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NOTES

Cloning, Expression, and Catabolite Repression of a Gene Encoding β-Galactosidase of *Bacillus megaterium* ATCC 14581

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A gene encoding β-galactosidase, designated *mbgA*, was isolated from *Bacillus megaterium* ATCC 14581. Chromosomal β-galactosidase production could be dramatically induced by lactose but not by isopropyl-β-D-thiogalactopyranoside (IPTG) and was subject to catabolite repression by glucose. Disruption of *mbgA* in the *B. megaterium* chromosome resulted in loss of lactose-inducible β-galactosidase production. A 27-bp inverted repeat was found to overlap the *mbgA* promoter sequence. Two partially overlapping catabolite-responsive elements (CREs) were identified within the inverted repeat. Base substitutions within CRE-I and/or CRE-II caused partial relief from catabolite repression. The results suggest that the 27-bp inverted repeat may serve as a target for a catabolite repressor(s).

Many catabolic enzymes in *Bacillus* spp. are subjected to catabolite repression by glucose or other rapidly metabolizable carbon sources (4, 11, 27, 33). The mechanism underlying catabolite repression in *Bacillus* spp. is quite different from the cyclic AMP (cAMP)-dependent positive regulatory mechanism operative in *Escherichia coli* (28). The latter involves a positive regulatory protein, cAMP receptor protein (19). In contrast, *Bacillus* does not contain detectable cAMP or cAMP receptor protein-like proteins (2, 13), and addition of exogeneous cAMP does not affect catabolite repression in *B. subtilis* (24). A negative transcription regulator, CcpA (7, 9, 10), which is a member of the LacI/GalR family (36), is believed to mediate catabolite repression by interacting with a *cis*-acting catabolite-responsive element (CRE) located in the regulatory region or coding region of catabolic-enzyme-encoding genes subjected to carbon catabolite repression (12, 35). The consensus sequences 5′-TGWNANCGNTNWCA-3′ (35), where W stands for adenine or thymine, and 5′-(T/A)GNAA(C/G)CGN(T/A)(T/A)NCA-3′ (12) have been proposed for the CRE. In this report, we describe the cloning, sequencing, expression, and regulation of the *B. megaterium* β-galactosidase gene (*mbgA*). A 27-bp inverted repeat was identified in the *mbgA* promoter region. Two CRE-like sequences within the 27-bp inverted repeat were found to be able to exert catabolite repression on β-galactosidase production in *B. megaterium*.

Cloning of the β-galactosidase gene. To clone the β-galactosidase gene, chromosomal DNA of *B. megaterium* ATCC 14581 was isolated as described previously (37) and was partially digested with the restriction enzyme *Sau*3AI. DNA fragments ranging in size from 6 to 9 kb were gel purified and ligated into *Bam*HI-cut and dephosphorylated vector pQE30 (Qiagen, Inc.). *E. coli lac* mutant JM109 (29) was transformed with the ligated DNA to generate a genomic library. The β-galactosidase gene was isolated by direct selection for enzyme activity on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates. One plasmid isolated from a blue colony was found to contain an insert of about 9 kb and was subjected to further analysis. A 5-kb *Pst*I fragment derived from the 9-kb insert was further subcloned into *Pst*I-cut pQE30 in both orientations. Both of the resulting plasmids, when introduced into *E. coli* JM109, still gave blue colonies on Luria-Bertani (LB) medium plates (29) containing X-Gal. These results suggest that the 5-kb *Pst*I fragment contains the cloned β-galactosidase gene and the promoter-like sequences from which transcription can start in *E. coli*.

