

Regulation of Hexuronate Utilization in *Bacillus subtilis*

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We have identified a locus essential for galacturonate utilization in *Bacillus subtilis*. Genes homologous to *Escherichia coli* and *Erwinia chrysanthemi* glucuronate and galacturonate metabolic genes were found in a cluster consisting of 10 open reading frames (ORFs) in the *B. subtilis* chromosome. A mutant of *B. subtilis* containing a replacement of the second and third ORFs was unable to grow with galacturonate as its primary carbon source. Galacturonate induced expression from a σ^A -dependent promoter, *exuP1*, located upstream from the first ORF. The eighth ORF in this cluster (the *exu* locus) encodes a LacI and GalR homolog that negatively regulated expression from *exuP1*. A 26-bp inverted repeat sequence centered 15 bp downstream from the *exuP1* start point of transcription acted in *cis* to negatively regulate expression from *exuP1* under noninducing conditions. Expression from the *exuP1* promoter was repressed by high levels of glucose, which is probably mediated by CcpA (catabolite control protein A). A σ^E -dependent promoter, *exuP2*, was localized between the second and third ORFs and was active during sporulation.

Bacillus subtilis, an endospore-forming bacterium naturally found in soil (36), is capable of using many compounds as sources of carbon and energy. Some strains of *B. subtilis* can use glucuronate and galacturonate as primary carbon sources (10). Polymethylgalacturonate, or pectin, is a constituent of plant cell walls and thus is found in the soil. Extracellular pectate lyases, produced by *Erwinia chrysanthemi* and many other bacteria, including *B. subtilis*, convert pectin into oligogalacturonate (18, 28). Oligogalacturonate can be metabolized into D-galacturonate by enzymes produced by *Erwinia* sp. (6). In addition, free galacturonate and glucuronate enter *Escherichia coli* and *Erwinia* sp. via the *exuT* transport system. The *uxuA*, *uxuB*, *uxaA*, *uxaB*, and *uxaC* genes of *E. coli* and *E. chrysanthemi* encode enzymes that degrade intracellular galacturonate and glucuronate into 2-keto 3-deoxygluconate (KDG), which is further metabolized to pyruvate and 3-phosphoglycerinaldehyde (Fig. 1). The expression of these genes, including *exuT*, is negatively regulated by the *exuR* gene product in these organisms (18, 35). The ability to degrade pectin plays an important role in the pathogenicity of *Erwinia* sp., which is the causative agent of soft-rot disease in plants (7). Some pectate-lyase-producing species of *Bacillus* also cause soft-rot disease under certain conditions (see reference 30 and references therein).

In a recent report, a cluster of genes encoding putative proteins that are homologous to *E. coli* and *E. chrysanthemi* genes involved in the metabolism of hexuronates (glucuronate and galacturonate) was identified in *B. subtilis* (38). This cluster (*yjmA* through *yjmJ*) is located at 99° in the *B. subtilis* chromosome and includes 10 open reading frames (ORFs) (Fig. 1). These encode putative proteins that appear to be homologs of *uxaC*, *uxuA*, *exuT*, *uxaB*, and *uxaA* in *E. coli* and *E. chrysan-*

themi. This finding suggests that these genes are necessary for the utilization of hexuronates by *B. subtilis*.

In this report, we show that the *B. subtilis* homologs of *E. coli* and *E. chrysanthemi* genes known to be involved in hexuronate metabolism are essential for growth of *B. subtilis* on galacturonate as the sole carbon source. We identified two promoters that direct transcription of these genes. The first promoter, *exuP1*, is a σ^A -dependent promoter located upstream from the first gene. The other promoter, *exuP2*, is a σ^E -dependent promoter located upstream from the third gene. We found that transcription from *exuP1* is induced by galacturonate and is repressed by the product of the *exuR* gene encoded within the *exu* locus. We also found that *exuP1* activity is repressed by glucose.

MATERIALS AND METHODS

Bacterial strains and general cloning procedures. With the exception of ZB307A, all other *B. subtilis* strains in this study are congenic derivatives of the sporulation-proficient strain MB24 (Table 1). *E. coli* DH5 α (Bethesda Research Laboratories, Bethesda, Md.) was used for routine molecular cloning procedures. Luria-Bertani (LB) medium (42) was used for routine growth of *E. coli* and *B. subtilis*. Difco sporulation medium (DSM) was used for sporulation of *B. subtilis* (32). Tris-Spizizen salts minimal liquid medium (TSS) was prepared as described previously (9), except that glucose was omitted. Glucuronic acid and galacturonic acid (Sigma Chemical Co.) were added to TSS to a final concentration of 2.5% (wt/vol). Where necessary, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to liquid and agar medium at a final concentration of 1 mM. Antibiotic-resistant *B. subtilis* strains were selected at the following concentrations: chloramphenicol, 5 μ g/ml; spectinomycin, 100 μ g/ml; kanamycin, 10 μ g/ml; and erythromycin, 1 μ g/ml. Antibiotic-resistant *E. coli* strains were selected at the following concentrations: ampicillin, 75 μ g/ml; and kanamycin, 40 μ g/ml. Restriction endonuclease cleavage reactions and ligations were done as described previously (42). The PCR protocol was done as described in the GeneAmp Kit instructions (Perkin-Elmer Cetus, Norwalk, Conn.). PCR products were either purified with Promega Wizard PCR Prep columns (Promega, Madison, Wis.) or eluted from agarose gels by using QuikPick (Stratagene, La Jolla, Calif.).

