JOURNAL OF BACTERIOLOGY, Oct. 2001, p. 5877–5884 Vol. 183, No. 20
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.20.5877–5884.2001

Involvement of Two Distinct Catabolite-Responsive Elements in Catabolite Repression of the *Bacillus subtilis myo*-Inositol (*iol*) Operon

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Received 29 May 2001/Accepted 13 July 2001

The *Bacillus subtilis* inositol operon (*iolABCDEFGHIJ*) is involved in *myo*-inositol catabolism. Glucose repression of the *iol* operon induced by inositol is exerted through catabolite repression mediated by CcpA and the *iol* induction system mediated by IolR. In this study, we identified two *iol* catabolite-responsive elements (*cre*'s), to which CcpA complexed with P-Ser-HPr or P-Ser-Crh probably binds. One is located in *iolB* (*cre-iolB*, nucleotides +2397 to +2411; +1 is the transcription initiation nucleotide), which was the only *cre-iol* found in the previous *cre* search of the *B. subtilis* genome using a query sequence of WTGNAANCGNWNNCW (W stands for A or T, and N stands for any base). Deletion and base substitution analysis of the *iol* region indicated that *cre-iolB* functions even if it is located far downstream of the *iol* promoter. Further deletion and base substitution analysis revealed another *cre* located between the *iol* promoter and the *iolA* gene (*cre-iiolA*, nucleotides +86 to +100); the prefix "i" indicates a location in the intergenic region. Both *cre-i**iolA* and *cre-iolB* appeared to be recognized to almost the same extent by CcpA complexed with either P-Ser-HPr or P-Ser-Crh. Sequence alignment of the six known *cre*'s, including *cre-i**iolA*, which were not revealed in the previous *cre* search, exhibited another consensus sequence of WTGAAARCGYTTWWN (R stands for A or G, and Y stands for C or T); the right two thymines (TT) were found to be essential for the function of *cre-i**iolA* by means of base substitution analysis. A *cre* search with this query sequence led to the finding of 14 additional putative *cre*'s.**

myo-Inositol is abundant in nature, especially in soil. Various microorganisms, including *Bacillus subtilis*, are able to grow on *myo*-inositol as the sole carbon source. The *B. subtilis iol* divergon consisting of the *iolABCDEFGHIJ* and *iolRS* operons is involved in inositol catabolism (29, 31). These two *iol* operons are induced upon the addition of inositol to the medium (29). This induction is negatively regulated by a repressor (IolR) belonging to the DeoR family through its unique interaction with the extended binding regions close to their promoters (31). Inositol dehydrogenase encoded by *iolG* catalyzes the first reaction of inositol catabolism by *B. subtilis* (19, 29). The synthesis of this enzyme induced by inositol was repressed on the addition of glucose to the medium (18, 29). Very recently, DNA microarray analysis implied that not only the expression of *iolG* but also that of the other 11 *iol* genes was under glucose repression (30).

The well-characterized mechanisms underlying glucose repression are those of catabolite repression and inducer exclusion. Recently, the mechanism underlying catabolite repression in *B. subtilis* was extensively investigated. These studies revealed that *Bacillus*, as well as other low-GC gram-positive bacteria, possesses a negative regulatory mechanism for catabolite repression, which is very different from the positive regulatory mechanisms of enteric bacteria involving cyclic AMP and cyclic AMP receptor protein (20, 25). In low-GC gram-positive bacteria, negative regulation of the transcription of catabolite-repressive genes occurs through the binding of the catabolite control protein (CcpA) (10), which interacts with allosteric effectors such as P-Ser-HPr (6) and P-Ser-Crh (7), to their *cis*-acting catabolite-responsive elements (*cre*'s) (15).

DNA microarray analysis revealed that the expression of the *iolABCDEFGHIJ* and *iolRS* operons was under glucose repression, which was partially CcpA dependent (30). The glucose repression of the synthesis of inositol dehydrogenase (Idh) is dependent on both CcpA and IolR (8, 16, 29, 30), implying that catabolite repression and the induction system mediated by IolR are involved in glucose repression of the *iolABCDEFG HIJ* operon. Since almost no glucose repression was observed in a doubly mutated strain with respect to *ccpA* and *iolR* (30), IolR-independent repression is likely to be exerted through catabolite repression mediated by CcpA. When *cre* sequences were searched for in the *B. subtilis* genome using a query sequence of WTGNAANCGNWNNCW (W and N stand for A or T and for any base, respectively), 126 putative and known *cre*'s were found (15). One of them is located within the *iolB* gene, that is, *cre-iolB*, which has been found to function as a *cre* in an in vivo *cre* test system (15).

In this work, we found on deletion and base substitution analysis of the *iol* region that *cre-iolB*, which is located far downstream of the *iol* promoter (P*iol*), functioned as a *cre*. Further deletion and base substitution analysis revealed another functional *cre*, which is located between P*iol* and *iolA* and named *cre-i**iolA*; the prefix "i" indicates the location of *cre* in the intergenic region. Thus, the CcpA-dependent catabolite repression of the *iolABCDEFGHIJ* operon was due to the two *cre*'s functioning independently. Interestingly, the sequence of

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TABLE 1. Bacterial strains used in this work

