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Expression of the *Bacillus subtilis acsA* Gene: Position and Sequence Context Affect *cre*-Mediated Carbon Catabolite Repression

JILL M. ZALIECKAS, LEWIS V. WRAY, JR., AND SUSAN H. FISHER*

Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts 02118

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In *Bacillus subtilis*, carbon catabolite repression (CCR) of many genes is mediated at *cis*-acting carbon repression elements (*cre*) by the catabolite repressor protein CcpA. Mutations in transcription-repair coupling factor (*mfd*) partially relieve CCR at *cre* sites located downstream of transcriptional start sites by abolishing the Mfd-mediated displacement of RNA polymerase stalled at *cre* sites which act as transcriptional roadblocks. Although the *acsA cre* is centered 44.5 bp downstream of the *acsA* transcriptional start site, CCR of *acsA* expression is not affected by an *mfd* mutation. When the *acsA cre* is centered 161.5 bp downstream of the transcriptional start site for the unregulated *tms* promoter, CCR is partially relieved by the *mfd* mutation. Since CCR mediated at an *acsA cre* centered 44.5 bp downstream of the *tms* start site is not affected by the *mfd* mutation, the inability of Mfd to modulate CCR of *acsA* expression most likely results from the location of the *acsA cre*. Higher levels of CCR were found to occur at *cre* sites flanked by A+T-rich sequences than at *cre* sites bordered by G and C nucleotides. This suggests that nucleotides adjacent to the proposed 14-bp *cre* consensus sequence participate in the formation of the CcpA catabolite repression complex at *cre* sites. Examination of CCR of *acsA* expression revealed that this regulation required the Crh and seryl-phosphorylated form of the HPr proteins but not glucose kinase.

In *Bacillus subtilis*, carbon catabolite repression (CCR) of many genes is mediated at a *cis*-acting site called a carbon repression element (*cre*) (20). The *cre* sites for the *acsA* (15), *xyl* (22), *gnt* (11), and *hut* (31, 43) genes are downstream of the transcriptional start site, while the *cre* sites for the *lev* (26), *bglPH* (23), *acu* (15), *amyE* (42), and *mmg* (3) genes lie within or adjacent to the promoter region (Table 1). While the *cre* is generally considered to be a 14-bp sequence with dyad symmetry (20, 42), most *cre* sites are flanked by A+T-rich sequences (Table 1). CCR mediated at all known *B. subtilis cre* sites is relieved by inactivation of the *ccpA* gene, which encodes the CCR repressor protein (3, 7, 10, 15, 19, 23, 26, 43). CcpA is a member of the LacI/GalR family of regulatory proteins (19, 41). The *cre* sites have significant sequence similarity to the operators for other proteins belonging to the LacI/GalR family (20). Recently, CCR of *gnt* and *xyl* expression was reported to be partially relieved by inactivation of the *ccpB* gene, which encodes a CcpA homolog (5). Interestingly, CcpB-dependent regulation was observed only in cells grown in liquid cultures with "low aeration" or on solid medium.

Multiple factors, including the protein homologs HPr and Crh as well as glucose-6-phosphate (Glc-6-P), have been proposed to function as corepressors for CcpA binding to *cre* sites (11, 12, 14, 27). The HPr protein is a signal transduction component of the phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) (33). In gram-positive bacteria, HPr can be phosphorylated at two residues, His-15 by enzyme I of the PTS and Ser-46 by the ATP-dependent HprK kinase (13, 34, 35). It has been demonstrated in vitro that the seryl-phosphorylated form of HPr (HPr-ser-P) enhances binding of CcpA to the downstream *cre* sites in the *B. subtilis gnt* and the *Bacillus megaterium xyl* operons (11, 14, 27). The in vivo role of HPr in *B. subtilis* CCR has been studied in strains that contain a mutation in the HPr-encoding *ptsH* gene that replaces the Ser-46 codon with an alanine codon. This allele, *ptsH1*, produces a mutant form of HPr that cannot be phosphorylated by HprK (8, 13, 34, 35). The *ptsH1* mutation relieves CCR of the *bglPH* (23), *gnt* (8, 27), *iol* (12), *lev* (26), and *xyl* (7) genes but does not affect glucose repression of the *amyE* gene (40). Crh (*crh*), an HPr homolog, has also been found to be involved in *B. subtilis* CCR (12). It was reported that complete relief of CCR for inositol dehydrogenase, levanase, and β-xylosidase expression required both *ptsH1* and *crh* mutations (12). Since Crh is phosphorylated at a seryl residue by the HprK kinase, the phosphorylated form of Crh has also been proposed to function as a corepressor for the binding of CcpA (12, 13).

Glc-6-P enhances the in vitro binding of CcpA to multiple *cre* sites within the *B. megaterium xyl* and *B. subtilis gnt* operons (14, 27). The primary *cre* in the *B. megaterium xyl* operon is centered 130.5 bp downstream of the *xyl* transcriptional start site. In the presence of Glc-6-P, CcpA binds cooperatively to this downstream *xyl cre* and two auxiliary *cre* sites, one of which is located within the *xyl* promoter region (14). In contrast, HPr-ser-P enhances noncooperative binding to the primary *xyl cre* site and the downstream *gnt cre* site (14, 27). In vivo evidence that Glc-6-P is directly involved in mediating CCR has been suggested by the observation that a loss-of-function mutation in the *B. megaterium* gene encoding glucose kinase (*glk*) partially relieves CCR of *xyl* operon expression (38).