Primer extension of RNA. Total RNA was prepared from *B. subtilis* EU8702 harvested during the exponential growth phase in LB broth with or without IPTG, as described previously (2, 37). Total RNA from strains MB24 and EUKM9804 was prepared from cells grown in DSM broth or DSM plus 2.5% (wt/vol) galacturonate as previously described (2, 37) and harvested at the times indicated. The primer E20BAM (5'-CCAAACGGATCCTTTGCTTCTTCAGC TG-3'), complementary to nucleotides +102 to +74 relative to the mapped start point of transcription from *exuP2*, was used in primer extension reactions of that promoter. The primer YjmExt3 (5'-CATAATTGTGATAGAGGCTGACAGC G-3'), complementary to nucleotides +119 to +93 relative to the mapped start

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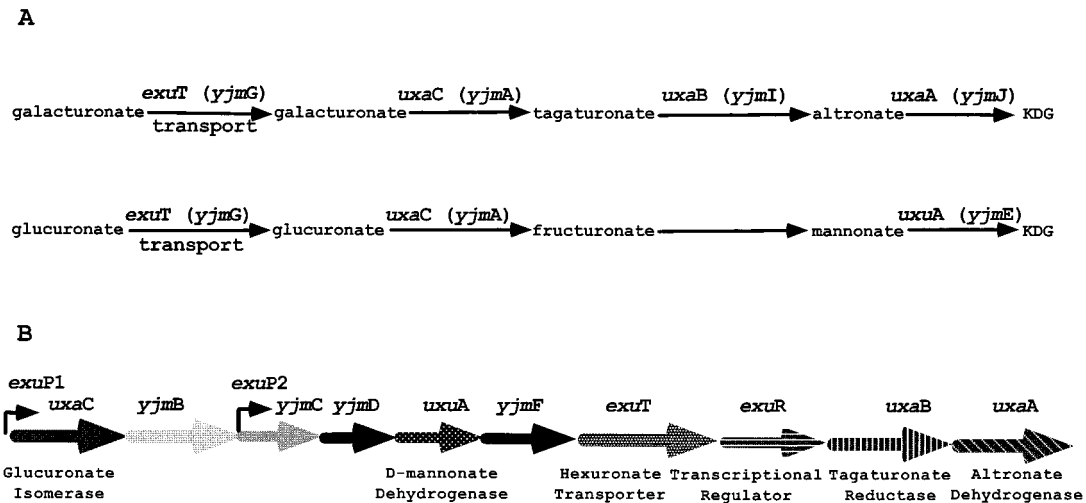


FIG. 1. Hexuronate metabolism and the *exu* locus. (A) Shown are the pathways of glucuronate and galacturonate utilization in *E. coli*, *Erwinia* sp. (19), and probably *B. subtilis*. *E. coli* and *Erwinia* sp. genes that catalyze the different steps are listed to the left of the names of *B. subtilis* genes as designated in the SubtiList database (29). The gene products are as follows: *exuT*, aldohexuronate transport system; *uxaC*, uronate isomerase; *uxaB*, altronate oxidoreductase; *uxuB*, mannonate oxidoreductase; *uxaA*, altronate hydrolase; *uxuA*, mannonate hydrolase. (B) Map of the *exu* locus in *B. subtilis*. Bent arrows show the indicated transcriptional start sites. The straight arrows indicate the relative length and orientation of the genes. Putative gene products are listed beneath the straight arrows. Genes of unknown function are labeled according to the nomenclature in the SubtiList database (29).

point of transcription of *exuP1*, was used in primer extension reactions of that promoter. Primers were end labeled with [γ - 32 P]dATP by using T4 polynucleotide kinase. Approximately 10 ng of labeled primer was annealed to 50 μ g of total RNA from *B. subtilis* at 55°C. Primer extension reactions were carried out with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals). The same oligonucleotides were used in parallel to prime sequencing reactions so that the size of the transcript could be read directly from the sequencing ladder.

Mutagenesis of *exuP1* and *exuO*. Mutagenesis was carried out in vitro by using a multistep PCR procedure described by Cormack (8). The first step was PCR amplification of MB24 chromosomal DNA by using primer YjmA2BamUS (5'-GCGTAAACATTGGATCCCTCAAAAAAGAGATTGATCCC-3'), which contains sequence changes to create a *Bam*HI site (underlined bases), and a reverse primer that overlapped the region to be mutagenized, which contained the appropriate base substitutions. The second step was PCR amplification from MB24 chromosomal DNA with YjmA2HindDS (5'-GGTCACCATACAGCCAA GCTTCCGTGATG-3'), which contains a base substitution to create a *Hind*III site (underlined), and a forward primer that overlapped the reverse primer and contained the appropriate base substitutions. The PCR products from both reactions were purified and used in a reaction with the two outside primers,

YjmA2BamUS and YjmA2HindDS, so the entire 290-bp region was amplified, including the base substitutions. These PCR products were digested and cloned into *Bam*HI- and *Hind*III-digested pTKlac (22). The resulting constructs placed the various derivatives of the promoter in front of the promoterless derivative of the *lacZ* gene and adjacent to the *trpA* terminator in order to prevent expression from upstream promoters. The plasmids were sequenced to ensure that the proper mutations were present. The pTK constructs were linearized with *Sca*I and crossed into an SP β prophage. The SP β phage was induced from the chromosome of each strain by heat, generating phage lysates of the wild type and mutant *exuP1-lacZ* fusions. These specialized transducing phage were used to transduce strains MB24, EUKM9804, EUKM9810, and EUX9510 to chloramphenicol resistance. The resulting strains were grown to either mid-exponential phase, and samples were taken from each culture. β -Galactosidase activity assays were performed with the culture samples as described previously (23). In each case, two independent transductants were analyzed, and the level of β -galactosidase produced by each varied by less than 13%. Miller units were calculated by the formula described by Miller (27): 1 unit = 1.000 \times [optical density at 420 nm (OD₄₂₀)/(time \times volume \times OD₆₀₀)].

Insertional disruption of *exuR*. The primers YjmHindUS (5'-GCCATAAGT GAGGATTACAACGTTTATGTGCGGC-3') and YjmHBamDS (5'-CCGAA

