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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* operator 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 α-amylase promoter of *Lactobacillus amylovorus* by demonstrating that glucose repression of the chloramphenicol acetyltransferase gene under control of the α-amylase 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 xylulose, and conversion of xylulose to xylulose-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 Xyl 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*, called 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 α-amylase promoter of *L. amylovorus.*

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TABLE 1. Plasmids used in this study

Plasmid	Marker(s)	Source or reference
pIN15E	Ap^r, Ery^r	This study
pGEM-3	Ap^r	Promega
pWH1509E	Ap^r, Ery^r, Tc^r	26
pXH50A	Ap^r, 3′-*xylP xylQ xylR* 5′-*xylA*	20
pIN15E-xylR	Ap^r, Ery^r, Δ*xylR*	This study
pLP3537-xyl	Ap^r, Ery^r, *xylR xylA xylB*	22
pLP3537-xyl*	Ap^r, Ery^r, *xylR xylA xylB*	This study
pLP3537-ΔxylR	Ap^r, Ery^r, Δ*xylR xylA xylB*	This study
pXH37A	Ap^r, 3′-*xylA xylB*	20
pLP3537-17	Ap^r, Ery^r, Δ*xylR* 5′-*xylA*	19
pLP3537HP45	Ap^r, Ery^r, *xylR* 5′-*xylA*	22
pLP3537	Ap^r, Ery^r	23
pLP3537-9	Ap^r, Ery^r, *xylR-xylA* intergenic region, 5′-*xylA*	This study
pEI2-ccpA	Ap^r, Ery^r, Δ*ccpA*	This study
pEI2	Ap^r, Ery^r	23
pRB-α-amy	Ap^r, Ery^r, Cm^r	This study
pRBE1	Ap^r, Ery^r, Cm[a]	1

[a] Promoterless *cat-86* 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 *Lactobacillus* strain in complementation experiments. The *Lactobacillus* strains were routinely cultivated at 37°C in MRS medium (Difco) or in M medium as described previously (19). The following energy sources were used at a final concentration of 1% (wt/vol): glucose, xylose, or a combination of 1% xylose and 1% glucose. For plating, the media were solidified with 1.5% (wt/vol) agar.

Plasmids and plasmid constructions. The plasmids used in this study are listed in Table 1. Plasmid pIN15E was constructed by cloning the multicloning site of pGEM-3, present on a *Hin*dIII-*Sca*I fragment, into plasmid pWH1509E (this plasmid, which harbors a temperature-sensitive replicon for gram-positive bacteria, is similar to pWH1509C and pWH1509K [25] and was kindly provided by C. Hueck and W. Hillen). Integration vector pIN15E-xylR was constructed as follows. By using pXH50A (comprising the *xylR* gene, the *xylR-xylA* intergenic region, and the first 795 bp of the *xylA* gene), a 404-bp *xylR* 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*I and *Hin*dIII restriction sites to allow direct cloning of the PCR product into pIN15E. The resulting plasmid, pIN15E-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 pLP3537-17 contains the *xylR* gene with a deletion of 220 bp followed by the *xylR-xylA* intergenic region and the first 795 bp of *xylA* (19). Plasmid pLP3537-ΔxylR was made by cloning the 3.7-kb *Hin*dIII 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*I-*Hin*dIII fragment from pXH50A 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 *xylA*, into pLP3537. The integration vector pEI2-ccpA was made by cloning of an 875-bp *Pst*I-*Hin*dIII 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 pRB-α-amy was constructed by cloning a 2-kb chromosomal DNA fragment from *L. amylovorus*, containing a part of the α-amylase 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 pIN15E-xylR, colonies were selected on MRS plates with 5 μg of erythromycin per ml at 30°C. Two individual transformants harboring the correct plasmid 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 subcultivation of bacteria.

DNA and RNA isolation. *Lactobacillus* plasmid and 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.

