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A σ^E-Dependent Operon Subject to Catabolite Repression during Sporulation in *Bacillus subtilis*

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To identify genes expressed at intermediate stages of *Bacillus subtilis* sporulation, we screened for σ^E-dependent promoters. One promoter that we found drives expression of an operon consisting of at least five open reading frames (ORFs). The predicted products of the first three ORFs are very homologous to enzymes involved in fatty acid metabolism, including acetyl coenzyme A (acetyl-CoA) acetyltransferase (thiolase), 3-hydroxybutyryl-CoA dehydrogenase, and acyl-CoA dehydrogenase, respectively. We showed that the fourth ORF encoded a third isozyme of citrate synthase in *B. subtilis*. Genetic evidence and primer extension results showed that transcription of this operon is directed by the mother cell compartment-specific sigma factor, σ^E, and so the operon was named *mmg* (for mother cell metabolic genes). Furthermore, we found that a sequence (*mmgO*) with homology to a catabolite-responsive element mediates glucose repression of *mmg* promoter activity during sporulation and that this repression was lost in a *ccpA* mutant.

At the end of exponential growth, *Bacillus subtilis* begins a series of morphological changes resulting in the formation of a dormant endospore. This starvation-induced differentiation process is linked to changes in the metabolic state of the cell. The Krebs cycle enzymes, for example, are induced during the transition from exponential to stationary phase, and inactivation of any of the enzymes in this pathway results in a decrease in sporulation efficiency (6, 13, 22). This sporulation defect is apparently not due solely to the lack of energy and biosynthetic precursors provided by an intact Krebs cycle. Loss of the first three enzymes of the Krebs cycle appears to prevent the phosphorylation of a key regulator of sporulation initiation, Spo0A, to its active form (21). Phosphorylation of Spo0A by an alternate route allows this checkpoint to be bypassed, and early sporulation genes are transcribed. This suggests that a signal is transmitted by the Krebs cycle enzymes, allowing the initiation of sporulation (21).

While there are many examples of metabolic enzymes induced in early stationary phase (16, 23, 40), very little is known about the effectors of metabolism in the later stages of sporulation. Two examples of metabolic gene products expressed at later stages of sporulation are glucose dehydrogenase and enzymes for the production of glycogen. The *gdh* operon is transcribed by $E\sigma^G$ and encodes a glucose dehydrogenase, which is produced in the forespore compartment of sporulating cells (36). In the mother cell compartment, $E\sigma^E$ drives transcription of genes for glycogen synthesis from the *bvx* promoter (15, 25). No readily detectable sporulation phenotype is associated with mutations of either transcription unit. It is also possible that the lack of easily detectable phenotypes has left other sporulation-associated metabolic genes unidentified.

In an effort to identify genes expressed at intermediate stages of sporulation, we screened for σ^E-dependent promoters. One promoter that we found drives expression of an operon consisting of at least five open reading frames (ORFs). The predicted products of the first three of these ORFs bear striking similarity to fatty-acid-metabolizing enzymes. We showed that the fourth ORF encoded a citrate synthase, the third found in *B. subtilis*. A sequence with homology to a catabolite-responsive element (CRE) was demonstrated to mediate glucose repression from this promoter. This provided the first direct evidence of catabolite repression of a promoter dependent on a sporulation-induced sigma factor. The transcription of these genes is apparently dependent on the mother-cell-specific sigma factor, σ^E, and so the operon was named *mmg* (for mother cell metabolic genes).

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MATERIALS AND METHODS

Strains. *B. subtilis* and *Escherichia coli* strains used in this work are listed in Table 1.

Subcloning of the *mmg* region. Chromosomal DNA isolated from strain EUX19 was used to transform *B. subtilis* CU1050(pTV17) to chloramphenicol resistance, thus selecting for transfer of the Cm^r marker and its associated chromosomal fragment to pTV17 by homologous recombination, creating pTVE19. Cleavage of pTVE19, with *Sal*I, released a 3.2-kb chromosomal fragment, which was cloned into pUS19 to create pUSE19. Plasmid pUS19 (obtained from W. Haldenwang) is a pUC19 derivative containing a spectinomycin resistance cassette (27). The pUSE19 insert, with chromosomal-fragment-encoded *Sau*3AI termini rather than the library-encoded *Sal*I sites, is shown in Fig. 1B. This insertion is oriented so that the end which was proximal to the library *lacZ* gene is abutting the *Hin*dIII side of the multicloning region. Plasmid pUSE19ΔE (Fig. 1B) was made by using vector and insert *Eco*RI sites to delete most of *mmgA* and upstream DNA. A Campbell-type integration of pUSE19ΔE into the wild-type MB24 chromosome followed by *Hin*dIII cleavage, ligation, and transformation of *E. coli* yielded pUSE19H.

Plasmid pTKlac (24) was used to construct *lacZ* reporter fusions to DNA fragments from the *mmg* operon region (Fig. 1C). The pE19lac1 insert was amplified by PCR using the E19ECO oligonucleotide (5′-TAAGGGAATTCC GTATACAGTCAATCGTCC-3′) and the E19H3 oligonucleotide (5′-CCGAA TAAGCTTAATGGAGTTCTTGCAGCAC-3′), containing sequence changes (underlined bases) to create *Eco*RI and *Hin*dIII sites, respectively. The pE19lac2 fragment was obtained from pUSE19 by using the chromosomal *Eco*47III site and the 3′ *Hin*dIII site to clone into a *Sma*I- and *Hin*dIII-digested pTKlac plasmid. The *Spe*I-*Hin*dIII fragment was cloned *Xba*I to *Hin*dIII into pTKlac, creating pE19lac9. Restriction sites created via PCR mutagenesis are indicated in parentheses next to those sites in Fig. 1C.

Three versions of pE19lac10 were constructed in pTKlac to test *mmgO* operator function (see Fig. 6D). The wild-type operator version of the plasmid (pE19lac10-WT) contains a PCR fragment amplified with oligonucleotides E19ECO (see above) and E19III (5′-GAACCTGCACTAAGACGGCGTC-3′) and then cleaved with *Eco*RI and *Hin*dIII. The strategy for constructing mutant operators took advantage of the *Eco*47III cleavage site, which bisected the *mmgO* site. Plasmid pE19lac10-CC was created by PCR amplifying the promoter region with the E19ECO oligonucleotide along with the E19COM3 oligonucleotide (5′-AGATAGACAGCGCTTGGAATTTC-3′), which creates the GT-to-CC substitution (complementary positions underlined) at positions 2 and 3 of

