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Catabolite Regulation of the *Bacillus subtilis* *ctaBCDEF* Gene Cluster

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***Bacillus subtilis* cytochrome *c* oxidase caa_3 is encoded by the *ctaCDEF* genes at the *ctaABCDEF* locus, with the *ctaBCDEF* genes organized as an operon-like unit. A dyad symmetry sequence and a catabolite response element homolog can be recognized in the 240-bp intercistronic region between *ctaB* and *ctaC*. *ctaB′-lacZ* and *ctaBCD′-lacZ* transcriptional fusions integrated at the native locus were used to study catabolite effects on transcription of the *ctaB* and *ctaCDEF* genes. In Schaeffer's medium lacking glucose, *ctaBCD′-lacZ* was expressed at a very low level during the exponential phase, and expression increased about 30-fold 2 h after entry into the stationary phase. In the presence of 0.5% glucose, *ctaBCD′-lacZ* expression was totally repressed. In contrast to *ctaBCD′-lacZ*, *ctaB′-lacZ* was constitutively expressed regardless of carbon source. The *ctaCDEF* genes were separated from *ctaB* by insertion of plasmids carrying selectable markers in such a way that the *ctaCDEF* and *ctaB* transcription units remained intact. Enzymatic assays of caa_3 with these constructs, showed that *ctaCDEF* was not expressed independently of *ctaB*. Also, when a *′ctaB-ctaC′-lacZ* fusion (containing the *ctaB-ctaC* intercistronic region) was placed at a remote nonessential locus, β-galactosidase activity could not be detected. The absence of a promoter in the *ctaB-ctaC* intercistronic space also was indicated by the inability to detect *ctaC*-specific transcripts with RNase protection assays, primer extension, and rapid amplification of 5′ cDNA ends. Direct mRNA measurements showed that, in the presence of 0.5% glucose, *ctaBCDEF* transcripts terminated at the 3′ end of the putative stem-loop structure and the distal portion was down-regulated. A possible mechanism for *ctaCDEF* gene regulation is suggested. Catabolite repression of *ctaBCD′-lacZ* was partly dependent on CcpA but was independent of HPr. The expression of *ctaBCDEF* also appears to require the *strC*, *ctaA*, and *resD-resE* gene products.**

When a variety of carbon sources are available, *Bacillus subtilis* preferentially utilizes glucose. While glucose is present, genes encoding enzymes for nonfermentable carbon source utilization are usually down-regulated, a process called catabolite repression (CR) (2). During glycolysis, end products such as acetoin are formed (28), secreted into the medium, and not utilized by the cell until glucose is exhausted. Energy production through consumption of nonfermentable carbon sources can be obtained only via oxidative respiration, for which an active tricarboxylic acid (TCA) cycle and a functional respiratory chain are required. Terminal oxidases are enzymes that catalyze the final step of respiration, reducing one molecule of oxygen to two molecules of water. The free energy available from this reaction is used by the oxidase complexes to pump protons from the cytoplasm to the cell exterior (5, 13, 30, 45). Both biochemical and genetic evidence has shown that there are two heme A-containing terminal oxidases (*a*-type cytochromes) in *B. subtilis* (21, 29). One is quinol oxidase aa_3, encoded by the *qoxABCD* operon located at kb 3913 to 3917 on the *B. subtilis* genetic map (20, 35). The other is cytochrome *c* oxidase caa_3, encoded by the *ctaCDEF* genes located at kb 1560 to 1563 (20, 37). At the *cta* locus, there are six genes, *ctaABCDEF*. *ctaA* and *ctaB* encode two enzymes required for the biosynthesis of heme A, the prosthetic group of both aa_3 and caa_3 (26, 27, 42).

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It was first thought that terminal oxidase caa_3 was primarily expressed when cells were grown at a low rate (1, 43). In an early spectral analysis, Chaix and Petit (1) reported that the absorption peak at 600 nm (aa_3) was shifted to 605 nm (caa_3) and the peak at 548 nm corresponding to cytochrome *c* increased when cultures grown in glucose-containing minimal medium were compared with cultures grown in succinate-containing minimal medium. Biochemical evidence has shown that both *a*-type terminal oxidases are present in succinate-grown cells, whereas caa_3 is undetectable in glucose-grown cells (21). Quantification of aa_3 expression was monitored with a *qoxA′-lacZ* transcriptional fusion, and it was shown that the level of expression of the *qoxABCD* operon was higher in rich medium (Luria-Bertani [LB] medium) and glucose-containing minimal medium than in succinate-containing minimal medium (35). There have not been molecular studies on how *ctaCDEF* is regulated in response to carbon sources prior to this report.

Mueller and Taber (26, 27) have studied *ctaA* extensively and have shown that the expression of *ctaA* is active in exponential cultures and is elevated postexponentially in a relatively high concentration of glucose (1.0%). The promoter for *ctaA* (*ctaAp*) has been identified; transcription of *ctaA* occurs in the sense opposite that of *ctaBCDEF* (26, 27). Thus, *ctaB* must possess a specific promoter (*ctaBp*) in order to be transcribed. CtaB, as well as CtaA, has an important role in heme A biosynthesis and is required for the formation of both *a*-type terminal oxidases (42). Because aa_3 is constitutively expressed regardless of culture conditions (21, 35) and because of the linked enzymatic function of CtaB and CtaA, *ctaB* is very likely transcribed with kinetics similar to those of *ctaA* or at least is

not repressed by glucose. Between the *ctaB* and *ctaC* open reading frames, there is a 240-bp intercistronic region, sufficient for a specific promoter capable of activating *ctaCDEF* transcription; however, such a promoter has not been experimentally demonstrated. It is also possible that, in the absence of a *ctaCDEF*-specific promoter, the *ctaBCDEF* genes are organized as an operon unit and that *ctaCDEF* transcription depends on the upstream promoter *ctaBp*. In this case, a glucose-sensitive regulatory element should occur in the *ctaB-ctaC* intercistronic region to allow the differential expression of *ctaB* and *ctaCDEF*.

Three components involved in CR of gene expression in *B. subtilis* have been identified (2, 11, 17, 18). A 14-bp palindromic sequence was first identified as necessary for CR of α-amylase (*amyE*) expression in *B. subtilis*, and a consensus sequence was subsequently proposed for the catabolite response element (CRE) based on a mutational analysis of this region (46). Sequences similar to the CRE consensus sequence have been demonstrated to mediate CR in a number of other genes originating from *B. subtilis*, *Bacillus megaterium*, and *Staphylococcus xylosus* (11 and references therein). The identity of the critical sequence of the CRE was further strengthened by use of point mutations in various *B. subtilis* genes, e.g., *acsA*, *acuA*, and *hutP*, leading to increased or decreased repression efficiencies (15, 47). CR of most genes containing the CRE is also affected by the *trans*-acting factors CcpA and HPr at the level of transcription initiation (11). However, a *ptsH1* strain with an alanine substitution at Ser-46 had no effect on the repression of *amyE* (44) and only partially relieved the repression of *iol* (6) and *levD* (23). A *ptsH1-crh* (*crh* encodes an HPr-like protein [12]) double mutant almost completely relieved these repressions.

Several genes are known to influence the synthesis or assembly of *a*-type cytochromes. The most striking are *strC* and *resD-resE*; *strC* mutants were first isolated as spontaneous streptomycin-resistant colonies (38) and contained only 40% of the wild-type complement of *a*-type cytochromes (24). The two-component signal transduction system *resD-resE* is a global regulator of *B. subtilis* respiration; a *resD-resE* mutant completely lacked *a*-type terminal oxidases (41). Both *strC* and *resD-resE* are required for the postexponential activation of *ctaA* (27, 41).