The 5-kb *Pst*I fragment was further cloned into *Pst*I-cut pBluescript KS(+) (Stratagene, Inc.) in both orientations for DNA sequencing by the dideoxy-chain termination method (30). The restriction map is shown in Fig. 1. One complete open reading frame (ORF) and two incomplete ORFs were identified. Based on its sequence homology to several published β-galactosidase genes, this complete ORF is assumed to be the structural gene for the β-galactosidase of *B. megaterium* and is designated here *mbgA* (*megaterium* β-galactosidase gene). This position of *mbgA* in the 5-kb *Pst*I fragment is reinforced by assaying for changes in the β-galactosidase activity of various deletion derivatives of the *Pst*I fragment shown in Fig. 1. pGS240, a modified form of pBluescript KS(+) which loses α-complementation ability (29), was constructed by inversion of the 0.45-kb *Pvu*II fragment containing part of the coding sequence of the *lacZ* α fragment of pBluescript KS(+). Insertion of the 5-kb *Pst*I fragment which contains the cloned *mbgA* gene into the *Pst*I site of pGS240 yields plasmid pGS242. pGS246 was constructed by digestion of plasmid pGS242 with *Sal*I to delete a 2.3-kb fragment, followed by religation. pGS281 was constructed by digestion of plasmid pGS242 with *Eco*RV to delete a 0.8-kb fragment, followed by religation. pGS249 was constructed by unidirectional deletion (6) with exonuclease III of a 1.2-kb fragment downstream of the cloned β-galactosidase gene.

Sequence analysis revealed that *mbgA* consists of 3,102 bp, which corresponds to a protein monomer of 1,034 amino acids with a calculated molecular mass of 118,088 Da (data not shown). Comparison of the deduced amino acid sequence encoded by the *mbgA* gene with those of the β-galactosidase of *E.*

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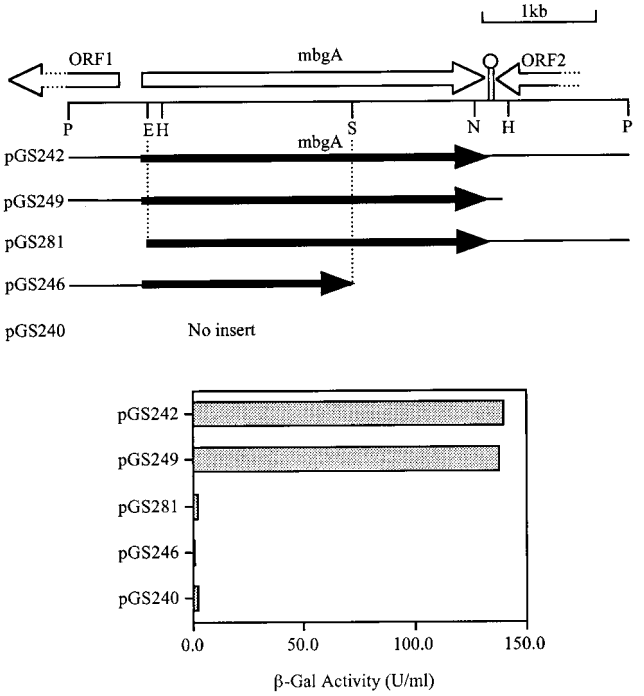

FIG. 1. Restriction map and genetic organization of the *B. megaterium* β-galactosidase gene (*mbgA*) and β-galactosidase activities of deletion derivatives of the 5-kb *Pst*I fragment in *E. coli*. E, *Eco*RV; H, *Hin*dIII; N, *Nae*I; P, *Pst*I; S, *Sal*I.

