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

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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 accA cre is centered 44.5 bp downstream of the accA transcriptional start site, CCR of accA is not affected by an mfd mutation. When the accA cre is centered 161.5 bp downstream of the transcriptional start site for the accB operon, CCR is partially relieved by the mfd mutation. Since CCR mediated at an accA cre centered 44.5 bp downstream of the accB start site is not affected by the mfd mutation, the inability of Mfd to modulate CCR of accA expression most likely results from the location of the accA 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 accA 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 *accA* (15), *xyl* (22), *gnt* (11), and *hut* (31, 43) genes are downstream of the transcriptional start sites. 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 CcpA 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 HprK-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-xylulose dehydrogenase gene 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 gnt 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 gnt and gnt oper-
ons act as transcriptional roadblocks for RNA polymerase and that Mfd enhances CCR mediated at these sites by displacing RNA polymerase stalled at CCR roadblocks 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 transferred into strain 168 (tcp2) by using plasmid DNA, as previously described (43). The mfd22-Tn10 insertion was transferred by transformation with selection for transposon-encoded chloramphenicol resistance. Transformation with selection for spectinomycin resistance (spc) was resistance to the glcK-cre site located within the glcK promoter (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 promotorless lacZ gene from pSFL6 or pSFL7 integrated at the amyE site.

### 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 at 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 elongation...
tion would be expected to terminate 28 nucleotides downstream of the \textit{acsA} transcriptional start site (assuming that the \textit{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 \textit{acsA cre}. CCR at \textit{cre} sites centered 161.5 bp downstream of the transcriptional start site for an unregulated promoter. It is possible that the CcpA-ac\textit{csA cre} complex can function as a transcriptional roadblock but that some unique characteristics of the \textit{acsA cre}, such as the proximity of the \textit{acsA cre} to the \textit{acsA promoter}, prevents Mfd from interacting with RNA polymerase. To test this hypothesis, the \textit{acsA cre} was placed downstream of the \textit{tms} promoter in a P\textit{tms}::\textit{lacZ} transcriptional fusion and CCR of \textit{b-galactosidase expression was examined in wild-type and \textit{mfd} mutant strains. Expression of the \textit{tms} promoter is not regulated by CCR in wild-type or \textit{mfd} mutant cells (TMS922 \textit{lacZ} fusion [Table 3]). The HUT924 \textit{lacZ} fusion (44) contains the \textit{het cre} site centered 161.5 bp downstream of the transcriptional start site for the \textit{tms} promoter and is regulated by CCR (Table 3). Oligonucleotide-directed mutagenesis was used to convert the \textit{het cre} in HUT924 to an \textit{acsA cre} by making three nucleotide changes within the 14-bp central \textit{het cre}. The resulting TMS940 \textit{lacZ} fusion contains the central 14-bp sequence of the \textit{acsA cre} (Fig. 1). \textit{b-Galactosidase expression from the TMS940 \textit{lacZ} fusion was repressed 23-fold by glucose in the wild-type strain but only 8.8-fold in the \textit{mfd}22 mutant (Table 3). These results indicate that Mfd can modulate CCR mediated at a 14-bp \textit{acsA cre} centered 161.5 bp downstream of a transcriptional start site.

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

The low level of CCR observed with the TMS942 \textit{lacZ} fusion suggested that nucleotides flanking the 14-bp core \textit{cre} sequence can affect the ability of the \textit{cre} to mediate CCR. To test this hypothesis, three nucleotides flanking the 14-bp \textit{het cre} in the HUT924 \textit{lacZ} fusion were altered so that they matched the flanking nucleotides present in the \textit{acsA cre}. The resulting \textit{lacZ} fusion, TMS948, contains the 14-bp \textit{het cre} flanked by the \textit{G} and \textit{C} nucleotides present in the \textit{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 \textit{lacZ} fusion (2.6-fold) is lower than that seen with the HUT924 fusion (14-fold), the activity of a \textit{cre} appears to be dependent upon its flanking nucleotides.

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

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**Table 3. \textit{b-Galactosidase expression from \textit{lacZ} fusions in wild-type and \textit{mfd} mutant strains}**

<table>
<thead>
<tr>
<th>\textit{lacZ} fusion</th>
<th>Relevant genotype</th>
<th>cre position (bp)</th>
<th>\textit{b-Galactosidase sp act (U/mg of protein) in cells grown on Гlucose, Citrate}</th>
<th>Glucose repression ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS7 \textit{mfd}</td>
<td>+44.5</td>
<td>3.6</td>
<td>62.1</td>
<td>17</td>
</tr>
<tr>
<td>TMS922 \textit{mfd}</td>
<td>+44.5</td>
<td>4.6</td>
<td>88.1</td>
<td>19</td>
</tr>
<tr>
<td>HUT924 \textit{mfd}</td>
<td>+161.5</td>
<td>2.5</td>
<td>34.8</td>
<td>14</td>
</tr>
<tr>
<td>TMS940 \textit{mfd}</td>
<td>+161.5</td>
<td>1.1</td>
<td>25.0</td>
<td>23</td>
</tr>
<tr>
<td>TMS942 \textit{mfd}</td>
<td>+161.5</td>
<td>8.5</td>
<td>32.1</td>
<td>3.8</td>
</tr>
<tr>
<td>TMS948 \textit{mfd}</td>
<td>+161.5</td>
<td>12.9</td>
<td>33.7</td>
<td>2.6</td>
</tr>
<tr>
<td>TMS951 \textit{mfd}</td>
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<td>0.5</td>
<td>15.9</td>
<td>32</td>
</tr>
<tr>
<td>TMS952 \textit{mfd}</td>
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<td>5.9</td>
<td>30.7</td>
<td>5.2</td>
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<tr>
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<td>+44.5</td>
<td>4.0</td>
<td>35.4</td>
<td>177</td>
</tr>
</tbody>
</table>

\textit{a} All strains are derivatives of 168, with the indicated \textit{lacZ} fusion integrated as a single copy at the amyE locus.

\textit{b} Position relative to the transcriptional start site (+1).

\textit{c} Data are averages of three or more determinations which did not vary by more than 20%.

\textit{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.
Role of CcpB, glucose kinase, HPr, and Crh in 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 in liquid cultures of wild-type and ccpB mutant strains containing the ACS7 lacZ fusion integrated as a single copy at the amyE locus.

The glucose repression ratio was calculated by dividing the enzyme activity (b-galactosidase sp act) for wild-type and mutant cells grown on minimal medium (12). In vitro studies have shown that HPr-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 site located 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 ofiol, lev, and b-xylosidase expression in cells grown in minimal medium (12). In vitro studies have shown that HPr-seryl-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 do not affect the activity of CcpA.
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 accA cre mediates higher levels of CCR when centered 44.5 bp downstream of the tms start site in the TMS952 fusion (5,2-fold) than when this sequence is centered either 44.5 bp downstream of the tms start site in the TMS942 (3.8-fold). One explanation for this result is that the accA 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 accA promoter in response to carbon availability. Indeed, we have observed that CodY contributes to the regulation of accA expression in response to carbon availability (9).

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