Here we report the transcriptional regulation of the *ctaBCDEF* gene cluster in response to growth phase, carbon sources, the catabolite repression regulators CcpA and HPr, and the *ctaA* regulator genes *strC* and *resD-resE*.

MATERIALS AND METHODS

Bacterial strains and media. The *B. subtilis* strains and plasmids used in this study are listed in Table 1. RB1 (*trpC2*) is a derivative of wild-type *B. subtilis* 168. Growth supplements and antibiotics were obtained from Sigma Chemical Company, and media were obtained from Difco Laboratories. *B. subtilis* strains were maintained on LB medium. *B. subtilis* strains containing integrative plasmids conferring resistance to chloramphenicol, tetracycline, and spectinomycin were grown on LB agar plates containing 5 μg of chloramphenicol, 5 μg of tetracycline, and 60 μg of spectinomycin per ml, respectively. *Escherichia coli* strains containing plasmids conferring resistance to ampicillin were grown on MacConkey agar plates or in LB broth containing 50 μg of ampicillin per ml. Promoter activity indicated by *lacZ* fusions in *B. subtilis* colonies was detected by spraying plates with 4-methylumbelliferyl-β-D-galactoside to identify fluorescent colonies under UV light.

For the study of *ctaB* or *ctaCDEF* gene expression in response to various carbon sources, *B. subtilis* strains were grown in Schaeffer's sporulation medium (9) containing no glucose, 0.1 or 0.5% glucose (Sigma), 20 mM acetoin (Fluka), or 20 mM malate (Sigma). The medium (liquid-to-flask volume ratio of 1:10) was inoculated with 1 ml of an overnight culture to yield an initial optical density at 600 nm of approximately 0.02, as measured with a UV-visible spectrophotometer (Pharmacia Biotech). The flasks were shaken in a 37°C water bath at 250 rpm, and culture samples were taken every 15 min in the exponential phase and every 30 or 60 min in the stationary phase. The time points chosen for the collection of cell samples were relative to T_0, the point at which the departure from exponential growth was first observed in the growth curve.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
B. subtilis		
RB1	*trpC2*	This laboratory
RB95	*strC2*	This laboratory
RB996	*trpC2 ctaA*	This laboratory
RB1263a	*trpC2 resD-resE*	M. Hulett
RB1298	RB1Ω pDIA5333	This study
RB1301	RB1263a Ω pDIA5333	This study
RB1303	RB95 Ω pDIA5333	This study
RB1305	RB996 Ω pDIA5333	This study
RB1319A	RB1 Ω pAI195	This study
RB1320	RB1 Ω pAI196	This study
RB1321	RB1 Ω pAI197	This study
RB1323	*trpC2 ccpA*::*spc*	J. Stülke
RB1325	RB1 Ω pDIA5334	This study
RB1327	*ptsGHI*::*tet*	J. Stülke
RB1330	RB1298 Ω RB1323	This study
RB1331	RB1298 Ω PB1327	This study
E. coli		
JM107	Δ(*lac-proAB*) *thi endA1 gyrA96 hsdR17 relA1* λ^- *supE44* (*F′ traD36 proAB lacI*^{q}ZΔM15)	This laboratory
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ^- *thi-1*	GIBCO BRL
Plasmids		
pDIA5333	*ctaBCD′-lacZ* Amp^r Cm^r	36
pDIA5334	*ctaB′-lacZ* Amp^r Cm^r	36
pSGMU38	*lacZ* Amp^r Cm^r 7.8 kb	J. Errington
pAI195	pSGMU38 with a 444-bp *ctaB-ctaC* fragment fused to *lacZ*	This study
pAI196	pSGMU38 with a 324-bp *ctaB-ctaC* fragment fused to *lacZ*	This study
pAI197	pSGMU38 with a 760-bp *ctaB-ctaC* fragment fused to *lacZ*	This study

In vitro DNA manipulations and bacterial cell transformation. Restriction digestion, ligation, small-scale plasmid isolation from *E. coli*, genomic DNA isolation from *B. subtilis* strains, and subcloning were performed by either standard protocols (16, 22) or by following the manufacturer's instructions for Wizard Miniprep kits (Promega) and Puregene (Gentra System, Inc.). Enzymes were obtained from the following sources: United States Biochemical Corp., Sigma, New England Biolabs, and Amersham Life Science. *B. subtilis* was transformed by the method of Piggot et al. (31); *E. coli* was transformed by the method of Hanahan (16) or by following the GIBCO BRL protocol for DH5α transformation.

Construction of integration plasmids. Integration plasmids pAI195, pAI196, and pAI197 were constructed by cloning the *B. subtilis ctaB-ctaC* fragment generated by PCR into the *Pst*I-*Hin*dIII restriction site of pSGMU38 (8). pAI195, pAI196, and pAI197 contain 444-, 324-, and 760-bp PCR products, respectively, generated by use of the primer pairs XL16 (5′-CGTACGAAGCTTCTTCTATTTA-3′) and XL19 (5′-GACTAGCTGCAGACTGCAACAACAACCACC-3′), XL24 (5′-CGTACGAAGCTTCAGGCGGCTTTACTTTTAAC-3′) and XL19, and XL16 and XL26 (5′-GACTAGCTGCAGTGTCGGTACAATCAGCTCC-3′), respectively; the underlined sequences are the engineered *Pst*I (CTGCAG) and *Hin*dIII (AAGCTT) restriction enzyme sites. Each reaction contained 100 pmol of each primer and 20 ng of linearized plasmid pAI536 (26). PCR was performed on a Perkin-Elmer Gene Amp PCR System 9600 as follows: cycle 1, 5 min at 94°C, 2 min at 50°C, and 3 min at 72°C; cycles 2 to 29, 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C; and cycle 30, 1 min at 94°C, 2 min at 50°C, and 10 min at 72°C. The *ctaBCD′-lacZ* and *ctaB′-lacZ* fusions were constructed by inserting a *Bgl*II-*Eco*RI fragment containing the first 244 codons of *ctaA*, all of *ctaB* and *ctaC*, and the first 51 codons of *ctaD* and a *Bgl*II-*Nco*I fragment encompassing the first 244 codons of *ctaA* and the first 299 codons of *ctaB*, respectively, into the multiple cloning site of pJM783 (36). The fusions were introduced into the *B. subtilis* chromosome by Campbell-type recombination events, and the integrations were confirmed by Southern blotting (data not shown).

