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Expression of the *Bacillus subtilis xyl* Operon Is Repressed at the Level of Transcription and Is Induced by Xylose

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Expression of xylose isomerase was repressed in *Bacillus subtilis* strains W23, 168, and BR151 and could be induced in the presence of xylose. The expression was also glucose repressed in strains 168 and BR151, although this effect was not observed with W23. A *xyl-cat* fusion gene was constructed on a multicopy plasmid, from which the *xyl* promoter located on a 366-base-pair (bp) DNA fragment derived from W23 directed the expression of chloramphenicol resistance. The regulation of expression was not very pronounced in this multicopy situation. The *xyl* promoter is a strong signal for transcription initiation. The 5′ sequence of the *xyl* mRNA was identified by nuclease S1 mapping. The promoter consisted of the −10 sequence TAAGAT, the −35 sequence TTGAAA spaced by 17 bp, and an upstream poly(A) block with 14 As out of 17 bp. To study the regulation, a *xyl-lacZ* fusion gene was constructed and integrated as a single copy into the *amy* gene of *B. subtilis* 168. This strain grows blue on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) indicator plates in the presence of xylose and white in the presence of glucose. Quantitatively, the induction of β-galactosidase by xylose was 100-fold. In the presence of xylose plus glucose, the expression of the indicator gene was repressed to 30% of the fully induced level. About 25 to 60% of the maximal *lacZ* expression was obtained with this strain when the 366-bp *xyl* DNA fragment was provided in *trans* on a multicopy plasmid. This result indicates that repression in the absence of xylose is mediated in *trans* by a soluble factor which is expressed at a low level in *B. subtilis* 168. The xylose effect depended on negative regulation. The estimations of mRNA amounts by dot blot analysis showed unambiguously that the induction by xylose occurs at the level of transcription. The possible molecular mechanisms are discussed with respect to the nucleotide sequence of the 366-bp *xyl* regulatory DNA.

D-Xylose is an abundant pentose which can be utilized by many microorganisms. In *Escherichia coli* and *Salmonella typhimurium* this process involves four activities, namely xylose transport and the actions of xylose isomerase, xylulose kinase, and a proposed positive regulator (3, 32). The genes encoding the *xyl* activities are organized in operons. In *S. typhimurium* and *E. coli*, the expression of the *xyl* operons seems to be regulated by a positive control mechanism (32) depending on the presence of xylose and by catabolite repression exerted by glucose (8). In addition, a regulatory effect of the xylose isomerase itself has been described for *E. coli* (3).

The *xyl* operons from *E. coli* (5, 9), *S. typhimurium* (14), and the gram-positive organisms *Bacillus subtilis* (36) and *Streptomyces violaceoniger* (22) have been cloned. It has been suggested that the organization of the genes in operons is similar among these organisms. Nucleotide sequence analysis of the *B. subtilis* xylose isomerase gene revealed extensive homology of the encoded amino acid sequence to that of xylose isomerase derived from *E. coli* (17, 37). However, no data regarding the regulation of expression of the genes encoding the xylose-utilizing proteins in gram-positive organisms are available as yet.

In this article, we define the *xyl* promoter in *B. subtilis* by S1 mapping and describe the construction of a *xyl-lacZ* transcriptional fusion and its single-copy integration into the *B. subtilis* chromosome. Both the xylose isomerase activity expressed from the wild-type gene and the β-galactosidase activity expressed from the *xyl-lacZ* fusion were regulated at the level of transcription and were induced by xylose. Glucose repression was observed in *B. subtilis* 168 and BR151 but not in *B. subtilis* W23. Possible regulatory mechanisms are discussed.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* RRI and *B. subtilis* W23, 168, and BR151 were generally used for transformations as described previously (6, 7). We constructed pWH331 (Fig. 1) from pCPP3 (2) by first eliminating the *Bam*HI site by cutting, filling in the protruding ends, religating to yield pWH330, and then inserting the polylinker from pIC20R (23) into the single *Eco*RI site to yield pWH331. During the cloning procedure, the *cat*-proximal *Eco*RI site was lost when the protruding ends were removed.

