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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

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Catabolite repression (CR) of xylose utilization by *Bacillus subtilis* involves a 14-bp *cis*-acting element (CRE) located in the translated region of the gene encoding xylose isomerase (*xylA*). Mutations of CRE making it more similar to a previously proposed consensus element lead to increased CR exerted by glucose, fructose, and glycerol. Fusion of CRE to an unrelated, constitutive promoter confers CR to β-galactosidase expression directed by that promoter. This result demonstrates that CRE can function independently of sequence context and suggests that it is indeed a generally active *cis* element for CR. In contrast to the other carbon sources studied here, glucose leads to an additional repression of *xylA* expression, which is independent of CRE and is not found when CRE is fused to the unrelated promoter. This repression requires a functional *xylR* encoding Xyl repressor and is dependent on the concentrations of glucose and the inducer xylose in the culture broth. Potential mechanisms for this glucose-specific repression are discussed.

Many genes in *Bacillus subtilis* are transcriptionally controlled by catabolite repression (CR), in which the presence of a rapidly metabolizable carbon source inhibits expression of other genes mainly encoding carbon catabolic enzymes (for reviews, see references 2, 5, and 28). A cyclic AMP-cyclic AMP receptor protein-mediated mechanism mediating CR as observed for *Escherichia coli* is not likely to be functional in *B. subtilis* since (i) cyclic AMP can be detected only in low amounts under oxygen stress conditions, and its concentration does not vary with the strength of catabolite repression (18), (ii) cyclic AMP receptor protein has not been detected in gram-positive bacteria so far, and (iii) CR in *B. subtilis* involves negative rather than positive regulation of target genes (36).

The *cis*-active CR-mediating elements in a number of genes in *B. subtilis* have been analyzed. Two different sequence elements were shown to be active in CR according to mutational analyses: a 15-bp element with dyad symmetry centered at position −66 of the *citB* regulatory region (6) and a 14-bp sequence of nearly palindromic structure at position −3 to +11 in *amyE* (numbers are given with respect to the transcriptional start sites) (23, 39).

Sequences similar to the *cis* element of *amyE* were found in the translated regions of *xylA*, *gntR*, and *hutP* of *B. subtilis*, in which they mediate CR of these genes (13, 22, 22a, 25) and in a number of CR-regulated genes in other gram-positive bacteria (12, 27, 32, 33, 39). The similarity of these sequences has led to the hypothesis of a generally *cis*-active element for CR (CRE), as was originally proposed by Weickert and Chambliss (39). It has further been demonstrated for CR of *gntR* that expression from a constitutive promoter becomes subject to CR when this promoter is fused to CRE-containing fragments of *gntR* (22).

It has been shown for a number of inducible, CR-regulated genes in *E. coli* that inducer exclusion occurs in addition to the cyclic AMP-cyclic AMP receptor protein-mediated regulation. This mechanism reduces the cytoplasmic inducer concentration and leads to suboptimal induction (reviewed in reference 29). An inducer exclusion type of repression has not yet been demonstrated in *B. subtilis.*

We have analyzed the regulation of expression of the xylose utilization operon in *B. subtilis* and found that it is inducible by xylose via a Xyl repressor-mediated mechanism (7, 14) and is subject to CR (13). The CRE of the *xyl* operon is located 134 bp downstream from the transcription start site in the xylose isomerase reading frame (13). In this article, we demonstrate that CRE of *xyl* confers CR when fused to an unrelated, constitutive promoter. Mutations of CRE that make it more similar to the consensus sequence lead to increased CR. These results demonstrate that CRE is a *cis*-active element which functions independently of sequence context. Glucose leads to an additional inducer exclusion type of repression of the *xyl* operon, which requires a functional *xylR*. This additional repression is dependent on the concentrations of glucose and xylose in the culture broth, whereas no such concentration dependency of *xyl* repression is observed for fructose or glycerol.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used and constructed in this study are listed in Tables 1, 2, and 3. *E. coli* DH5α was used as a general cloning host. *B. subtilis* 168 was the parental strain for all *B. subtilis* strains constructed in this study.

Culture and growth conditions. *B. subtilis* strains were grown in S minimal medium [15 mM $(NH_4)_2SO_4$, 80 mM K_2HPO_4, 44 mM KH_2PO_4, 3.4 mM Na-citrate, 1 mM $MgSO_4$ (pH 7.4)] supplemented with 0.5% Na-succinate, 0.05% yeast extract, and 40 mg of tryptophan per liter. Additional carbon sources were supplied at different concentrations as indicated in the text. Cells were grown under vigorous aeration at 37°C.

General methods. Recombinant DNA techniques were performed as described previously (19). Oligonucleotide-directed mutagenesis was done according to the method of Kunkel et al.