Strain	Genotype	Reference
GM122	*trpC2 sacB′-′lacZ*	4
SA003	*trpC2 sacB′-′lacZ ptsH1*	4
1A250	*trpC2 alsR1 ilvΔ1*	16
1A147	*ccpA1 trpC2 alsR1 ilvΔ1*	16
168	*trpC2*	2
YF244	*trpC2 metC7 iolR*::*cat*	29
QB5223[a]	*trpC2 ptsH1*	12
QB7096[a]	*trpC2 crh*::*aphA3*	Not published
QB7102[a]	*trpC2 ptsH1 crh*::*aphA3*	Not published
FU704	*trpC2 sacB′-′lacZ iolR*::*neo*	This work
FU706	*trpC2 alsR1 ilvΔ1 iolR*::*neo*	This work
FU707	*ccpA1 trpC2 alsR1 ilvΔ1 iolR*::*neo*	This work
FU709	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol-iolAB′*(−107/+2474)-*lacZ*]	This work
FU713	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol-iolAB′*(−107/+2270)-*lacZ*]	This work
FU715	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol-iolAB′*(−107/+2474,+2405G→T)-*lacZ*]	This work
FU716	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol-iolAB′*(−107/+2474,+2410C→T)-*lacZ*]	This work
FU719	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol-iolA′*(−107/+1192)-*lacZ*]	This work
FU720	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol-iolA′*(−107/+712)-*lacZ*]	This work
FU721	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol-iolA′*(−107/+298)-*lacZ*]	This work
FU722	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol*(−107/+110)-*lacZ*]	This work
FU723	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol*(−107/+82)-*lacZ*]	This work
FU724	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol*(−107/+47)-*lacZ*]	This work
FU726	*trpC2 amyE*::[*cat* P*spac*-(*cre-iiolA*)-*lacZ*]	This work
FU727	*trpC2 amyE*::[*cat* P*spac*-(*cre-iolB*)-*lacZ*]	This work
FU728	*trpC2 ptsH1 amyE*::[*cat* P*spac*-(*cre-iiolA-lacZ*)]	This work
FU729	*trpC2 ptsH1 amyE*::[*cat* P*spac*-(*cre-iolB*)-*lacZ*]	This work
FU734	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol-iolA′*(−107/+298,+91A→G)-*lacZ*]	This work
FU735	*trpC2 sacB′-′lacZ iolR*::*neo amyE*::[*cat* P*iol-iolA′*(−107/+298,+94G→T)-*lacZ*]	This work
FU738	*trpC2 sacB′-′lacZ amyE*::[*cat* P*spac*-(*cre-iiolA*)-*lacZ*]	This work
FU742	*trpC2 alsR1 ilvΔ1 amyE*::[*cat* P*spac*-(*cre-iiolA*)-*lacZ*]	This work
FU743	*ccpA1 trpC2 alsR1 ilvΔ1 amyE*::[*cat* P*spac*-(*cre-iiolA*)-*lacZ*]	This work
FU744	*trpC2 alsR1 ilvΔ1 amyE*::[*cat* P*spac*-(*cre-iolB*)-*lacZ*]	This work
FU745	*ccpA1 trpC2 alsR1 ilvΔ1 amyE*::[*cat* P*spac*-(*cre-iolB*)-*lacZ*]	This work
FU748	*trpC2 crh*::*aphA3 amyE*::[*cat* P*spac*-(*cre-iiolA*)-*lacZ*]	This work
FU749	*trpC2 crh*::*aphA3 amyE*::[*cat* P*spac*-(*cre-iolB*)-*lacZ*]	This work
FU750	*trpC2 ptsH1 crh*::*aphA3 amyE*::[*cat* P*spac*-(*cre-iiolA*)-*lacZ*]	This work
FU751	*trpC2 ptsH1 crh*::*aphA3 amyE*::[*cat* P*spac*-(*cre-iolB*)-*lacZ*]	This work
FU752	*trpC2 sacB′-′lacZ amyE*::[*cat* P*spac*-(*cre-iiolA*,+96T→G)-*lacZ*]	This work
FU753	*trpC2 sacB′-′lacZ amyE*::[*cat* P*spac*-(*cre-iiolA*,+96T→A)-*lacZ*]	This work
FU754	*trpC2 sacB′-′lacZ amyE*::[*cat* P*spac*-(*cre-iiolA*,+97T→G)-*lacZ*]	This work
FU758	*trpC2 iolR*::*cat*	This work
FU759	*trpC2 iolR*::*cat ptsH1*	This work
FU760	*trpC2 iolR*::*cat crh*::*aphA3*	This work
FU761	*trpC2 iolR*::*cat ptsH1 crh*::*aphA3*	This work

[a] Obtained from I. Martin-Verstraete (Institut Pasteur, Paris, France).

cre-iiolA does not match the 3′ part of the query sequence used for the genome-wide *cre* search. *cre-iiolA* was found to belong to a group of *cre*'s exhibiting similar but distinct consensus sequences.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains used are listed in Table 1. Strains FU758, FU759, and FU760 carrying *iolR*::*cat* were obtained by the transformation of strains 168, QB5223, and QB7096 with DNA of strain YF244 to chloramphenicol (5 μg/ml) resistance on tryptose blood agar base (TBAB) plates (Difco), respectively. Strain FU761 was obtained by the transformation of strain FU759 with DNA of strain QB7096 to kanamycin (10 μg/ml) resistance on TBAB plates, because strains such as strain QB7102 carrying both the *ptsH1* and *crh*::*aphA3* mutations were not transformable.

Strains FU704, FU706, and FU707 carrying *iolR*::*neo* were constructed as follows. Plasmid pIOLR1::*neo* was first obtained by disruption of *iolR* encoded in plasmid pIOLR1 (29) with insertion of the neomycin resistance cassette derived from plasmid pBEST513 (11). Plasmid pIOLR1 was digested with *Eco*RV, ligated with a cassette which had been prepared by *Eco*RI digestion and subsequent blunt ending of plasmid pBEST513, and was used for the transformation of *Escherichia coli* strain JM109 (21) to kanamycin resistance (25 μg/ml) on Luria-Bertani (LB) plates (21). The resultant plasmid, pIOLR1::*neo*, was used for the double-crossover transformation to neomycin (15 μg/ml) resistance of strains GM122, 1A250 and 1A147, resulting in strains FU704, FU706, and FU707, respectively. Construction of the other strains listed in Table 1 is described below.

Construction of strains for deletion analysis of *cre*'s of the *iol* operon. To construct strains FU709 and FU713 carrying *iolR*::*neo* and transcriptional fusions of *iol* regions (nucleotides [nt] −107 to +2270 and +2474, respectively; +1 is the transcription initiation nucleotide of the *iol* operon) to *lacZ*, we first replaced the *Eco*RI site of plasmid pCRE-test (15) with an *Xba*I site, which is absent from the *iol* region (nt −107 to +2474). This was done through its digestion with *Eco*RI, blunt ending, attachment of an *Xba*I linker, digestion with *Xba*I, and subsequent self-ligation, resulting in plasmid pCRE-test2.

