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Suppression of *ctc* Promoter Mutations in *Bacillus subtilis*

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Transcription from the *Bacillus subtilis ctc* promoter is induced as cells enter stationary phase under conditions in which the enzymes of the tricarboxylic acid cycle are repressed. This transcription requires the presence of a secondary form of RNA polymerase, $E\sigma^B$, that is found in exponentially growing cells and in early-stationary-phase cells. Starting with a defective *ctc* promoter that had either a base substitution at position −15 or a base substitution at position −36, we were able to identify four independent second-site mutations within these mutated promoters that suppressed the effect of the original mutations and thereby restored function to the *ctc* promoter. Three of these mutated promoters had an additional base substitution(s) at positions −5, −9, or both −5 and −9 that enhanced their utilization in vivo by $E\sigma^B$, whereas one of the promoters had a single-base-pair deletion in the −15 region that placed it under a completely different form of regulation than that of the wild-type *ctc* promoter. In addition to mutations in the *ctc* promoter region, we also isolated three classes of mutants that exhibited increased *ctc* expression. The effects of the mutations in these strains were not allele specific, since they increased expression from both mutant and wild-type *ctc* promoters. One class of mutants which affected expression from the *ctc* promoter carried mutations that blocked the activity of the tricarboxylic acid cycle. A second class of mutations mapped near *cysA* and was unable to sporulate. Three-factor transformation crosses and complementation analysis indicated that one of these mutations was an allele of *spo0H*. The third class of mutations is closely linked to *dal* and may define a regulatory gene for *sigB*, the sigma B structural gene.

In exponentially growing *Bacillus subtilis*, there are several forms of RNA polymerase that differ only by their sigma subunits, the proteins that associate with the core moiety of RNA polymerase and provide its specificity for promoter recognition. The most abundant form of RNA polymerase in vegetative cells is $E\sigma^{43}$, which contains a 43-kilodalton sigma subunit. One of several other forms of RNA polymerase that are also present in exponentially growing cells contains a 30-kilodalton sigma subunit and is referred to as $E\sigma^B$. Recently, the sigma B structural gene was cloned and sequenced (1).

One gene that is transcribed by the secondary RNA polymerase $E\sigma^B$ is the *ctc* gene of *B. subtilis* (9, 18). Earlier studies indicated that the *ctc* promoter is not utilized in vitro by the major RNA polymerase in vegetative cells, $E\sigma^{43}$, but instead is used in vitro by the secondary RNA polymerase $E\sigma^B$ (9, 17). Single-base substitutions in the *ctc* promoter that decrease utilization of the promoter in vitro by $E\sigma^B$ also decrease utilization of the promoter in vivo, and numerous base substitutions in the promoter that have no effect on its utilization in vitro also have no effect in vivo (18). Moreover, mutations in *sigB*, the structural gene for $E\sigma^B$, have been shown to block transcription of *ctc* in vivo (10). These results strongly support the hypothesis that $E\sigma^B$ uses the *ctc* promoter in vivo.

$E\sigma^B$ is present in exponentially growing cells, but *ctc* is not expressed until the beginning of stationary phase under conditions in which enzymes of the tricarboxylic acid (TCA) cycle are repressed (11). Therefore, additional factors may be involved in the regulation of *ctc* transcription. To identify genes that affect utilization of the *ctc* promoter and to determine which nucleotides are important for this utilization, we have identified both intergenic and intrapromoter mutations that suppress the effects of mutations in the *ctc* promoter. The intrapromoter mutations identify two base pairs within the promoter that affect *ctc* transcription and also indicate that the spacing between the −10 and −35 regions of the *ctc* promoter is important for the proper regulation of *ctc* expression. In addition, the mutations in the strains carrying intergenic suppressors of *ctc* mutations identify several genes whose products affect *ctc* transcription.

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MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The *B. subtilis* strains and phages used are listed in Table 1. Plasmid pLC4 and its derivatives containing single point mutations in the *ctc* promoter have been described previously (18). Plasmid DNA was isolated from *B. subtilis* and *Escherichia coli* by alkaline lysis followed by centrifugation in cesium chloride-ethidium bromide density gradients (2).

Transformations. DNA was introduced into *B. subtilis* by transformation of competent cells (8). Drug-resistant transformants were selected on LB agar (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 1.5% agar) supplemented either with 5 μg of chloramphenicol, 5 μg of rifampin, or 25 μg of lincomycin per ml plus 1 μg of erythromycin per ml. Dal^+ transformants were selected on LB agar. Plasmid DNA was introduced into *E. coli* TB1 (obtained from Bethesda Research Laboratories, Inc.), JM83 (obtained from Bethesda Research Laboratories, Inc.), or HB101 (15) by the $CaCl_2$ procedure of Mandel and Higa (14) or by the $CaCl_2$/RbCl procedure of Kushner (13). Transformants were selected on LB agar containing 60 μg of ampicillin per ml.