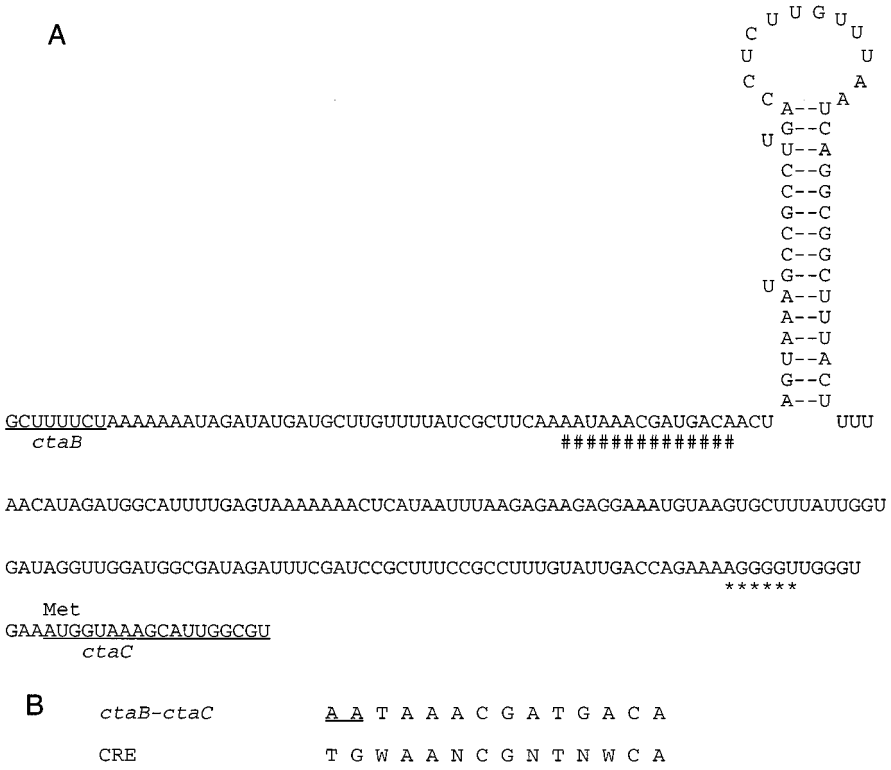

FIG. 1. Sequence of the *ctaB-ctaC* intergenic region showing the CRE and the presumed *rho*-independent terminator structure. (A) The CRE homologue (#) and the putative ribosome binding site (*) are marked, the *ctaB* and *ctaC* open reading frames are underlined, and the CtaC translational start site is labeled at AUG (methionine). Secondary structure prediction was performed by use of the Squiggles option of the Plotfold program of Genetics Computer Group sequence analysis software. (B) In the CRE consensus sequence, N represents any nucleotide, and W represents either A or T. The underlined sequence in the *ctaB-ctaC* CRE homologue represents the mismatch compared with the consensus sequence.

RNase protection assays. Total cellular RNA from *B. subtilis* was isolated with an RNaid Plus kit (Bio 101, Inc.) by following the supplier's protocol. Two DNA fragments were amplified by PCR to serve as templates for in vitro transcription of the cRNA probes. The PCR procedure was performed in the same way as for the amplification of the *ctaB-ctaC* intergenic region described above, except that different primer pairs were used. The template for the long probe (473 nucleotides), spanning from within *ctaB* to within the *ctaC* open reading frame, was amplified with primers XL1 (5′-TCTATTTCGTTGCCATGG-3′) and XL2 (5′-GGATCCTAATACGACTCACTATAGGGAGGACTGCAACAACAACCAC CR-3′); the underlined sequence is the T7 promoter. The short probe (364 nucleotides), spanning from the right arm of the stem-loop structure (see Fig. 1) to within the *ctaC* open reading frame, was amplified with primers XL1 and XL3 (5′-TCTTGTTTAATCAGGCGG-3′). With the purified PCR fragments as templates, [α-^{32}P]UTP (Dupont)-labelled cRNA probes were generated with an Ambion, Inc., MAXIscript T7/T3 in vitro transcription kit by following the supplier's protocol. The full-length cRNA probes were isolated from polyacrylamide gels and hybridized to *B. subtilis* or yeast total cellular RNA. RNase protection assays were performed as described by Driscoll and Taber (7) with an RPA kit (Ambion). Autoradiography was carried out on the dried gels with intensifying screens (Kodak) at −70°C overnight.

RT-PCR assay. Total cellular RNA from *B. subtilis* grown with or without glucose was isolated with an RNeasy Total RNA kit (Qiagen). Extracts were treated with RNase-free DNase I from the MAXIscript kit at 37°C for 1 h to destroy any possible DNA contamination. Samples were then precipitated with 0.5 M ammonium acetate and 2.5 volumes of ethanol, washed with 70% ethanol, and resuspended in 50 μl of nuclease-free water. RNA concentrations were read as the absorbance at 260 nm in a Genequant II UV spectrometer (Pharmacia Biotech). The reverse transcription (RT) reactions were performed by use of tubes containing You-Prime First-Strand Beads (Pharmacia Biotech), 4.2 μg of *B. subtilis* total cellular RNA, and 15 pmol of synthetic oligonucleotide ctaCB1 (5′-ACAGTTCTTCACCCTGCTTAGCC-3′) by following the bead supplier's protocol. H_2O was used as one negative control. The other negative control was 4.2 μg of total cellular RNA aliquots without the You-Prime First-Strand Beads. The positive control was 27 ng of plasmid pAI536 containing the *ctaABCD* sequence (26). Five-microliter aliquots of cDNA or controls were then amplified with Ready To Go PCR Beads (Pharmacia Biotech) by following the supplier's protocol. Primers ctaCB1 and ctaCF1 (5′-CAAGCCAGGAGCTGATTGTAC C-3′), each at 20 pmol, were included in all reactions and controls. Following RT-PCR, 10-μl aliquots were electrophoresed in a 1% agarose gel (FMC BioProducts) containing 0.5 μg of ethidium bromide (Life Technologies) per ml. The relative intensities of DNA bands were photographed with an AlphaImager (Alpha Innotech Corporation).

Enzymatic activity assays. The determination of β-galactosidase activities in cultures of *B. subtilis* was performed by the method of Zuber and Losick (48). One-milliliter aliquots of cells were removed from cultures, flash frozen in liquid nitrogen, and stored at −70°C overnight for enzymatic activity assays. β-Galactosidase activity was expressed in Miller units as described previously (25).

TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine) plate assays were performed as described previously (26). TMPD oxidation-positive colonies became blue within less than 5 min, whereas TMPD oxidation-negative colonies remained white.

RESULTS

Genetic and transcriptional organization of the *ctaBCDEF* locus. *ctaBCDEF* is organized as an operon-like unit in which only two regions have sufficient sequence to accommodate promoters: 5′ to *ctaB* and the 240-bp *ctaB-ctaC* intercistronic region. In the latter region (3′ to *ctaB*), a dyad symmetry sequence could be detected, and mRNA could form a stem-loop secondary structure with a ΔG of −16 kcal/mol (Fig. 1). This potential RNA structure might serve as a *rho*-independent terminator. Three nucleotides upstream of the dyad symmetry sequence, a potential CRE homologue (with the first 2 nucleotides mismatched to the consensus sequence) could be detected (Fig. 1). The second of the two mismatches occurred at one of the five critical bases of the optimal CRE sequence deduced from the *amyE* mutational analysis (46). However, CREs with weak similarity to the optimal sequence, especially at the first two bases, are still able to function in CR of *acuABC* (15) and *hut*, *gnt*, and *xyl* (47 and references therein). The

