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Regulation of Expression of the Lactobacillus pentosus xylAB Operon

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The xylose cluster of Lactobacillus pentosus consists of five genes, two of which, xylAB, form an operon and code for the enzymes involved in the catabolism of xylose, while a third encodes a regulatory protein, XylR. By introduction of a multicopy plasmid carrying the xyl operon and by disruption of the chromosomal xylR gene, it was shown that L. pentosus xylR encodes a repressor. Constitutive expression of xylAB in the xylR mutant is repressed by glucose, indicating that glucose repression does not require XylR. The xylR mutant displayed a prolonged lag phase compared to wild-type bacteria when bacteria were shifted from glucose to xylose medium. Differences in the growth rate in xylose medium at different stages of growth are not correlated with differences in levels of xylAB transcription in L. pentosus wild-type or xylR mutant bacteria but are positively correlated in Lactobacillus casei with a plasmid containing xylAB. Glucose repression was further investigated with a ccpA mutant. An 875-bp internal fragment of the ccpA gene of L. pentosus was isolated by PCR and used to construct a ccpA knockout mutant. Transcription analysis of L. pentosus xylA showed that CcpA is involved in glucose repression. CcpA was also shown to be involved in glucose repression of the xylR operon of Lactobacillus amylolovorus by demonstrating that glucose repression of the chloramphenicol acetyltransferase gene under control of the xylR promoter is strongly reduced in the L. pentosus ccpA mutant strain.

Xylose fermentation in bacteria involves the transport of xylose into the cell, isomerization to xululose, and conversion of xylulose to xylose-5-phosphate, followed by further degradation resulting in the formation of equimolar amounts of lactate and acetate as final products. At least five proteins in Lactobacillus pentosus MD353 are responsible for xylose fermentation. They are encoded by the xylose cluster of genes, comprising xylP, xylQ, xylR, xylA, and xylB. The first two genes code for proteins that are presumably involved in the regulation of transport of xylose, while the other genes code for the regulatory protein of the cluster and for xylose isomerase and xylulose kinase, respectively. Expression of the xyl genes, which is induced by xylose and repressed by glucose, is regulated at the transcriptional level (see Fig. 1) (19, 20).

The deduced amino acid sequence of XylR is homologous to that of XylR of Bacillus subtilis (17), Bacillus megaterium (26), Bacillus licheniformis (30), and Staphylococcus xylosus (31). The repressor function of XylR has been unambiguously demonstrated in these organisms (15, 17, 29, 32). Recent studies have established that XylR of B. subtilis is involved not only in repression of the xyl operon in the absence of the inducer xylose but also in glucose repression (4, 16).

L. pentosus XylR most probably also functions as a repressor protein, based on the following considerations. (i) L. pentosus XylR is homologous to the XylR repressors described above, (ii) an operator-like element is present downstream of the L. pentosus xylA promoter, and (iii) introduction into L. pentosus of multiple copies of a DNA fragment containing the xylR-xylA intergenic region with the putative xyl operator and flanking sequences results in xylA expression in the absence of xylose (19, 20). However, some differences in the expression of xylR between L. pentosus and other bacterial species have been noticed. For example, Northern blot analyses showed that L. pentosus xylR is expressed from its own promoter in medium containing glucose but lacking xylose, whereas under inducing conditions it is also part of a larger transcript. Besides xylR, this transcript comprises xylP and xylQ. From a comparison of the amounts of transcript, xylR is at least 10-fold more efficiently transcribed under inducing conditions than under noninducing conditions (19). In S. xylosus, the xylR gene is constitutively expressed (31), whereas in B. megaterium (26) and B. licheniformis (30), xylR is monocistronic and induced by xylose.

Transcription of the xyl genes of L. pentosus is repressed by glucose. The mechanism underlying glucose repression or catabolite repression (CR) in lactobacilli is not known. However, an element which is homologous to the consensus sequence for glucose repression in B. subtilis, the catabolite-responsive element (CRE) (13, 33), and which overlaps the −35 sequence of the xylA promoter has been identified, suggesting that CR in L. pentosus might occur by a similar mechanism (19). CR in Bacillus and Staphylococcus is mediated by a protein, called CcpA. This protein is believed to negatively control transcription by interaction with the CRE. Genes encoding CcpA have been identified in B. subtilis, B. megaterium (13), S. xylosus (6) and L. casei (21a).