Culture and growth conditions. Bacilli were grown in the following media: (i) LB medium (10 g of peptone per liter, 5 g of yeast extract per liter, 10 g of NaCl per liter [pH 7.4]). (ii) Spizizen minimal medium (1), and (iii) Penassay broth (antibiotic medium no. 3; Oxoid Ltd. Basingstoke, England). Plates were prepared with 1% agar (Oxoid). The sugar concentration in minimal medium was 2%, and the required amino acids were supplemented at 40 μg/ml. *lacZ* indicator plates contained 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml. The final concentrations of the antibiotics were 5 or 30 mg of chloramphenicol per liter when selecting for a chromosomally located *cat* gene or for plasmid-coded Cm^r, respectively, 30 mg of kanamycin per liter, and 100 mg of ampicillin per liter. We tested α-amylase expression by growing colonies overnight on an LB plate containing 1% starch and staining the plate with iodine as described elsewhere (31).

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant marker(s)	Source or reference
B. subtilis strains		
168	*trpC2*	BGSC[a] 1A1
BR151	*trpC2 lys-3 metB10*	BGSC 1A40
W23	*thr*	BGSC 2A1
512	*amyE*::(P_{xyl} *spoVG-lacZ'* Cm^r)	This study
E. coli		
RRI	F^- *hsdS20* (r_B^- m_B^-) *recA*$^+$ *ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44*	21
Plasmids		
pCPP3[b]	Km^r	2
pWH300[b]	Km^r	This study
pWH331[b]	Km^r	This study
pWH416[b]	Km^r Cm^r	This study
pWH418[c]	Ap^r	This study
pIW11[c]	Ap^r	36
pIC20R[c]	Ap^r	23
pDH32[c]	Ap^r	33; D. Henner, personal communication

[a] BGSC, *Bacillus* Genetic Stock Center.
[b] The hosts for these plasmids were *B. subtilis* and *E. coli*.
[c] The host for these plasmids was *E. coli*.

General methods. The preparation of plasmid DNA from *E. coli* (15, 19) and *B. subtilis* (19), radioactive labeling (11, 24), DNA sequencing (24), elution of DNA from polyacrylamide and agarose gels (21, 24), and Southern hybridization (35) were done as described earlier. All other recombinant DNA techniques were done as previously described (21). *B. subtilis* protoplasts were regenerated on CR5 medium (29).

Chromosomal DNA from *B. subtilis* was prepared from 100-ml cultures grown to an optical density (600 nm) of 1. The cells were pelleted by centrifugation and suspended in 10 ml of 50 mM EDTA–50 mM NaCl–30 mM Tris hydrochloride (pH 7.9). Lysozyme (80 mg) was added, and the mixture was incubated at 37°C for 30 min. Then sodium dodecyl sulfate to a final concentration of 1% and 5 mg of proteinase K were added, and incubation was continued for 30 min at 37°C. The mixture was extracted once with 10 ml of phenol and once with 10 ml of chloroform-isoamylalcohol (24:1 vol/vol). The aqueous phase was then added drop by drop to 3 volumes of ethanol, and the precipitate was spooled on a glass rod and dissolved in 5 ml of 10 mM Tris hydrochloride (pH 8.0)–0.1 mM EDTA. RNase A (0.4 mg), which had been preboiled for 10 min in 0.1 M sodium acetate (pH 5.0), was added, and the solution was incubated for 30 min at 37°C and treated with 2.5 mg of proteinase K in 1% sodium dodecyl sulfate for 1 h at 45°C. The solution was extracted five times with 5 ml of phenol, five times with 5 ml of chloroform-isoamylalcohol (24:1 vol/vol), and five times with ether. Finally, the DNA was precipitated by the addition of 0.5 volume of 30% polyethylene glycol 6000 in 1.5 M NaCl and incubation for 30 min at 0°C. The mixture was centrifuged, and the DNA was washed twice with ethanol and redissolved in 1 ml of 10 mM Tris hydrochloride (pH 8.0)–0.1 mM EDTA. The yield varied typically between 0.5 and 1 mg.

