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Glucose Kinase-Dependent Catabolite Repression in *Staphylococcus xylosus*

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By transposon Tn*917* mutagenesis, 16 mutants of *Staphylococcus xylosus* were isolated that showed higher levels of β-galactosidase activity in the presence of glucose than the wild-type strain. The transposons were found to reside in three adjacent locations in the genome of *S. xylosus*. The nucleotide sequence of the chromosomal fragment affected by the Tn*917* insertions yielded an open reading frame encoding a protein with a size of 328 amino acids with a high level of similarity to glucose kinase from *Streptomyces coelicolor*. Weaker similarity was also found to bacterial fructokinases and xylose repressors of gram-positive bacteria. The gene was designated *glkA*. Immediately downstream of *glkA*, two open reading frames were present whose deduced gene products showed no obvious similarity to known proteins. Measurements of catabolic enzyme activities in the mutant strains grown in the presence or absence of sugars established the pleiotropic nature of the mutations. Besides β-galactosidase activity, which had been used to detect the mutants, six other tested enzymes were partially relieved from repression by glucose. Reduction of fructose-mediated catabolite repression was observed for some of the enzyme activities. Glucose transport and ATP-dependent phosphorylation of HPr, the phosphocarrier of the phosphoenolpyruvate:carbohydrate phosphotransferase system involved in catabolite repression in gram-positive bacteria, were not affected. The cloned *glkA* gene fully restored catabolite repression in the mutant strains in *trans*. Loss of GlkA function is thus responsible for the partial relief from catabolite repression. Glucose kinase activity in the mutants reached about 75% of the wild-type level, indicating the presence of another enzyme in *S. xylosus*. However, the cloned gene complemented an *Escherichia coli* strain deficient in glucose kinase. Therefore, the *glkA* gene encodes a glucose kinase that participates in catabolite repression in *S. xylosus*.

The presence of a rapidly metabolizable carbon source, especially glucose, results in a coordinated change of metabolic functions in many bacteria. Regulation is achieved by altering the activity of a number of proteins, which then leads to the differential expression of operons encoding metabolic enzymes, a process termed carbon catabolite repression (CR). In *Escherichia coli* (for reviews, see references 47–49), the regulatory cascade starts with the glucose-specific EIIA protein ($EIIA^{Glc}$) of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) as the allosteric effector. Nonphosphorylated $EIIA^{Glc}$ prevents the uptake of alternative carbon sources, either indirectly by inhibition of a catabolic enzyme (glycerol kinase) or directly by inhibition of sugar permeases (e.g., lactose and melibiose permease), a phenomenon designated inducer exclusion. In both cases, sugar-specific induction of the respective catabolic genes is hindered. Phosphorylated $EIIA^{Glc}$, however, activates adenylate cyclase, the cyclic AMP (cAMP)-generating enzyme. An enhanced level of cAMP serves as the signal for a global alteration of gene expression, which is finally exerted by the catabolite gene activator protein, also called cAMP receptor protein.

In gram-positive bacteria with low GC content, one central regulatory pathway also depends on a PTS component, the phosphocarrier protein HPr (42, 43). HPr, phosphorylated at a serine residue by an ATP-dependent, metabolite-activated protein kinase (13), allosterically controls sugar permeases in *Lactobacillus brevis* (64, 66) and a sugar-phosphate phosphatase of *Lactococcus lactis* (65), which results in inducer exclusion and inducer expulsion, the rapid efflux of preaccumulated sugars or sugar metabolites (41). HPr(serine-phosphate) [HPr(Ser-P)] also interacts with a pleiotropic regulator (11), the catabolite control protein CcpA, first recognized in *Bacillus subtilis* (23). In this organism, CcpA negatively controls a number of genes or operons, whose expression is subject to catabolite repression (12, 25). In addition to HPr(Ser-P), another phosphorylated form of HPr, HPr(histidine-P), generated by the PTS protein enzyme I, regulates non-PTS enzymes by phosphorylation. The glycerol kinase from *Enterococcus faecalis* is activated by this phosphorylation (14), whereas the activity of the lactose transport protein of *Streptococcus thermophilus* is inhibited (39). At least in *B. subtilis*, there are several genes that are regulated by CR independently of the HPr(Ser-P)-CcpA control system (12, 18).

In the high-GC gram-positive genus *Streptomyces*, HPr(Ser-P)- or cAMP-dependent CR has not been demonstrated so far (59). A gene encoding a glucose kinase has been implicated in CR of agarase and glycerol kinase in *Streptomyces coelicolor* (1, 2, 29). In glucose kinase mutants of *S. coelicolor*, transcription of the agarase gene is no longer subject to glucose repression (1), but details about molecular mechanisms of the signal transduction are missing.

We were interested in carbohydrate-specific regulation of gene expression in the low-GC gram-positive bacterium *Staphylococcus xylosus* (53). We chose *S. xylosus*, since it is applied in meat fermentation processes (22) and can therefore serve as a nonpathogenic host to study staphylococcal gene expression. The organism may also be of considerable biotechnological

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interest, since it is able to secrete peptides and proteins into the culture medium but produces very little extracellular protease. To start with the analysis of gene regulation in *S. xylosus*, we had cloned genes for sucrose and maltose utilization (8, 15, 63). The latter is especially subject to catabolite repression, but no further molecular details have been worked out. Although substantial biochemical data concerning CR have been accumulated for a number of low-GC gram-positive organisms, little information is available about the genes involved in CR, except for the *ccpA* genes from *B. subtilis* and *Bacillus megaterium* (23, 27). Therefore, we sought to isolate mutants of *S. xylosus* altered in CR by transposon mutagenesis. In this study, we report on the characterization of *S. xylosus* CR mutants harboring transposons in a gene with similarity to glucose kinase of *S. coelicolor.*