***mutD* mutagenesis.** *E. coli* LE30 (*mutD*) (obtained from Cold Spring Harbor Laboratory) was transformed with plasmid pCR15, a pLC4 derivative containing the *ctc* promoter

TABLE 1. Bacterial strains and phages

Strain or phage	Genotype	Source
B. subtilis strains		
BS50	*spoIIG41*	W. Haldenwang (23)
JH642	*trpC2 pheA1*	J. Hoch
JH648	*trpC2 pheA1 spo0B*	J. Hoch
JH651	*trpC2 pheA1 spo0H81*	J. Hoch
IS233	*trpC2 pheA1 spo0HΔHind*	I. Smith (25)
IS1142	*trpC2 recE4 spo0HΔHin*d (pIS11a)	I. Smith (25)
QB928	*aroI906 purB33 dal-1 trpC2*	C. Truitt (24)
QB944	*purA16 cysA14 trpC2*	C. Truitt (24)
LS3	*rfm-3Y*	A. L. Sonenshein (21)
EU300	*trpC2 pheA1 socA1*	This paper
EU301	EU300 *rfm-3Y*	This paper
EU900	*aroI906 purB33 trpC2 dal*$^+$	This paper
CR101	*trpC2 pheA1 ΔsigB::erm*	This paper
Phages		
PBS1	*B. subtilis* generalized transducing phage	K. Bott
SPβ *ctc-lacZ*Δ798	SPβ*c2 del2*::Tn*917*(cat) *ctc*::*lacZ*Δ798	Igo and Losick (10)

with a base substitution at nucleotide −15 (18). The transformation mixture was allowed to grow overnight at 37°C in LB containing 60 μg of ampicillin per ml. Plasmid DNA was isolated by the method of Birnboim and Doly (2) and was used to transform *B. subtilis* JH642 to chloramphenicol resistance. Transformants were selected on LB containing 5 μg of chloramphenicol per ml and were screened for *xylE*-producing colonies. Plasmid DNA from yellow colonies was isolated by alkline lysis and was introduced into JH642 to confirm that the mutation allowing *xylE* expression was plasmid borne. The *Eco*RI-*Hin*dIII fragments containing the *ctc* promoters from these mutant plasmids were removed and ligated between the *Eco*RI and *Hin*dIII sites of M13mp9 for nucleotide sequencing.

Hydroxylamine mutagenesis. Plasmids pCR36 (18) and pCR15 (18) were digested with *Hin*dIII and *Eco*RI, and the 143-base-pair DNA fragments containing the *ctc* promoter were isolated from a 6% polyacrylamide gel (16). Mutagenesis of these fragments was carried out at 73°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) that contained 0.2 μg of DNA and 50 mM hydroxylamine in a total volume of 50 μl. The hydroxylamine was made as a 2 M stock in 0.1 M sodium pyrophosphate–2 mM NaCl (pH 6.0) and was diluted to 0.1 M in 0.1× SSC before it was added to the reaction. After 30 min, 25 μl of the reaction mixture was stopped by ethanol precipitation, whereas the remainder was precipitated after 2 h of hydroxylamine treatment. The mutagenized fragments were ligated to pLC4 that had been digested with *Hin*dIII and *Eco*RI, and the ligation was used to transform *E. coli* HB101 to ampicillin resistance. The transformation mixture was grown overnight at 37°C in 4 ml of LB containing 60 μg of ampicillin per ml, and plasmid DNA was isolated by alkaline lysis (2). The plasmids were used to transform *B. subtilis* JH642 to chloramphenicol resistance. Transformants were selected on LB containing 5 μg of chloramphenicol per ml and then were screened for *xylE* production by spraying the colonies with 0.5 M catechol. Plasmid DNA was isolated from the yellow *xylE*-producing colonies and was analyzed as described above for the mutants isolated after LE30 mutagenesis.

Construction of CR101. The erythromycin resistance gene (*erm*) from plasmid pTV39, a derivative of pTV8 (27), was excised as a *Bam*HI-*Sal*I fragment and was ligated to pUC18 which had been digested with *Bam*HI and *Sal*I (12). The *erm* gene was then excised as an *Eco*RI-*Hin*dIII fragment, and the ends were made blunt with S1 nuclease. Plasmid pML11, which contains the *B. subtilis sigB* gene, was digested with *Eco*RV and *Stu*I to remove 211 base pairs of the *sigB*-coding sequence. The deleted bases were replaced with the *erm* gene. This new plasmid was linearized with *Sca*I and transformed into *B. subtilis* JH642. Transformants were selected on LB containing 25 μg of lincomycin and 1 μg of erythromycin per ml, and insertion of *sigB*::*erm* into the chromosome was verified by Southern hybridization (22) with the *sigB*::*erm* plasmid as a probe. The probe was labeled with [α-^{32}P]dATP by the method of Feinberg and Vogelstein (7).

DNA sequencing. The *ctc* promoters carrying second-site base substitutions were removed from pLC4 by digestion with *Eco*RI and *Hin*dIII and were then ligated between the *Hin*dIII and *Eco*RI sites of M13mp9. Bacteriophages were propagated in the *E. coli* host strain 71.18 (26). Nucleotide sequences were determined by the dideoxy chain termination method, using [α-^{32}P]dATP. The primer was no. 1200, obtained from New England BioLabs, Inc.

Transductions. PBS1 transducing phage was prepared by three successive passages through the donor strain by a plate lysate procedure. When donor cells became motile, 0.3 ml of cells were mixed with different dilutions of a PBS1 phage stock in 0.75% soft agar, and the mixture was poured onto fresh tryptose blood agar base plates (Difco Laboratories). After overnight incubation at 37°C, 3 ml of antibiotic medium 3 (Difco) was added to plates that showed confluent lysis and the plates were refrigerated for 1 h. The antibiotic medium 3 and top agar were scraped off the plates and centrifuged at 10,000 × *g* for 15 min at 4°C to pellet the debris. Phage stocks were treated with 20 μg of DNase per ml in the presence of 5 mM $MgSO_4$ and filter sterilized. PBS1 transductions were performed by mixing motile recipient cells with phage and incubating at 37°C for 20 min. Cells were centrifuged and washed with DSG [2× Davis salts (Difco) containing 0.5% glucose and 2.5 mM $MgSO_4$]. After centrifugation, cells were suspended in DSG and plated on the appropriate selective agar plates (5).