TABLE 1. Strains used in this study

Strain	Genotype, phenotype, and/or other description	Source (reference)
E. coli		
DH5α	F$^-$ φ80d*lacZ*DM15 *D*(*lacZYA-argF*)*U169 recA1* hsdR17 (r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA relA1*	Laboratory stock
W620	*thi-1 pyrD36 gltA6 galK30 rpsL129*	CGSC[a]
B. subtilis		
MB24	*trpC2 metC3*	P. Piggot (33)
ZB307A	Spβ *c*2del2::Tn*917*::pSK10Δ6 Mls^r	Laboratory stock; P. Zuber
CU1051	*leuA8 metB5 thrA5 sup-3 pla-1* SPβ^s (pTV17)	Laboratory stock (3)
EU101	*spoIIGBD*::*erm chr*::Tn*917*ΩHU160::Pspac-*spoIIGB* Pm^r Em^r	Laboratory stock (44)
EU8702	*trpC2 pheA1 spoIIGBD*::*erm* (pDG180) Pm^r Em^r	Laboratory stock (3)
EUR9030	*aroI916 purB33 trpC2 spoIIAC*::*erm* Em^r	Laboratory stock (8)
SJB67	*pheA1* Δ*citA*::*neo* Δ*citZ471* Nm^r	A. L. Sonenshein (22)
1A722	*trpC2 bfmB*::Tn*917* Em^r	BGSC[b]
AH62	*trpC2 metC3 spoIIIA*::Tn*917* Em^r	Laboratory stock
BG314	*metC3* ΩpBG15 (5)	P. Youngman
WLN-29	*trpC2 aroG932 gra*::Tn*917*	G. Chambliss (17)
EUX19	EU101 (SPβE19) Em^r Pm^r Cm^r	This study
EUX9401	*trpC2 metC3* (SPβE19lac2) Cm^r Em^r	This study
EUX9402	*trpC2 metC3* ΩpE19lac2 Cm^r	This study
EUX9403	*trpC2 metC3* (SPβE19lac1) Cm^r Em^r	This study
EUX9407	*trpC2 metC3* ΩpE19lac9 Cm^r	This study
EUX9409	*trpC2 metC3* ΩpE19lac9 Δ*mmgAB*::*kan* Cm^r Km^r	This study
EUX9412	*trpC2 metC3* Δ*mmgABCD*::*kan* Km^r	This study
EUX9501	*trpC2 metC3* Δ*mmgABCD*::*erm* Em^r	This study
EUX9505	*trpC2 metC3* (SpβspoIID-*lacZ*) Cm^r Em^r	This study
EUX9506	*trpC2 metC3* (SPβE19lac10-WT) Cm^r Em^r	This study
EUX9507	*trpC2 metC3* (SpβE19lac10-CC) Cm^r Em^r	This study
EUX9508	*trpC2 metC3* (SpβE19lac10-GG) Cm^r Em^r	This study
EUX9509	*trpC2 metC3* (Spβ*spoIIID-lacZ*) Cm^r Em^r	This study
EUX9510	*trpC2 metC3 gra26*::Tn*917* Em^r	This study
EUX9511	*trpC2 metC3 gra26*::Tn*917* Em^r (SPβE19lac10-WT) Cm^r Em^r	This study
EUX9512	*trpC2 metC3 gra26*::Tn*917* Em^r (SPβE19lac10-CC) Cm^r Em^r	This study

[a] CGSC, *E. coli* Genetic Stock Center.
[b] BGSC, *Bacillus* Genetic Stock Center.

the operator (see Fig. 6D). Cleavage of this PCR fragment with *Eco*RI and *Eco*47III released a 212-bp fragment, containing the GT-to-CC operator mutation, which was used in a triple ligation with *Eco*RI- and *Hin*dIII-digested pTKlac and the 982-bp *Eco*47III-*Hin*dIII fragment containing the downstream half of *mmgO*. The same strategy was used to make the pE19lac10-GG plasmid, except that the downstream half of the operator was modified by PCR mutagenesis. The downstream fragment was amplified with oligonucleotide E19III (see above) and oligonucleotide E19COM4 (5′-GAAATTGTAAGCGCTGGGTATCTTCT-3′), creating a TC-to-GG substitution (mutated positions underlined) at positions 12 and 13 of *mmgO* (see Fig. 6D). A triple ligation was then done with the mutant *Eco*47III-*Hin*dIII fragment, a wild-type *Eco*RI (PCR-created site)-*Eco*47III fragment, and *Eco*RI-*Hin*dIII-digested pTKlac to create pE19lac10-GG.

Plasmids for making chromosomal insertion-deletions were pUS19 derivatives, with insertions as shown in Fig. 1D. To make pE19Δ1, a 1,984-bp *Eco*47III-*Stu*I fragment was deleted from pUSE19 and replaced with a 1.5-kb *Sma*I fragment containing a kanamycin cassette (45) from pKD102 (obtained from W. Haldenwang). The pE19Δ2 deletion plasmid was constructed by deleting the 3,042-bp *Eco*RI-*Pst*I fragment from pUSE19H and cloning in the *Eco*RI (vector site)-*Pst*I (pKD102 site) fragment from pE19Δ1. Plasmid pE19Δ3 was constructed by cloning the 2.3-kb *Sma*I-*Pst*I erythromycin resistance cassette fragment from pUC18ERM (24) into an *Eco*47III- and *Pst*I (vector site)-cleaved pUSE19 plasmid. This plasmid was then cleaved with *Pst*I and *Hin*dIII to clone in the 820-bp *Pst*I-*Hin*dIII fragment from pUSE19H.

Complementation of citrate synthase mutants was done with pSpacmmgD, which contains *mmgD* under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible P_{spac} promoter (47). The parent vector, pDH88 (18), was cleaved with *Sma*I and *Xba*I, and then the 1,312-bp *Nru*I-*Spe*I fragment of pUSE19H, containing *mmgD*, was inserted. These vectors are integrational in *B. subtilis* and replicative in *E. coli*. The P_{spac} promoter is functional in both *B. subtilis* and *E. coli.*

General methods and growth conditions. Methods for restriction digests, ligations, *E. coli* transformations, and the preparation of Luria-Bertani (LB) medium and M9-glucose medium were those of Sambrook et al. (37). The PCR protocol is described in the GeneAmp kit instructions (Perkin-Elmer Cetus, Norwalk, Conn.). PCR products were purified by using the Wizard PCR Preps Kit (Promega, Madison, Wis.). When necessary, DNA restriction fragments were purified by using the GENECLEAN II kit (Bio 101, La Jolla, Calif.). The chromatogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used on plates at a concentration of 40 μg/ml. Where necessary, IPTG was added to liquid and agar medium at a final concentration of 1 mM. Antibiotic resistance markers were selected at the following concentrations for *E. coli*: ampicillin, 100 μg/ml; spectinomycin, 100 μg/ml; and kanamycin, 40 μg/ml. Antibiotic resistance markers were selected in *B. subtilis* at the following concentrations: chloramphenicol, 5 μg/ml; neomycin, 4 μg/ml; spectinomycin, 100 μg/ml; kanamycin, 12.5 μg/ml; erythromycin, 1 μg/ml; and lincomycin, 20 μg/ml. Sporulation was carried out either in Difco sporulation medium (DSM) (31, 38) or by resuspension in defined media by the method of Sterlini and Mandelstam (41). Tris-Spizizen salts minimal agar (TSSA) was prepared as described previously (9), except that we did not add trisodium citrate. Tris-Spizizen salts (TSS) liquid medium was prepared similarly except that agar was not added. Heat and chloroform tests of *B. subtilis* cultures (31), *B. subtilis* transformation (9), SPβ transduction methods (9), and the β-galactosidase activity assay (24) were also described previously.

Genetic mapping of *mmgABCDE*. Mapping was done by two-factor crosses via PBS1 transduction (9, 10). By using strain EUX9412 (Δ*mmgABCD*::*kan*) as the donor, a series of crosses was done with recipient strains 1A722 (*bfmB*::Tn*917*), EUR9030 (*spoIIAC*::*erm*), AH62 (*spoIIIA*::Tn*917*), and BG314 (containing a Campbell insertion of pBG15 [5] at *spo0A*). The results are presented as percents cotransduction. Map positions obtained from the above-described crosses were confirmed by reciprocal crosses using EUX9412 (Δ*mmgABCD*::*kan*) as the recipient with 1A722 (*bfmB*::Tn*917*) and EUR9030 (*spoIIAC*::*erm*) as donors (data not shown).

