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Catabolite Repression of the *xyl* Operon in *Bacillus megaterium*

THOMAS RYGUS AND WOLFGANG HILLEN*

Lehrstuhl für Mikrobiologie, Institut für Mikrobiologie und Biochemie der Friedrich-Alexander Universität Erlangen-Nürnberg, Staudtstr. 5, 8520 Erlangen, Germany

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We characterized catabolite repression of the genes encoding xylose utilization in *Bacillus megaterium*. A transcriptional fusion of *xylA* encoding xylose isomerase to the *spoVG-lacZ* indicator gene on a plasmid with a temperature-sensitive origin of replication was constructed and efficiently used for single-copy replacement cloning in the *B. megaterium* chromosome starting from a single transformant. In the resulting strain, β-galactosidase expression is 150-fold inducible by xylose and 14-fold repressed by glucose, showing that both regulatory effects occur at the level of transcription. Insertion of a kanamycin resistance gene into *xylR* encoding the xylose-dependent repressor leads to the loss of xylose-dependent regulation and to a small drop in the efficiency of glucose repression to eightfold. Deletion of 184 bp from the 5′ part of the *xylA* reading frame reduces glucose repression to only twofold. A potential glucose-responsive element in this region is discussed on the basis of sequence similarities to other glucose-repressed genes in *Bacillus subtilis*. The sequence including the glucose-responsive element is also necessary for repression exerted by the carbon sources fructose and mannitol. Their efficiencies of repression correlate to the growth rate of *B. megaterium*, as is typical for catabolite repression. Glycerol, ribose, and arabinose exert only a basal twofold repression of the *xyl* operon, which is independent of the presence of the *cis*-active glucose-responsive element within the *xylA* reading frame.

Gene regulation in gram-positive bacteria has been mainly studied in *Bacillus subtilis*, focusing on developmentally regulated cell differentiation processes. In particular, genetic competence (3) and sporulation (1, 21) have been studied in great detail. Much effort has also been focused on the regulation of vegetatively expressed genes for the utilization of different carbon sources in *B. subtilis* (6). Other members of the bacilli, e.g., *B. amyloliquefaciens*, *B. licheniformis*, and *B. megaterium*, play important roles in the industrial production of enzymes and for the expression of heterologous proteins with high yields (2, 9, 11, 18, 22, 24, 27). *B. megaterium* has been used for protein expression (18, 26) and as a host with improved stability of plasmids compared with *B. subtilis* (27, 32, 34, 35). *B. megaterium* strains lack natural competence, and most of them are less efficiently transformable than *B. subtilis*. Therefore, integration of markers by homologous recombination with the chromosome has as yet only been demonstrated as Campbell-type integration of plasmids (4, 31). In a different approach, the transducing bacteriophage MP13 has been used for marker transfer and mapping (33). To study the regulation of *xyl* operon expression by different carbon sources in a single-copy situation, we developed an efficient method for replacement of chromosomal markers in *B. megaterium*. This is achieved by using plasmids providing DNA sequences identical to the *B. megaterium* chromosome and a temperature-sensitive origin of replication to divide the one-step process of transformation with linear DNA into three easily screenable or selectable steps: (i) transformation; (ii) single-crossover recombination; (iii) excision of the carrier replicon. Transformation efficiencies and recombination rates play only minor roles in the final yields in this approach. In this study, we made use of this generally applicable method of marker exchange on the *B. megaterium* chromosome to study catabolite repression of the *xyl* operon encoding xylose utilization (27). While catabolite repression in bacilli at the level of enzymatic activities (5) was described some time ago, it has only recently been established that catabolite repression for several genes in *B. subtilis* is mediated at the level of transcription (6, 12). Different *cis*-acting catabolic control sequences have been proposed for a number of operons (7, 15, 20, 36). Catabolite repression of *xyl* in *B. megaterium* is more efficient than that in *B. subtilis* W23 (8, 15, 16), thus facilitating the study of effects of other carbon sources repressing less effectively than glucose.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are described in Table 1. *B. megaterium* WH320, a derivative of strain DSM319, was constructed by ethyl methanesulfonate mutagenesis (27) and has no detectable β-galactosidase activity, whereas the wild-type strain shows low β-galactosidase activity in the media used in this study. *Escherichia coli* RR1 *lacZ*ΔM15 was generally used for transformations as described previously (17). *B. megaterium* WH320 was transformed by using protoplasts as described previously (23). pWH1505 was constructed by first inserting the 3.0-kbp *Bgl*II (nucleotide positions 1 to 2131 in the *xyl* sequence [27]) fragment from pWH1500 into the single *Bgl*II site (27) of pWH1503, a pIC20H derivative containing a promoterless *spoVG-lacZ* fusion within the *Sma*I site of the pIC20H polylinker (27); this was followed by insertion of the *Bam*HI (nucleotide position 2103 in the *xyl* sequence [27])-*Pst*I fragment from pWH1500 into the respective sites on pWH1503. This results in a *xylA1-spoVG-lacZ* fusion flanked by DNA originating from the *xyl* operon of *B. megaterium*.

