trans-Acting Factors and cis Elements Involved in Glucose Repression of Arabinan Degradation in Bacillus subtilis

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In Bacillus subtilis, the synthesis of enzymes involved in the degradation of arabinose-containing polysaccharides is subject to carbon catabolite repression (CCR). Here we show that CcpA is the major regulator of repression of the arabinases genes in the presence of glucose. CcpA acts via binding to one cre each in the promoter regions of the abnA and xsa genes and to two cres in the araABDLMNQ-abfA operon. The contributions of the coeffectors HPr and Crh to CCR differ according to growth phase. HPr dependency occurs during both exponential growth and the transitional phase, while Crh dependency is detected mainly at the transitional phase. Our results suggest that Crh synthesis may increase at the end of exponential growth and consequently contribute to this effect, together with other factors.

Bacillus subtilis secretes a vast number of polysaccharide-degrading enzymes that are able to hydrolyze plant cell wall material (30). Arabinose and xylose are the two most abundant pentoses in nature and often occur associated with hemicellulosic substrates, such as arabinoxylans, arabinoxylans (25, 26, 29). B. subtilis synthesizes enzymes, namely, endo-1,5-α-arabinanases (ABN; EC 3.2.1.99) and α-L-arabinofuranosidases (AF; EC 3.2.1.55), capable of releasing arabinosyl oligomers and arabinose from the plant tissue homoglycan arabian (1, 13, 14, 35). In previous studies, we characterized the transcriptional regulation of three arabinan-degrading enzyme genes, abnA (ABN), xsa (AF), and abfA (AF), which are clustered with genes encoding enzymes that further catabolize L-arabinose (23). Expression of these genes is (i) induced by arabinose and arabian, (ii) negatively controlled by AraR, the key regulator of the arabinan regulon, and (iii) repressed in the presence of glucose (6, 12, 23). The last phenomenon, carbon catabolite repression (CCR), is a global regulatory mechanism that ensures the selection of rapidly metabolized carbon and energy sources, such as glucose, for optimal bacterial growth (3, 30). CcpA is the master regulator of CCR in Bacilli and other gram-positive bacteria with low GC contents and regulates approximately 10% of the B. subtilis genome (2, 18). This global regulator binds to cres (catabolite-responsive elements) either upstream of or in the promoter region or within coding regions of target genes (17, 34). This interaction is modulated by phosphorylation of HPr, a phosphocarrier protein of the phosphoenolpyruvate phosphotransferase system (PTS), or of Crh (an HPr-like protein) (4, 30, 33). In this work, we identified trans-acting factors and cis elements involved in glucose repression of arabinan-degrading enzyme genes. Moreover, we address the question of temporal regulation of these genes (23) by determination of the contributions of CcpA and the coeffectors HPr and Crh to glucose repression at different growth stages (exponential and postexponential [transitional] growth).

cis-Regulatory elements in the promoter regions of abnA and xsa. The involvement of two cres present in the araABDLMNQ-abfA operon (cre araA and cre araB) (Fig. 1) in CCR was shown in previous studies (12, 17). Both cre araA, located between the promoter region and the araA gene, and cre araB, placed 2 kb downstream within the araB gene, are independently functional, and both contribute to glucose repression (12). Moreover, the increase of glucose repression, 1,0-fold, measured in a strain bearing an abfA-lacZ reporter fusion (IQB450) (23; see below) was only 1,6-fold higher than that observed with a strain bearing an araAB-lacZ fusion, i.e., 1,3-fold (12), suggesting the absence of other cre active sites in glucose-mediated CCR of abfA expression. Expression of abnA and xsa is also subject to glucose repression (23). Potential cre sequences, namely, cre abnA (+83-TGTAAGCGCTTTCT [SubtiList position 2949135]) (Fig. 1) (17) and cre xsa (+1-TAAAAGCGCT 3-Ccis [SubtiList position 2914316]) (Fig. 1) (18), are present in the promoter regions of the genes. To assess the functionality of these putative regulatory sites, we introduced a single-base-pair substitution which destroyed the central symmetry in both cre abnA (+89 C→A) and cre xsa (+7 C→A) (Fig. 1). The two promoter regions, together with the respective mutated creS, were fused to the lacZ reporter gene and introduced into the amylE locus. In the resulting B. subtilis strains, IQB472 and IQB473 (Table 1), respectively, the levels of accumulated β-galactosidase activity in the absence or presence of the inducer arabinose and under repressing conditions (arabinose plus glucose) were determined. Glucose repression was almost abolished in the mutants compared to that in strains bearing the wild-type promoters (IQB410 and IQB405) (23) (Table 2). These results indicate that both cre abnA and cre xsa are functional and that the designed mutation prevented binding of CcpA (see below) in the presence of glucose. Thus, cre abnA and cre xsa are the cis-acting elements involved in glucose-mediated CCR of abnA and xsa expression at the transcrip-
impaired HPr Ser46 phosphorylation (5), partially abol-