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TABLE 1. Bacterial strains used in this study[a]

Strain	Marker	Source or reference
E. coli DH5α	*recA1 endA1 gyrA96 thi relA1 hsdR17*(r_K^- m_K^+) *supE44*	19
B. subtilis 168	*trpC2*	BGSC1A1[b]
B. subtilis WH335	*amyE*::(*Pxyl*$_{396bp}$*Eco*RI-*Hin*dIII-*spoVG-lacZ* Cm^r) *trpC2*	This study
B. subtilis WH340	*B. subtilis* WH335 *xylR* mutant *xylR*::Erm^r	This study
B. subtilis WH343	*amyE*::(*tetP*$_A$-*xylA*$_{43bp}$*Hpa*I-*Hin*dII-*spoVG-lacZ* Cm^r) *trpC2*	This study

[a] For further strains derived from the ones listed in this table, see Table 3.
[b] BGSC, *Bacillus* Genetic Stock Center.

(16). DNA blot hybridization analysis was performed by the method of Southern (34). Plasmid DNA from *E. coli* was prepared with the Qiagen plasmid kit (Diagen, Düsseldorf, Federal Republic of Germany). Chromosomal DNA from *B. subtilis* was prepared as described previously (40). *B. subtilis* strains were transformed by natural competence according to the method of Hardy (10). β-Galactosidase activity was determined according to the method of Miller (21) with modifications described previously (30). Xylosidase activity was determined as follows. Cells were grown to an optical density of 0.5, collected by centrifugation, resuspended in Z buffer (21), and disrupted by sonication. The reaction mixture contained 1 mg of *p*-nitrophenyl-β-D-xylopyranoside per ml and 0.05 M potassium phosphate (pH 6.8). Xylosidase activity was determined according to the increase in A_{410} at 25°C. Amylase production was determined by growing colonies overnight on Luria broth plates containing 1% starch. The plates were stained with 1% iodine solution and observed for light halos around the colonies.

TABLE 2. Plasmids used in this study

Plasmid	Characteristics	Reference
pWH421		14
M13mp10		20
pDH32	Integrational plasmid for *B. subtilis*	31
pDH32/M	pDH32 with three successive stop codons in *spoVG*	This study
pWH343	Shuttle vector for *E. coli* and *Bacillus* species, *tetP*$_A$ promoter	9
pWH416		8
pWH456	pWH343 with *tetP*$_A$-*xylA*$_{43bp}$*Hpa*I-*Hin*dIII	This study
pWH477	pWH456 mutation in CRE (3-G→T)	This study
pWH478	pWH456 mutation in CRE (10-A→T)	This study
pWH479	pWH456 mutation in CRE (3-G→T, 10-A→T)	This study
pWH484	pDH32/M with *Pxyl*$_{369bp}$*Eco*RI-*Hin*dIII-*spoVG-lacZ*	This study
pWH485	pWH484 mutation in CRE (3-G→T)	This study
pWH486	pWH484 mutation in CRE (10-A→T)	This study
pWH487	pWH484 mutation in CRE (3-G→T, 10-A→T)	This study
pWH488	pWH484 deleted for CRE *Pxyl*$_{353bp}$*Eco*RI-*Hpa*I-*spoVG-lacZ*	This study
pWH489	pDH32/M with *tetP*$_A$-*xylA*$_{43bp}$*Hpa*I-*Hin*dIII-*spoVG-lacZ*	This study
pWH490	pWH489 mutation in CRE (G→T)	This study
pWH491	pWH489 mutation in CRE (A→T)	This study
pWH492	pWH489 mutation in CRE (G→T, A→T)	This study
pWH493	pWH489 deleted for CRE *tetP*$_A$-*spoVG-lacZ*	This study
pIW11	YRP7 with 5.8-kbp chromosomal DNA from *B. subtilis* W23	40
pIW11*xylR* mutant	pIW11 *xylR*::Erm^r	This study

Construction of CRE mutants. pWH421, which contains a 205-bp fragment of the *B. subtilis* W23 *xyl* operon including the *xylA* promoter region, was restricted with *Nsi*I-*Hin*dIII, and the resulting 107-bp *xylA* fragment was ligated to M13mp10 cut with *Pst*I-*Hin*dIII. This construct was used to introduce the double-base-pair substitution in the CRE sequence as well as the two single substitutions (16). All mutations were confirmed by sequencing. The 43-bp *Hpa*I-*Hin*dIII fragments with the CRE mutations were inserted into pWH416 by replacing the plasmid-borne *Hpa*I-*Hin*dIII fragment.

All promoter-CRE-*lacZ* fusions were integrated in the *amyE* locus of *B. subtilis* 168. For chromosomal integration in *amyE*, we constructed pDH32/M. pDH32 (31) should contain three successive stop codons in all three reading frames located 49 to 36 bp upstream of the *spoVG*::*lacZ* start codon. However, the plasmid carries a 2-bp deletion which moves the position of one stop codon to +14 in the *spoVG*::*lacZ* reading frame (13). To regenerate the third stop codon, pDH32 was linearized with *Bam*HI, the recessed ends were filled in, and the resulting DNA was ligated to a 14-bp linker with the sequence CTACGTAAGCTTAA. Thereby, a single *Bam*HI site was restored, and a single *Sna*BI site and a single *Hin*dIII site were introduced. The construct was confirmed by sequencing. The orientation of the linker, with respect to the restriction sites, is 5′-*Bam*HI-*Sna*BI-*Hin*dIII-3′. The resulting plasmid was called pDH32/M.

pWH416 (containing wild-type [WT] CRE), pWH474 (3-G→T), pWH475 (10-A→T), and pWH476 (3-G→T, 10-A→T) were cut with *Eco*RI and *Hin*dIII (the *Hin*dIII site is located 20 bp downstream from CRE), and the resulting 396-bp fragment was ligated to pDH32/M cut with *Eco*RI and *Hin*dIII. This procedure yielded plasmids pWH484 to pWH487, having fusions of the different mutant CREs to *spoVG*::*lacZ*. These fusions contain the entire intergenic region between *xylR* and *xylA*. A CRE deletion (pWH488) was obtained by restriction of pDH32/M with *Hin*dIII, the recessed ends were filled in, and the plasmid was restricted with *Eco*RI and ligated with a 353-bp *Eco*RI-*Hpa*I fragment from pWH484.