Culture and growth conditions. Bacilli and *E. coli* were grown in LB medium (10 g of tryptone, 5 g of NaCl, 5 g of yeast extract per liter of deionized water; pH 7.3). MOPSO medium was used as the minimal medium for bacilli (1). If necessary, media were solidified by the addition of 1.5% agar (Oxoid, Wesel, Germany). Diauxic growth curves and gen-

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genetic marker(s)[a]	Source or reference
Strains		
B. megaterium WH320	*lac*	27
B. megaterium WH321	*lac xylR* Kn^r	This study
B. megaterium WH322	*lac* Kn^r *xylR xylA1-spoVG-lacZ*	This study
B. megaterium WH323	*lac xylA1-spoVG-lacZ*	This study
B. megaterium WH324	*lac xylA2-spoVG-lacZ*	This study
B. megaterium WH325	*lac* Kn^r *xylR xylA2-spoVG-lacZ*	This study
B. subtilis BR151	*trpC2 lys-3 metB10*	BGSC 1A40[b]
E. coli RR1 *lacZ*ΔM15	*lacZ*ΔM15 F^- *hsdS20* (r_B^- m_B^-) *ara-14 proA2 leu lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44*	25
Plasmids		
pBEST501	Kn^r Ap^r	14
pBR327	Ap^r Tc^r	29
pTV32ts	Cm^r Ery^r Tn*917-lac*	37
pWH1500	Ap^r Cm^r *xylAB xylR*	27
pWH1503	Ap^r *spoVG-lacZ*	27
pWH1505	Ap^r *xylR xylA1-spoVG-lacZ*	This study
pWH1505K	Ap^r Kn^r *xylA1-spoVG-lacZ*	This study
pWH1509C	Ap^r Tc^r Cm^r	This study
pWH1509K	Ap^r Tc^r Kn^r	This study
pWH1518	Tc^r Kn^r *xylR xylA1-spoVG-lacZ*	This study
pWH1519	Tc^r Kn^r Cm^r *xylA1-spoVG-lacZ*	This study
pWH1518ΔSB	Tc^r Kn^r *xylR xylA2-spoVG-lacZ*	This study
pWH1519ΔSB	Tc^r Kn^r Cm^r *xylA2-spoVG-lacZ*	This study

[a] Ery^r, erythromycin resistance.
[b] BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus.

eration times were determined with a Biostat M fermentor (Braun, Melsungen, Germany).

General methods. Plasmid DNA from *E. coli* was prepared with the Qiagen ›plasmid‹ kit (Diagen, Düsseldorf, Germany). Preparation of plasmid DNA (10) and of total DNA from bacilli (8) and Southern blot analyses (30) were done as described before. The DNA probe for Southern blot analysis was labeled with biotin-7-dATP under the conditions recommended by the supplier (GIBCO/BRL, Eggenstein, Germany). All other general methods were done as described previously (10, 13, 17, 27).

β-Galactosidase assays. Cells were grown in MOPSO medium supplemented with 0.05% Casamino Acids, 0.05% yeast extract, and the respective regulatory carbon sources described in the Results and Discussion section to an optical density of 0.4 or 0.6 at 600 nm. β-Galactosidase activities were assayed on the same day by the method of Miller (19) with modifications described previously (28).

