques for propagating pBS constructions as phage in *E. coli* and isolating virion were carried out by using helper virus R408 as described by Short et al. (41). Supercoiled plasmid templates for DNA sequencing were prepared as described by Mierendorf and Pfeffer (29) and were sequenced as described by Chen and Seeburg (8). Sequencing reaction products were run on 5% polyacrylamide–7 M urea wedge gels (0.4 to 0.8 mm). *ctaA*-specific oligonucleotide primers were purchased from Operon Technologies (San Pablo, Calif.).

**Cloning of the *ctaA* alleles.** Chromosomal DNA (5 μg) from strains RB816 and RB819 was digested to completion with *Hin*dIII and *Eco*RI, respectively. One μg was ligated overnight under dilute DNA concentrations to favor recircularization of linear plasmid molecules. The ligation products were precipitated with ethanol and suspended in 20 μl of sterile distilled water prior to transformation of competent *E. coli* strain JM107. Positive clones were identified by restriction enzyme analysis and the inability to correct the mutant allele by marker rescue analysis. The *ctaA* alleles were sequenced by using denatured plasmid DNA and *ctaA*-specific oligonucleotides. The DNA sequence for each allele was obtained from two independent clones.

## RESULTS

**Rationale and isolation of cytochrome $aa_3$-deficient mutants.** A selection strategy to isolate cytochrome $aa_3$-deficient mutants of *B. subtilis* was based on the observation that *B. subtilis* strains phenotypically deficient in cytochrome $aa_3$ do not accumulate streptomycin (2, 28). Growth conditions have been established that repress or enhance the cellular levels of the enzyme (28). Systematic increases in cytochrome $aa_3$ levels brought about by the addition of vitamin-free Casamino Acids (Difco) to growth media result in a corresponding increase in growth inhibition by streptomycin and the rate of uptake of the antibiotic (28). Cytochrome $aa_3$ deficiency has been observed in a specific class of streptomycin-resistant (Str$^r$) mutants (*strC*) of *B. subtilis* (28, 43, 48). The Str$^r$ phenotype is the result of a decrease in the rate of streptomycin uptake and has been correlated with the deficiency in cytochrome $aa_3$ (2, 28). The *strC* mutation appears to be conditional, since supplementation of complex or minimal salts growth media with vitamin-free Casamino Acids results in the production of wild-type levels of cytochrome $aa_3$ and an increased rate of streptomycin uptake (28). These observations suggest that the uptake of streptomycin into cells of *B. subtilis* is dependent on the cytoplasmic membrane content of cytochrome $aa_3$ (2, 50) and provide a rationale for a mutant selection scheme in which loss of cytochrome $aa_3$ would render the mutant cell resistant to streptomycin. Mutations in the structural genes for the protein subunits of the apocytochrome, the biosynthetic enzymes for heme *a*, or regulatory genes necessary for the expression or assembly of the holoenzyme could be expected to reduce or abolish steptomycin uptake.

To distinguish known mutations with Str$^r$ phenotypes (i.e., *rpsL*[*strA*], *strB*, and *strC*) from mutations that confer a Cta$^-$ (cytochrome $aa_3$-deficient) phenotype, spontaneous Str$^r$ mutants were screened for the ability to oxidize the artificial electron donor TMPD. The Cta phenotype of the Str$^r$ mutants was scored by a modification of the TMPD agar plate test (19) as described in Materials and Methods. Wild-type strains (Cta$^+$) and previously isolated *str* mutants oxidized TMPD and stain blue.

Strain RB1 was grown at 37°C in LB medium containing 1% vitamin-free Casamino Acids. These nutritional conditions enhance the cellular levels of cytochrome $aa_3$ (28; J. Mueller, unpublished data) and reduce the chance of isolating mutants of the *strC* type. The culture was incubated overnight at moderately high aeration to allow adequate time for spontaneous mutants deficient in cytochrome $aa_3$ formation to undergo cell division and dilute out preexisting enzyme. This was necessary since approximately 50% of the normal levels of cytochrome $aa_3$ will allow sufficient streptomycin uptake to cause growth inhibition (28). The cells were harvested by centrifugation and suspended in 4 ml of LB medium. To isolate Str$^r$ mutants, samples were plated on LB agar containing 1% vitamin-free Casamino Acids and 100 μg of streptomycin per ml to produce approximately 300 single colonies per plate. The plates were incubated overnight at 37°C. Approximately 12,000 spontaneous Str$^r$ colonies were screened with TMPD; 136 Str$^r$ mutants were deficient in TMPD oxidase activity. Each mutant was examined for cytochrome $aa_3$ content by low-temperature spectroscopic analysis of whole-cell preparations (47). A total of 97 Str$^r$ TMPD$^-$ mutants contained levels of cytochrome $aa_3$ between 20 to 30% of the normal level, indicating that the threshold of cytochrome $aa_3$ activity that can be detected by the TMPD colony assay is approximately 30% of the activity of the wild-type strain. The remaining 39 mutants were identified as possible *cta* mutants by the complete lack of cytochrome $aa_3$. Mutants deficient in cytochrome *c* or *o* were not detected among the TMPD$^-$ isolates. This observation probably reflects the specificity of the streptomycin enrichment.

The 39 *cta* mutants were divided into nine classes based on cytochrome profiles and were subdivided into 21 classes after evaluation of each mutant for growth on several nonfermentable carbon sources (i.e., glycerol, lactate, malate, and succinate), utilization of glutamate as the sole carbon source, accumulation of organic acids, and the formation of chloroform-resistant spores. Because these mutations were not derived from independent cultures, a single mutant with a specific block in cytochrome $aa_3$ was chosen for further study and was designated RB676 (*cta-676*). Preliminary genetic analysis indicated that strain RB676 carried two unlinked mutations. The *cta-676* mutation was transferred by transformation from strain RB676 to RB888, creating strain RB934. The *cta-676* mutation conferred a cytochrome $aa_3$-deficient, asporogenous phenotype, while the second mutation suppressed the sporulation defect imposed by *cta-676*.

A second independent *cta* mutation was isolated by plating strain RB1 on minimal glucose media containing 1% vitamin-free Casamino Acids medium and 100 μg of streptomycin per ml. A total of 500 spontaneous Str$^r$ colonies were screened for TMPD oxidase activity. A total of 13 TMPD$^-$ mutants were isolated and examined spectroscopically for their cytochrome $aa_3$ content. All 13 mutants contained low levels of cytochrome $aa_3$. One mutant (RB642; *cta-642*) contained approximately 5% of the wild-type cytochrome $aa_3$ content and was chosen for further study.