FIG. 1. Organization of the B. subtilis chromosome region comprising the arabinan-degrading enzyme genes abnA, abfA, and xsa. The three genes are represented by dotted arrows pointing in the direction of transcription. abfA belongs to the araABDLMNPO-abfA metabolic operon, abnA is located immediately upstream, and xsa is positioned 23 kb downstream of the metabolic operon. Hairpin structures denote terminators. The dotted boxes below the physical map represent extensions of the inserts fused to the lacZ reporter gene in the indicated plasmids. Plasmid pSA1 was integrated into the host chromosome by means of a single-crossover (Campbell-type) recombinational event that occurred in the region of homology of the resulting strain (Table 1). Linearized DNAs from plasmids pSN40, pZI26, pZI27, and pRIT1 were used to transform B. subtilis strains (Table 1), and the fusions were integrated into the chromosome via double recombination at the amyE locus. Above the map, the DNA sequences of cre abnA and cre xsa are depicted, and the numbers indicate the positions of the abnA and xsa genes relative to the transcriptional start site. The single nucleotide change (C→A) introduced into each cre is shown.

tional level. In addition, the results suggest that only one cre in each promoter is responsible for this phenomenon.

A major role of CcpA and different contributions of effectors HPr and Crh to CCR. The arabinan-degrading enzyme genes are subject to temporal regulation, since the levels of expression of abfA, xsa, and abnA increase at early postexponential phase in response to arabinose and arabinan (23). Here we analyzed this effect in the presence of glucose. The expression of abfA-lacZ, xsa-lacZ, and abnA-lacZ transcriptional fusions, integrated into the chromosomes of strains IQB450, IQB405, and IQB410, respectively (Table 1) (23), was analyzed as described above, and samples were collected 2 and 4 h after induction (t2 and t4, respectively), corresponding to the middle exponential and early postexponential (transitional) growth phases, respectively (Fig. 2A). Although expression of abfA-lacZ, xsa-lacZ, and abnA-lacZ increased at t4, the glucose repression indexes, measured as ratios between expression in the presence of arabinose and the values obtained in the presence of arabinose plus glucose, were similar during exponential growth and transitional phase (Fig. 2B, C, and D). These results suggest that the effect of glucose repression under these conditions is not affected over time.

To identify the trans-acting factors involved in CCR of the arabinan-degrading enzyme genes, we examined the expression of abfA-lacZ, xsa-lacZ, and abnA-lacZ transcriptional fusions, as described above, in B. subtilis ccpA, pstH1, crh, hprK, and araR mutant backgrounds (Table 1). In a wild-type background, the addition of glucose caused 21.8-, 19.3-, and 6.5-fold repression of abfA-lacZ, xsa-lacZ, and abnA-lacZ expression, respectively (strains IQB450, IQB405, and IQB410, respectively) during exponential growth phase (23). Disruption of the ccpA gene led to a complete loss of glucose-mediated CCR of expression from the abfA-lacZ (IQB474), xsa-lacZ (IQB423), and abnA-lacZ (IQB422) fusions (Fig. 2), revealing its major role in glucose-mediated CCR of the arabinan-degrading enzyme genes. The pstH1 mutation (HPr Ser46 to Ala), which impaired HPr Ser46 phosphorylation (5), partially abol-

ished glucose repression of abfA-lacZ (IQB475), xsa-lacZ (IQB467), and abnA-lacZ (IQB466) fusion expression (Fig. 2). In contrast, disruption of the crh gene caused almost no effect on glucose repression of expression from the abfA-lacZ, xsa-lacZ, and abnA-lacZ fusions (IQB476, IQB469, and IQB468, respectively) during exponential growth phase (t2). Accordingly, in the pstH1 crh double mutant background, the levels of glucose repression, at t2, of abfA-lacZ (IQB477), xsa-lacZ (IQB471), and abnA-lacZ (IQB470) fusions were only slightly lower than those observed in the single pstH1 mutant (Fig. 2), suggesting no or a small contribution of Crh to CCR mediated by glucose. These results are in agreement with previous observations that both CcpA and HPr, but not Crh, participate in glucose repression of the araE and araAB genes (12). However, in the crh-null mutant background, a significant relief of glucose repression of abfA-lacZ and xsa-lacZ expression, of 6.4-fold and 2.1-fold, respectively, was observed at transitional phase (t4), suggesting a contribution of Crh to glucose-mediated CCR at late growth phases (discussed below). The disruption of the hprK gene, encoding the bifunctional HPr kinase/phosphorylase, which reversible phosphorylates HPr and Crh (8, 16, 24), also caused a decrease of glucose repression (in IQB478, IQB479, and IQB480) (Fig. 2).