Isolation and dot blot hybridization of RNA. Total RNA for the S1 mapping experiment was isolated from *B. subtilis* BR151 with either pWH331 or pWH416 grown in 1 liter of LB medium at 37°C. For the dot blot hybridization, RNA was isolated from *B. subtilis* 512 grown in minimal medium supplemented with 0.05% yeast extract and either 2% xylose or 2% glucose to an optical density (600 nm) of approximately 0.8, exactly as described previously (34). RNA was denatured with glyoxal, and samples were dotted onto nitrocellulose filters (Schleicher & Schüll, Dassel, Federal Republic of Germany). The filters were baked, prehybridized, hybridized, and washed as previously described (38).

S1 mapping. The DNA probe containing the *xyl* promoter was isolated from pWH416 by digestion with *Dde*I, dephosphorylation of the fragments with calf intestine phosphatase, and elution of the 2,073-base-pair (bp) DNA from a 1% agarose gel. After 5'-end labeling with [γ^{32}P]ATP, the DNA was cleaved with *Eco*RI, and the 580-bp DNA probe was eluted from a 5% polyacrylamide gel. The probe (12,000 cpm) and 150 μg of RNA were ethanol precipitated, dried in vacuo, dissolved in 30 μl of 40 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); pH 6.4]–1 mM EDTA–0.4 M NaCl–80% formamide, heated to 75°C for 10 min, and allowed to cool overnight to 30°C. Then, 300 μl of 0.28 M NaCl–0.05 M sodium acetate (pH 4.6)–4.5 mM $ZnSO_4$ and 250 U of nuclease S1 were added, and the mixture was incubated for 30 min at 37°C. The reaction was terminated by two phenol extractions followed by two ether extractions. The nucleic acids were then ethanol precipitated, dissolved in 4 μl of 98% formamide–0.05% bromophenol blue–0.05% xylenecyanol, and run on a 6% sequencing gel. The gel was exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens at −70°C.

Enzyme assays. For all enzyme assays, the cells were grown in either LB medium or minimal medium supplemented with 0.05% yeast extract. For induction experi-

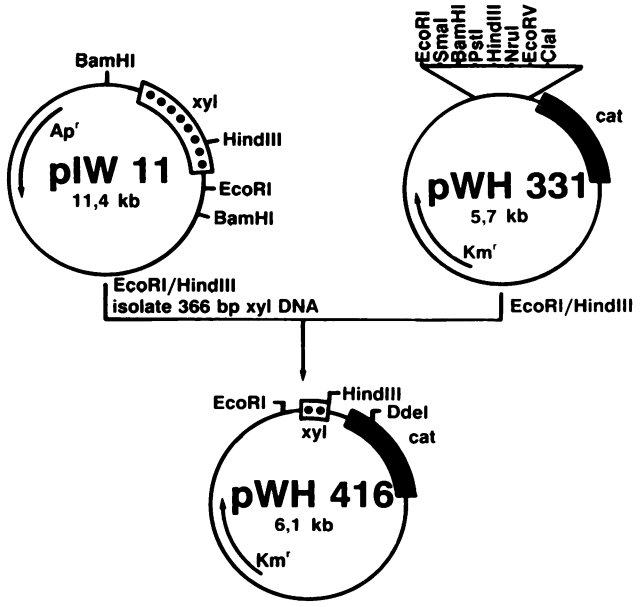

FIG. 1. Construction of a multicopy *xyl-cat* transcriptional fusion. pWH331 is a derivative of pCPP3 (2) carrying a promoterless *cat* gene (filled box) and the polylinker sequence from pIC20R (23). Some sites are indicated. pIW11 was a gift from M. Wilhelm and carries the xylose isomerase gene (dotted box) from *B. subtilis* W23. The 366-bp *xyl* DNA carries the putative *xyl* promoter and the N-terminal coding region of the xylose isomerase gene (37). It was cloned into pWH331 to give pWH416. The construction was done in *E. coli*. pWH416 transformed *B. subtilis* BR151 and W23 to a chloramphenicol resistance phenotype.

ments, 2% xylose, 2% glucose, or both sugars at 2% were added. The chloramphenicol acetyltransferase (CAT) (30) and xylose isomerase assays (4, 36) and the determination of protein concentrations (30) were done exactly as described previously. β-Galactosidase assays were done by the method of Miller (25) and modified as described previously (33).

