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Regulation of Lactose Utilization Genes in *Staphylococcus xylosus*

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The lactose utilization genes of *Staphylococcus xylosus* have been isolated and characterized. The system is comprised of two structural genes, *lacP* and *lacH*, encoding the lactose permease and the β-galactosidase proteins, respectively, and a regulatory gene, *lacR*, coding for an activator of the AraC/XylS family. The lactose utilization genes are divergently arranged, the *lacPH* genes being opposite to *lacR*. The *lacPH* genes are cotranscribed from one promoter in front of *lacP*, whereas *lacR* is transcribed from two promoters of different strengths. Lactose transport as well as β-galactosidase activity are inducible by the addition of lactose to the growth medium. Primer extension experiments demonstrated that regulation is achieved at the level of *lacPH* transcription initiation. Inducibility and efficient *lacPH* transcription are dependent on a functional *lacR* gene. Inactivation of *lacR* resulted in low and constitutive *lacPH* expression. Expression of *lacR* itself is practically constitutive, since transcription initiated at the major *lacR* promoter does not respond to the availability of lactose. Only the minor *lacR* promoter is lactose inducible. Apart from lactose-specific, LacR-dependent control, the *lacPH* promoter is also subject to carbon catabolite repression mediated by the catabolite control protein CcpA. When glucose is present in the growth medium, *lacPH* transcription initiation is reduced. Upon *ccpA* inactivation, repression at the *lacPH* promoter is relieved. Despite this loss of transcriptional regulation in the *ccpA* mutant strain, β-galactosidase activity is still reduced by glucose, suggesting another level of control.

The lactose operon of *Escherichia coli* is a paradigm for gene regulation (for a review, see reference 33). Studying *lac* regulation led to fundamental concepts of how a set of genes may be coordinately regulated depending upon the concentration of metabolizable compounds in the growth medium. Soon after repression of the *lac* operon was established, the universality of that regulatory mode was challenged by the analysis of the arabinose and maltose systems in *E. coli*, where positive control was realized (for a review, see reference 43). Molecular characterization of sugar utilization systems has also provided valuable knowledge on gene regulation in bacteria other than *E. coli*. Examples include the complex sucrose metabolism of *Bacillus subtilis*, *lac* systems in several AT-rich gram-positive bacteria, and global control by carbon catabolite repression (reviewed in references 9, 41, and 46).

In *Staphylococcus xylosus* (44), an AT-rich gram-positive bacterium used in meat fermentations (18), the regulation of maltose, sucrose, and xylose catabolic genes has been studied in some detail (10, 13, 45). In addition, two genes encoding proteins involved in carbon catabolite repression in this organism have been isolated (11, 51). One of the genes, the glucose kinase gene *glkA*, has been detected by transposon mutagenesis and screening for altered β-galactosidase activity in the presence of glucose. It was of interest, therefore, to clone the β-galactosidase gene of *S. xylosus* and to analyze its regulation.

In this communication, we report on the isolation and characterization of the lactose utilization genes of *S. xylosus* and their transcriptional regulation.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and phage DNAs. The staphylococcal strains used in this study are listed in Table 1. *E. coli* TG1 [*supE hsdΔ5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was used to screen the *S. xylosus* library (8) for phage M13 and plasmid cloning. The *S. xylosus* library had been constructed in pBR322. Genes to be introduced into *S. xylosus* were cloned in *E. coli* TG1 by using the shuttle vector pRB473 (8), which is a derivative of pRB373 (6). Plasmid pTV1Ts harboring transposon Tn*917* (55) served for transposon mutagenesis.

Transposon mutagenesis. The transposon mutagenesis was performed as described previously (51). Cells were plated on agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal [100 μg/ml]), erythromycin (2.5 μg/ml), and glucose (1%) and incubated at 37°C for about 48 h.

DNA manipulations, sequencing, transformation, and transfection. DNA manipulations, plasmid DNA isolation and sequencing, Southern blot analysis, transformation and transfection of *E. coli*, and preparation of media and agar plates for bacterial growth were done by standard procedures (42). Isolation of chromosomal DNA from *S. xylosus* and the construction of the genomic library of *S. xylosus* in *E. coli* have been described previously (8). Plasmids were introduced into *S. xylosus* by electroporation (7). PCR was carried out with Vent DNA polymerase (New England Biolabs) or r*Tth* DNA polymerase XL (Perkin-Elmer) in accordance with the instructions of the suppliers.

