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Structure and Expression of the Cytochrome aa_3 Regulatory Gene *ctaA* of *Bacillus subtilis*

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Mutations that define the *ctaA* gene of *Bacillus subtilis* block cytochrome aa_3 formation and sporulation. We have recently described the isolation and initial characterization of the *ctaA* locus. Analysis of in vivo mRNA transcripts by RNase protection experiments located the 5′ and 3′ termini of the *ctaA* transcript, confirming a monocistronic structure. By using a nuclease protection assay, an increase in the abundance of steady-state *ctaA* mRNA was observed during the initiation of sporulation, followed by a decrease during subsequent stages. Transcripts originating from the *ctaA* gene were most abundant 2.0 h after the end of exponential growth. This pattern of *ctaA* mRNA accumulation was confirmed by coupling the transcription of the *ctaA* gene to *lacZ* in an integrative plasmid vector. Expression of *ctaA* was not repressed by glucose and was independent of the *spo0A* and *spo0H* (*sigH*) gene products. Postexponential expression was found to be dependent on the product of the *strC* gene. The expression of *ctaA* appears to be regulated in a growth stage-specific manner. The transcriptional start site, identified by high-resolution S1 nuclease protection experiments, was preceded by a single σ^A-dependent promoter sequence.

Bacillus subtilis responds to nutrient deprivation by undergoing a series of metabolic and morphological changes that culminate in formation of a dormant endospore (13, 14), a process that requires a normally regulated and functional system of energy metabolism (24a). Sufficient perturbation of any of these metabolic functions abolishes sporulation proficiency (24a). It is of interest to determine if changes in energy-generating processes are under the control of regulators that mediate the initiation of sporulation.

Genes for biosynthesis of the electron transfer component menaquinone (*menCD* [20]) and for several tricarboxylic acid cycle enzymes (*citB* [6], *citG* [10], and *sdh* [17]) are controlled at the level of transcription. Expression of these genes increases during the transition stage from exponential- to stationary-phase growth; however, each is influenced by somewhat different metabolic conditions. Genes encoding electron transport components, tricarboxylic acid cycle enzymes, and sporulation functions may share common regulatory features, and the metabolic context in which sporulation is initiated is in part defined by components of the respiratory chain. Significant variations in the composition and organization of the *B. subtilis* electron transport chain are observed in response to environmental conditions (5, 9, 26–28). Furthermore, there are differences between forespore and mother cell membranes in their relative cytochrome contents (28, 29).

Several genes are known to influence the synthesis or assembly of cytochrome aa_3 (27). The most striking is *strC* (25), a mutation which causes a strong defect in cytochrome aa_3 synthesis (3, 27). The reduction in cytochrome aa_3 does not appear to affect normal respiration or sporulation (3, 27). To study the regulation and assembly of the cytochrome aa_3 complex, we have initiated studies to isolate genes whose products are required for cytochrome aa_3 formation. In the accompanying paper, we reported on the isolation and DNA sequence of the *ctaA* gene (22). Of interest is the observation that *ctaA* mutants not only fail to express a functional membrane-associated cytochrome aa_3 complex but are also asporogeneous. A rare pseudorevertant, designated *sca*, was isolated which restores all defects conferred by a *ctaA* deletion mutation (22). This strongly suggests that the *ctaA* gene does not encode a structural gene for apocytochrome aa_3 or heme *a* biosynthesis but is necessary at some step in the expression, biogenesis, or both, of the oxidase complex. The block in sporulation conferred by *ctaA* mutations is independent of the effect on cytochrome aa_3 formation, as suggested by the isolation of compensatory mutations in at least one locus, *scs* (22).

Here we report on the identification of the *ctaA* promoter and transcriptional studies on the timing and regulation of its activation during the course of growth and sporulation. The *ctaA* promoter is active in vegetative cells and fully active at $T_{2.5}$. Although *ctaA* is required for sporulation and activation of *ctaA* expression occurs at the onset of sporulation, expression of *ctaA* was not prevented by mutations that block sporulation at stage 0. Glucose-containing medium did not repress expression from the *ctaA* promoter but inhibited the normal decline in activity after $T_{2.5}$. A *strC* mutant, proposed to be a regulator of cytochrome aa_3 synthesis (3), reduced the normal postexponential expression to vegetative levels. The presumptive RNA polymerase-binding region of the *ctaA* promoter displayed similarity to the consensus sequences for the σ^A-associated RNA polymerase.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this work are listed in Table 1. An isogenic strain carrying the *spo0HΔHind* null mutation (RB1016) was constructed by transformation of RB1012 to macrolide-lincosamide-streptogramin B resistance, taking advantage of the transformational linkage of *chr*::Tn*917*HU146 (85%) to *spo0HΔHind*. Macrolide-lincosamide-streptogramin B-resistant transformants were selected and screened for a Spo$^-$ phenotype. An isogenic strain carrying the *spo0A12* mutation was constructed by transforming strain RB1012 with saturating