During exponential growth of cells with an araR-null mutant background, we observed a twofold derepression of expression of xsa-lacZ (IQB406) and abfA-lacZ (IQB453) in the presence of glucose compared to that of the wild type (Fig. 2B and C). AraR is a negative regulator of the arabinose-inducible genes (6, 7, 20), and this effect was previously observed with araE-lacZ and araAB-lacZ fusions (12). The AraR binding sites and cre sequences are located close to each other in the promoter regions of the araABDLMNPO-abfA, araE, and xsa genes (12, 19, 23). Since under particular physiological conditions both CcpA and AraR might be bound to the DNA in close proximity, a possible interaction (cooperation) between the two proteins in the negative control of these genes was proposed (12, 19). Interestingly, this effect was not observed
for the abnA’-lacZ fusion in an araR-null mutant background (strain IQB411) (Fig. 2D; Table 1). This observation is in agreement with a different localization of the araR binding site in the abnA promoter and also to a weak control of abnA expression by AraR as a result of a distinct mechanism of action (23).

**Crb expression increases at transitional growth phase.** The contribution of both effectors HPr and Crh to CCR by glucose can depend on the growth conditions, as shown for the hut operon, which responds to HPr in rich LB medium and to both HPr and Crh in minimal medium (36). Crh is the major effector for CCR of the citM gene in a medium containing succinate and glutamate (32). In connection with this, it was recently shown that the nature of the carbon source influences ptsH and crh expression (9). Using ptsH’-lacZ and crh’-lacZ transcription and translational fusion analysis, it was suggested that the different contributions of Crh and HPr to CCR might be due to the drastic differences in their synthesis rates under the conditions that cause CCR. In the presence of PTS carbohydrates, such as glucose, the synthesis level of HPr during exponential growth is higher than that of Crh (14), which is consistent with the observation that Crh is a major effector of CCR of the citM gene in a medium containing succinate and glutamate.

### TABLE 1. B. subtilis strains, plasmids, and oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Plasmid, strain, or oligonucleotide</th>
<th>Relevant construction, genotype, or sequence (5′-3′)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJM783</td>
<td>Integrative plasmid; promoterless lacZ preceded by rbs&lt;sub&gt;pts&lt;/sub&gt; and multicloning site (MCS); cat bla</td>
<td>22</td>
</tr>
<tr>
<td>pSN32</td>
<td>Promoterless lacZ preceded by rbs&lt;sub&gt;pts&lt;/sub&gt; and MCS; cat bla; flanked by amyE-5′ and amyE-3′</td>
<td>20</td>
</tr>
<tr>
<td>pMutin4</td>
<td>Promoterless lacZ preceded by rbs&lt;sub&gt;pts&lt;/sub&gt; and MCS; lacI bla ery Ery&lt;sub&gt;Pspac&lt;/sub&gt;</td>
<td>31</td>
</tr>
<tr>
<td>pRTT1</td>
<td>pSN32 containing a 281-bp fragment of the &lt;sub&gt;pts&lt;/sub&gt;H promoter region in the MCS</td>
<td>23</td>
</tr>
<tr>
<td>pSN40</td>
<td>pSN32 carrying a 292-bp fragment of the abnA promoter region in the MCS</td>
<td>23</td>
</tr>
<tr>
<td>pSA1</td>
<td>pJM783 containing a 687-bp fragment comprising the abfA 5′ region in the MCS</td>
<td>23</td>
</tr>
<tr>
<td>pZI26</td>
<td>Same as pSN40, but with a single-base-pair substitution at position +89 (C→A)</td>
<td>This work</td>
</tr>
<tr>
<td>pZI27</td>
<td>Same as pRTT1, but bearing a single-base-pair substitution at position +7 (C→A)</td>
<td>This work</td>
</tr>
<tr>
<td>pGP211</td>
<td>pBSK&lt;sup&gt;a&lt;/sup&gt; derivative that contains the spec gene inserted into the hprK gene</td>
<td>10</td>
</tr>
<tr>
<td>pZI45</td>
<td>Identical to pBGM6 (9), pMutin4 bearing a crh-lacZ fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pZI48</td>
<td>Identical to pLF2 (9), pMutin4 bearing a ptsH-lacZ fusion</td>
<td>This work</td>
</tr>
</tbody>
</table>