Cloning of the DNA region upstream of *lacP*. Since plasmid pBG303, a representative of the β-galactosidase plasmids from the *S. xylosus* library (11), contained a truncated putative lactose transporter gene upstream of the β-galactosidase gene, this region was isolated from the chromosome. Southern blot analysis with a *lacH*-specific probe revealed a second *Sst*I restriction site about 8 kb upstream of the one within *lacH* (Fig. 1). Chromosomal DNA of *S. xylosus* was digested with *Sst*I, ligated, and used for inverse long-range PCR with the following primers: 5′-CCAATTCGTAATATCCCCGCTCC (positions 2982 to 2960 at the 3′ end of *lacP*) and 5′-CACTAACGGTCCCATCGGTTTGG (positions 3590 to 3612 at the 5′ end of *lacH*). Restriction of the PCR product with *Hpa*I produced two *Hpa*I fragments of 2.5 and 2.8 kb in size. The 2.8-kb fragment, located next to *lacH* (Fig. 1), was cloned into pUC18, generating plasmid pBG304 (Fig. 1), which was used for further analysis.

Construction of a *lacP* deletion mutant of *S. xylosus* by gene replacement. To construct a *lacP* deletion mutant, two PCR fragments were produced. The first, a 1.1-kb fragment, included the whole *lacR* gene and the *lacP* region encoding the first 20 amino acids of LacP. The second fragment, 3.5 kb in length, contained the last 20 codons of *lacP* together with the complete *lacH* gene. The primers for these amplifications were as follows. For the *lacR-lacP*′ fragment, 5′-GACGGATCCGGGCAGAAAACCAATGGAAG (positions 897 to 916; *Bam*HI restriction site underlined) and 5′-GCAGTCGACCTTACCGATGGCACCGAATCC (positions 2027 to 2006; *Sal*I restriction site underlined); for the *′lacP-lacH* fragment, 5′-GCAGTCGACACGGATATTGAAAAGACATTACAG (posi-

TABLE 1. *S. xylosus* strains used

Strain	Genotype[a]	Reference or source
C2a	Wild type	16
TX258	*lacR*::Tn*917*	This work
TX259	*lacH*::Tn*917*	This work
TX260	Δ*lacP*	This work
TX140	*glkA*::Tn*917* Δ*tnpA*; Derivative of TX14	51
TX154	*ccpA*::*ermB*	11

[a] All strains listed are derivatives of *S. xylosus* DSM 20267 (44) cured of the endogenous plasmid pSX267 (16).

tions 3293 to 3316; *Sal*I restriction site underlined) and 5′-GACGCTAGCGTA GGTATTGGAGGCGCAGG (positions 6763 to 6744; *Nhe*I restriction site underlined). After restriction with the appropriate restriction enzymes, both PCR fragments were cloned in one step as a *Bam*HI-*Sal*I fragment and a *Sal*I-*Nhe*I fragment in the *Bam*HI-*Nhe*I-restricted temperature-sensitive shuttle vector pBT2 (7), generating plasmid pBT2RΔPH2. On this plasmid, about 92% of *lacP* was removed. The plasmid was introduced into *S. xylosus* C2a, and appropriate dilutions of an overnight culture in B medium with chloramphenicol (20 μg/ml), incubated at 30°C, were plated on lactose utilization test plates (32) and incubated at 37°C for about 48 h. White colonies (lactose negative) that integrated the Δ*lacP* gene into their chromosome by double crossover and that were chloramphenicol sensitive could be detected. One representative colony, designated *S. xylosus* TX260 (Δ*lacP*), was shown to carry the expected Δ*lacP* mutation based on PCR analysis (data not shown).

Growth of *S. xylosus* to monitor expression of the lactose utilization genes. *S. xylosus* was grown in B medium, which consisted of 1% peptone (Gibco BRL), 0.5% yeast extract, 0.5% NaCl, and 0.1% $K_2HPO_4 \cdot 3H_2O$. Carbohydrates were added to a final concentration of 25 mM, if required. The complex growth medium was used since no suitable minimal medium is available for *S. xylosus*. Fermentation of carbohydrates by *S. xylosus* was monitored on agar plates (32) containing 0.5% of the respective sugar.

For the determination of lactose transport and β-galactosidase activity, and to prepare RNA for primer extension analysis, the following growing conditions were applied. To test for inducibility, cells were grown in B medium without additional carbohydrate to an optical density at 578 nm (OD_{578}) of 1.2. Sugars (25 mM) were added, and the cultures were incubated for 1 additional hour and harvested (OD_{578}, 2 to 2.5). The culture without added carbohydrate grown for the same time period served as the uninduced control. To measure glucose repression, lactose and glucose were added concomitantly to cultures grown as described above.

Measurements of lactose uptake. Transport of lactose was measured by using whole cells. The harvested cells were washed with ice-cold MT buffer (100 mM MOPS [morpholinepropanesulfonic acid; pH 7.0], 0.5 mM $MgSO_4$, 10 mM NaCl) and resuspended in the same buffer to yield an OD_{578} of 3.0. These cells were kept on ice until use. One milliliter of the cell suspension was preincubated for 2 min at 30°C, and then 200 μM lactose (25 μM [^{14}C]lactose [57.0 mCi/mmol]) was added. Samples 0.15 ml in size were taken after 1, 2, 4, 8, and 15 min, collected on cellulose nitrate disks (pore size, 0.45 μm), and washed with 5 ml of MT buffer. Filters were dried at 80°C for 30 min, and the radioactivity was determined by liquid scintillation counting. Uptake rates are expressed in picomoles of lactose accumulated per minute per milligram of cell protein. The amount of protein was determined by the method of Bradford (3).