**Phenotypic characteristics of *cta* mutants.** To establish that the *cta* mutants were indeed deficient in cytochrome $aa_3$, we assayed wild-type and mutant cytoplasmic membranes for

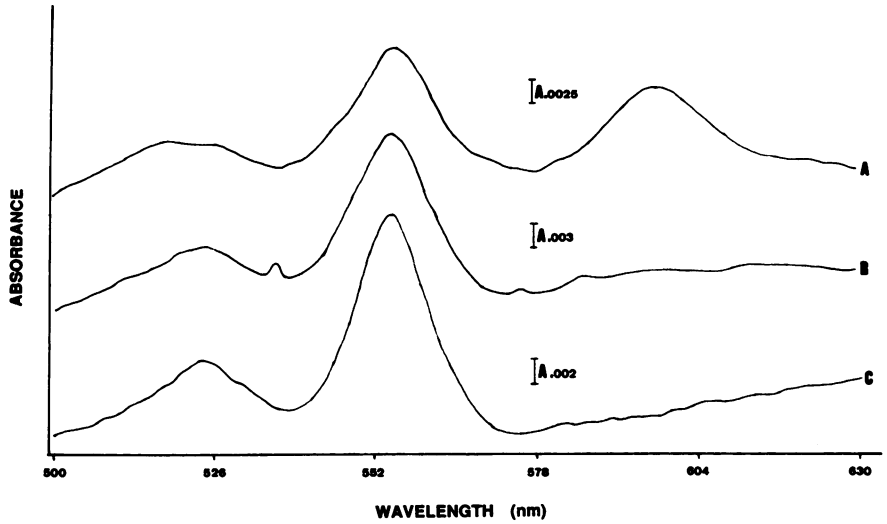


FIG. 1. Room-temperature (reduced minus oxidized) difference spectra of solubilized *B. subtilis* cytoplasmic membranes. A, *cta*$^+$; B, *cta-642*; C, *cta-676*. Cells were grown in LB medium plus 1.0% vitamin-free Casamino Acids. The protein concentration in each case was 1.0 mg/ml.

cytochrome content by room-temperature difference absorption spectrophotometry. Each mutant lacked the easily recognized maximum at 602 nm, confirming the absence of cytochrome $aa_3$ (Fig. 1). Cytochrome spectra of whole cells at liquid nitrogen temperature revealed that the *cta-642* mutation actually conferred a leaky phenotype, producing approximately 5% of the normal cellular complement of the oxidase (Table 2; data not shown). The spectrophotometric data presented in Fig. 1, together with low-temperature spectroscopic observations (data not shown), show that the mutations do not cause pleiotropic loss of cytochromes *c* or *b* and therefore do not decrease cytochrome $aa_3$ synthesis indirectly through an effect on early steps in heme biosynthesis.

Phenotypes associated with the *cta* mutations are presented in Table 2. Both mutants were found to be sporulation deficient, more severely so when the cells were grown in liquid 2× NS medium than when the bacteria were grown as colonies on solid 2× NS medium, as judged by phase-contrast microscopy. The specific block in sporulation has not been determined biochemically or morphologically; however, the phenotypic characteristics and microscopic examination of the mutants suggest a block at stage 0. The *cta* mutants had growth rates during logarithmic growth in sporulation and minimal glucose salts medium similar to those of the wild-type strain RB1. The mutants were also tested for production of several sporulation (late-growth)-associated products. Each mutant was found to produce amylase, protease, and antibiotic to about the same extent as the wild-type strain, as judged by zone diameters in plate tests (Table 2).

TABLE 2. Phenotypic properties of *cta* mutants

| Strain and relevant genotype | % Cox[a] | Presence (+) or absence (−) of[b]: | | | | | % Spo[c] |
|---|---|---|---|---|---|---|---|
| | | Lct | PA | AB | PR | Amy | |
| RB1 *cta*$^+$ | 100 | + | − | + | + | + | 100 |
| RB642 *cta-642* | 5 | − | + | + | + | + | 0 |
| RB934 *cta-676* | 0 | − | + | + | + | + | 0 |

[a] Cox, Cytochrome $aa_3$ relative to the wild type (RB1).

[b] Abbreviations: Lct, growth in minimal lactate medium; PA, accumulation of organic acids in glucose-containing medium; AB, production of sporulation-associated antibiotics; PR, production of sporulation-associated protease; Amy, production of alpha-amylase.

[c] Spo, heat-resistant spores, indicated as percent survivors relative to a wild-type control (RB1); 0 = <0.0001%.

Neither mutant was able to utilize lactate (Lct$^-$) as the sole carbon and energy source, suggesting that the sporulation defect may be an indirect consequence of a metabolic deficiency; e.g., mutations that block tricarboxylic acid (TCA) cycle function cause the Lct$^-$ phenotype and prevent cells from sporulating (18). To test whether the apparent metabolic block in the *cta* mutants was caused by a primary or secondary effect on TCA cycle function, both strains were tested initially for the accumulation of organic acids on purification agar plates (6). Both *cta* mutants accumulated organic acids, providing further evidence for a block in TCA metabolism (Table 2). This result, however, contradicted nutritional studies which indicated that neither mutant contains a severe block in TCA cycle function. *B. subtilis* strains deficient in the TCA cycle enzymes citrate synthase, aconitase, and isocitrate dehydrogenase are glutamate auxotrophs. Mutants with lesions in alpha-ketoglutarate dehydrogenase, succinate dehydrogenase, and fumarase cannot grow on media containing glutamate as the sole carbon source. Neither *cta* mutant required glutamate for growth on minimal glucose media, and both utilized glutamate as the sole source of carbon. In support of these findings, cell extracts from a *cta* deletion strain (RB996) showed normal levels of two TCA cycle enzymes, aconitase and fumarase (H. Taber, data not shown). Acidification of the medium may explain the Spo$^-$ phenotype of these strains, but the nature of the metabolic block associated with the *cta* mutations remains obscure.

Strains carrying the *cta-642* or *cta-676* mutation appeared morphologically distinct from *cta*$^+$ strains on solid LB or 2× NS medium. The *cta* mutants formed small (2-mm), smooth colonies, while the wild-type strain formed large (10- to 15-mm), rough, granular colonies. By microscopic examination, the *cta* mutants appeared as normal motile gram-positive rods. In the latter stages of the genetic experiments,

the small-colony phenotype was used to screen for the Cta phenotype. It is clear that both *cta* mutations confer similiar phenotypes, suggesting that *cta-642* and *cta-676* may be allelic. The only obvious phenotypic difference was a low level of cytochrome $aa_3$ biosynthesis associated with the *cta-642* mutation.