We have extended our studies on the role of XylR in expression of the xyl genes of L. pentosus by analyzing the effect of XylR on bacterial growth in xylose medium and by determining the effect of XylR on transcription of xylAB at various growth stages in xylose medium and under conditions of glucose repression. We have performed similar studies with a Lactobacillus species which cannot ferment xylose, L. casei, after introduction of the xylRAB genes. We also report on the identification of the ccpA gene of L. pentosus and on the effect of a ccpA knockout mutation on expression of the xylAB genes of L. pentosus and of the xylR promoter of L. amylolovorus.

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TABLE 1. Plasmids used in this study

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* Promoterless cat<sup>86</sup>-gene.

**MATERIALS AND METHODS**

**Bacterial strains and media.** *L. pentosus* MD353 (20) was used for the isolation of part of the ccpA gene and as a host for the titration experiments. *L. pentosus* MD363 (kindly provided by M. A. Daeschel) was used to construct mutant strains with a disrupted xylR (363ΔxylR) or ccpA (363ΔccpA) gene. *E. coli* JM109 was used for the construction of recombinant DNA plasmids. *L. casei* ATCC 393 (3) was used as a D-xylose-nonfermenting bacteria, is similar to pWH1509C and pWH1509K (25) and was kindly provided by C. Hueck and W. Hillen). Integration vector plIN15E-xylR was constructed as follows. By using pXH50A (comprising the xylR gene, the xylR-xylA intergenic region, and the first 582 bp of the xylA gene), a 408-bp xylA fragment missing 178 bp at the 5′ end and 582 bp at the 3′ end of the gene was obtained by PCR. The primers were elongated with Pst<sup>I</sup> and HindIII restriction sites to allow direct cloning of the PCR product into plIN15E. The resulting plasmid, plIN15E-xylR, was used to disrupt the chromosomal xylR gene by a single crossover event. Plasmids pLP3537-xyl and pLP3537-ΔxylR were used for repressor titration experiments. Plasmid pLP3537-xyl, which carries the xylR, xylA, and xylB genes (22), was used for complementation experiments. Plasmid pLP3537-9 contains the xylR gene with a deletion of 220 bp followed by the nlr-xylA intergenic region and the first 795 bp of xylA (19). Plasmid pLP3537-ΔxylR was made by cloning the 3.7-kb HindIII fragment of pXH37A (20), containing the remaining 3′ part of xylA followed by the complete xylB gene, into pLP3537-17. In this way, an intact xylA gene was restored. Plasmids pLP3537HP45 and pLP3537-9 were used for complementation of *L. casei*. Plasmid pLP3537HP45 was constructed by cloning the 2.4-kb Pst<sup>I</sup>-HindIII fragment from pXH30A into pLP3537 (22, 23). Plasmid pLP3537-9 was constructed by cloning a PCR fragment, containing the xylR-xylA intergenic region followed by the first 180 bp of xyl<sup>B</sup>, into pLP3537. The integration vector pEI2-ccepA was made by cloning of an 875-bp Pst<sup>I</sup>-HindIII PCR fragment, containing an internal fragment of the *L. pentosus* ccpA gene, into plasmid pEI2, a derivative of pUC19 carrying the erythromycin gene from pE194 (23). Plasmid pRBE1-fo-amy was constructed by cloning a 2.4-kb chromosomal DNA fragment from *L. amylovorus*, containing a part of the o-amylose gene with promoter sequences (7), into the promoter-screening vector pRBE1, carrying a promoterless chloramphenicol resistance gene (19).