Determination of β-galactosidase activity in cell extracts. Crude extracts were prepared by vortexing cells repeatedly with glass beads in β-galactosidase buffer (T4) containing 0.1 M Tris (pH 8.0), 0.5 M KCl, 1 mM $MgSO_4$, 0.4 mM $MnCl_2$, and 4 mM dithiothreitol. The assays were performed at 30°C with *p*-nitrophenyl-β-D-galactopyranoside (7.5 mM) as the substrate and 15 to 400 μg of cellular protein. The release of nitrophenol was monitored at 405 nm. Specific β-galactosidase activity is expressed in nanomoles of nitrophenol released per minute per milligram of protein. Protein concentrations in the cell extracts were determined by the method of Bradford (3).

RNA preparation and primer extension analysis. Isolation of total RNA (except 5S RNA and tRNAs) was performed with the RNeasy Midi Kit (Qiagen). Five to 8 milliliters of the culture, harvested at an OD_{578} of 2.0 to 2.5, were washed with 5 ml of ice-cold EDTA solution (0.5 M, pH 8.0), and the pellet was resuspended for cell disruption in a mixture of 1 ml of lysostaphin solution (0.5 mg/ml of H_2O) and 10 μl of 100× TE buffer (1 M Tris [pH 8.0], 0.1 M EDTA) and incubated at 37°C. By using large amounts of lysostaphin, the cells lysed rather quickly, within about 2 min. After cell lysis occurred, preparation of the RNA was continued in accordance with the RNeasy protocol for isolation of total RNA from bacteria, with the larger volumes of buffers and solutions given in the protocol always used. The final RNA solution was concentrated to a volume of 25 μl, which contained 2 to 10 μg of RNA/μl. Primer extension experiments were performed with avian myeloblastosis virus reverse transcriptase (Stratagene). The following primers yielded reverse transcripts: for *lacR*, 5′-CCTACATTCG GTACGCC (positions 1705 to 1721); and for *lacPH*, 5′-CATCCTTACCGATG GCAC (positions 2030 to 2013). The 5′-end ^{32}P-labeled oligonucleotides were used in primer extension reactions with 15 μg of cellular RNA. Reverse transcripts were resolved on 5% urea-containing polyacrylamide gels. DNA sequenc-

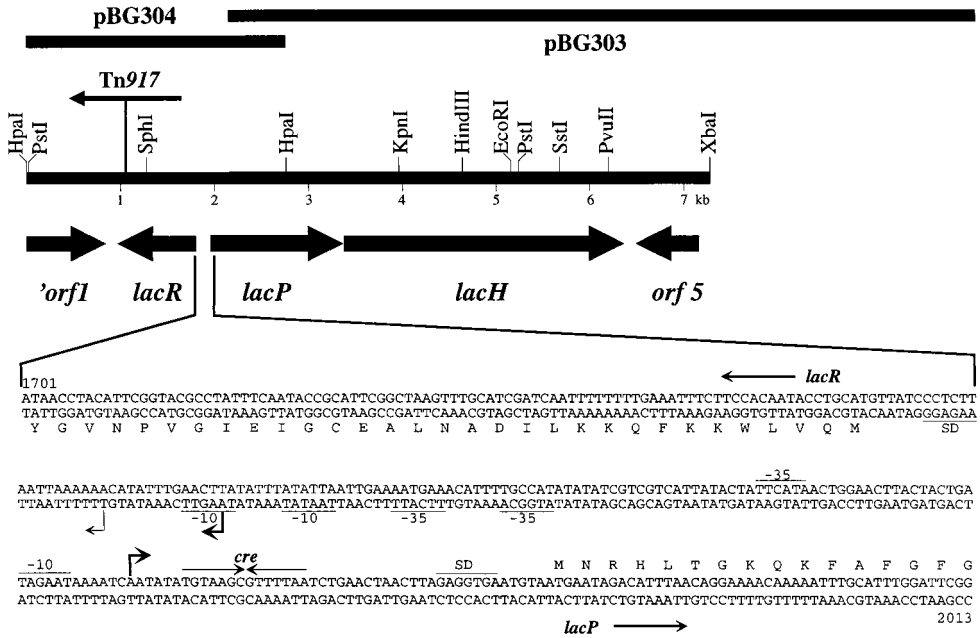

FIG. 1. Genetic organization of the lactose utilization genes of *S. xylosus* and nucleotide sequence of the *lacR-lacPH* promoter region. The *lac* region that has been sequenced is shown. The size and orientation of the genes were deduced from the nucleotide sequence. The position and orientation of Tn*917* in the *lacR* mutant strain TX258 are marked. The sequence of the *lacR-lacP* intergenic region is shown. Numbering refers to the complete *lac* sequence available from the EMBL database under accession no. Y14599. The transcriptional start sites of the *lac* promoters and an inverted repeat resembling *cre* are indicated by arrows. The major transcriptional start site of the *lacR* promoter is symbolized by a boldface arrow. Putative RNA polymerase- and ribosome-binding sites are underlined.

ing reactions using the same oligonucleotide were used for sizing the primer extension products.