Mutations in *mfd*, which encodes transcription-repair coupling factor, partially relieve CCR mediated at the downstream *cre* sites in the *hut* and *gnt* operons but not at *cre* sites located in promoter regions of the *bglPH*, *gnt*, and *amyE* genes (44). Mfd promotes strand-specific DNA repair by displacing RNA polymerase stalled at a nucleotide lesion and recruiting the (A)BC exinuclease to the DNA damage site (36). These results suggest that the downstream *cre* sites in the *hut* and *gnt* oper-

* Corresponding author. Mailing address: Department of Microbiology, Boston University School of Medicine, 715 Albany St., Boston, MA 02118. Phone: (617) 638-5498. Fax: (617) 638-4286. E-mail: shfisher@bu.edu.

TABLE 1. Sequence alignment and positions of established carbon repression elements[a]

Gene or operon[b]	DNA Sequence[c]	Position (bp)[d]
lev	TAACAA TGAAAACGCTTAAC ACAACT	−45.5
gnt (*cre*$_{up}$)	TAGAAA TGAAAGTGTTTGCA TAAAAG	−37.5
bglPH	CAAAAA TGAAAGCGTTGACA TCTCAC	−36.5
acu	CATTGT TGAAAACGCTTTAT AATTTG	−26.5
amyE	TTTAAA TGTAAGCGTTAACA AAATTC	+4.5
mmg	AGAAAT TGTAAGCGCTGTCT ATCTTC	+21.5
acsA	TGAACT TGAAAGCGTTACCA GCAATA	+44.5
xyl	CTATTT TGGAAGCGTAAACA AAGTGG	+140.5
gnt (*cre*$_{down}$)	TCTGAT TGAAAGCGGTACCA TTTTAT	+147.5
hut	CGCAAT TGAAACCGCTTCCA AAAAGA	+209.5
Consensus	WWWW TGWAARCGYTWNCW WWWW	

[a] References for each *cre* are given in the text.
[b] *cre*$_{up}$ and *cre*$_{down}$ denote *gnt cre* upstream and downstream sites, respectively.
[c] Symbols for ambiguous nucleotides in the consensus sequence are as follows: W represents A or T; R represents A or G; Y represents C or T; and N represents A, C, G, or T.
[d] The location of each *cre* is given as a center position relative to the transcriptional start site.

ons act as transcriptional roadblocks for RNA polymerase and that Mfd enhances CCR mediated at these sites by displacing RNA polymerase stalled at CcpA-*cre* complexes in the *hut* and *gnt* operons.

Surprisingly, CCR of *acsA* expression, which requires a *cre* centered 44.5 bp downstream of the *acsA* transcriptional start site, is not altered in the *mfd* mutant (15). In this report, we demonstrate that the location of the *acsA cre* is most likely responsible for the inability of Mfd to modulate CCR mediated at this *cre*. Interestingly, nucleotides adjacent to the proposed 14-bp *cre* consensus sequence were found to contribute to the level of CCR mediated at *cre* sites.

MATERIALS AND METHODS

Bacterial strains. Table 2 lists *B. subtilis* strains used in this study. All *lacZ* transcriptional fusions were transformed into strain 168 (*trpC2*) by using plasmid DNA, as previously described (43). The *mfd22*::Tn*10* insertion was transferred by transformation with selection for transposon-encoded chloramphenicol resistance. Transformation with selection for spectinomycin (*spc*) resistance was used to transfer the *glcK*::*spc*, *ccpB*::*spc*, and *crh*::*spc* mutations. The *ptsH1* mutation was transferred by transformation with selection for the genetically linked chloramphenicol resistance gene. Transformants containing the *ptsH1* mutation were identified by lack of growth on mannitol minimal medium plates containing ammonium as the nitrogen source (8).

The *lacZ* α-complementation *Escherichia coli* strain TOP10 (Invitrogen Corp.) was used as the host for DNA cloning experiments with plasmid pMTL21P (4). *E. coli* MC1061 contains a deletion of the chromosomal *lac* genes and was used for the construction of *lacZ* fusions. A derivative of MC1061 containing the plasmid copy number mutation *pcnB80* (25) was used as the host for plasmids containing the *B. subtilis glcK* gene.

TABLE 2. *B. subtilis* strains used in this study

Strain	Genotype[a]	Reference, source, and/or derivation[b]
168	*trpC2*	This laboratory
QB7097	*trpC2 crh*::*spc*	I. Martin-Verstraete
GM1222	*trpC2 pheA1* Δ(*bgaX*) *ptsH1*(*cat*) Δ*amyE*::(*gntRK′*-*lacZ*)	J. Deutscher (8)
JZ6	*trpC2 glcK*::*spc*	168 × pGLK6
JZ7	*trpC2 ccpB*::*spc*	168 × pCCP105
SF13CDH	*trpC2 ccpA*::Tn*10*	This laboratory
SF22CDH	*trpC2 mfd22*::Tn*10*	44

[a] Genotypic designations are those of Biaudet et al. (2) with the addition of *crh* (12) and *glcK* (37).
[b] Strains were derived by transforming the indicated strain with DNA from the indicated plasmid.