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
B. subtilis		
RB1	*trpC2*	This laboratory
RB47	*lys-3 metB10 trpC2*	This laboratory
RB95	*strC2*	This laboratory
RB784	*pheA1 spo0HΔHind chr*::Tn*917*HU146	P. Zuber
RB874	*spo0A12 pheA1 trpC2*	BGSC[a]
RB972	RB1 Φ(*ctaA'-lacZ*) *cat*	This work
RB1012	RB47 Φ(*ctaA'-lacZ*) *cat*	This work
RB1015	RB1012 *chr*::Tn*917*HU146	This work
RB1016	RB1015 *spo0HΔHind*	This work
RB1019	*lys-3* Φ(*ctaA'-lacZ*) *cat trpC2*	This work
RB1020	RB1019 *spo0A12*	This work
RB1021	RB95 Φ(*ctaA'-lacZ*) *cat*	This work
E. coli		
JM107	Δ(*lac-proAB*) *thi endA1 gyrA96 hsdR17 relA1* λ⁻ *supE44* (F' *traD36 proAB lacI*[q]*ZΔM15*)	This laboratory
JM108	JM109 F⁻	R. Colinas
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ⁻ Δ(*lac-proAB*) [F' *traD36 proAB lacI*[q]*ZΔM15*]	M. Belfort
Plasmids		
pSGMU32	*bla*⁺ *cat*⁺ *lacZ*⁺ 7.8 kb[b]	J. Errington; 8
pBS+/−	*bla*⁺ 3.2 kb	M. Belfort

[a] BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus, Ohio.
[b] kb, Kilobases.

amounts of RB874 DNA. Met⁺ transformants were selected and screened for acquistion of the Spo⁻ phenotype by congression. *spo0A* mutants were routinely checked for protease production to confirm the presence or absence of *abr* partial suppressor mutations.

Culture media and genetic techniques. LB medium was used for routine culture of *B. subtilis* and *Escherichia coli*. Growth and sporulation in 2× nutrient sporulation (NS) broth were carried out as described by Miller et al. (20). Competent cells of *B. subtilis* were prepared and transformed as described by Piggot et al. (24). Transformations of *E. coli* strains were carried out as described by Hanahan (11). Selections for antibiotic resistances were carried out as described previously (22).

In vitro manipulation of DNA. All DNA manipulations were carried out as previously described (22). Isolation of chromosomal DNA from *B. subtilis* strains and preparative isolation of plasmid DNA from *E. coli* by the alkaline lysis procedure were as described previously (22). Restriction enzymes and DNA modification enzymes were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and used as recommended by the supplier.

Purification of RNA. Samples (80 ml) were removed during exponential growth and at hourly intervals beginning with the end of the exponential phase of growth from a 1.0-liter culture of strain RB1 cells growing in 2× NS medium. Total RNA was prepared from the samples as described by Miller et al. (20).

RNA-RNA hybridizations. All plasmid templates used in RNase protection experiments carried their respective fragments in the appropriate orientation to synthesize complementary (antisense) RNA from the T3 promoter in plasmid pBS−. Purified plasmid templates suitable for the production of in vitro transcripts were prepared by alkaline lysis by the method of Melton et al. (18). Each plasmid was linearized with *Eco*RI and used as a template for RNA synthesis by T3 RNA polymerase with a kit and protocol from Stratagene Cloning Systems, Inc. (La Jolla, Calif.). Mixtures of radiolabeled probe (500,000 cpm) and 40 μg of *B. subtilis* RNA were coprecipitated with ethanol and suspended in 30 μl of formamide hybridization buffer. The mixture was heated to 85°C for 5 min and cooled slowly to 37°C overnight. A mixture of RNase A (40 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and RNase T_1 ([Sigma] 2 μg/ml) was added and incubated at 30°C for 30 min. The nuclease-resistant hybrids were purified, denatured, and subjected to electrophoresis in 5% polyacrylamide–7 M urea sequencing gels (18). For kinetic experiments, dried gels were exposed to film at −70°C without intensifying screens. The probe was determined to be present in excess under these conditions.

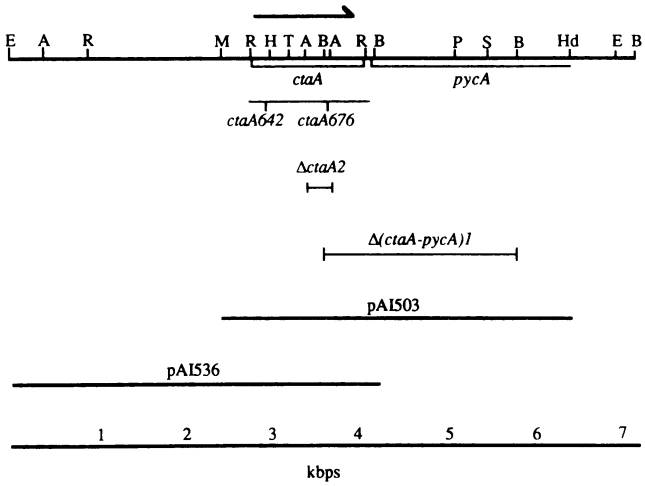

FIG. 1. Genetic and physical map of the *B. subtilis* chromosome in the region of the *ctaA* gene. The arrow above the map shows the location and orientation of the *ctaA* transcriptional unit. The positions of the various *ctaA* alleles and the plasmids representing a portion of this region are listed below the restriction map. The flanking map was determined by Southern blot hybridization. Abbreviations: Hd, *Hin*dIII; E, *Eco*RI; R, *Rsa*I; B, *Bgl*II; A, *Acc*I; T, *Taq*I; P, *Pst*I; S, *Sac*I; M, *Mbo*I; H, *Hpa*II; kbps, kilobase pairs.