Chloramphenicol 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 chloramphenicol 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-^{35}S-dATP (Amersham) or with a LI-COR infrared automated DNA sequencer.

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* transcription 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 thermosensitive 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),

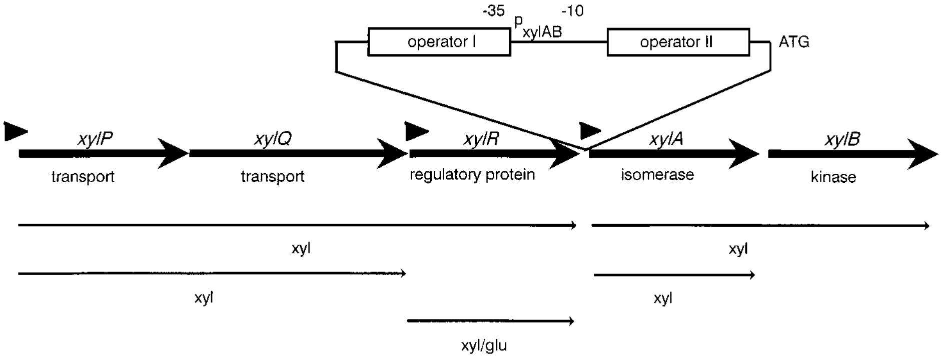

FIG. 1. Schematic representation of the genetic and transcriptional organization of *xyl* genes of *L. pentosus* MD353. The *xyl* genes and their function are depicted. Transcripts found after Northern blot analysis are indicated by thin arrows. The growth conditions under which the transcripts were found and their sizes are indicated (xyl, xylose; glu/xyl, xylose or glucose, 1% [wt/vol]). Triangles indicate mapped transcription initiation sites. The intergenic region between *xylR* and *xylA* with the two operator sequences and the promoter of the *xylAB* operon is enlarged.

whereas in the wild-type strain no *xylA* transcript could be detected under these conditions, indicating that *xylR* codes for a repressor protein. Furthermore, in the presence of xylose and the absence of glucose, at least a fivefold increase in *xylA* transcription was observed in the mutant strain compared to wild-type bacteria. The amount of *xylA* transcript in 363ΔxylR in the presence of glucose plus xylose is nearly the same as that in wild-type bacteria but considerably smaller than that in 363wt in the presence of xylose, suggesting that glucose repression does not depend on XylR. Similar observations have been made for the *S. xylosus xyl* operon (32). XylR, however, is required for glucose repression in *B. subtilis* (4, 16).

Effect of XylR on the growth and transcription of *xylAB* in the presence of xylose. To determine whether the observed effect of *xylR* disruption on *xylA* expression had an influence on xylose fermentation, we compared the growth behavior of 363wt and 363ΔxylR after a shift from glucose medium to xylose medium. Wild-type bacteria reproducibly showed a lag phase of ~48 h, whereas the *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 *xylA* and *xylB* at various stages of growth, RNA was isolated from 363wt and 363ΔxylR 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 *xylA* and 3,000-bp *xylAB* transcripts were present in both strains, 363wt and 363ΔxylR, in a 1:1 ratio (Fig. 3). As previously observed, the amount of transcript was larger in the *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 *xylA* or *xylAB* (Fig. 1) transcript could be detected in the wild-type strain and only weak *xylA* transcription was observed in 363ΔxylR after 24 h. A *xylA* transcript was detected again during growth after 72 h in the wild-type strain and after 96 h in the *xylR* mutant strain. At least 10 times more *xylA* transcript was detected than in 363wt during the exponential phase of growth. Surprisingly, no *xylAB* transcript could be detected during the exponential phase of growth in either 363wt or 363ΔxylR. To verify that exponentially growing bacteria ferment xylose, we measured the enzyme activities of *xylA* and *xylB*. Significant amounts of xylose isomerase and xylulose kinase activity were detected in extracts from wild-type and ΔxylR 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