Cell growth, media, and enzyme assays. The methods used for bacterial cultivation have been previously described (1). Minimal liquid cultures were grown in the morpholinepropanesulfonic acid (MOPS) minimal medium of Neidhardt et al. (29). Glucose and glutamine were added to final concentrations of 0.5 and 0.2%, respectively, to MOPS minimal medium.

Extracts for enzyme assays were prepared as previously described (1). Cells grown in minimal medium were harvested during exponential growth (75 to 85 Klett units). β-Galactosidase was assayed as described previously (1). One unit of β-galactosidase activity produced 1 nmol of *o*-nitrophenol per min. β-Galactosidase activity was always corrected for endogenous β-galactosidase activity present in *B. subtilis* 168 cells containing the promoterless *lacZ* gene from pSFL6 or pSFL7 integrated at the *amyE* site.

Plasmids and *lacZ* fusions. pSFL6 and pSFL7 are neomycin resistance *lacZ* transcriptional fusion vectors that integrate into the *amyE* locus and contain promoterless *trpA-lacZ* and *spoVG-lacZ* genes, respectively (44). The TMS922 *lacZ* fusion contains the *tms* promoter (28) cloned into pSFL7 (44). The HUT924 *lacZ* fusion is a derivative of pSFL7 that contains a DNA fragment with the *hut cre* located downstream of the *tms* promoter (44).

B. subtilis SF13CDH contains a Tn*10* transposon insertion in the *ccpA* gene (9). Chromosomal DNA adjacent to the transposon insertion was cloned by plasmid rescue (39) and used for directed chromosomal walking to obtain DNA downstream of *ccpA* that contained the *acsA* gene (16). Plasmid pACS6 was constructed by inserting a *Sal*I-*Eco*RV DNA fragment containing the *acsA* promoter and *cre* into pJDC9 (6). The ACS7 *lacZ* fusion contains the *acsA* DNA fragment from pACS6 inserted into pSFL6.

Inactivation of the chromosomal *ccpB* and *glcK* genes. A 1,680-bp DNA fragment containing the *ccpB* gene was obtained by PCR amplification of *B. subtilis* chromosomal DNA with *Pwo* DNA polymerase (Boehringer Mannheim) by using primers CCPB1 (5′-GAAAAAAGGATATTCCGGCACAG) and CCPB2 (5′-TCATTCGCTCTAAATCATTGACCC). Plasmid pCCP104 contains a 1,537-bp *Pst*I-*Nsi*I DNA fragment from the PCR-generated DNA cloned into pMTL21P (4). The *ccpB* coding sequence contains an *Hpa*I site that overlaps codons 78 and 79. pCCP105 contains a spectinomycin resistance gene cassette (18) inserted into this *Hpa*I site of pCCP104. The chromosomal *ccpB* gene was disrupted by transforming *B. subtilis* cells with linearized pCCP105 DNA.

PCR amplification with primers GLKA1 (5′-TTCGCCTTCACACCAGGAGTC) and GLKA2 (5′-CCGTTCATTTTTCGTTGAGGG) was used to obtain a 2,247-bp DNA fragment containing the *B. subtilis glcK* coding region (37). A 2,039-bp *Bcl*I-*Hin*dIII DNA fragment from the PCR-generated DNA was cloned into pJDC9 to construct pGLK3. Plasmid pGLK6 contains a spectinomycin resistance gene cassette (18) inserted into an *Ehe*I site located within the *glcK* coding sequence of pGLK3. The chromosomal *glcK* gene was disrupted by transforming *B. subtilis* cells with linearized pGLK6 DNA.

Oligonucleotide mutagenesis. Plasmid pTMS902 contains the *tms* promoter cloned into the polylinker region of pALTER-1 (Promega Corp.). pTMS949 and pTMS950 were constructed by oligonucleotide-directed mutagenesis of pTMS902 by a protocol provided by the supplier of pALTER-1 (Promega Corp.) to place the *acsA cre* sequence at +44.5 relative to the *tms* promoter transcriptional start site. The *Eco*RI-*Hin*dIII fragments from pTMS949 and pTMS950 were cloned into pSFL7 to construct the TMS951 and TMS952 *lacZ* fusions. pHUT904 was constructed by inserting a *hut cre Mun*I-*Bsr*BI DNA fragment downstream of the *tms* promoter in pTMS902. Mutations in the *hut cre* of pHUT904 were generated by oligonucleotide mutagenesis. *Eco*RI-*Hin*dIII DNA fragments containing the *tms* promoter and mutated *hut cre* were cloned into pSFL7 to construct the TMS940, TMS942, and TMS948 *lacZ* fusions.

RESULTS

CCR of *acsA* expression in an *mfd* mutant strain. To determine if the Mfd protein is involved in mediating CCR at the *acsA cre*, expression of an *acsA-lacZ* fusion (ACS7) was examined in wild-type and *mfd* mutant strains. Although the *acsA cre* site is centered 44.5 bp downstream of the *acsA* transcriptional start site (15), the *mfd* mutation does not alter CCR of *acsA* expression (Table 3). This result was unexpected because several lines of evidence suggested that Mfd would be involved in CCR at a *cre* located in this position. First of all, Mfd can stimulate transcription-dependent repair at nucleotide lesions located 15 nucleotides downstream of a promoter start site (36). Secondly, when the *E. coli lac* repressor functions as a transcriptional roadblock in vivo, transcription elongation by RNA polymerase terminates 16 nucleotides upstream of the center of the *lac* operator (17). If this observation for the *lac* operator is applied to the *acsA cre*, then transcription elonga-