TABLE 1. Characteristics of the *B. subtilis* strains used in this study

Strain	Genotype and phenotype and/or description	Source or reference
MB24	<i>trpC2 metC3</i>	P. Piggot (33)
ZB307A	SP β <i>c2del2::Tn917::pSK10Δ6 MLS^r</i>	Laboratory stock; P. Zuber
CU1050	<i>leuA8 metB5 thrA5 sup-3 pla-1</i> SP β ^s	Bacillus Genetic Stock Center
CU1051	<i>leuA8 metB5 thrA5 sup-3 pla-1</i> SP β ^s (pTV17)	Laboratory stock (1)
EU101	<i>spoIIIGBD::erm chr::Tn917ΩHU160::Pspac-spoIIIGB</i> Pm ^r Em ^r	Laboratory stock (43)
EU8702	<i>trpC2 pheA1 spoIIIGBD::erm</i> (pDG180) Pm ^r Em ^r	Laboratory stock (1)
EUX20	EU101 (SP β E20) Pm ^r Em ^r Cm ^r	This study
EUX9510	<i>trpC2 metC3 gra26::Tn917</i> Em ^r	Laboratory stock (5)
EUKM9801	<i>trpC2 metC3 yjmB::pE20::kan</i> Km ^r	This study
EUKM9802	<i>trpC2 metC3</i> SP β <i>exuP1 wt-lacZ</i> MLS ^r	This study
EUKM9803	<i>trpC2 metC3</i> SP β <i>exuP1(O₁)-lacZ</i> MLS ^r	This study
EUKM9804	<i>trpC2 metC3 exuR::pΔExuR</i> Sp ^r	This study
EUKM9805	<i>trpC2 metC3 exuR::pΔExuR</i> SP β <i>exuP1 wt-lacZ</i> MLS ^r Sp ^r	This study
EUKM9806	<i>trpC2 metC3 exuR::pΔExuR</i> SP β <i>exuP1(-1/-2GG)-lacZ</i> MLS ^r Sp ^r	This study
EUKM9807	<i>trpC2 metC3 exuR::pΔExuR</i> SP β <i>exuP1(-7/-8GG)-lacZ</i> MLS ^r Sp ^r	This study
EUKM9808	<i>trpC2 metC3 exuR::pΔExuR</i> SP β <i>exuP1(O₁)-lacZ</i> MLS ^r Sp ^r	This study
EUKM9809	<i>trpC2 metC3 gra26::Tn917</i> SP β <i>exuP1(O₁)-lacZ</i> MLS ^r Em ^r	This study
EUKM9810	<i>trpC2 metC3 gra26::Tn917 exuR::pΔExuR</i> Sp ^r Em ^r	This study
EUKM9811	EUKM9810 SP β <i>exuP1(O₁)-lacZ</i> MLS ^r Sp ^r Em ^r	This study

TABLE 2. Doubling times in TSS minimal medium

Strain	Doubling time (h) in:	
	TSS-glucuronate	TSS-galacturonate
MB24	>24	2.5
EUKM9804	6.33	ND ^a
EUKM9801	ND	>24

^a ND, not determined.

TATCATCAGGATCCCGCAGGCCCTTTGCG-3'), which contain engineered *Hind*III and *Bam*HI sites, respectively (underlined bases), were used to amplify an internal fragment of *yjmH* (*ExuR*). This fragment was ligated to *Bam*HI-*Hind*III-digested pUS19. pUS19 (obtained from W. Haldenwang) is a pUC19 derivative containing a spectinomycin resistance cassette (3). The resulting plasmid, pΔ*ExuR*, was used to transform wild-type MB24 to Sp^r. Spectinomycin-resistant transformants arose from a single reciprocal recombination event between the homologous *exuR* sequences in the plasmid and the chromosome (Campbell-type integration), creating a truncated version of the gene.

Km^r cassette replacement within the *exu* operon. Chromosomal DNA isolated from strain EUX20 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 pTVE20. Cleavage of pTVE20 with *Sa*I released a 3-kb chromosomal fragment, which was cloned into pUS19 to create pUSE20. pUSE20 was digested with *Cla*I and subsequently filled in with Klenow DNA polymerase, creating blunt ends. A 1.5-kb Km^r cassette was released from pKD102 (obtained from W. Haldenwang) with *Sma*I and cloned into the blunt ends created in pUSE20. The resulting plasmid, pE20::Kan, was digested with *Sca*I and used to transform MB24 to Km^r. The resulting transformant, EUKM9801, contains replacement of DNA sequence spanning ORF2 and ORF3 by a Km cassette resulting from a double-crossover recombination event between homologous sequences within the chromosome and pUSE20::Kan.

Assay for glucose repression of *exuP1*. Strain EUKM9810 (ΔCcpA Δ*ExuR*) was made by using chromosomal DNA isolated from strain EUKM9804 to transform EUX9510 to Sp^r. Strains tested for glucose repression were grown to the exponential phase in DSM supplemented with 1% glucose. Samples of the cultures were taken and assayed for β-galactosidase activity as described previously. Because strain EUX9510 gives a background level of β-galactosidase activity, the specific activity in EUX9510 was subtracted when any strains derived from EUX9510 were assayed.

RESULTS

Genes in the *exu* cluster are essential for galacturonate utilization. To determine whether the *exu* locus was necessary for the utilization of galacturonate, we isolated a mutant strain (EUKM9801) in which much of *yjmB* and *yjmC* was replaced with a Km^r cassette containing a transcriptional terminator. Wild-type MB24 was able to grow in liquid TSS-galacturonate minimal media (Table 2) and on TSS-galacturonate agar plates (data not shown). In contrast, strain EUKM9801 was unable to grow in minimal medium with galacturonate as the primary carbon source, indicating that at least one of the genes in this locus is necessary for wild-type *B. subtilis* to utilize galacturonate (Table 2). Therefore, we named the hexuronate utilization genes in the same manner as their *E. coli* and *E. chrysanthemi* homologs as indicated in Fig. 1.

Genes involved in utilization of galacturonate are transcribed from a σ^A-dependent promoter, *exuP1*. We used primer extension analysis to locate the promoter of the *exu* locus. In wild-type *E. coli* and *E. chrysanthemi*, genes of the hexuronate system are only expressed when an appropriate intermediate in the pathway (galacturonate, glucuronate, tagaturonate, or fructuronate) is present. Therefore, to identify the *exu* transcript, total RNA was isolated from MB24 grown in DSM or DSM supplemented with 2.5% galacturonate, an inducer of *uxuA* expression in *E. chrysanthemi* and of the whole hexuronate regulon in *E. coli* (18, 35). A single extension product was produced from the RNA isolated from cells grown with galacturonate. The size of this transcript suggests that this