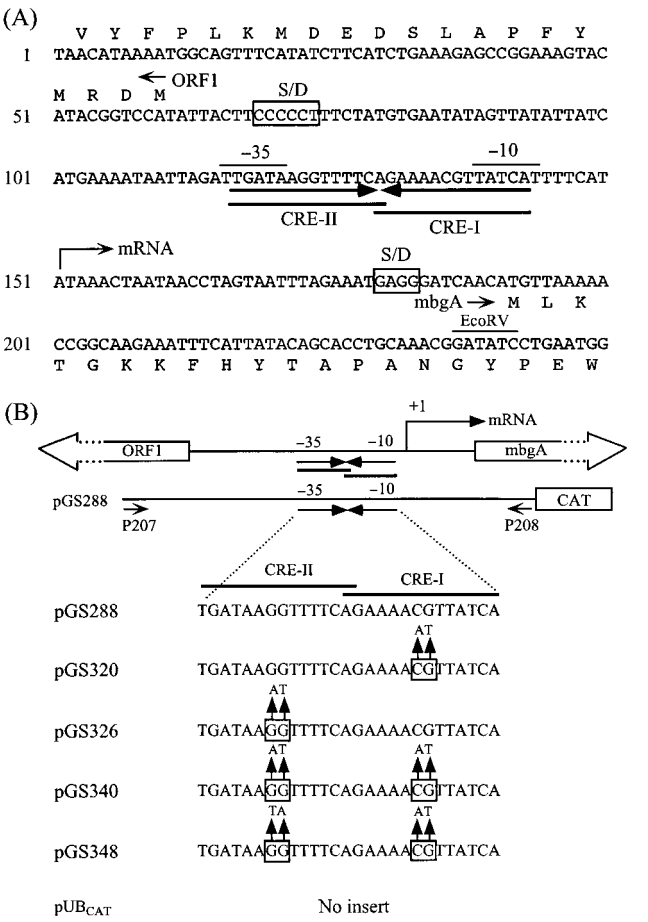

FIG. 2. Nucleotide sequence of the regulatory region of the *B. megaterium* *mbgA* gene and base substitutions in CREs. (A) The σ^A-like promoter sequence is overlined. The 27-bp inverted repeat that overlaps the promoter sequence is indicated by a pair of solid inverted arrows. Two partially overlapping CREs which are contained in the 27-bp inverted repeat are designated CRE-I and CRE-II. The N-terminal amino acids of ORF1 and *mbgA* are also shown. The putative ribosome-binding sites (S/D) are boxed. (B) pUB_{CAT}-based plasmids pGS288, pGS320, pGS326, pGS340, and pGS348 contain a *cat* reporter gene transcriptionally fused to the *mbgA* promoter. pGS288 carries the wild-type *mbgA* promoter, whereas pGS320, pGS326, pGS340, and pGS348 carry various base substitutions in CREs. The positions of the two flanking primers, P207 (5′-GCGGGTCGACAAGCTCGCCTCTTCCTT-3′) and P208 (5′-GCGGAAG CTTTGGTAGTAAACAGA-3′), are also indicated.

coli (15), *Klebsiella pneumoniae* (3), and *Streptococcus thermophilus* (31) revealed 43.7, 45.4, and 49.5% overall similarity, respectively. Immediately downstream of *mbgA* is an inverted repeat with the sequence AAGAAGGACTTTCATTTGAAA GTCCTTTTT, which may serve as a putative ρ-independent transcription terminator. Further downstream is an incomplete ORF transcribed in the opposite direction (Fig. 1). Further upstream is an incomplete ORF transcribed in the direction opposite to that of the *mbgA* gene (Fig. 1). The genetic organization of these ORFs suggests that *B. megaterium* contains a β-galactosidase gene that exists in a monocistronic operon. This is unlike the case in many other bacteria, where the β-galactosidase gene and the lactose permease gene usually coexist in an operon (15, 18, 26, 38). The nucleotide sequence of the *mbgA* regulatory region is shown in Fig. 2.

Identification of the transcription initiation site of *mbgA*. To determine the precise transcription initiation site of *mbgA*, RNA was isolated from log-phase *B. megaterium* cells harboring plasmid pGS288 (Fig. 2). A 16-mer oligonucleotide (5′-C GTTTGCAGGTGCTGT-3′) complementary to a region extending from position +71 to position +86 relative to the transcription start site of the *mbgA* gene was used as the primer. Primer extension was carried out as described previously (29), and the result is shown in Fig. 3. Only one major extension product was detected, indicating that the 5′ end of the mRNA for *mbgA* is located 40 bp upstream of its translation start site (Fig. 2). High-resolution S1 nuclease mapping (1) was also performed to further confirm this result. Two major protected fragments with only a one-base difference in length were observed (Fig. 3). One of them corresponds to a transcription initiation site which is identical to that identified by primer extension analysis. This transcription initiation site is at an appropriate distance from a putative promoter sequence (TTGATA for the −35 box and TATCAT for the −10 box) that may be recognized by the *B. megaterium* equivalent of *B. subtilis* σ^A (23). A 27-bp inverted repeat overlaps the −35 and −10 regions of this putative promoter (Fig. 2). Within the 27-bp inverted repeat are two partially overlapping sequences (CRE-I and CRE-II) that exhibit considerable sequence similarity to the CRE consensus sequence (12).