DNA sequencing. Plasmid templates were sequenced according to the double-stranded DNA method from the Sequenase protocol (United States Biochemical). Initial sequence was obtained with an oligonucleotide which annealed to sequence outside of the multicloning region. Further sequence was obtained by using oligonucleotides synthesized on a Beckman Oligo 1000 DNA Synthesizer (Beckman Instruments, Inc., Palo Alto, Calif.). Both strands of relevant DNA were sequenced in this manner. Approximately 2.5 kb of DNA sequence was obtained from the 3.2-kb pUSE19 (Fig. 1B) chromosomal insert, including sequence from 224 bp upstream of the *mmg* transcriptional start site down to the

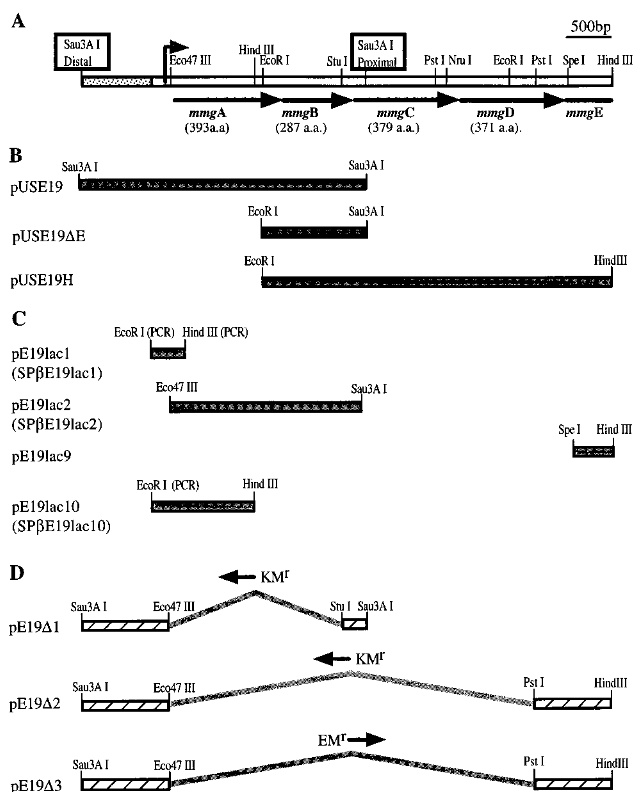

FIG. 1. (A) Restriction map of the *mmg* region. Restriction enzymes are listed above their cleavage sites. Only the terminal *Sau*3AI sites of the original library clone are indicated, and these are labeled as proximal and distal relative to the *lacZ* fusion point of the original clone. The bent arrow shows the transcriptional start site. The straight arrows indicate the sizes and orientations of the ORFs. Putative genes and the expected amino acid (a.a.) lengths of their products are listed beneath the straight arrows. The *mmgE* sequence is incomplete, and no a.a. length is given. The stippled region is either unsequenced DNA or DNA sequenced on only one strand. (B) Bars represent fragments cloned into vector pUS19 for sequencing and further subcloning. *Sau*3AI sites in pUSE19 represent actual chromosomal endpoints but are not indicative of vector sites into which they are cloned (see Materials and Methods). (C) Bars represent fragments cloned into pTKlac for *lacZ* fusion studies. Beneath the plasmid name is the name of the SPβ phage (if any) constructed with it. (D) Bars represent insertions into pUSE19. Hatch marks are regions of homology with the *mmg* chromosomal fragment shown in panel A. Bent bars represent deleted regions with either a kanamycin (KM^r) or an erythromycin (EM^r) resistance cassette insertion. Transcriptional orientations of cassettes are shown by arrows. The restriction sites of the fragments are equivalent to the chromosomal sites shown in panel A and do not necessarily reflect vector and antibiotic marker sites.

library *Sau*3AI site (Fig. 3, position 2487). Further downstream sequence was obtained from pUSE19H (Fig. 1B).

Complementation of *E. coli* and *B. subtilis* citrate synthase mutants. The *E. coli* citrate synthase mutant W620 (Table 1) was transformed with plasmid pSpacmmgD (described above) or its parent, pDH88 (18, 47), and ampicillin-resistant colonies were selected. W620 and its transformants were grown in LB medium-ampicillin to late exponential phase (optical density at 600 nm [OD_{600}] = 1.3 to 1.5), at which time serial dilutions (10^{-1} to 10^{-7}) were made in saline solution (NaCl, 9 g/liter) and 0.1-ml samples of each dilution were plated on M9-glucose agar (containing uracil [150 μg/ml], thiamine [50 μg/ml], and ampicillin [100 μg/ml]) with or without IPTG. The plates were incubated at 37°C for 36 h, after which time the titer of CFU per milliliter was determined.

A similar experiment was done with the *B. subtilis* citrate synthase mutant strain SJB67. This strain was transformed to chloramphenicol resistance by integration of pSpacmmgD by homologous recombination to create EUX9504. The integrant and the parent strain were grown in LB medium-chloramphenicol until late exponential phase (OD_{600} = 1.3 to 1.5). Dilutions were then made in saline solution, as described above, and 0.1-ml samples were plated onto TSSA-glucose (containing 20 μg of phenylalanine per ml) with or without IPTG. The plates were incubated at 37°C for 36 h, after which time the titer of CFU per milliliter was determined. Culture doubling times for SJB67 and EUX9504 were tested in TSS-glucose liquid minimal media (containing 20 μg of phenylalanine per ml) with no additions, with glutamate (200 μg/ml), or with IPTG.

Primer extension of RNA. Total EU8702 RNA was prepared from exponential-phase cells grown in LB broth with or without IPTG, as described previously (3, 35). Total MB24 RNA was prepared from cells grown in DSM broth, as previously described (4, 35), and harvested at the times indicated below. The primer E19EXT (5′-CAAAACTCCGCCGAATTTGCCAAATGGAG-3′), complementary to bases 309 to 337 of the sequence shown in Fig. 3, was 5′ end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase as described previously (30). Approximately 10 ng of labeled primer was annealed to either 50 μg of total RNA from strain EU8702 or 15 μg of total RNA from strain MB24. Extensions were then done with avian myeloblastosis virus reverse transcriptase, as previously described (30, 35).

Assay for glucose repression of a σ^E-dependent promoter. Strains were grown in DSM broth at 37°C with shaking (150 rpm) and with a culture-to-flask volume ratio of about 1:10. At 1.5 h after the end of the exponential growth phase ($T_{1.5}$), each culture was split into two flasks. Glucose was added to one flask at a final concentration of 1%, while no glucose was added to the other flask. Culture samples were taken at the times indicated below and assayed for β-galactosidase activity as described previously (24). Miller units were calculated by the formula described by Miller (29): units = 1,000 × OD_{420}/(time × volume × OD_{600}).

Plasmid pTKlac derivatives (pE19lac10-WT, -CC, and -GG) were used to create specialized SPβ transducing phages, as described previously (24). The SPβ constructs containing the spoIID and spoIIID *lacZ* fusions have been described previously (11, 44). These phages were used to transduce strains MB24 and EUX9510 (*ccpA* mutant) to chloramphenicol resistance. EUX9510 was constructed by transforming MB24 to erythromycin resistance with chromosomal DNA from strain WLN-29 (17), containing a Tn*917* insertion at *ccpA*. A low level of β-galactosidase activity was noted with EUX9510, and this background activity was subtracted from the final Miller units of the strain's derivatives. No background activity was observed with MB24 or its derivatives.

Nucleotide sequence accession number. The sequence of the *mmgABCDE* operon here reported has been deposited in the GenBank database and assigned accession number U29084.

RESULTS

Cloning of the *mmg* operon. A screen for σ^E-dependent promoters, described previously (3, 4, 19), was used to isolate the promoter and associated genes characterized in this work. A lysate of a specialized SPβ phage, containing a *lacZ* fusion library of random chromosomal fragments from *B. subtilis* (provided by P. Zuber) (3, 4), was used to transduce *B. subtilis* EU101. A chloramphenicol resistance (Cm^r) marker within the specialized transducing phage was used to select lysogens. The lysogens were streaked on DSM agar containing X-Gal or X-Gal plus IPTG. Exposure of strain EU101 to IPTG results in the production of pro-σ^E, which is processed to active σ^E during sporulation. Therefore, colonies which remained white on DSM agar in the presence of X-Gal alone but turned blue in the presence of X-Gal and IPTG were expected to contain σ^E-dependent promoters fused to *lacZ*. One isolate, designated EUX19, was assayed for expression of β-galactosidase during growth and sporulation in liquid DSM. When P_{spac}-spoIIGB was induced in mid-exponential phase by the addition of IPTG, activity appeared by the second hour after the start of sporulation (T_2) and was maximal at about T_5 (Fig. 2A). In the absence of IPTG, P_{spac}-*spoIIGB* was not induced and β-galactosidase activity was not detected at any time point tested (Fig. 2A). This result demonstrated that this transcriptional activity is dependent on σ^E and restricted to the stationary phase, during which the cells are forming endospores.