RESULTS AND DISCUSSION

Construction of temperature-sensitive shuttle plasmids for *E. coli* and bacilli. pTV32ts (37) carrying the temperature-sensitive origin of replication from pE194ts was restricted with *Bam*HI-*Pst*I and blunt ended by treatment with DNA polymerase I from *E. coli*. After ligation of *Eco*RI linkers, the fragment containing the origin of replication was eluted from the gel and ligated into the single *Eco*RI site of pBR327. The resulting plasmid was called pWH1509C and is able to replicate in *E. coli* and at temperatures below 35°C also in *B. subtilis* and *B. megaterium*. Its physical and genetic structure is shown in Fig. 1. It confers resistance to ampicillin (Ap^r) and tetracycline (Tc^r) in *E. coli* and to chloramphenicol (Cm^r) in bacilli. The chloramphenicol resistance phenotype was not very useful in our hands since it had to be selected at 5 mg of chloramphenicol per liter in solid medium, and background growth appeared under these conditions when 10^4 cells were plated. A single copy of the plasmid integrated in the *B. megaterium* WH320 genome confers resistance to just 1 or 2 mg of chloramphenicol per liter, which is suitable for screening but not for selection. Thus, we constructed pWH1509K carrying the kanamycin resistance (Kn^r) (*neo*) cassette from pBEST501 instead of the *cat* fragment. pBEST501 was restricted with *Sma*I, and the DNA fragment containing *neo* was eluted from the gel and ligated into pWH1509C cleaved with *Hpa*I and *Pvu*II. The resulting plasmid was called pWH1509K and mediates resistance against 10 mg of kanamycin per liter in *B. megaterium*. Its physical and genetic structure is also shown in Fig. 1. Selection on agar plates containing 4 mg of kanamycin per liter resulted in growth of *B. megaterium*(pWH1509K) without background at high plating densities.

Construction of plasmids for single-copy cloning in the *xyl* operon on the chromosome of *B. megaterium*. We aimed to

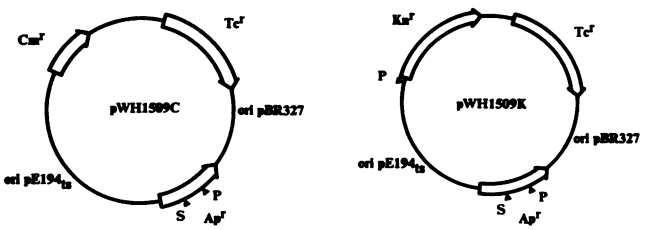

FIG. 1. Physical structures of the temperature-sensitive shuttle plasmids pWH1509C and pWH1509K. The *ori* from pBR327 for *E. coli* and from pE194ts for the *Bacillus* species are indicated. The resistance genes for tetracycline (Tc^r) and ampicillin (Ap^r) for *E. coli* and chloramphenicol (Cm^r) and kanamycin (Kn^r) for the *Bacillus* species are shown with their directions. Restriction sites used in the construction of integrative plasmids are *Sca*I (S) and *Pst*I (P).

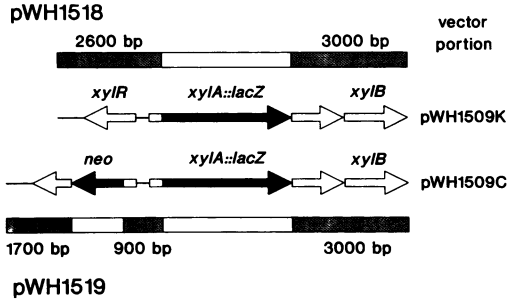

FIG. 2. Genetic constructions of pWH1518 and pWH1519. The two central figures show the genetic constructions of pWH1518 (top) and pWH1519 (bottom). Reading frames of *B. megaterium xyl* genes are indicated as open arrows with their designations. Both plasmids contain a *xylA1-spoVG-lacZ* fusion shown as solid arrows. The kanamycin resistance cassette (*neo*) is shown as a hatched arrow. The vectors used for the construction of integrative plasmids are indicated on the right side. The boxes below and on top indicate the sequences identical to (hatched) and different from (open) the *B. megaterium xyl* operon.