RESULTS

Regulation of expression of xylose isomerase in *B. subtilis*. Table 2 shows the xylose isomerase activities of *B. subtilis* W23 and 168 and its derivative BR151 (Table 1) under different growth conditions. In minimal medium supplemented with 0.05% yeast extracts, the three strains grew slowly and did not express any detectable xylose isomerase activity. Strain W23 showed no activity in minimal medium supplemented with 2% glucose, whereas 168 and BR151 expressed xylose isomerase at low levels under these conditions. All three strains showed clearly induced xylose isomerase expression when grown in minimal medium supplemented with 2% xylose. In the presence of both xylose and glucose, W23 fully expressed xylose isomerase, whereas for strains 168 and BR151, the expression was glucose repressed. The induction of xylose isomerase expression was at least sevenfold (for 168 and BR151) or greater (for W23).

Nuclease S1 mapping of the *xyl* promoter and expression of a multicopy *xyl-cat* fusion. Part of the *B. subtilis* W23 *xyl* operon has been cloned in *E. coli*, and the regulatory sequence has been determined previously (36, 37). To map the transcription start nucleotide in *B. subtilis*, the *Eco*RI-*Hin*dIII *xyl* DNA fragment containing the putative promoter and the start of the xylose isomerase gene was cloned from pIW11 (36) in front of the *cat* gene on pWH331 derived from pCPP3 (2). The cloning experiment and the structure of the resulting plasmid, pWH416, are outlined in Fig. 1.

B. subtilis BR151 was transformed with pWH416, and *cat* gene expression under transcriptional control of the *xyl* sequence was determined (Table 3). *B. subtilis* BR151 without a plasmid showed no detectable CAT activity, whereas the same strain transformed with pWH331 carrying the promoterless *cat* gene expressed 30 to 50 U of CAT (30) under all growth conditions. BR151 transformed with pWH416 expressed 118,000 U of CAT in the presence of 2% xylose. In the presence of 2% glucose, the expression was reduced to 36,000 U, and with both glucose and xylose, 42,000 U of CAT was measured. When the bacteria were grown in LB medium, CAT expression was approximately 25,000 U under all conditions (data not shown). Strain W23 was also transformed with pWH416. When grown in LB medium, this strain expressed 25,000 U of CAT also, indicating that the 366-bp *xyl* DNA contains identical activities for transcription initiation in strains BR151 and W23. This result further indicates that the *xyl* promoter is indeed located on the *Eco*RI-*Hin*dIII fragment, as suggested previously (37). Furthermore, it appeared to be a strong signal for transcription initiation on the scale of *B. subtilis* promoters (26, 27). The regulation of expression described above for the xylose isomerase activity was found only to a very small extent on this multicopy construction. The xylose-grown cells expressed about three times as much CAT as did the glucose-grown cells, and cells grown in the presence of both glucose and xylose showed a threefold repression of expression by glucose. Therefore, pWH416 is useful for the determination of the promoter sequence when the regulatory properties are studied using a single-copy gene fusion, as described below.

TABLE 2. Inducibility of xylose isomerase activities in *B. subtilis* strains

Strain	Sp act[a] of xylose isomerase (mM/mg · h) after growth in minimal medium[b] supplemented with:		
	2% Xylose	2% Glucose	2% Xylose and 2% glucose
W23	5.9 ± 0.7	None[c]	7.5 ± 0.5
168	9.9 ± 1.4	1.0 ± 0.9	1.2 ± 1.2
BR151	6.8 ± 1.2	0.5 ± 0.5	0.9 ± 0.3

[a] The activities were determined in four independent experiments, as previously described (4). Values are means ± standard deviations.

[b] Minimal medium was supplemented with 0.05% yeast extract and did not have any other carbon source. When no sugar was added, no xylose isomerase activity was detectable.

[c] None, No detectable xylose isomerase activity.

TABLE 3. CAT expression from a multicopy *xyl-cat* fusion

B. subtilis strain	Sp act of CAT (nmol/min · mg) after growth in medium supplemented with[a]:		
	2% Xylose	2% Glucose	2% Xylose and 2% glucose
BR151	None	None	None
BR151(pWH331)	54	28	29
BR151(pWH416)	118,000	36,000	42,500

[a] Values are means from three independent experiments (standard deviation, ±10%). The units are defined elsewhere (30). Measurements were done in minimal medium supplemented with 0.05% yeast extract. CAT activity in medium without sugar supplement was not determined. None, No CAT activity found.