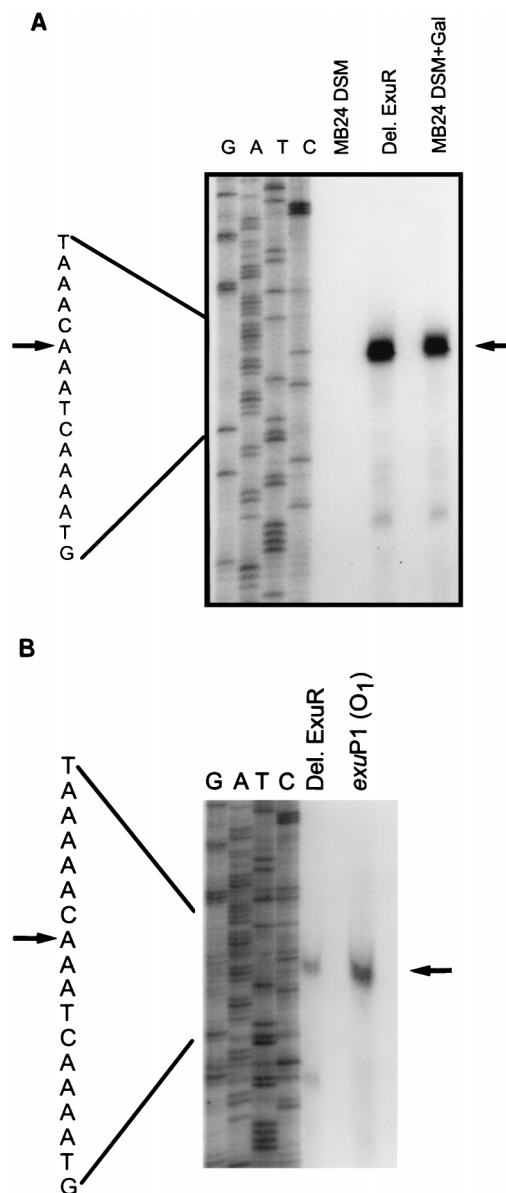


FIG. 2. (A) Mapping the transcriptional start site of the *exuP1* promoter. An oligonucleotide complementary to a region in the *uxaC* gene was used to prime cDNA synthesis from total RNA. RNA was prepared from a mid-log-phase culture of MB24 grown in DSM (MB24 DSM), from MB24 grown in DSM supplemented with 2.5% galacturonate (MB24 DSM+Gal), or from a mid-log-phase culture of EUKM9804 grown in DSM (Del. *ExuR*). The same oligonucleotide was used in dideoxy sequencing of plasmid pTK-*exuP1* (wild type). Shown is an autoradiograph of the primer extension and sequencing products run on a 6% polyacrylamide-urea gel. The sequence is labeled as the reverse complement for ease of comparison with other sequence data. The arrows indicate the location of the major primer extension products. (B) Mapping of the transcriptional start site of *exu* in strain EUKM9803. RNA was prepared from a mid-log-phase culture of EUKM9804 (Del. *ExuR*) or EUKM9804 [*exuP1* (*O*₁)-*lacZ*] grown in DSM. The same oligonucleotide used in panel A was used to prime cDNA synthesis from these RNAs and to create the sequencing ladder from pTK-*exuP1* (wild type). Shown is an autoradiograph of the primer extension and sequencing products run on a 6% polyacrylamide-urea gel. The sequence ladder is labeled as in panel A.

cluster of genes is transcribed from a promoter that starts at an A residue 53 bp upstream from the initiation codon for *uxaC* (Fig. 2). The apparent 5' end of the transcript was immediately downstream from a sequence with strong similarity to a

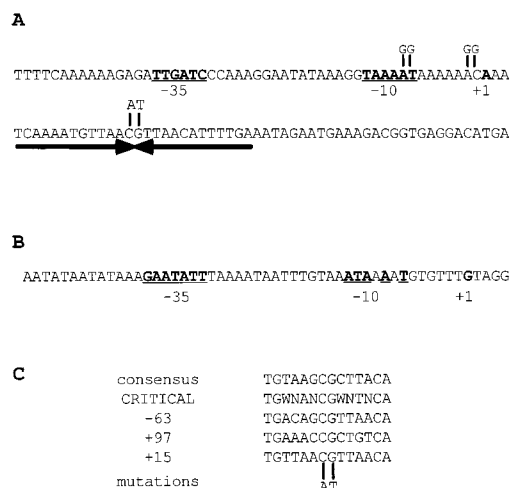


FIG. 3. (A) Promoter region of *exuP1*. Shown is the sequence of the non-transcribed strand. The sequences corresponding to the -35 and -10 regions recognized by σ^A RNA polymerase as well as the transcriptional start site (+1) are underscored and labeled. The AT-to-GG change at positions -7 and -8 indicated created *exuP1* ($-7/-8GG$). The AC-to-GG change at positions -1 and -2 indicated *exuP1* ($-1/-2GG$). The arrows indicate the 26-bp perfect inverted repeat designated *exuO*. The CG-to-AT change indicated in this repeat created *exuP1* (O_1). (B) Promoter region of *exuP2*. The predicted -35 and -10 recognition sites for σ^E RNA polymerase are underscored and labeled. The mapped transcriptional start site is indicated (+1). (C) Comparison of sequences in the *exuP1* region with consensus and critical (genetically defined as most essential bases) versions of a CRE. The nontranscribed strand is shown. The position of the center of the sequence relative to the start point of transcription of *exuP1* is indicated to the left of the sequence. The ambiguity codes are W = A or T and N = A, C, G, or T. The *exuP1* (O_1) CG-to-AT mutation made at positions 7 and 8 in the +15 sequence is indicated.

consensus σ^A -dependent promoter (13). To test whether this sequence functioned as a promoter for the *exu* genes, we constructed three transcriptional fusions of the putative σ^A promoter to a promoterless derivative of *lacZ*. The first of these constructs contained a 262-bp DNA fragment that included the wild-type sequence of the promoter, including 52 bp upstream and 210 bp downstream from the putative start point of transcription. Two additional constructs contained otherwise identical DNA fragments with a 2-bp GG substitution at positions -7 and -8 , or a 2-bp GG substitution at positions -1 and -2 relative to the mapped transcriptional startpoint (Fig. 3). An SP β specialized transducing phage was used to carry these constructs into the chromosome of strain EUKM9804 (Δ ExuR). The resulting strains, EUKM9805 (wild-type [wt] *exuP1*), EUKM9806 ($-1/-2GG$ *exuP1*), and EUKM9807 ($-7/-8GG$ *exuP1*), were assayed for β -galactosidase activity. Mutations at -7 and -8 in the hypothetical -10 recognition sequence of the σ^A promoter severely reduced the amount of β -galactosidase synthesized from the promoter when compared to those of the wild type and $-1/-2$ GG versions of the promoter fusion (Fig. 4). This result indicates that the sequence at position -7 to -8 is required for promoter activity. We refer to this promoter as *exuP1*.