Nucleotide sequence accession number. The nucleotide sequence is available from the EMBL database under accession no. Y14599.

RESULTS

Cloning of the lactose utilization genes of *S. xylosus*. To clone the β-galactosidase gene, aliquots of an amplified *S. xylosus* library that was stored as plasmid pools (11) were introduced into *E. coli* TG1 and the transformants were checked for β-galactosidase activity on X-Gal-containing agar plates. Blue colonies were obtained from six plasmid pools. Subsequently, the plasmid contents of four representative transformants of each pool were analyzed. Plasmids originating from one pool were found to be identical. In addition, two plasmids from different pools showed the same restriction patterns. Therefore, five distinct β-galactosidase-expressing plasmids were isolated from the amplified library. Comparative restriction analysis of these plasmids revealed a common region of about 4 kb. Plasmid pBG303 containing an insertion of about 8 kb was chosen for further analysis. By Southern blot analysis of chromosomal *S. xylosus* DNA, it was verified that the insertion represented a continuous segment of the genome. Partial sequencing of the cloned DNA identified an open reading frame with high sequence similarity to various β-galactosidases. In addition, an incomplete open reading frame that resembled sugar transporters of the GPH family was detected upstream of the β-galactosidase gene (Fig. 1) (37). Since none of the plasmids from the library contained the complete putative lactose transporter gene, the gene was isolated from the *S. xylosus* genome by a different approach.

The missing information was cloned on plasmid pBG304 by inverse PCR as described in Materials and Methods. Several attempts to obtain the whole lactose utilization region, as depicted in Fig. 1, in *E. coli* or *Staphylococcus carnosus* (15) failed. Subsequently, the lactose utilization region of *S. xylosus* was sequenced by using the plasmids pBG303 and pBG304.

Nucleotide sequence of the lactose utilization genes. The nucleotide sequence was determined for both strands from the *Hpa*I restriction site within *orf1* to the unique *Xba*I restriction site (Fig. 1). It comprises 7,206 bp and contains 5 open reading frames, one of which (*orf1*) is truncated at the 5′ end. The largest open reading frame, encoding a protein of 994 amino acids with a calculated molecular mass of 115.246 kDa, constitutes the β-galactosidase gene and is designated *lacH*. The β-galactosidase protein of *S. xylosus* has the highest degree of similarity to the β-galactosidase protein of *Actinobacillus pleuropneumoniae* (2), with almost 40% identical residues. With the β-galactosidase protein from *E. coli* (27), LacH has 33% amino acids in common. Several conserved regions, which appear to be important for the hydrolytic activity in β-galactosidases (26), are also present in the *S. xylosus* enzyme.

The *lacP* gene, located upstream of *lacH*, encodes a protein of 462 amino acids (51.667 kDa) that shows significant similarity to members of the GPH protein family that transport galactosides, pentoses, and hexunorides (37). Within this family, the melibiose permeases of enteric bacteria (17, 31, 54) are most similar to the *S. xylosus* protein (37 to 39% identical residues). Hydropathy analysis (28) and structural predictions (22) have suggested that LacP is a membrane protein with 11 transmembrane segments. Therefore, *lacP* appears to encode the lactose permease of *S. xylosus.*

Upstream of *lacP* and on the opposite strand of the DNA, an open reading frame, named *lacR*, with a coding capacity of 279 amino acids is found. The deduced LacR protein has a molecular mass of 32.339 kDa and resembles regulatory proteins of the AraC/XylS family (12). LacR of *S. xylosus* has 31% identical residues to the raffinose operon activator from *Pediococcus pentosaceus* (Swiss-Prot no. P43465) and 24% to both AraC from *E. coli* (52) and XylS from *Pseudomonas putida* (25). The helix-turn-helix DNA binding motif of the regulator family, which is located in the C-terminal portion of the proteins, is present at the appropriate position in LacR (amino acids 190 to 209). Therefore, *lacR* probably encodes the regulator of the lactose utilization genes.