Plasmids pWH484 to pWH488 were linearized with *Sca*I and transformed into *B. subtilis* 168, and the resulting clones were selected for chloramphenicol resistance. Correct integration into the *amyE* locus was confirmed by demonstrating an amylase-minus phenotype and by Southern blot analysis (data not shown). The orientation of the *xylA*::*lacZ* fusion is opposite to the transcriptional direction of *amyE*. Integration of pDH32/M without a promoter in front of *lacZ* resulted in no detectable β-galactosidase activity. The names of the respective strains are listed in Tables 1 and 3.

Construction of fusions of mutant CREs to the *tetP*$_A$ promoter. WT and mutant CREs were fused to the unregulated *tetP*$_A$ promoter. pWH343 (9) was cleaved with *Sma*I and ligated with a 43-bp *Hpa*I-*Hin*dIII (filled-in) fragment from pWH416 and pWH474 to pWH476, yielding *tetP*$_A$::CRE fusions. The resultant plasmids were called pWH456 (WT CRE) and pWH477 to pWH479 (mutant CREs [Table 2]). These plasmids were cut with *Eco*RI, and the *tetP*$_A$::CRE-containing 213-bp fragments were ligated with pDH32/M cut with *Eco*RI, yielding *tetP*$_A$::CRE::*lacZ* transcriptional fusions. The correct orientations of the inserts were confirmed by restriction analyses with *Eco*RV and *Sal*I. The plasmids were designated pWH489 to pWH492 (Table 2). A *tetP*$_A$::*lacZ* fusion without

TABLE 3. *B. subtilis* strains with different CREs used in this study

CRE	CRE sequence	*B. subtilis* strain with:		
		xylA::*lacZ*	*tetP*$_A$::*lacZ*	*xylA*::*lacZ* *xylR* mutant
WT	TGGAAGCGTAAACA	WH335	WH343	WH340
3-G→T	TGTAAGCGTAAACA	WH336	WH344	WH350
10-A→T	TGGAAGCGTTAACA	WH337	WH345	WH351
3-G→T, 10-A→T	TGTAAGCGTTAACA	WH338	WH346	WH341
With deletion		WH339	WH347	WH342

CRE was cloned by ligating the 170-bp *Eco*RI fragment from pWH343 into the *Eco*RI site of pDH32/M. The plasmid was called pWH493.

The plasmids with the different *tetP*$_A$::*lacZ* fusions were integrated into *amyE* of *B. subtilis* 168, and integrations were confirmed by Southern blot analyses. The designations of the respective strains are listed in Tables 1 and 3.

Insertional inactivation of *xylR*. We have inactivated the chromosomally encoded Xyl repressor in strains with different mutant CREs by insertion of an erythromycin resistance (Erm^r) gene into *xylR*. pIW11 (40), containing the entire *xyl* operon from *B. subtilis* W23, was cut with *Mlu*I and ligated with a 1.4-kbp *Taq*I fragment from pE194 to yield the pIW11*xylR* mutant (*xylR'*::*erm*::*'xylR*). The pIW11*xylR* mutant was cut with *Pst*I or, alternatively, with *Sal*I and transformed into *B. subtilis* WH335, WH336, WH337, WH338, and WH339. Colonies were selected on Luria broth plates containing 10 μg of erythromycin and screened for blue color on Luria broth plates with 40 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per liter, without xylose because of constitutive expression of *xylA*::*lacZ* or, alternatively, were screened for constitutive expression of xylosidase. The designations of the resultant *xylR* mutant strains are listed in Tables 1 and 3.

RESULTS

Construction of *B. subtilis* strains for CR analysis. The relevant genotypes of the constructed *B. subtilis* strains are listed in Tables 1 and 3, and details of the regulatory elements employed in these strains are outlined in Fig. 1. Single-copy *xylA*::*spoVG*::*lacZ* transcriptional fusions, in which the CRE sequence was either altered by oligonucleotide-directed mutagenesis or deleted, were constructed by integration into *amyE* (31). Since the WT CRE sequence of *xyl* deviates in 2 bp from the consensus sequence, each of these and both base pairs were changed to yield a better or perfect fit with the consensus element (39). This constitutes a set of five isogenic strains differing only in the CRE. In the next set of constructions, these four CREs were combined with the *E. coli tetP*$_A$ promoter, which had been previously modified to yield constitutive promoter activity in *B. subtilis* (9). Together with the respective construct lacking CRE, this constituted another set of five strains, in which the functionality of CRE in a new, unrelated sequence context was determined. Finally, the endogenous *xylR* gene was inactivated by insertion of an *erm* cassette in the five *xylA*::*spoVG*::*lacZ*-containing strains. These constructs were used to establish the participation of a functional *xylR* in CR.