DNA-RNA hybridizations. RNA was isolated from cells of strain RB1 harvested 2 h after the end of exponential growth in 2× NS broth (T_2). Single-stranded pAI558 DNA containing the sense strand of the 227-base-pair (bp) *Rsa*I-*Hpa*II fragment spanning the *ctaA* promoter and 5′ coding region was used as template DNA (Fig. 1). With primer extension, a uniformly labeled antisense strand was synthesized with modified T7 DNA polymerase (Sequenase) (4). The resultant double-stranded DNA was digested with *Eco*RI and *Hin*dIII, and the probe was separated from the vector by gel electrophoresis in a 1.0% low-melting-point agarose gel (SeaPlaque; FMC Corp., Marine Colloids Div., Rockland, Maine). The double-stranded probe fragment was purified by phenol extraction, and the radioactivity was estimated by Cerenkov counting. An additional 19 nucleotides of the pBS− polylinker sequence were present at the 5′ end, and 11 nucleotides were present at the 3′ end of the probe fragment. The conditions for hybridization and S1 nuclease digestion were as described by Miller et al. (20). Each hybridization reaction contained 40 μg of RNA and 50,000 cpm of uniformly labeled double-stranded probe. Hybridization was for 3 h at

42°C. S1 digestion was for 30 min at 37°C with 2,000 U of S1 nuclease (Boehringer Mannheim) per ml. S1 nuclease-resistant DNA fragments were denatured and electrophoresed on 5% polyacrylamide–7 M urea wedge gels (23) beside a dideoxy sequencing ladder prepared from the same DNA template used to synthesize the probe as described by Aldea et al. (2). A restriction enzyme digestion of the sequencing reaction products was performed immediately after the termination reaction by the addition of 2 U of *Pst*I. The reaction was terminated by the addition of 4 μl of dye mix and stored at −20°C. Since the pAI558 sequencing reaction products digested with *Pst*I leave three additional nucleotides with respect to the corresponding protected fragment, a correction must be made to determine the precise start point (2).

Construction of a *ctaA'-lacZ* transcriptional fusion. Plasmid pAI515 (22) was digested with *Taq*I and filled in with Klenow enzyme to generate blunt-end fragments. The 830-bp *Taq*I-*Taq*I (blunt) fragment containing the *ctaA* promoter was gel purified and inserted into the *lacZ-cat* vector pSGMU32 (8) that had been digested with *Sma*I. The resulting plasmid, pAI600, contained the 5′ end of *ctaA* gene upstream of a promoterless *lacZ* gene using the translational signals of the *B. subtilis spoIIAA* gene. The *ctaA'-lacZ* transcriptional fusion was inserted into the chromosome by single-reciprocal (Campbell-like) recombination at the *ctaA* locus by transformation of competent cells of the strain RB1 with plasmid pAI600. Transformants were selected on LB agar plates containing chloramphenicol (5 μg/ml) and 4-methylumbelliferyl β-D-galactopyranoside (20 μg/ml). In all cases, Southern blot hybridization confirmed that pAI600 had integrated correctly and in single copy at the *ctaA* locus. All subsequent growth and manipulations were carried out in the absence of chloramphenicol selective pressure to maintain the copy number at one.

Measurement of β-galactosidase activity. Bacteria were cultured for assay in highly aerated 2× NS broth at 37°C. Cells were grown overnight on LB plates at ambient temperature and used to inoculate 50 ml of 2× NS broth in 1.0-liter flasks to give an optical density at 600 nm of ~0.05. In this way, even samples taken at the early stages of log-phase growth would consist uniformly of bacteria in the vegetative phase of growth. At 30-min intervals during growth and sporulation, 1.0-ml samples were withdrawn, centrifuged, and frozen in liquid nitrogen. The specific activity of β-galactosidase was determined as described by Miller (19) with the substrate *o*-nitrophenyl-β-D-galactopyranoside. One unit of enzyme hydrolyzes 1 μmol of *o*-nitrophenyl-β-D-galactopyranoside per min per optical density unit (600 nm). In determining levels of *ctaA*-directed β-galactosidase synthesis, the background level (2 to 3 U) of *o*-nitrophenyl-β-D-galactopyranoside-hydrolyzing activity observed in isogenic parent cells lacking a gene fusion was subtracted from the levels of enzyme activity measured in the fusion-bearing strain.

RESULTS

Mapping the 5′ terminus of *ctaA* mRNA by nuclease hybridization experiments. Figure 1 shows a detailed genetic and physical map of the *ctaA* region of the *B. subtilis* chromosome. Preliminary S1 analysis with strand-specific probes localized the 5′ end of the *ctaA* transcription unit to the *Rsa*I-*Taq*I fragment of pAI532 previously designated as one end of the *ctaA* transcriptional unit by integration analysis (22; Fig. 1). The site of *ctaA* transcription initiation was mapped by RNase protection experiments with two antisense RNA probes (22; Fig. 2). One hybridization probe contained nucleotide sequences extending from the *Hpa*II site within the *ctaA* open reading frame to a *Rsa*I site preceding the *ctaA* coding sequence (probe B; Fig. 1 and 2). The second hybridization probe contained sequences extending from the *Taq*I site within the *ctaA* open reading frame to the same *Rsa*I site (probe A). Uniformly labeled antisense RNA probes were hybridized to total RNA purified from cells harvested at an early stage of sporulation (T_2). The resulting hybrids were then treated with ribonucleases A and T_1, denatured, and subjected to polyacrylamide gel electrophoresis. Hybridization to total cellular RNA predominantly protected from nuclease action probe species of about 125 bases in length for probe B and about 370 to 375 bases for probe A (Fig. 2, lanes 2 and 5). Neither sense strand probe protected a detectable RNA species (data not shown). From the sizes of the protected RNAs, we calculated that the 5′ terminus of the *ctaA* mRNA orginates from a site located approximately 25 bp upstream of the *ctaA*

