Regulation of xylanolytic enzymes in Bacillus subtilis

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The synthesis of the xylanolytic enzymes β-xylanase and β-xylosidase of Bacillus subtilis was studied. In contrast to many catabolic extracellular enzymes, β-xylanase was synthesized constitutively during exponential growth and was not repressed by glucose. β-Xylosidase synthesis was induced 100-fold by xylose and repressed 100-fold by glucose. Carbon catabolite repression was abolished in a ccpA mutant. Titration experiments using a multicopy operator sequence responsible for carbon catabolite repression indicated that the gene encoding β-xylosidase is part of the same carbon catabolite repression regulon as the amyE and bglS genes.

Keywords: Bacillus subtilis, β-xylanase, β-xylosidase, regulation, glucose repression

INTRODUCTION

Xylan, a 1,4-β-glycoside-linked polymer of D-xylose (Biely, 1985), is one of the most widespread carbohydrates (Timell, 1967). The polymer can be catabolized by the successive action of β-xylanase, β-xylosidase (Hollenberg & Wilhelm, 1987), D-xylose isomerase and xylulokinase. The resulting xylulose 5-phosphate is further metabolized via the pentose phosphate pathway (Jeffris, 1983).

The regulation of the xyl operon of Bacillus subtilis, encoding d-xylose isomerase and xylulokinase, is well understood. It is induced by xylose and repressed by glucose. Induction is mediated by release of the xyl repressor from its operator DNA in front of the xyl operon upon binding of xylose to the repressor (Gärtn er et al., 1988). So far, nothing is known about the uptake of xylose.

Roncero (1983) mapped the genes involved in xylan degradation in B. subtilis. These genes are linked at 48° on the B. subtilis chromosomal map. The xynA and xynB genes, encoding β-xylanase and β-xylosidase, respectively, have been cloned and sequenced (Bernier et al., 1983; Hastrup, 1988; Paice et al., 1986). The xynB gene was shown to form an operon with the xynC gene, which encodes a putative xyloside permease. While the xynCB operon is known to be induced by xylose and repressed by glucose (Hastrup, 1988), no information is available about the regulation of β-xylanase. It was therefore the aim of this study to characterize the regulation of β-xylanase and to increase our understanding of the regulation of β-xylosidase synthesis.

METHODS

Bacterial strains and plasmids. The B. subtilis strains and plasmids used in this study are listed in Table 1. The genetic markers of the strains were periodically confirmed.

Media and growth conditions. B. subtilis cells were grown in amino acid starvation medium (ASM) (Stülke et al., 1993) at 37 °C under vigorous agitation. Cultures were inoculated from exponential-phase overnight cultures. For experiments dealing with P-xylanase regulation, the overnight cultures were centrifuged prior to inoculation. Growth rates were measured using modified ASM as described earlier (Stülke et al., 1993). If necessary, antibiotics were added to 1 μg ml⁻¹ (erythromycin) and 5 μg ml⁻¹ (chloramphenicol).

Determination of enzyme activities. β-Xylanase activities were

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>B. subtilis</td>
<td>trpC2</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>BG314</td>
<td>trpC2 aroD120 bgl-35</td>
<td>Borris et al. (1986)</td>
</tr>
<tr>
<td>BGW2</td>
<td>trpC2 hys-3 cep.A::Tn917</td>
<td>Krüger et al. (1993)</td>
</tr>
<tr>
<td>IS58</td>
<td>trpC2 hys-3</td>
<td>Smith et al. (1980)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHV33</td>
<td>Tc' Ap' Cm'</td>
<td>Primrose &amp; Ehrlich (1981)</td>
</tr>
<tr>
<td>pEC042</td>
<td>Tc' Cm' bglS</td>
<td>Krüger et al. (1993)</td>
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assayed by determination of reducing sugar liberated from birch wood xylan using the dinitrosalicylic acid method as described previously (Stülke et al., 1993). One enzyme unit was defined as 1 mol xylose equivalent produced per OD₅₄₀ unit ml⁻¹ and s⁻¹ (≈ 1 kat).