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. xylosus* C2a, a derivative of *S. xylosus* DSM20267 (53) that had been cured of its endogenous plasmid, pSX267 (21), was used for the isolation of chromosomal DNA. Subcloning for sequencing was performed in *E. coli* TG1 [*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)]. Heterologous *glkA* expression was measured in *E. coli* ZSC112 (*ptsG pstM glk* [9]). The shuttle plasmid pRB473, a derivative of pRB373 (6) conferring chloramphenicol resistance, served as the vector for gram-positive hosts. The transposon Tn*917*, along with surrounding chromosomal *S. xylosus* DNA, was cloned in *Staphylococcus carnosus* (20). The partial gene library of *S. xylosus* was constructed in pBR322 (57). For construction of nested deletions, the phagemid pBluescript (Stratagene) was used. Plasmid pTV1Ts (67) was applied for transposon delivery in *S. xylosus.*

Transposon mutagenesis. The plasmid pTV1Ts was chosen for transposon delivery. The plasmid contains the transposon *Tn*917 and shows temperature-sensitive replication in gram-positive hosts (67). A culture of *S. xylosus* cells harboring pTV1Ts was grown at 41°C for about 15 generations in B medium containing 2.5 μg of erythromycin per ml and 1% glucose. Dilutions were plated onto 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%). After incubation at 37°C for about 36 h, 16 blue clones were detected among about 7,000 colonies. The mutant colonies were all chloramphenicol sensitive, resulting from plasmid curing at high temperatures. Thus, they acquired the Tn*917* in their genome.

Growth media, DNA manipulations, and transformation. DNA manipulations, plasmid DNA isolation, Southern blot analysis, transformation of *E. coli*, and preparation of media and agar plates for bacterial growth were performed according to standard procedures (50). Chromosomal DNA from *S. xylosus* was isolated by the method of Marmur (32). Plasmids were introduced into *S. xylosus* and *S. carnosus* by protoplast transformation (20). DNA sequencing was done by the dideoxy chain termination method (51). Analytical PCR (35) analysis was performed with *Taq* polymerase (Boehringer Mannheim GmbH); PCR for cloning was carried out with Vent polymerase (New England Biolabs). *S. xylosus* was grown in B medium, which consisted of 1% peptone (no. 140; Gibco BRL), 0.5% yeast extract, 0.5% NaCl, and 0.1% K_2HPO_4. To test catabolite repression, 1% of each of the respective sugars was added. Fermentation of carbohydrates by *S. xylosus* was monitored on agar plates (34) containing 0.5% of the respective sugar.

Primer extension analysis. To determine the transcriptional start point of *glkA*, the wild-type strain was grown under the same conditions as the cultures to measure enzyme activities. Total RNA was isolated as described previously (56), and primer extension experiments (63) were performed with a synthetic oligonucleotide covering nucleotide positions 1003 to 1026 (see Fig. 2).

Determination of enzyme activities in cell extracts. To assay enzymatic activities in *S. xylosus*, cultures were grown in B medium with 1% carbohydrate, if required, to an optical density at 578 nm (OD_{578}) of 1.5. *E. coli* strains were grown in Luria-Bertani medium to an OD_{578} of 1.5. Crude extracts were prepared by repeated vortexing of the cells with glass beads as described previously (8). The enzyme assays contained 50 to 150 μg of cellular protein. Determination of the activities of gluconate kinase (36), mannitol-1-phosphate dehydrogenase (37), glucitol dehydrogenase (37), glucose kinase (54), and α-glucosidase (11) was done according to published procedures. The activity of β-glucuronidase was measured according to the α-glucosidase protocol (11) with *p*-nitrophenyl-β-*d*-glucuronide as the substrate. Since it turned out that the β-galactosidase activity was readily lost in crude extracts, the enzyme was measured in toluenized cells by the method of Miller (33).

The ability of HPr kinase to phosphorylate HPr in vitro was tested as described by Deutscher and Engelmann (10) with HPr from *B. subtilis*. Five micrograms of purified HPr was incubated with 40 μg of *S. xylosus* extracts in the presence of ATP and fructose-1,6-bisphosphate for 30 min at 37°C. To identify HPr kinase-dependent phosphorylation of HPr unambiguously, the doubly phosphorylated HPr had to be produced, because HPr phosphorylated at His-15 by enzyme I or at Ser-46 by HPr kinase cannot be separated on gels. Therefore, phosphoenolpyruvate was added, and the mixture was incubated for 5 min at 37°C. The different forms of HPr were separated on nondenaturing 15% polyacrylamide gels. The amount of doubly phosphorylated HPr was taken as a measure of HPr kinase activity.

Protein concentrations in cell extracts were determined by the method of Bradford (5).

Measurements of glucose uptake. Transport of glucose was measured according to the method of Romano et al. (44) with whole cells grown in B medium supplemented with 1% glucose. Cells were harvested at an OD of 1.5, washed in 0.1 M Tris-HCl (pH 7.4) buffer, and resuspended in the same buffer to a final density of 10^8 cells per ml. After addition of 0.2 mM [^{14}C]glucose (2.5 μCi/μmol) to 0.6 ml of prewarmed cells, the cells were incubated at 25°C, and 0.15-ml samples were taken at intervals, collected on membrane filters (pore size, 0.45 μm), and washed with 10 ml of 0.9% NaCl. Filters were dried at 37°C, and the radioactivity was determined by liquid scintillation counting. Uptake rates are expressed in nanomoles of glucose per minute per milligram of cellular protein.

Nucleotide sequence accession number. The nucleotide sequence is stored in the EMBL database under accession number X84332.

RESULTS

Isolation of *S. xylosus* mutants with alterations in CR. For the isolation of *S. xylosus* mutants with alterations in CR, expression of a β-galactosidase gene was chosen as the screening system. The activity of the enzyme is easily visualized on B medium agar plates containing X-Gal (100 μg/ml). After 24 h of incubation at 37°C, *S. xylosus* colonies appear blue, but they stay white for at least 48 h if glucose (1%) is added. Thus, it seemed feasible to generate CR mutants and detect them as blue colonies in the presence of glucose. Since CR in low-GC gram-positive bacteria appeared to be mediated by negative control (25), transposon mutagenesis was applicable. By using transposon Tn*917*, 16 blue colonies were isolated from agar plates that contained erythromycin (2.5 μg/ml), X-Gal (100 μg/ml), and glucose (1%). The colonies were indistinguishable from wild-type cells on media containing X-Gal but no glucose. They also showed the same color as the wild-type strain on glucose fermentation test plates, indicating that no obvious defect in glucose utilization occurred. Consequently, the mutant colonies, like the wild type, did not grow in the presence of deoxyglucose (10 mM). Additionally, the mutants and the wild type showed the same growth rates in liquid cultures with B medium supplemented with glucose. The mutant phenotype, glucose-resistant β-galactosidase expression (Grb), was stably maintained after repeated streaking of the cells. The mutants were designated *S. xylosus* TX1 to TX16 according to the corresponding mutations *grb-1*::Tn*917* to *grb-16*::Tn*917*. Their chromosomal DNAs were isolated and subjected to molecular analysis.