construct several deletion and insertion mutants of the *xyl* operon. The fusion of the *spoVG-lacZ* indicator gene to the first 196 bp of the *xylA* reading frame on the *Pst*I-*Nru*I fragment from pWH1505 (see Materials and Methods) was inserted into the *Pst*I and *Sca*I sites in the *bla* gene (Ap^r) of pWH1509K (Fig. 1). This results in a *xylA1-lacZ* transcriptional fusion flanked by 2.6 and 3.0 kbp from *B. megaterium xyl* on either side, respectively. This construction was called pWH1518 and is shown in Fig. 2. In the next step, the *xylR* gene was inactivated by inserting a Kn^r cassette (14). For that purpose, the *Sma*I fragment containing *neo* from pBEST501 was inserted into the *Stu*I (nucleotide position 1228 in the *xyl* sequence [27]) site in *xylR* on pWH1505, yielding pWH1505K. A fragment from that plasmid resulting from complete digestion with *Nru*I and partial restriction with *Pst*I (because of an additional *Pst*I site in the *neo* cassette) was cloned into pWH1509C cleaved with *Sca*I and *Pst*I. This plasmid was named pWH1519 and is shown in Fig. 2. This construct contains 900 bp from *B. megaterium xyl* between *neo* and *lacZ* and 1.7 and 3.0 kbp, respectively, on the flanking sides. Thus, this plasmid should yield three possible replacement clones resulting from different subsequent recombination events.

Since it has recently been demonstrated that the *cis* element for glucose repression of *xylA* in *B. subtilis* is located in the reading frame (15), we aimed to delete a respective homologous element in *xylA* from *B. megaterium* (see below). Therefore, we deleted the *Sca*I (nucleotide position 1926 in the *xyl* sequence [27])-*Bam*HI (nucleotide position 2103 in the *xyl* sequence [27]) fragment from pWH1518 and pWH1519 by digestion, filling in, and religation. The resulting plasmids pWH1518ΔSB and pWH1519 ΔSB contain transcriptional fusions of *lacZ* to the first 15 bp of the *xylA* reading frame and are called *xylA2-lacZ*.

Single-copy replacement cloning of the *xyl* constructs in *B. megaterium*. *B. megaterium* WH320 was transformed with 0.5 pmol of pWH1518, pWH1519, or pWH1505K. After outgrowth at 30°C, the protoplasts were regenerated overnight at either the permissive (30°C) or nonpermissive (40°C) temperature. About 2×10^4 transformants were obtained with pWH1518 and pWH1519 at 30°C, while none for pWH1519 and only 4 for pWH1518 were obtained at the nonpermissive temperature. This result shows that integration does not frequently accompany transformation. In fact, pWH1505K, which carries the complete *xyl* operon from *B. megaterium*, but no origin of replication for that strain, did not yield any kanamycin-resistant strain. This indicates clearly the low frequency of recombination during protoplast transformation.

Kanamycin-resistant transformants of both plasmids, pWH1518 and pWH1519, were transferred to minimal plates with 1% xylose and 40 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per liter and incubated at 30 and 40°C. Of 100 transformants with either plasmid, all gave blue colonies at 40°C, indicating that at least single-crossover integration of the plasmid into the chromosome had occurred.