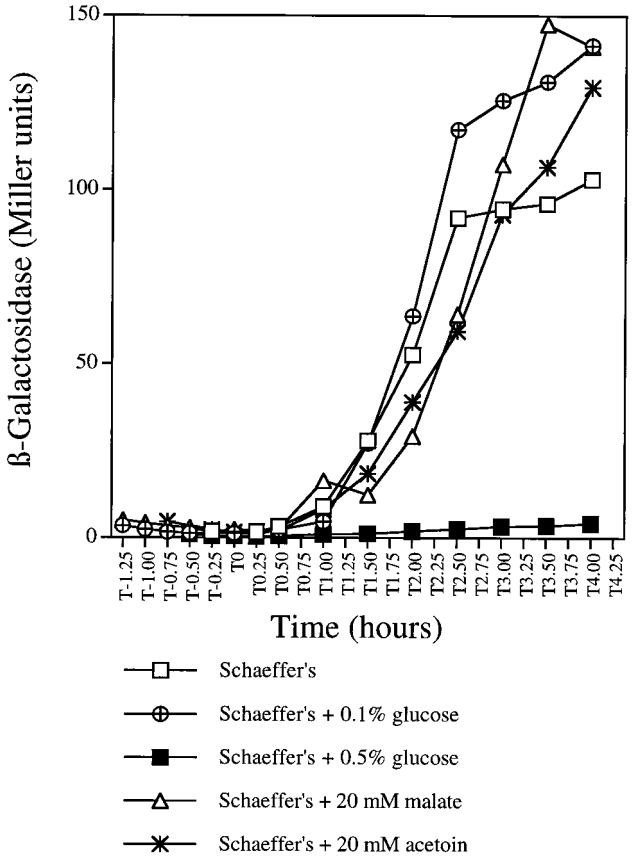

FIG. 2. Expression of *ctaBCD'-lacZ* in the presence of different carbon sources. β-Galactosidase activity in strain RB1298 (containing the *ctaBCD'-lacZ* fusion) grown in Schaeffer's medium supplemented with different carbon sources is indicated. β-Galactosidase activity was plotted against the time at which the cell samples were collected. T0 indicates the end of exponential growth.

observation that the first two base mismatches are always accompanied by less similarity to the remaining optimal CRE makes it difficult to exclude the potential role of the CRE identified in the *ctaB-ctaC* intercistronic region. Combined with the adjacent CRE, the putative stem-loop structure might play a role in the regulation of the *ctaBCDEF* operon.

Effects of glucose, glycerol, and secondary carbon sources on *ctaBCDEF* expression. Evidence for the regulation of *ctaCDEF* expression has not been reported, except for limited biochemical and spectroscopic evidence, which suggests that *B. subtilis* contains caa_3 only during growth on nonfermentable carbon sources (1, 21, 43). In order to further study the expression of *ctaCDEF* and its relationship to the expression of *ctaB*, a plasmid (pDIA5333) containing a *ctaBCD'-lacZ* transcriptional fusion (36) was integrated as a single copy at the native locus of wild-type strain RB1, and strain RB1298 was obtained. The expression of the *ctaBCDEF* genes was tested by growing strain RB1298 in Schaeffer's medium alone or supplemented with different concentrations of glucose or with 20 mM acetoin or malate. In Schaeffer's medium without additional carbon sources, *ctaBCD'*-directed β-galactosidase expression was less than 4 Miller units during the exponential phase, began to increase at the initiation of the stationary phase (T_0), and was maintained at about 100 Miller units from T_2 to T_4 (Fig. 2). In contrast, when a high concentration of glucose (0.5%) was present in the medium, β-galactosidase activity was totally lacking throughout both exponential and postexponential phases. These results demonstrate that the expression of *ctaBCDEF* is growth phase dependent and is subject to glucose repression. However, at a lower concentration of glucose (0.1%), which would be exhausted and converted into glycolytic end products by T_0, glucose repression was not present at T_1, and the *ctaBCD'*-directed expression level was eventually substantially higher (about 150 Miller units) than that in glucose-free medium (Fig. 2). This result was presumably due to the stimulatory effects of glycolytic end-product reutilization in the postexponential period. In order to test this conclusion, 20 mM acetoin (a glycolytic end product) or 20 mM malate (a TCA cycle intermediate) was added directly to the medium to mimic their production via glycolysis. Results similar to those obtained with 0.1% glucose were observed (Fig. 2). These data suggest that the transcription of *ctaBCDEF* can also be postexponentially stimulated by secondary carbon sources.

In gram-positive bacteria, the glycerol effect on catabolite-repressible genes is not well understood, but it seems to involve a mechanism different from that for glucose repression (2). Experiments similar to those shown in Fig. 2 were carried out with strain RB1298, except that the effect of growth in glycerol-supplemented Schaeffer's medium was measured. As with glucose, high concentrations of glycerol abolished postexponential-phase expression of the *ctaBCD'-lacZ* fusion, and low concentrations of glycerol activated expression to the same extent as low concentrations of glucose. The kinetics of expression in the presence of glycerol were similar to those in the presence of glucose in terms of timing. However, the response to glycerol was more sensitive than that to glucose, since the glycerol repressive effect was still present at 0.05% (5.4 mM) glycerol, while 0.1% (5.5 mM) glucose was no longer repressive (Fig. 2). It is not known whether this result is due to the inherent differences in transcriptional responses to the two substrates, to differences in uptake efficiency, or to growth phase-dependent differences in rates of substrate utilization.

Glucose-independent expression of *ctaB*. The heme A-containing cytochrome aa_3 is present when cells are grown in glucose-containing media (21). Because heme A synthesis requires CtaB, the *ctaB* gene is therefore expected not to be subject to glucose repression. In order to monitor *ctaB* expression, the β-galactosidase activity of a *ctaB'-lacZ* transcriptional fusion in strain RB1325 was measured. In Schaeffer's medium alone, *ctaB'-lacZ* was actively expressed in growing cells (Fig. 3), and expression increased postexponentially to a level comparable to that of the *ctaBCD'-lacZ* fusion (Fig. 2). In contrast to that of *ctaBCD'-lacZ*, *ctaB'-lacZ* expression was not repressed during growth in 0.5% glucose but rather reached a level (approximately 150 Miller units) similar to that of *ctaBCD'-lacZ* expression in 0.1% glucose or in the presence of the secondary carbon source acetoin or malate. This result may have been due in part to the stimulatory effect of glycolytic end products and TCA cycle intermediates, but the more important results is that, unlike transcription in the *ctaCDEF* gene cluster (as measured with the *ctaBCD'-lacZ* fusion), the expression of *ctaB* is glucose insensitive. Direct measurement of *ctaB* mRNA in RNase protection assays with a *'ctaB-ctaC'* probe (see the long probe in Fig. 5) transcribed from a PCR fragment showed accumulated *ctaB* transcripts when cultures were grown in the presence or absence of 0.5% glucose (data not shown).

***ctaCDEF* is not expressed when separated from *ctaB*.** Based on sequence analysis, two possible regulatory mechanisms could be responsible for the differential expression of *ctaCDEF* in response to various carbon sources: (i) *ctaCDEF* has a specific promoter that regulates its expression or (ii) *ctaCDEF* transcription relies on the *ctaB* promoter, and catabolite-sensitive regulation occurs in the *ctaB-ctaC* intercistronic region to