TABLE 3. β-Galactosidase expression from *lacZ* fusions in wild-type and *mfd* mutant strains

lacZ fusion[a]	Relevant genotype	*cre* position (bp)[b]	β-Galactosidase sp act (U/mg of protein) in cells grown on[c]:		Glucose repression ratio[d]
			Glucose	Citrate	
ACS7	Wild type	+44.5	3.6	62.1	17
	mfd	+44.5	4.6	88.1	19
TMS922	Wild type		30.5	30.4	1.0
	mfd		51.2	49.7	1.0
HUT924	Wild type	+161.5	2.5	34.8	14
	mfd	+161.5	13.3	68.5	5.1
TMS940	Wild type	+161.5	1.1	25.0	23
	mfd	+161.5	5.1	45.0	8.8
TMS942	Wild type	+161.5	8.5	32.1	3.8
	mfd	+161.5	25.0	45.0	1.8
TMS948	Wild type	+161.5	12.9	33.7	2.6
TMS951	Wild type	+44.5	0.5	15.9	32
	mfd	+44.5	0.2	35.4	177
TMS952	Wild type	+44.5	5.9	30.7	5.2
	mfd	+44.5	8.0	53.7	6.7

[a] All strains are derivatives of 168, with the indicated *lacZ* fusion integrated as a single copy at the *amyE* locus.

[b] Position relative to the transcriptional start site (+1).

[c] Data are averages of three or more determinations which did not vary by more than 20%. Cultures were grown in MOPS minimal medium containing glutamine as the nitrogen source and the indicated carbon source.

[d] The glucose repression ratio was calculated by dividing the enzyme activity found in cultures grown with citrate by the enzyme activity found in glucose-grown cultures.

lacZ Fusion	DNA Sequence
consensus *cre*	WWWW TGWAARCGYTWNCW WWWW
HUT924 (*hut cre*)	ATGAAT TGAAACCGCTTCCA AAAAGA
TMS940	ATGAAT TGAAAGCGTTACCA AAAAGA
TMS942	ATGACT TGAAAGCGTTACCA GCAAGA
TMS948	ATGACT TGAAACCGCTTCCA GCAAGA
ACS7 (*acsA cre*)	TGAACT TGAAAGCGTTACCA GCAATA
TMS951	GATAAT TGAAAGCGTTACCA TTTAGC
TMS952	GATACT TGAAAGCGTTACCA GCTAGC
TMS922 (*tms*)	GATAAG CGGTTTGCAGTTGT TTTAGC

FIG. 1. *cre* DNA sequences. The HUT924 sequence corresponds to the *hut cre* and flanking DNA present in the HUT924 *lacZ* fusion (P_{tms}-*hut cre-lacZ*). The ACS7 sequence corresponds to the *acsA cre* with its flanking DNA present in ACS7 (*acsA-lacZ*) and is shown in reverse text. HUT940, HUT942, and HUT948 were derived from HUT924; the nucleotides shown in reverse text denote the positions within the *hut cre* that were changed to match the sequence of the *acsA cre* (ACS7). The TMS922 sequence corresponds to DNA located 44.5 bp downstream of the *tms* promoter (30). TMS951 and TMS952 were derived from TMS922; the nucleotides shown in reverse text denote nucleotides downstream of the *tms* promoter that were changed to match the sequence of the *acsA cre*.

tion would be expected to terminate 28 nucleotides downstream of the *acsA* transcriptional start site (assuming that the *acsA cre* functions as a roadblock). Taken together, these observations suggest that the Mfd protein should be able to recognize and dissociate RNA polymerase from a transcriptional roadblock at the *acsA cre*.

CCR at *cre* sites centered 161.5 bp downstream of the transcriptional start site for an unregulated promoter. It is possible that the CcpA-*acsA cre* complex can function as a transcriptional roadblock but that some unique characteristics of the *acsA cre*, such as the proximity of the *acsA cre* to the *acsA* promoter, prevents Mfd from interacting with RNA polymerase. To test this hypothesis, the *acsA cre* was placed downstream of the *tms* promoter in a P_{tms}-*lacZ* transcriptional fusion and CCR of β-galactosidase expression was examined in wild-type and *mfd* mutant strains. Expression of the *tms* promoter is not regulated by CCR in wild-type or *mfd* mutant cells (TMS922 *lacZ* fusion [Table 3]). The HUT924 *lacZ* fusion (44) contains the *hut cre* site centered 161.5 bp downstream of the transcriptional start site for the *tms* promoter and is regulated by CCR (Table 3). Oligonucleotide-directed mutagenesis was used to convert the *hut cre* in HUT924 to an *acsA cre* by making three nucleotide changes within the 14-bp central *hut cre*. The resulting TMS940 *lacZ* fusion contains the central 14-bp sequence of the *acsA cre* (Fig. 1). β-Galactosidase expression from the TMS940 *lacZ* fusion was repressed 23-fold by glucose in the wild-type strain but only 8.8-fold in the *mfd22* mutant (Table 3). These results indicate that Mfd can modulate CCR mediated at a 14-bp *acsA cre* centered 161.5 bp downstream of a transcriptional start site.