### Plasmids

- **pJM783**: Integrative plasmid; promoterless lacZ preceded by rbs<sub>pts</sub> and multicloning site (MCS); cat bla.
- **pSN32**: Promoterless lacZ preceded by rbs<sub>pts</sub> and MCS; cat bla; flanked by amyE-5′ and amyE-3′.
- **pMutin4**: Promoterless lacZ preceded by rbs<sub>pts</sub> and MCS; lacI bla ery Ery<sub>Pspac</sub>.
- **pRTT1**: pSN32 containing a 281-bp fragment of the <sub>pts</sub>H promoter region in the MCS.
- **pSN40**: pSN32 carrying a 292-bp fragment of the abnA promoter region in the MCS.
- **pSA1**: pJM783 containing a 687-bp fragment comprising the abfA 5′ region in the MCS.
- **pZI26**: Same as pSN40, but with a single-base-pair substitution at position +89 (C→A).
- **pZI27**: Same as pRTT1, but bearing a single-base-pair substitution at position +7 (C→A).
- **pGP211**: pBSK<sup>a</sup> derivative that contains the spec gene inserted into the hprK gene.
- **pZI45**: Identical to pBGM6 (9), pMutin4 bearing a crh-lacZ fusion.
- **pZI48**: Identical to pLF2 (9), pMutin4 bearing a ptsH-lacZ fusion.

### Strains

- **16ST**: Prototroph.
- **QB5223**: trpC2 ptsH1.
- **QB7097**: trpC2 crh:spc.
- **WLN29**: trpC2 araG932 ccpA::Tn917.
- **IOB405**: amyE::xsa-lacZ cat.
- **IOB406**: amyE::xsa-lacZ cat araR::km.
- **IOB410**: amyE::abnA-lacZ cat.
- **IOB411**: amyE::abnA-lacZ cat araR::km.
- **IOB422**: amyE::abnA-lacZ cat ccpA::Tn917.
- **IOB423**: amyE::xsa-lacZ cat ccpA::Tn917.
- **IOB450**: abfA::pSAI(abfA-lacZ cat).
- **IOB453**: abfA::pSAI(abfA-lacZ cat) araR::km.
- **IOB466**: amyE::abnA-lacZ cat ptsH1.
- **IOB467**: amyE::xsa-lacZ cat ptsH1.
- **IOB468**: amyE::abnA-lacZ cat crh:spc.
- **IOB469**: amyE::xsa-lacZ cat crh:spc.
- **IOB470**: amyE::abnA-lacZ cat ptsH1 crh:spc.
- **IOB471**: amyE::xsa-lacZ cat ptsH1 crh:spc.
- **IOB472**: amyE::abnA (+89 C→A)-lacZ cat.
- **IOB473**: amyE::xsa (+7 C→A)-lacZ cat.
- **IOB474**: abfA::pSAI(abfA-lacZ cat) ccpA::Tn917.
- **IOB475**: abfA::pSAI(abfA-lacZ cat) ptsH1.
- **IOB476**: abfA::pSAI(abfA-lacZ cat) crh:spc.
- **IOB477**: abfA::pSAI(abfA-lacZ cat) ptsH1 crh:spc.
- **IOB478**: abfA::pSAI(abfA-lacZ cat) hprK:spc.
- **IOB479**: amyE::abnA-lacZ cat hprK:spc.
- **IOB480**: amyE::xsa-lacZ cat hprK:spc.
- **IOB495**: crh::pZI45(crh-lacZ erm::pMutin4).
- **IOB496**: ptsH::pZI48(ptsH-lacZ erm::pMutin4).

### Oligonucleotides

- **ARA201**: −8-GGTTACTTTAAAAGACTCTTATTACATGC+21 (xsa).
- **ARA205**: GCATGAATTAAGCTCTTTTTAGAATACC.
- **ARA206**: +66-GGATAAATATTTAATTTGTAAAGGTTTCTTTAAATAAGGC+106 (abnA).
- **ARA207**: CCTTTATTTTAGAAGCCCTTCAAATTATTTACCC.

<sup>a</sup> Arrows indicate transformation, performed as previously described (27), and point from donor DNA to the recipient strain.

<sup>b</sup> Transformation was carried out with linearized plasmid DNA.