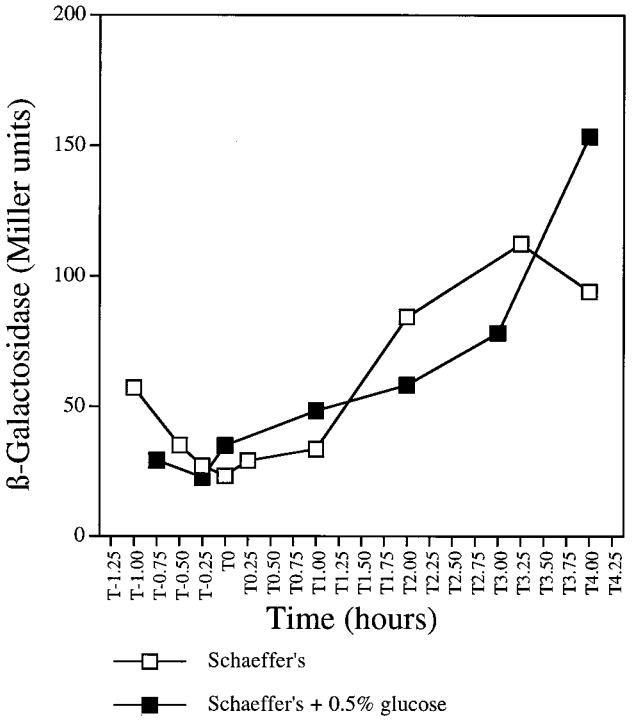

FIG. 3. Glucose effect on the expression of *ctaB′-lacZ*. *ctaB′*-directed β-galactosidase synthesis of strain RB1325 grown in Schaeffer's medium alone or supplemented with 0.5% glucose was measured and plotted as described in the legend to Fig. 2.

reduce *ctaCDEF* transcription in the presence of glucose. In order to test for the existence of a *ctaCDEF*-specific promoter, plasmids pAI195, pAI196, and pAI197 carrying chloramphenicol markers were integrated into the chromosome of strain RB1 by a single-crossover recombination event. By selection for Cm^r colonies, strains RB1319A, RB1320, and RB1321 were obtained. In these integrants (Fig. 4), the continuity of *ctaBCDEF* transcription through the *ctaB-ctaC* intercistronic region is disrupted by the chloramphenicol resistance cassette. This cassette is transcribed in an opposite sense compared to the transcription of *ctaBCDEF*, but transcription within both *ctaB* and *ctaCDEF* would not be interrupted. Gene sequences downstream from the inserted plasmid pSGMU38 were initiated from different positions in the different integrants. In strains RB1319A and RB1321, *′ctaBCDEF* was initiated from the last eight codons of *ctaB*. In strain RB1330, *′ctaCDEF* was initiated from the second half of the dyad symmetry sequence (Fig. 1 and 4). The formation of the caa_3 oxidase in the integrants would be completely dependent on a *ctaCDEF*-specific promoter within the *ctaB-ctaC* intercistronic region if such a promoter existed. Measurement of caa_3 activity in intact cells was performed with TMPD, which can serve as an artificial electron donor for cytochrome *c* oxidase caa_3 but not for quinone oxidase aa_3 (21, 29).

Table 2 shows that caa_3 was no longer present under either growth condition after *ctaCDEF* was separated from *ctaB* by the integration events. Also, upstream of the chloramphenicol selectable marker in these constructs, *ctaC′-lacZ* fusions occurred at different sites within *ctaC* open reading frames. RB1319A, RB1320, and RB1321 contain the putative first 55, 55, and 160 amino acids of CtaC, respectively, according to the sequence published by Saraste et al. (37). β-Galactosidase activity fold increases (that in Schaeffer's medium divided by that in Schaeffer's medium plus 0.5% glucose) measured with these integrants were comparable to that of RB1298 (data not shown).

The lack of a *ctaCDEF*-specific promoter was also supported by the following two experiments. First, when the intact *ctaB-ctaC* intercistronic sequence was cloned in front of promoterless *lacZ* at the *amyE* locus, as either an in-frame translational or a transcriptional fusion, no β-galactosidase activity could be detected (data not shown). However, the same translational construct has been successfully used as an expression indicator for $menp_1$, the major promoter in menaquinone biosynthesis (32). Second, when primer extension and rapid amplification of 5′ cDNA ends were carried out with primers complementary to the 5′ region of the *ctaC* open reading frame, no *ctaC′* transcriptional start sites were found. The absence of *ctaC′*-initiated transcripts was also indicated by the direct measurement of transcripts in RNase protection assays (see below).

Low-resolution mapping of the *ctaB* transcript 3′ terminus. The absence of a *ctaCDEF*-specific promoter pointed to the likelihood of catabolite-sensitive transcription termination in the *ctaB-ctaC* intercistonic region. To map the approximate 3′ ends of *ctaB* transcripts, independent RNase protection assays were performed with two cRNA probes initiating at the same nucleotide in the *ctaC* open reading frame (nucleotide 1731, according to reference 37) (Fig. 5). If a promoter exists in the *ctaB-ctaC* intercistronic region, both probes should protect a common fragment of the 5′ end of *ctaC* mRNA. However, the 3′ terminus of *ctaB* mRNA would be protected uniquely by each of the probes. Total cellular RNA was isolated from RB1 cells grown in LB medium to growth stage T_0. [^{32}P]UTP-labelled probes were hybridized to increasing amounts of *B. subtilis* or yeast RNA, and unprotected RNA was digested with RNase A and RNase T_1. Protected RNA species were only observed when the long probe was tested (Fig. 5; the 30-nucleotide protected fragment of the short probe was not visualized due to its small size). This approximately 140-nucleotide long fragment maps to the 3′ end of the stem-loop structure, indicating that *ctaB* transcripts terminate at the stem-loop structure. Neither the long probe nor the short probe was able to detect any *ctaC′* mRNA. This result confirms that no transcripts were initiated in the *ctaB-ctaC* intercistronic space. As a control, neither probe protected any fragment in yeast total cellular RNA (data not shown). Cells grown in Schaeffer's medium with or without glucose to T_0 were also tested, and *ctaB* mRNA was observed to terminate at the same site (data not shown).

Glucose effect on the transcription of *ctaCDEF*. The results described above suggested that the absence of the terminal oxidase caa_3 in exponential-phase cultures and postexponential-phase cultures in the presence of high glucose concentrations was due to down-regulation of the distal portion of *ctaBCDEF* transcripts. In order to directly measure *ctaC* transcripts, RT-PCR assays were carried out. Cellular RNA was extracted from cells grown in Schaeffer's medium with or without glucose at growth stages $T_{-0.5}$ to T_4. First-strand cDNA was reverse transcribed with primer ctaCB1 hybridized to *ctaC* mRNA (beginning at nucleotide 2360, according to reference 34), and a 338-bp *ctaC* open reading frame fragment was subsequently amplified with primer pair ctaCB1-ctaCF1 (starting t nucleotide 2022, according to reference 37) (Fig. 4). As shown in Fig. 6, in the absence of glucose, *ctaC* mRNA abundance was undetectable during the exponential phase, began to increase at T_0, and reached a maximum after T_2. When cells were grown with 0.5% glucose added to the culture, *ctaC* mRNA levels were barely detectable. DNA contamination was monitored by PCR amplification of RNA aliquots without reverse transcriptase treatment, and no contamination was ob-