Molecular characterization of the mutants. To localize the transposon in the genome of the mutants, Southern blot analysis was performed with Tn*917* DNA as a probe. Hybridization patterns obtained with several restriction enzymes indicated that 14 of the 16 mutants were identical. The transposon, along with surrounding DNA of *S. xylosus* TX14 (*grb-14*::Tn*917*), a representative of the mutants with identical transposon insertions, was cloned on an 8.2-kb *Eco*RI-*Pst*I fragment in the shuttle vector pRB473 by directly selecting erythromycin resistance specified by Tn*917* in *S. carnosus*. The chromosomal DNAs around the Tn*917* insertion sites of *S. xylosus* TX15 (*grb-15*::Tn*917*) and TX16 (*grb-16*::Tn*917*) were cloned by the same approach with *Eco*RI-*Pst*I (TX15) and *Eco*RI (TX16). With the cloned genomic DNAs adjacent to Tn*917* as hybridization probes, we found that the transposon integrated within about 1 kb of the same chromosomal region in all mutants (Fig. 1). In *S. xylosus* TX15, Tn*917* is found about 0.4 kb apart from the insertion site in TX14, and the distance between *Tn*917 in TX14 and TX16 was 0.6 kb. In addition, a deletion of

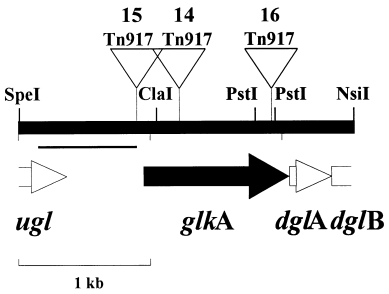

FIG. 1. Physical map of the *grb* region and location of the transposons. The sizes and orientations of the ORFs in the *grb* region and the locations of the transposons were deduced from the nucleotide sequence. The numbers above the transposons refer to the *S. xylosus* Grb mutant strains TX14, TX15, and TX16. The bar spanning the intergenic region between *ugl* and *glkA* indicates the deletion that occurred in strain TX15. The ORFs with unknown function (*ugl*, *dglA*, and *dglB*) were designated according to their positions relative to the glucose kinase gene (*glkA*).

about 0.8 kb occurred in TX15. Therefore, it seemed that only one genetic locus was inactivated in the Grb mutants, and it was sufficient to clone and analyze the *S. xylosus* wild-type region spanning all three transposon insertion sites (Fig. 1).

Nucleotide sequence of the *grb* region. To clone the wild-type *grb* region, a partial library of *S. xylosus* DNA enriched for *Eco*RI fragments of about 5 to 8 kb was constructed in *E. coli* with plasmid pBR322. The *grb* fragment was identified in pools of plasmids by Southern blot hybridization with genomic DNA next to the *grb-14* insertion as a probe. Plasmids which hybridized contained a 6.3-kb *Eco*RI fragment. By restriction analysis of one representative plasmid, pGRB141, a 2.5-kb *Spe*I-*Nsi*I fragment (Fig. 1) was found spanning the transposon insertion sites in the Grb mutants. In the nucleotide sequence of the fragment (Fig. 2), four open reading frames (ORFs) were found on one strand. The first ORF is truncated at its 5′ end, and the fourth was truncated at the 3′ end. ORF2, the longest ORF on the fragment, encodes a protein with a size of 328 amino acids (aa) and with a calculated molecular mass of 35 kDa, and ORF3 encodes a polypeptide with a size of 107 aa (12 kDa), if one counts from the ATG codons at nucleotide positions 973 and 1959, respectively (Fig. 2). Both start codons are preceded by potential Shine-Dalgarno (SD) sequences (55), although complementarity to the 16S rRNA of the ORF2 SD sequence seems to be relatively weak for genes of gram-positive origin (61). Another potential SD sequence is found in front of the start codon of the truncated ORF4 (Fig. 2).

Similarity searches in databases yielded the fact that the gene product of ORF2 resembled glucose kinase of *S. coelicolor* (2); fructokinases of *Zymomonas mobilis* (68), *Pediococcus pentosaceus* (GenBank, L32093), and *Streptococcus mutans* (52); NagC (38); three ORFs of unidentified functions from *E. coli* (4, 40 [GenBank, U18997]); an ORF close to the gene encoding a hyaluronidase of *Clostridium perfringens* (GenBank, M81878); and several xylose repressors from gram-positive bacteria (28, 30, 46, 56 [GenBank, L18965]). Comparison of the proteins individually suggested that ORF2 encodes a sugar kinase rather than a transcriptional repressor. The size of the deduced ORF2 protein (328 aa) is similar to that of the kinases ranging from 293 to 317 aa, and identical residues are found throughout the whole protein. Additionally, a stretch of about 80 aa found in NagC and the xylose repressors that contains a helix-turn-helix DNA binding motif is lacking in the ORF2 protein as well as in the kinases. The ORF2 protein shares 33% identical residues with glucose kinase of *S. coelicolor*, 29% identical residues with the fructokinases, and about 25% identical residues with the xylose repressors, if the 80-aa stretch mentioned above is not considered. The degrees of identity of the ORFs of unknown function from *E. coli* and *C. perfringens* varied between 25 and 28%. Therefore, ORF2 was designated *glkA*. No significant similarities were found for the other ORFs encoded on the sequenced fragment. They were given provisional names according to their position relative to *glkA* (Fig. 2), *ugl* (upstream of glucose kinase), *dglA*, and *dglB*, respectively.