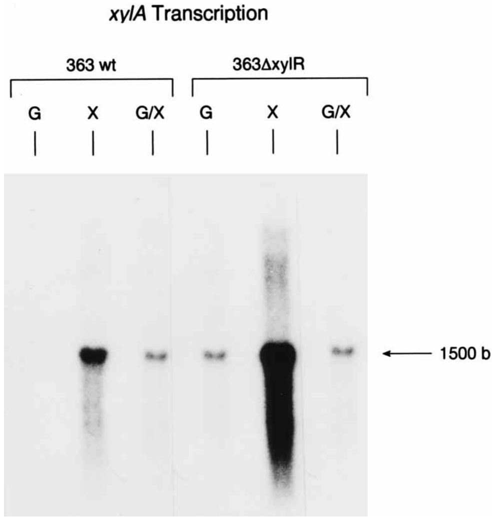

FIG. 2. Northern blot analysis of *xylA* mRNA from *L. pentosus* MD363 (363wt) and the *xylR* disruption mutant (363ΔxylR), isolated after a 2-h incubation in the presence of 1% glucose (G), 1% xylose (X), or 1% glucose plus 1% xylose (G/X). Induction was performed by procedure 1 (see Materials and Methods). A 600-bp *Eco*RI *xylA* fragment was used as a probe.

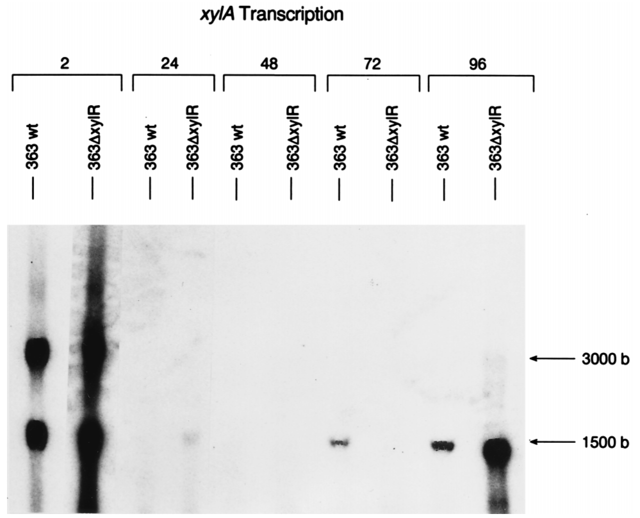

FIG. 3. Northern blot analysis of *xylA* mRNA isolated from *L. pentosus* MD363 (363wt) and the *xylR* disruption mutant (363ΔxylR) after 2, 24, 48, 72, and 96 h of cultivation in the presence of xylose. Induction was performed by procedure 2 (see Materials and Methods). A 600-bp *Eco*RI *xylA* fragment was used as a probe.

fivefold 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 TGTAAT 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-

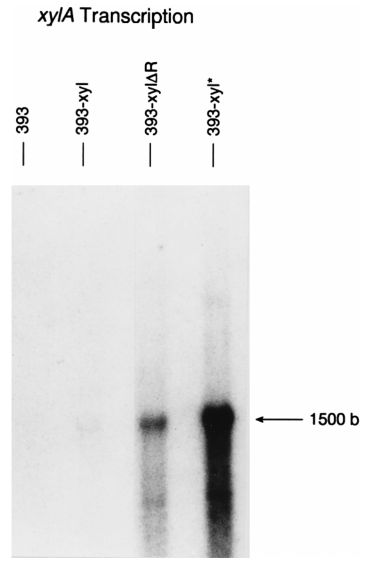

FIG. 4. Northern blot analysis of *xylA* mRNA isolated after 24 h of cultivation in the presence of 1% (wt/vol) xylose from *L. casei* 393 (393) and *L. casei* transformants harboring plasmid pLP3537-xyl (393-xyl), pLP3537-ΔxylR (393-xylΔR), or pLP3537-xyl* (393-xyl*). Induction was performed by procedure 2 (see Materials and Methods). A 600-bp *Eco*RI *xylA* fragment was used as a probe.