The two *iol* regions were amplified by PCR using chromosomal DNA of strain 168 and appropriate *iol*-specific primer pairs, which had been designed to produce 5′ and 3′ flanking *Xba*I and *Bam*HI sites, respectively (Fig. 1). The PCR products were doubly digested with *Xba*I and *Bam*HI and then ligated with the *Xba*I-*Bam*HI arm of plasmid pCRE-test2 from which P*spac* had been eliminated. When the *iol* regions were cloned into plasmid pCRE-test2 in *E. coli*, unexpected mutations were frequently introduced into the cloned regions, probably due to

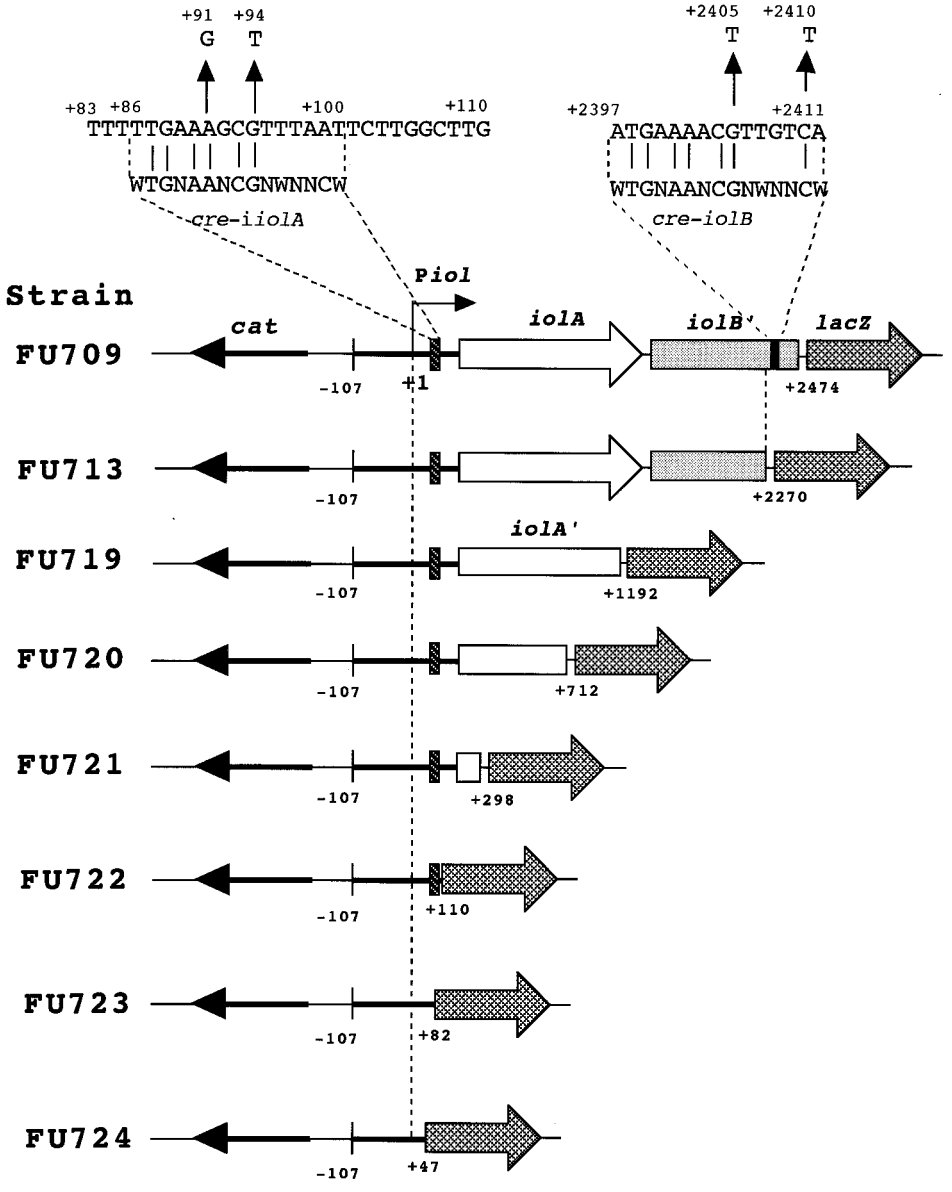

FIG. 1. Location of *cre-iiolA* and *cre-iolB*. The upper part of the figure shows the sequences of *cre-iiolA* and *cre-iolB*, which are aligned with a consensus sequence (15). The base substitutions of the indicated bases in the *cre* sequences which caused the knockout of their function are shown; nt +1 is the *iol* transcription initiation base. Strains carrying a series of deletion derivatives of P*iol* (*iol* promoter)-*iolAB'-lacZ* in the *amyE* locus, used for identification of *iol-cre*'s, were constructed as described in the text except for the following. The primers for amplifying the respective *iol* regions by PCR for construction of strains FU709, FU713, FU719, FU720, FU721, FU722, FU723, and FU724 were a forward one (nt −105 to −85) with an adapter sequence of GTCCTCTAGA and reverse primers (+2448 to +2474, +2250 to +2270, +1173 to +1192, +693 to +712, +279 to +298, +91 to +110, +58 to +82, and +27 to +47) with an adapter sequence of GATAGGATCC; the underlined sequences are *Xba*I and *Bam*HI sites, respectively.

high expression of *iolA* from the *iol* promoter, which is harmful to this bacterium. This gene codes for a protein exhibiting high similarities to methylmalonate-semialdehyde dehydrogenases of various species (29). So, the ligated DNAs were digested with *Pst*I and then used directly for the double-crossover transformation into the *amyE* locus of *B. subtilis* strain FU704 to chloramphenicol (5 μg/ml) resistance on TBAB plates, resulting in strains FU709 and FU713 carrying the *iol* regions (nt −107 to +2474 and +2270) between P*iol* and *lacZ*, respectively. Their correct construction was confirmed by sequencing of the inserted *iol* regions.

Strains FU719, FU720, FU721, FU722, FU723, and FU724 carrying a series of further deletions of the *iol* region (nt −107 to +2270) between P*iol* and *lacZ* in *amyE* of strain FU713 were constructed as follows. Each of the *iol* regions (nt −107 to +1192, +712, +298, +110, +82, and +47) was amplified by PCR using DNA of strain 168 and its *iol*-specific primer pair to generate flanking *Xba*I and *Bam*HI sites (Fig. 1). The PCR products were digested with *Xba*I and *Bam*HI and then ligated with the *Xba*I-*Bam*HI arm of plasmid pCRE-test2. The ligated DNAs were used for the transformation of *E. coli* strain JM109 to ampicillin (50 μg/ml) resistance on Luria-Bertani plates. The correct construction of the P*iol-iol-lacZ* fusions in the resultant plasmids was confirmed by sequencing. The plasmids were linearized with *Pst*I and then used for the double-crossover transformation of strain FU704 carrying *iolR*::*neo* to chloramphenicol resistance, strains FU719, FU720, FU721, FU722, FU723, and FU724 being produced.