**Chromosomal integration.** After transformation of *L. pentosus* MD363 by electroporation (19) with plIN15E-xylR, colonies were selected on MRS plates with 5 μg of erythromycin per ml at 30°C. Two individual transformants harboring the plasmid pLP3537 were used for chromosomal integration. MRS medium with 2.5 μg of erythromycin per ml was inoculated with cells from the respective transformants. Cultures were incubated for 5 h at the nonpermissive temperature (38°C), followed by temperature shifts of 2 h at 42°C and 1 h at 38°C. Dilutions of the cultures were plated on MRS plates containing 2.5 μg of erythromycin per ml and were incubated for 16 h at 38°C. Southern blot analysis of chromosomal DNA isolated from the putative integrants showed that approximately 80% of the colonies contained a disrupted xylR gene. Knockout ccpA mutants were constructed by direct integration of the suicide vector pEI2-ccpA. The genetic stability of mutant strains was verified by Southern blot analysis after overnight subculture of bacteria.

**DNA and RNA isolation.** *Lactobacillus* chromosomal DNA was isolated by previously described procedures (20, 23). RNA was isolated as previously described (24). Prior to induction, bacteria (10 ml) were cultivated overnight in glucose-containing M medium (19), harvested, washed twice with M medium without sugar (M1 medium), and resuspended in 1/10 of the original volume of M1 medium. Two different induction procedures were used. In procedure 1, 10 ml of M medium−1% (wt/vol) glucose and/or 1% (wt/vol) xylose was inoculated with 330 μl of washed cells. After a 2-h incubation at 37°C, the cells were harvested and RNA was isolated. In procedure 2, 10 ml of M medium−1% (wt/vol) glucose and/or 1% (wt/vol) xylose was inoculated with 10 μl of washed cells. The cells were incubated at 37°C, and RNA was isolated after different incubation periods. In hybridization experiments, equal amounts of RNA were used as determined by spectrophotometric analysis of RNA samples prior to electrophoresis.

**Chromophenolic acetyltransferase assay.** Cells were cultivated in 10 ml of M medium supplemented with 1% (wt/vol) glucose or 1% (wt/vol) galactose and harvested at an optical density at 595 nm of 0.4. Preparation of bacterial extracts and determination of chromophenolic acetyltransferase (CAT) activity were as described by Lokman et al. (19). The protein concentrations were determined by a protein assay (Bio-Rad Laboratories GmbH) with bovine serum albumin as a standard.

**Other techniques.** Transformation of *E. coli*, plasmid DNA isolation from *E. coli*, DNA and RNA transfer to Hybond N filters, and filter hybridizations were performed by standard procedures (27). Nucleotide sequencing was performed by the dideoxy chain termination method (28), with the T7 DNA polymerase sequencing system of Pharmacia and α<sup>-35</sup>S-dATP (Amersharm) or with a LI-COR infrared automated DNA sequence.

**RESULTS**

**Titration experiments in *L. pentosus* MD353.** The structure of the xyl cluster and operator sequences present in the xylR-xylA intergenic region is shown in Fig. 1. Previously, we have demonstrated that a repression factor is titrated when the xylR-xylA intergenic region and flanking sequences are introduced on a multicopy plasmid in *L. pentosus* MD353, as determined by expression of xylAB in the absence of xylose. To investigate whether the factor that is titrated is XylR, we repeated the titration experiments by transforming *L. pentosus* MD353 with a plasmid containing the xylR-xylA intergenic region and xylR (pLP3537HP45) and with a plasmid harboring the intergenic region only (pLP3537-9). Compared to wild-type MD353, a more than 10-fold increase in xylA expression was observed with the transformant harboring the plasmid lacking xylR, while the amounts of xylA were only marginally increased in transformants with the plasmid harboring xylR (results not shown). This result indicates that XylR is titrated under these conditions.

**Disruption of the chromosomal xylR gene.** To further study the role of xylR in regulation of the expression of the xylAB operon, we have disrupted xylR on the chromosome of *L. pentosus* MD363 (similar to but more transformable than strain MD353) by integration of a plasmid with a thermostable origin of replication harboring part of xylR (see Materials and Methods for details). Southern and Northern blot analysis had demonstrated that with respect to the organization and expression of the xyl genes, *L. pentosus* MD363 is the same as *L. pentosus* MD353 (not shown). The resulting mutant, designated 363ΔxylR, contained two truncated xylR genes, one missing 582 bp at the 3′ end and the other missing 178 bp at the 5′ end.