The deduced polypeptide of the truncated *orf1* (Fig. 1) had similarity to regulators of the LysR family (19), whereas the product of *orf5* did not have significant similarity to proteins in databases. It appears that neither gene takes part in lactose utilization in *S. xylosus.*

TABLE 2. Lactose transport and β-galactosidase activity in the *S. xylosus* wild type, C2a, and in the *lacR* mutant, TX258

Strain	Growth conditions[a]	Lactose transport[b] (pmol of lactose transported/min/mg of protein)	β-Galactosidase activity[c] (nmol of nitrophenol produced/min/mg of protein)
C2a	B	371 ± 99	9 ± 2
	B + lactose	5,143 ± 187	98 ± 14
	B + galactose	578 ± 94	7 ± 2
TX258 (*lacR*)	B	78 ± 8	<1
	B + lactose	83 ± 8	<1

[a] The strains were grown in B medium (B) to an OD_{578} of 1.2. The indicated sugar was added at 25 mM. After 1 h of growth, the cells were harvested. Cultures without additional sugar were grown and harvested accordingly.

[b] Lactose transport was determined by using 200 μM [^{14}C]lactose (7.12 mCi/mmol) and 1 ml of washed cells at an OD_{578} of 3. The values (means ± standard deviations) represent the initial uptake rates within the first 4 min of the experiment. They were taken from two measurements, each from three cultures.

[c] The values (means ± standard deviations) represent two determinations, each with bacterial extracts from three cultures.

Isolation of transposon-induced lactose utilization mutants of *S. xylosus*. In a previous study intended to isolate *S. xylosus* mutants altered in global catabolite repression, Tn*917* transposon mutagenesis was performed, using β-galactosidase expression for screening. Besides several dark blue colonies, which were catabolite repression mutants (51), one white and one pale blue colony were isolated. Both mutant strains lost the ability to ferment lactose as determined by acid production on lactose-containing indicator plates (32). By PCR analysis using transposon- as well as *lac*-specific primers, Tn*917* was localized to *lacR* in *S. xylosus* TX258 (Fig. 1) and to *lacH* in *S. xylosus* TX259. Due to the integration of Tn*917* into the *lacH* gene, no β-galactosidase activity was detectable in TX259 (data not shown). Therefore, the strain was not further analyzed. In the mutant, TX258, however, the exact location of Tn*917* in *lacR* was determined by DNA sequencing. Tn*917* was found to have integrated 120 bp apart from the end of *lacR* (Fig. 1). To analyze the consequences of *lacR* inactivation for *lac* gene expression, lactose transport and β-galactosidase activity were determined in the wild type and the mutant strain.

Lactose transport and β-galactosidase activity in the wild-type and *lacR* mutant strains. To measure activities specified by proteins encoded by the *lac* genes, strains were grown in complex medium with lactose or galactose or without additional sugar. As summarized in Table 2, lactose transport and β-galactosidase activity in the wild-type strain are induced by the addition of lactose to the growth medium, whereas galactose has no effect. Induction of lactose transport was found to be about 14-fold and β-galactosidase activity was stimulated

TABLE 3. β-Galactosidase activity in the *S. xylosus* wild type, C2a, and in the catabolite repression mutants TX140 (*glkA*) and TX154 (*ccpA*)

Strain	Growth conditions[a]	β-Galactosidase activity[b] (nmol of nitrophenol produced/min/mg of protein)
C2a	B	9 ± 2
	B + lactose	98 ± 14
	B + lactose + glucose	5 ± 1
TX140 (*glkA*)	B	9 ± 1
	B + lactose	83 ± 10
	B + lactose + glucose	20 ± 5
TX154 (*ccpA*)	B	8 ± 2
	B + lactose	119 ± 31
	B + lactose + glucose	28 ± 3

[a] The strains were grown in B medium (B) to an OD_{578} of 1.2. The indicated sugars were added at 25 mM. After 1 h of growth, the cells were harvested. Cultures without additional sugar were grown and harvested accordingly.

[b] The values (means ± standard deviations) represent two determinations, each with bacterial extracts from three cultures.