The exact location of Tn*917* in the mutants was determined by DNA sequencing with transposon-specific primers. In TX14 and TX16, Tn*917* integrated in the *glkA* reading frame (Fig. 2), but it is found upstream of *glkA* in TX15. A deletion of 783 bp occurred in TX15, removing most of the intergenic region between *glkA* and *ugl* and part of *ugl*. Therefore, the *grb-14* and *grb-16* mutations should result in the production of truncated GlkA proteins, whereas the *grb-15* mutation should most likely affect *glkA* expression.

Activity of catabolic enzymes in the wild-type and Grb mutant strains. Mutations in genes participating in CR should affect several bacterial functions concomitantly. In order to determine whether the Tn*917* integration into *glkA* resulted in a pleiotropic phenotype, a number of enzymatic activities were

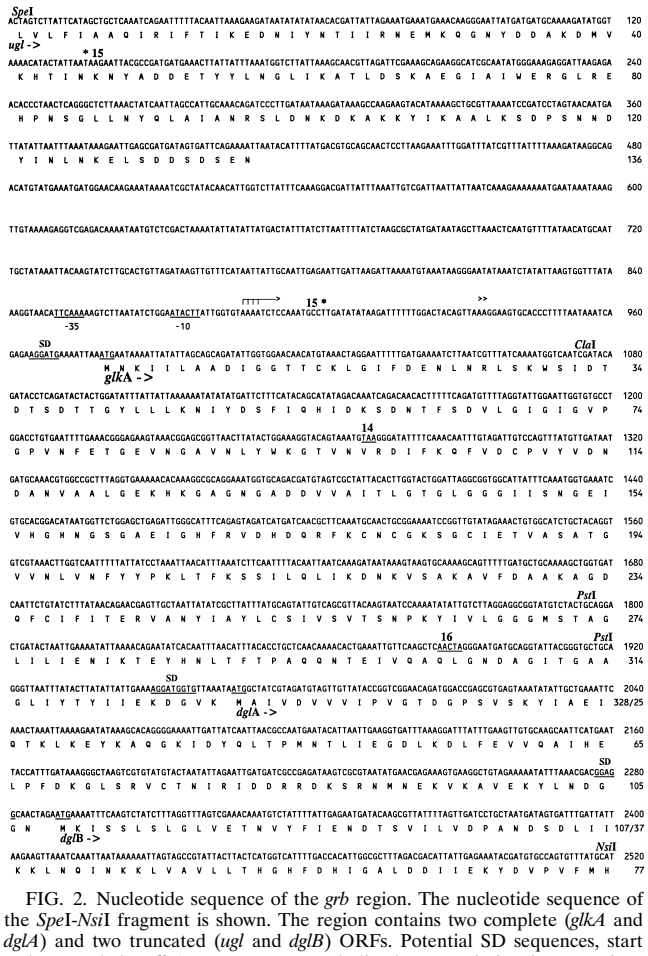

FIG. 2. Nucleotide sequence of the *grb* region. The nucleotide sequence of the *Spe*I-*Nsi*I fragment is shown. The region contains two complete (*glkA* and *dglA*) and two truncated (*ugl* and *dglB*) ORFs. Potential SD sequences, start codons, and the *glkA* promoter are underlined. Transcriptional start points, deduced from primer extension experiments, are indicated by arrows. The nucleotides which were duplicated by the transposon Tn*917* insertion in the Grb mutant strains *S. xylosus* TX14 and TX16 are underlined and marked by the respective numbers. The extent of the deletion found in strain TX15 is indicated by asterisks.

TABLE 1. Catabolic enzyme activities in the *S. xylosus* wild type, C2a, and the Grb mutant strains TX14, TX15, and TX16

Enzyme	Growth conditions[a]	Enzyme activity[b] in strain			
		C2a	TX14	TX15	TX16
β-Galactosidase	B + lactose	153.8 ± 17.3	148.3 ± 19.8	157.2 ± 33.6	134.5 ± 27.7
	B + lactose + glucose	23.3 ± 5.9	75.1 ± 9.3	86.2 ± 12.3	67.3 ± 6.9
	B + lactose + fructose	45.2 ± 8.9	92.7 ± 11.7	101.4 ± 9.8	86.2 ± 13.7
	B + lactose + mannitol	109.8 ± 12.1	134.8 ± 15.8	125.1 ± 14.3	117.9 ± 12.3
α-Glucosidase	B	11.9 ± 2.7	15.8 ± 2.4	12.3 ± 3.5	10.8 ± 1.9
	B + glucose	1.5 ± 1.0	4.4 ± 1.1	5.1 ± 1.7	3.8 ± 2.1
	B + fructose	7.5 ± 1.4	5.7 ± 2.1	5.2 ± 2.0	6.0 ± 1.7
	B + mannitol	8.4 ± 1.3	9.9 ± 1.2	10.2 ± 1.2	8.9 ± 2.4
β-Glucuronidase	B	22.5 ± 4.7	24.9 ± 2.4	21.3 ± 5.7	20.8 ± 3.9
	B + glucose	5.5 ± 1.1	9.8 ± 1.2	10.1 ± 3.2	8.3 ± 4.2
	B + fructose	7.4 ± 1.3	12.5 ± 2.1	13.8 ± 5.1	10.2 ± 2.7
	B + mannitol	13.0 ± 2.1	17.9 ± 1.8	18.4 ± 3.1	16.9 ± 3.9
Gluconate kinase	B + gluconate	141.7 ± 30.1	164.6 ± 34.0	148.4 ± 18.1	137.5 ± 35.1
	B + gluconate + glucose	44.0 ± 16.2	131.1 ± 14.3	115.2 ± 16.2	103.2 ± 24.8
	B + gluconate + fructose	65.3 ± 6.1	137.6 ± 16.9	119.2 ± 16.7	119.6 ± 23.3
	B + gluconate + mannitol	138.9 ± 20.4	ND[c]	ND	ND
Mannitol-1-P dehydrogenase	B + mannitol	71.3 ± 5.4	28.4 ± 12.3	19.5 ± 7.8	31.1 ± 12.9
	B + mannitol + glucose	<1	1.4 ± 2.0	0.8 ± 1.1	2.1 ± 0.1
Glucitol dehydrogenase	B + glucitol	8.6 ± 1.9	10.0 ± 1.1	8.8 ± 2.6	8.2 ± 2.3
	B + glucitol + glucose	3.6 ± 0.9	8.9 ± 0.3	8.9 ± 2.7	7.6 ± 0.6