**Cloning and characterization of the *ctaA* locus.** In order to clone the wild-type gene designated by the *cta-676* allele, a plasmid library of *B. subtilis* DNA that contained partial *Mbo*I fragments of approximately 2.0 to 5.0 kb was constructed and used to screen for the presence of *cta* by the appropriate integration of *cta* sequences into the chromosome of mutant *cta-676*, restoring cytochrome $aa_3$ function (32). To introduce the *E. coli* plasmid replicon pSGMU2 into the chromosome at a site adjacent to *cta*, we transformed competent cells of strain RB676 (*cta-676*) with a plasmid pool from about 8,000 recombinant clones, selecting for $Cm^r$. $Cm^r$ $Cta^+$ transformants were expected to arise by integration of pSGMU2 into the chromosome adjacent to an intact copy of the *cta* locus. $Cm^r$ recombinants were screened for cytochrome $aa_3$ by the TMPD plate assay. A single $Cm^r$ $Cta^+$ transformant was isolated and designated strain RB704. To verify that the integrant was not the result of congression or reversion of the *cta-676* allele, genetic linkage of the integrated *cat* marker to *cta-676* was checked by transformation of strain RB676 to $Cm^r$ with chromosomal DNA from strain RB704. Approximately 85% cotransfer was found between the integrated *cat* gene and *cta-676*. Comparable transformation analysis with strain RB642 showed 95% cotransfer between the integrated *cat* gene and the *cta-642* mutation, indicating that *cta-642* and *cta-676* were closely linked and possibly allelic. These two mutations were considered to define the *ctaA* locus.

To obtain a plasmid carrying *ctaA* sequences, chromosomal DNA from strain RB704 containing the integrated copy of plasmid pSGMU2 was digested with *Hin*dIII, ligated at a dilute concentration, and used to transform *E. coli* JM109 cells to $Ap^r$. The resulting plasmid, pAI503, contained a *B. subtilis* chromosomal insert of approximately 4.0 kb (Fig. 2). That the insert in pAI503 contained a contiguous segment of DNA was confirmed by Southern blot hybridization (data not shown). Transformation of both *ctaA* mutants with plasmid pAI503 restored cytochrome $aa_3$ and sporulation proficiency to wild-type levels, indicating that the cloned fragment contained at least the portion of the *ctaA* locus designated by these alleles (see Fig. 4).

A restriction map of the *ctaA* region of the *B. subtilis* chromosome is depicted in Fig. 2. This region represents ~6.9 kb of DNA, the structure of which has been established by Southern blot analysis. pAI503 and pAI536 comprise 6.4 kb of DNA from this region of the chromosome. The fragment carried by pAI536 was isolated by subcloning the internal 600-base-pair (bp) *Bgl*II fragment of pAI503 into the *Bam*HI site of pSGMU2, and the resulting plasmid (designated pAI516) was integrated into the RB1 chromosome (Fig. 2). Chromosomal DNA from one integrant (RB811) was digested with *Eco*RI, ligated at low DNA concentration, and introduced by transformation into *E. coli* strain JM109. Clones were obtained that carried an overlapping DNA fragment extending 2.4 kb upstream of our initial insert (Fig. 2).

**Chromosomal location of the *ctaA* locus.** The chromosomal location of *ctaA* was determined by phage PBS1-mediated transduction. A strain containing a Tn*917* insertion near the *ctaA* locus was isolated (RB648) and found to be 85% linked by transduction to *ctaA642*. A phage lysate derived from

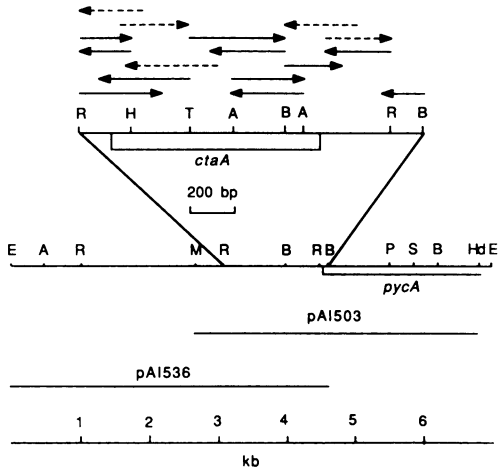


FIG. 2. Physical map and sequencing strategy of the *ctaA* region of *B. subtilis* chromosome. The potential open reading frames are indicated. The arrows above the restriction map show the direction and extent of each sequencing reaction. The regions indicated by the solid arrows were sequenced by using either the T3 or T7 primer, while regions sequenced with *ctaA*-specific oligonucleotides are indicated with the dashed arrows. Lines below the restriction map indicate the extent of the DNA carried by various plasmid clones. Abbreviations: H, *Hpa*II; Hd, *Hin*dIII; E, *Eco*RI; R, *Rsa*I; B, *Bgl*II; T, *Taq*I; P, *Pst*I; M, *Mbo*I; A, *Acc*I; S, *Sac*I. The chromosomal *Mbo*I site was converted to a *Bam*HI site during cloning of the *ctaA* locus.

strain RB648 was used to transduce to macrolide-lincosamide-streptogramin B resistance the kit of genetically marked *B. subtilis* strains described by Dedonder et al. (9). Linkage could only be demonstrated between the transposon-encoded drug marker and a single marker in each of two strains: 50% to *pyrD1* in RB353 and 26% to *metC3* in strain RB359.

To confirm this assignment, phage were propagated on *B. subtilis* strain RB811 containing the plasmid pAI516 integrated adjacent to the *ctaA* locus. A phage lysate was used to transduce strains RB353 and RB359 to $Cm^r$. The *cat* gene was found to cotransduce with *pyrD1* and *metC3* at frequencies of 52 and 6%, respectively, and with *cysC* at a frequency of 15%, suggesting that the *cat* determinant (and therefore *ctaA*) lie counterclockwise from *pyrD* on the *B. subtilis* chromosome (35). Additional linkages of *cat* to *spo0E* (37%) and *pycA* (95%) provided further evidence for the position of the *ctaA* locus between *pyrD* and *metC* (35).

By using markers in the *pyrD-metC* region, transformation crosses were performed in an attempt to define more precisely the position of the *ctaA* locus. Two-factor transformation data are summarized in Fig. 3. DNA was prepared from strain RB811 and used to transform to $Cm^r$ several strains containing mutations previously mapped to this region of the chromosome. Linkage was observed with *spoVD* (29%), *pycA* (75%), *citL* (25%), and *dapE* (12%) (Fig. 3). No transformation or transduction linkage was observed with *strB*, *fruB*, or *spoIIF*. Both *ctaA* mutations and the *pycA19* allele were used in reciprocal transformation crosses. The *ctaA* locus was found to be linked tightly to the *pycA* locus, placing *ctaA* at approximately 127 degrees on the *B. subtilis* chromosome (Fig. 3) (35).