SPβ transductions were performed by mixing host cells and phage and incubating at 37°C (11). After 15 min, LB containing 0.5 μg of chloramphenicol per ml was added and the cells were aerated for 90 min at 37°C. Transductants were selected by plating dilutions on LB agar containing 5 μg of chloramphenicol per ml.

Assay of $CatO_2$ase and β-galactosidase. Catechol 2,3-diox-

ygenase ($CatO_2$ase) activities were measured as described previously (18). For β-galactosidase assays, 150 ml of LB broth containing 5 μg of chloramphenicol per ml, 0.5% glucose, and 0.05% glycine was inoculated with cells to an initial optical density of 0.05 to 0.10 at 550 nm. Cells were shaken at 37°C and 200 rpm, and the optical density at 550 nm was monitored hourly. Starting between the middle of the exponential growth phase (mid-log) and t_0 (the end of exponential growth phase), 5-ml samples were collected hourly and centrifuged. The cells were washed with 1 ml of 50 mM Tris hydrochloride (pH 8.0) and centrifuged, and the cell pellets were frozen overnight at −20°C. Pellets were suspended in 1 ml of Z buffer (16) containing 20 mM sodium EDTA (pH 8.0) and 1 mg of lysozyme per ml and incubated for 20 min at 37°C. After addition of 10 μl of 10% Triton X-100, cells were placed on ice for 15 min. Extracts were centrifuged for 5 min at 10,000 rpm and 4°C to remove cellular debris. β-Galactosidase activities were determined spectrophotometrically (16) at 28°C in a total volume of 3 ml which contained 25 to 250 μl of extract, 2.15 to 2.38 ml of Z buffer and 0.6 ml of 4 mg of ONPG (*o*-nitrophenyl-β-D-galactopyranoside) per ml in 0.1 M phosphate buffer (pH 7.0). The increase in A_{420} was monitored over time. One unit corresponds to the formation of 1 nmol of *o*-nitrophenol per min at 28°C. Protein concentrations were measured by the Bio-Rad assay with bovine serum albumin as the standard. Production of β-galactosidase by colonies was monitored on LB agar supplemented with 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml.

Assay of chloramphenicol acetyl transferase (CAT). CAT activity was determined by using the cultures grown for $CatO_2$ase assays. At $t_{1\text{-}2}$ and $t_{2\text{-}3}$ (see Table 2, footnote *c*), 15- to 50-ml samples were isolated and centrifuged. Cells were washed with 3 to 10 ml of 50 mM Tris hydrochloride–1 M NaCl (pH 8.0), and the pellets were frozen overnight at −20°C. Extracts were prepared by the method of Truitt et al. (24). CAT activity was measured spectrophotometrically (20) at 37°C in a total volume of 3 ml which contained 3 to 5 μl of extract, 100 mM Tris hydrochloride (pH 8.0), 0.1 mM acetyl coenzyme A, 1.2 mg of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and 0.1 mM chloramphenicol. One unit corresponds to the acetylation of 1 μmol of chloramphenicol per min at 37°C. Protein concentrations were determined as described for β-galactosidase assays.

Determination of sporulation efficiency. 10 ml of DSM (19) was inoculated with a single colony of the strain to be tested, and cells were shaken for 20 to 24 h at 37°C. Dilutions were plated on LB agar before and after heating 1 ml of culture at 80°C for 10 min.

Nitrosoguanidine mutagenesis. *B. subtilis* ZB278 cells were incubated overnight on LB agar. Cells from the plate were then inoculated into two 250-ml Klett flasks containing 25 ml of PAB medium plus methionine and tryptophan and were grown to a Klett value of 70 to 75 at 37°C. The cells were then harvested and suspended in 25 ml of MG. Half of the cells were suspended in MG containing approximately 100 μg of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) per ml, whereas the other half were suspended in regular MG as a control. These cells were then incubated at 37°C for 30 min in a shaking water bath. After the 30-min incubation, the cells were pelleted and suspended in 25 ml of MG to remove the NTG. The cells were pelleted again, suspended in 25 ml of LB containing 15% glycerol, and stored at −70°C (modified from S. Zahler, personal communication). *B. subtilis* containing pCR derivatives was mutagenized by the procedure of Carlton and Brown (3).

RESULTS

Intragenic suppressors of *ctc* promoter mutations. We previously described the multicopy plasmid pLC4 into which the wild-type *ctc* promoter and mutant derivatives of the *ctc* promoter had been cloned to produce transcriptional (operon) fusions so that these promoters directed transcription of *xylE*, the structural gene for $CatO_2$ase (18). In *B. subtilis* JH642 containing the pLC4 derivative that carried the wild-type *ctc* promoter (pCR31), *xylE* was expressed at a high level (30 mU of $CatO_2$ase per mg of protein) when the cells entered stationary phase (18). In contrast, *B. subtilis* JH642 cells that contained plasmid pCR15, which differed from pCR31 only by a single-base transition at position −15 of the *ctc* promoter, expressed *xylE* only at low levels (<1 mU of $CatO_2$ase per mg of protein) (18). Another plasmid, pCR36, differed from pCR31 by a transition at position −36 of the *ctc* promoter and also produced only low levels of $CatO_2$ase (18). Colonies that expressed *xylE* could be identified on plates by their yellow appearance after they were sprayed with an aqueous solution of catechol. Colonies that contained pCR15 remained white after the application of catechol, whereas those containing pCR36 were pale yellow after catechol treatment (18).