[a] The strains were grown in B medium (B) containing 1% of the indicated sugar(s) or without additional carbohydrate to an OD_{578} of 1.5.
[b] The assays were performed with aliquots of extracts prepared from 40 ml of cells. Mean values were taken from three measurements, each with bacterial extracts from two cultures. Enzyme activity is expressed as follows: β-galactosidase, Miller units (33); α-glucosidase and β-glucuronidase, nanomoles of nitrophenol per minute per milligram of protein; gluconate kinase, nanomoles of NADPH per minute per milligram of protein; mannitol-1-P dehydrogenase and glucitol dehydrogenase, nanomoles of NADH per minute per milligram of protein.
[c] ND, not determined.

measured in the *S. xylosus* wild type and in the Grb mutant strains. Under nonrepressed conditions, cells were grown in complex B medium supplemented with 1% of each of the sugars which induced expression of the respective enzymes. Since no inducing substrates are known for β-glucuronidase and α-glucosidase expression, these activities were determined in cultures without additional carbohydrate. To measure catabolite repression, 1% glucose, fructose, or mannitol was added to the cultures. Complex growth media were used, since no minimal medium is available for *S. xylosus*. As summarized in Table 1, all catabolic enzymes tested exhibited sensitivity to catabolite repression in the *S. xylosus* wild type. The repressive effect of glucose varied from over 70-fold for mannitol-1-P dehydrogenase to only 2-fold for glucitol dehydrogenase. The other activities showed intermediate repression factors ranging from eightfold (α-glucosidase) to threefold (gluconate kinase). Inhibition caused by fructose in the growth medium was found to be threefold for glucuronidase and about twofold for the other enzymes that were tested. Reduction of enzymatic activities in mannitol-grown cells was generally less than twofold. No repressive effect of mannitol was found on gluconate kinase.

The same measurements were also performed for the Grb mutants. A general relief from catabolite repression by glucose was observed in all three Grb strains (Table 1). The enzyme activities determined in the individual mutants did not differ significantly among each other. Therefore, their enzyme activities are considered as the result of one mutational event and will be discussed jointly rather than individually.

In the mutants, glucitol dehydrogenase was no longer subject to glucose repression and gluconate kinase repression was virtually lost, but residual reduction of β-galactosidase, β-glucuronidase, and α-glucosidase levels was still detectable. Glucose in the growth medium reduced the activity of β-galactosidase in the mutants twofold and reduced that of α-glucosidase threefold, which is considerably less than the sixfold (β-galactosidase) and eightfold (α-glucosidase) repression observed in the wild-type strain. The effect of the *grb* mutation was weakest for β-glucuronidase, which showed only a minor relief from repression. Comparison of mannitol-1-P dehydrogenase values of wild-type and mutant strains was impossible, since the activity was below the detection level in glucose-grown wild-type cells. However, measurable levels in the mutants suggested that glucose repression of mannitol-1-P dehydrogenase was also reduced.

In contrast to the general reduction in glucose repression in the mutant strains, the *grb* mutation altered fructose-mediated regulation of some but not all enzymatic activities. Repression of β-galactosidase, gluconate kinase, and β-glucuronidase was about half as strong as that in the wild type, whereas α-glucosidase activity was not affected.

The effect of the *grb* mutation on mannitol-mediated regulation was less obvious, since the sugar repressed only weakly in the wild-type strain. It seemed that repression of β-galactosidase and β-glucuronidase is diminished in the mutants, but α-glucosidase was expressed at wild-type levels.

Glucose kinase activities in the wild type and in the Grb mutant strains. In the Grb mutants, transposon Tn*917* is located within (TX14 and TX16) or upstream (TX15) of the *glkA* gene, whose deduced gene product resembles glucose kinase of *S. coelicolor*. Therefore, glucose kinase activities in the wild type and in the mutant strains were compared. Cell extracts were prepared from cultures grown in B medium in the presence or absence of glucose. As summarized in Table 2, *S. xylosus* C2a possesses glucose kinase activity, which was at a slightly higher level in glucose-grown cells. Surprisingly, the mutant strains showed only a minor reduction in glucose kinase activity. They expressed about 75% of the wild-type level. The *glkA* gene is interrupted in TX14 and TX16. It seems unlikely that the truncated proteins that can be produced in

TABLE 2. Glucose kinase activities in the *S. xylosus* wild type, C2a, and the Grb mutant strains TX14, TX15, and TX16

Growth conditions[a]	Glucose kinase activity[b] (nmol of NADPH produced/min/mg of protein) in strain			
	C2a	TX14	TX15	TX16
B	423.4 ± 29.3	318.5 ± 43.1	344.2 ± 57.2	327.9 ± 32.1
B + glucose	507.8 ± 45.7	361.5 ± 37.1	367.3 ± 26.2	343.6 ± 27.9

[a] Strains were grown in B medium (B) without or with 1% glucose to an OD_{578} of 1.5.

[b] The assays were carried out with aliquots of extracts of 40 ml of cells. Mean values were taken from two measurements, each with cell extracts from two cultures.

these mutants mediate the high level of residual glucose kinase activity. Therefore, the glucose kinase activity found in the mutant strains should be due to another enzyme. Since the in vitro activity of enzymes depends on the assay conditions, the observed ratio between the different kinase activities may not exactly reflect their in vivo distribution. However, it is clear that *S. xylosus* has a strong glucose kinase distinct from GlkA. The amounts of glucose kinase produced by the three mutants were about the same, although the *glkA* ORF remained intact in TX15. Obviously, the transposon insertion inactivated the *glkA* promoter in this strain.