Dependence of CR of *xylA* expression on xylose and glucose concentrations. The effects of the concentrations of various sugars on expression of *xylA*::*lacZ* in strain WH335 (WT CRE) were determined. Xylose-dependent induction reached a plateau of 200-fold between 8.4 and 33.4 mM xylose and de-

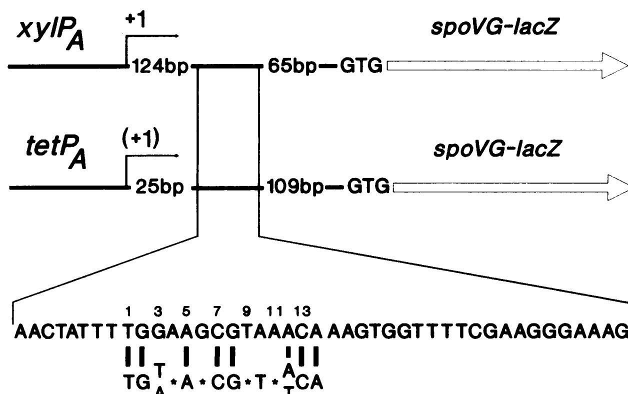

FIG. 1. Genetic constructions employed in this study. The localization and sequence of the 43-bp *Hpa*I-*Hin*dIII CRE-containing fragment with respect to the *xylA* promoter and the *tet* promoter are shown. Distances from the transcriptional start points (putative for *tet*) to the 43-bp fragment and from this fragment to the start codon (GTG) of the *spoVG*::*lacZ* fusion are given in base pairs. The WT CRE from *xylA* is aligned with the consensus sequence (Weickert and Chambliss [39]), and matches are indicated by bars. Stars indicate random bases in the consensus sequence. The numbers on top of the CRE refer to the designations of base pairs used in this article.

creased slightly at higher concentrations (Fig. 2A). Unless indicated otherwise, 16.7 mM xylose was therefore used in further experiments.

The concentration dependence of glucose repression in WH335 was also determined. The results are shown in Fig. 2B. The optimal (~40-fold) repression was achieved between 7 and 13.9 mM glucose, which decreased to ~20-fold repression at 111 mM glucose. This surprising result demonstrates that the glucose concentration is critical for the efficiency of glucose repression.

The effects of various xylose concentrations on the efficiency of repression at 13.9 mM glucose in strain WH335, together with the induction curve of xylose alone, are shown in Fig. 2A. Glucose repression appears to be most effective at low concentrations of xylose. At increased ratios of xylose over glucose, the inducer xylose is able to overcome glucose repression to a large extent.

The efficiency of CRE increases with better similarity to the consensus sequence. The expression of β-galactosidase from the single-copy *xylA*::*lacZ* fusions containing CRE with mutations, making it more similar to the consensus sequence (Fig. 1), was used to determine the efficiency of glucose-mediated CR. The results are shown in Table 4. All of the constructs are inducible by xylose with roughly the same efficiencies, although the maximal expression levels decreased with the increased similarity of CRE to the consensus sequence, which might be due to a weak repression exerted by xylose itself. Since this effect is slightly more pronounced for the basal levels than for the xylose-induced expression levels, the induction factor increases somewhat from 200-fold in WH335 to 270-fold in WH338. In contrast, the repression exerted by glucose depends to a large extent on the CRE sequence, because both single mutations exhibit a 100-fold repression, an ~3-fold stronger effect than that of the WT. The double mutation in WH338 leads to glucose repression about sevenfold that of the WT CRE in WH335. Surprisingly, the deletion of CRE in WH339 does not abolish glucose repression completely. Instead, a 13-fold repression is still observed in that strain.