FIG. 5. Northern blot analysis of *L. pentosus* MD353 *ccpA* mRNA, isolated from cells cultivated in the presence of 1% xylose (X) or 1% glucose (G). The 875-bp PCR fragment containing a part of the *L. pentosus ccpA* gene was used as a probe.

script of about 10 kb was detected, suggesting that the *L. pentosus ccpA* gene is part of an operon. In *B. megaterium* and *B. subtilis*, *ccpA* is also part of an operon. In the presence of glucose, an additional 1-kb transcript was formed, which was present in large excess compared to the 10-kb transcript. In *B. subtilis*, *ccpA* was also expressed in glucose medium (21). The size of the 1-kb transcript is in agreement with that of the *ccpA* gene, if *L. pentosus ccpA* was the same size as the other *ccpA* 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-amylase 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-amylase promoter of *L. amylovorus* in the presence of glucose. As found for the *L. pentosus xylA* promoter, the −35 sequence of the α-amylase 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-amylase 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-amylase 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-amylase 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-amylase 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-

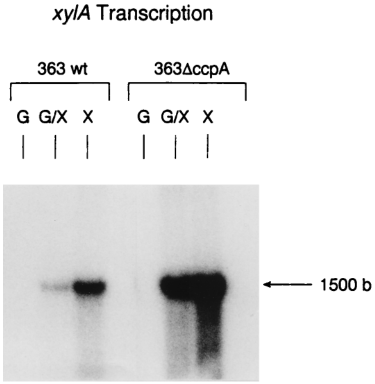

FIG. 6. Northern blot analysis of *xylA* mRNA isolated from *L. pentosus* MD363 and 363Δ*ccpA* after 2 h of cultivation in the presence of the indicated sugars (1%): glucose (G), xylose (X), or glucose plus xylose (G/X). Induction was performed by procedure 1 (see Materials and Methods). A 600-bp *Eco*RI *xylA* fragment was used as a probe.

Consensus CRE TGWAANCGNTNWCA TGWAANCGNTNWCA

-35 **-10**

TTTTCGATTTTTATGAAAACGG**TTGCA**AAGAAGTTAGCAAAAATA**TATAAT**TTCTTTTGAAATTGTTCACTTG

CRE1 **CRE2**

GCCAAGCTGCAGTTTCAATATTTTAATAA**AGGGGG**CAGTAAAAA**GTG**

RBS **START α-Amy**

FIG. 7. Sequence of the α-amylase promoter, with the putative CREs indicated. RBS, ribosome binding site.

pressed expression of the *xylAB* operon in wild-type bacteria even under inducing conditions. Full expression of the *xylAB* operon may lead to levels of catabolic intermediates of xylose that are toxic to the cells. The observation that a 363ΔxylR 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 (363ΔxylR) 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 363ΔxylR 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 antitermination (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 ΔxylR 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 2. CAT activities of *L. pentosus* MD363 and strain 363ΔccpA harboring the indicated plasmids

Strain	CAT activity ($U \cdot mg^{-1}$) with[a]: Glucose	Galactose	Repression (%)
L. pentosus MD363(pRBE1)	<0.01	0.01	
L. pentosus MD363(pRB–α-amy)	0.03	0.26	89
L. pentosus 363ΔccpA(pRB–α-amy)	0.16	0.22	27

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

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 absence of growth defects of *ccpA* mutants in glucose and xylose medium suggests that CcpA affects glycolysis rather 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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REFERENCES