Base substitutions in *cre* sequences within the P*iol-iol-lacZ* fusions. Strains FU715 and FU716 carrying base substitutions of +2405G→T and +2410C→T in the *cre-iolB* sequence (Fig. 1) within the P*iol-iolAB'*(nt −107 to +2474)-*lacZ* fusion of strain FU709 were constructed as follows. Introduction of base substi-

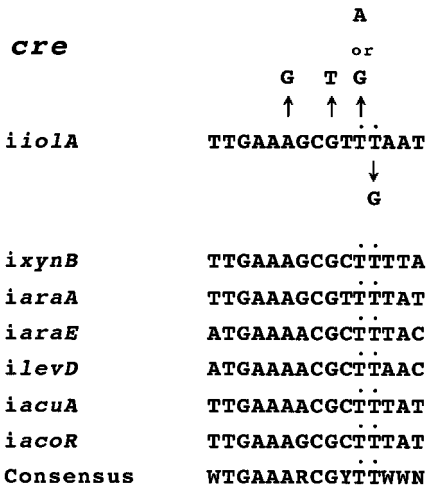

FIG. 2. Alignment of the *cre*-i*iolA* sequence with known *cre* sequences that were not revealed with a query sequence of WTGNAA NCGNWNNCW. The upper part of the figure shows the knockout substitutions of the *cre*-i*iolA* sequence. The sequences of five known *cre* sequences that were not revealed with a query sequence of WTGNA ANCGNWNNCW (15) are aligned with that of *cre*-i*iolA*; the known *cre*'s are *cre*-i*xynB* (7), *cre*-i*araA* (22), *cre*-i*araE* (23), *cre*-i*levD* (12), *cre*-i*acuA* (9), and *cre*-i*acoR* (1). The consensus sequence carries two thymines (TT) in the consensus sequence, which are indicated with a dot in this alignment. R represents A or G, and Y represents C or T.

tutions was performed by means of recombinant PCR using DNA of strain 168 and the following primers, as described previously (31). The common upstream and downstream primers were 5′-GTCCTCTAGACCTTCTCTTACTTCTCTT ACTTG-3′ (the *Xba*I site is underlined) and 5′-GATAGGATCCTCATTTAA TTAGTAGGATGTGTATCCG-3′ (the *Bam*HI site is underlined), respectively. The overlapping primers for +2405G→T were 5′-GGGAAATGAAAACTTTG TCATCG-3′ and 5′-CGATGACAAAGTTTTCATTTCCC-3′, and those for +2410C→T were 5′-AACGTTGTTATCGTTCCTGCGG-3′ and 5′-CCGCAG GAACGATAACAACGTT-3′ (each substituted base is underlined). The resultant recombinant PCR products were digested with *Xba*I and *Bam*HI and then ligated with the *Xba*I-*Bam*HI arm of plasmid pCRE-test2. The resultant plasmids were linearized with *Pst*I and then integrated into *amyE* of strain FU704 through a double-crossover event, resulting in strains FU715 and FU716 being produced.

Strains FU734 and FU735 carrying base substitutions (+91A→G and +94G→T) in the *cre*-i*iolA* sequence (Fig. 1) within the *iol* region (nt −107 to +298) were constructed as follows. Introduction of base substitutions was performed by recombinant PCR using DNA of strain 168 and the following primers, as described above. The common upstream and downstream primers were 5′-G TCCTCTAGACCTTCTCTTACTTCTCTTACTTG-3′ (the *Xba*I site is underlined) and 5′-GATAGGATCCCATAGCACTTCTTTCGTCGC-3′ (the *Bam*HI site is underlined), respectively. The overlapping primers for +91A→G were 5′-GGTGTTTTTGAAGGCGTTTAATTCTTGGC-3′ and 5′-GCCAAGAATT AAACGCCTTCAAAAACACC-3′, and those for +94G→T were 5′-GGTGTT TTTGAAAGCTTTTAATTCTTGGC-3′ and 5′-GCCAAGAATTAAAAGCTT TCAAAAACACC-3′ (each substituted base is underlined).

Strain construction for further functional analysis of *cre*-i*iolA* and *cre*-i*olB*. The respective *iol* regions containing *cre*-i*iolA* and *cre*-i*olB* (nt +63 to +121, and +2375 to +2430) were amplified by PCR using DNA of strain 168 and the following two primer pairs to generate flanking *Bam*HI sites. For amplification of the *cre*-i*iolA* region, the upstream and downstream primers were 5′-GATAGG ATCCCGCCATTTATTTTTTTGGTG-3′ (nt +63 to +82) and 5′-GATAGGA TCCCCACTTTTCAGCAAGCCAAG-3′ (nt +102 to +121), and for that of the *cre*-i*olB* sequence, they were 5′-GATA*GGATCC*GACGAGACAATGACTGTG GG-3′ (nt +2375 to +2394) and 5′-GATAGGATCCTGGTATCCCGCAGGA ACGAT-3′ (nt +2411 to +2430) (the respective *Bam*HI sites are underlined). The PCR products were digested with *Bam*HI and then cloned into the *Bam*HI site of plasmid pCRE-test (15). The ligated DNA was used for the transformation of *E. coli* strain JM109 to ampicillin resistance. After confirming the correct orientations and sequences of the P*spac*-*cre*-*lacZ* fusions in the resulting plasmids pCRE-i*iolA* and -*iolB* by sequencing, they were linearized with *Pst*I and used for the double-crossover transformation of strains GM122, 168, QB5223, QB7096, 1A250, and 1A147 to chloramphenicol resistance. The respective resultant strains (FU738, FU726, FU728, FU748, FU742, and FU743) carried the P*spac*-(*cre*-i*iolA*)-*lacZ* fusion in their *amyE* locus, whereas the other strains (FU727 from 168, FU729 from QB5223, FU749 from QB7096, FU744 from 1A250, and FU745 from 1A147) contained the P*spac*-(*cre*-i*olB*)-*lacZ* fusion. Strains FU750 and FU751 were obtained by the transformation of strains FU728 and FU729 with DNA of strain QB7096 to kanamycin (10 μg/ml) resistance on TBAB plates, respectively.