Effects of lactose, glucose, and IPTG on β-galactosidase production in wild-type *B. megaterium* and the *mbgA* mutant. The effects of lactose, glucose, and IPTG on β-galactosidase production were examined by using *B. megaterium* cells grown in LB medium. β-Galactosidase activities were measured at 37°C with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate by the method of Miller (20). It was found that 2% lactose could increase β-galactosidase production about 18-fold (Table 1). In the presence of 2% lactose plus 2% glucose, about 25-fold repression exerted by glucose was observed. Treatment with either 1 or 6 mM IPTG caused only a slight increase in β-galactosidase production compared to 2% lactose treatment. These results suggest that β-galactosidase produc-

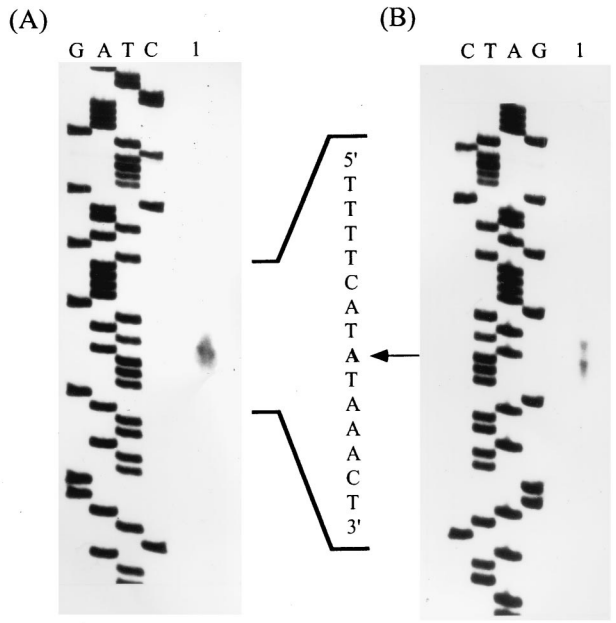

FIG. 3. Determination of the transcription initiation site for *mbgA* by primer extension analysis and S1 nuclease mapping. (A) Primer extension analysis of transcripts was performed with RNA from *B. megaterium* cells harboring pGS288. Lane 1 shows the result of this analysis. (B) S1 nuclease mapping was carried out with RNA isolated from the same source as that used for panel A. Lane 1 shows the protected products. For both panels, dideoxy sequencing ladders obtained with the same primer used to make the probes for S1 nuclease mapping and primer extension experiments are shown. The sequence indicated is complementary to that read from the ladder.

tion in *B. megaterium* is lactose inducible and is subject to catabolite repression by glucose. To examine the contribution of *mbgA* to lactose-mediated induction of β-galactosidase production in *B. megaterium*, we disrupted the *mbgA* gene in the *B. megaterium* chromosome by using integrative plasmid pGS278, which was constructed as follows. A 600-bp *Eco*RI-*Hin*dIII fragment carrying an internal sequence of the *mbgA* gene was generated by PCR using synthetic oligonucleotides P194 (5′-GCCAAAGCTTAAATGGAAGC-3′) and P195 (5′-GCGGAATTCAGCTGAAACGCTAAGTT-3′). This fragment was cloned into *Eco*RI- and *Hin*dIII-cut plasmid pDG1515 (5) to get plasmid pGS278. Disruption of the *mbgA* gene by a Campbell-like single-crossover recombination event was confirmed by Southern blot analysis (29) (data not shown). The *mbgA* mutant was then grown in LB medium in the absence or presence of 2% lactose to an optical density at 600 nm of 0.6 for β-galactosidase activity assay. As shown in Table 1, disruption of the *mbgA* gene in the *B. megaterium* chromosome abolished the lactose-mediated induction of β-galactosidase production. This suggests that the *mbgA* gene is the only lactose-inducible β-galactosidase gene in *B. megaterium*.