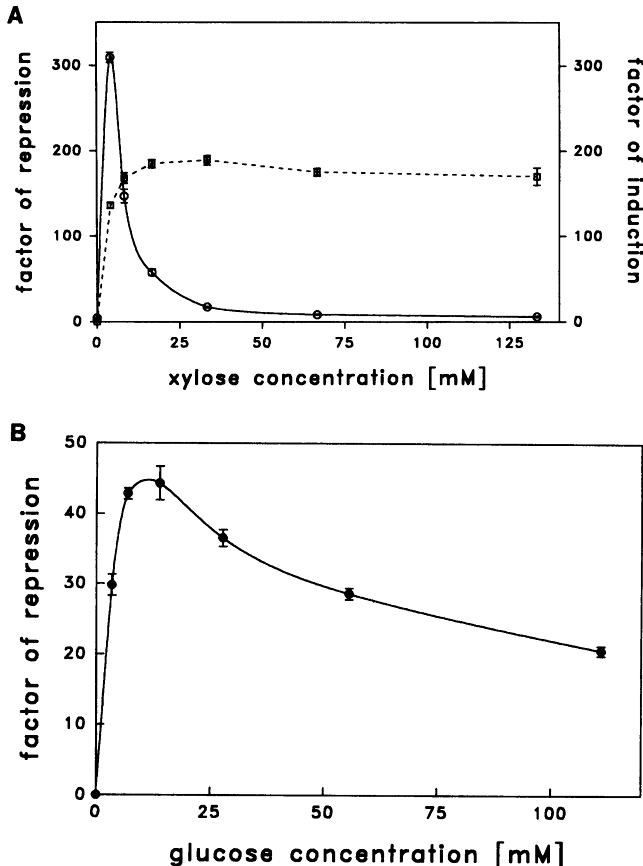

FIG. 2. Dependence of β-galactosidase expression in WH335 (WT CRE, *xylR*$^+$) on the concentrations of xylose and glucose. (A) Induction by xylose (right ordinate, □) and repression by glucose (13.9 mM constant glucose concentration, left ordinate, ○) assayed for various xylose concentrations. The maximal induction corresponds to 1,100 β-galactosidase units. (B) Dependence of repression on the concentration of glucose. The concentration of xylose was 16.7 mM. The repression factor is the β-galactosidase activity given as a percentage of the respective activity without glucose.

Participation of *xylR* in glucose repression of the *xyl* operon. To establish a mechanism for the apparent glucose repression in the absence of CRE, we have inactivated *xylR* in the strains with CRE mutations and determined glucose-mediated repression of *xylA*::*lacZ* in these strains as well. These results are also presented in Table 4. It is apparent that inactivation of *xylR* leads to a severe reduction in glucose repression in all strains compared with glucose repression of the respective *xylR*$^+$ strains. Repression is only 4-fold with WT CRE; it increases to 7- to 9-fold in the single-base-pair mutations of CRE and increases to 13-fold in the strain with the optimal CRE. This result confirms the participation of CRE and demonstrates the importance of a *xylR*-mediated mechanism for glucose repression. The strain lacking both *xylR* and CRE does not show significant glucose repression (1.3-fold) anymore.

The glucose repression mediated by CRE is independent of sequence context. The WT and mutant CREs were combined with a new sequence context by placing them 25 bp downstream of the (putative) transcription start of a modified *tet* promoter with constitutive activity (9) in *B. subtilis* (Fig. 1). This element drives expression of β-galactosidase from a *spoVG*::*lacZ* translational fusion, which is used to determine CR mediated by glucose. The results of this experiment are given in Table 5. The absolute expression levels are quite low because of the weak activity of the *tet* promoter in *B. subtilis*. In the absence of CRE, an almost twofold increase in β-galactosidase expression is found in the presence of 13.9 mM glucose. Insertion of the WT CRE from the *xyl* operon leads to about threefold repression on the basis of unregulated level. The two single mutations increase the repression efficiency to ~10-fold, whereas the double mutation is repressed 23-fold. Thus, the repression, which is even higher when based on the glucose-induced expression (numbers in parentheses in Table 5), is considerably stronger than those found in the *xylA*::*lacZ* strains when *xylR* is inactivated (compare with results in Table 4). Inactivation of *xylR* in WH343 showed the same glucose repression as that in WH343 (data not shown). These observations confirm that CRE is functional in different sequence contexts.

The concentration dependence of glucose repression is dependent on *xylR*. Strain WH340, with the WT CRE and the inactivated *xylR*, and strain WH339, lacking CRE but having WT *xylR*, have been used to study the concentration dependence of glucose repression. The results shown in Fig. 3 indicate that the *xylR*-mediated portion of glucose repression gives rise to concentration dependence, whereas the CRE-mediated portion of glucose repression is independent of the glucose concentration in the range studied here.

CR exerted by other carbon sources. The effect of fructose on CR was compared with the glucose repression for strains WH335, WH340, WH338, and WH339. The results shown in Fig. 4 indicate that fructose does not show the *xylR*-mediated repression effect obtained from the comparison of WH335 with WH340. In the latter strain with the inactivated *xylR*, both sugars lead to roughly the same level of repression. The consensus CRE in WH338 shows increased repression by fructose compared with that of the WT CRE in WH335. This resembles the effect exerted by glucose. When CRE is deleted in strain WH339, an insignificantly small reduction in β-galactosidase expression is seen in the presence of fructose. The same set of strains was assayed for repression by glycerol, leading to qualitatively identical results, except that there was a somewhat lower total level of repression (Fig. 3). No concentration dependence of CR was found for fructose or glycerol as shown in Fig. 3. These results indicate that glucose, fructose, and glycerol trigger the CRE-mediated response, whereas only glucose triggers the *xylR*-mediated response in addition to the CRE-dependent route.

Several other carbon sources were checked for their ability to confer CR to β-galactosidase expression from the *xylA*::*lacZ* fusion in WH335. The respective results are given in Fig. 5. The compounds employed here show much weaker CR than glucose. The phosphotransferase system (PTS) compounds mannitol and fructose yielded almost fourfold repression. The non-PTS compounds glycerol (threefold) and glucitol (twofold) showed reduced repression and the non-PTS sugar arabinose showed no CR. These results indicate that CR is independent of PTS-mediated uptake.