To determine the location of the *mmg* promoter, several DNA fragments from this region were examined for promoter activity by being fused to a promoterless derivative of *lacZ* (see Materials and Methods). The SPβ lysogen EUX9401, containing a 2.2-kb fragment of the original 3.2-kb isolate (Fig. 1C; equivalent to the insert in pE19lac2), showed no β-galactosidase activity during vegetative growth or sporulation in DSM liquid. Since strain EUX9402, containing a Campbell-type insertion of pE19lac2 at the locus corresponding to its insert DNA (Fig. 1C), showed a sporulation-dependent pattern of β-galactosidase expression (Fig. 2B), all or part of the *mmg*

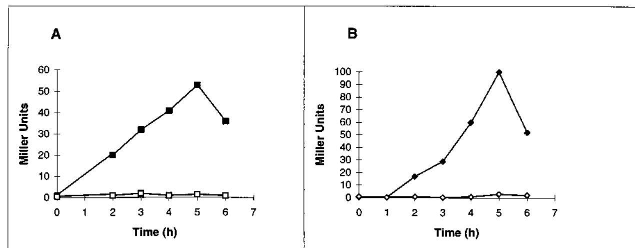

FIG. 2. β-Galactosidase activity from *mmg-lacZ* transcriptional fusions. (A) *B. subtilis* EUX19, containing the original 3.2-kb library isolate fused to a *lacZ* reporter, was grown in DSM to mid-log phase, and then the culture was split. IPTG was added to half the culture for P_{spac}-*sigE* induction (filled squares), and no IPTG was added to the other half (open squares). Cultures were grown to stationary phase, and samples were harvested for β-galactosidase assays at the indicated times after the end of the exponential growth phase. (B) *B. subtilis* EUX9407 (filled diamonds) and EUX9409 (open diamonds) both contained a *lacZ* fusion integrated within *mmgE* by a Campbell-type insertion. EUX9409 also contained a Km^r cassette which replaced *mmgA* and *mmgB*. Both were grown in DSM, and samples for β-galactosidase assays were harvested hourly after the end of the exponential growth phase.

promoter is upstream of the *Eco*47III site (Fig. 1A). A 300-bp fragment, containing the region upstream from the *Eco*47III site, was PCR amplified with oligonucleotides designed to create convenient restriction sites for cloning into pTKlac. The specialized SPβ phage made with this construct (Fig. 1C, pE19lac1) was used to create strain EUX9403. β-Galactosidase expression from this 300-bp fragment was similar to that shown for the primary isolate in Fig. 2A, suggesting that a promoter was probably within this region (data not shown).

To determine the length of the transcription unit that starts within the 300-bp fragment, we isolated plasmid pUSE19H, containing approximately 2.7 kb of DNA downstream of the original 3.2-kb isolate, and subcloned a small segment of that region in pTKlac, creating pE19lac9. Strain EUX9407 is strain MB24 with a Campbell-type integrant of plasmid pE19lac9 (Fig. 1C), creating a *lacZ* fusion approximately 5 kb downstream from the promoter. Strain EUX9409 was isogenic to EUX9407 except that it contained a kanamycin resistance (Km^r) cassette between the promoter and the *lacZ* fusion (Fig. 1D, pE19Δ1). The pattern of β-galactosidase expression in EUX9407 (Fig. 2B) was similar to that of the original library isolate, EUX19 (Fig. 2A). The Km^r insertion, however, abolished β-galactosidase activity from the distal *lacZ* fusion (Fig. 2B). These data, along with the analysis of the nucleotide sequence (see below), suggest that the transcription unit extends at least 5 kb.

The *mmg* operon may encode several metabolic enzymes. The sequence of the transcription unit revealed several ORFs with putative ribosome binding sites, as shown in Fig. 3. For reasons described below, these ORFs were designated *mmgA* to *mmgE*.

The nucleotide and predicted amino acid sequences of each ORF were used to search databases by using the FastA program provided in the Genetics Computer Group package (14). The results of these searches suggested that the operon had a metabolic function, since putative products of four of the five ORFs were very similar to well-characterized metabolic enzymes. The predicted products of *mmgA*, *mmgB*, and *mmgC* were most similar to three enzymes known to be involved in fatty acid metabolism. The *mmgA* gene could encode a predicted protein with 49% identity and 68% similarity to an acetyl coenzyme A (acetyl-CoA) acetyltransferase (thiolase) from the bacterium *Alcaligenes eutrophus* (accession no.

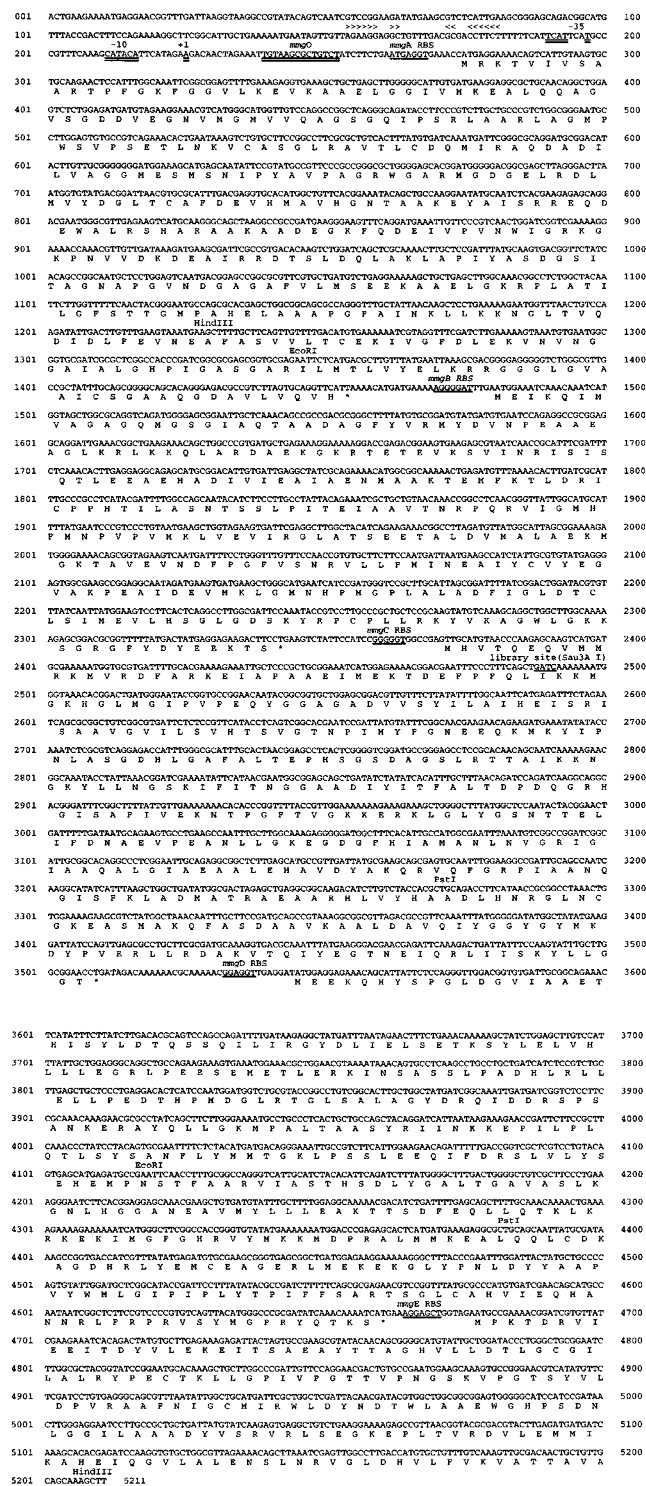

FIG. 3. Nucleotide sequence of the nontemplate strand of the *mmg* region. A putative factor-independent terminator is overscored with arrows. The −10 and −35 regions, the +1 transcriptional start, and the *mmgO* CRE are labeled and double underlined. The amino acid sequences (one-letter code) of the ORFs are beneath the parent strand of DNA. Expected ribosome binding sites are underlined and are labeled with the gene name plus RBS. The original library *lacZ* fusion site is also underlined. Stop codons are marked with asterisks.