TABLE 1. Effects of lactose, glucose, and IPTG on β-galactosidase production in wild-type *B. megaterium* and an *mbgA* mutant

B. megaterium strain	Addition[a]	Mean β-galactosidase activity[b] (U/ml) ± SD
Wild type	None	0.31 ± 0.03
	Lac	5.54 ± 0.39
	Lac + Glu	0.22 ± 0.02
	1 mM IPTG	0.39 ± 0.04
	6 mM IPTG	0.35 ± 0.05
mbgA mutant	None	0.15 ± 0.02
	Lac	0.21 ± 0.02

[a] Abbreviations: Lac, lactose; Glu, glucose.
[b] *B. megaterium* cells were grown in LB medium to an optical density at 600 nm of 0.6 in the absence or presence of 2% sugar or IPTG, as specified. β-Galactosidase activities of the chloroform-treated *B. megaterium* cells were determined as described previously (20). Each value is the mean of four experiments.

TABLE 2. Effects of base substitutions in CREs on catabolite repression of *mbgA* promoter-*cat* transcriptional fusions

Plasmid in *B. megaterium*[a]	Addition[b]	Mean CAT sp act[c] (mU/mg of protein) ± SD	Fold repression[d]
pGS288	None	1,310 ± 67	
	Lac	1,300 ± 82	
	Lac + Glu	140 ± 21	9.3
pGS320	None	1,290 ± 59	
	Lac	1,310 ± 73	
	Lac + Glu	730 ± 34	1.8
pGS326	None	2,210 ± 117	
	Lac	1,880 ± 110	
	Lac + Glu	1,320 ± 61	1.4
pGS340	None	400 ± 28	
	Lac	380 ± 24	
	Lac + Glu	180 ± 17	2.1
pGS348	None	1,340 ± 69	
	Lac	1,420 ± 75	
	Lac + Glu	1,090 ± 58	1.3
pUB_{CAT}	None	90 ± 18	
	Lac	85 ± 14	
	Lac + Glu	89 ± 12	

[a] The plasmids listed are shown in Fig. 2.
[b] Abbreviations: Lac, lactose; Glu, glucose.
[c] At least two independent clones for each construct were chosen for CAT activity assays. *B. megaterium* cells were grown in LB medium to an optical density at 600 nm of 1.0 in the absence or presence of 2% sugar, as specified. After sonication and centrifugation, crude extracts were subjected to CAT assays (32). Each value is the mean of at least six determinations in separate experiments.
[d] Lac/(Lac + Glu).

Effects of mutations in CREs on catabolite repression of the *mbgA* promoter-*cat* transcriptional fusions. To assess the contributions of the two CRE-like sequences to catabolite repression of β-galactosidase production, we firstly introduced a DNA fragment carrying the *mbgA* promoter and CRE-like sequences into promoter probe vector pUB_{CAT} (37). The resulting plasmid, pGS288, bears an *mbgA* promoter-*cat* transcriptional fusion (Fig. 2). *B. megaterium* cells transformed with plasmid pGS288 were grown in LB medium in the absence or presence of lactose or lactose plus glucose. Chloramphenicol acetyltransferase (CAT) activities were measured at 37°C by the spectrophotometric method of Shaw (32). It was found that 2% glucose could still exert about ninefold catabolite repression (Table 2).

We also made some *mbgA* promoter-*cat* transcriptional fusion constructs in which CRE-I, CRE-II, or both were mutated by the two-step PCR method (8) (Fig. 2). We then examined the responses of these fusions to lactose and glucose. For each construct, at least two independent clones were chosen for CAT activity assays in order to obtain consistent results. The results showed that mutations in either of the two CREs caused partial relief of catabolite repression (Table 2). Muta-