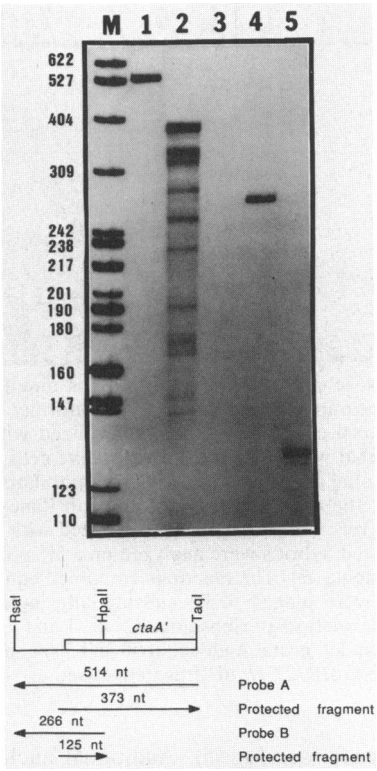

FIG. 2. Low-resolution RNase mapping of the 5′ terminus of *ctaA* mRNA. Shown at the bottom is a schematic illustration of cRNA probes and fragments protected by cellular RNA. All cRNA probes were uniformly labeled with [α-^{32}P]CTP and prepared as described in Materials and Methods. Probe A is complementary to mRNA and is 514 nucleotides (478 of *B. subtilis* and 36 nucleotides of pBS− polylinker). Probe B is complementary to mRNA and is 266 nucleotides (227 nucleotides of *B. subtilis* and 39 nucleotides of pBS− polylinker). Shown at the top is an autoradiograph with probes A and B. The radioactive probes (500,000 cpm) were hybridized with 40 μg of RNA isolated from wild-type cells harvested 2 h after the end of exponential-phase growth in 2× NS broth and treated with RNases A and T_1. Lanes: 1, probe A (500 cpm) alone; 2, probe A hybridized with *B. subtilis* RNA; 3, probe A hybridized with 40 μg of yeast RNA; 4 and 5, identical to lanes 1 and 2, except that probe B was used; M, radiolabeled single-stranded DNA size markers (pBR322 digested with *Hpa*II).

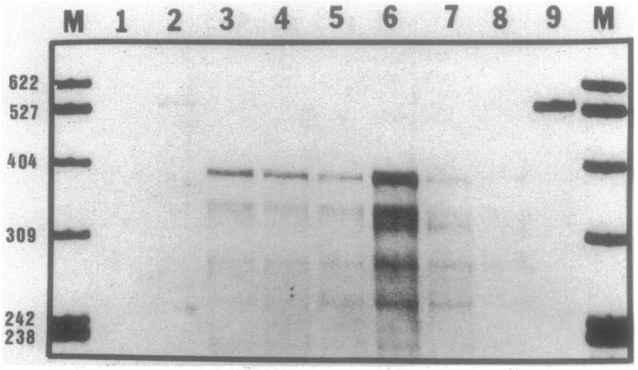

FIG. 3. RNase protection assay used for monitoring the time course of appearance of *ctaA* mRNA. Uniformly labeled cRNA probe A (500,000 cpm) (Fig. 2) was hybridized with 40 μg of *B. subtilis* RNA that was isolated from vegetative cells (lane 3), at the end of exponential growth (lane 4), or from sporulating cells at 1.0-h intervals after the end of exponential growth (lanes 5 through 8). Hybridization reaction products were digested with RNases A and T_1, and protected hybrids were analyzed on a 5% polyacrylamide–7 M urea sequencing gel. The reactions contained equal quantities of RNA, which were judged to be substantially undegraded by gel electrophoresis analysis of ribosomal RNAs. Lane 1, 40 μg of yeast RNA; lanes 2 and 9, probe A alone, at 50 and 500 cpm, respectively; M, radiolabeled pBR322 *Hpa*II fragments used as size markers.