Plasmids pAI503 and pAI536 failed to correct by recombination mutations in loci adjacent to *ctaA* including *spo0E*, *spoVD*, *spoVE*, *fruA-B*, *dapE*, *citL*, and *strB*, indicating that

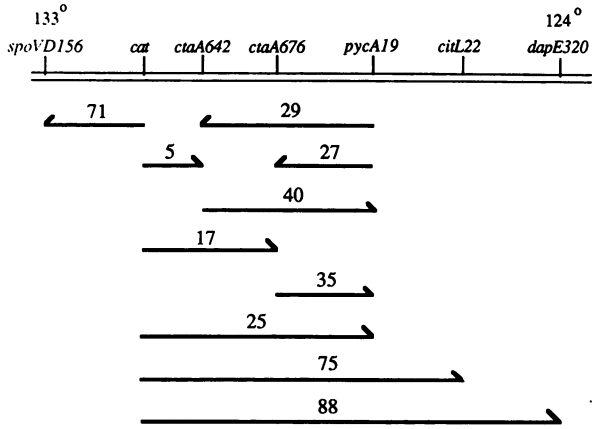


FIG. 3. Linkage map of the *B. subtilis* chromosome surrounding the *ctaA* locus. Arrows point from the selected to the unselected marker. For each selected marker, at least 400 transformants were purified and screened for the presence or absence of the unselected marker; *cat* designates the chloramphenicol acetyltransferase gene carried on the integrational plasmid in strain RB811 (RB1ΩpAI516). Values indicated are 100% minus the percent cotransformation. The positions of the loci are provisional and represent the order of these markers as presented in the most recent genetic map of the *B. subtilis* chromosome (35). The majority of these markers have not been ordered relative to outside markers by three-factor crosses.

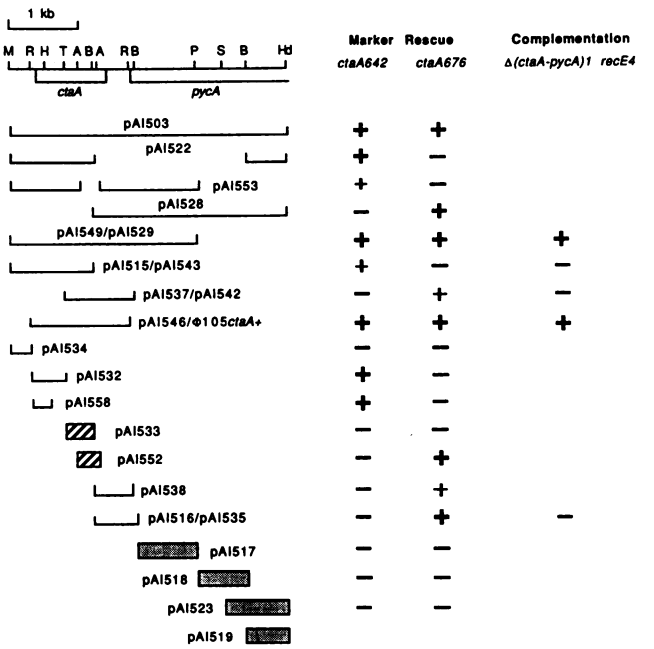


FIG. 4. Marker rescue and gene disruption analysis of the *ctaA* and *pycA* loci. A restriction map of cloned sequences is shown at the top. Segments of the *ctaA* region were subcloned into pSGMU2 by using the indicated endonuclease restriction sites and the designations of derivative plasmids given. Marker rescue represents the ability of recombinant plasmids to rescue *ctaA* mutants for growth on solid MLT medium. Integration (gene disruption) data represent the outcome of plasmid insertion into the chromosome of wild-type strain RB1 (CtaA$^+$ Pyc$^+$) by selection for Cm$^r$ integrants and scoring recombinants for the oxidation of TMPD (CtaA$^+$/CtaA$^-$) by plate assay and for growth on solid MGT medium with and without 400 μg of aspartate (Pyc$^+$/Pyc$^-$ [Asp$^+$/Asp$^-$]) per ml. Hatched boxes indicate that plasmid integration resulted in a CtaA$^-$ Pyc$^+$ phenotype. Stippled boxes indicate that plasmid insertion resulted in a CtaA$^+$ Pyc$^-$(Asp$^-$) phenotype. Solid lines indicate that the phenotype remained Cta$^+$ Pyc$^+$. The column on the far right of the diagram indicates the ability (+) or inability (−) of respective recombinant phage or plasmids to complement in *trans* the Δ(*ctaA-pycA*)*1* allele. Complementation was assumed to have occurred if the merodiploid exhibited the CtaA$^+$ Spo$^+$ phenotype of the wild-type strain. Abbreviations: M, *Mbo*I; R, *Rsa*I; H, *Hpa*II; Hd, *Hin*dIII; T, *Taq*I; B, *Bgl*II; P, *Pst*I; A, *Acc*I; S, *Sac*I.

the wild-type allele for each of these genes is not closely linked to *ctaA*.

**Fine-structure genetic mapping of the *ctaA642* and *ctaA676* mutations by marker rescue.** To localize *ctaA* on the cloned fragments, we tested the capacity of subcloned segments to correct the *ctaA642* and *ctaA676* mutations by transformation (Fig. 4). Subclones were generated in pSGMU2 by use of the indicated endonuclease restriction cleavage sites. The experiments of Fig. 4 show that the *ctaA642* mutation was rescued by the 230-bp *Rsa*I-*Hpa*II fragment of the insert contained in plasmid pAI552, whereas the *ctaA676* mutation was corrected by the 75-bp *Bgl*II-*Acc*I fragment carried by plasmids pAI538 and pAI552. This indicates that the maximum distance between the two point mutations on the chromosome was no more than 1.0 kb (Fig. 4).

**Use of integrational plasmids to define the transcriptional unit of the *ctaA* locus.** Integrational plasmids were used to localize the *ctaA* transcriptional unit on the cloned fragment and determine whether the fragment contained the entire *ctaA* transcriptional unit or only a part of it (34). This method exploits the ability of transformation-competent *B. subtilis* cells to incorporate exogenous DNA into homologous chromosomal sequences and provides a useful genetic test for the extent of a transcription unit. Integrational plasmids cannot replicate in *B. subtilis* but can express a selectable marker, usually drug resistance, when integrated into the chromosome by a single reciprocal crossover event in the region of homology (34). If the region of homology lies completely within the *ctaA* transcriptional unit, integration would lead to a disruption of the unit and a CtaA$^-$ phenotype. Since the cloned internal fragment does not contain an intact 5′ or 3′ end, both duplicated genetic regions flanking the plasmid fail to constitute an intact gene. However, if the region of homology extends beyond the transcriptional unit, at least one intact unit will result from the crossover, and the phenotype of the wild-type strain is retained.