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          1                                                   50
B.s.MmgD  MEEKQHYSPG LDGVIAAETH ISYLDTQSSQ ILIRGYDLIE LSETKSYLEL
B.c.CtsA  MVNTNQFIPG LEGVIASETK ISFLDTVNSE IVIKGYDLLA LSKTKGYLDI
B.s.CitZ  ....MTATRG LEGVVATTSS VSSI..IDDT LTYVGYDIDD LTENRSFEEI
B.s.CitA  .....MVHYG LKGITCVETS ISHIDGEKGR LIYRGHHAKD IALNHSFEEA

          51                                                 100
B.s.MmgD  VHLLLEGRLP EESEMETLER KINSASSLPA DHLRLLELLP .EDTHPMDGL
B.c.CtsA  VHLLLEGTIP NEAEKQHLEE TLKQEYDVPD EIIQVLSLLP .KTAHPMDAL
B.s.CitZ  IYLLWHLRLP NKKELEELKQ QLAKEAAVPQ EIIEHFKSYS LENVHPMAAL
B.s.CitA  AYLILFGKLP STEELQVFKD KLAAERNLPE HIERLIQSLP .NNMDDMSVV

          101                                                150
B.s.MmgD  RTGLSALAGY DRQIDDRSPS ANKERAYQLL GKMPALTAAS YRIINKKEPI
B.c.CtsA  RTGVSVLASF DTELLNREHS TNLKRAYQLL GKIPNIVANS YHILHSEEPV
B.s.CitZ  RTAISLLGLL DSEADTMNPE ANYRKAIRLQ AKVPGLVAAF SRIRKGLEPV
B.s.CitA  RTVVSALGEN TYTFHPKTEE .....AIRLI AITPSIIAYR KRWTRGEQAI

          151                                                200
B.s.MmgD  LPLQTLSYSA NFLYMMTGKL PSSLEEQIFD RSLVLYSEHE MPNSTFAARV
B.c.CtsA  QPLQDLSYSA NFLYMITGKK PTELEEKIFD RSLVLYSEHE LPNSTFTARV
B.s.CitZ  EPREDYGIAE NFLYTLNGEE PSPIEVEAFN KALILHADHE LNASTFTARV
B.s.CitA  APSSQYGHVE NYYYMLTGEQ PSEAKKKALE TYMILATEHG MNASTFSARV

          201                                                250
B.s.MmgD  IASTHSDLYG ALTGAVASLK GNLHGGANEA VMYLLLEAKT TSDFEQLLQT
B.c.CtsA  IASTLSDLYG ALTGAVASLK GHLHGGANEA VMEMLQDAQT VEGFKHLLHD
B.s.CitZ  CVATLSDIYS GITAAIGALK GPLHGGANEG VMKMLTEIGE VENAEPYIRA
B.s.CitA  TLSTESDLVS AVTAALGTMK GPLHGGAPSA VTKMLEDIGE KEHAEAYLKE
                                   *

          251                                                300
B.s.MmgD  KLKRKEKIMG FGHRVYMKKM DPRALMMKEA LQQLCDKAGD HRLYEMCEAG
B.c.CtsA  KLSKKEKIMG FGHRVYMKKM DPRAAMMKEA LKELSAVNGD DLLLQMCEAG
B.s.CitZ  KLEKKEKIMG FGHRVY.KHG DPRANDLKEM SKRLTNLTGE SKWYEMSIRI
B.s.CitA  KLEKGERLMG FGHRVY.KTK DPRAEALRQK AEEV...AGN DRDLDLALHV
                                  *

          301                                                350
B.s.MmgD  E.......RL MEKEKGLYPN LDYYAAPVYW MLGIPIPLYT PIFFSARTSG
B.c.CtsA  E.......QI MREEKGLFPN LDYYAAPVYW KLGIPIPLYT PIFFSSRTVG
B.s.CitZ  E.......DI VTSEKKLPPN VDFYSASVYH SLGIDHDLFT PIFAVRRMSG
B.s.CitA  EAEAIRLLEI YKPGRKLYTN VEFYAAAVMR AIDFDDELFT PTFSASRMVG
                                 *                      *   *

          351                      379
B.s.MmgD  LCAHVIEQHA NNRLFRPRVS YMGPRYQTK. .......
B.c.CtsA  LCAHVMEQHE NNRIFRPRVL YTGARNLRVE D......
B.s.CitZ  WLAHILEQYD NNRLIRPRAD YTGPDKQKFV PIEERA.
B.s.CitA  WCAHVLEQAE NNMIFRPSAQ YTGAIPEEVL S......
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FIG. 4. Alignment of MmgD with three other citrate synthases. The *B. coagulans* (B.c.) citrate synthase (CtsA) and two *B. subtilis* (B.s.) citrate synthases (CitA and CitZ) are compared with MmgD. Bold letters indicate matches of at least two out of three to the equivalent MmgD positions. Highly conserved positions, demonstrated to be involved in the active sites of pig heart and *E. coli* citrate synthases, are marked with asterisks.

P14611). The expected product of *mmgB* is 55% identical and 70% similar to the 3-hydroxybutyryl-CoA dehydrogenase of *Clostridium acetobutylicum* (accession no. A43723). The third gene, *mmgC*, could encode a protein which is 52% identical and 68% similar to the short-chain acyl-CoA dehydrogenase of *Megasphaera elsdenii* (accession no. L04528), while a putative *B. subtilis* acyl-CoA dehydrogenase (accession no. Z49782) is 55% identical and 72% similar to the predicted product of *mmgC*.

The predicted product of *mmgD* was most similar to citrate synthases. The *Bacillus coagulans* citrate synthase protein, CtsA (accession no. M74818), was most similar to the *mmgD* gene product, having 66% identity and 80% similarity. Since *B. subtilis* was already known to have two genes encoding citrate synthases (22), comparisons with these proteins were also made. The *citA* gene product (accession no. U05256) showed 35% identity and 56% similarity to MmgD, while the *citZ* gene product (accession no. U05257) was 42% identical and 61% similar to MmgD. CitA and CitZ are 42% identical and 63% similar to each other. An alignment of these citrate synthase sequences with the MmgD sequence is shown in Fig. 4. Six amino acids involved in the active-site region of pig heart and *E. coli* citrate synthases (1, 28, 32) were also compared with the corresponding amino acids in MmgD. The equivalent six positions in MmgD were highly conserved, and these amino acids are underscored by asterisks in Fig. 4. These comparisons indicated that MmgD was probably a citrate synthase.

The full extent of the *mmgE* ORF was not determined. A FastA search of the protein database revealed two proteins with strong similarity to the predicted partial *mmgE* product. The first was the product of an incomplete ORF (108 amino acids) found immediately downstream of the *B. coagulans ctsA* gene. The amino-terminal 108 amino acids of this ORF (accession no. B43936) are 77% identical and 87% similar to the amino-terminal 99 amino acids of MmgE. The product of a *Saccharomyces cerevisiae* ORF of unknown function (accession no. S52815) showed 59% identity and 75% similarity to MmgE over 178 amino acids at the amino terminus of the yeast protein. Interestingly, the yeast homolog of MmgE is also located immediately downstream of a putative citrate synthase gene.