The next screen made use of the xylose utilization property of *B. megaterium* WH320::pWH1518. A second recombination could yield wild-type *B. megaterium* WH320 or WH323. The former is *lacZ xylA*$^+$, and the latter is *lacZ*$^+$ *xylA*, rendering it unable to grow on xylose as a sole carbon source. The *xylA* mutant phenotype leads to blue minicolonies on plates containing 1% xylose as well as 0.02% Casamino Acids and yeast extract which can be easily distinguished from colonies of normal size. This screen was performed after overnight growth of *B. megaterium* WH320::pWH1518 in minimal medium with glycerol as the sole carbon source. Of eight independent screening experiments, five yielded minicolonies at a rate of 1 in 10^4. Using the same protocol, the strains *B. megaterium* WH322, WH324, and WH325 were constructed from plasmids pWH1519, pWH1518ΔSB, and pWH1519ΔSB, respectively (Fig. 3). *B. megaterium* WH321 was obtained as large, white, and kanamycin-resistant colonies on minimal plates with 1% xylose, 40 mg of X-Gal per liter, and 4 mg of kanamycin per liter. The markers of these strains as listed in Table 2 were verified. Final proof for the proposed recombinations was obtained from a Southern analysis of *Bgl*II-restricted chromosomal DNA from each strain, using pWH1519 as a probe. The results of that experiment are shown in Fig. 3. *B. megaterium* WH320 yields three signals originating from the 2,100-bp DNA with *xylR* and part of *xylA* and the two flanking fragments of 1,400 and 10,500 bp. Insertion of the *spoVG-lacZ* fusion into *xylA* yields either a 5,400-bp fragment for *xylA1-lacZ* or a 5,200-bp fragment for *xylA2-lacZ*. Integration of the Kn^r cassette into *xylR* introduces a new *Bgl*II site and yields two signals of 1,400 and 2,000 bp instead of the 2,100-bp band for the *xylR* wild-type strains and 5,000 or 5,200 bp for the two *xylA-lacZ* fusion strains. The results in Fig. 3 demonstrate clearly that the respective expected fragments were found for each constructed strain. Thus, the single-copy replacement cloning of all *xyl* derivatives occurred in the genetic arrangements shown in Fig. 3.

The replacement cloning system developed here on the basis of plasmids with a temperature-sensitive origin of replication for *B. megaterium* worked well and conveniently for chromosomal cloning in the *xyl* operon. Our results showed that the majority of single-crossover recombination events take place during growth of the transformed strain rather than during the transformation event. The fact that no single recombination with pWH1505K was obtained indicates that a double recombination of a linear DNA during transformation is very unlikely. In the method described above, the double-recombination event necessary for integration of linear DNA is split up into two single-recombination events. This method should be successful for any given locus on the *B. megaterium* chromosome provided that

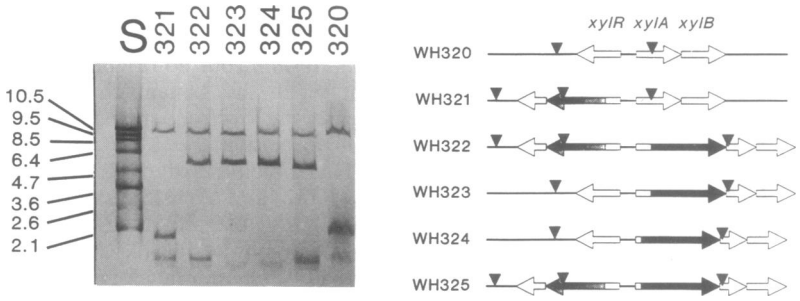

FIG. 3. Southern blot analysis and genetic organization of the *B. megaterium* strains constructed by insertional cloning. Total DNA from *B. megaterium* WH320, WH321, WH322, WH323, WH324, and WH325 was restricted with *Bgl*II and probed with pWH1519 DNA. The result is shown on the left, and the corresponding constructs are depicted on the right side. The lanes are designated with the strain numbers. Lane S contains size markers; fragment sizes in kilobase pairs are given on the left side. The triangles in the constructs indicate *Bgl*II sites. The *xyl* genes are shown as open arrows, the neomycin resistance cassette is shown as a hatched arrow, and the *spoVG-lacZ* fusion is shown as a solid arrow. The expected fragment sizes in kilobase pairs for the strains are WH320, (10.5), 2.1, (1.3); WH321, (10.5), 1.9, 1.4, (1.3); WH322, (10.5), 5.3, 1.4, (1.3); WH323, (10.5), 5.4, (1.3); WH324, (10.5), 5.2, (1.3); WH325, (10.5), 5.1, 1.4, (1.3). Flanking fragments not influenced by the insertions are given in parentheses. *B. megaterium* WH320(pWH1519) fragments are 10.5, 10.5, 5.3, 2.1, 1.4, and 1.3 kbp (data not shown).

selections or screens for the integrated and disrupted genes are available. Thus, this protocol considerably facilitates the construction of stable, recombinant, and plasmid-free strains of *B. megaterium*. It is conceivable that this approach would also work for other bacilli, in particular, those species with a low transformation rate, since only one transformant is needed.