β-Xylosidase activities were measured in permeabilized cells by assaying the nitrophenol liberated from p-nitrophenylxylopyranoside as described by Roncero (1983). One unit of β-xylosidase was defined as the amount of enzyme which produces an increase of 0.01 A₄₅₀ unit in 1 min. Repression ratios are quotients of β-xylosidase activities in unpressed and repressed cultures (5.5 h after the onset of stationary phase). Each assay was repeated twice. Experiments were performed in duplicate or triplicate; the deviation of results of different experiments was up to 5%. Representative results are shown in the Figures and Tables.

**Isolation and amplification of DNA.** xynA-specific DNA was amplified by the PCR method using oligonucleotides deduced from the published sequence of the *B. subtilis* xynA gene (Paice et al., 1986). The sequences of the primers are given below:

5'-d(CCTGATTAAGGAAGATCTGTTACC)-3'
5'-d(CTGAATTCGTGGTATTATACTGAAGG)-3'

Chromosomal DNA was isolated as described by Meade et al. (1982). PCR was carried out using Taq polymerase (Promega). Amplification of the xynA DNA fragment from PstI-digested chromosomal DNA was achieved by adding 200 ng template DNA, 50 mM KCl, 10 mM Tris/HCl pH 9.0, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 0.1% (v/v) Triton X-100, 200 µM of each dNTP, 25 pmol of each primer and 2.5 units of Taq polymerase in a final volume of 100 µl. The samples were overlaid with 60 µl light mineral oil (Sigma). PCR conditions were as follows: denaturation 1 min, 94 °C; annealing 1 min, 56 °C; extension 2 min, 72 °C; 30 cycles. The products of the reactions were analysed on an agarose gel containing 1% (w/v) agarose and 0.5 µg ethidium bromide ml⁻¹ in Tris/borate buffer (90 mM Tris/borate, 1 mM EDTA pH 8.0).

**RNA isolation, slot-blot hybridization and detection.** RNA was isolated by a rapid procedure as described by Ladin et al. (1992). Dilution series (1.25 µg, 2.5 µg, 5 µg, 10 µg) of the different RNA preparations were spotted onto a Hybond-N nylon filter (Amersham) using the Bio-Dot SF microfiltration apparatus (Bio-Rad). Hybridization was performed using a DNA labelling and detection kit (Boehringer Mannheim). xynA-specific DNA was labelled with digoxigenin dUTP. The filter was baked, prehybridized and hybridized with the labelled DNA probe and the bound probe detected as recommended by the manufacturer. The intensities of the hybridization signals were compared using the personal densitometer SCAN v0.90 BETA and the Image Quant software. All data were calculated such that the results expressed the relative amount of xynA-specific mRNA in 1 µg of total RNA. The relative amounts of xynA mRNA are given as a percentage of the xynA mRNA of the sample from exponential-phase cells.

**RESULTS**

**Growth of *B. subtilis* on xylose and xylan**

Since no information was available on the utilization of xylose and xylan, the growth of *B. subtilis* 168 on agar plates containing either xylose or xylan as single carbon sources was tested. While the bacteria grew slowly on xylan, they did not grow on xylose.

In order to verify the lack of utilization of xylose, bacteria were grown in ASM (without glucose, but containing 0.1% xylose). Growth was monitored and the concentration of reducing sugar measured. Again, utilization of xylose was not detectable.

**Regulation of β-xylanase and β-xylosidase synthesis by xylose and xylan**

 Cultures of *B. subtilis* 168 were grown in ASM with or without 0.1% xylose or xylan and the activities of β-xylanase and β-xylosidase were assayed, in the culture supernatants and in permeabilized cells, respectively. The synthesis of β-xylanase was not influenced by the presence of xylose or xylan in the medium (data not shown), while β-xylosidase synthesis was induced by both substrates (Fig. 1). Without an inducer there was only basal β-xylosidase activity of 0.2-1 unit. However, if the growth medium contained 0.1% xylose, activity increased from a basal level of 0.5 to about 100 units (Fig. 1). The induction was about 100-fold with xylose and 40-fold with xylan. However, β-xylosidase synthesis was delayed in the presence of xylan.