Galacturonate and glucuronate induce expression from *exuP1*. Genetic studies of hexuronate metabolism in *E. coli* and *E. chrysanthemi* show that intermediates in galacturonate and glucuronate metabolism induce expression of the genes involved in their metabolism (18, 31, 40). Our primer extension results (Fig. 2A) suggested that *exuP1* activity is induced by galacturonate. To test whether glucuronate or galacturonate induces expression from *exuP1*, we grew strain EUKM9802, which harbors an *exuP1-lacZ* transcriptional fusion, in DSM (a

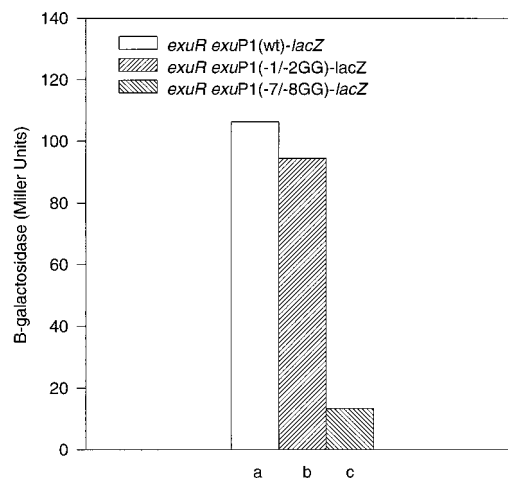


FIG. 4. Effect of promoter mutations on *exuP1* activity. Shown is the specific activity of β -galactosidase (average of four samples) accumulated in cultures of EUKM9805 [a; Δ ExuR with *exuP1-lacZ*], EUKM9806 [b; Δ ExuR with *exuP1* ($-1/-2GG$)-*lacZ*], and EUKM9807 [c; Δ ExuR with *exuP1* ($-7/-8GG$)-*lacZ*] grown in DSM. β -Galactosidase activity is given in Miller units.

non-catabolite-repressing medium) or DSM supplemented with glucuronate or galacturonate. We monitored β -galactosidase activity accumulation in EUKM9802 grown in these different conditions. Galacturonate induced *exuP1* activity 20-fold, whereas glucuronate induced *exuP1* activity only 5-fold (Fig. 5).

Expression from *exuP1* is repressed by a regulator encoded within the operon. In *E. coli* and *E. chrysanthemi*, *exuR* regulates expression of all the genes necessary for the metabolism of glucuronate and galacturonate into KDG (18, 35). The eighth ORF in the *exu* locus is homologous to known transcriptional regulators of the LacI-GalR family. To test the hypothesis that ORF8 regulates expression from *exuP1*, we isolated a strain that harbors an insertional disruption of ORF8 (EUKM9804 [see Materials and Methods]), which we refer to as ExuR. We hypothesized that if this gene encodes a regulator for the

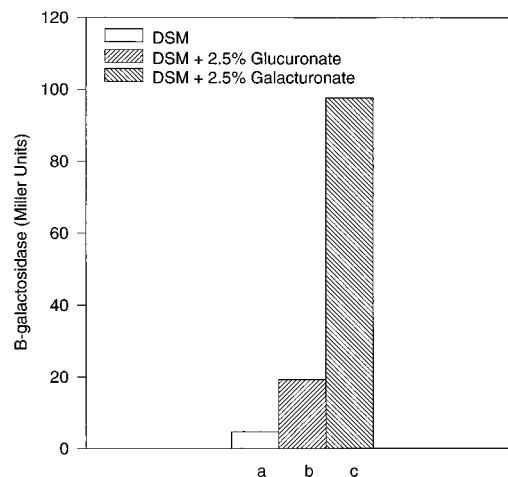


FIG. 5. Induction of *exuP1* expression by glucuronate and galacturonate. Shown is the specific activity of β -galactosidase accumulated in cultures of EUKM9802 (MB24 with *exuP1-lacZ*) grown in DSM (a), DSM supplemented with 2.5% glucuronate (b), or DSM supplemented with 2.5% galacturonate (c). β -Galactosidase activity is given in Miller units.

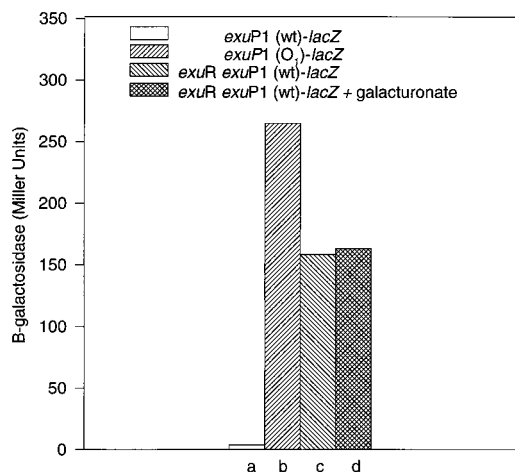


FIG. 6. Effect of regulator and operator mutations of *exuP1* expression. Shown is the specific activity of β -galactosidase accumulated in cultures of EUKM9802 (a; MB24 with *exuP1-lacZ*), EUKM9805 (c; Δ ExuR with *exuP1-lacZ*), or EUKM9803 [b; MB24 with *exuP1(O₁)-lacZ*] grown in DSM. Samples were also taken from EUKM9802 (MB24 with *exuP1-lacZ*) grown in DSM plus 2.5% galacturonate (d). β -Galactosidase activity is given in Miller units.

operon, then constitutive expression of the operon may result from disruption of this gene. We introduced the *exuP1-lacZ* fusion into MB24 and EUKM9804 to determine the effect of the ORF8 mutation on expression from *exuP1*. High-level constitutive expression of *exuP1-lacZ* resulted from the ExuR mutation in EUKM9805 (Fig. 6).