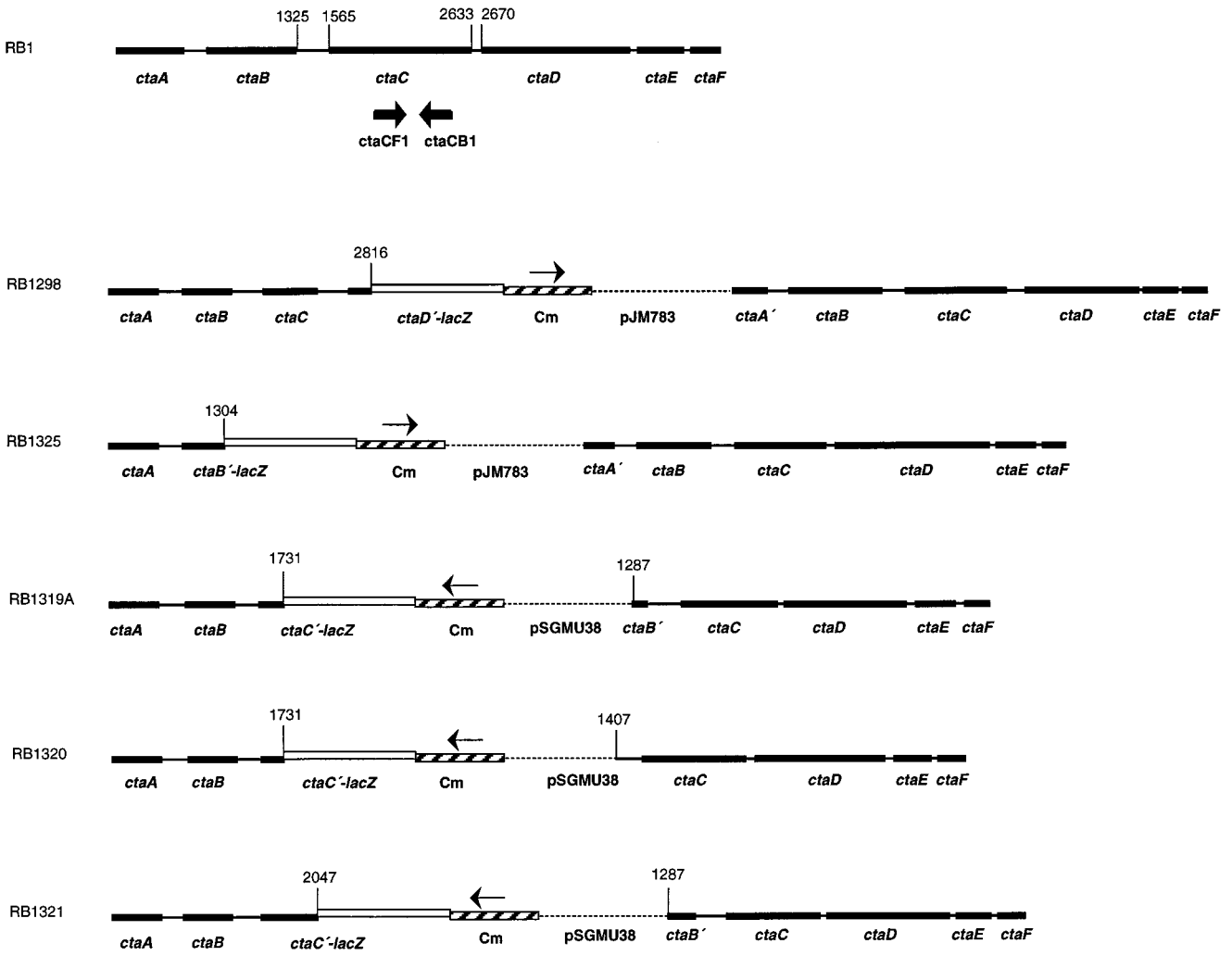

FIG. 4. Gene organization of the *cta* locus in the wild-type strain and strains carrying integrated plasmids. Genes *ctaA*, *ctaB*, and *ctaCDEF* are indicated as black boxes. Nucleotide numbering is based on the sequence determined by Saraste et al. (37). The approximate position of primer pair ctaCB1-ctaCF1 is indicated by arrows in the RB1 gene locus map. *lacZ*, chloramphenicol resistance gene (Cm), and plasmid sequences are indicated by open boxes, hatched boxes, and broken lines, respectively. The transcription orientations of Cm are represented by arrows. In strains RB1319A, RB1320, and RB1321, the initiation codon and the ribosome binding site (RBS) of the *lacZ* gene originate from the *B. subtilis spoIIA* gene (8). In strains RB1298 and RB1325, the initiation codon and the RBS of the *lacZ* gene originate from the *B. subtilis spoVG* gene (36). The maps are not drawn to scale.

served (data not shown). This result indicates that the absence of transcription through *ctaC* was associated with the down-regulation of caa_3 synthesis when the cells were grown with high concentrations of glucose. However, in the absence of glucose, *ctaBCDEF* transcripts proceeded across the *ctaB-ctaC* intercistronic region and into the *ctaCDEF* coding region.

Expression of the *ctaBCDEF* operon in *ccpA* and *ptsH* mutants. When either the *ccpA* or the *ptsH* gene (encoding HPr) is inactivated by integration of antibiotic resistance cassettes, the integrants lose the glucose repression of many catabolic genes (11, 18). To investigate the role of CcpA and HPr in CR of the *ctaBCDEF* operon, either a *ptsHI* (*ptsHI*::*tet*) or a *ccpA* (*ccpA*::*spc*) gene disruption (40) was introduced into strain RB1298 carrying the *ctaBCD'-lacZ* transcriptional fusion (Table 1). CcpA effects were assessed by measuring the β-galactosidase activity of strain RB1330 (*ccpA*::*spc ctaBCD'-lacZ*) in the presence of high concentrations of glucose. In the *ccpA* mutant background, glucose repression was partially lifted, such that *ctaBCD'-lacZ* activity was about one-third that observed in the wild type (Fig. 7). Partial relief of glucose repres-

TABLE 2. Phenotypic properties of *ctaBCDEF* transcriptional continuity disruption mutants

Strain and relevant genotype	caa_3 content[a] in the following medium:	
	Schaeffer's	Schaeffer's + glucose
RB1 (wild type)	Blue	White
RB996 Δ*ctaA*	White	White
RB1298 *ctaBCD'-lacZ* Cm^{rb} *ctaBCDEF*[c]	Blue	White
RB1319A *ctaBC'-lacZ* Cmrd *ctaCDEF*[c]	White	White
RB1320 *ctaBC'-lacZ* Cmrd *ctaCDEF*[c]	White	White
RB1321 *ctaBC'-lacZ* Cmrd *ctaCDEF*[c]	White	White

[a] caa_3 activity measured with TMPD as a substrate. Blue, caa_3 positive; white, caa_3 not detectable.

[b] The chloramphenicol resistance cassette is transcribed in the same direction as *ctaBCDEF*.

[c] Genotypes of integrants show the positions of chloramphenicol resistance cassette and *lacZ* fusion plasmid integration.

[d] The chloramphenicol resistance cassette is transcribed in the direction opposite that of *ctaBCDEF*.