tions in CRE-I of pGS320 and CRE-II of pGS326 decreased catabolite repression from about 9-fold in the wild type to about 1.8-fold and 1.4-fold, respectively. These results suggest that both CRE-I and CRE-II can function as an active CRE for catabolite repression of the β-galactosidase gene of *B. megaterium*. When CRE-I and CRE-II were mutated together (pGS340 and pGS348 in Fig. 2), catabolite repression was still not completely abolished (Table 2). These results suggest that either another mechanism(s) is involved in catabolite repression of *mbgA* expression or another CRE-like sequence(s) is located in the coding region of the *mbgA* gene. Previous studies indicated that, in addition to a promoter-proximal CRE, a *cis*-acting site required for catabolite repression was also identified in the coding regions of the *hutP* (25, 39), *gntR* (21, 22), and *xylA* (14) genes of *B. subtilis*. We cannot exclude the possibility that base substitutions in the central CG or GG residues of the two CREs could only partially reduce the affinity of the catabolite repressor for these two sites. However, previous in vitro studies indicated that alterations of the central residues could prevent binding of the catabolite repressor to CREs identified in some *Bacillus* genes (17, 22). It remains to be experimentally determined whether two catabolite repressor molecules can bind to these two CREs simultaneously. Investigation by in vitro gel mobility shift assays and in vivo studies of the possible role of CcpA in catabolite repression of *mbgA* expression via the two CREs should help to clarify these issues.

On the other hand, mutations of CRE-I, CRE-II, or both had differential effects on basal expression from the *cat* reporter gene. Mutations in CRE-I or pGS320 had little or no effect on basal expression, whereas mutations in CRE-II of pGS326 caused about a 1.7-fold increase in the basal expression (Table 2). The estimated ΔG values for the potential hairpin structure of the 27-bp inverted repeat (an 11-bp stem and a 5-base loop) in pGS288, pGS320, pGS326, pGS340, and pGS348 are −5.8, −3.6, −3.6, −9.8, and −3.6 kcal/mol, respectively, according to the method of Tinoco et al. (34). Site-directed mutagenesis in pGS348 reduced the dyad symmetry ($\Delta G = -3.6$ kcal/mol) of the 27-bp inverted repeat, whereas mutagenesis in pGS340 enhanced the dyad symmetry ($\Delta G = -9.8$ kcal/mol) in such a way that a perfect 27-bp inverted repeat was obtained (Fig. 2). Mutations in both CRE-I and CRE-II of pGS348 had little or no effect on the basal expression, whereas mutations in pGS340, which strengthen the hairpin, caused about a 3.3-fold reduction in the basal expression. Base substitutions in either half of the 27-bp inverted repeat did not directly change the sequences in the −35 and −10 boxes of the *mbgA* promoter. However, it was still possible that alterations of their flanking sequences might affect RNA polymerase recognition and binding, thus altering the promoter strength.

In contrast to the dramatic induction of chromosomal β-galactosidase production by lactose, *cat* expressions from the various *mbgA* promoter-*cat* transcriptional fusions on the pUB110-derived high-copy plasmids (16) (ca. 50 copies per chromosome) were not lactose inducible (Table 2). One possible reason is that the concentration of the putative lactose-responsive repressor (not the catabolite repressor) in *B. megaterium* is relatively very low. A limited amount of the repressor might efficiently repress expression of the single-copy *mbgA* gene in the chromosome (lactose inducible) but might not be enough to repress expression of multiple copies of the *mbgA* gene on a high-copy plasmid (not lactose inducible). Another possibility is that the lactose-responsive *cis*-acting element is not contained in the *mbgA* promoter region mentioned above.

In conclusion, we have identified two CRE-like sequences within the 27-bp inverted repeat which can exert catabolite repression on β-galactosidase production in *B. megaterium*. To our knowledge, this is the first example of two functional overlapping CREs that exist in the promoter region of a *Bacillus* gene. In CRE-II, the central two residues are GG instead of the highly conserved CG residues. The sequencing markers shown in Fig. 3 clearly indicated that this was not a sequencing error. Moreover, this is not unprecedented; it has been reported that a functional CRE identified in the promoter region of *gntR* contains central TG residues (22). Although we have demonstrated that the β-galactosidase of *B. megaterium* is capable of hydrolyzing ONPG and X-Gal, the physiological substrates for the β-galactosidase of *B. megaterium* remain undetermined. Work using the purified β-galactosidase of *B. megaterium* is needed to determine if it can use lactose as a substrate.

Nucleotide sequence accession number. The nucleotide sequence reported here has been assigned GenBank accession no. AF047824.

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