**RNA analysis of the xylR deletion mutant.** The effect of a xylR disruption on xylA expression was analyzed by Northern blotting with total RNA isolated from cells cultivated in medium containing glucose, xylose, or glucose plus xylose. As shown in Fig. 2, xylA transcription in 363ΔxylR also occurred under noninducing conditions (in the presence of glucose,
Whereas in the wild-type strain no \textit{xylA} transcript could be detected under these conditions, indicating that \textit{xylR} codes for a repressor protein. Furthermore, in the presence of xylose and the absence of glucose, at least a fivefold increase in \textit{xylA} transcription was observed in the mutant strain compared to wild-type bacteria. The amount of \textit{xylA} transcript in 363\textit{ΔxylR} in the presence of glucose plus xylose is nearly the same as that in wild-type bacteria but considerably smaller than that in 363\textit{wt} in the presence of xylose, suggesting that glucose repression does not depend on XylR. Similar observations have been made for the \textit{S. xylosus} \textit{xyl} operon (32). XylR, however, is required for glucose repression in \textit{B. subtilis} (4, 16).

\textbf{Effect of XylR on the growth and transcription of \textit{xylAB} in the presence of xylose.} To determine whether the observed effect of \textit{xylR} disruption on \textit{xylA} expression had an influence on xylose fermentation, we compared the growth behavior of 363\textit{wt} and 363\textit{ΔxylR} after a shift from glucose medium to xylose medium. Wild-type bacteria reproducibly showed a lag phase of \(\sim 48\) h, whereas the \textit{xylR} mutant had a lag phase of approximately 75 h. Wild-type and mutant bacteria showed the same generation time (7 h) in xylose medium and reached the same final cell density. The generation time of mutant and wild-type bacteria in glucose-containing medium was the same (1 h), and a lag period of only 3 h was observed. We have verified that the lag phase phenomenon was due to physiological adaptation and not to a spontaneous mutation. For this wild-type and mutant bacteria harvested during the exponential phase of growth in xylose medium were cultivated overnight in glucose medium and subsequently used to inoculate a xylose-containing medium. For the wild-type and mutant strains, growth resumed after lag periods of 48 and 75 h, respectively. When xylose-growing cells were directly transferred to xylose medium, the specific growth rates were unchanged but the lag phase was no longer observed. Xylose-grown bacteria yielded equal numbers of colonies when plated on glucose or xylose plates. Moreover, the plating efficiency of individual colonies on xylose or glucose plates was the same, irrespective of whether such colonies were isolated from glucose- or xylose-containing plates, ruling out mutations as a cause of the long lag phase after the shift from glucose to xylose medium.

To determine the transcription of \textit{xylA} and \textit{xylB} at various stages of growth, RNA was isolated from 363\textit{wt} and 363\textit{ΔxylR} cultures 2, 24, 48, 72, and 96 h after the shift from glucose medium to xylose medium. After 2 h of cultivation in xylose medium, the 1,500-bp \textit{xylA} and 3,000-bp \textit{xylAB} transcripts were present in both strains, 363\textit{wt} and 363\textit{ΔxylR}, in a 1:1 ratio (Fig. 3). As previously observed, the amount of transcript was larger in the \textit{xylR} deletion mutant. After 24 and 48 h of cultivation, a period in which no cell growth was observed, degradation of rRNA was observed (results not shown). No \textit{xylA} or \textit{xylAB} (Fig. 1) transcript could be detected in the wild-type strain and only weak \textit{xylA} transcription was observed in 363\textit{ΔxylR} after 24 h. A \textit{xylA} transcript was detected again during growth after 72 h in the wild-type strain and after 96 h in the \textit{xylR} mutant strain. At least 10 times more \textit{xylA} transcript was detected than in 363\textit{wt} during the exponential phase of growth. Surprisingly, no \textit{xylAB} transcript could be detected during the exponential phase of growth in either 363\textit{wt} or 363\textit{ΔxylR}. To verify that exponentially growing bacteria ferment xylose, we measured the enzyme activities of \textit{xylA} and \textit{xylB}. Significant amounts of xylose isomerase and xylulose kinase activity were detected in extracts from wild-type and \textit{xylR} mutant cells harvested during the exponential phase of growth, after a shift from glucose to xylose medium. The activity of both enzymes was four- to
fivfold higher in ΔxylR mutant bacteria than in wild-type bacteria (results not shown).