Regulation of *xylAB* transcription by xylose and glucose. The regulation of *xyl* operon transcription had previously been studied in the multicopy situation, in which a 150-fold induction of expression was observed in response to the inducer xylose (26, 27). The strains constructed here enabled us to study the regulation of *xylA* expression in the more relevant single-copy situation by determining β-galactosidase expression. The results are shown in Table 3. The wild-type strain WH320 has no detectable β-galactosidase activity, whereas β-galactosidase expression is 150-fold inducible by xylose in WH323, confirming the results obtained in the multicopy situation (26, 27). In the presence of glucose and xylose, β-galactosidase expression is 14-fold repressed. Even the noninduced level without xylose is 10-fold repressed by glucose. Strain WH322 carries the inactivated *xylR* gene, and consequently, β-galactosidase is constitutively expressed with regard to xylose. Addition of glucose to the growth medium yields a roughly eightfold repression in the presence and absence of xylose. This result shows that glucose repression is, for the most part, not dependent on a functional *xylR* allele. When 177 bp from the *xylA* reading frame are deleted, as in WH324 and WH325, only a marginal glucose repression occurs. The reduced level of β-galactosidase activity expressed in these strains may be a result of the different fusions but is of no relevance for glucose repression since only expression differences of identical strains in dependence on glucose are interpreted. Therefore, only the very small residual glucose effect seen in WH325 is neither due to a functional *xylR* allele nor due to an active *cis* site in *xylA*. Taken together, the results shown in Table 3 demonstrate clearly that (i) glucose repression operates on the RNA level since *xylA-lacZ* fusions are transcriptional fusions; (ii) the major portion of glucose repression is independent of a functional *xylR* allele; (iii) glucose repression depends on a *cis*-acting site within the 177-bp deletion in the *xylA* reading frame; (iv) the xylose-dependent regulation is mediated by the *xylR* gene.

These results for glucose repression are reminiscent of the ones obtained for the *xyl* operon of *B. subtilis* (15), in which a glucose-responsive element with similarity to a consensus element in other catabolite-repressed genes has been identified (36). Inspection of the 177 bp containing the glucose-responsive element of *xyl* from *B. megaterium* shows indeed that this consensus element is present. A comparison of the sequences is shown in Fig. 4. Although rigorous proof for the

TABLE 2. Phenotypes of recombinant *B. megaterium* strains

B. megaterium strain	Color on X-Gal plates[a]		Kanamycin resistance phenotype[b]	Growth on xylose as sole carbon source
	1% xylose	No sugar		
WH320::pWH1518	Blue	White	r	+
WH320::pWH1519	Blue	White	r	+
WH320	White	White	s	+
WH321	White	White	r	+
WH322	Blue	Blue	r	−
WH323	Blue	White	s	−
WH324	Blue	White	s	−
WH325	Blue	Blue	r	−

[a] β-Galactosidase expression was determined on reduced agar plates containing 0.05% yeast extract, 0.05% Casamino Acids, and 1% xylose or no additive sugar.
[b] r, resistant; s, sensitive.

TABLE 3. β-Galactosidase activities of recombinant *B. megaterium* strains

B. megaterium strain	β-Galactosidase activity[a] after growth in minimal medium supplemented with 0.05% yeast extract–Casamino Acids and:			
	No sugar	1% glucose	0.5% xylose	1% glucose–0.5% xylose
WH320	0	0	0	0
WH322	1,400 ± 70	190 ± 12	1,500 ± 66	190 ± 9
WH323	10 ± 0.3	1 ± 0.5	1,500 ± 36	110 ± 5
WH324	1 ± 0.3	4 ± 0.3	430 ± 19	280 ± 8
WH325	390 ± 4	290 ± 5	450 ± 2	330 ± 20

[a] Cells were grown to an optical density of 0.6 at 600 nm, and the activities are given in units (± standard deviation) as defined by Miller (19).