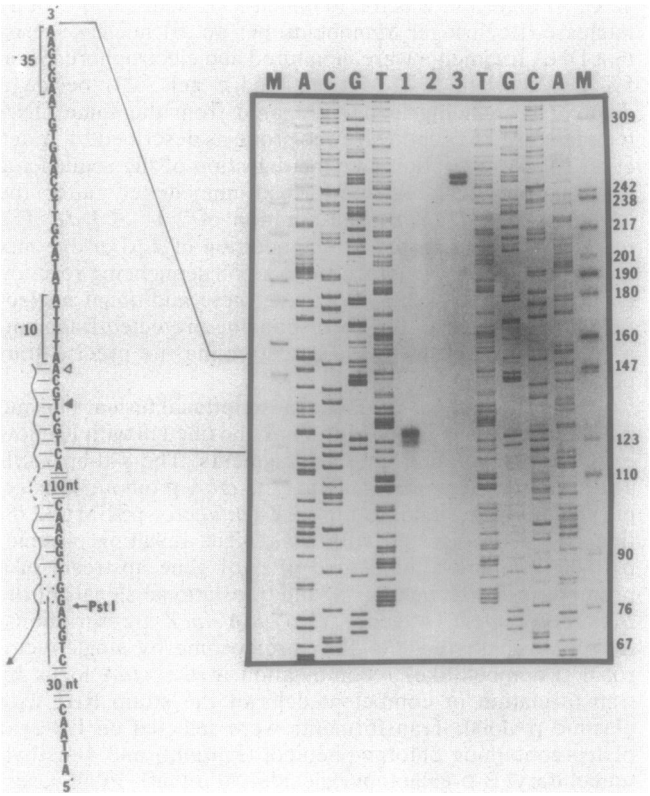

FIG. 4. High-resolution S1 nuclease mapping of the 5′ terminus of the *ctaA* mRNA. Lanes: 1, protected fragments obtained by hybridizing DNA probe (50,000 cpm) made from single-stranded pAI558 and 40 μg of T_2 RNA from strain RB1 after digestion with S1 nuclease (2,000 U/ml); 2, probe plus yeast RNA; 3, probe alone (500 cpm); A, C, G, and T, dideoxynucleotide sequencing reactions derived from the single-stranded form of pAI558 after digestion with *Pst*I; M, a pBR322 *Hpa*II ladder as size markers. The DNA sequence of the transcribed strand is shown at the left to indicate the position of the protected fragment (open arrowhead) and the −35 and −10 regions of the *ctaA* promoter. The mRNA is indicated by a wavy line to show the three nucleotide difference between the 5′ ends of the protected fragments and the *Pst*I-digested sequence reaction products. After making this correction, the position of the transcription start point is as indicated (closed arrowhead). The untreated probe DNA (lane 3) appears as two bands because of fill in of the 3′ recessed ends by T7 DNA polymerase during preparation of the probe DNA.

coding sequence (see Fig. 5). Additional nuclease-resistant signals smaller than the predominant species were detected, which we attributed to hybridization of abortive transcripts present in the RNA probe (Fig. 2, lane 2).

The use of the RNase protection hybridization assay in a time course experiment showed that steady-state transcripts originating from the *ctaA* promoter were present in vegetative cells and were most abundant 2 h after the onset of sporulation (Fig. 3). The increased *ctaA* mRNA concentration could result either from increased transcription initiation or decreased degradation of the mRNA.

High-resolution S1 nuclease mapping experiments were used to map the 5′ end of the *ctaA* transcript more precisely. RNA was isolated from sporulating cells (T_2), and a uniformly labeled double-stranded DNA probe was allowed to hybridize to the mRNA. The 5′ end of the *ctaA* mRNA was obtained by comparing the migration of the S1-resistant fragments with the migration of products of a dideoxy sequencing reaction done on the same single-stranded DNA template used to synthesize the probe (Fig. 4). The 5′ terminus was determined to be at an adenine residue approximately 28 bases upstream from the *ctaA* translation initiation codon (Fig. 4 and 5). A minor band 3 nucleotides smaller in size was occasionally observed. We suppose that *ctaA* mRNA might have a tendency to hybridize to the three adjacent bases of the plasmid polylinker sequence contained in the 5′ end of the DNA probe. The two-of-three-bp match between the *ctaA* transcript and probe polylinker DNA is moderately stable because of the G/C content in the respective region of each sequence. This interpretation is supported by the observation that changes in the temperature of the S1 nuclease digestion markedly increased (30°C) or reduced (42°C) the presence of this band (data not shown).

The DNA sequence immediately upstream of the *ctaA* gene is shown in Fig. 5. The position of the 5′ end of the mRNA is indicated with an arrow. There are sequences that resemble $E\sigma^A$ promoters upstream of the 5′ end of the *ctaA* mRNA: (−77)-TTGCCA-N_{17}-TACACT-(−49) and (−60)-TTCGCT-N_{17}-TAAAAT-(−32). The results of the S1 nuclease protection experiment suggest that the latter promoter consensus sequences are functionally active in vivo (Fig. 5). The sequences corresponding to the −10 and −35 components of the σ^A RNA polymerase binding site exhibit 3/6 and 5/6 homology, respectively, with the consensus structures for these sites (21; Fig. 5). The weak similiarity between the putative *ctaA* promoter −35 sequence and consensus for this region may be a sufficent explanation for our observation that this promoter appears to be used relatively inefficiently by $E\sigma^A$ in vivo. Whether the upstream promoter structure has any effect on sequestering $E\sigma^A$ from productively interacting with the identified *ctaA* σ^A promoter remains to be determined. No sequences corresponding to the recognition sites for minor forms of RNA polymerase were identified by computer analysis (7).

The nucleotide sequence -TTTTGTGAACAAAA- was identified upstream from the putative −35 sequence, at

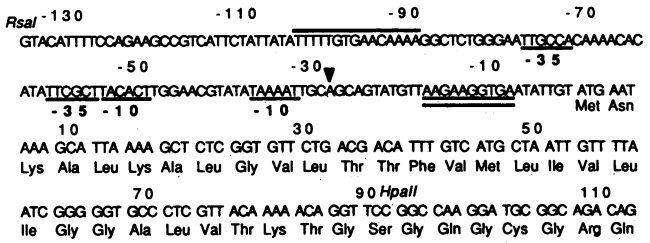

FIG. 5. The DNA sequence of the *ctaA* promoter region. The noncoding strand is shown. The first base of the *ctaA* initiation codon is numbered 0. The deduced amino acid sequence is indicated below the DNA sequence. The start site for the *ctaA* mRNA is marked with an arrowhead. Possible $E\sigma^A$ promoter −35 and −10 sequences located upstream of the start of *ctaA* mRNA are underlined. The putative ribosome-binding site is double underlined. A sequence previously identified in the *sdh*, *citG* and *menCD* promoter regions is indicated by overlining (24a). Restriction enzyme cleavage sites used for cloning and transcript mapping are indicated. The sequence shown was determined by Mueller and Taber (22).

position −96. This sequence structure has been identified previously in the promoter regions of the *citG* gene, *sdh* operon, and the *menCD* transcriptional unit (15, 20, 24a). The significance of this structure is not yet known, although it is of interest that all of these genes are involved in cell energy metabolism.