To identify the nucleotides important for transcription from the *ctc* promoter, we generated second-site mutations in the weak *ctc* promoters and screened for the abilities of these mutated promoters to direct *xylE* expression in *B. subtilis* JH642. After mutagenesis of pCR15 by passage in *E. coli* LE30 (*mutD*) and transformation of *B. subtilis* JH642 with the mutagenized plasmids, 1 of 500 colonies screened was yellow when sprayed with catechol. Plasmid DNA was isolated from this colony, and the *ctc* promoter was cloned into M13mp9 for DNA sequencing. The *ctc* promoter from this plasmid was found to contain the original transition at position −15 plus an additional transition at position −9. To ensure that the −9 transition and not another mutation on the plasmid was responsible for suppressing the −15 transition, the *ctc* promoter was removed from M13mp9, recloned between the *Eco*RI and *Hin*dIII sites of pLC4, and transformed into *B. subtilis* JH642. Because the transformants were yellow when sprayed with catechol, we concluded that the transition at nucleotide −9 suppressed the effect of a transition at position −15 of the *ctc* promoter. This mutant promoter was designated 15M6, and the pLC4 derivative containing it was designated pCR15M6.

Additional suppressor mutations were generated in the mutant *ctc* promoter by hydroxylamine mutagenesis. After in vitro mutagenesis of pCR15, the promoters were cloned between the *Eco*RI and *Hin*dIII sites of pLC4, amplified in *E. coli* HB101, and transformed into *B. subtilis* JH642. After spraying the plates with catechol, 5 of 1,000 colonies containing mutagenized promoters from pCR15 were yellow. In addition to the original −15 transition, two of the mutant promoters had transitions at nucleotide −9, one had a transition at position −5, and two had transitions at both −5 and −9 (Fig. 1). These results indicated that transitions at position −5 or −9, or both, could suppress the effects of a transition at position −15 of the *ctc* promoter. These new *ctc* promoters containing the −15 substitution plus substitutions at −5 or both −5 and −9 were designated 15M12 and 15M11, respectively, and the pLC4 derivatives containing these promoters were designated pCR15M12 and pCR15M11, respectively.

Expression of *xylE* from promoters 15M6, 15M11, and 15M12. To quantitate the amount of transcription occurring

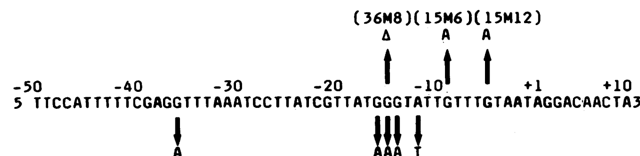

FIG. 1. Structure of the *ctc* promoter region. Shown is the nontranscribed strand of the *ctc* promoter region. Transcription proceeds from left to right, with the start point of transcription indicated as nucleotide +1. Beneath the sequence are indicated the base changes in the mutant promoters that were used to isolate the intragenic and intrapromoter suppressor mutations. Above the sequence are indicated the base changes that occurred in the intrapromoter suppressor mutations. Promoter 15M6 has transitions at nucleotides −15 and −9, 15M11 has transitions at positions −15, −9 and −5, 15M12 has transitions at nucleotides −15 and −5, and promoter 36M8 has a deletion at one of the three G residues in the −15 region.

from promoters 15M6, 15M11, and 15M12, the pLC4 derivatives containing each promoter were transformed into *B. subtilis* JH642 and $CatO_2$ase synthesis was monitored hourly after inoculation into LB-glucose-chloramphenicol medium. *xylE* expression was induced from all the mutant *ctc* promoters at the beginning of stationary phase, the time when transcription was induced from the wild-type *ctc* promoter. The promoter with the transition at −9 was utilized fivefold more efficiently than was the parental *ctc* promoter with a transition at −15, whereas the promoter with the transition at −5 was utilized threefold more efficiently (Table 2). In addition, the double mutant with transitions at both −5 and −9 increased utilization of the parental promoter 17-fold (Table 2). These results cannot be accounted for by alterations in plasmid copy number, since expression of the plasmid-encoded CAT gene was similar for all pLC4 derivatives in each host background (data not shown). Furthermore, the observation that promoters 15M6, 15M11, and 15M12 had low levels of activity in CR101 (a strain in which part of the sigma B structural gene had been deleted) indicated that at least 82% of the transcription from these pseudorevertant promoters was due to $E\sigma^B$ and that the additional base changes in these promoters acted by allowing $E\sigma^B$ to utilize a *ctc* promoter with a transition at the −15 position (Table 2).

A change in regulation of the *ctc* promoter. We also identified a mutation that suppressed the effects of a transition at the −36 position of the *ctc* promoter. The mutant promoter from pCR36 was isolated and treated in vitro with hydroxylamine. These promoters were cloned into pLC4, and the plasmids were amplified and screened for *xylE* activity as described above. Of 1,000 colonies screened, 3 became yellow when sprayed with catechol. The promoters from these plasmids were cloned into M13mp9, and their nucleotide sequences were determined. All three mutant promoters contained the original −36 transition as well as a deletion of one of three G residues centered at the −15 position (Fig. 1). Although these may not have been independent isolates, this result indicated that a deletion of a G in the −15 region was able to suppress the effect of a transition at the −36 position of the *ctc* promoter. This mutant promoter was designated 36M8, and the pLC4 derivative containing it was designated pCR36M8.