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B.subtilis      TATTTTGGAAGCGTAAACAAAGTGG
                || | ||  |||
                  T     T
consensus       TG·A·CG·T·CA
                  A     A
                ||| | ||  |||
B.megaterium    CATTTTGAAAGCGCAAACAAAGTTT
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FIG. 4. Sequence comparison of the *cis*-active element mediating glucose repression of the *xyl* operon in *B. subtilis* W23 (15) with the putative target sequence for catabolite repression of *xyl* in *B. megaterium*. Both sequences are compared to a consensus sequence based on glucose repression of the *B. subtilis amyE* gene (36). Vertical bars indicate identity of the consensus sequence with the respective *xyl* element. A dot in the consensus sequence indicates no preference for a particular base pair at this position.

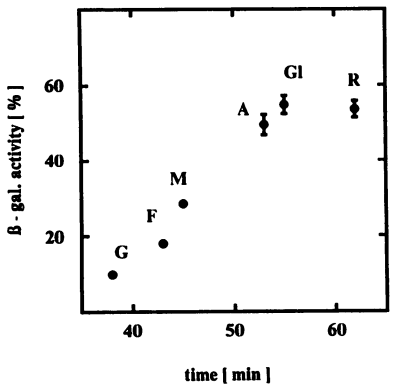

FIG. 5. Plot of the efficiency of catabolite repression versus generation time for different carbon sources in *B. megaterium* WH322. The β-galactosidase (β-gal) activity determined in 0.5% xylose and 1% of the indicated carbon source is given as the percent expression in 0.5% xylose. The generation times were determined for *B. megaterium* WH320 in minimal medium with the respective sole carbon source. Abbreviations: G, glucose; F, fructose; M, mannitol; A, arabinose; Gl, glycerol; R, ribose.

assumption that this is the *cis*-acting element for glucose repression in *B. megaterium* is not yet available, this hypothesis is nevertheless very likely. It can be taken from Fig. 4 that the *B. megaterium* element has one more match with the consensus sequence than does the *B. subtilis* element. This correlates with the 14-fold glucose repression of *xylA1-lacZ* in *B. megaterium* WH323 compared with the 3-fold effect found in *B. subtilis xyl* (15). This more pronounced glucose repression enables us to study the effects of other sugars on *xyl* expression in *B. megaterium*. Another consensus element published for the *gnt* genes of *B. subtilis* is found partially overlapping with the *amy* motif if one mismatch is allowed (Fig. 4). Under the same conditions, the consensus sequence 5'-ATTGAAAG-3' is found six times within the sequenced part of the *xyl* genes (27), curiously with two perfect matches outside the ΔSB fragment, while the *amy* consensus sequence is present only in one copy. Although we cannot exclude any influence of this motif on catabolite repression, it is not very likely that a sequence occurring as frequently as the *gnt* consensus element should be the target for the *xyl* genes. In *B. subtilis*, the *gnt* consensus is also present in the *xyl* genes; it has been shown that deletion of part of this motif does not influence the glucose repression of the *xyl* genes in this organism (15).

Catabolite regulation of *xylAB* expression by various carbon sources. We determined the repression exerted by the carbon sources fructose, mannitol, arabinose, ribose, and glycerol on *xylAB* expression to characterize the glucose effect as catabolite repression. The β-galactosidase activities of *B. megaterium* WH322 to WH325 were quantified after growth in media containing 0.5% xylose alone or in combination with 1% of the sugars given above. The results are presented in Table 4 and show that each of these carbon sources leads to a clear repression. The repression efficiencies vary between 5 and 55% of the fully induced level for glucose and glycerol, respectively. A graph of the generation times of *B. megaterium* in these carbon sources versus their repression efficiencies (Fig. 5) shows a correlation for glucose, fructose, mannitol, arabinose, and glycerol: increasing generation time leads to decreasing efficiency of repression, which is typical for catabolite repression. Only ribose shows a larger repression, as would be anticipated from the long generation time.