Strains FU752, FU753, and FU754 carrying the respective base substitutions of the *cre*-i*iolA* sequence (+96T→G, +96T→A, and +97T→A) (Fig. 2) were constructed as follows. Introduction of base substitutions was performed by recombinant PCR using DNA of plasmid pCRE-i*iolA* as the template and the following primer pairs, as described above. The common upstream and downstream primers were 5′-TGTAAAACGACGGCCAGTTAAAGGATTTGAGC GTAGCG-3′ and 5′-CAGGAAACAGCTATGACCATTACGCCAGCTGGCG AAAG-3′, where the underlined sequences are located in the *cat* and *lacZ* genes of plasmid pCRE-i*iolA*, respectively. The overlapping primers for the +96T→G substitution were 5′-GAAAGCGGTAATTCTTGG-3′ and 5′-CCAAGAATTA CACGCTTTC-3′, those for +96T→A were 5′-GAAAGCGTATAATTCTTG G-3′ and 5′-CCAAGAATTATACGCTTTC-3′, and those for +97T→G were 5′-GAAAGCGTTGAATTCTTGG-3′ and 5′-CCAAGAATTCAACGCTTTC-3′ (each substituted base is underlined). The resulting recombinant PCR products were digested with *Bam*HI and then cloned into the *Bam*HI site of plasmid pCRE-test. After linearization of the resulting plasmids with *Pst*I, strain GM122 was transformed, resulting in strains FU752, FU753, and FU754.

Idh and β-Gal assay. Cells were grown to an absorbance level at 600 nm (A_{600}) of 0.6 in S6 medium (5) containing 0.5% Casamino Acids (Difco) and supplemented with required amino acids (50 μg/ml) with and without a 10 mM concentration of inositol and/or glucose. In addition, neomycin (15 μg/ml), chloramphenicol (5 μg/ml), and kanamycin (5 μg/ml) were added to the media for the growth of strains carrying *iolR*::*neo*, *iolR*::*cat* or with *cat* integration into *amyE*, and *crh*::*aphA3*, respectively. The cells (A_{600} unit = 3.6) were harvested and then lysed by lysozyme treatment and brief sonication (18). Idh activity in crude cell lysates was spectrophotometrically assayed as described previously (18). β-Galactosidase (β-Gal) activity in crude cell extracts was spectrophotometrically assayed as previously described (15). The amounts of protein in cell extracts were determined by the method of Bradford (3) with bovine serum albumin as a standard.

RESULTS

***B. subtilis* genes involved in glucose repression of Idh synthesis.** Glucose repression of the synthesis of Idh encoded by *iolG* is known to occur through catabolite repression mediated by CcpA (8, 16, 30) as well as a regulation involving IolR, probably through inducer exclusion (29, 30). We first investi-

TABLE 2. Effects of the *iolR*, *ptsH*, *crh*, and *ccpA* mutations on catabolite repression of inositol dehydrogenase (Idh) synthesis[a]

Strain	Relevant genotype	Idh activity (nmol/min/mg)			Repression ratio[b]
		−Iol	+Iol	+Iol and Glc	
168	Wild type	13	922	<2	>461
QB5223	*ptsH1*	23	1,031	13	79
QB7096	*crh*::*aphA3*	23	1,147	<2	>470
QB7102	*ptsH1 crh*::*aphA3*	19	827	113	7.3
FU758	*iolR*::*cat*	1,440	1,227	254	4.8
FU759	*ptsH1 iolR*::*cat*	1,455	1,118	678	1.6
FU760	*crh*::*aphA3 iolR*::*cat*	1,575	1,226	324	3.8
1A147	*ccpA1*	16	760	155	4.9
FU706	*iolR*::*neo*	914	810	133	6.1
FU707	*ccpA1 iolR*::*neo*	963	878	793	1.1

[a] Cells of *B. subtilis* strains were grown with (+) and without (−) *myo*-inositol (Iol) and with *myo*-inositol and glucose (Glc), and Idh activities in crude extracts were determined as described in the text. Idh activities are expressed as averages of the values obtained for at least three independent experiments.

[b] Ratio of +Iol to +Iol and Glc.

TABLE 3. Deletion and base substitution analyses for *cre*'s of the *iol* operon to monitor *lacZ* expression under the control of the *iol* promoter and *cre*('s) in the background of *iolR*::*neo*[a]

Strain[b]	Fusion	*iol* region	β-Gal activity (nmol/min/mg)		Repression ratio[c]
			−Glc	+Glc	
FU709	P*iol-iolAB′-lacZ*	−107 to +2474	1,217	349	3.5
FU715	P*iol-iolAB′-lacZ*	−107 to +2474, +2405G→T	1,123	448	2.5
FU716	P*iol-iolAB′-lacZ*	−107 to +2474, +2410C→T	942	358	2.6
FU713	P*iol-iolAB′-lacZ*	−107 to +2270	2,615	1,077	2.4
FU719	P*iol-iolA′-lacZ*	−107 to +1192	1,046	449	2.3
FU720	P*iol-iolA′-lacZ*	−107 to +712	4,418	1,921	2.3
FU721	P*iol-iolA′-lacZ*	−107 to +298	5,380	2,232	2.4
FU722	P*iol-lacZ*	−107 to +110	751	307	2.4
FU723	P*iol-lacZ*	−107 to +82	1,813	1,927	0.9
FU724	P*iol-lacZ*	−107 to +47	1,649	1,753	0.9
FU734	P*iol-iolA′-lacZ*	−107 to +298, +91A→G	7,332	9,276	0.8
FU735	P*iol-iolA′-lacZ*	−107 to +298, +94G→T	6,712	7,148	0.9

[a] Cells of *B. subtilis* strains were grown with (+) and without (−) glucose (Glc), and β-Gal activities in crude extracts were determined as described in the text. β-Gal activities are expressed as averages of the values obtained for at least three independent experiments.
[b] All the *B. subtilis* strains assayed carried an *iolR* disruption (*iolR*::*neo*).
[c] Ratio of −Glc to +Glc.