Since the GlkA protein weakly resembled fructokinases, the cell extracts prepared to measure glucose kinase activity were also tested for fructokinase. No difference was detectable between the wild type and the mutant strains (data not shown).

Glucose transport in the wild type and in the Grb mutant strains. Since the transposon was found within a chromosomal region encoding a protein most likely involved in glucose catabolism, it was conceivable that *glkA* inactivation indirectly affected glucose transport, which would then lead to the loss of catabolite repression. The wild type and the mutant strains were grown in B medium containing 1% glucose, and [^{14}C]glucose uptake was measured. The wild type and the mutant strains took up glucose at comparable rates. After 10 min, about 11.5 nmol of [^{14}C]glucose per mg of protein was incorporated in each strain. Thus, glucose transport was not affected by the *glkA* mutation.

HPr kinase activity in the wild type and in the Grb mutant strains. Since HPr(Ser-P) plays a central role in mediating CR in other gram-positive low-GC bacteria, the activity of HPr kinase or the expression of its gene would be a likely target to modulate the regulatory response according to carbon source availability. To test whether the *grb* mutation affected HPr kinase activity, cultures of the wild type and of the Grb mutant strains were grown in the presence of glucose, and cell extracts were assayed for HPr kinase with HPr purified from *B. subtilis*. As shown in Fig. 4, the doubly phosphorylated form of HPr is produced by crude extracts of *S. xylosus* in the presence of fructose-1,6-bisphosphate and ATP and subsequent phosphoenolpyruvate-dependent phosphorylation. This form of HPr is not detected in the absence of ATP and fructose-1,6-bisphosphate (data not shown). Therefore, *S. xylosus* has HPr kinase activity. If one compares the amounts of doubly phosphorylated HPr that are produced by the wild-type and mutant strains (Fig. 3), no obvious difference is detectable. Apparently, the *grb* mutation did not affect the in vitro activity of HPr kinase, indicating that comparable amounts of the enzyme are produced in the wild type and in the Grb mutant strains. However, a change in the activity of HPr kinase in vivo cannot be completely ruled out.

Complementation of the *grb* mutation by *glkA*. If one considers the slight loss of glucose kinase activity in the Grb mutant strains, it may appear that the CR resistance phenotype is not due to the inactivation of the *glkA* gene. The genomic organization of the *glkA* region (Fig. 2) suggests that the two genes downstream of *glkA*, *dglA* and *dglB*, could be transcriptionally coupled to *glkA*. Overlapping stop and start codons of *glkA* and *dglA* may even indicate additional translational coupling. One would predict that the transposon insertions in the mutant strains exert strong polar effects on the expression of the ORFs downstream of *glkA*. To test whether the phenotype of the Grb mutants was indeed caused by loss of GlkA function, a plasmid that expressed *glkA* without the downstream genes was constructed.

For that purpose, the shuttle vector pRB473 was used. In this plasmid, the multiple cloning site is flanked by transcriptional terminators (6). Expression of cloned genes is thus dependent on promoters that are on the inserted fragments (15). To test, whether the *glkA* gene has its own promoter, the transcriptional start point of *glkA* was determined by primer extension analysis. RNA was isolated from *S. xylosus* wild-type cells that were grown in B medium with glucose. A strong reverse transcript localizing the start point to a stretch of four A's from nucleotide position 881 to 884 (Fig. 2) was obtained (Fig. 4). A smaller, less-intense product was also detected. Practically the same amounts of these transcripts were found with RNA from cells grown without glucose (data not shown). It seems that *glkA* expression is not appreciably induced by glucose under our experimental conditions.

The localization of the *glkA* promoter to a region around nucleotide position 860 (Fig. 2) is perfectly consistent with the deletion that occurred in the TX15 mutant. In this strain, the region upstream of nucleotide position 897 (Fig. 2) up to the *ugl* reading frame is missing. The second apparent start site inferred from the minor reverse transcript would be located outside of the TX15 deletion. However, it seems possible that it was produced by degradation of the larger transcript.

To obtain *glkA* including its promoter but without the downstream genes, a DNA fragment was amplified by PCR (positions 608 to 1963 [Fig. 2]) and cloned by means of newly created restriction sites (*Sal*I and *Kpn*I) into pRB473. The resulting plasmid, pGRB144, was introduced into *S. xylosus* TX14, TX15, and TX16. To test whether the cloned *glkA* gene

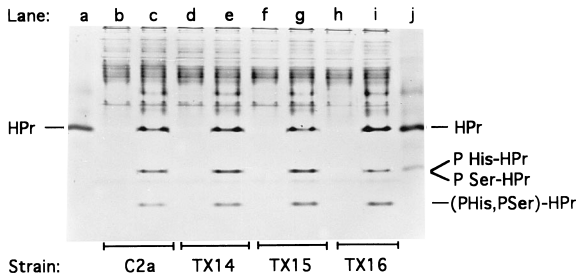

FIG. 3. HPr kinase activity in the *S. xylosus* wild type, C2a, and the Grb mutant strains TX14, TX15, and TX16. The different phosphorylated forms of HPr were separated on nondenaturing 15% polyacrylamide gels. The proteins were visualized by Coomassie blue staining. HPr kinase activity was assessed by comparison of the intensities of the doubly phosphorylated forms that appeared upon incubation of 5 μg of purified *B. subtilis* HPr with 40 μg of *S. xylosus* cell extracts. Lanes c, e, g, and i contained cell extracts from the *S. xylosus* wild type, C2a, and the Grb mutant strains TX14, TX15, and TX16 together with purified *B. subtilis* HPr. In lanes b, d, f, and h, the same cell extracts without HPr were loaded. Lane a contained 3 μg of purified *B. subtilis* HPr, and lane j contained 5 μg of HPr partially phosphorylated at His-15 by purified enzyme I of *B. subtilis*. The positions of the different HPr forms on the gel are indicated on the right.