When the cells were grown in LB-glucose-chloramphenicol medium, the promoter in plasmid pCR36M8, which contained a single-base-pair deletion in the −15 region as well as the original −36 transition, directed three times less $CatO_2$ase synthesis than did the parental *ctc* promoter with only a −36 transition (Table 2; Fig. 2). This result was not expected, since pCR36M8 appeared to produce more $CatO_2$ase than did pCR36 when colonies were sprayed with catechol. Because the primary difference between the plate assays and the liquid assays was that the liquid medium was supplemented with 0.5% glucose, it was possible that glucose repressed transcription from promoter 36M8. To address this question, cultures grown in LB-chloramphenicol medium in the absence of glucose were assayed for $CatO_2$ase production. Under these conditions, pCR31 and pCR36 produced approximately 10% of the $CatO_2$ase activity seen when the cultures were grown in the presence of glucose (Table 3). In contrast, the expression of *xylE* from pCR36M8 increased fivefold over that observed when the cultures were grown in the presence of glucose (Table 3; Fig. 2). Moreover, the $CatO_2$ase activity from pCR36M8 in *B. subtilis* CR101, which did not produce $E\sigma^B$, did not differ significantly from that in JH642 (Tables 2 and 3), implying that most of the transcription from promoter 36M8 was not due to $E\sigma^B$. Because the *ctc* promoter in pCR36M8 did not appear to be transcribed by $E\sigma^B$ and was not utilized in the presence of glucose, the deletion of one of three G's centered at −15 appeared to have placed this promoter under a different form of regulation than that of the wild-type *ctc* promoter.

To determine what factors were necessary for expression from the 36M8 promoter, we introduced pCR36M8, pCR36, and pCR31 into strains that were deficient in various regulatory functions. These strains were grown in LB-chloramphenicol medium in the absence of glucose, and their levels of *xylE* expression were determined. Many genes in *B. subtilis* that are repressed by glucose and that are induced as the cells enter stationary phase are under sporulation control. 36M8 had not come under any known sporulation control, since its utilization did not depend on the gene products of either *spo0H* or *spo0B*, which control the expression of many genes that are essential for sporulation (Table 3). The 36M8 promoter was also not dependent on the

TABLE 2. Effect of *sigB* on *xylE* activities from mutant *ctc* promoters in media with glucose

Strain	Plasmid[a]	*ctc* promoter[b]	$CatO_2$ase sp act (mU/mg)[c]		Activity ratio[d]
			t_{1-2}	t_{2-3}	
JH642	pCR31	WT	25.8	32.8	1.00
CR101	pCR31	WT	<0.1	<0.1	<0.01
JH642	pCR15	−15	0.5	1.0	0.02
CR101	pCR15	−15	<0.1	<0.1	<0.01
JH642	pCR36	−36	8.3	12.7	0.32
CR101	pCR36	−36	<0.1	<0.1	<0.01
JH642	pCR15M6	−15, −9	2.8	4.5	0.11
CR101	pCR15M6	−15, −9	0.6	0.8	0.02
JH642	pCR15M11	−15, −9, −5	8.9	12.4	0.34
CR101	pCR15M11	−15, −9, −5	1.6	3.0	0.06
JH642	pCR15M12	−15, −5	1.9	2.6	0.07
CR101	pCR15M12	−15, −5	0.1	0.1	<0.01
JH642	pCR36M8	−36, ΔG	3.1	3.8	0.12
CR101	pCR36M8	−36, ΔG	2.0	2.8	0.08

[a] Plasmid that was added to strain in first column.
[b] Indicates wild-type (WT) *ctc* promoter, *ctc* promoters with transitions at −36, −15, −9, or −5, or *ctc* promoter with a deletion (Δ) of one of three G's centered at −15.
[c] Average from two experiments. The cells were grown in LB-glucose-chloramphenicol medium. The first sample (t_{1-2}) was taken within the first hour of stationary phase, and the second sample (t_{2-3}) was taken 1 h later.
[d] Ratio of $CatO_2$ase activity at t_{1-2} for the indicated strain divided by the activity of $CatO_2$ase from JH642(pCR31) at t_{1-2}.

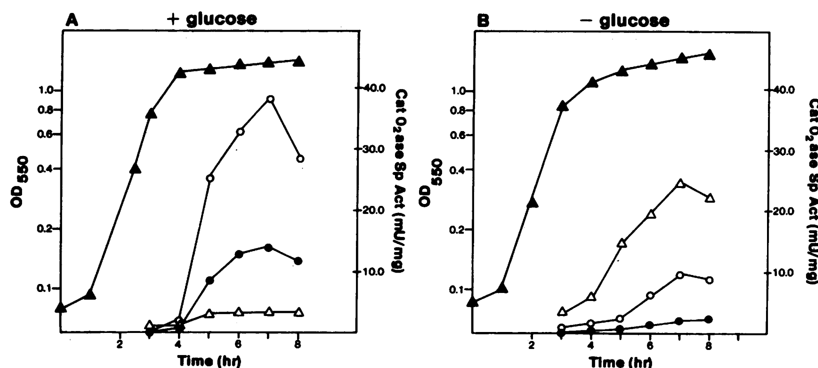

FIG. 2. Time course of *ctc-xylE* induction. Shown are the specific activities of $CatO_2$ase from JH642 containing different pLC4 derivatives. The cells were grown in LB-chloramphenicol medium in the presence (A) or absence (B) of glucose, and growth was measured by optical density at 550 nm (OD_{550}) (▲). The pLC4 derivative pCR31 (○) contains the wild-type *ctc* promoter, pCR36 (●) contains the *ctc* promoter with a transition at position −36, and pCR36M8 (△) contains a *ctc* promoter with a transition at nucleotide −36 as well as a single-base-pair deletion in the −15 region. Sp Act, Specific activity.

sporulation-induced sigma factor σ^E for its utilization, since pCR36M8 expressed *xylE* in a strain deficient in σ^E (Table 3). Changes in plasmid copy number could not account for the levels of $CatO_2$ase activity seen, since the plasmid-borne CAT gene was expressed at similar levels from pCR31, pCR36, and pCR36M8 in each host background and under each condition used for the $CatO_2$ase assays (data not shown). Therefore, the deletion in the 36M8 promoter placed the transcription from this promoter under a different form of regulation, in response to glucose. Moreover, we conclude that the spacing between the −10 and −35 regions of the CTC promoter is important for the recognition of this promoter by $E\sigma^B$.