<sup>c</sup> Substituted residues in oligonucleotides are underlined. The position relative to the transcriptional start site of the xsa and abnA genes is indicated in the forward primer. Site-directed mutagenesis was performed using a QuikChange kit (Stratagene).
growth is 100-fold higher than that of Chr, which may explain the minor role of the latter in certain circumstances (9). Moreover, CcpA binds Chr(Ser-P) more weakly than HPr(Ser-P) (28). We have previously shown the absence of a Chr contribution to glucose repression of the araABDLMNPQ-abfA and araE genes in both C minimal medium supplemented with casein hydrolysate and CSK minimal medium (12). To further investigate the lack of Chr dependency during exponential growth and its possible contribution to glucose repression of the arabinan-degrading enzyme genes during the transitional growth phase, we analyzed the expression of both ptsH-lacZ and chr-lacZ transcriptional fusions at different growth stages. The constructed transcriptional fusions are identical to those described by Görke et al. (9). In B. subtilis strains bearing the fusions, the complete ptsH and chr coding regions are restored downstream from the lacZ insertion cassette. Their expression is directed by the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible Pspac promoter in order to prevent polar effects caused by insertions into their natural chromosomal loci (Table 1). Expression from the chr-lacZ (IQB495) (Table 1)
and ptsH-lacZ (IQB496) (Table 1) transcripational fusions during exponential growth phase (t2) was comparable in both C minimal medium supplemented with casein hydrolysate and CSK minimal (Table 3). The presence of arabinose, a non-PTS sugar, did not influence crh and ptsH expression (Table 3). In addition, the β-galactosidase activities obtained for crh-lacZ and ptsH-lacZ in CSK minimal medium plus glucose (29 and 229, respectively) (Table 3) were very similar to those obtained by Görke et al. (9). Interestingly, at transitional phase (29 and 229, respectively) (Table 3) were very similar to those obtained by Görke et al. (9). Interestingly, at transitional phase. Thus, this difference in synthesis level may contribute to the glucose repression relief effect on abfA-lacZ and xsa-lacZ expression in the crh-null mutant background (Fig. 2). However, due to a still expected higher concentration of HPr than Crh under these conditions, other factors, such as intracellular variation of certain metabolites which may influence Crh(Ser-P) phosphorylation and/or CcpA-Crh(Ser-P) complex formation, are likely to contribute to Crh(Ser-P)-mediated glucose repression at transitional phase.

Concluding remarks. CCR of the arabinan-degrading enzyme genes is mediated by binding of CcpA to one cre located downstream near the promoter regions of abnA and xsa and to two cre present in the araABDLMNPO-abfA operon, with cre araA located near the promoter and cre araB positioned within the araB gene. Our data suggest that during exponential growth phase, glucose-mediated CCR is mediated by CcpA-Crh(Ser-P) and towards the end of exponential growth, at transitional phase, glucose repression is achieved by both CcpA-HPr(Ser-P) and CcpA-Crh(Ser-P) complexes.

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TABLE 3. Expression of crh-lacZ and ptsH-lacZ fusions in minimal media at different growth stages

<table>
<thead>
<tr>
<th>Medium</th>
<th>Strain (promoter fusion)</th>
<th>Timeb</th>
<th>β-Galactosidase activity (Miller units)c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Ara +Ara +Ara +Glc +Glc</td>
</tr>
<tr>
<td>C</td>
<td>IQB495 (crh-lacZ)</td>
<td>t2</td>
<td>36.0 ± 3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t4</td>
<td>37.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>IQB496 (ptsH-lacZ)</td>
<td>t2</td>
<td>97.4 ± 14.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t4</td>
<td>87.5 ± 7.1</td>
</tr>
<tr>
<td>CSK</td>
<td>IQB495 (crh-lacZ)</td>
<td>t2</td>
<td>31.4 ± 3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t4</td>
<td>28.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>IQB496 (ptsH-lacZ)</td>
<td>t2</td>
<td>59.7 ± 5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t4</td>
<td>55.0 ± 5.9</td>
</tr>
</tbody>
</table>

a Strains containing different promoter-lacZ fusions were grown on C minimal medium (21) supplemented with casein hydrolysate or in CSK minimal medium (9) with IPTG (1 mM) in the absence of sugar (−Ara), in the presence of arabinose (+Ara), in the presence of arabinose plus glucose (+Ara +Glc), and in the presence of glucose (+Glc). Antibiotics used as selective markers were added as appropriate.

b Samples were analyzed 2 h (t2) and 4 h (t4) after the addition of sugars, which correspond to exponential growth phase and transitional phase, respectively.

c The levels of accumulated β-galactosidase activity represent the averages of two measurements each from three independent experiments.

REFERENCES