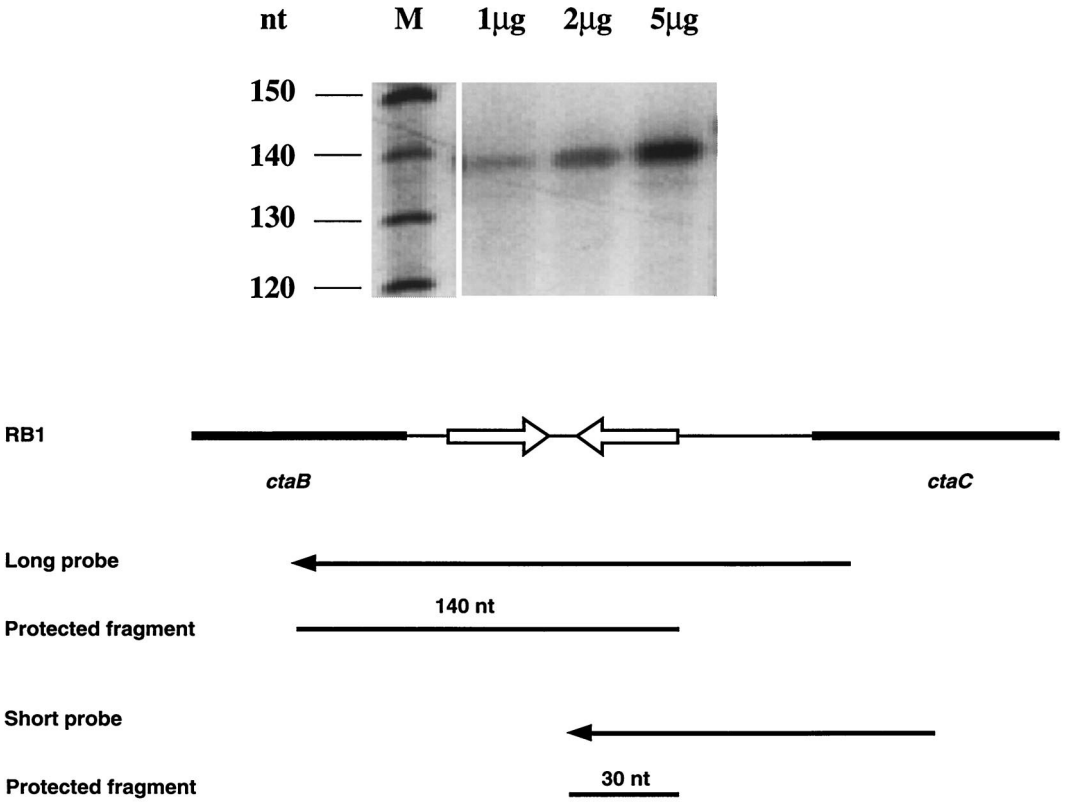

FIG. 5. Low-resolution mapping of the *ctaB* transcript 3′ terminus by RNase protection assays. In lane M, the size marker SequaMark (Research Genetics) was used by following the supplier's protocol. Sizes in nucleotides (nt) are indicated on the left. The other lanes contained 1, 2, and 5 μg of total cellular RNA isolated from cells at T_0, hybridized to the long probe, and treated with 0.025 U of RNase A and 1 U of RNase T_1. Protected hybrids were separated by electrophoresis on a 5% polyacrylamide gel containing 8 M urea. An autoradiograph of the dried gel is shown. The structures of the long probe and the short probe are shown.

sion in the *ccpA* strain could occur if CcpA were not the only effector of glucose repression (see Discussion). The strain containing the *ptsHI*::*tet* gene disruption remained completely repressible by glucose and showed normal growth phase-dependent regulation (data not shown), suggesting that HPr is not required for specific regulation of the *ctaBCDEF* operon.

Effects of *strC*, *resD-resE*, and *ctaA* mutations on *ctaBCD′-lacZ* expression. To examine the possible effects of *strC* and *resD-resE* on *ctaBCDEF* expression, the *ctaBCD′-lacZ* transcriptional fusion was integrated into strains RB95 and RB1263a, creating strains RB1303 and RB1301, respectively. β-Galactosidase activity was measured with Schaeffer's medium. Strain RB1305 (Δ*ctaA ctaBCD′-lacZ*) was also tested, since this *ctaA* deletion mutant is *a*-type cytochrome deficient (26). The expression of *ctaBCD′-lacZ* in the *resD-resE* and *ctaA* strains was reduced to about 10 to 25% that in the wild type (Fig. 7). An additional effect was observed: *strC* caused a delay of about 1.5 h in the postexponential-phase increase in *ctaBCD′-lacZ* expression. Evidently, the products of *strC* and *resD-resE* are necessary (directly or indirectly) for optimal postexponential-phase transcriptional activation of *ctaBCDEF*. The effect of *strC* or *resD-resE* mutations on the synthesis or assembly of the terminal oxidase caa_3 complex may be mediated through their negative effect on *ctaA* expression.

DISCUSSION

Under the culture conditions used in this study, it was found that genes encoding the terminal oxidase caa_3 are expressed by *B. subtilis* principally during the postexponential growth period and are subject to glucose repression. The rate of growth during the postexponential period in Schaeffer's medium is low, and we suggest that this low growth rate provides one type of signal for the expression of *ctaCDEF*. The nature of this signal is not known, but the effect has been observed during exponential growth in defined medium with succinate as a carbon

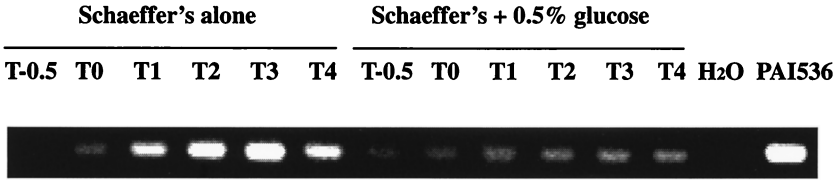

FIG. 6. Direct measurement of *ctaC* mRNA. A culture of wild-type strain RB1 was grown in Schaeffer's medium with or without glucose, and total cellular RNA was isolated at $T_{-0.5}$, T_0, T_1, T_2, T_3, and T_4. The reverse-transcribed first-strand cDNA from primer ctaCB1 was amplified by PCR with primer pair ctaCB1-ctaCF1. The location of the primers is shown in Fig. 4. The 338-bp amplified *ctaC* fragments were separated by electrophoresis on a 1% agarose gel containing 0.5 μg of ethidium bromide per ml. As controls, plasmid pAI536 and H_2O were treated as total cellular RNA was. The growth conditions and timing are indicated.

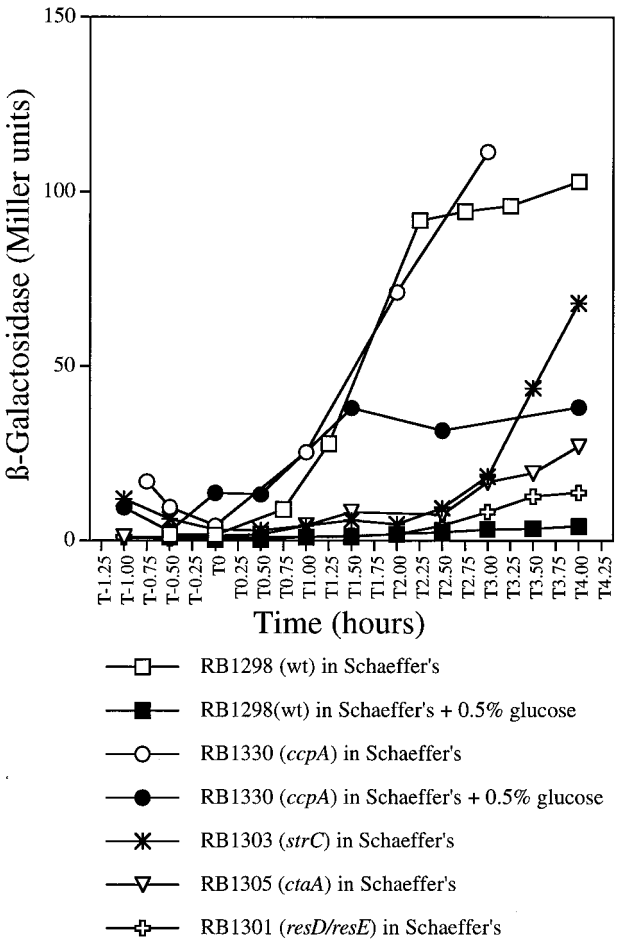

FIG. 7. Effect of *ccpA*, *strC*, *resD-resE*, and *ctaA* mutations on the expression of *ctaBCD′-lacZ*. β-Galactosidase activities of the *ctaBCD′-lacZ* fusion in strains RB1298 and RB1330 grown in Schaeffer's medium alone or supplemented with 0.5% glucose or in strains RB1301, RB1303, and RB1305 grown in Schaeffer's medium alone were measured and plotted as described in the legend to Fig. 2. wt, wild type.