Suppressor mutation at *spo0H*. Recently, we used enhanced expression of *xylE* from the mutant *ctc* promoter in pCR15 to identify a spontaneous intergenic suppressor of *ctc* promoter mutations, *socB1* (10). The *socB1* mutation was mapped to a gene that is adjacent and downstream from *sigB*. This mutation is thought to affect *sigB* activity or expression (6, 10). We next attempted to isolate a mutant with an alteration at a locus other than *socB1* that would suppress the effect of a mutation in the *ctc* promoter. After treatment with nitrosoguanidine (2), strain JH642 that contained pCR15 was plated on LB-chloramphenicol agar and sprayed with catechol. A pale yellow colony was isolated and designated EU300. The mutation in EU300 that caused the increased expression of *xylE* from pCR15 was shown to be located on the chromosome by curing the strain of the plasmid and retransformation with pCR15. The expression of *xylE* from pCR15 was about twofold greater in EU300 than in the parental strain (Table 4), whereas expression of *xylE* from pCR31 was about sixfold greater in EU300 than in the parental strain (Table 4). The increased expression of *xylE* in EU300 seemed to be due to increased transcription from the *ctc* promoter, since the single-base substitution in the *ctc* promoter of pCR15 reduced the level of *xylE* expression in EU300 when that level was compared with the level of *xylE* expression in EU300 from pCR31, the wild-type *ctc* promoter (Table 4). The mutation in EU300 also increased expression from other weakened *ctc* promoters (pCR12 and

TABLE 3. *xylE* activities from pCR36M8 in media without glucose

Strain	Plasmid[a]	Relevant host mutation	*ctc* promoter[b]	$CatO_2$ase sp act (mU/mg)[c]		Activity ratio[d]
				t_{1-2}	t_{2-3}	
JH642	pCR31	WT	WT	2.6	6.2	1.00
	pCR36	WT	−36	0.5	1.4	0.19
	pCR36M8	WT	−36, ΔG	15.2	19.5	5.8
CR101	pCR31	*sigB*::*erm*	WT	0.2	1.0	0.08
	pCR36	*sigB*::*erm*	−36	0.6	0.7	0.23
	pCR36M8	*sigB*::*erm*	−36, ΔG	23.6	29.5	9.08
IS233	pCR31	*spo0H*	WT	80.4	103.3	30.9
	pCR36	*spo0H*	−36	13.8	17.2	5.3
	pCR36M8	*spo0H*	−36, ΔG	19.0	48.5	7.3
JH648	pCR31	*spo0B*	WT	35.6	48.2	13.7
	pCR36	*spo0B*	−36	8.0	9.8	3.08
	pCR36M8	*spo0B*	−36, ΔG	17.0	32.6	6.54
BS50	pCR31	*spoIIG41*	WT	2.2	3.5	0.85
	pCR36	*spoIIG41*	−36	0.6	1.4	0.23
	pCR36M8	*spoIIG41*	−36, ΔG	20.6	22.6	7.9

[a] Plasmid that was added to strain in first column.
[b] Indicates wild-type (WT) *ctc* promoter, *ctc* promoter with a transition at −36, or *ctc* promoter with a transition at −36 and a deletion of one of three G's centered at −15.
[c] All except values for BS50 are the averages of two experiments. The cells were grown in LB-chloramphenicol medium. The first sample (t_{1-2}) was taken within the first hour of stationary phase, and the second sample was taken 1 h later.
[d] Ratio of $CatO_2$ase at t_{1-2} for the indicated strain divided by the activity of $CatO_2$ase from JH642(pCR31) at t_{1-2}.

TABLE 4. *ctc*::*xylE* expression in intergenic suppressor strains

Strain	Plasmid[a]	*ctc* promoter[b]	$CatO_2$ase sp act (mU/mg)[c]		Activity ratio[d]
			t_{1-2}	t_{2-3}	
JH642	pCR31	WT	5	7	1.0
JH642	pCR15	−15	0.8	1	0.16
EU300	pCR31	WT	25	42	5.0
EU300	pCR15	−15	2	2	0.4
JH651	pCR31	WT	19	33	3.8
JH651	pCR15	−15	1	3	0.2

[a] Plasmid that was added to strain in first column.
[b] Indicates wild-type (WT) *ctc* promoter or *ctc* promoter with a transition at position −15.
[c] Average from two experiments. The first sample (t_{1-2}) was taken within hour 1 of stationary phase, and the second sample (t_{2-3}) was taken 1 h later. These cells were grown in LB glucose medium.
[d] Ratio of $CatO_2$ase activity at t_{1-2} from test strain divided by the activity of $CatO_2$ase from JH642(pCR31) which was measured in the same experiment.

pCR36); therefore, the mutation in EU300 did not affect use of the *ctc* promoter in an allele-specific manner.