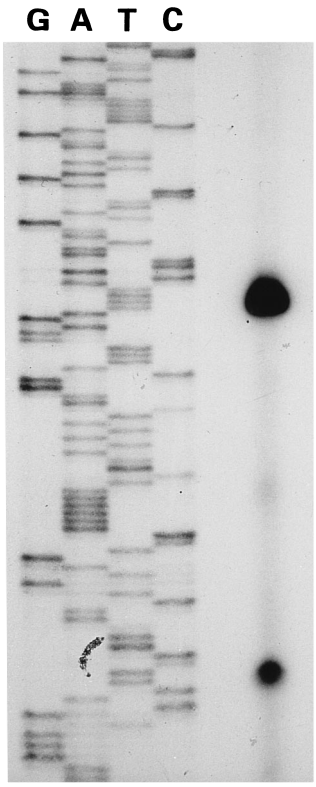

FIG. 4. Determination of the transcriptional start point of *glkA* by primer extension. An autoradiograph of a 6% polyacrylamide urea gel of reverse transcripts obtained with a ^{32}P-end-labeled *glkA*-specific primer and 10 μg of total RNA isolated from the *S. xylosus* wild type, C2a, grown in B medium with glucose (1%) is shown. Lanes G, A, T, and C contained the respective sequencing reaction mixtures with the same primer.

restored CR in the mutants, the transformants were first checked on X-Gal–glucose plates for β-galactosidase expression. The mutant strains harboring the *glkA* plasmid pGRB144 were indistinguishable from wild-type cells containing the shuttle vector (data not shown). Since all previous experiments with the mutant strains gave identical results, only the transformed TX14 strain as the representative of the Grb mutants was analyzed further. The activities of β-galactosidase, β-glucuronidase, and α-glucosidase were measured in cultures grown with or without glucose. As shown in Table 3, the *glkA* gene alone cloned on a plasmid and expressed from its own promoter could complement the *grb-14* mutation. Thus, the phenotype of the mutants is indeed a consequence of the loss of GlkA function. The levels of enzyme activity obtained during these measurements were slightly lower than those in the previous determinations. These differences may reflect the lower growth rate of the plasmid-containing strains. Nevertheless, the results clearly demonstrate that the cloned *glkA* gene completely restored CR in the mutant strain.

The *glkA* plasmid pGRB144 was also introduced into the wild type in order to test if this would lead to glucose kinase overproduction and, perhaps, altered CR. The level of glucose kinase activity in the pGRB144-containing strain was only about twofold higher than that in cells with the vector alone. Accordingly, β-galactosidase and α-glucosidase activities showed wild-type regulation (Table 3).

Complementation of an *E. coli glk* mutation by *glkA* of *S. xylosus*. The high level of residual glucose kinase activity found in crude extracts of the Grb mutant strains indicated that GlkA does not constitute the major glucose kinase in *S. xylosus*. However, it does contribute to the total glucose-phosphorylating activity in the organism and should be able to complement the glucose kinase mutation in *E. coli* ZSC112 (*ptsG ptsM glk*). In this strain, the presence of an active glucose kinase can be monitored on glucose MacConkey agar supplemented with fucose. Since the *E. coli glk* mutant is deficient in PTS-dependent glucose uptake, fucose must be added to the plates to induce galactose permease-mediated glucose transport. Under these conditions, colonies of the mutant strain appear white. With the *glkA* plasmid pGRB144, which complemented the regulatory defect of the Grb mutants, red colonies developed. The pGRB144-containing *E. coli* strain could also grow in minimal medium with glucose and fucose. It reached an OD_{578} of 2.3 after 24 h of incubation at 37°C, whereas ZSC112 harboring the shuttle vector pRB473 remained at an OD_{578} of 0.07, the initial OD of both cultures. In extracts of ZSC112 (pGRB144), glucose kinase activity was about 15-fold higher

TABLE 3. Catabolic enzyme activities in the Grb mutant strain TX14 harboring *glkA* cloned on plasmid

Enzyme	Growth conditions[a]	Plasmid	Enzyme activity[b] in *S. xylosus* strain	
			C2a	TX14(*grb-14*)
β-Galactosidase	B + lactose	pRB473	135.3 ± 17.2	141.3 ± 12.9
	B + lactose + glucose	pRB473	21.4 ± 3.7	74.3 ± 7.2
	B + lactose	pGRB144(*glkA*)	120.3 ± 12.1	129.5 ± 10.4
	B + lactose + glucose	pGRB144(*glkA*)	19.6 ± 6.7	25.3 ± 5.8
α-Glucosidase	B	pRB473	10.8 ± 2.2	11.3 ± 3.7
	B + glucose	pRB473	1.1 ± 0.9	5.2 ± 2.1
	B	pGRB144(*glkA*)	14.8 ± 4.2	12.4 ± 3.4
	B + glucose	pGRB144(*glkA*)	1.1 ± 0.9	1.9 ± 1.0
β-Glucuronidase	B	pRB473	17.3 ± 4.9	20.6 ± 5.3
	B + glucose	pBR473	3.8 ± 1.6	9.0 ± 2.8
	B	pGRB144(*glkA*)	ND[c]	19.5 ± 3.2
	B + glucose	pGRB144(*glkA*)	ND	2.7 ± 1.8

[a] The strains were grown in B medium (B) containing 20 μg of chloramphenicol, 1% of the indicated sugar(s), or no carbohydrate to an OD_{578} of 1.5.
[b] Assays were performed with aliquots of extracts prepared from 40 ml of cells. Mean values were taken from two measurements, each with cell extracts from two cultures. Enzyme activity is expressed as follows: β-galactosidase, Miller units (33); α-glucosidase and β-glucuronidase, nanomoles of nitrophenol per minute per milligram of protein.
[c] ND, not determined.

than in the strain without *glkA*. Thus, the *glkA* gene is expressed in *E. coli* and encodes a glucose kinase that functions as such in vivo.

The *E. coli glk* mutant strain also provided the opportunity to test whether the cloned *grb-14*::Tn*917*, *grb-15*::Tn*917*, and *grb-16*::Tn*917* regions would mediate residual glucose kinase activity. None of these plasmids containing truncated (TX14 and TX16) or promoterless (TX15) *glkA* genes complemented the *glk* mutation. Therefore, the glucose kinase activity found in the Grb mutant strains must be due to another enzyme.