Wild-type strain RB1 was transformed to Cm$^r$ with DNA from each of the integrational plasmids illustrated in Fig. 4. The transformants from any one cross were phenotypically homogeneous, being either entirely CtaA$^+$ or CtaA$^-$ as judged by TMPD staining and spectroscopic measurement of cytochrome $aa_3$. Two plasmids, pAI533 and pAI552, gave transformants that were CtaA$^-$ (Fig. 4). Accordingly, the fragments of DNA contained within these two plasmids lie entirely within the *ctaA* transcriptional unit. Transformants made with pAI532 and pAI538 were CtaA$^+$ (Fig. 4); the fragments in these plasmids therefore designate the boundaries of the *ctaA* transcriptional unit and localize the *ctaA* coding sequence within the 1.35-kb *Rsa*I fragment in the cloned DNA. These results suggest that the *ctaA* transcript is monocistronic and about 1.0 kb in size. In addition, both mutations mapped within the *ctaA* transcriptional unit, providing additional evidence that *ctaA642* and *ctaA676* are allelic (Fig. 4). Southern hybridizations (data not shown) confirmed that integration of the pSGMU2 derivatives occurred by the expected Campbell mechanism.

**Localization of the *pycA* locus adjacent to *ctaA*.** Genetic experiments established that the *pycA19* allele was 60%

linked by transformation to both *ctaA* point mutations (Fig. 3). *B. subtilis pycA* (pyruvate carboxylase) mutants are aspartate auxotrophs ($Asp^-$) on minimal glucose media because of an inability to convert pyruvate to oxaloacetate (5). It seemed plausible that the *pycA* locus might lie within the 6.5 kb of chromosomal DNA contained in pAI536 and pAI503. Plasmids pAI503 and pAI536 were used to transform strain RB772 (*pycA19*), and the selection was made for $Asp^+$. Neither plasmid was able to rescue the *pycA19* mutation, indicating that the wild-type *pycA* allele was not present on the cloned sequences. However, integration of certain pSGMU2 subclones derived from pAI503 into the chromosome of strain RB1 yielded transformants requiring aspartate for growth on minimal glucose media (Fig. 4). These results suggest that one end of the *pycA* transcriptional unit lies within the 600-bp *Bgl*II fragment carried by pAI516 shown to contain an end of the *ctaA* gene. Integrants constructed with pAI519 were $Asp^-$ $CtaA^+$, which implies that pAI503 contains only a portion of the *pycA* locus. The integrant strains containing disruptions of the *pycA* locus sporulate normally, as previously observed with a *pycA* point mutant (14). Additionally, *ctaA* gene disruptions (e.g., with pAI533 [Fig. 4]) do not have a polar effect on expression from the *pycA* locus. This result provides additional genetic evidence that *ctaA* is monocistronic, assuming that transcription occurs left to right in Fig. 4.

**The *ctaA* locus encodes a diffusable, *trans*-acting product.** As an independent test that the 1.35-kb *Rsa*I fragment encoded an intact and fully functional *ctaA* gene product and to examine whether it could complement in *trans* a strain lacking this transcriptional unit, *B. subtilis* strains were constructed in which a portion of the transcriptional unit was deleted (see Materials and Methods). The phenotype of cells containing the Δ(*ctaA-pycA*)*1* or Δ*ctaA2* mutation were indistinguishable from that of *ctaA676* mutant cells. Overlapping restriction fragments encompassing the designated *ctaA* region of the cloned material were subcloned into the shuttle vector pMK4 (pAI529, pAI535, pAI542, and pAI543) (46) and tested in *trans* for complementation of the various *ctaA* alleles by selecting for $Cm^r$ transformants and screening with the TMPD plate assay. The results of these experiments are summarized in Fig. 4; for clarity, only the Δ(*ctaA-pycA*)*1* allele is shown. Plasmids that complemented the deletion strain also complemented *recE4* derivatives of the Δ*ctaA2*, *ctaA642*, and *ctaA676* alleles (data not shown). It is apparent from these results that the recombinant plasmids carrying the *Rsa*I fragment do encode an intact and fully functional *ctaA* gene capable of complementing in *trans* the various *ctaA* mutational defects. Plasmids that complemented the cytochrome $aa_3$ deficiency also restored the ability to sporulate at wild-type levels, recover from acid accumulation, and utilize lactate as the sole carbon and energy source. Complementation with multicopy plasmids did not result in gene dosage effects, since spectroscopic studies of several $CtaA^+$ recombinants showed normal levels of cytochrome $aa_3$ (data not shown).

Spontaneous $Cm^s$ segregants of the complemented strains were isolated by overnight growth in the absence of chloramphenicol, plating for individual colonies and replica plating to LB medium with and without chloramphenicol. The segregation frequency was approximately 20% for all strains tested. In all cases, 100% of the $Cm^s$ segregants cured of the recombinant plasmid exhibited the parental $CtaA^-$ phenotype, verifying that complementation was dependent on plasmid sequences.

A recombinant phage was constructed by using the temperate phage vector ϕ105J23 (22). The recombinant phage ϕ105*ctaA*$^+$, containing the 1.35-kb *Rsa*I fragment, was used to transduce the *ctaA* alleles to $Cm^r$. Transductants were picked onto selective media and scored for their CtaA phenotype by the TMPD method. Phage ϕ105*ctaA*$^+$ complemented all *ctaA* alleles in *trans* (Fig. 4; data not shown). This recombinant phage also provided a simple method to determine whether any of our remaining 38 *cta* mutants mapped within the *ctaA* locus, by a cross-streak technique as described by Jones and Errington (22). This analysis defined a second *cta* complementation group. Of the *cta* mutants, 11 were not complemented by the specialized transducing phage, indicating that minimally, mutations in at least one other unlinked locus, *ctaB*, result in a cytochrome $aa_3$-deficient phenotype (C. Gustafson and J. Mueller, unpublished data).

TABLE 3. Phenotypic properties of *ctaA* pseudorevertants

| Strain and relevant genotype | % Cox[a] | Presence (+) or absence (−) of[b]: | | % Spo[c] |
|---|---|---|---|---|
| | | Lct | PA | |
| RB1 *ctaA*$^+$ | 100 | + | − | 100 |
| RB642 *ctaA642* | 5 | − | + | 0 |
| RB944 *ctaA642 scs-2* | 5 | + | − | 39 |
| RB934 *ctaA676* | 0 | − | + | 0 |
| RB945 *ctaA676 scs-4* | 0 | + | − | 22 |
| RB829 Δ(*ctaA-pycA*)*1* | 0 | − | + | 0 |
| RB996 Δ*ctaA2* | 0 | − | + | 0 |
| RB971 Δ(*ctaA-pycA*)*1 scs-5* | 0 | + | − | 24 |
| RB923 Δ(*ctaA-pycA*)*1 sca-3* | 100 | + | − | 70 |

[a] Cox, Cytochrome $aa_3$ relative to the wild type (RB1).

[b] Abbreviations: Lct, growth in minimal lactate medium containing 400 μg of aspartate per ml; PA, accumulation of organic acids in glucose-containing medium.

[c] Spo, Heat-resistant spores, indicated as percent survivors relative to a wild-type control (RB1); 0 = <0.0001%.