As noted previously, most *cre* sequences contain a 14-bp core sequence flanked by A or T nucleotides (Table 1). The *acsA cre*, which contains G or C nucleotides at three of the four positions directly adjacent to the 14-bp core *cre* sequence, is a notable exception to this generalization (Table 1). To examine the contribution of the flanking G and C nucleotides to the activity of the *acsA cre* (Table 1), six bases of the *hut cre* in the HUT924 *lacZ* fusion were altered so that they matched the *acsA cre* sequence. The resulting 18-bp *acsA cre* in the TMS942 *lacZ* fusion contains the 14-bp core *acsA cre* sequence and its flanking nucleotides (Fig. 1). Interestingly, β-galactosidase expression from the TMS942 *lacZ* fusion was repressed only 3.8-fold by glucose in the wild-type cells (Table 3). Expression of the TMS942 fusion was partially relieved by the *mfd* mutation (Table 3). Although the level of CCR observed with the TMS942 *lacZ* fusion is surprisingly low, Mfd modulates CCR observed with the TMS942 fusion.

The low level of CCR observed with the TMS942 *lacZ* fusion suggested that nucleotides flanking the 14-bp core *cre* sequence can affect the ability of the *cre* to mediate CCR. To test this hypothesis, three nucleotides flanking the 14-bp *hut cre* in the HUT924 *lacZ* fusion were altered so that they matched the flanking nucleotides present in the *acsA cre*. The resulting *lacZ* fusion, TMS948, contains the 14-bp *hut cre* flanked by the G and C nucleotides present in the *acsA cre* (Fig. 1). Expression of TMS948 was repressed only 2.6-fold by glucose in the wild-type strain (Table 3). Since the level of CCR observed with the TMS948 *lacZ* fusion (2.6-fold) is lower than that seen with the HUT924 fusion (14-fold), the activity of a *cre* appears to be dependent upon its flanking nucleotides.

CCR at *cre* sites centered 44.5 bp downstream of the transcriptional start site for an unregulated promoter. To determine whether Mfd can modulate CCR mediated at an *acsA cre* centered 44.5 bp downstream of the transcriptional start site of a heterologous promoter, oligonucleotide-directed mutagenesis was used to place an *acsA cre* 44.5 bp downstream of the *tms* promoter. The TMS951 *lacZ* fusion contains the 14-bp core *acsA cre* sequence flanked by A+T-rich nucleotides (Fig. 1). The 14-bp core *acsA cre* sequence in the TMS952 *lacZ* fusion is flanked by the three G and C bases found adjacent to the *acsA cre* site (Fig. 1). In wild-type cells, lower levels of CCR were observed with the TMS952 *lacZ* fusion (5.2-fold) than with the TMS951 *lacZ* fusion (32-fold) (Table 3). CCR of

TABLE 4. β-Galactosidase expression from an *acsA-lacZ* fusion in wild-type and mutant strains

Relevant genotype[a]	β-Galactosidase sp act (U/mg of protein) in cells grown on[b]:		Glucose repression ratio[c]
	Glucose	Citrate	
Wild type	3.6	57.8	16
ptsH1	7.9	59.8	7.6
crh	4.0	58.5	15
crh ptsH1	35.2	56.3	1.6
glcK	3.7	58.4	16
ccpB	4.2	55.3	13

[a] All strains are derivatives of 168 containing the ACS7 *lacZ* fusion integrated as a single copy at the *amyE* locus.

[b] Data are averages of three or more determinations which did not vary by more than 20%. Cultures were grown in MOPS minimal medium containing glutamine as the nitrogen source and the indicated carbon source.

[c] The glucose repression ratio was calculated by dividing the enzyme activity found in cultures grown with citrate by the enzyme activity found in glucose-grown cultures.

neither the TMS951 nor the TMS952 *lacZ* fusion was relieved by the *mfd* mutation (Table 3).

Role of CcpB, glucose kinase, HPr, and Crh in *acsA* catabolite repression. One possible explanation for why Mfd does not modulate *acsA* CCR is that a unique catabolite repressor complex binds to the *acsA cre* and functions as an Mfd-independent transcriptional roadblock. This hypothesis was tested by determining whether factors known to participate in CCR at other *cre* sites are involved in CCR of *acsA* expression.

The *B. subtilis ccpB* gene encodes a CcpA homolog which has been reported to mediate CCR of *gnt* and *xyl* expression in cells grown on solid medium and in liquid cultures grown with low aeration (5). Although CCR of *acsA* expression has been shown to be dependent upon CcpA in cells grown in nutrient sporulation medium (15), CcpB may participate in *acsA* CCR under our growth conditions. Similar levels of CCR for ACS7 were observed in liquid cultures of wild-type and *ccpB* mutant cells grown in minimal medium (Table 4). In addition, no difference in the color of colonies formed by wild-type and *ccpB* mutant cells containing the ACS7 fusion was observed on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) minimal plates containing glucose and glutamine as carbon and nitrogen sources (data not shown). Taken together, these results indicate that CcpB does not participate in CCR of *acsA* expression under these growth conditions.