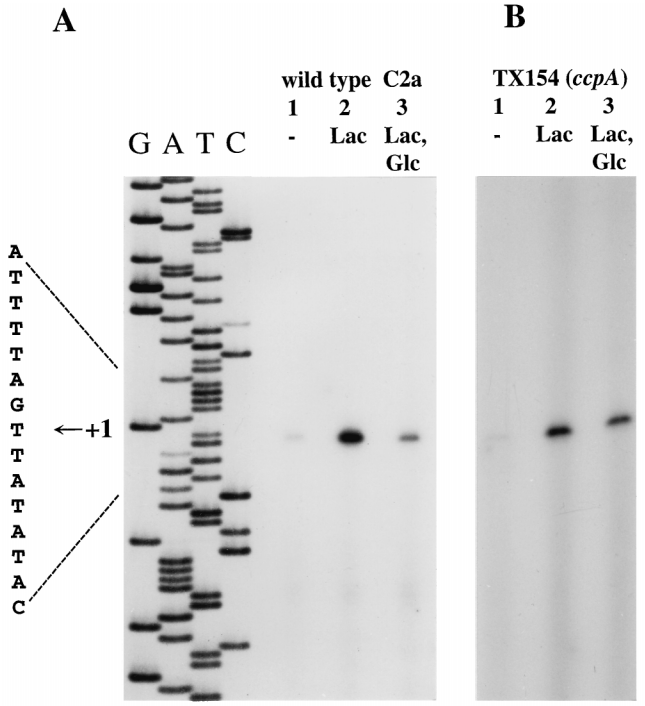

FIG. 2. Primer extension analysis of *lacPH* transcription. (A) Analysis of *lacPH* transcription in the *S. xylosus* wild type, C2a. Total RNA was prepared from *S. xylosus* wild-type cells grown in B medium containing no additional sugar (lane 1), 25 mM lactose (lane 2), or 25 mM each lactose and glucose (lane 3). Fifteen micrograms of RNA and a ^{32}P-labeled *lacP*-specific primer were used for the primer extension reactions. One-fourth of each reaction mixture was separated on a 5% polyacrylamide–urea gel, together with a sequencing reaction mixture obtained with the same primer. The autoradiograph and the sequence interpretation around the +1 site are shown. (B) Analysis of *lacPH* transcription in the *S. xylosus ccpA* mutant, TX154. Total RNA was prepared from *S. xylosus* cells grown in B medium containing no additional sugar (lane 1), 25 mM lactose (lane 2), or 25 mM each glucose and lactose (lane 3). Fifteen micrograms of RNA and a ^{32}P-labeled *lacP*-specific primer were used in the primer extension reactions. One-fourth of each reaction mixture was separated on a 5% polyacrylamide–urea gel. The autoradiograph of the gel is shown.

11-fold, indicating coordinated expression of both genes. In the *lacR* mutant strain, TX258, both activities were unregulated and much lower than in the wild type (Table 2). The low noninducible expression of the *lac* genes in the *lacR* mutant strongly suggests that LacR functions as an activator.

Inducibility of β-galactosidase activity in a *lacP* mutant strain. To determine whether a functional lactose permease is required for induction of the system, a *lacP* mutant strain was constructed (see Materials and Methods). In that strain, TX260, an in-frame deletion within *lacP* removed the coding region for 422 amino acids, leaving the *lacR-lacP* as well as the *lacP-lacH* intergenic region intact. The *lacP* deletion strain was lactose negative, as determined on utilization test plates, transported background levels of lactose (50 pmol lactose/min/mg of protein), and showed a noninducible β-galactosidase activity of about 5 U. Therefore, LacP cannot be replaced by another transporter and is required for induction. In the sucrose utilization system of *S. xylosus*, inactivation of the sucrose permease gene did not result in the loss of sucrose-mediated induction of gene expression (50).

Glucose-mediated repression of β-galactosidase activity in the wild type and in catabolite repression mutants. In previous studies on catabolite repression in *S. xylosus*, we have characterized two genes which are involved in this global regulatory process. *glkA*, the first gene that was isolated, encoded a glucose kinase (51). Inactivation of *glkA* resulted in a partial loss of glucose-specific repression of several catabolic enzymes, including β-galactosidase. The second gene encoded the catabolite control protein CcpA (11). Disruption of *ccpA* relieved some catabolic enzymes completely from repression by sugars, such as glucose, sucrose, or fructose. However, part of the glucose-mediated repression of β-galactosidase activity persisted. Since growing conditions used in the previous studies were slightly different from those in the *lac* induction experiments reported above, glucose repression of β-galactosidase activity was reexamined.

In the wild type, the addition of glucose prevents lactose-mediated induction, reducing β-galactosidase activity about 20-fold (Table 3). As expected, glucose repression of β-galactosidase activity was partially relieved in the mutants. The residual reduction was about fourfold in the glucose kinase mutant, TX140, and in the *ccpA* mutant strain, TX154 (Table 3). In both cases, β-galactosidase activity in the presence of lactose appeared to be slightly different than that in the wild type. In TX140, it reached about 85% of the wild-type level, whereas the wild-type value was exceeded by about 20% in the *ccpA* mutant strain. The new determinations are in good agreement with the β-galactosidase activities obtained earlier (11, 51). Obviously, neither mutations in *glkA* nor those in *ccpA* lead to a complete loss of glucose repression of β-galactosidase activity.

Analysis of *lacPH* transcription in the wild type, C2a. To localize the transcriptional start site(s) of the *lac* genes and to analyze *lac* regulation at the transcriptional level, RNA was isolated from induced and noninduced *S. xylosus* cells and reverse transcription experiments were performed with *lacH*- and *lacP*-specific primers. As shown in Fig. 2A, reverse transcripts were obtained with a *lacP* primer. No transcriptional start site between *lacP* and *lacH* was detected. This result is consistent with *lacH* subcloning experiments, which indicated that *lacH* does not possess its own promoter (data not shown). Therefore, *lacP* and *lacH* are cotranscribed, forming a bicistronic operon.