Mapping the 3′ terminus of *ctaA* mRNA by RNase protection hybridization. Based on the DNA sequence 3′ to *ctaA*, we proposed the existence of a *rho*-independent terminator immediately following the *ctaA* gene (22). The 3′ end of the *ctaA* mRNA was found at the predicted site (Fig. 6, lane 5) by RNase mapping in vivo RNA with an antisense RNA probe synthesized from a plasmid containing the 3′ end of the *ctaA* gene. We never observed a larger species of *ctaA* mRNA, suggesting that the majority of transcripts terminated at this site in vivo. The complementary sense strand RNA probe failed to detect any RNA species (Fig. 6, lane 2).

The positions of the 5′ and 3′ ends of the *ctaA* mRNAs, genetic analysis, and the DNA sequence of the region indicate that *ctaA* is the only gene in its transcriptional unit. The *ctaA* transcript stops before reaching the *pycA* gene, which lies just downstream of *ctaA* (22).

Production of β-galactosidase from *ctaA′-lacZ* fusion under sporulation conditions. To investigate further *ctaA* expression and monitor *ctaA* transcription more easily as a function of growth and sporulation, a *ctaA′-lacZ* transcriptional fusion was constructed by using the integrative transcription fusion vector pSGMU32 (8). We fused the *ctaA* promoter region to the structural gene of *lacZ* by insertion of the 800-bp *Taq*I fragment from pAI515 (22) at the *Sma*I site of pSGMU32. The resulting integrative plasmid, pAI600, was then used to transform competent cells of *B. subtilis* strain RB1, followed by selection for the plasmid-borne *cat* gene. Cm^r transformants were expected to arise by integration of pAI600 into the chromosome via Campbell recombination between the 800 bp of *B. subtilis* DNA contained on pAI600 and the *ctaA* promoter region in the chromosome. Such an integration event would leave the *ctaA* transcriptional unit intact adjacent to a single copy of pAI600.

Figure 7A shows that the *ctaA′-lacZ* fusion was active in growing cells and increased during the second hour of spore formation. The maximum level of β-galactosidase occurred between 2 and 3 h after the onset of sporulation, after which time the activity decreased. The activity of β-galactosidase increased with kinetics similiar to the levels of *ctaA* mRNA

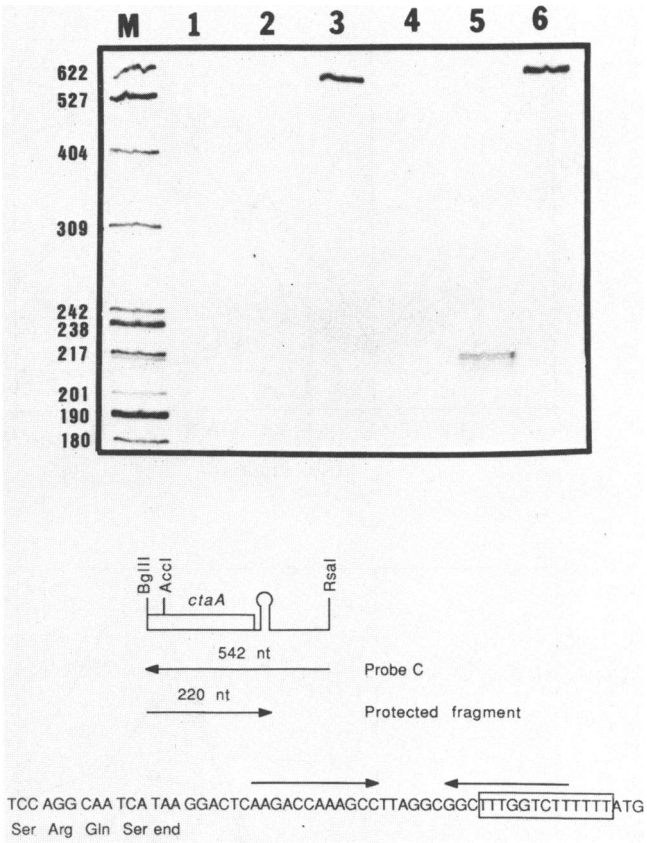

FIG. 6. Identification of the 3′ end of *ctaA* mRNA by RNase protection. The schematic diagram shows the strategy for mapping the 3′ end of *ctaA* transcripts. The 542-nucleotide (nt) antisense cRNA probe includes 485 nucleotides of *B. subtilis* and 57 nucleotides of pBS− polylinker spanning the 3′ end of the *ctaA* coding region. Probe C (500,000 cpm) or its complement (sense strand cRNA probe) was hybridized to 40 μg of *B. subtilis* or yeast RNA. Lanes: 1, sense strand probe hybridized to yeast RNA and treated with RNases A and T_1; 2, sense strand probe hybridized to *B. subtilis* RNA from T_2 cells grown in 2× NS broth and treated with RNases A and T_1; 3, sense strand probe alone (500 cpm); 4 through 6, identical to lanes 1 through 3, except that probe C (antisense cRNA probe) was used. The samples were run along with radiolabeled *Hpa*II fragments of pBR322, and the sizes shown are in base pairs (lane M). The probable locations of the 3′ ends are indicated by a box around the sequence (22), which also shows the region capable of forming a stem and loop structure (converging arrows).

observed during growth and sporulation (Fig. 3). This suggests, but does not prove, that an increase in transcription initiation from the *ctaA* promoter occurs during the second hour after the onset of the stationary-growth phase.