To determine the location of the mutation in EU300 that caused the increased expression from the *ctc* promoter, we used generalized transduction by phage PBS1. We found that the mutation (designated *socA1*) that caused increased expression from the *ctc* promoter was 14% linked to *purA* and 90% linked to *cysA*. Three-factor transformation crosses were used to precisely locate *socA1*. Chromosomal DNA from EU301, a rifampin-resistant derivative of EU300, was used to transform a *cysA* mutant strain that contained pCR15. From the data in Table 5, we concluded that *socA1* was located between *cysA* and *rpoB* (*rfm-3Y*) (49% cotransformed with *cysA* and 35% cotransformed with *rfm-3Y*).

Because of the location of *socA1* and because the presence of *socA1* caused these strains to sporulate inefficiently (Table 6), we considered it likely that *socA1* was an allele of the sporulation-essential gene *spo0H*. We assessed the relationship of *socA1* and *spo0H* by comparing the levels of *ctc*::*xylE* expression in EU300 to those in strains known to carry mutations in *spo0H*. *B. subtilis* JH651, which carries the *spo0H81* allele, and IS233, which carries a *spo0H* deletion, were transformed with pCR31 and pCR15, and the levels of expression of *ctc-xylE* were assayed in liquid culture. JH651 increased transcription from both the wild-type and mutant *ctc* promoters to approximately the same degree as did EU300 (Table 4). Moreover, the deletion of *spo0H* resulted in more than a 30-fold increase in expression from the *ctc* promoter when the cells were grown in liquid medium without glucose (Table 3). These results supported the idea that *socA1* was an allele of *spo0H*.

Complementation tests also were used to characterize *socA1*. The plasmid pIS11a contains a wild-type *B. subtilis spo0H* allele and can complement the *spo0H* deletion carried by IS233 (25). pIS11a also complemented the Spo$^-$ phenotype of EU300 (Table 6). Since this Spo$^-$ phenotype was associated with *socA1* in the mapping experiments and can be complemented by a wild-type *spo0H* gene, and since a mutation in *spo0H* increased use of the *ctc* promoter (described above), it is likely that *socA1* is an allele of *spo0H*.

Suppressor mutations at other loci. To isolate additional mutations that affect *ctc* expression and that may have been missed when the *xylE* plasmids were used, we also looked for mutations by using a *ctc-lacZ* fusion which is present in single copy in the *B. subtilis* chromosome. For these experiments, an SBβ-sensitive derivative of strain JH642 (ZB278) was grown to mid-exponential phase and treated with nitrosoguanidine, and the mutagenized cells were then infected at a low multiplicity of infection with the specialized transducing phage SBβ *ctc-lacZ*. Transductants, which were identified by their ability to grow on selective concentrations of chloramphenicol, were screened on media containing the indicator dye X-Gal to identify colonies that exhibited altered levels of *ctc-lacZ* expression. Out of the approximately 20,000 colonies screened, 74 colonies seemed more blue than did the wild-type colonies and were thus picked as putative *ctc*-overproducing (*cop*) mutants.

TABLE 5. Mapping *socA1* by three-factor transformation crosses[a]

Selection (sample size)	Recombinant class			No. of recombinants	Suggested order
	rfm-3Y	*cysA*	*socA*		
Rif^r (200)	1	1	1	50	*rfm-3Y socA1 cysA*
	1	0	1	28	
	1	1	0	13	
	1	0	0	109	
Cys$^+$ (199)	1	1	1	62	*rfm-3Y socA1 cysA*
	1	1	0	4	
	0	1	1	85	
	0	1	0	48	

[a] Recipient (000) strain QB944(pCR15)*cysA* was crossed with donor (111) strain EU301 *rfm-3Y socA1*.

TABLE 6. Efficiency of spore formation

Strain	No. of cells/ml	No. of spores/ml[a]	Spore frequency
JH642	4.5×10^8	3.8×10^8	8.4×10^{-1}
IS233	9×10^7	8×10^2	8.9×10^{-6}
IS233(pIS11a)	1.4×10^8	3×10^5	2.1×10^{-3}
EU300	8×10^7	1×10^3	1.2×10^{-5}
EU300(pIS11a)	1.7×10^8	2×10^6[b]	1.2×10^{-2}

[a] CFU that were resistant to 80°C (see Materials and Methods).
[b] Loss of the plasmid caused these cells to revert to a Spo$^-$ phenotype in 19 of 22 cases examined.

One class of mutations which should cause the cell to overexpress the *ctc-lacZ* fusions are mutations that affect the activity of the TCA cycle. Previous studies indicated that *ctc* expression is higher in cells that carry TCA cycle (*cit*) mutations and that this increase in expression can be seen on X-Gal indicator plates (11). Since mutations that affect the TCA cycle prevent cells from growing on minimal medium containing lactate as the sole carbon source, we decided to screen the various *cop* mutants for a TCA cycle deficiency. On the basis of this test, the 74 mutants could be broken down into three groups. The first group consisted of 10 mutants that grew well on both minimal glucose-glutamine medium and minimal lactate medium. These strains clearly did not contain mutations affecting the activity of the TCA cycle. The 11 mutants which made up the second group were unable to grow on either medium. As a result, it was not possible to score their TCA cycle phenotype by using this method. The third class consisted of 53 mutants that were able to grow on minimal medium supplemented with glucose but were unable to grow on minimal medium supplemented with lactate. Therefore, it seems likely that these 53 mutants contain a mutation that affects the activity of the TCA cycle and that the increase in *ctc-lacZ* expression is due to this mutation.