β-Xylanase was synthesized during exponential growth. Since it is rather unusual that extracellular enzymes of *B. subtilis* are synthesized during exponential growth, the levels of xynA mRNA during growth were analysed. Bacteria were grown in ASM, and RNA was extracted several times. Total cellular RNA was hybridized with labelled xynA DNA as probe and the intensities of the hybridization signals were quantified. The results confirmed the conclusion that xynA is expressed mainly during exponential growth (Table 2).

**Carbon catabolite repression of xylanolytic enzymes**

To analyse the influence of glucose on the synthesis of the xylanolytic enzymes, *B. subtilis* 168 was grown in ASM containing either 0.1% (the normal concentration) or...
Xylanolytic enzymes in *B. subtilis*

Table 2. Amounts of *xynA* mRNA in *B. subtilis* 168

*B. subtilis* 168 was grown in ASM. At the times indicated (relative to the onset of stationary phase) aliquots of the culture were harvested for RNA extraction. Total RNA was hybridized with the labelled *xynA* DNA probe as described in Methods.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>OD&lt;sub&gt;650&lt;/sub&gt;</th>
<th>Xylanase activity (nkat)</th>
<th>Relative amount of mRNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>0.45</td>
<td>0.61</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>0.96</td>
<td>0.74</td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>1.13</td>
<td>0.74</td>
<td>26</td>
</tr>
<tr>
<td>2.5</td>
<td>1.19</td>
<td>0.8</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>1.31</td>
<td>0.8</td>
<td>29</td>
</tr>
</tbody>
</table>

0.3% glucose. The presence of 0.3% glucose in the medium did not influence the synthesis of β-xylanase (Fig. 2). Again, the glucose-resistant synthesis of β-xylanase is rather unusual for extracellular catabolic enzymes. mRNA measurements indicated that there was no decrease in the amount of *xynA* mRNA even in the presence of 1.0% glucose (data not shown).

The induced level of β-xylosidase synthesis in *B. subtilis* 168 was approximately 100-fold repressed in the presence of 0.3% glucose compared to a culture containing 0.1% glucose. The effect of glucose on β-xylosidase synthesis was also tested in a mutant insensitive to carbon catabolite repression with respect to α-amylase (Henkin *et al.*, 1991) and β-glucanase (Krüger *et al.*, 1993). β-Xylosidase activities were assayed in the isogenic strains ISS8 and BGW2 (*ccpA*) grown in ASM containing 0.1% xylose and 0.1% or 0.3% glucose. β-Xylosidase synthesis was represented 100-fold in the wild-type strain ISS8 (Fig. 3a) but was not repressed in the *ccpA* mutant BGW2 (Fig. 3b). Furthermore, β-xylosidase was synthesized in the exponential phase in strain BGW2. To test the possibility that the lack of β-xylosidase synthesis during exponential growth in wild-type strains was due to carbon catabolite repression even at a very low glucose concentration, *B. subtilis* ISS8 was grown in ASM containing 0.1% xylose, either without glucose (Δ, ▲), with 0.1% glucose (○, ●) or with 0.3% glucose (▽, ▼).
P-xylanase is synthesized constitutively. In contrast to pEC042.

DISCUSSION

In this study it was shown that in B. subtilis 168 the synthesis of the first two enzymes of xylan degradation is regulated in completely different ways with respect to both induction and carbon catabolite repression.