Glc-6-P has been proposed to serve as a corepressor for the cooperative binding of CcpA to the *B. megaterium xyl cre* sites (14). There are two pathways for Glc-6-P synthesis in *B. subtilis*. Glc-6-P is the product of glucose transport by the PTS system (33). Alternatively, glucose can also be directly transported into the cell by the GlcP glucose permease (32) and phosphorylated by glucose kinase to produce Glc-6-P. Two observations indicate that GlcP plays a significant role in glucose transport in *B. subtilis*. First, the uptake rate of glucose is 30% lower in a *glcP* mutant than in wild-type cells (32). Second, CCR of *gnt* expression is partially relieved in *B. subtilis glcP* mutants (32). Since *glcK* encodes the only glucose kinase enzyme present in *B. subtilis* (37), Glc-6-P levels are likely to be reduced in *glcK* mutants because glucose transported by GlcP cannot be phosphorylated. Because CCR of *xyl* expression is partially relieved in a *B. megaterium* strain lacking glucose kinase (37), a *B. subtilis glcK* mutant strain was used to examine the possibility that Glc-6-P participates in CCR of *acsA* expression. No difference in the levels of β-galactosidase expression from the ACS7 fusion (*acsA-lacZ*) was seen in wild-type and *glcK* cells (Table 4).

The seryl-phosphorylated forms of HPr and Crh have been proposed to function as corepressors for the noncooperative binding of CcpA to *cre* sites (11, 12, 14). To determine whether HPr and Crh are required for *acsA* CCR, expression of the ACS7 fusion was examined in *B. subtilis* strains containing the *ptsH1* and *crh* mutations. In cells grown in minimal medium, the *ptsH1* mutation partially relieved CCR of *acsA* expression while the *crh* mutation did not affect *acsA* regulation (Table 4). In a *ptsH1 crh* double mutant, CCR of *acsA* expression was almost completely relieved (Table 4).

DISCUSSION

Mfd modulates CCR of gene expression when the *acsA cre* is centered 161.5 bp, but not 44.5 bp, downstream of the transcriptional start site. This suggests that the location of the *cre* relative to the transcriptional start site, rather than the promoter or *cre* sequence, determines whether Mfd participates in CCR mediated at a *cre* site. It is not clear why Mfd is unable to modulate CCR at a *cre* centered 44.5 bp downstream of the transcriptional start site. RNA polymerase stalled at a *cre* site located in this position may have a conformation that prevents Mfd-dependent displacement. Alternatively when the *acsA cre* is centered 44.5 bp downstream of the transcriptional start site, CcpA may inhibit the initiation of transcription by a cooperative interaction involving DNA looping between the downstream *cre* site and an unidentified upstream *cre* site in the promoter region. If the inability of Mfd to modulate CCR at *cre* sites centered 44.5 bp downstream of both the *tms* and *acsA* promoters results from the cooperative binding by CcpA, then these promoter regions must contain unrecognized CcpA binding sites that participate in this cooperative binding.

Both HPr and Crh participate in CCR of *acsA* expression. In cells grown in minimal medium, mutations in both the *ptsH* and *crh* genes are required to significantly relieve CCR of *acsA* expression. This agrees with previously published results showing that both Crh and HPr are required for wild-type levels of CCR of *iol*, *lev*, and β-xylosidase expression in cells grown in minimal medium (12). In vitro studies have shown that HPr-ser-P promotes noncooperative binding of CcpA to *cre* sites (14, 27), while Glc-6-P triggers cooperative binding of CcpA to the downstream *xyl cre* and an auxiliary *cre* site located within the *xyl* promoter region of the *B. megaterium xyl* operon (14). The observation that CCR of *acsA* expression is partially dependent upon HPr, but not glucose kinase, is consistent with the hypothesis that CcpA binds noncooperatively to the *acsA cre* and acts as a transcriptional roadblock.

The *cre* site is generally considered to be a 14-bp DNA sequence with dyad symmetry (20, 42). With the exception of the *acsA cre* site, all known *B. subtilis cre* sites are surrounded by A+T-rich sequences (Table 1). Mutational analysis of the nucleotides adjacent to the 14-bp *cre* site showed that the sequence context affects the level of CCR mediated at a *cre* site. When the *hut* and *acsA cre* sites are positioned downstream of the *tms* promoter, higher levels of CCR are seen at *cre* sites flanked by A+T-rich DNA regions than at sites containing the same 14-bp core sequence flanked by G and C nucleotides. This suggests that CcpA interacts with base pairs immediately adjacent to the 14-bp core *cre* sequence and argues that the *cre* consensus sequence should include these flanking sequences. Examination of the in vitro interactions between CcpA and the *amyE cre* revealed that CcpA contacts three phosphate groups at each end of the 14-bp core *cre* sequence (21). Our results suggest that optimal interactions

between CcpA and these phosphate groups occur when the *cre* core sequence is flanked by A+T-rich sequences. Replacement of the A+T-rich sequences adjacent to the *cre* core sequence with G and C nucleotides could cause subtle DNA conformation changes that diminish these interactions and reduce the affinity of CcpA for the *cre* site. Similar observations have been made for the binding sites for other members of the LacI/GalR family of regulatory proteins. Analysis of the ideal *lac* operator revealed that nucleotides flanking the 14-bp operator can affect both the in vivo repression level and the in vitro binding affinity of *lac* repressor (24).