**Functional analysis of xylR in L. casei 393.** To shed more light on the relationship between xylA expression and growth, we analyzed xylA transcription and growth, in the presence of xylose, of L. casei bacteria transformed with a plasmid harboring the xylAB operon. Southern blot analysis with xylA DNA as a probe revealed that L. casei 393 did not contain genes for xylose catabolism and was unable to ferment xylose (data not shown). Moreover, L. casei lacks the proteins XylP and XylQ, which affect the expression of xylA and the growth rate of L. pentosus, presumably by regulation of the transport of xylose (2). The inability of L. casei to ferment xylose could be complemented by introduction of plasmid pLP3537-xyl carrying the xylRAB genes (22). The resulting L. casei transformant is designated 393-xyl. L. casei 393-xyl grew very slowly (doubling time, ~10 h) on M medium containing 1% xylose. An L. casei transformant harboring the xylAB plasmid with a deleted xylR, designated 393-xylΔR, showed faster growth (doubling time, ~8 h) than 393-xyl and was probably able to ferment xylose more efficiently.

Interestingly, one of the L. casei 393 transformants harboring pLP3537-xyl grew much faster (doubling time, 3 h) on M medium containing xylose. Transformation of L. casei 393 with the plasmid isolated from this fast-growing transformant, designated 393-xyl+, yielded transformants with the same fast-growing phenotype. This indicated that the different growth behavior was plasmid encoded. Sequence analysis of the xylR-xylA intergenic region showed that in pLP3537-xyl* the –10 sequence of the xylA promoter has changed from TGTAAAT to the consensus –10 sequence, TATAAT (8). The transformants showed no detectable lag phase in xylose medium, nor was a difference observed in growth behavior between wild-type bacteria and transformants in medium supplemented with glucose (data not shown).

Northern blot analysis of RNA isolated from 393, 393-xyl, 393-xylΔR, and 393-xyl+ showed a positive correlation between growth rate and the xylA transcription levels found 24 h after the shift from glucose to xylose medium (Fig. 4). Almost no xylA transcript was observed in the transformant harboring plasmid pLP3537-xyl, which corresponds to the very slow growth in xylose-containing medium. The increase in the growth rate of 393-xylΔR compared to 393-xyl is accompanied by an increase in xylA transcription. The largest amount of xylA transcript was obtained in L. casei transformants harboring plasmid pLP3537-xyl*, which showed the fastest growth (Fig. 4).

**Identification of the L. pentosus MD353 ccpA gene.** Expression of the xylAB operon is not only controlled by XylR but is also subject to glucose repression (19). To unravel the mechanism of glucose repression in lactobacilli, we first established the presence of a ccpA-like gene in L. pentosus by heterologous hybridization with an internal DNA fragment of the B. megaterium ccpA gene as a probe. Since attempts to isolate the ccpA gene from a library of L. pentosus chromosomal DNA in E. coli with the same probe were unsuccessful, part of the ccpA gene was isolated by PCR. Based on the nucleotide sequence of the B. megaterium ccpA gene, a PCR fragment with the expected size (875 bp) was synthesized with L. pentosus MD353 chromosomal DNA as a template. The deduced amino acid sequence of the sequenced PCR product exhibited similarity to CcpA of B. subtilis (75%), B. megaterium (74%), S. xylosus (67%), and L. casei (83%), confirming that the synthesized product originated from the ccpA gene of L. pentosus. Assuming that the lengths of L. pentosus ccpA and L. casei ccpA are similar, the L. pentosus PCR product is missing 45 bp at the 5' end and 90 bp at the 3' end of the ccpA gene. As a consequence, the L. pentosus ccpA PCR product lacks the region corresponding to the first α-helix of the helix-turn-helix (HTH) DNA-binding domain predicted for the amino-terminal region of B. subtilis CcpA (9).