The increased expression from *exuP1* in strain Δ ExuR (EUKM9804) was confirmed by primer extension analysis. Similar extension products were produced from RNA isolated from EUKM9804 grown under noninducing conditions and from RNA isolated from MB24 grown in DSM plus 2.5% galacturonate (Fig. 2A). We conclude that ORF8 encodes a negative regulator of *exuP1*, and we refer to the gene product of ORF8 as ExuR, for *exu* repressor.

In *E. coli*, glucuronate and fructuronate induce expression of the whole hexuronate system, which includes the *exu* regulon and the *uxuAB* operon, whereas galacturonate and tagaturonate induce expression of *uxaCA*, *uxaB*, and *exuT* (17, 39). In *E. chrysanthemi* 3937 and B374, galacturonate induces expression of the *exu* regulon, whereas glucuronate induces expression of *uxuA*, but not *exuT* (necessary for its transport) or *uxuB* (specific for glucuronate metabolism). Consequently, these strains of *E. chrysanthemi* cannot utilize glucuronate as a carbon source for growth. *E. chrysanthemi* mutants that can grow on glucuronate have been isolated. One class of these mutants disrupt *exuR*, the regulator of the *exu* regulon. The *exuR* mutants express *exuT* and *uxaCBA* constitutively (18). We determined that MB24 cannot grow in TSS-glucuronate minimal medium (Table 2). However, strain EUKM9804, in which *exuR* is disrupted, was able to grow with glucuronate as the sole carbon source (Table 2).

***exuO* is involved in repression of *exuP1*.** Centered 15 bp downstream from the putative *exuP1* transcriptional start site is a 26-bp perfect inverted repeat sequence. To test whether this sequence functions as an operator for *exuP1* regulation, we constructed an *exuP1* transcriptional fusion to *lacZ* that contains a 2-bp AT substitution in the center of the inverted repeat, creating *exuP1 (O₁)-lacZ* (Fig. 3). We measured β -galactosidase accumulation in strains EUKM9802 and EUKM9803, in which the wild-type *exuP1-lacZ* and *exuP1 (O₁)-lacZ*

fusions were introduced into the MB24 background (Fig. 6). Expression from *exuP1 (O₁)-lacZ* in an otherwise wild-type strain was 20-fold higher than expression of *exuP1-lacZ* (Fig. 6). Primer extension analysis showed that the *exuP1 (O₁)-lacZ* mutation had not changed the start point of transcription (Fig. 2B). Therefore, it is unlikely that the mutation had created a new promoter. Since *exuP1 (O₁)-lacZ* was expressed similarly in the ExuR mutant (EUKM9808) and MB24 (EUKM9803) (Fig. 7, lanes a and c), *exuO* appears to be required for ExuR to exert its negative effect on *exuP1*-directed transcription. We conclude that this sequence is a *cis*-acting regulatory site that controls *exuP1* expression, and we refer to it as *exuO*, for *exu* operator. The *exuO* mutation may also have a second effect, since expression of *exuP1 (O₁)-lacZ* was higher than that of *exuP1-lacZ* when ExuR was mutated or when galacturonate was added.

Expression from *exuP1* is repressed by glucose. Expression of many genes that encode enzymes involved in metabolizing alternative carbon sources is repressed in the presence of carbon sources that are metabolized more rapidly (15, 41). Centered at bp -63 , $+15$, and $+97$ relative to the *exuP1* start point of transcription are 14-bp sequences that have similarity to a group of *cis*-acting catabolite-responsive elements (CREs). Based on its homology to known CREs, Rivolta et al. suggested that the inverted repeat centered at $+15$ from the start point of transcription could mediate catabolite repression of this operon (38). To test for catabolite repression of the *exuP1* promoter, we examined *exuP1-lacZ* expression in strain EUKM9805 (Δ ExuR *exuP1-lacZ*) grown in DSM and DSM supplemented with 1% glucose. This fusion showed an 8.7-fold reduction in activity when grown in DSM containing glucose compared to that with DSM alone (Fig. 7). We then tested whether the DNA sequence around $+15$ functioned as a CRE. To test whether this sequence had a role in glucose repression of *exuP1*, we grew EUKM9808 [Δ ExuR *exuP1 (O₁)-lacZ*] in DSM and DSM plus 1% glucose. The 2-bp O_1 TA mutation in the *exuO* region of this transcriptional fusion alters the sequence of the CRE-like element; therefore, this mutation would be expected to prevent CRE function. β -Galactosidase activity from strain EUKM9808 was 7.4-fold lower when

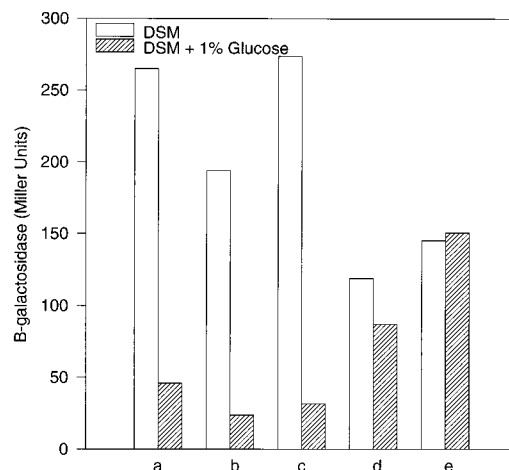


FIG. 7. Glucose repression of *exuP1*. Shown is the specific activity of β -galactosidase accumulated in cultures of EUKM9803 [a; MB24 with *exuP1(O₁)-lacZ*], EUKM9805 (b; Δ ExuR with *exuP1-lacZ*), EUKM9808 [c; Δ ExuR with *exuP1(O₁)-lacZ*], EUKM9809 [d; Δ CcpA with *exuP1(O₁)-lacZ*], and EUKM9811 (e; Δ CcpA Δ ExuR with *exuP1-lacZ*) grown in DSM or DSM supplemented with 1% glucose. β -Galactosidase activity is given in Miller units.

grown in the presence of 1% glucose (Fig. 7). Therefore, *exuO* did not have a large effect on glucose repression of *exuP1* under these conditions.