Complementation of *E. coli* and *B. subtilis* citrate synthase mutants with *mmgD*. The product of *mmgD* was demonstrated to function as a citrate synthase by complementation of an *E. coli* citrate synthase (*gltA*) mutant strain, W620. Strain W620 was transformed to ampicillin resistance by pSpacmmgD, in which *mmgD* is under the control of the inducible P_{spac} promoter (47), or by its parent plasmid, pDH88. When the transformants were plated on M9-glucose agar containing IPTG, only cells that received pSpacmmgD formed colonies. These transformants also formed colonies on plates without IPTG, but growth in the presence of IPTG was more robust. Thus, expression of *mmgD* overcame the growth defect of the *gltA* mutant *E. coli*.

B. subtilis was previously found to have two citrate synthase genes (*citA* and *citZ*) (22). Strain SJB67 (*citA*::*neo* Δ*citZ471*) contains mutations in both of these genes. Strain EUX9504 is isogenic to SJB67 except that pSpacmmgD is integrated into the chromosome. Strains SJB67 and EUX9504 were grown to late exponential phase in LB medium, diluted in saline, and plated on TSSA-glucose (see Materials and Methods) with or without IPTG. EUX9504 gave approximately 1×10^8 CFU/ml on TSSA-glucose plates with IPTG after 36 h of incubation and only about 5×10^3 CFU/ml on the no-IPTG plates—about a 20,000-fold increase in plating efficiency in the presence of IPTG. No colonies of SJB67 were found on the plates in the presence or absence of IPTG at any dilution tested.

Complementation of the SJB67 growth defect by *mmgD* was also assessed by growth in liquid media. EUX9504 and SJB67 had doubling times of >10 h in TSS-glucose liquid minimal medium. Addition of glutamate to the minimal medium increased the growth rates of both strains (producing doubling times of 1.2 and 1.4 h, respectively). In TSS-glucose minimal media containing IPTG but not glutamate, the doubling time of EUX9504 was 2.5 h whereas the doubling time of SJB67 was >10 h. These results show that the induction of *mmgD* expression from the P_{spac} promoter complemented the growth defect of SJB67, indicating that the *mmgD* product can function as a citrate synthase in the Krebs cycle.

The *mmgABCDE* operon maps at approximately 217° on the *B. subtilis* genetic map. By two-factor crosses using PBS1 generalized transducing phage, we found that *mmgABCDE* is 89 to 96% cotransduced with *bfmB* (100 transductants tested), 62 to 66% cotransduced with *spoIIAC* (100 transductants tested), 72% cotransduced with *spoIIIA* (100 transductants tested), and 100% cotransduced with *spo0A* (50 transductants tested). A comparison of our *mmgABCDE* sequence with the previously known sequence of the *spo0A* region (accession no. M10082) showed no overlap. These results indicate that *mmgABCDE* is located in the 217° region of the *B. subtilis* chromosome.

The *mmg* promoter is used by σ^E RNA polymerase. Primer extension analyses were done with total RNA prepared from *B. subtilis* MB24 harvested at designated times during growth and sporulation in liquid DSM to map the 5′ end of the *mmg* transcript (Fig. 5, lanes g to l). The oligonucleotide primer

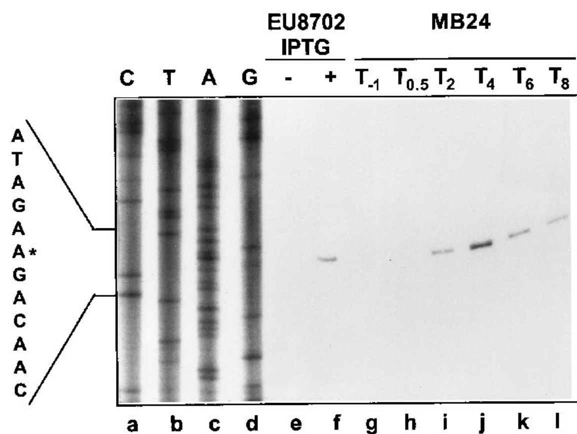

FIG. 5. Mapping the transcriptional start site of the *mmg* promoter. An oligonucleotide primer (E19EXT) complementary to a region within the *mmgA* ORF was used to prime cDNA synthesis from total RNA. RNA was prepared from mid-log-phase LB medium cultures of *B. subtilis* EU8702 grown in the presence or absence of IPTG (lanes e and f). In strain EU8702, the wild-type *sigE* allele is replaced by a plasmid-encoded, IPTG-inducible *sigE* allele that produces a vegetatively active version of σ^E. RNA was also prepared from wild-type *B. subtilis* (MB24) grown in DSM and harvested at 1 h before (T_{-1}) the end of stationary-phase growth and at several times after the end of exponential growth ($T_{0.5}$, T_2, T_4, T_6, and T_8). Fifty micrograms of total RNA was used for primer extensions in lanes e and f, whereas 15 μg of total RNA was used for primer extensions in lanes g to l. The same oligonucleotide (E19EXT) was used for dideoxy sequencing of plasmid pUSE19 (lanes a to d). Shown is an autoradiograph of the primer extension and sequencing products after they were subjected to electrophoresis on a 6% polyacrylamide–urea gel. The sequence is labeled as the reverse complement for ease of comparison with other sequence data. The asterisk indicates the start point of transcription.

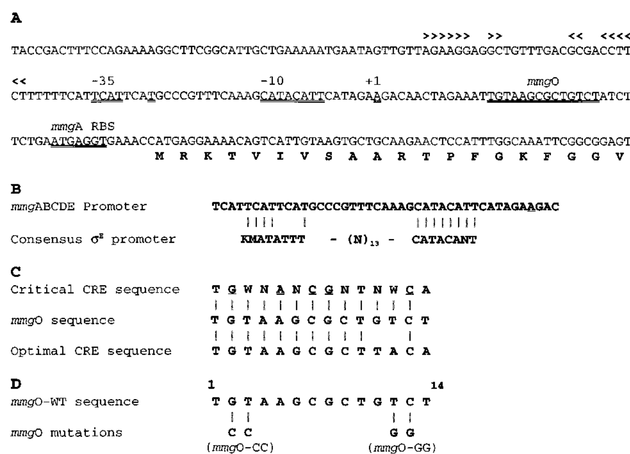

FIG. 6. (A) Promoter region of *mmg*. The putative factor-independent terminator is overscored by arrowheads. Other features are double underlined and labeled. The initial 20 amino acids of MmgA are beneath the corresponding DNA triplets. (B) Comparison of the *mmg* promoter with a consensus σ^E promoter. Matching bases are indicated by vertical bars. Ambiguity codes are as follows: K = G or T; M = A or C; and N = A, C, G, or T. (C) Comparison of *mmgO* with critical and optimal versions of a CRE (7, 46). Matching bases are indicated by vertical bars. Ambiguity codes are as follows: W = A or T and N = A, C, G, or T. (D) Mutations made in *mmgO*. The wild-type sequence is *mmgO*-WT. The GT-to-CC change at positions 2 and 3 creates *mmgO*-CC. The TC-to-GG change at positions at 12 and 13 creates *mmgO*-GG.

E19EXT is complementary to a region within the first ORF of the *mmg* transcription unit. The 5′-end-labeled E19EXT primer gave an extension product of 113 bases which mapped the apparent 5′ end of *mmg* mRNA to the adenine at position 225 of the nucleotide sequence presented in Fig. 3. The transcript began to accumulate at about T_2, with maximal product accumulation at T_4 and subsequent decreasing accumulation at T_6 and T_8 (Fig. 5, lanes i to l). No extension product was observed at vegetative or early-stationary-phase time points (Fig. 5, lanes g and h). The timing of expression was similar to that described previously for other σ^E-dependent promoters (3, 4, 26).