Rapidly metabolizable carbon sources (i.e., glucose) repress cytochrome aa_3 formation (26). To examine whether catabolite repression acts on *ctaA* expression, β-galactosidase accumulation was assayed in sporulation media containing 1.0% glucose. These results are shown in Fig. 7A, which shows that *ctaA*-driven β-galactosidase activity remained at a high level in the presence of glucose and did not decline after $T_{2.5}$, in contrast to that observed in the absence of glucose. Physiologically, these conditions inhibited the normal postexponential recovery from organic acid accumulation and sporulation as assayed after overnight growth. An identical response was observed in sporulation media containing 0.5% glucose (data not shown). This response has

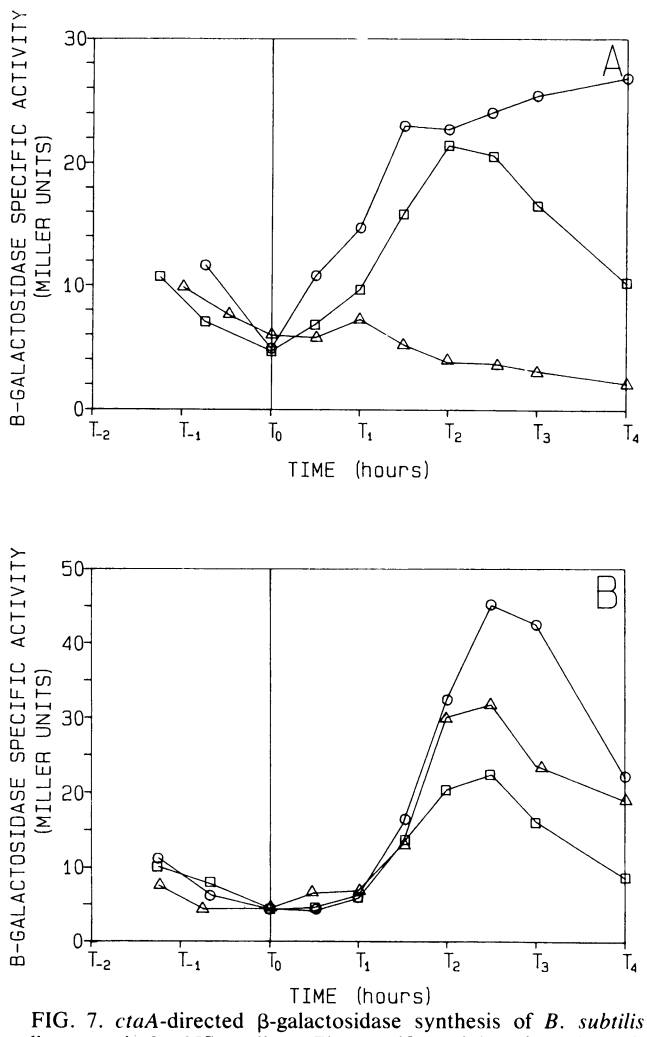

FIG. 7. *ctaA*-directed β-galactosidase synthesis of *B. subtilis* cells grown in 2× NS medium. The specific activity of β-galactosidase was measured for strains containing chromosomally inserted pAI600. T_0 represents the end of the exponential growth phase. (A) Symbols: □, RB972; ○, RB972 in 2× NS medium supplemented with 1.0% glucose; △, RB1021 (*strC2*). (B) Symbols: □, RB1015 (or RB1019) (*spo*$^+$); ○, RB1016 (*spo0HΔHind*); △, RB1020 (*spo0A12*).

also been observed in minimal salts-glucose medium with approximately 50% of the activity presented in Fig. 7A during all stages of growth (data not shown).

The *strC2* mutation reduces the level of *ctaA'-lacZ* fusion activity. *strC* mutants were isolated as spontaneous streptomycin-resistant colonies (25) and have been found to contain only 40% of the wild-type complement of cytochrome aa_3 (16, 27). The streptomycin-resistant phenotype has been correlated with the unique deficiency in cytochrome aa_3 (3, 16). To examine any possible relationship between *strC* and *ctaA*, the *ctaA'-lacZ* transcriptional fusion was integrated into strain RB95 (*strC2*), creating strain RB1021, and β-galactosidase activities were measured in 2× NS broth during growth and sporulation. *ctaA'-lacZ* activity remained at vegetative levels during postexponential growth in the *strC* mutant, i.e., the normal postexponential activation which occurred at T_1 in wild-type cells was not observed (Fig. 7A). Evidently the product of the *strC* gene, which is necessary for optimal synthesis of cytochrome aa_3, is required directly or indirectly for postexponential induction of *ctaA* expression. The effect of *strC* mutations on the synthesis or assembly of the cytochrome aa_3 complex may be mediated through its negative effect on *ctaA* expression.

Effect of early sporulation genes on expression of *ctaA*. Potential controlling factors contributing to activation of expression near the end of growth could include products of the *spo0* genes. To relate expression of *ctaA* to the beginning of sporulation, we tested *ctaA* expression in two mutants known to be blocked at the onset of sporulation. Isogenic strains carrying mutations in the *spo0A* and *spo0H* (*sigH*) loci were transformed with pAI600, with selection for Cm^r. The resulting strains were induced to sporulate in 2× NS medium, and samples were taken to measure fusion-directed β-galactosidase synthesis. Time course experiments were carried out in parallel with the corresponding isogenic *spo*$^+$ strains. Figure 7B shows that neither the *spo0A* nor the *spo0H* (*sigH*) mutation impaired transcription of *ctaA*, as judged by the use of the *ctaA'-lacZ* fusion. This implies that *ctaA* transcription is independent of the functions of the wild-type *spo0A* and *spo0H* (*sigH*) genes. This result can be interpreted to mean that *ctaA*, while essential for sporulation (22), is not a sporulation-specific gene. Alternatively, expression of *ctaA* may be dependent on signals that occur prior to early sporulation gene function, as previously suggested for *citB* transcription (6). Although the level of *ctaA* transcription differed between the *spo0*$^+$ and *spo0* mutant cells, the temporal pattern of *ctaA*-directed β-galactosidase synthesis observed in mutant and wild-type bacteria was the same (Fig. 7B). The elevated level of *ctaA* expression observed in both *spo0* backgrounds did not result in a relative increase in cytochrome aa_3 levels as determined by low-temperature spectroscopy (data not shown).