**The *ctaA* locus does not encode a structural gene for apocytochrome $aa_3$ or a heme *a* biosynthetic enzyme.** During construction of the *ctaA-pycA* deletion strain, three classes of $Cm^s$ segregants were recovered. The largest class was phenotypically $Cm^s$ $CtaA^-$ $Pyc^-$ $Spo^-$, as expected. The second class consisted of strains which were phenotypically $Cm^s$ $CtaA^-$ $Pyc^-$ $Spo^+$. A single clone representing the third class was isolated with a $Cm^s$ $CtaA^+$ $Pyc^-$ $Spo^+$ phenotype. Because RB828 is a deletion strain, true reversion was not possible. The genetic results suggested the isolation of two unique compensatory mutations in the deletion background. Examples of the latter two classes retained the expected deletion as determined by DNA blot hybridization (data not shown).

The phenotypic characteristics of the suppressor mutants are presented in Table 3. One class of suppressor mutants, referred to as *scs* (suppressor of *ctaA*-associated sporulation) restored the ability of the isogenic *ctaA* deletion mutant to utilize lactate as a nonfermentable carbon source for growth and the capacity of the deletion mutant to sporulate; however, cytochrome $aa_3$ formation was not restored. Partial revertants (*scs*) have also been isolated in both *ctaA* point mutant backgrounds by selection on MLT solid medium (Table 3). The *scs* mutations appeared at high frequency regardless of whether they were isolated in a point mutant or deletion genetic background [Δ(*ctaA-pycA*)*1* and Δ*ctaA2*], suggesting possible loss-of-function mutations. As discussed previously, the original mutant strain RB676 was

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RsaI
GTACATTTTCCAGAAGCCGTCATTCTATTATATTTTTGTGAACAAAAGGCTCTGGGAATTGCCACAAAACAC  72

ATATTCGCTTACACTTGGAACGTATATAAAATTGCAGCAGTATGTTAAGAAGGTGAATATTGT ATG AAT  141
                                                                Met Asn

AAA GCA TTA AAA GCT CTC GGT GTT CTG ACG ACA TTT GTC ATG CTA ATT GTT TTA  195
Lys Ala Leu Lys Ala Leu Gly Val Leu Thr Thr Phe Val Met Leu Ile Val Leu
    A (ctaA642)                        HpaII
ATC GGG GGT GCC CTC GTT ACA AAA ACA GGT TCC GGC CAA GGA TGC GGC AGA CAG  249
Ile Gly Gly Ala Leu Val Thr Lys Thr Gly Ser Gly Gln Gly Cys Gly Arg Gln

TGG CCG CTG TGT CAC GGC CGT TTT TTC CCT GAA CTG AAT CCG GCT TCA ATT ATT  303
Trp Pro Leu Cys His Gly Arg Phe Phe Pro Glu Leu Asn Pro Ala Ser Ile Ile

GAA TGG AGC CAC CGA TTC GCA AGC GGA ATC TCT ATT ATC CTT GTG CTA AGC CTT  357
Glu Trp Ser His Arg Phe Ala Ser Gly Ile Ser Ile Ile Leu Val Leu Ser Leu

GCG TTT TGG TCA TGG AGA AAA ATC ACG CCG ATT TTT CGT GAA ACA ACG TTT CTC  411
Ala Phe Trp Ser Trp Arg Lys Ile Thr Pro Ile Phe Arg Glu Thr Thr Phe Leu

GCG ATC ATG TCA ATT ATC TTT TTA TTT CTT CAG GCA TTG CTT GGC GCA TTG GCT  465
Ala Ile Met Ser Ile Ile Phe Leu Phe Leu Gln Ala Leu Leu Gly Ala Leu Ala
              TaqI
GTC GTA TTC GGT TCG AAC GCG CTG ATT ATG GCG CTT CAC TTC GGC ATC TCA TTA  519
Val Val Phe Gly Ser Asn Ala Leu Ile Met Ala Leu His Phe Gly Ile Ser Leu

ATT TCT TTT GCT TCA GTG CTT ATT TTA ACA TTG CTC ATA TTT GAA GCT GAT AAA  573
Ile Ser Phe Ala Ser Val Leu Ile Leu Thr Leu Leu Ile Phe Glu Ala Asp Lys

TCA GTC AGA ACA CTG GTT AAG CCG CTT CAA ATC GGC AAA AAG ATG CAA TTT CAC  627
Ser Val Arg Thr Leu Val Lys Pro Leu Gln Ile Gly Lys Lys Met Gln Phe His
                                              AccI
ATG ATA GGA ATT TTA ATA TAT TCC TAT ATC GTT GTG TAT ACA GGC GCA TAT GTA  681
Met Ile Gly Ile Leu Ile Tyr Ser Tyr Ile Val Val Tyr Thr Gly Ala Tyr Val

AGA CAC ACT GAA TCA AGT CTG GCA TGT CCT AAT GTG CCG CTG TGC AGC CCG CTG  735
Arg His Thr Glu Ser Ser Leu Ala Cys Pro Asn Val Pro Leu Cys Ser Pro Leu

AAC AAT GGA CTT CCG ACC CAA TTC CAT GAA TGG GTG CAA ATG GGC CAC AGA GCA  789
Asn Asn Gly Leu Pro Thr Gln Phe His Glu Trp Val Gln Met Gly His Arg Ala

GCA GCC TTA CTT TTA TTT GTA TGG ATT ATT GTT GCC GCT GTT CAT GCT ATT ACT  843
Ala Ala Leu Leu Leu Phe Val Trp Ile Ile Val Ala Ala Val His Ala Ile Trp
                  BglII                  A (ctaA676)
TCC TAT AAA GAT CAA AAA CAG ATC TTT TGG GGA TGG ATC TCA TGT CTT ATT TTC  897
Ser Tyr Lys Asp Gln Lys Gln Ile Phe Trp Gly Trp Ile Ser Cys Leu Ile Phe
                                          AccI
ATT ACA TTA CAG GCA CTG TCC GGT ATT ATG ATC GTA TAC TCT GAA CTG GCT CTG  951
Ile Thr Leu Gln Ala Leu Ser Gly Ile Met Ile Val Tyr Ser Glu Leu Ala Leu

GGC TTT GCA CTT GCC CAC TCG TTC TTT ATT GCC TGT CTG TTT GGC GTT CTA TGC 1005
Gly Phe Ala Leu Ala His Ser Phe Phe Ile Ala Cys Leu Phe Gly Val Leu Cys

TAC TTC TTA TTA TTG ATT GCT CGT TTC CGT TAT GAA TCC AGG CAA TCA TAA GGA 1059
Tyr Phe Leu Leu Leu Ile Ala Arg Phe Arg Tyr Glu Ser Arg Gln Ser  *