DISCUSSION

The mutagenesis of CRE in *xylA* to make it more similar or identical to the consensus sequence confirms and extends previous results obtained from a deletional analysis of this element (13). While deletion led to loss of glucose repression, the improvement of CRE leads to increased repression by glucose, fructose, and glycerol. This demonstrates unambiguously that CR of *xylA* is mediated by CRE.

TABLE 4. β-Galactosidase activities of *xylA*::*lacZ* fusions with different CREs

CRE	Sugar[a]	*xylR*+ strain	β-Galactosidase activity (Miller units)	Factor of regulation[b]	*xylR* mutant strain	β-Galactosidase activity (Miller units)	Factor of regulation[b]
WT	–	WH335	5.2 ± 0.2		WH340	1,078.0 ± 57.0	
	Xyl		1,022.0 ± 37.0	I, 197		1,166.0 ± 62.0	I, 1.1
	Xyl + Glc		27.0 ± 1.6	R, 38		278.0 ± 10.0	R, 4
3-G→T	–	WH336	4.2 ± 0.4		WH350	901.0 ± 37.0	
	Xyl		979.0 ± 43.0	I, 233		1,012.0 ± 27.0	I, 1.1
	Xyl + Glc		9.0 ± 0.3	R, 109		115.0 ± 1.2	R, 9
10-A→T	–	WH337	2.4 ± 0.1		WH351	574.0 ± 15.0	
	Xyl		594.0 ± 37.0	I, 248		603.0 ± 45.0	I, 1.1
	Xyl + Glc		5.7 ± 0.3	R, 104		85.0 ± 4.0	R, 7
3-G→T, 10-A→T	–	WH338	1.9 ± 0.2		WH341	581.0 ± 48.0	
	Xyl		509.0 ± 37.0	I, 268		535.0 ± 22.0	I, 1
	Xyl + Glc		2.0 ± 0.1	R, 255		42.0 ± 1.0	R, 13
None	–	WH339	5.7 ± 0.3		WH342	1,013.0 ± 29.0	
	Xyl		1,083.0 ± 25.0	I, 190		1,147.0 ± 24.0	I, 1.1
	Xyl + Glc		84.0 ± 9.0	R, 13		869.0 ± 25.0	R, 1.3

[a] Medium containing succinate. –, xylose and glucose absent.
[b] I, factor of induction (β-galactosidase activity in the presence of xylose divided by activity in the absence of xylose); R, factor of repression (activity in the presence of xylose divided by activity in the presence of xylose and glucose).

The location of CRE 134 bp downstream of the transcription start site of *xylA* is unusual for a negatively *cis*-acting element. In most other cases, they either overlap directly with the regulated promoter or fold back to additional sites close to the promoter by a DNA looping mechanism induced by protein binding. The latter mechanism has been demonstrated for, e.g., *lacI*-mediated control in *E. coli* (1). A search of the *xylA* promoter sequence does not yield any site with significant similarity (more than 6 matches out of 10 defined positions) to CRE. Because CRE exerts full repression in a new sequence context, a DNA looping mechanism is not very likely for CRE-mediated repression. However, the *tet* promoter sequence contains a CRE-like element overlapping the –10 region. This element shows two mismatches to the consensus sequence (TGATAGAGTTATAA). Although this sequence might be the target for a potential fold back mechanism, it does not mediate glucose repression when not combined with the *xyl* CRE. Furthermore, the mismatches to the consensus sequence occur at important positions which are highly conserved in a large number of potential CREs (12). Mutations at the same positions almost abolish glucose repression of *amyE* (39). Another conceivable mechanism is termination of the elongating RNA polymerase at CRE when occupied by an as yet unidentified protein, as has been demonstrated for the *lac* repressor-operator complex in some instances (4). However, no direct evidence clarifying the mechanism of repression has been obtained so far. The sensitivity of the *tet* promoter CRE fusion-driven expression to CR demonstrates that CRE does not have to be in a translated region to be functional. This result is in agreement with the function of CRE in *amyE*.

The consensus sequence CRE leads to a 13-fold glucose repression of *xylA* expression in the absence of *xylR*. The expression of *amyE* from *B. subtilis*, which also contains a consensus sequence CRE, is repressed 14.5-fold by glucose (39). The CRE in *xylA* of *Bacillus megaterium*, which shows one deviation from the consensus sequence at position 10 (T is

TABLE 5. β-Galactosidase activities of $tetP_A$::*lacZ* fusions with different CREs

Strain	CRE	Glucose[a]	β-Galactosidase activity (Miller units)	Factor[b]
WH347	None	–	37.0 ± 1.0	
		+	62.0 ± 5.0	0.6 (1)
WH343	WT	–	64.0 ± 8.0	
		+	22.0 ± 2.3	3 (5)
WH344	3-G→T	–	30.0 ± 0.7	
		+	3.0 ± 0.1	10 (17)
WH345	10-A→T	–	16.0 ± 0.5	
		+	1.7 ± 0.1	9 (16)
WH346	3-G→T, 10-A→T	–	11.0 ± 0.6	
		+	0.5 ± 0.1	23 (37)

[a] –, absent; +, present.
[b] β-Galactosidase activity in the absence of glucose divided by β-galactosidase activity in the presence of glucose. Factors in parentheses are calculated on the basis of the glucose-induced activity (multiplied by 1.67).