**Expression of the ccpA gene.** To analyze transcription of the L. pentosus ccpA gene, RNA was isolated from L. pentosus MD353 cells cultivated in the presence of 1% glucose and subsequently grown for 2 h in 1% xylose or 1% glucose, as described in Materials and Methods. Transcription of the ccpA gene was analyzed by Northern blotting with the ccpA PCR fragment as a probe (Fig. 5). Under both conditions, a tran-
Furthermore, in the ccpA with xylose is completely relieved in the isolated after a 2-h cultivation in the presence of glucose, a probe. 875-bp PCR fragment containing a part of the L. pentosus ccpA gene was used as a probe.

To provide further evidence for a role of CcpA in catabolite repression in L. amylovorus, the size of the 1-kb transcript is in agreement with that of the genes analyzed so far.

**Disruption of the chromosomal ccpA gene.** To analyze the function of the ccpA gene in more detail, we disrupted the chromosomal gene of MD363 by integration of the E. coli vector pEI2 (23) containing the L. pentosus ccpA fragment (see Materials and Methods for details). The disruption mutant contained two truncated copies of the ccpA gene. The copy missing part of the 3' end is not expected to be functional, since in B. megaterium a C-terminal deletion of 7 amino acids resulted in an inactivated protein (15a). The second truncated gene is deprived from its promoter and is missing part of the putative HTH motif for DNA binding. Therefore, functionality of this copy can most probably also be excluded (9). The mutant is designated 363ΔccpA.

The growth behavior of the ccpA mutant in M medium containing 1% glucose, 1% xylose, or 1% glucose plus 1% xylose was analyzed. Under these conditions, the growth rate of the ccpA mutant was the same as that of wild-type bacteria but the lag phase in xylose medium was considerably increased (from 48 to 70 h).

**Effect of ccpA disruption on xylA expression.** RNA of wild-type L. pentosus MD363 and the ccpA mutant, 363ΔccpA, was isolated after a 2-h cultivation in the presence of glucose, xylose, or xylose plus glucose (1%, wt/vol), as described in Materials and Methods. The strong repression of xylA transcription in wild-type bacteria when glucose is present together with xylose is completely relieved in the ccpA mutant (Fig. 6). Furthermore, in the ccpA mutant, a significant increase in xylA transcription, compared to that in wild-type bacteria, was observed in the presence of xylose.

**Effect of ccpA disruption on the α-amylose promoter of L. amylovorus.** To provide further evidence for a role of CcpA in catabolite repression in Lactobacillus, we have assessed the effect of disruption of the ccpA gene on expression from the α-amylose promoter of L. amylovorus in the presence of glucose. As found for the L. pentosus xylA promoter, the −35 sequence of the α-amylose promoter partly overlaps with a sequence (CRE1) that shows homology to the consensus sequence for glucose repression (CRE) (12, 33) (Fig. 7). A second putative CRE was identified downstream from the −10 promoter sequence (CRE2). Expression of the α-amylose gene is repressed by glucose but not by galactose or cellobiose, both in L. amylovorus and in L. casei (13a). To verify whether CcpA is involved in glucose repression of the α-amylose promoter, we introduced plasmid pRB-α-amy into L. pentosus MD363 and into the ccpA mutant 363ΔccpA. pRB-α-amy contains part of the L. amylovorus α-amylose gene, including the promoter, followed by a promoterless CAT. CAT activity was analyzed after cultivation of wild-type 363 and 363ΔccpA, both harboring plasmid pRB-α-amy, in the presence of glucose or galactose. Strong repression (89%) was observed when the L. pentosus transformant harboring pRB-α-amy was cultivated in the presence of glucose. This repression was reduced to 27% when the ccpA gene was disrupted (Table 2). This result clearly shows that L. pentosus CcpA controls not only the expression of the xyl operon but also the transcription of the cat gene directed by the promoter sequences of the α-amylose gene of L. amylovorus.