CTCAAGACCAAAGCCTTAGGCGGCTTTGGTCTTTTTTATGTCTTGTTTTTTGGCGCTTTATGCGTTTCAAT 1130

CGCCAGCCTTCGCCGAACTTGATGATCAGTGAGCGGCTCACTTCGGTCTGTCCCCTTTCTCATCGTGATTA 1201

TAGAAATTTCATATGGATCTATAGCTCGCGAACATAAGTATTAAAGCCTTTTAAATCAGATAGTGCTTAAA 1272

GAAATATGCACGGCATACTACAAAGCATCTAAAATATTGGCTGTATAAGCCTCTTTTTATTTTGGGCTTGT 1343
    RsaI
GACAGTACTCTATAATCTCTGAAGTCCCCTTATATCTCTTCTAACCATCTGTTTCACTCCACATTTTCTAT 1414

ATAAAAAACAAGAGTGTATATCATTGATATACACTCTTTGTTTTTTATGCTTTTTCAATTTCAAGGAGCAG 1485
BglII
ATCT                                                                    1489
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FIG. 5. Nucleotide sequence of the nontranscribed strand of *ctaA* and flanking DNA. Endonuclease restriction sites correspond to those shown in Fig. 2. The predicted amino acid sequence of CtaA is shown below the DNA sequence. The potential ribosome-binding site (double underline) and sequences involved in the putative stem-loop structure of transcription terminators (converging arrows) are indicated. The locations of the *ctaA642* and *ctaA676* mutations are shown. These sequence data have been submitted to the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession number M23915.

isolated as lacking cytochrome $aa_3$ but capable of efficient sporulation in 2× NS medium. This phenotype was later shown by two-factor transformation crosses with the *pycA* locus to be conferred by two unlinked mutations. This strain carries a point mutation in the *ctaA* gene plus a second mutation (*scs*) which suppresses the sporulation defect imposed by the *ctaA* lesion. Microscopic examination of *scs* cells after growth overnight in 2× NS broth revealed an accumulation of both intact and lysed cells, as well as a significant number of apparently normal spores, suggesting oligosporogeny. The *scs* partial suppressor strains are pigmented on sporulation plates and form a higher frequency of phase-bright spores than the parent strain does; however, the frequency of heat-resistant spores is only ~30% that of the wild type. This suggests tentatively that the majority of the cell population is blocked at stages IV or V of sporogenesis. Each suppressor strain was lysogenized with the $\phi 105 ctaA^+$ specialized transducing phage, restoring cytochrome $aa_3$ and sporulation proficiency to wild-type levels for all *scs* lysogens. The second class of suppressor mutant, referred to as *sca* (suppressor of *ctaA*), restored all phenotypic defects conferred by the null mutation (Table 3).

In general, the suppressor mutations appeared to bypass the requirement for CtaA in cytochrome $aa_3$ biosynthesis and sporulation. None of the *scs* and *sca* mutations conferred a detectable phenotype on an otherwise wild-type strain. As mentioned above, gene disruptions of the *pycA* locus do not manifest any of the phenotypes conferred by mutations at the *ctaA* locus. Additionally, *scs* partial suppressors have been isolated in strain RB996 carrying an internal *ctaA* deletion (J. Mueller, unpublished data).

**Nucleotide sequence analysis of the *ctaA* gene.** The nucleotide sequence of the 1,489-bp *Rsa*I-*Bgl*II fragment including the *ctaA* gene was determined by the dideoxy-chain termination method by using the sequencing strategy shown in Fig. 2. The entire sequence was determined on both strands and across all restriction sites used in the subcloning of fragments except for 138 nucleotides on the 3′ end. Unambiguous sequence from this region was determined with two independent clones. The complete sequence with the predicted amino acid sequence is shown in Fig. 5. In the region where the *ctaA* mutations map (Fig. 4), a single open reading frame of 918 nucleotides was identified starting with an ATG codon at position 136 and ending with a TAA stop codon at position 1054. The initiation codon is preceded by a potential ribosome-binding site with high complementarity to the 3′ end of *B. subtilis* 16S rRNA (20). This sequence could base pair with 16S rRNA with a calculated binding energy of −15.2 kcal/mol (52). The open reading frame consists of 306 amino acids with a predicted molecular weight of 34,065 and is in the orientation and location expected for the *ctaA* gene. We tentatively conclude that this open reading frame in fact represents the *ctaA* gene, since insertion and deletion mutations that interrupt this coding sequence cause a CtaA$^-$ phenotype (Fig. 4). The *ctaA* gene is followed by a region of dyad symmetry centered at nucleotide positions 1077 and 1078 with a potential to form a stem and loop secondary structure of $\Delta G$ equaling −24.6 kcal/mol (Fig. 5). The stem is followed by a stretch of T (U) residues, resembling a *rho*-independent transcription termination site (38).

The predicted amino acid sequence of CtaA as translated from the nucleotide sequence implies a very hydrophobic protein (polarity index, 29.8%). Plotting of the average residue hydrophobicity as a function of residue number reveals the presence of several internal regions of high average hydrophobicity according to the method of Kyte and Doolittle (23; Fig. 6). These hydrophobic sequences are separated by hydrophilic residues that frequently contain clustered positive charges. In Chou-Fasman predictions, the internal hydrophobic sequences are predicted to be alpha-

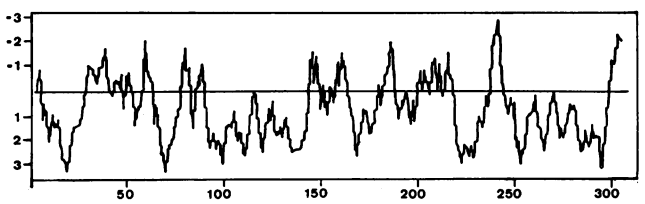


FIG. 6. Hydropathy profile of CtaA based on the data of Kyte and Doolittle (23). The window length in the calculation was 9. The scale on the abscissa represents amino acid residues. Values below the horizontal line represent hydrophobic regions, and values above the line represent hydrophilic regions.

helical (data not shown) and therefore may designate transmembrane regions.

Computer-assisted searches of protein sequence data banks showed no significant similarity to any sequence contained in these banks and, in particular, no similiarity to the highly conserved bacterial and mitochondrial cytochrome $aa_3$ polypeptides (37, 42, 45). This finding is also consistent with the isolation of a compensatory (bypass) mutation in a *ctaA* null mutant that restores cytochrome $aa_3$ (Table 3) and confirms the inference that *ctaA* does not encode a cytochrome $aa_3$ subunit.

**Cloning the *ctaA642* and *ctaA676* alleles.** Cloning of chromosomal fragments carrying the mutations in strains RB642 and RB676 was done as described in Materials and Methods. Sequence analysis was carried out for the *ctaA642* and *ctaA676* alleles. The *ctaA642* mutation that mapped upstream of the *Hpa*II site (Fig. 4) was determined to be a single base pair G-199 to A transition (Fig. 5). The resulting missense mutation converts glycine-22 to an arginine residue (Fig. 5). The *ctaA676* allele, which maps within the 74-bp *Bgl*II-*Acc*I fragment (Fig. 4), was found to be a similiar transition, G-878 to A, that produced a chain-terminating codon TAG (Trp-248 to stop; Fig. 5). The fact that one of the alleles is a nonsense mutation indicates that the pleiotropic phenotype of mutations in *ctaA* must be caused by the loss of the gene product of this locus and that the missense mutation in strain RB642 probably causes a change that impairs the biological activity or stability of the protein.