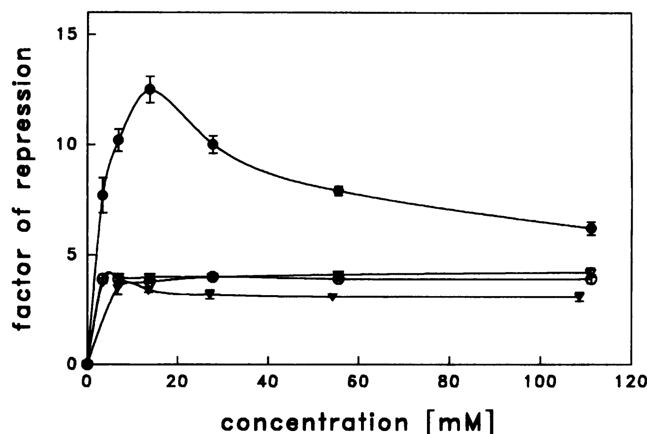

FIG. 3. Effects of carbon source concentration on catabolite repression of *xylA*. ●, repression in strain WH339 (no CRE, *xylR*+) as a function of the glucose concentration; ○, repression in strain WH340 (WT CRE, *xylR* mutant) as a function of the glucose concentration; △, CR in strain WH335 (WT CRE, *xylR*+) as a function of the fructose concentration; ▲, CR in strain WH335 (WT CRE, *xylR*+) as a function of the glycerol concentration. All determinations were done with 16.7 mM xylose in the broth. The factor of repression is given as the percentage of β-galactosidase expression in the presence of 16.7 mM xylose.

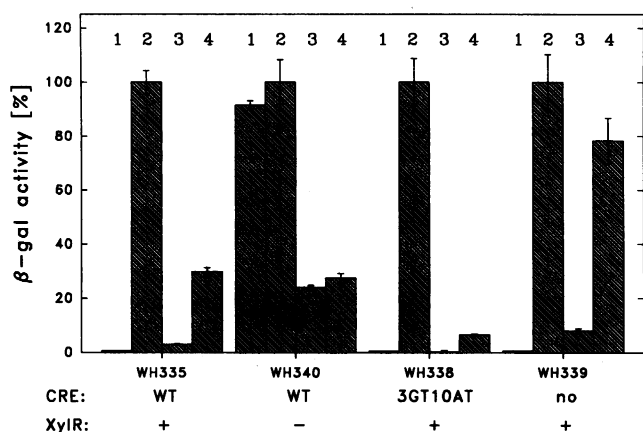

FIG. 4. Influence of *xylR* and CRE on repression of β-galactosidase (β-gal) expression by glucose or fructose. The relevant genetic elements of the four tested strains are indicated below the horizontal axis. β-Galactosidase activity is given as the percentage of the fully induced level (bars 2), which varies from ~1,100 U (WH335, WH339, and WH340) to ~500 U (WH338) (Table 4). The bars are marked with numbers from 1 to 4 to indicate the different sugars used: 1, no regulatory carbon source; 2, xylose at 16.7 mM; 3, xylose at 16.7 mM and glucose at 13.9 mM; 4, xylose at 16.7 mM and fructose at 13.9 mM.

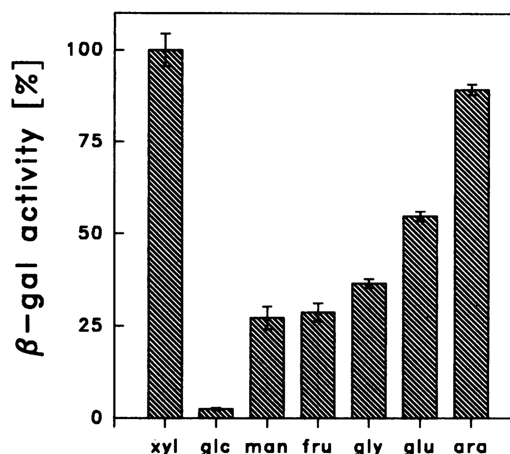

FIG. 5. Catabolite repression of β-galactosidase (β-gal) expression in strain WH335 (WT CRE, $xylR^+$). Residual β-galactosidase activities obtained with various sugars are given as percentages of the fully induced level (about 1,100 U). Media contained xylose at 16.7 mM (xyl) or xylose at 16.7 mM plus either glucose at 13.9 mM (glc), mannitol at 13.7 mM (man), fructose at 13.9 mM (fru), glycerol at 27.2 mM (gly), glucitol at 13.7 mM (glu), or arabinose at 16.7 mM (ara).

replaced by A), leads to a 10-fold repression in a *xylR* mutant strain (27), which is almost identical to the 9-fold repression in WH350 (3-G→T, *xylR* mutant). The similarities of these values show that repression efficiencies vary according to the deviations of the respective CREs from the consensus sequence but that they are the same for comparable CREs in their WT sequence contexts for different genes in different species. The level of repression of $tetP_A$-driven expression is higher than the level of repression of the three genes mentioned above (Table 5). This effect is not due to *xylR*, because no decrease in repression was observed in a *xylR* mutant strain. The higher degree of repression was observed for all four different CREs and might, therefore, reflect an effect which depends on the specific position of these CREs in the fusion with the *tet* promoter. Repression efficiencies varying from 11- to 4-fold have also been observed to be dependent on the distances between the promoter and CRE for CR of *gntR* (22). However, repression efficiencies of the $tetP_A$::CRE::*lacZ* fusions increase with increasing identity of CRE to the consensus sequence even in this new sequence context. It is thus quite clear that, at least for the positions tested here, the consensus sequence of CRE derived from many genes (39) is the optimally repressing sequence and that CRE is a general *cis*-acting element mediating CR.