It is perplexing that the 18-bp *acsA cre* mediates higher levels of CCR when centered 44.5 bp downstream of the *acsA* start site in the ACS7 fusion (17-fold) than when this sequence is centered either 44.5 bp downstream of the *tms* start site in the TMS952 fusion (5.2-fold) or 161.5 bp downstream of the *tms* start site in TMS942 (3.8-fold). One explanation for this result is that the *acsA* promoter region contains sequence determinants which enhance CCR at the *cre* site and that these sequence determinants are not present in the *tms* promoter region. It is also possible that in addition to CcpA, other *trans*-acting factors may regulate expression of the *acsA* promoter in response to carbon availability. Indeed, we have observed that CodY contributes to the regulation of *acsA* expression in response to carbon availability (9).

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REFERENCES

1. **Atkinson, M. R., L. V. Wray, Jr., and S. H. Fisher.** 1990. Regulation of histidine and proline degradation enzymes by amino acid availability in *Bacillus subtilis*. J. Bacteriol. **172:**4758–4765.
2. **Biaudet, V., F. Samson, C. Anagnostopoulos, S. D. Ehrlich, and P. Bessières.** 1996. Computerized genetic map of *Bacillus subtilis*. Microbiology **142:**2669–2729.
3. **Bryan, E. M., B. W. Beall, and C. P. Moran, Jr.** 1996. A σ^E-dependent operon subject to catabolite repression during sporulation in *Bacillus subtilis*. J. Bacteriol. **178:**4778–4786.
4. **Chambers, S. P., S. E. Prior, D. A. Barstow, and N. P. Minton.** 1988. The pMTL nic^- cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. Gene **68:**139–149.
5. **Chauvaux, S., I. T. Paulsen, and M. H. Saier, Jr.** 1998. CcpB, a novel transcription factor implicated in catabolite repression in *Bacillus subtilis*. J. Bacteriol. **180:**491–497.
6. **Chen, J., and D. A. Morrison.** 1987. Cloning of *Streptococcus pneumoniae* DNA fragments in *Escherichia coli* requires vectors protected by strong antiterminators. Gene **55:**179–187.
7. **Dahl, M. K., and W. Hillen.** 1995. Contributions of XylR, CcpA and HPr to catabolite repression of the *xyl* operon in *Bacillus subtilis*. FEMS Microbiol. Lett. **132:**79–83.
8. **Deutscher, J., J. Reizer, C. Fischer, A. Galinier, M. H. Saier, Jr., and M. Steinmetz.** 1994. Loss of protein kinase-catalyzed phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system, by mutation of the *ptsH* gene confers catabolite repression resistance to several catabolic genes of *Bacillus subtilis*. J. Bacteriol. **176:**3336–3344.
9. **Fisher, S. H., J. M. Zalieckas, and L. V. Wray, Jr.** 1997. Unpublished data.
10. **Fujita, Y., and Y. Miwa.** 1994. Catabolite repression of the *Bacillus subtilis gnt* operon is mediated by the CcpA protein. J. Bacteriol. **176:**511–513.
11. **Fujita, Y., Y. Miwa, A. Galinier, and J. Deutscher.** 1995. Specific recognition of the *Bacillus subtilis gnt cis*-acting catabolite-responsive element by a protein complex formed between CcpA and seryl-phosphorylated HPr. Mol. Microbiol. **17:**953–960.
12. **Galinier, A., J. Haiech, M.-C. Kilhoffer, M. Jaquinod, J. Deutscher, and I. Martin-Verstraete.** 1997. The *Bacillus subtilis crh* gene encodes a HPr-like protein involved in carbon catabolite repression. Proc. Natl. Acad. Sci. USA **94:**8439–8444.
13. **Galinier, A., M. Kravanja, R. Engelmann, W. Hengstenberg, M.-C. Kilhoffer, J. Deutscher, and J. Haiech.** 1998. New protein kinase and protein phosphatase families mediate signal transduction in bacterial catabolite repression. Proc. Natl. Acad. Sci. USA **95:**1823–1828.
14. **Gösseringer, R., J. Deutscher, A. Galinier, and W. Hillen.** 1997. Cooperative and non-cooperative DNA binding modes of catabolite control protein CcpA from *Bacillus megaterium* result from sensing two different signals. J. Mol. Biol. **266:**665–676.
15. **Grundy, F. J., A. J. Turinsky, and T. M. Henkin.** 1994. Catabolite regulation of *Bacillus subtilis* acetate and acetoin utilization genes by CcpA. J. Bacteriol. **176:**4527–4533.
16. **Grundy, F. J., D. A. Waters, T. Y. Takova, and T. M. Henkin.** 1993. Identification of genes involved in utilization of acetate and acetoin in *Bacillus subtilis*. Mol. Microbiol. **10:**259–271.
17. **Guérin, M., M. Leng, and A. R. Rahmouni.** 1996. High resolution mapping of *E. coli* transcription elongation complex *in situ* reveals protein interactions with the non-transcribed strand. EMBO J. **15:**5397–5407.
18. **Guérout-Fleury, A.-M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene **167:**335–336.
19. **Henkin, T. M., F. J. Grundy, W. L. Nicholson, and G. H. Chambliss.** 1991. Catabolite repression of α-amylase gene expression in *Bacillus subtilis* involves a *trans*-acting gene product homologous to the *Escherichia coli lacI* and *galR* repressors. Mol. Microbiol. **5:**575–584.