gated the effects of the *ptsH1* mutation causing the replacement of Ser46 of HPr with alanine and *crh*::*aphA3* disruption on glucose repression of Idh synthesis. As shown in Table 2, Idh synthesis was severely repressed by glucose in strain 168*trpC2* (wild type) (repression ratio, >461). Idh synthesis was not relieved from this glucose repression in strain QB7096 (*crh*::*aphA3*) at all (repression ratio, >470), but it was partially relieved from catabolite repression in strain QB5223 (*ptsH1*) (repression ratio = 79). Idh synthesis was more relieved from glucose repression in strain QB7102 carrying the *ptsH1* and *crh*::*aphA3* mutations (repression ratio = 7.3) and strain 1A147 carrying the *ccpA1* mutation (repression ratio = 4.9). Moreover, this CcpA-independent glucose repression observed in strain 1A147 (*ccpA1*) was almost completely abolished in strain FU707 carrying the *ccpA1* and *iolR*::*neo* mutations (repression ratio = 1.1) (Table 2). Part of the results are consistent with those reported by Galinier et al. (8), who found that Crh involvement in catabolite repression of Idh synthesis was solely observed in the *ptsH1* background, because HPr alone is likely to be sufficient to cause this catabolite repression. However, it was reported that either the *ptsH crh* double mutant or the *ccpA*::*spec* mutant was completely relieved from glucose repression of Idh synthesis (8). We cannot explain this discrepancy properly, but it might possibly be due to the difference in the media for cell cultivation; we used S6 (this work; 16, 30) or DSM medium (30), whereas they used CSK medium (8).

To more clearly demonstrate the involvement of either HPr or Crh in the CcpA-dependent catabolite repression of Idh synthesis, we constructed *ptsH1* and *crh*::*aphA3* isogenic mutants in the *iolR*::*cat* background and examined their effect on this catabolite repression (Table 2). Idh synthesis was considerably relieved from glucose repression in strain FU758 carrying *iolR*::*cat* (repression ratio = 4.8). The remaining repression is likely to be due to catabolite repression mediated by CcpA, because Idh synthesis was released from glucose repression (repression ratio = 6.1) in strain FU706 carrying *iolR*::*neo* and was almost completely released in strain FU707 carrying *ccpA1* and *iolR*::*neo* (repression ratio = 1.1). This CcpA-dependent catabolite repression, which was slightly decreased in strain FU760 carrying *crh*::aphA3 and *iolR*::*cat* (repression ratio = 3.8), appeared to be still present in strain FU759 carrying *ptsH1* and *iolR*::*cat* (repression ratio = 1.6), suggesting that Crh might be involved in this repression. However, we could not investigate catabolite repression of Idh synthesis in strain FU761 carrying triple defects of *ptsH1*, *crh*::*aphA3*, and *iolR*::*cat*, because this strain could not grow normally in the medium with glucose. The results suggest that Crh as well as HPr is involved in the CcpA-dependent catabolite repression of Idh synthesis.

Actual involvement of *cre-iolB* in catabolite repression of the *iol* operon. Upon a search for *cre* sequences in *B. subtilis*, 126 putative and known *cre*'s were revealed (15). Among them, *cre-iolB* was found to function as a *cre* in an in vivo *cre* test system (15). The *iol* operon is most likely transcribed from only one promoter, P*iol* (29). Hence, to determine whether or not *cre-iolB*, which is located approximately 2,400 bp downstream of P*iol*, is actually involved in the catabolite repression of the *iol* operon, we constructed a transcriptional fusion, P*iol-iolA-iolB′-lacZ*, possessing an *iol* region (nt −107 to +2474) with *cre-iolB* (nt +2397 to +2411), which expresses *lacZ* under the direction of P*iol* (Fig. 1), and integrated it into the chromosomal *amyE* locus of strain FU704 carrying *iolR*::*neo*. The resulting strain, FU709, produced a high level of β-Gal constitutively even on growth without inositol, which was repressed 3.5-fold on the addition of glucose to the medium (Table 3).

To determine whether or not *cre-iolB* actually functions in the P*iol-iolA-iolB′*(nt −107 to +2474)-*lacZ* transcriptional fusion, we introduced the base substitutions of +2405G→T and +2410C→T in the *cre-iolB* sequence, which resulted in strains FU715 and FU716 (Fig. 1), with the G and C corresponding to conserved bases (positions 9 and 14 of the query sequence [WTGNAANCGNWNNCW]) for the genome-wide *cre* search (15). As shown in Table 3, β-Gal synthesis in strains FU715 and FU716 was partially relieved from catabolite repression (repression ratio = 2.5 and 2.6, respectively). We also deleted a *cre-iolB* region (nt +2271 to 2474) from the P*iol-iolA-iolB′-lacZ* fusion, which resulted in strain FU713. This deletion

decreased the catabolite repression ratio of β-Gal synthesis to 2.4, which is similar to the levels observed for the cases of base substitutions in *cre-iolB* (Table 3). These results indicate that *cre-iolB* is actually involved in catabolite repression of the transcription from approximately 2,400 bp downstream of its own promoter (P*iol*), although it appeared to mediate a low level of catabolite repression. Furthermore, even if *cre-iolB* had been deleted from or mutated in the P*iol-iolA-iolB′-lacZ* fusion, β-Gal synthesis was still partially under catabolite repression. This suggests that another *cre* responsible for this residual catabolite repression exists between nt −107 and +2270 of the *iol* region, although the genome-wide *cre* search with the query sequence (WTGNAANCGNWNNCW) failed to reveal any *cre* candidate (15).

Identification of another *cre* for catabolite repression of the *iol* operon. To find another *cre* for the *iol* operon, which might be located between nt −107 and +2270, we further constructed a series of deletion derivatives of strain FU713, from nt +2270 toward the 5′ direction in the P*iol-iolA-iolB′-lacZ* fusion (Fig. 1), and determined the catabolite repression ratios of β-Gal synthesis (Table 3). The catabolite repression ratios of β-Gal synthesis (2.3 to 2.4) obtained with strains FU719, FU720, FU721, and FU722 carrying *iol* regions from nt −107 to nt +1192, +712, +298, and +110, respectively, were almost the same as that with strain FU713 carrying one from nt −107 to +2270 (2.4) (Table 3), indicating that no other *cre* is located in the *iol* region between nt +111 and +2270 covering *iolA* and *iolB′*. However, β-Gal synthesis was completely relieved from catabolite repression in strains FU723 and FU724 carrying *iol* regions from nt −107 to +82 and +47, respectively (repression ratio = 0.9). These results suggest that another *cre* for the *iol* operon is located between nt +83 and +110.