To test whether the CcpA protein mediated the glucose repression of *exuP1*, we introduced the *exuP1* (O_1)-*lacZ* fusion into strain EUX9510, which contains a Tn917 insertion in the gene encoding CcpA, creating strain EUKM9809. In addition, we introduced the wild-type *exuP1-lacZ* fusion into strain EUKM9810, which contains the *exuR* disruption and the *ccpA* disruption, to create strain EUKM9811. EUKM9809 and EUKM9811 were grown in DSM and DSM plus 1% glucose. The levels of β -galactosidase activity in strain EUKM9809 grown in DSM plus 1% glucose were only slightly lower (approximately 1.4-fold) than those obtained when grown in DSM, whereas β -galactosidase accumulated to slightly higher levels in strain EUKM9811 when grown in DSM plus 1% glucose compared to DSM alone. Therefore, it appears that the *ccpA* mutation partially relieves the glucose repression of *exuP1* promoter activity. However, the *ccpA* mutation also reduced *exuP1* activity in cells grown in DSM that was not supplemented with glucose. These results raise the possibility that CcpA may have indirect effects on *exuP1* activity, possibly by affecting cellular metabolism. Therefore, it is not possible to conclude from these experiments whether CcpA plays a direct role in glucose repression of *exuP1* activity.

A σ^E -dependent promoter, *exuP2*, is located between *yjmB* and *yjmC*. We previously described a screen in which we isolated σ^E -dependent promoters from a library of random chromosomal fragments of *B. subtilis* which were fused to a promoterless version of *lacZ* (1, 2, 16). An isolate from this screen, strain EUX20, contained a 3-kb chromosomal segment of library DNA with apparent σ^E -dependent promoter activity. To further characterize this isolate, EUX20 was grown under sporulating conditions with P_{spac} -*sigE* (IPTG-inducible form of the gene encoding σ^E) either induced or uninduced. β -Galactosidase activity appeared by the second hour after the start of sporulation (T_2) and was maximal at about T_5 (data not shown) in the presence of IPTG. In the absence of IPTG, P_{spac} -*sigE* was not induced, and β -galactosidase activity was not detected at any time point tested (data not shown). This result demonstrated that this transcriptional activity is dependent on σ^E and is restricted to the stationary phase when the cells are forming endospores. To localize the promoter activity, several subclones of the original 3-kb fragment were used to make transcriptional fusions to a promoterless derivative of *lacZ* and tested for accumulation of β -galactosidase activity throughout sporulation. Using this method, we were able to localize promoter activity with a profile identical to that of promoters expressed by σ^E to a 300-bp region of DNA (data not shown).

Primer extension analysis was performed to locate the promoter more precisely. RNA samples isolated from *B. subtilis* MB24 harvested at the indicated times during growth and sporulation in DSM liquid were used as the template for the first set of extension reactions. In this way, we mapped the 5' end of the *exuP2* transcript to a guanine residue 55 bp upstream from the initiation codon for *yjmC* (Fig. 8). We also noted that *exuP2*-specific transcript begins to accumulate by 2 h into sporulation and that the accumulated product is maximal at about 4 h of sporulation (Fig. 8, lanes i and j). This is reminiscent of the pattern of expression seen with other σ^E -dependent genes (1, 2, 25). We also did primer extension analysis of total RNA isolated from mid-exponential-phase broth cultures of *B. subtilis* EU8702. Strain EU8702 has a deletion of the chromosomal *sigE* allele, but carries a plasmid-borne P_{spac} -*sigE* allele producing an IPTG-inducible active form of σ^E . RNA from exponential-phase cells, grown without

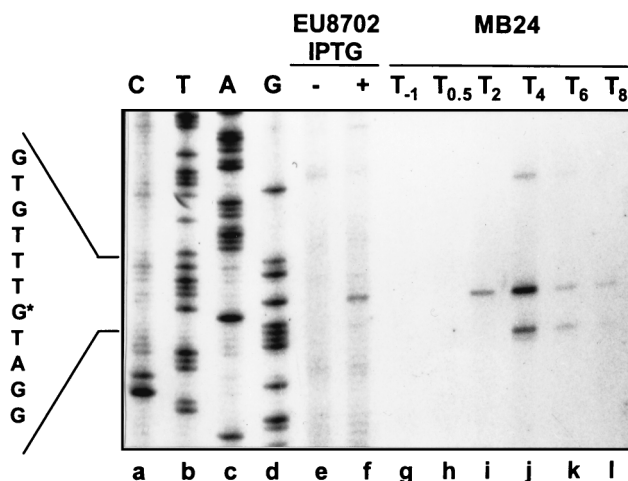


FIG. 8. Mapping of the transcriptional start site of the *exuP2* promoter. An oligonucleotide primer (E20BAM), complementary to a region within the *yjmC* gene was used to prime cDNA synthesis from total RNA. RNA was prepared from mid-log-phase LB medium cultures of *B. subtilis* EU8702, grown in the 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 the exponential growth phase 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 g to l. The same oligonucleotide (E20BAM) was used for dideoxy sequencing of plasmid pUSE20 (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, and the asterisk indicates the putative start of the *exuP2* transcript. The longer extension product seen in lane j was not reproducibly found, and its origin is unknown.

inducer, gave no extension product equivalent to that described above (Fig. 8, lane e). RNA from an exponential-phase culture induced with IPTG showed a single extension product (Fig. 8, lane f) of the same size as that seen in the T_2 -through- T_8 samples from MB24. These results located the apparent 5' end of the *exuP2* transcript between the second and third ORFs in the *exu* locus, within the 300-bp region believed to contain σ^E -dependent promoter activity. Moreover, this location of the 5' end of the transcript is immediately downstream from a sequence with similarity to a consensus σ^E promoter (16).