The influence of the 177-bp deletion containing the *cis*-acting site for glucose repression on the effect exerted by the other sugars is evident when strains WH323 and WH324 are compared. WH323 contains the glucose-responsive element, while WH324 does not (Fig. 3). These results indicate the presence of two effects; one is a general reduction of expression observed with all carbon sources in the absence of the glucose operator. However, repression is only twofold and no distinction between the different carbon sources is found in WH324. In the presence of the glucose-responsive element, on the other hand, glucose exerts the highest repression, then fructose and mannitol, while glycerol, arabinose, and ribose lead to almost the same repression as in the absence of the *cis* element. These results show that *cis*-acting sites for fructose- and mannitol-mediated repression are also located in the 177 bp at the 5' end of the *xylA* reading frame. While it is not formally proven, it seems very likely

TABLE. 4. Caabolic repression of the *xylA-lacZ* fusions by different carbon sources

Carbon source	β-Galactosidase activity[a] in *B. megaterium* strains			
	WH322	WH323	WH324	WH325
Xylose	100 ±3.8	100 ± 5.6	100 ± 7.5	100 ± 4.5
Xylose + glucose	9.9 ± 1.3	4.7 ± 0.3	47.0 ± 2.2	55.3 ± 2.6
Xylose + fructose	18.0 ± 0.8	16.0 ± 1.4	50.8 ± 3.8	52.3 ± 3.0
Xylose + mannitol	28.5 ± 1.0	25.8 ± 1.8	52.7 ± 2.2	54.3 ± 2.3
Xylose + glycerol	54.8 ± 2.4	53.4 ± 3.9	65.6 ± 5.4	66.9 ± 5.6
Xylose + arabinose	49.5 ± 2.7	46.1 ± 4.2	62.5 ± 4.2	63.8 ± 4.5
Xylose + ribose	53.6 ± 2.2	52.8 ± 3.8	60.6 ± 3.3	59.5 ± 5.5

[a] Cells were grown in minimal medium supplemented with 0.05% yeast extract–0.05% Casamino Acids and the respective carbon source to an optical density of 0.4 at 600 nm. β-Galactosidase activities are given in units (± standard deviation) as defined by Miller (19).

that the glucose-responsive element mediates regulation exerted by fructose and mannitol as well.

Influence of a functional *xylR* gene on catabolite repression and diauxic growth of *B. megaterium*. *B. megaterium* WH320 exhibits diauxic growth in a medium containing glucose and xylose as the sole carbon sources. The generation time of the first log phase is the same as that in glucose minimal medium (40 min), indicating that this sugar is metabolized in that growth phase. The lag phase lasts 70 min before the culture enters the second log phase with a generation time of 75 min, typical for growth on xylose minimal medium. The same experiment was conducted with *B. megaterium* WH321, which is a *xylR* mutant, to check the contribution of Xyl repressor to the diauxic growth behavior (data not shown). The results indicate that diauxic growth does not depend on a functional *xylR* gene. While the growth curve is clearly diauxic with conserved generation times, the lag phase is reduced to about 40 min. Thus, a functional *xylR* gene leads to a longer lag phase but is not the sole cause for diauxic growth. We assume that glucose repression mediated by the *cis* sequence as discussed above may be the major cause for diauxic growth. A comparison of the regulatory effects of different carbon sources on $xylR^+$ and *xylR* mutant strains (Table 4) shows that repression by all carbon sources is somewhat more efficient in the $xylR^+$ strains. This suggests also a small contribution of the Xyl repressor to catabolite repression, possibly via inducer exclusion.

In conclusion, the results presented in this article indicate catabolite repression of the *xylAB* operon on the level of transcription exerted by various sugars to different extents. At least three different mechanisms seem to contribute to catabolite repression. (i) The largest and most differential effect for various sugars depends on the presence of the *cis*-acting element; (ii) a functional *xylR* gene contributes to a small and differing extent to repression; (iii) a basic twofold repression is independent of both elements.

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