**DISCUSSION**

Two elements, present in the xylR-xylA intergenic region of L. pentosus, appear to be involved in binding of regulatory factors in such a way that transcription of the xylAB operon is tightly regulated. One element is the operator of the xylAB operon, the target site for the XylR repressor. The second element has been implicated in catabolite repression (19). The titration experiments presented here and in an earlier study (19) show that XylR is titrated when multiple copies of the xylR-xylA intergenic region are present. Disruption of the chromosomal xylR gene of L. pentosus MD363 or of the xylR gene in L. casei transformed with plasmid xylRAB resulted in xylA transcription in the absence of xylose (Fig. 2 and 4), reinforcing the conclusion that xylR encodes a repressor protein. The increased xylA transcription, in xylose medium, of an L. pentosus strain in which the xylR gene was disrupted or which carried multiple copies of the xylR-xylA intergenic region, compared to that in wild-type bacteria, indicated that XylR partially re-
pressed expression of the xylAB operon in wild-type bacteria even under inducing conditions. Full expression of the xylAB operon might lead to levels of catabolic intermediates of xylose that are toxic to the cells. The observation that a 363ΔxylIR mutant displays a significantly increased lag phase is consistent with this hypothesis.

In wild-type and xylR mutant L. pentosus, there was no correlation between the growth rate and xylA transcription. L. pentosus showed a very long lag period after a shift from glucose medium to xylose medium, although xylAB was efficiently transcribed during the first few hours after the shift. Transcription of xylA in L. pentosus was found already 5 min after the shift and reached a maximum value approximately 4 h later (our unpublished results). Between 6 and 48 h after the shift, no xylA transcription could be detected. Moreover, degradation of rRNA was observed, suggesting that RNA and protein synthesis had stopped. In contrast, for L. casei bacteria with a multicopy plasmid containing xylAB, a positive correlation between the level of expression of xylA and the growth rate was found. Moreover, no lag phase was observed for L. casei bacteria transformed with a plasmid containing the xylAB genes when shifted from glucose medium to xylose medium. That no correlation between xylA transcription and the growth rate is observed in L. pentosus, in contrast to L. casei, may be explained by the presence in the former but not the latter organism of a protein that influences the uptake of xylose into the cells, making the growth dependent on transport. Preliminary uptake experiments show that xylose uptake is positively correlated with the rate of growth of L. pentosus (2).

Like the ΔccpA mutant and ΔxylP/Q mutants (2), repressor mutants (363ΔxylIR) have a considerably longer lag phase (75 h) than do wild-type bacteria (48 h). Despite constitutive expression of xylAB during the first few hours after xylose induction, no cellular growth takes place until 96 h. Evidently, expression of xylAB is not the limiting factor for growth under these conditions. Similar long lag periods have been observed when L. pentosus 363wt is shifted from glucose to ribose medium. The cause of the exceptionally long lag phase in growth when the cells are shifted to xylose or ribose medium is not yet understood. The longer lag period of 363ΔxylIR compared to 363wt most probably is due to an increased xylAB expression. The observation that the ccpA mutant and the ΔxylP/Q mutants (2), which displayed an increased lag phase compared to wild-type bacteria after a shift from glucose to xylose medium, also showed markedly increased levels of xylAB transcription during the lag period is consistent with this conclusion.

xylA mRNA was found as a monocistronic messenger and as a bicistronic messenger together with xylB mRNA 2 h after L. pentosus was shifted from glucose to xylose medium (Fig. 3). Surprisingly, no xylAB mRNA could be detected during the exponential phase of growth (Fig. 3 and 4) or when glucose-grown bacteria were shifted to xylose medium at a much higher initial cell density (Fig. 2 and 6). Under these conditions, xylB mRNA might be more susceptible to nucleolytic degradation than xylA mRNA, or xylB might be less efficiently transcribed.