The transcriptional start site of the *lacPH* promoter was found 46 bp upstream of the *lacP* start codon (Fig. 1). The

amount of reverse transcript obtained with RNA isolated from lactose-grown cells (Fig. 2A, lane 2) is much larger than that obtained with RNA from uninduced cultures (Fig. 2A, lane 1). The results of the primer extension experiments are in accordance with the lactose uptake and β-galactosidase activities measured under these conditions. They demonstrate that initiation of *lacPH* transcription is regulated in a lactose-dependent manner. Induction of *lacPH* transcription is dependent on LacR, since no *lacPH* transcript was detectable in the *lacR* mutant strain TX258 (data not shown).

Primer extension experiments with RNA isolated from cells that were grown with lactose and glucose yielded a less intense band than those with RNA from lactose-induced cells (Fig. 2A, lane 3). Therefore, glucose prevents efficient initiation of transcription at the *lacPH* promoter.

Analysis of *lacPH* transcription in catabolite repression mutants. A palindromic sequence that could serve as an operator for the catabolite control protein CcpA is located in the *lacPH* promoter region (Fig. 1) (11, 20, 24). It resembles catabolite responsive elements (*cres*) (23), which have been found to be essential for CcpA-mediated catabolite repression in a number of AT-rich gram-positive bacteria and which constitute the binding sites for CcpA. It was therefore of interest to determine the consequences of *ccpA* inactivation on glucose-mediated repression of *lacPH* transcription.

As shown in Fig. 2B, lactose induced *lacPH* transcription initiation in the *ccpA* mutant as it did in the wild type. The reverse transcript obtained from RNA prepared from glucose-grown cells (Fig. 2B, lane 3) had nearly the same intensity as the band from induced cells (Fig. 2B, lane 2). Therefore, glucose repression of *lacPH* transcription initiation is mainly, but not exclusively, due to the action of CcpA.

The same primer extension experiments were performed with RNA isolated from the glucose kinase mutant, TX140. Inducibility of *lacPH* transcription by lactose was the same as that in the other tested strains (data not shown). In contrast to the reduced repression in the *ccpA* mutant, the primer extension product from glucose-repressed TX140 cells had about the same intensity as that from the wild type grown under the same conditions (data not shown). Apparently, glucose-mediated regulation of transcription initiation at the *lacPH* promoter is not significantly altered by the *glkA* mutation.

Transcriptional analysis of *lacR*. To determine the transcriptional start site of *lacR*, the same RNAs as those for the *lacPH* analysis were used in reverse transcription experiments with a *lacR*-specific primer. As shown in Fig. 3, a strong reverse transcript was observed, localizing the site of initiation 35 bp from the *lacR* start codon (Fig. 1). The bands were equally strong with RNAs from cells grown in the presence or absence of lactose. Therefore, initiation at this site is not inducible by lactose. However, a smaller, less intense primer extension product appeared when RNA from lactose-grown cells was used (Fig. 3, lane 2). Transcription at this second *lacR* promoter is initiated 22 bp upstream of the *lacR* start codon (Fig. 1). Therefore, *lacR* is transcribed from two promoters which differ in strength and inducibility. Transcription initiated at the major promoter, P1, occurs independently from lactose in the growth medium, whereas initiation at the minor promoter, P2, relies on lactose for induction. Accordingly, primer extension experiments in the *lacR* mutant, TX258, showed that the major transcript is present, whereas the minor one could not be detected (data not shown). Therefore, transcription starting from P2 requires a functional LacR activator.

Glucose repression of *lacR* expression would be a conceivable mechanism to prevent induction of the *lacPH* operon. Therefore, *lacR* transcription in the presence of glucose was studied. As shown in Fig. 3, lane 3, transcription at P1 is hardly affected by glucose. Initiation at this promoter is constitutive with respect to the carbon source in the medium. On the other hand, the minor transcript from P2 is subject to glucose repression. No P2-specific reverse transcript was obtained with RNA from cells grown in the presence of lactose and glucose (Fig. 3, lane 3).