DISCUSSION

Three transposon insertion mutants (TX14, TX15, and TX16) of *S. xylosus* showing Grb expression were characterized. Their phenotypes were identical, and the transposon integrated within or upstream of the same gene (*glkA*) in each strain, strongly indicating that the transposons caused the mutations. This could be confirmed by introducing a transposase-deficient derivative of the cloned *grb-14*::Tn*917* region of *S. xylosus* TX14 into the wild-type genome by homologous recombination. The resulting strain was phenotypically indistinguishable from the original Grb mutants (data not shown).

The *grb* (*glkA*) mutation is clearly pleiotropic, affecting glucose repression of all catabolic enzymes tested. However, relief of repression is not strictly glucose specific, since a weak but detectable reduction in fructose-mediated repression was observed for some enzymatic activities. With one exception (glucitol dehydrogenase), a residual repression persisted in the mutant strains. In conclusion, we consider the phenotype of the *grb* (*glkA*) mutation to be a partial loss of catabolite repression especially by glucose. Therefore, we will restrict the following discussion to glucose-mediated regulation.

The transposon insertions in the mutant strains identified a gene of *S. xylosus* encoding a glucose kinase that belongs to the recently recognized ROK protein family (58). This group of proteins consists of repressors, ORFs with unidentified functions, and sugar kinases. With respect to the work presented here, the most interesting member of the family is the glucose kinase of *S. coelicolor* (1, 2). Mutations in the corresponding gene (*glkA*) resulted in 2-deoxyglucose resistance, deficiency in glucose repression, and an inability to utilize glucose. A specific regulatory role has been proposed for the enzyme of *S. coelicolor*, since several observations suggested that glucose phosphorylation is not sufficient for glucose repression (1, 29). Suppressor mutants of a *glkA* deletion strain capable of glucose utilization were isolated. These showed virtually wild-type levels of glucose kinase activity. However, glucose repression remained defective in these strains (1). Moreover, introduction of a cloned glucose kinase gene from *Z. mobilis* into the *glkA* mutant restored growth on glucose but not glucose repression (1). Interestingly, the *Z. mobilis* glucose kinase does not belong to the ROK protein family (3). The *glkA* mutant strain also shows relief from repression by sugars other than glucose, corroborating the specific regulatory function of glucose kinase in *S. coelicolor* (29).

The situation in *S. coelicolor* artificially achieved by mutation or cloning resembles the wild-type conditions in *S. xylosus*. In the *glkA* mutant strain, 75% of the wild-type level of glucose kinase activity persists, glucose utilization is not detectably affected, and the cells remain 2-deoxyglucose sensitive. Obviously, GlkA constitutes only a minor glucose kinase of *S. xylosus*. However, the relatively subtle loss of glucose phosphorylation in the mutants markedly impairs glucose repression. It seems that the glucose kinase activity of a special enzyme, namely GlkA, is needed to sustain regulation in *S. xylosus*. This may indicate that the GlkA protein is able to exert a specific regulatory function that cannot be fulfilled by the other glucose kinase(s). It may even suggest that the catabolic activity of GlkA is dispensable for mediating regulation.

Such two distinct functions, a regulatory function and a catalytic function, residing in different protein domains have been proposed for hexokinase PII of the yeast *Saccharomyces cerevisiae*. The enzyme participates in glucose repression and constitutes the major glucose kinase activity in this organism (19, 60). Earlier evidence suggested that PII can mediate repression even without being capable of glucose phosphorylation (16). However, recent results correlated regulation with catalytic activity (31, 45). Interestingly, the enzyme has protein kinase activity in vitro (17, 24) and is phosphorylated in vivo (62). The biological significance of both findings remains to be elucidated.

Considering the results obtained in *S. cerevisiae*, *S. coelicolor*, and *S. xylosus*, one can conclude that phosphorylation of glucose by a special enzyme is needed to create a signal finally leading to catabolite repression. The nature of the signal, however, remains obscure.

In *S. xylosus*, the situation is further complicated by the PTS and the HPr(Ser-P)-dependent catabolite control pathway. We have shown that *S. xylosus* does possess HPr kinase activity (Fig. 4), and catabolite responsive elements, the likely targets for CcpA, were found in several *S. xylosus* genes by sequence comparison (15, 26) and functional analysis (26). Therefore, one would anticipate finding modulation of enzyme activities by HPr(Ser-P) and HPr(Ser-P)–CcpA-mediated regulation of gene expression in *S. xylosus*. When glucose enters the cells as glucose-6-phosphate by the PTS, it is difficult to imagine how glucose kinase would participate in regulation. Under these circumstances, HPr(Ser-P) and HPr kinase most likely come into play. The residual repression in the *glkA* mutant strains may be due to this control pathway. On the other hand, the high level of glucose kinase activity in *S. xylosus* suggests that glucose may enter the cell also by a non-PTS transport system or systems. Indeed, a *ptsI* mutant of *S. xylosus* constructed by gene replacement is hardly affected in glucose utilization, but it has a sucrose$^-$ and mannitol$^-$ phenotype (7). It is quite conceivable that two signalling pathways exist, depending on the route of glucose entry. The efficiency with which the PTS and the non-PTS transport systems take up glucose may strongly depend on growth conditions and sugar concentrations. The use of complex media and relatively high glucose concentrations in our mutagenesis procedure may have biased our screening towards *glkA*, encoding a central component of the non-PTS signalling pathway.

Several questions need to be answered by future research. First, it should be determined whether the observed phenotype of the *glkA* mutation is due to altered gene expression or enzyme activity. For this purpose, target genes, especially the β-galactosidase gene, should be cloned and their transcriptional regulation should be analyzed. Second, other genes encoding proteins involved in catabolite repression (e.g., the *ccpA* or the HPr kinase gene) should be cloned to establish dependence patterns and possible interactions of the gene products.

Although the mechanism by which glucose kinase creates a signal that triggers glucose repression in *S. xylosus* is far from being understood, the identification of *glkA* as a novel component in the regulatory network of a low-GC gram-positive bacterium may be of significance for other members within this group of organisms.

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