## DISCUSSION

We have isolated mutants of *B. subtilis* lacking the cytochrome $aa_3$ terminal oxidase complex by exploiting the observation that loss of cytochrome $aa_3$ results in a profound decrease in streptomycin accumulation (2, 50). Strains that contained low concentrations or no cytochrome $aa_3$ were identified by their failure to oxidize the artificial electron donor TMPD, which apparently donates electrons primarily to the cytochrome $aa_3$ branch of the respiratory chain. The phenotype of *ctaA* mutant cells includes the lack of spectrophotometrically detectable levels of cytochrome $aa_3$. Inactivation of *ctaA* has a striking pleiotropic effect on spore formation, utilization of nonfermentable carbon and energy sources, and accumulation of organic acids during growth in glucose-containing medium. This observation suggested that cytochrome $aa_3$ was necessary for sporulation; however, the isolation of compensatory mutations that uncouple the phenotypes suggests that *ctaA* is involved in two distinct pathways, one in the formation of cytochrome $aa_3$ and the other in sporulation. Although *ctaA* mutants appear to contain a functional TCA cycle, the loss of cytochrome $aa_3$ may uncouple electron transport from oxidative phosphorylation, resulting in the inability to regenerate reducing equivalents (NADH, NADPH, and FADH) for oxidation by the TCA cycle. The possible bioenergetic defects imposed by loss of the terminal oxidase could disrupt normal membrane function, affecting metabolic activities required during the early stages of sporulation. Since *B. subtilis* membranes contain one and possibly two additional terminal oxidases, such hypotheses would predict that neither cytochrome *o* or cytochrome *d* could compensate efficiently for the functional loss of cytochrome $aa_3$.

As a first step in the characterization of *ctaA*, we have cloned the gene. The genetic organization of *ctaA* appears to be monocistronic. Complementation of a *ctaA* null mutant by introduction of the wild-type *ctaA* gene on a phage or multicopy plasmid vector restored cytochrome $aa_3$, TMPD oxidase activity, and utilization of nonfermentable carbon sources for growth and sporulation. Experiments in which we disrupted the *ctaA* gene demonstrate that the pleiotropic effects are the consequence of the absence of CtaA rather than the lack of expression of one or more downstream genes in the *ctaA* transcriptional unit. In addition, we also cloned a portion of the *B. subtilis* pyruvate carboxylase structural gene.

Nucleotide sequence analysis of the *ctaA* region identified a single open reading frame in the correct orientation and location. The putative protein is not homologous to any electron transport proteins such as bacterial or mitochondrial cytochromes and thus represents a novel gene product. Formally, CtaA plays the role of a positive effector of cytochrome $aa_3$ biosynthesis, and its sequence suggests that it is an integral membrane protein, with a predicted charge index of 11.5. One remarkable feature of this polypeptide is an abundance of cysteine (seven residues) and histidine (nine residues). These residues do not conform to the consensus determined for finger structures characteristic of nucleic acid-binding proteins. The *ctaA* gene product may function as a carrier polypeptide that acts as an obligatory membrane-bound cofactor for cytochrome $aa_3$ synthesis or assembly of the oxidase holoenzyme into the cytoplasmic membrane. Alternatively, *ctaA* may encode an integral membrane protein whose loss from the cytoplasmic membrane disrupts normal membrane biogenesis and integrity. In theory, this may nonspecifically inhibit insertion of cytochrome $aa_3$, disturb the functioning of other membrane-active components such as lactate dehydrogenase, and block formation of the asymmetric septum during sporogenesis.

Isolation of pseudorevertants that suppress the effects of a *ctaA* null mutation indicates that the role of CtaA in cytochrome $aa_3$ biosynthesis and sporulation can be bypassed. The isolation of the *sca* pseudorevertant in a *ctaA* deletion background provides strong genetic evidence that the *ctaA* gene is not a structural gene of the *B. subtilis* apocytochrome $aa_3$ complex or a gene for a heme *a* biosynthetic enzyme. The *sca* bypass mutation also implies that cytochrome $aa_3$ and CtaA are dispensable for growth and sporulation in a laboratory environment. This latter conclusion is perhaps not surprising considering the redundancy in terminal oxidases in most procaryotic systems (36). It is possible that the *sca* gene product is a component common to the regulation of cytochrome $aa_3$ and metabolic events necessary for the initiation of the developmental process; however, we have not as yet determined whether the *sca* phenotype is conferred by a single mutation. The isolation of *scs* bypass mutations, which uncouple the sporulation and cytochrome $aa_3$ phenotypes from each other, suggests that the roles of CtaA in sporulation and expression of cytochrome $aa_3$ constitute distinct and separate pathways and that the pleiotropic phenotype conferred by mutations in *ctaA* (with the obvious exception of cytochrome $aa_3$ expression) is a consequence of a single metabolic block. The *scs* mutations may designate alternate pathways in the metabolic regulation of sporulation. Speculation on the underlying mechanisms governing cytochrome $aa_3$ biogenesis and the coupling of *ctaA* to sporogenesis will be guided by the nature of mutations that bypass the requirements for the *ctaA* gene product.

In summary, we have identified a new gene in *B. subtilis*, *ctaA*, which appears to be required for the genetic expression, posttranscriptional biogenesis, or both, of the *B. subtilis* cytochrome $aa_3$ terminal oxidase complex. To date, the only genetic information on any bacterial cytochrome $aa_3$

complex at the molecular level has been the cloning of the cytochrome $aa_3$ structural genes from *P. denitrificans* and the gram-positive thermophilic bacillus PS3 (37, 42, 45). Recently, a cytochrome $aa_3$ gene from *Bradyrhizobium japonicum* has been cloned, although its function is unknown (33). To the best of our knowledge, this is the first report of the cloning of a gene involved in the regulation of a bacterial cytochrome $aa_3$. Additional experiments are necessary to determine whether the effect of *ctaA* mutations occurs at the level of subunit synthesis, stability, or assembly. Further studies will focus on the role of the *ctaA* gene product in the regulation of cytochrome $aa_3$ biogenesis and metabolic functions critical to the early stages of sporulation.

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## ADDENDUM

While this paper was under review, the isolation and characterization of a *B. subtilis* cytochrome $aa_3$-deficient mutant was report by W. S. James, F. Gibson, P. Taroni, and R. K. Poole (FEMS Microbiol. Lett. **58**:277–282, 1989). Their data agree with our observations of the gene identified herein as *ctaA*.

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