The CRE-mediated repression responds not only to the presence of glucose but also to a number of other carbon sources. The ones studied here differ in their uptake mechanisms as well as in their intracellular degradation pathways. Glucose, fructose, and mannitol are substrates for the phosphoenolpyruvate-sugar PTS (26), while glycerol and glucitol are not transported via the PTS (17, 35). The result that they all lead to catabolite repression of *xylA* indicates that CR is not coupled to a specific uptake system. It is rather likely that the stringency of repression exerted by each of these sugars is directly proportional to the growth rate on that sugar, as has been demonstrated for CR of *xylA* in *B. megaterium* (27). It has been reported that only glycolytic sugars exhibit CR of *bglS* in *B. subtilis* (15). Furthermore, CR of gluconate kinase and inositol dehydrogenase expression is dependent on the intracellular formation of fructose-1,6-diphosphate, glyceraldehyde-3-phosphate, and 3-phosphoglycerate or dihydroxyacetone-P or both (24). Thus, the sugar-specific concentration of an intracellular metabolite, most likely from glycolysis, might also trigger CR of *xylA*. The detailed molecular mechanism by which CR is mediated in *B. subtilis* remains to be elucidated.

One gene involved in the signalling chain from the presence of glucose to the regulatory effect exerted at CRE is *ccpA* (11, 12a), which encodes a putative DNA-binding protein that belongs to the *lacI-galR* family of bacterial repressors (38). Because CRE shows sequence similarities to those of *lac* and *gal* operators (23), it has been suggested that CcpA might directly interact with CRE. However, the specificity of CcpA binding to CRE has yet to be demonstrated.

A different mechanism for CR of inducible genes like *xylA* is the exclusion of inducer from its interaction with the respective repressor protein, either by reducing the cytoplasmic inducer concentration or by mechanisms involving the repressor (described below). We use inducer exclusion for any mechanism which depends on a functional repressor protein. The results presented here are, to our knowledge, the first demonstration of an inducer exclusion type of glucose repression in *B. subtilis*. This phenomenon contributes to a large extent to glucose repression of *xylA* but does not contribute to the general CR exerted by other carbon sources. This finding seems to contradict our previously published results, in which *xylR* inactivation did not influence glucose repression of *xylA* (13). The critical difference in the present study is a result of the dependence of the *xylR*-mediated effect on the concentrations of glucose and xylose. If both sugars are supplied at 2% (133.2 mM for xylose, 111.1 mM for glucose), as done in the previous study, we do indeed not find a *xylR*-dependent repression (Fig. 2); however, at the lower concentrations employed here, this effect becomes apparent. We have redetermined glucose repression in strains WH335 (WT CRE, $xylR^+$), 340 (WT CRE, *xylR* mutant), 339 (CRE deletion, $xylR^+$), and 342 (CRE deletion, *xylR* mutant) at a concentration of 2% of both sugars and found a repression factor of fivefold in WH335 and WH340 and no glucose

repression in WH339 and WH342 (data not shown). The lower sugar concentrations employed in this study are probably closer to the conditions in the natural habitats of the *Bacillus* spp. Therefore, we regard the *xylR*-mediated effect as physiologically very relevant.

The *xylR*-mediated effect is specific for glucose and does not occur with fructose or glycerol. The same is found for the concentration-dependent repression of *xylA*, which is seen for glucose but not for fructose or glycerol. The glucose-specific PTS could be a potential sensor site for inducer exclusion as demonstrated for inhibition of lactose permease and maltose permease activities by the unphosphorylated form of enzyme IIa^{Glc} in enteric bacteria (29). Similarly, nonphosphorylated enzyme IIa^{Glc} could inhibit xylose uptake by a not yet identified permease in *B. subtilis*. It could also be possible that xylose uptake may be inhibited by a direct interaction of glucose with xylose permease because of the structural similarities of both sugars. In another conceivable mechanism, glucose and xylose might compete for binding to the Xyl repressor. This would be in agreement with the anti-inducing effect of glucose observed in vitro for the *Bacillus licheniformis* Xyl repressor (30). It is quite feasible that both sugars, which differ only in the CH_2-OH moiety at position 6 being replaced by a proton in xylose, bind to the same proteins. This effect would require that free glucose is taken up by a non-PTS mechanism as described for *B. licheniformis* (37). However, all of these assumptions cannot explain the reduced repression at elevated glucose concentrations. The lower level of repression under these conditions could be due to glucose blocking the turnover of xylose by binding to xylose isomerase (3) and thereby increasing the intracellular inducer concentration. Taken together, it is quite possible that multiple mechanisms contribute to the complex regulatory effects observed for different concentrations of glucose.

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