The palindromic sequence between xylA and xylB, which can form a stem-loop structure, might control either the stability of xylAB mRNA (20) or its formation by a mechanism involving attenuation. An antiterminator mechanism of regulation for the xylAB operon, as described for the bgl and sac operons of B. subtilis, is not very likely, since the palindromic structure and flanking sequences between xylA and xylB showed no homology to the specific RNA binding sequences involved in attenuation (5, 10, 18). Despite the absence of detectable levels of xylAB RNA, significant levels of xylose isomerase and xylulose kinase activity were detected in extracts from wild-type and ΔxylIR mutant cells harvested during the exponential phase of growth, after a shift from glucose to xylose medium, in accordance with the expectations. Moreover, a positive correlation was observed between the kinase activity and the growth rate (our unpublished observations). Further research is needed to explain the absence of xylB RNA in growing cells.

In the ccpA disruption mutant, glucose repression of the xylAB promoter of L. pentosus was completely relieved and that of the α-amylase promoter of L. amylovorus was largely relieved, demonstrating that CcpA is involved in glucose repression in lactobacilli (Fig. 5; Table 2). Also in a B. subtilis ccpA mutant, α-amylase expression was still partially (50%) repressed in the presence of glucose (9). Furthermore, in B. subtilis, catabolite repression of inositol dehydrogenase and histidase was only partially relieved by a ccpA mutation, implying the presence of other CR control mechanisms beside the one involving CcpA. This suggests that factors other than CcpA contribute to glucose repression in bacilli and lactobacilli. Compared to wild-type bacteria, xylA transcription of the ccpA mutant was increased in the presence of xylose, indicating that CcpA is partially repressing xylA transcription even in the absence of a rapidly metabolizable sugar like glucose.

Analysis of the growth behavior of the ccpA disruption mutant demonstrated that in xylose medium the doubling time was similar to that of wild-type bacteria. In contrast, greatly impaired growth was observed for a B. megaterium ccpA deletion mutant in the presence of a variety of energy sources, such as glucose, fructose, glucitol, and glycerol (13). B. subtilis ccpA mutants exhibited a complete growth defect on minimal medium with glucose as the sole carbon source. This defect was

<table>
<thead>
<tr>
<th>Strain</th>
<th>CAT activity (U-α-amy)</th>
<th>Repression (%)</th>
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</thead>
<tbody>
<tr>
<td>L. pentosus MD363(pRBε1)</td>
<td>0.01 0.01</td>
<td></td>
</tr>
<tr>
<td>L. pentosus MD363(pRB-α-amy)</td>
<td>0.03 0.26</td>
<td>89</td>
</tr>
<tr>
<td>L. pentosus 363ΔccpA(pRB-α-amy)</td>
<td>0.16 0.22</td>
<td>27</td>
</tr>
</tbody>
</table>

* Mean CAT activities of two independent determinations are given. The energy sources used in the growth medium are indicated.

Fig. 7. Sequence of the α-amylase promoter, with the putative CREs indicated. RBS, ribosome binding site.
partially restored by the addition of citrate or other tricarboxylic acid cycle intermediates (34). Hueck and Hillen proposed that the growth defects indicate that CcpA might specifically influence the upper part of the glycolytic pathway, which may, in turn, lead to CR (11). Interestingly, the growth of a B. megaterium ccpA deletion strain on xylose, which is degraded via the pentose phosphate cycle, is almost identical to that of wild-type bacteria (11). Since L. pentosus can ferment glucose either by glycolysis or by the pentose phosphate pathway (14), the harboring the xyl positively correlated with changes of the growth rate of L. pentosus than the pentose phosphate cycle.

In conclusion, our results show that XylR is a repressor in L. pentosus and that CcpA, but not XylR, is involved in CR in this organism. Determination of the levels of transcription of xylA indicates that changes in the level of xylA transcription do not correlate with corresponding changes of the growth rate in wild-type and xylR and ccpA mutant L. pentosus strains but are positively correlated with changes of the growth rate of L. casei harboring the xyl genes.

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