source by Lauraeus et al. (21). Before the modern distinction between terminal oxidases aa_3 and caa_3 was recognized, Chaix and Petit (1) systematically studied by spectral means the influence of carbon sources on the formation of the 600-nm (aa_3) and 605-nm (caa_3) components in intact cells. The latter was present in the exponential phase only when the growth rates in defined media were low. In the current studies, exponential growth rates did not vary widely when Schaeffer's medium, which is an undefined, broth-based medium, was augmented with a variety of carbon sources. Thus, for example, the addition of malate did not result in the transcription of *ctaCDEF* during the exponential phase, because the cells were not dependent on this nonfermentable carbon source for growth.

Earlier studies from our laboratory addressed the growth-dependent regulation of respiratory chain components when *B. subtilis* cultures were grown in Schaeffer's medium. Expression of the *ctaA* gene was shown to be maximal 2 hours after the onset of the stationary phase (T_2) (27), while $menp_1$-initiated transcript formation was maximal from the exponential phase (T_{-1}) to the early stationary phase (T_1) and then declined rapidly (32). In contrast to the data for *ctaA* and $menp_1$, the data presented here show that both the terminal oxidase caa_3 genes *ctaCDEF* and the heme A biosynthesis gene *ctaB* are activated and maintained at maximal transcription levels after T_2. Overall, the transcription of *ctaA*, *ctaB*, and $menp_1$ is maintained at substantial levels after T_2, even though not all of these genes are expressed at maximal levels. A sufficient supply of the heme A prosthetic group and the reducing substrate (menaquinone) may allow the cells to switch on the caa_3 respiration branch in order to cope with limited nutrient availability during the late stationary phase.

When grown with low concentrations of glucose, *B. subtilis* reutilizes accumulated glycolytic end products after glucose is exhausted. As shown here, the level of *ctaBCDEF* transcription in the late postexponential phase was higher in the presence of a nonfermentable carbon supplement or a low concentration (0.1%) of glucose than in glucose-free Schaeffer's medium. A similar stimulatory effect was also observed for $menp_1$-initiated transcripts in the stationary phase (32). This effect on $menp_1$ was abolished in mutants blocked in secondary carbon reutilization: *acuA* (acetoin utilization) and *acsA* (acetyl coenzyme A synthesis; acetate utilization). Therefore, nonfermentable carbon sources may serve as or induce a positive signal for *B. subtilis* to coordinately regulate genes involved in energy production. A TGAAA sequence motif previously described for menaquinone biosynthesis genes (*menB* and *menE*) and for the heme A biosynthesis gene *ctaA* (7) also appears in the *ctaB-ctaC* intercistronic region. This sequence may serve as a *cis*-acting element for such regulation.

From sequence analysis alone, it appears that *ctaCDEF* may possess its own specific promoter in the *ctaB-ctaC* intercistronic region (37). However, genetic disruption experiments described in this work excluded the possibility of *ctaCDEF* being transcribed independently of *ctaB*. The downstream portion of the polycistronic transcript (*ctaBCDEF*) initiated from *ctaBp* provides information for the translation of the caa_3 subunit peptides. Another prokaryotic *ctaBCDEF* homologue is the alkaliphilic *Bacillus firmus* OF4 *cta* operon, in which *ctaCDEF* encodes pH-regulated cytochrome caa_3 (33). The *cta* gene organizations in *B. firmus* OF4 and *B. subtilis* are identical and exhibit 54% overall amino acid sequence identity. Northern blot analysis revealed a 5-kb (*ctaBCDEF*) message when the *B. firmus* OF4 total cellular RNA was probed with a *ctaB* probe, with no sign of any shorter transcripts (*ctaB*) (33). We suggest that, as with *B. firmus* OF4, *B. subtilis* initiates a polycistronic mRNA spanning *ctaB* to *ctaF*.

Many *B. subtilis* genes that respond to CR are regulated by CcpA, which binds to the CRE located in the respective promoter regions or in the 5′-terminal regions of their open reading frames (11, 18, 19). Thus, the frequency of transcription initiation is regulated. Our present data do not easily fit into such a regulator-operator model because of the lack of a *ctaCDEF*-independent promoter. However, a less common transcriptional termination-antitermination mechanism has been identified for a number of catabolic genes in *B. subtilis* (34, 39). One of the best-studied models is the *B. subtilis glpD* gene, encoding glycerol-3-phosphate (G3P) dehydrogenase (10, 14). The operon in which *glpD* is located belongs to the *glp* regulon, which is involved in the uptake and metabolism of glycerol and G3P. The expression of *glpD* is induced by G3P and repressed by glucose. An inverted repeat has been identified as a transcription terminator in the leader region of the *glpD* gene, and spontaneous mutants with deletions or insertions in the inverted repeat produce G3P dehydrogenase constitutively in the presence of glucose. Transcriptional antitermination is effected by the upstream gene product, GlpP, in conjunction with G3P. It has been suggested (14) that the GlpP protein interacts directly with the terminator to control *glpD* mRNA stability. Consequently, *glpP* mutants fail to grow on glycerol as a sole carbon and energy source (14).

A similar antitermination mechanism could also occur in *ctaCDEF* gene regulation, with the stem-loop structure in the *ctaB-ctaC* region acting as a terminator. This hypothesis leads to the speculation that antitermination also involves an interaction between a regulatory protein and the growing transcript. In the presence of glucose, the formation of this terminator could be enhanced by regulators such as CcpA. After glucose is exhausted, CcpA might not be able to stabilize the terminator, allowing the *ctaBp*-initiated transcription machinery to proceed through the dyad symmetry region into the *ctaCDEF* genes. The proximally located CRE might be recognized as an mRNA binding signal for the CcpA regulator. This model differs from the previously studied models in which the CRE is recognized as a DNA binding sequence for CcpA (18, 19). Recently, additional CcpA-like catabolite repressor proteins were reported (3, 4) and might be implicated in the residual CR of *ctaCDEF* transcription in the *ccpA*::*spc* background. Measuring *ctaCDEF* expression in *ccpB* and *ccpA-ccpB* strains (4) will assist in resolving this question.

Additional HPr-like proteins also appear to be involved in *B. subtilis* catabolite control. Recently, it was shown that, in a *B. subtilis* HPr-deficient strain, the synthesis of inositol dehydrogenase and β-xylosidase was not relieved from CR (12), as we have observed with the genes encoding terminal oxidase caa_3. In that study (12), an additional disruption in the *crh* gene, encoding an HPr-like protein, caused an almost complete loss of glucose repression. These results indicate that, in addition to CcpA and CcpB, both HPr and Crh participate in CR of certain genes in *B. subtilis*. It would be interesting to test whether caa_3 synthesis is also derepressed in a *crh* or a *crh-ptsH* double-mutation background, as is the expression of inositol dehydrogenase and β-xylosidase.

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