20. **Hueck, C. J., and W. Hillen.** 1995. Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the Gram-positive bacteria? Mol. Microbiol. **15:**395–401.
21. **Kim, J.-H., and G. H. Chambliss.** 1997. Contacts between *Bacillus subtilis* catabolite regulatory protein CcpA and *amyO* target site. Nucleic Acids Res. **25:**3490–3496.
22. **Kraus, A., C. J. Hueck, D. Gärtner, and W. Hillen.** 1994. Catabolite repression of the *Bacillus subtilis xyl* operon involves a *cis* element functional in the context of an unrelated sequence, and glucose exerts additional *xylR*-dependent repression. J. Bacteriol. **176:**1738–1745.
23. **Krüger, S., and M. Hecker.** 1995. Regulation of the putative *bglPH* operon for aryl-β-glucoside utilization in *Bacillus subtilis*. J. Bacteriol. **177:**5590–5597.
24. **Lehming, N., J. Sartorius, M. Niemöller, G. Genenger, B. Wilcken-Bergemann, and B. Müller-Hill.** 1987. The interaction of the recognition helix of *lac* repressor with *lac* operator. EMBO J. **6:**3145–3153.
25. **Lopilato, J., S. Bortner, and J. Beckwith.** 1986. Mutations in a new chromosomal gene of *Escherichia coli* K-12, *pcnB*, reduce plasmid copy number of pBR322 and its derivatives. Mol. Gen. Genet. **205:**285–290.
26. **Martin-Verstraete, I., J. Stülke, A. Klier, and G. Rapoport.** 1995. Two different mechanisms mediate catabolite repression of the *Bacillus subtilis* levenase operon. J. Bacteriol. **177:**6919–6927.
27. **Miwa, Y., K. Nagura, S. Eguchi, H. Fukuda, J. Deutscher, and Y. Fujita.** 1997. Catabolite repression of the *Bacillus subtilis gnt* operon exerted by two catabolite-responsive elements. Mol. Microbiol. **23:**1203–1213.
28. **Moran, C. P., N. Lang, S. F. J. LeGrice, G. Lee, M. Stephens, and A. L. Sonenshein.** 1982. Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol. Gen. Genet. **182:**339–346.
29. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:**736–747.
30. **Nilsson, D., B. Hove-Jensen, and K. Arnvig.** 1989. Primary structure of the *tms* and *prs* genes of *Bacillus subtilis*. Mol. Gen. Genet. **218:**565–571.
31. **Oda, M., T. Katagai, D. Tomura, H. Shoun, T. Hoshino, and K. Furukawa.** 1992. Analysis of the transcriptional activity of the *hut* promoter in *Bacillus subtilis* and identification of a *cis*-acting regulatory region associated from the site of transcription. Mol. Microbiol. **6:**2573–2582.
32. **Paulsen, I. T., S. Chauvaux, P. Choi, and M. H. Saier, Jr.** 1998. Characterization of glucose-specific catabolite repression-resistant mutants of *Bacillus subtilis*: identification of a novel hexose:H^+ symporter. J. Bacteriol. **180:**498–504.
33. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57:**543–594.
34. **Reizer, J., C. Hoischen, F. Titgemeyer, C. Rivolta, R. Rabus, J. Stülke, D. Karamata, M. H. Saier, Jr., and W. Hillen.** 1998. A novel protein kinase that controls carbon catabolite repression in bacteria. Mol. Microbiol. **27:**1157–1169.
35. **Reizer, J., S. L. Sutrina, L. Wu, J. Deutscher, P. Reddy, and M. H. Saier, Jr.** 1992. Functional interactions between proteins of the phosphoenolpyruvate: sugar phosphotransferase systems of *Bacillus subtilis* and *Escherichia coli*. J. Biol. Chem. **267:**9158–9169.
36. **Selby, C. P., and A. Sancar.** 1995. Structure and function of transcription-repair coupling factor. II. Catalytic properties. J. Biol. Chem. **270:**4890–4895.
37. **Skarlatos, P., and M. K. Dahl.** 1998. The glucose kinase of *Bacillus subtilis*. J. Bacteriol. **180:**3222–3226.
38. **Spath, C., A. Kraus, and W. Hillen.** 1997. Contribution of glucose kinase to glucose repression of xylose utilization in *Bacillus megaterium*. J. Bacteriol. **179:**7603–7605.
39. **Steinmetz, M., and R. Richter.** 1994. Easy cloning of mini-Tn*10* insertions from the *Bacillus subtilis* chromosome. J. Bacteriol. **176:**1761–1763.
40. **Voskuil, M. I., and G. H. Chambliss.** 1996. Significance of HPr in catabolite repression of α-amylase. J. Bacteriol. **178:**7014–7015.

41. **Weickert, M. J., and S. Adhya.** 1992. A family of bacterial regulators homologous to Gal and Lac repressors. J. Biol. Chem. **267:**15869–15874.
42. **Weickert, M. J., and G. H. Chambliss.** 1990. Site-directed mutagenesis of a catabolite repressor operator sequence in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**6238–6242.
43. **Wray, L. V., Jr., F. K. Pettengill, and S. H. Fisher.** 1994. Catabolite repression of the *Bacillus subtilis hut* operon requires a *cis*-acting site located downstream of the transcription initiation site. J. Bacteriol. **176:** 1894–1902.
44. **Zalieckas, J. M., L. V. Wray, Jr., and S. H. Fisher.** 1998. Transcription-repair coupling factor is involved in carbon catabolite repression of the *Bacillus subtilis hut* and *gnt* operons. Mol. Microbiol. **27:**1031–1038.