RNA was also purified from mid-exponential-phase LB broth cultures of *B. subtilis* EU8702. Strain EU8702 has the chromosomal *sigE* allele deleted but carries a plasmid-borne P_{spac}-*sigE* allele producing an IPTG-inducible, active form of σ^E (4). RNA from exponential-phase cells grown without an inducer gave no extension product (Fig. 5, lane e). RNA from an exponential-phase culture induced with IPTG showed a single extension product (Fig. 5, lane f) of the same size as that seen in the T_2 through T_8 samples from MB24. Time course and induction results were confirmed by primer extensions using a second primer (E19H3) which gave an expected 103-base product (results not shown). These results located the apparent 5′ end of the *mmg* transcript within the 300-bp region shown to contain σ^E-dependent promoter activity in the *lacZ* reporter strain EUX9403. Moreover, this location of the 5′ end of the transcript is immediately downstream from a sequence with strong similarity to a consensus σ^E promoter (Fig. 6B).

***mmgABCDE* transcription is regulated by *mmgO* and CcpA.** Centered at 22 bp downstream of the putative *mmgABCDE* transcriptional start site is a 14-bp sequence (*mmgO*) that has similarity to a group of *cis*-acting CREs. In cells growing in the presence of glucose or other rapidly metabolizable carbon sources, the CcpA protein, it is thought, binds to the CRE to decrease transcription from specific promoters (17). The best characterized of these operators is *amyO*, which is necessary for repression of the α-amylase gene (*amyE*) in response to glucose (7, 46). Weickert and Chambliss were able to deduce the critical and optimal 14-bp operator sequences by mutagenesis (46). A comparison of *mmgO* with the critical sequence revealed a match of 13 of 14 bp, while a comparison with the optimal sequence revealed a match of 11 of 14 bp (Fig. 6C).

To test for catabolite repression of the *mmg* promoter, we took advantage of the observation that between $T_{0.5}$ and T_1, *B. subtilis* becomes largely refractory to inhibitory effects of glucose on sporulation (25). Since σ^E does not become active until about T_2, there is a window of time during which the addition of glucose does not inhibit sporulation and σ^E is not yet active. We predicted that if glucose was added during this period, σ^E-dependent sporulation genes would be transcribed normally but a σ^E-dependent gene subject to catabolite repression would not be. As a preliminary test of this idea, cultures of a *spoIID-lacZ* fusion strain (EUX9505) and an *mmgE-lacZ* fusion strain (EUX9407) were split at $T_{1.5}$. Glucose was added to half of each culture. Samples for β-galactosidase assays were then taken at T_5. The cultures with no added glucose produced expected levels of β-galactosidase activity. The *spoIID-lacZ* strain with glucose added at $T_{1.5}$ gave a level of β-galactosidase activity that was slightly higher than that given by the sample with no added glucose. The *mmgE-lacZ* fusion, under the same conditions, showed a fivefold reduction in activity compared with the sample containing no glucose (data not shown). This apparent repression of *mmg* transcription encouraged us to further examine the role of the *mmgO* site in this glucose effect.

Three strains were constructed to test the role of *mmgO* in glucose repression of *mmg* transcription. One strain (EUX9506) contained a wild-type *mmg* promoter region, referred to as *mmgO*-WT, fused to the *lacZ* resident in a spe-

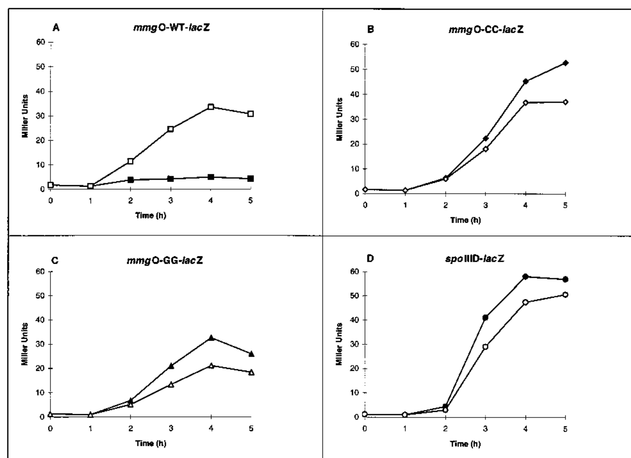

FIG. 7. Effect of *mmgO* on *mmg* promoter activity. Shown is the specific activity of β-galactosidase accumulated in cultures of strains containing *lacZ* fused to an *mmg* promoter with an *mmgO*-WT operator (A), an *mmgO*-CC mutant operator (B), or an *mmgO*-GG mutant operator (C) or to *spoIIID* (D). Specialized SPβ phages, carrying these promoters, were used to transduce *B. subtilis* MB24. The transductants were grown in DSM and at $T_{1.5}$ were split into two flasks. To half of each culture, glucose was added to a final concentration of 1% (filled symbols). The second half of each culture was the no-glucose control (open symbols). Samples were taken for β-galactosidase assays at hourly intervals from T_0 to T_5. β-Galactosidase activity is given in Miller units.

cialized SPβ prophage in an MB24 background. The second strain (EUX9507) was isogenic to the first except that it contained a GT-to-CC dinucleotide change at positions 2 and 3 of *mmgO* (Fig. 6D), producing a promoter region referred to as *mmgO*-CC. The third strain (EUX9508) was also isogenic to the first except that it contained a TC-to-GG dinucleotide change at positions 12 and 13 of *mmgO*, producing a promoter region referred to as *mmgO*-GG (Fig. 6D). A fourth strain (EUX9509), containing an *spoIIID-lacZ* fusion (44), was used as a control for the glucose repression, because *spoIIID* has a well-characterized σ^E promoter with levels of transcriptional activity similar to those of the *mmg* promoter. While it has been noted that a potential CRE lies near the translational start of the *spoIIID* gene (20), this site is deleted in our *spoIIID-lacZ* fusion (11, 44). The four strains were grown in DSM, and samples for β-galactosidase assays were taken at hourly intervals as indicated in Fig. 7. At $T_{1.5}$, cultures were split into two flasks and glucose was added to one flask at a final concentration of 1%. The addition of glucose to EUX9506 (*mmgO*-WT) resulted in a decrease in β-galactosidase activity compared with the no-glucose control (Fig. 7A), with a maximal difference of about sixfold at T_4. The no-glucose samples of the *mmgO* mutant strains EUX9507 (*mmgO*-CC) and EUX9508 (*mmgO*-GG) had β-galactosidase activity levels similar to those of EUX9506 (*mmgO*-WT). Expression of *mmgO*-CC–*lacZ*, *mmgO*-GG–*lacZ*, and *spoIIID-lacZ* was not repressed by glucose addition (Fig. 7B to D). From these data, we conclude that the CRE site is required for glucose repression of the *mmg* promoter during sporulation.

To test whether CcpA is required for repression of *mmg* promoter activity, we constructed two *ccpA* mutant strains containing *lacZ* fusions to the *mmgO*-WT (EUX9511) and *mmgO*-CC (EUX9512) promoter regions. Growth conditions, glucose addition, and sampling were as described above. Strain EUX9511 showed no repression of β-galactosidase expression in response to glucose (Fig. 8). Therefore, CcpA is required for glucose repression of *mmg* promoter activity. Furthermore, β-galactosidase expression was similar in the *ccpA-mmgO* double mutant (EUX9512) (Fig. 8), indicating that the effect of the *mmgO* mutation requires CcpA. This result is consistent with a model in which CcpA binds to *mmgO* to mediate glucose repression of *mmg* promoter activity.