1. **Brückner, R.** 1992. A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. Gene **122:**187–192.
2. **Chaillou, S., B. C. Lokman, R. J. Leer, P. W. Postma, and P. H. Pouwels.** Unpublished data.
3. **Chassy, B. M., and J. L. Flickinger.** 1987. Transformation of *Lactobacillus casei* by electroporation. FEMS Microbiol. Lett. **44:**173–177.
4. **Dahl, M. K., and W. Hillen.** 1995. Contributions of XylR, CcpA and HPr to catabolite repression of the *xyl* operon in *Bacillus subtilis*. FEMS Microbiol. Lett. **132:**79–83.
5. **Debarbouille, M., M. Arnaud, A. Fouet, A. Klier, and G. Rapoport.** 1990. The *sacT* gene regulating the *sacPA* operon in *Bacillus subtilis* shares strong homology with transcriptional antiterminators. J. Bacteriol. **172:**3966–3973.
6. **Egeter, O., and R. Brückner.** 1996. Catabolite repression mediated by the catabolite control protein CcpA in *Staphylococcus xylosus*. Mol. Microbiol. **21:**739–749.
7. **Fitzsimons, A., P. Hols, J. Jore, R. J. Leer, M. O'Connell, and J. Delcour.** 1994. Development of an amylolytic *Lactobacillus plantarum* silage strain expressing the *Lactobacillus amylovorus* α-amylase gene. Appl. Environ. Microbiol. **60:**3529–3535.
8. **Graves, M. C., and J. C. Rabinowitzo.** 1986. In vivo and in vitro transcription of the *Clostridium pasteurianum* ferredoxin gene. J. Biol. Chem. **261:**11409–11415.
9. **Henkin, T. M., F. J. Grundy, W. L. Nicholson, and G. H. Chambliss.** 1991. Catabolite repression of alpha-amylase gene expression in *Bacillus subtilis* involves a trans-acting gene product homologous to the *Escherichia coli lacI* and *galR* repressors. Mol. Microbiol. **5:**575–584.
10. **Houman, F., M. R. Diaz-Torres, and A. Wright.** 1990. Transcriptional antitermination in the *bgl* operon of *E. coli* is modulated by a specific RNA binding protein. Cell **62:**1153–1163.
11. **Hueck, C. J., and W. Hillen.** 1995. Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the gram-positive bacteria? Mol. Microbiol. **15:**395–401.
12. **Hueck, C. J., W. Hillen, and M. H. Saier.** 1994. Analysis of a cis-active sequence mediating catabolite repression in Gram-positive bacteria. Res. Microbiol. **145:**503–518.
13. **Hueck, C. J., A. Kraus, D. Schmiedel, and W. Hillen.** 1995. Cloning, expression and functional analyses of the catabolite control protein CcpA from *Bacillus megaterium*. Mol. Microbiol. **16:**855–864.

13a. **Jore, J., and B. Chassy.** Personal communication.

14. **Kandler, O.** 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie Leeuwenhoek **49:**209–224.
15. **Kauder, C., R. Allmansberger, D. Gartner, D. Schmiedel, and W. Hillen.** 1993. An operator binding-negative mutation of Xyl repressor from *Bacillus subtilis* is trans dominant in *Bacillus megaterium*. FEMS Microbiol. Lett. **109:**81–84.

15a. **Kraus, A.** Personal communication.