The 21 mutant strains from class 1 and class 2 were further classified on the basis of their ability to sporulate. For this analysis, the 21 strains were streaked on DSM sporulation medium and incubated for 1 week at 37°C. Mutants that were able to sporulate were identified on the basis of their ability to produce a sporulation-specific brown pigment and by their ability to produce heat-resistant spores. Of the 21 mutants, 9 were able to sporulate, whereas the other 12 mutants were sporulation deficient. (Of the 12 sporulation-deficient mutants, 9 could contain *cit* mutations, since they are members of class 2 and since mutations that affect the TCA cycle also prevent the cell from sporulating.)

To classify these mutations further, we next determined whether any of the *cop* mutations mapped near the chromosomal locations of either *spo0H* (*socA*) or *socB*, two loci which affect *ctc* expression. As indicated earlier, *spo0H* (*socA*) is linked by transformation to *cysA*, whereas *socB* is linked by transformation to *dal-1*. To determine whether any of the cop mutations could be within the *spo0H* gene, chromosomal DNA was prepared from each of the 21 mutants and was used to transform a *B. subtilis* strain that is

auxotrophic for *cysA* to *cys* prototropy. The resulting transformants were then screened on X-Gal indicator plates to detect increased expression of the *ctc-lacZ* fusion. Three of the mutants showed high linkage to *cysA*. Furthermore, these three strains were sporulation deficient, a result consistent with the idea that these three mutants contain a mutation in or near *spo0H*.

To determine whether any of the *cop* mutations could be in or near *socB*, the chromosomal DNA isolated from the 21 mutants was used to transform a *dal* mutant strain to D-alanine prototrophy and the resulting transformants were screened for increased *ctc-lacZ* expression on X-Gal indicator plates. Only one of the mutants that caused an increase in *ctc-lacZ* expression was linked to *dal-1*. This mutation, called *copB*, is similar to *socB* in its effect on *ctc* expression. However, unlike the *socB* mutation, the *copB* mutation does not cause a small colony size. The locations of the mutations that increase *ctc* expression in the remaining strains were not determined.

DISCUSSION

We previously described several base substitutions in the *ctc* promoter that decreased its utilization in vivo (18). The activities of these mutant *ctc* promoters were assayed by using these promoters to direct transcription of *xylE* in plasmid pLC4. In the present study, we isolated mutants that carried either intragenic or intergenic mutations that suppressed the effects of mutations in the *ctc* promoter. Four intragenic mutations were generated by mutagenesis of weak mutant *ctc* promoters and were identified by their abilities to direct *xylE* expression from plasmid pLC4. Three of these mutant *ctc* promoters (15M6, 15M11, and 15M12) were still utilized by $E\sigma^B$, since there was little or no *xylE* activity produced from these plasmids in the *sigB* mutant strain *B. subtilis* CR101. Furthermore, transcription of *xylE* was induced from the promoters at the beginning of stationary phase, the time when *xylE* was expressed from the wild-type *ctc* promoter. Since base substitutions at positions −5 and −9 enable $E\sigma^B$ to utilize these mutant *ctc* promoters, it is likely that $E\sigma^B$ interacts with the nucleotides at positions −5 and −9 of these promoters.

xylE also was induced from the fourth mutant promoter in pCR36M8 at the beginning of stationary phase, but two observations indicate that the single-base-pair deletion in this promoter placed it under a different type of regulation than that of the wild-type *ctc* promoter. First, the level of *xylE* expression from pCR36M8 in *B. subtilis* CR101 (*sigB*) was the same as that in a wild-type host background, indicating that this promoter was no longer dependent on $E\sigma^B$. Second, the amount of $CatO_2$ase produced from PCR36M8 decreased when the cells were grown in the presence of glucose, whereas glucose stimulated $CatO_2$ase production from the pLC4 derivative containing the wild-type *ctc* promoter. These results indicate that the sequence between the −10 and −35 regions of the *ctc* promoter is necessary for use of this promoter by $E\sigma^B$ and for its regulation in response to glucose.

We also have isolated mutants carrying intergenic mutations that increase expression of *xylE* from pLC4 derivatives containing mutant and wild-type *ctc* promoters in an allele nonspecific manner. One class of mutants is unable to use the TCA cycle. We do not yet understand how this enhances expression for the *ctc* promoter, but it is consistent with the previous findings that repression of the TCA cycle enhances *ctc* expression (11). A second class of mutants is represented by EU300. The mutation in EU300 was shown by three-factor transformation crosses to be located between *rfmY3*, an allele of *rpoB*, and *cysA*, a region which contains the sporulation-essential locus *spo0H*. The mutation at this location in EU300 is associated with a defect in sporulation, and this Spo⁻ phenotype can be complemented by a plasmid that carries the *spo0H* locus. Like the mutation in EU300, a previously characterized mutation in *spo0H* also caused an increase in utilization of the *ctc* promoter, a result that confirmed a similar observation by Igo and Losick (11). We propose that the mutation in EU300, referred to as *socA1* in Results, now be designated *spo0H300*, since it appears that this mutation is an allele of *spo0H*.

The *copB* and *socB* mutations are closely linked to *dal*. We recently cloned the *socB1* allele and showed by nucleotide sequencing that it contained a single-base-pair deletion in an open reading frame located immediately downstream from *sigB* (10). *socB1* and possibly *copB* may inactivate the structural gene for a negative regulator of *sigB* expression, or activity of sigma B (10). *socA1* was found to be an allele of *spo0H*, a gene which also encodes a sigma factor, sigma 30 (4; J. Weir, E. Dubnau, L. Carter, and C. P. Moran, unpublished results). The observation that the effects of *ctc* promoter mutations could be suppressed by mutations in genes involved in the activity of two different sigma factors raises the possibility that sigma factors compete for available core RNA polymerase.

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