***mmg* deletions have no obvious effects on sporulation or growth.** In an effort to determine the role of the *mmg* operon during sporulation, two deletion strains were created from *B. subtilis* MB24. EUX9412 (Δ*mmgABCD*::*kan*) was created by a chromosomal replacement of *mmgABCD* with the kanamycin resistance cassette from pE19Δ2 (Fig. 1D). EUX9501 (Δ*mmgABCD*::*erm*) was made by a similar replacement using pE19Δ3 (Fig. 1D). The ability of these strains to sporulate was determined by heat and chloroform tests of T_{18} samples of cultures in DSM. The untreated MB24 (parental) and deletion strains gave between 10^8 and 10^9 CFU/ml. The heat- and chloroform-treated samples gave approximately the same numbers, with less than twofold variation between counts for treated and untreated samples. Also, phase-contrast microscopy showed no obvious abnormalities of the deletion strains compared with the parental strain (data not shown). The parental and deletion strains were also tested for the ability to sporulate after resuspension in S1 medium (42) containing acetate, butyrate, or caproate (with and without glycine) and by the Sterlini and Mandelstam growth and resuspension method. The results from the heat and chloroform tests showed no significant variation from the wild type (data not shown).

Parental and *mmg* deletion strains were found to grow similarly on agar and liquid versions of a variety of media, including DSM, LB medium, Sterlini and Mandelstam growth medium, and TSS medium (containing various carbon sources, e.g., glucose, lactate, and glutamate). No growth was observed for either strain when TSS medium was supplemented solely with acetate, propionate, butyrate, or caproate.

DISCUSSION

We have isolated an operon (*mmgABCDE*) that is expressed during sporulation and probably encodes enzymes that are involved in carbon metabolism. The predicted gene products for *mmgA*, *mmgB*, and *mmgC* are similar to enzymes associated with the β-oxidation of fatty acids. The *mmgD* gene product functions as a citrate synthase. *B. subtilis* has two other isozymes of citrate synthases (CitA and CitZ) (22). The reason for multiple forms of citrate synthase is unknown, but one

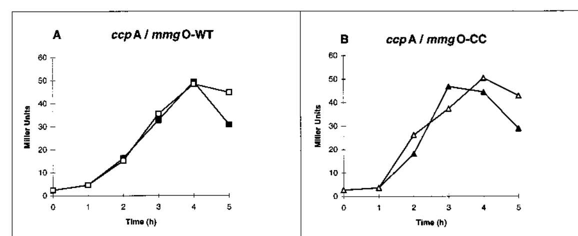

FIG. 8. Effect of *ccpA* on *mmg* promoter activity. Shown is the specific activity of β-galactosidase accumulated in cultures of strains representing a *ccpA* mutant strain containing *lacZ* fused to *mmgO*-WT (A) and *mmgO*-CC mutant (B) promoter regions. Specialized SPβ phages carrying these versions of the *mmg* promoter region were used to transduce strain EUX9510, which has a transposon insertion at *ccpA*. The transductants were grown in DSM, and at $T_{1.5}$ the cultures were split. Glucose was added to a final concentration of 1% to half of each culture (filled symbols), while no glucose was added to the other half (open symbols). Samples were taken for β-galactosidase assays at the times shown. β-Galactosidase activity is given in Miller units.

reason may be that the Krebs cycle involvement in both energy production and the production of metabolic intermediates requires a high degree of regulatory adaptability. This idea is supported by *lacZ* fusion studies of *B. subtilis citA* and *citZ* transcriptional regulation, which show that both genes are negatively regulated by glucose. A combination of glucose and glutamate, however, will synergistically repress *citZ-lacZ* but partially relieve glucose repression for *citA-lacZ* (23). This may allow *B. subtilis* to maintain a basal level of citrate synthase under various conditions. Whereas glucose affects transcription from the *mmg* promoter, the effect of glutamate has not been explored. The enzymatic activities of the three citrate synthases may also be differentially regulated by allosteric effectors. Citrate synthase is an important point for the regulation of the Krebs cycle and is often the rate-limiting step of the cycle (16). Some isozymes of citrate synthase are allosterically inhibited by high-energy compounds such as ATP (12). On the other hand, the *mmgD* product is most homologous to *ctsA* of *B. coagulans*, which is not inhibited by ATP or NADH (39).

An additional, but not exclusive, explanation for multiple citrate synthases is that they may each interact specifically with different groups of proteins. Barnes and Weitzman reported that five sequential enzymes of the Krebs cycle (fumarase, malate dehydrogenase, citrate synthase, aconitase, and isocitrate dehydrogenase) could be isolated as a complex (2). This may allow the efficient transfer of substrates from one enzyme to the next. A mitochondrial form of citrate synthase specifically interacts with a mitochondrial thiolase (43). The presence of a potential thiolase (*mmgA*) among the *mmg* ORFs could indicate that the *mmgD* citrate synthase interacts with this thiolase. The possibility that these enzymes interact leads us to suggest that the *mmg*-encoded enzymes are used to channel fatty acids through the steps of β-oxidation to produce acetyl-CoA, which is condensed by citrate synthase, along with oxaloacetate, into citrate. This pathway could be used to derive energy from internal or external fatty acids during sporulation.

Although we have not identified an environmental condition under which expression of this operon is essential for sporulation, it seems likely that such expression would affect the use of carbon sources by the mother cell, since the operon is transcribed from a promoter used by the mother-cell-specific σ^E RNA polymerase and this transcription is regulated in response to carbon source availability (i.e., catabolite repression). One of the best previously characterized examples of catabolite repression in *B. subtilis* is the α-amylase gene (*amyE*). This gene is negatively regulated by the CcpA protein (17) at a *cis*-acting CRE in response to glucose or another rapidly utilizable carbon source. Mutagenesis of the *amyE* CRE (*amyO*) revealed critical and optimal 14-bp sequences with twofold symmetry (7, 46). In the *mmg* promoter region we observed the presence of a CRE-like sequence, which we called *mmgO* (Fig. 6A and C). To test catabolite repression of *mmg*, we took advantage of the observation of Kiel et al. that glucose may be added to sporulating cultures at T_1 and later, without inhibiting sporulation (25). Our results demonstrate that *mmg* promoter activity is repressed by glucose and that a *cis*-acting CRE (*mmgO*) is necessary for this repression. Since the glucose repression of *mmg* promoter activity also requires CcpA and the levels of *mmg* promoter activity in the presence of glucose in strains that are singly or doubly mutated at *mmgO* and/or *ccpA* are similar, it is likely that CcpA binds *mmgO* to mediate catabolite repression of *mmg* expression.

Our results with the *mmg* promoter provide the first direct evidence for this type of repression of a σ^E-dependent promoter. Indirect evidence suggests that other σ^E-dependent promoters may be repressed by a similar mechanism. The glycogen operon of *B. subtilis* is transcribed from a σ^E-directed promoter (15, 25). Immediately upstream of this promoter we observed a sequence (TATAAGCGCTTTCA) which is homologous to a CRE. Moreover, the published effect on glycogen synthesis of glucose addition to sporulating cultures (25) is consistent with a model in which expression of these enzymes is repressed by glucose. The role of the glucose repression of σ^E-dependent transcription is not known, but we have considered two possibilities. There are reports that mother cells of sporulating *B. subtilis* can be induced to resume vegetative-type division by the addition of rich media (34). Although we have not seen evidence of this phenomenon in our experiments, it is possible that the rapid repression of some σ^E-dependent promoters would facilitate the nutrient-induced reprogramming of the mother cells. A second, and to us more likely, role for the CRE-mediated glucose repression of σ^E-dependent operons such as *mmg* is one of providing a mechanism for controlling gene expression in the mother cell in response to carbon source availability and thereby regulating the metabolic state of the mother cell during development of the endospore.

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