16. **Kraus, A., C. Hueck, D. Gartner, and W. Hillen.** 1994. Catabolite repression of the *Bacillus subtilis xyl* operon involves a *cis* element functional in the context of an unrelated sequence, and glucose exerts additional XylR-dependent repression. J. Bacteriol. **176:**1738–1745.
17. **Kreuzer, P., D. Gratner, R. Allmansberger, and W. Hillen.** 1989. Identification and sequence analysis of the *Bacillus subtilis xylR* gene and *xyl* operator. J. Bacteriol. **171:**3840–3845.
18. **Le-Coq, D., C. Lindner, S. Kruger, M. Steinmetz, and J. Stulke.** 1995. New β-glucoside (*bgl*) genes in *Bacillus subtilis*: the *bglP* gene product has both transport and regulatory functions similar to those of BglF, its *Escherichia coli* homolog. J. Bacteriol. **177:**1527–1535.
19. **Lokman, B. C., R. J. Leer, R. van Sorge, and P. H. Pouwels.** 1994. Promoter analysis and transcriptional regulation of the *Lactobacillus pentosus* xylose regulon. Mol. Gen. Genet. **245:**117–125.
20. **Lokman, B. C., P. van Santen, J. C. Verdoes, J. Kruse, R. J. Leer, M. Posno, and P. H. Pouwels.** 1991. Organization and characterization of three genes involved in D-xylose catabolism in *Lactobacillus pentosus*. Mol. Gen. Genet. **230:**161–169.
21. **Miwa, Y., M. Saikawa, and Y. Fujita.** 1994. Possible function and some properties of the CcpA protein of *Bacillus subtilis*. Microbiology **140:**2567–2575.

21a. **Perez-Martinez, G.** Personal communication.

22. **Posno, M., P. T. Heuvelmans, M. J. van Giezen, B. C. Lokman, R. J. Leer, and P. H. Pouwels.** 1991. Complementation of the inability of *Lactobacillus* strains to utilize D-xylose with D-xylose catabolism-encoding genes of *Lactobacillus pentosus*. Appl. Environ. Microbiol. **57:**2764–2766.
23. **Posno, M., R. J. Leer, N. van Luijk, M. J. F. van Giezen, P. T. H. M. Heuvelmans, and P. H. Pouwels.** 1991. Incompatibility of *Lactobacillus* vectors with replicons derived from small cryptic *Lactobacillus* plasmids and segregational instability of the introduced vectors. Appl. Environ. Microbiol. **57:**1822–1828.
24. **Pouwels, P. H., N. Van Luijk, M. Posno, and R. J. Leer.** 1994. Control of replication of the *Lactobacillus pentosus* plasmid p353-2—evidence for a mechanism involving transcriptional attenuation of the gene coding for the replication protein. Mol. Gen. Genet. **242:**614–622.
25. **Rygus, T., and W. Hillen.** 1992. Catabolite repression of the *xyl* operon of *Bacillus megaterium*. J. Bacteriol. **174:**3049–3055.
26. **Rygus, T., A. Scheler, R. Allmansberger, and W. Hillen.** 1991. Molecular cloning, structure, promoters and regulatory elements for transcription of the *Bacillus megaterium* encoded regulon for xylose utilization. Arch. Microbiol. **155:**535–542.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Scheler, A., and W. Hillen.** 1994. Regulation of xylose utilization in *Bacillus licheniformis*: Xyl repressor-xyl-operator interaction studied by DNA modification protection and interference. Mol. Microbiol. **13:**505–512.
30. **Scheler, A., T. Rygus, R. Allmansberger, and W. Hillen.** 1991. Molecular cloning, structure, promoters, and regulatory elements for transcription of the *Bacillus licheniformis* encoded regulon for xylose utilization. Arch. Microbiol. **155:**526–534.
31. **Sizemore, C., E. Buchner, T. Rygus, C. Witke, F. Götz, and W. Hillen.** 1991. Organization, promoter analysis and transcriptional regulation of the *Staphylococcus xylosus* xylose utilization operon. Mol. Gen. Genet. **227:**3042–3048.
32. **Sizemore, C., B. Wieland, F. Götz, and W. Hillen.** 1992. Regulation of *Staphylococcus xylosus* xylose utilization genes at the molecular level. J. Bacteriol. **174:**3042–3048.
33. **Weickert, M. J., and G. H. Chambliss.** 1990. Site-directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**6238–6242.
34. **Wray, L. V., F. K. Pettengill, and S. Fisher.** 1994. Catabolite repression of the *Bacillus subtilis hut* operon requires a *cis*-acting site located downstream of the transcription initiation site. J. Bacteriol. **176:**1894.