β-Xylanase is synthesized constitutively. In contrast to many other extracellular enzymes, β-xylanase is synthesized during exponential growth and only to a negligible extent during stationary phase. On the other hand, β-xylosidase synthesis was found to be strongly induced by xylose and xylan. Xylose induction for the genes of the xynCB and xylAB operons was described previously (Gärtner et al., 1988; Hastrup, 1988). The genes of the xynCB and xylAB operons are controlled by the xylR gene product (Hastrup, 1988; Kreuzer et al., 1989). It was suggested that xynA is subject to xylR regulation, too (Klier & Rapoport, 1988), but we found no induction of β-xylanase synthesis by xylose or xylan. A comparison of the DNA sequences of xynA and xynCB with that of xylAB is in agreement with this finding. While there are operator structures for XylR binding in front of the xylAB and xynCB operons (Hastrup, 1988), there is no similar structure present in the sequence of xynA.

Interestingly, β-xylosidase activities increase as cells enter the stationary phase even under non-repressing conditions. This may result from an accumulation of β-xylosidase after the cessation of growth or from an increased synthesis of the enzyme during stationary phase. Temporal activation of gene expression after the onset of stationary phase is a well-documented phenomenon in B. subtilis (e.g. amYE, Nicholson & Chambliss, 1987; bglS, Stülke et al., 1993; dogQ, Msadek et al., 1991). An important factor involved in the regulation of many post-exponentially expressed genes is the SpoOA protein (Boylan et al., 1988; Hulett & Jensen, 1988; Stülke et al., 1993). It is part of the signalling pathway from a decreased intracellular GTP concentration to the temporal activation of the bglS gene (Stülke et al., 1993). The same is true for β-xylosidase, the synthesis of which is delayed in a spo0A mutant by about 2 h compared with an isogenic wild-type strain (C. Lindner, unpublished results). These data suggest that β-xylosidase synthesis is temporally activated after the cessation of growth.

β-Xylosidase proved to be strongly repressed by glucose. While the glucose present in ASM (0.1%) does not repress the synthesis of either α-amylase or β-glucanase (Krüger et al., 1993), there is no β-xylosidase synthesis until the glucose concentration in the medium is as low as 0.01% (0.055 mM).

Glucose repression is completely abolished in a ccapA mutant. This suggests that carbon catabolite repression of β-xylosidase synthesis could be mediated by the same regulatory system as α-amylase (Henkin et al., 1991), levanase (Martin et al., 1989) and β-glucanase (Krüger et al., 1993). The ccapA gene product is homologous to different Escherichia coli repressor proteins such as GalR and LacI (Henkin et al., 1991; Weickert & Adhya, 1992). It is possible that this protein is the repressor of the genes that are glucose-resistant in a ccapA mutant.

In the presence of an optimized bglS operator on a multicopy plasmid, carbon catabolite repression of β-xylosidase synthesis is reduced. Therefore xynB probably belongs to the same carbon catabolite regulon as the bglS and amYE genes. Moreover, there is a sequence with high homology to the targets of catabolite repression of the amYE and bglS genes at the transcription start point of the xynCB operon (Weickert & Chambliss, 1990; Krüger et al., 1993). It is not completely identical to the consensus operator but is very similar to the bglS operator structure. These data indicate that α-amylase, β-glucanase and β-xylosidase are regulated coordinately with respect to catabolite repression. β-Xylanase synthesis is not influenced by a high glucose concentration. This is rather unusual for extracellular catabolic enzymes of B. subtilis, although levansucrase (Steinmetz & Åyemich, 1988) is also not repressed by glucose.

While the regulation of the xyl operons of different Bacillus spp. has been intensively studied (Gärtner et al., 1988; Rygus et al., 1991; Scheler et al., 1991), only limited information on the regulation of xylanolytic enzymes has hitherto been available. Interestingly, B. subtilis 168 is able to grow on xylan, but not on xylose, as a single carbon source. This may be due to the fact that there is no effective uptake system for xylose in B. subtilis. Small amounts of xylose that are necessary for induction are possibly taken up in an unspecific way.

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REFERENCES


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