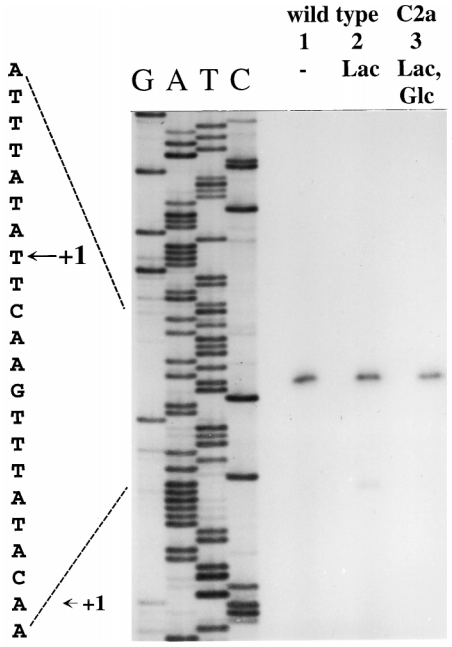

FIG. 3. Primer extension analysis of *lacR* transcription in the *S. xylosus* wild type, C2a. RNA was isolated from wild-type cells grown in B medium without additional sugar (lane 1), with lactose (lane 2), or with lactose and glucose (lane 3). Fifteen micrograms of RNA and a ^{32}P-labeled *lacR*-specific primer were used in the primer extension reactions. One-fourth of the reaction mixtures were separated on 5% polyacrylamide–urea gels next to a sequencing reaction mixture obtained with the same primer. The autoradiograph and the sequence interpretation around the +1 sites are shown.

DISCUSSION

The lactose utilization system of *S. xylosus* is comprised of a lactose permease of the GPH family of sugar transporters (37), a β-galactosidase, which belongs to family 2 of glycosyl hydrolases (21), and a regulator of the AraC/XylS group of proteins (12). The system clearly differs from that found in *Staphylococcus aureus*, where lactose uptake is mediated by a phosphoenolpyruvate-dependent phosphotransferase system and internalized lactose-phosphate is cleaved by a phospho-β-galactosidase (4, 5, 35). In *S. xylosus*, β-galactosidase produces glucose and galactose from incoming lactose. Therefore, utilization of lactose depends on the phosphorylation of glucose and galactose by respective kinases. A gene encoding glucose kinase has been characterized in *S. xylosus* (51), and a genomic fragment that complemented an *E. coli* galactokinase mutant has been identified but not further characterized (8). It appears that both kinases needed for lactose utilization are present in *S. xylosus.*

The *lacPH* genes of *S. xylosus* are positively controlled by LacR, which constitutes, to our knowledge, the first example of a member of the AraC/XylS family regulating *lac* genes. In *Staphylococcus aureus*, *Lactococcus lactis*, and *Streptococcus mutans*, these genes are negatively controlled by repressors

with similarity to DeoR of *E. coli* (9, 36, 38, 48, 49), whereas *lac* regulation in *Lactobacillus casei* is achieved by antitermination (1, 14, 40). Two other sugar catabolic operons in gram-positive bacteria appear to be controlled by AraC/XylS-type proteins, the multiple-sugar metabolism (*msm*) gene cluster in *Streptococcus mutans* (30, 39) and the raffinose utilization genes in *Pediococcus pentosaceus* (L32093).

The putative binding site for LacR in the *lacPH* promoter region could not be identified by sequence inspection. No sequences resembling operators for other AraC/XylS members could be detected (29, 34, 47, 53). In addition, no extended direct or inverted repeats are present in this area.

Apart from lactose-specific, LacR-mediated control, *lacPH* transcription is subject to CcpA-dependent carbon catabolite repression. The *cre*-like palindrome located from +7 to +20 with respect to the *lacPH* promoter is most likely the target for CcpA.

In the *ccpA* mutant strain, glucose still reduces β-galactosidase activity about fourfold (Table 3). Expression of *lacH* under control of a constitutive promoter showed that the activity of the enzyme is not affected by the carbon source in the medium (data not shown). Likewise, the β-galactosidase assay was not sensitive to the addition of glucose (data not shown). Therefore, the observed reduction of β-galactosidase activity is due to diminished *lacPH* expression.

It is well documented that glucose in the growth medium can reduce internal inducer concentrations by processes termed inducer exclusion and expulsion (41). Therefore, this regulatory mode should affect the activity of LacR in *S. xylosus*, resulting in less efficient initiation of transcription at the *lacPH* promoter. The comparable intensities of the reverse transcripts in the *ccpA* mutant strain (Fig. 2B, lanes 2 and 3) strongly indicate that inducer exclusion or expulsion plays a minor role, if any, in the CcpA-independent regulation of *lacPH* expression.

Additional evidence for *lacPH* expression control, which is not operating at the initiation of transcription, is provided by the β-galactosidase assays and *lacPH* primer extension analysis with the wild type. The β-galactosidase activity in glucose-repressed cells was lower than that in uninduced cells (Table 3). The opposite was true for intensities of the respective reverse transcripts, however (Fig. 2A).

Interestingly, this alternative level of glucose control seems to depend on a functional glucose kinase. In the glucose kinase mutant strain, initiation of transcription at the *lacPH* promoter is not altered but β-galactosidase activity in the presence of glucose is higher than in the wild type (Table 3). Premature termination of transcription, mRNA stability, or even posttranscriptional events could perhaps be affected by the presence of glucose and a functional glucose kinase. Further detailed analyses are needed to elucidate the role of glucose kinase in regulation. It appears that the *lac* genes constitute a good model system to study CcpA-dependent as well as CcpA-independent glucose repression in *S. xylosus.*

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