DISCUSSION

In this report we identify the *ctaA* gene promoter and present studies on its regulation. Transcription of the *ctaA* gene is initiated from a single σ^A-dependent promoter located approximately 28 nucleotides upstream of the proposed initiation codon for the *ctaA* open reading frame. The *ctaA* transcript stops at a putative *rho*-independent terminator located 30 to 40 bp downstream of the *ctaA* coding sequence. The conclusion that the *ctaA* transcript is monocistronic is consistent with the results obtained from gene disruption and complementation experiments (22). Expression of *ctaA* was evident during exponential growth and increased during the second and third hour of the stationary phase of growth. The finding that *ctaA* expression is not sensitive to catabolite repression or affected by mutations in two early regulatory genes for sporulation, which function at the earliest point in differentiation, suggests that increased expression of *ctaA* during stationary phase is not a sporulation-specific event. Moreover, a mutation (*strC*) that partially blocks cytochrome aa_3 synthesis was found to inhibit the postexponential increase in *ctaA*-directed β-galactosidase synthesis without any obvious perturbation of the sporulation process. Postexponential expression of *ctaA* may be necessary for the increase in cellular cytochrome aa_3 levels; however, regardless of the relationship of *ctaA* to cytochrome aa_3 formation, neither is an essential prerequisite for initiation and completion of the developmental process. Thus, it appears that the increase in *ctaA* expression reflects primarily the growth phase and not the developmental status of the cell. It is clear, however, that a functional *ctaA* gene product is necessary for sporulation to occur, at least when sporulation is induced by nutrient

depletion (22). We have recently discovered that induction of sporulation by the GMP synthetase inhibitor decoyinine is also blocked in a *ctaA* null mutant (J. Mueller, unpublished observation). Exactly how regulation of *ctaA* relates to initiation of sporulation remains uncertain.

We did not observe glucose-repressible expression of *ctaA* but instead found that *ctaA'-lacZ* activity remained at a maximum level in medium containing sporulation-inhibiting concentrations of glucose. A unique class of glucose stimulated genes has recently appeared in the literature (24a). The glucose enhancement phenotype of *ctaA* expression is also characteristic of the promoters for the *ctc* gene (12), some *com* (competence) genes (1), and the *menCD* (menaquinone) locus (20). Expression of these genes is induced at the start of stationary phase and is stimulated by glucose. Glucose inhibits the derepression of the tricarboxylic acid cycle in *B. subtilis*, resulting in the accumulation of organic acids produced during glycolysis. Further analysis of the *menCD* promoter has revealed that the maintenance of expression during the stationary phase is correlated with a decrease in extracellular pH. The acidic pH values are associated with high-glucose-containing media, or with strains carrying mutations in tricarboxylic acid cycle enzyme genes (K. Hill, J. Mueller, and H. Taber, submitted for publication). Whether *ctaA* expression is regulated by extracellular pH remains to be examined.

Regulation of *ctaA* bears some resemblance to that of the *menCD* operon (20). Neither is transcribed by minor forms of RNA polymerase, depends on the products of the *spo0A* or *spo0H* (*sigH*) genes, or exhibits stimulation by decoyinine (20; J. Mueller and H. Taber, unpublished data). There are differences, however, in the timing of their expression. During early postexponential growth (T_1), *menCD* promoter activity declines while *ctaA* activity increases. This may represent an indirect mechanism by which menaquinone (or an intermediate in the biosynthesis of menaquinone) down regulates cytochrome aa_3 formation (9). It is possible that the increases in menaquinone and CtaA are primarily required for the normal conversion in energy metabolism which occurs during the transition of growing cells from fast exponential growth to slow stationary-phase growth. These genes may designate a critical overlap between growth phase transition and sporulation. The molecular events that occur during this adaptation period may be prerequisite for the successful initiation of sporulation but occur prior to responses currently recognized as sporulation signals (i.e., *spo0* activity and decrease in the guanine nucleotide pools). Of interest is the observation that the promoter region of the *ctaA* gene shares a single region of sequence similarity with the *menCD* promoter. Conceivably, this region of dyad symmetry could represent a binding site for regulatory proteins to govern transcription of this class of genes. We are currently investigating whether this sequence reflects a binding site for a putative regulatory protein which is responsible for the common aspects of the regulation of these genes (glucose and growth phase activation).

A functional *strC* gene product appears to be required for postexponential transcription of *ctaA*, suggesting that the cytochrome aa_3 deficiency exhibited by *strC* strains is a consequence of decreased *ctaA* expression. The effect may be indirect; the growth of our *strC2*-containing strain is markedly perturbed in sporulation medium, with the mutant strain exiting exponential growth at a much lower cell density than the wild-type strain. This suggests that postexponential stimulation of *ctaA* expression may require a stationary-phase signal which is altered or abolished in *strC* mutants. It will be interesting to examine whether overexpression of *ctaA* can compensate for various physiological defects imposed by mutations at the *strC* locus. Further study of the *ctaA* and *strC* genes of *B. subtilis* should provide insight into the biology of cytochrome aa_3 expression and biogenesis.

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