DISCUSSION

We have identified a locus, *exu*, in *B. subtilis* that is required for utilization of galacturonate as a source of carbon. Since plant by-products that would be found in the soil can be converted into galacturonate, the metabolic pathway responsible for galacturonate utilization may be very important when *B. subtilis* is growing in this environment. Although genes predicted to have a role in both glucuronate and galacturonate utilization are found in the *exu* locus, MB24 grew very poorly in TSS-glucuronate minimal medium. One explanation for the poor growth on glucuronate compared to that on galacturonate is that glucuronate is a poor inducer of genes required for its transport and metabolism, whereas galacturonate is a much better inducer of expression of the *exu* locus. Constitutive expression of the *exu* locus in the Δ ExuR strain enabled *B. subtilis* to grow in TSS-glucuronate minimal media, supporting this hypothesis. Hugouvieux-Cotte-Pattat and Robert-Baudouy proposed the same explanation for the inability of some

E. chrysanthemi strains to utilize glucuronate for growth despite the presence of the genes necessary for glucuronate uptake and metabolism in those strains (18, 20).

Four genes in the *exu* locus encode proteins that may not be directly involved in the metabolism of galacturonate or glucuronate. *yjmB* shares homology with the *uidB* gene of *E. coli*, which encodes a β -glucuronide transporter (GenBank accession no. 2507412). In *E. coli*, β -glucuronidase, encoded by the *uidA* gene, converts β -glucuronide into glucuronate, which can be metabolized by the pathway described above (26). No β -glucuronidase has been described in *B. subtilis*, and it is not known whether *B. subtilis* is capable of utilizing β -glucuronides as a carbon source. The predicted protein encoded by *yjmF* is homologous to several oxidoreductases and has been proposed as a potential mannonate oxidoreductase (38), part of the glucuronate catabolic pathway. However, we found that *yjmF* shared more homology with the *kduD* genes of *E. coli* and *E. chrysanthemi* than with mannonate reductases from *E. coli* and *Erwinia* sp. KduD is involved in the intracellular metabolism of pectin by-products and converts 2,5-diketo-3-deoxygluconate into KDG (29). The other two genes in the *exu* locus, *yjmC* and *yjmD*, encode proteins that are homologous to dehydrogenases. YjmC is 39% identical to malate dehydrogenase from *Methanococcus jannaschii* (GenBank accession no. 2497860). Its function is unknown; however, it could, in theory, play a role in gluconeogenesis, or it could supplement the activity of the primary malate dehydrogenase, the product of *citH* (21), in the citric acid cycle. *yjmD* is similar to alcohol dehydrogenases, but its function is also unknown.

The predicted protein encoded by *exuR* is a member of the LacI-GalR family of transcriptional regulators. A hallmark of these proteins is a helix-turn-helix motif that has been shown in some cases to make sequence-specific contacts with the major groove of the DNA helix (4). Computer analysis of the ExuR amino acid sequence strongly suggests the presence of a helix-turn-helix motif that may be involved in interaction with DNA (data not shown). The amino acid sequence similarity shared between proteins in this family extends throughout the DNA binding motif, and it is thought that these structural similarities account for the fact that many of the operator sites bound by these proteins are similar (11). LacI and GalR proteins bind as homodimers to operator sites that have dyad symmetry. A candidate operator site in the *exuP1* promoter region is the 26-bp perfect inverted repeat that is centered at +15 relative to the transcriptional start site and is similar to operator sites shown to be bound by LacI and GalR proteins. We have shown that mutations in this sequence result in constitutive expression from *exuP1*. Therefore, although we have not demonstrated directly that ExuR binds to the *exuO* sequence, our genetic data suggest that this may be the mechanism by which ExuR regulates expression from the *exuP1* promoter. We have not shown that the entire *exu* locus is transcribed from the *exuP1* promoter, forming a single operon. However, reverse transcription-PCR analysis of RNA from cultures of wild-type cells grown in the presence or absence of inducer, and from the *exuR* mutant, showed that transcription of the first and seventh ORFs is coordinately regulated by both galacturonate and ExuR (data not shown).

Many genes involved in the utilization of alternative carbon sources are subject to catabolite control and are repressed by more rapidly metabolized carbon sources (33). In gram-positive bacteria, the CcpA protein plays a major role in catabolite control (14). In the presence of glucose or other rapidly metabolizable carbon sources, CcpA interacts with *cis*-acting CRE sites in the DNA. Mutagenesis of the *amyO* CRE site enabled Weikert and Chambliss to deduce the optimal and critical

14-bp operator sequences (Fig. 3) (44). Not surprisingly, we found that, in the presence of glucose, expression of the *exu* genes was repressed (Fig. 7). In the proximity of the *exuP1* promoter, there are three 14-bp sequences that match perfectly with the 14-bp critical CRE sequence. Any of these could potentially mediate the observed glucose repression. One of these sequences is overlapped by the 26-bp inverted repeat designated *exuO* that is involved in ExuR repression of *exuP1* in the absence of inducer. This observation raises the possibility that more than one regulator is binding to this sequence; i.e., both CcpA and ExuR may interact with this sequence. However, we have shown that a 2-bp substitution in this sequence that would be expected to abolish CcpA binding had only a small effect on glucose repression of *exuP1*. Therefore, CcpA binding to the *exuO* region is probably not solely responsible for glucose repression. Since CcpA is probably necessary for glucose repression of *exuP1*, but the inverted repeat centered at +15 is not essential for catabolite regulation, CcpA may interact with other potential CRE sequences near *exuP1*.

We have demonstrated that, in addition to transcription from the *exuP1* promoter during growth, another promoter, *exuP2*, is transcribed by σ^E RNA polymerase during sporulation. It is not known whether there are any conditions in which this locus is essential for sporulation. Strain EUKM9801 sporulated efficiently in DSM (data not shown). σ^E is involved in the transcription of other genes with metabolic functions (5, 12, 24). One possibility is that the function of the σ^E promoter is to ensure transcription of the *exuR* gene, so that, in the absence of galacturonate, ExuR-controlled genes would not be expressed. It is not known whether ExuR directly regulates genes other than those in the *exu* locus. A search of the *B. subtilis* chromosomal DNA sequence did not reveal any perfect matches to the 26-nucleotide *exuO* sequence. However, other potential *exuO*-like operators may be found after the critical bases required for operator function are defined. Therefore, it remains a possibility that ExuR binding sites exist in other parts of the chromosome and regulate other promoters.

ACKNOWLEDGMENTS

We thank A. Glasgow and I. Stojiljkovic for critical review of the manuscript.

K.M. was supported in part by a Patricia Roberts Harris fellowship. This work was supported by Public Health Service grant GM54395 to C.P.M. from the National Institutes of Health.

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