Careful examination of the nucleotide sequence of the *iol* region (nt +83 to +110) revealed that this region contains a *cre*-like sequence (nt +86 to +100) exhibiting high similarity to the 5′ side of the consensus sequence (WTGNAANCGNWNN CW) (Fig. 1). In a very recent publication dealing with whole genome analyses (17), this sequence has been also proposed to function as a *cre*. To determine whether or not the *cre*-like sequence is another *cre* for the *iol* operon, we introduced the base substitutions of +91A→G and +94G→T into this sequence in strain FU721 carrying the P*iol-iolA′*(nt −107 to +298)-*lacZ* fusion, which resulted in strains FU734 and FU735, respectively (Fig. 1). As shown in Table 3, these base substitutions completely abolished the catabolite repression of β-Gal synthesis in strains FU734 and FU735 (repression ratios = 0.8 and 0.9, respectively), indicating that the *cre*-like sequence functioned as a *cre* for the *iol* operon, and was designated as *cre-*i*iolA*.

Functional analysis of *cre-*i*iolA* by means of base substitutions. Fig. 2 lists several *cre*'s, such as *cre-*i*xynB* (7), *cre-*i*araA* (22), *cre-*i*araE* (23), *cre-*i*levD* (12), *cre-*i*acuA* (9), and *cre-*i*acoR* (1), which have been reported to function or proposed to function as *cre*'s but were not revealed in our previous *cre* search (15). Interestingly, the last C of the consensus sequence of WTGNAANCGNWNNCW is not conserved in the sequences of these *cre*'s as well as *cre-*i*iolA* (Fig. 2), nevertheless the substitution of this C abolished the *cre* function almost completely, as in the cases of C→T and G in *cre-*i*amyE* (27), C→T in *cre-gntR* (6), C→T in *cre-hutP* (28), and C→T in *cre-iolB* (Table 3). Instead, the WN bases underlined in the above sequence are TT in all of these *cre* sequences (Fig. 2). Thus, we examined whether or not the positioning of the TT bases is essential for *cre-*i*iolA* function.

TABLE 4. Base substitution analysis for *cre-*i*iolA* to monitor *lacZ* expression under the control of the *spac* promoter and *cre-*i*iolA*[a]

Strain	Base substitution in *cre-*i*iolA*	β-Gal activity (nmol/min/mg)		Repression ratio[b]
		−Glc	+Glc	
FU738	Wild type	226	53	4.3
FU752	+96T→G	219	240	0.9
FU753	+96T→A	203	207	1.0
FU754	+97T→G	232	272	0.9

[a] Cells of *B. subtilis* strains were grown with (+) and without (−) glucose (Glc), and β-Gal activities in crude extracts were determined as described in the text. β-Gal activities are expressed as averages of the values obtained for at least three independent experiments.
[b] Ratio of −Glc to +Glc.

We first constructed a P*spac*-(*cre-*i*iolA*)-*lacZ* fusion through cloning of an *iol* region (nt +63 to +121) containing *cre-*i*iolA* into the *Bam*HI site of plasmid pCRE-test (15) and integrated it into the *amyE* locus of strain GM122, which resulted in strain FU738. As shown in Table 4, β-Gal synthesis in strain FU738, which is directed by a constitutive *spac* promoter (P*spac*), was subjected to catabolite repression (repression ratio = 4.3) due to the presence of *cre-*i*iolA* between *Pspac* and *lacZ*. Then, we introduced three base substitutions of the TT of *cre-*i*iolA* (+96T→G, +96T→A, and +97T→G) into the P*spac*-(*cre-*i*iolA*)-*lacZ* fusion of strain FU738, which resulted in strains FU752, FU753, and FU754, respectively. As shown in Table 4, these base substitutions completely abolished the catabolite repression of β-Gal synthesis observed with strain FU738 (repression ratios = 0.9 for strains FU752 and FU754 and 1.0 for strain FU753). The results clearly indicate that the TT of *cre-*i*iolA* are essential for its function.

Analysis of HPr and Crh involvement in catabolite repression exerted through *cre-*i*iolA* and *cre-iolB*. Not only HPr but also Crh is involved in catabolite repression of Idh synthesis (Table 2) (8), which is likely exerted through *cre-*i*iolA* and *cre-iolB* (Table 2). The sequence of *cre-*i*iolA* was found to be distinct from that of *cre-iolB* (Fig. 2). So, we examined the HPr and Crh involvement in the catabolite repressions exerted through *cre-*i*iolA* and *cre-iolB*. We constructed a series of isogenic strains—FU726 (wild type), FU728 (*ptsH1*), FU748 (*crh*::*aphA3*), and FU750 (*ptsH1 crh*::*aphA3*)—carrying the P*spac*-(*cre-*i*iolA*)-*lacZ* fusion in the *amyE* locus and another series of isogenic strains—FU727 (wild type), FU729 (*ptsH1*), FU749 (*crh*::*aphA3*), and FU751 (*ptsH1 crh*::*aphA3*)—carrying the P*spac*-(*cre-iolB*)-*lacZ* fusion in this locus.

As shown in Table 5, β-Gal synthesis in strain FU726 (wild type) was under catabolite repression exerted through *cre-*i*iolA* (repression ratio = 3.2). This ratio was reduced to 1.5 in FU728 (*ptsH1*) but remained almost the same (repression ratio = 3.3) in strain FU748 (*crh*::*aphA3*). Also, this synthesis was completely relieved from catabolite repression in strain FU743 (*ptsH1 crh*::*aphA3*). In a similar manner, the catabolite repression ratios of β-Gal synthesis exerted through *cre-iolB* in strains FU727 (wild type), FU729 (*ptsH1*), FU749 (*crh*::*aphA3*), and FU751 (*ptsH1 crh*::*aphA3*) were found to be 4.3,

TABLE 5. Effects of the *ptsH, crh* and *ccpA* mutations on catabolite repression of β-Gal synthesis under the control of the *spac* promoter and *cre*-i*iolA* or *cre-iolB*

Strain	*cre*	Relevant genotype	β-Gal activity (nmol/min/mg)		Repression ratio[b]
			−Glc	+Glc	
FU726	i*iolA*	Wild type	212	67	3.2
FU728	i*iolA*	*ptsH1*	221	148	1.5
FU748	i*iolA*	*crh*::*aphA3*	240	73	3.3
FU750	i*iolA*	*ptsH1 crh*::*aphA3*	278	333	0.8
FU742	i*iolA*	Wild type	291	95	3.1
FU743	i*iolA*	*ccpA1*	337	370	0.9
FU727	*iolB*	Wild type	226	53	4.3
FU729	*iolB*	*ptsH1*	196	147	1.3
FU749	*iolB*	*crh*::*aphA3*	183	42	4.4
FU751	*iolB*	*ptsH1 crh*::*aphA3*	259	279	0.9
FU744	*iolB*	Wild type	307	70	4.4
FU745	*iolB*	*ccpA1*	320	328	1.0

[a] Cells of *B. subtilis* strains were grown with (+) and without (−) glucose (Glc), and β-Gal activities in crude extracts were determined as described in the text. β-Gal activities are expressed as averages of the values obtained for at least three independent experiments.

[b] Ratio of −Glc to +Glc.

1.3, 4.4, and 0.9, respectively (Table 5). Furthermore, isogenic strains FU742 (wild type) and FU743 (*ccpA1*) carrying the P*spac*-(*cre*-i*iolA*)-*lacZ* fusion exhibited catabolite repression ratios of 3.1 and 0.9, respectively, while isogenic strains carrying the P*spac*-(*cre-iolB*)-*lacZ* fusion exhibited the ratios of 4.4 and 1.0. These results clearly indicate that the catabolite repression exerted through *cre*-i*iolA* and that exerted through *cre-iolB* occur independently of each other; the latter repression (repression ratios = 4.3 and 4.4 for the wild type) seemed somewhat severer than the former (repression ratios = 3.2 and 3.1). These findings also suggest that HPr is likely to be involved in catabolite repression exerted by both *cre*-i*iolA* and *cre-iolB* to almost the same extents, and if HPr is deficient, Crh can compensate for the HPr function partially.

DISCUSSION

Glucose repression of the *iol* operon is known to be exerted through catabolite repression mediated by CcpA and a regulation system involving IolR, probably through inducer exclusion (30). We investigated the catabolite repression of the *iol* operon under experimental conditions where the involvement of IolR in its repression was eliminated. Deletion and base substitution analysis allowed us to identify two *cre*'s of the *iol* operon (*cre*-i*iolA* and *cre-iolB*) (Fig. 1 and Tables 3 and 4). *cre*-i*iolA* is located between the *iol* promoter and the *iolA* gene (nt +86 to +100), while *cre-iolB* is in *iolB* (nt +2397 to +2411). The presence of two *cre*'s has been reported in the *gnt* (14), *ackA* (26), *ara* (22), and *rbs* (15, 24) operons. Our previous in vivo results (14) implied that catabolite repression exerted by *cre*-i*gntR* (cre_{up}) was probably independent of that exerted by *cre-gntR* (cre_{down}). This study also revealed that *cre*-i*iolA* and *cre-iolB* likely function independently (Table 5). In addition, *cre-iolB* was found to function at the original location (nt +2397 to +2411), i.e., far downstream of the transcription initiation site (Table 3). Although two *cre*'s of each of the other operons have not been characterized well, it is notable that the relative locations of the two *cre*'s of the *ara* and *rbs* operons are very similar to those of *iol*. These results are well consistent with the idea that the mechanism underlying catabolite repression can be explained by a transcription roadblock if *cre* is located downstream of the transcription initiation site (6, 13).

Our previous genome-wide *cre* search using a query sequence of WTGNAANCGNWNNCW (15) failed to reveal *cre*-i*iolA* as well as six known *cre*'s (*cre*-i*xynB* [7], *cre*-i*araA* [22], *cre*-i*araE* [23], *cre*-i*levD* [12], *cre*-i*acuA* [9], and *cre*-i*acoR* [1]), although it revealed 126 putative and known *cre*'s, including *cre-iolB*. Alignment of the sequences of the six *cre*'s not revealed by the search led to another consensus sequence of WT GAAARCGYTTWWN (Fig. 2). The 5′ part of this consensus sequence perfectly coincides with the previous query sequence, but the 3′ one does not match it well. It is notable that the 3′ part of the latter consensus sequence includes conserved TT but is devoid of the last CW of the former consensus sequence. Actually, base substitution analysis of the TT of *cre*-i*iolA* ind icated that they are indispensable for its function (Fig. 2 and Table 4). Although the sequence of *cre*-i*iolA* appeared to be distinct from that of *cre-iolB* in conserved bases, a protein complex of CcpA with either P-Ser-HPr or P-Ser-Crh likely binds to both *cre*'s to similar extents (Table 5).

Recent analysis of *B. subtilis cre* sequences led to the following three conclusions (15). (i) Lower mismatching of *cre* sequences with the query sequence (WTGNAANCGNWNNCW) is required for *cre* function. (ii) Although *cre* sequences are partially palindromic, lower mismatching in the same direction as that of transcription of the target genes is more critical for *cre* function than that in the inverse direction. (iii) Yet, a more palindromic nature of *cre* sequences is desirable for a better function. Comparison of the above two consensus sequences also implied that the 5′ part of *cre* sequences should be well conserved for their efficient function and that a protein complex of CcpA with P-Ser-HPr or P-Ser-Crh recognizes this part. The last CW (preferably CA) of the query sequence of WTG NAANCGNWNNCW is likely to be required for pairing with TG, resulting in proper binding of the complex. However, this pairing might be compensated for by another pairing of TT of the second consensus sequence of WTGAAARCGYTTWWN with AA. Of course, both pairings appear to be more desirable for efficient *cre* function.

We searched for *cre* sequences in the *B. subtilis* genome with the currently deduced consensus sequence of WTGAAARCG YTTWW through the DNA pattern search program of the SubtiList Web Server (http://genolist.pasteur.fr/SubtiList/). This search revealed 14 more putative *cre*'s without any mismatch: *cre*-i*ybgJ*, *cre*-i*ycbF*, *cre*-i*yceK*, *cre*-i*ydaA*, *cre-yebB*, *cre*-i*opuE*, *cre*-i*slp*, *cre*-i*yqkI*, *cre-levR*, *cre*-i*ysfC*, *cre-yusL*, *cre*-i*yvbQ*, *cre*-i*yvfK*, and *cre*-i*yycE*. Our present study implies that a genome-wide search for certain *cis*-acting elements with a single query sequence will not reveal most of the elements in question because of additional features due to their secondary structure, such as a palindromic nature (this work) and sequence periodicity (31). In the case of a *cre* search, a genome-wide search with these two query sequences appears to be able to reveal almost all of them.

ACKNOWLEDGMENTS

We thank K. Adachi, S. Yamada, M. Takatani, M. Abe, and S. Iijima for their help in the experiments.

This work was supported by a grant, JSPS-RFTF96L00105, from the Japan Society for the Promotion of Science.

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