Total RNA prepared from BR151 transformed with either pWH331 or pWH416 (Fig. 1) was used to determine by S1 mapping the start site of transcription from the *xyl* promoter in the heterologous host BR151 (Fig. 2). The DNA probe used is described in the legend to Fig. 2, and the relevant restriction sites are shown in Fig. 1. BR151 transformed with pWH331 did not synthesize RNA that will protect the DNA probe from degradation by nuclease S1. BR151 transformed with pWH416 contained an RNA which protected the DNA probe up to a single site located 250 bp away from the end label. The sequence interpretation of the S1 mapping result is also given in Fig. 2. The sequence identified a *xyl* promoter suggested previously on the basis of sequence homology to other promoters in *B. subtilis* (37). The −10 and −35 regions, along with the upstream A block of the *xyl* promoter, are depicted in Fig. 3. The resolution of the gel (Fig. 2) did not allow the identification of the start nucleotide precisely; however, it is clear that the transcription of the *xyl* mRNA started in the AAT sequence (Fig. 3). It should be pointed out that no further site of transcription initiation was detectable in this experiment.

Construction and regulation of expression of a single-copy *xyl-lacZ* fusion in *B. subtilis*. To study the regulation of *xyl* expression in *B. subtilis*, we constructed a fusion of the *Eco*RI-*Hin*dIII *xyl* promoter DNA with the *spoVG-lacZ* fusion gene contained on plasmid pDH32 (18, 33; D. Henner, personal communication) and integrated the fusion via double recombination with the *amy* gene stably into the chromosome of *B. subtilis* 168. For that purpose, pDH32 was digested with *Bam*HI, the protruding ends were filled in, and the DNA was redigested with *Eco*RI. pIW11 was digested with *Hin*dIII, the protruding ends were filled in, and the

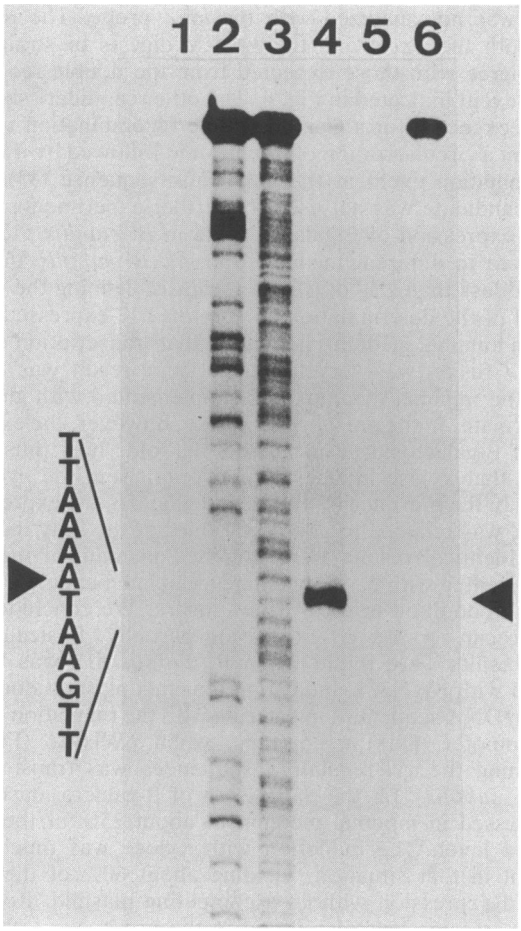

FIG. 2. S1 nuclease mapping of transcription start from *xyl* promoter. An autoradiograph of a sequencing gel is shown. Lanes 2 and 3 show G and G+A sequencing reactions, respectively, of the 580-bp DNA probe extending from the *Dde*I site in the *cat* gene (Fig. 1) to the *Eco*RI site flanking the *xyl* DNA (Fig. 1). The *Dde*I end was labeled at the 5′ end. Other lanes: 1, result of an S1 experiment with RNA from *B. subtilis* BR151 transformed with pWH331; 4 an S1 map with RNA from *B. subtilis* transformed with pWH416; 5, DNA probe incubated with S1 without RNA; 6, 580-bp DNA probe without S1 treatment. The length of the hybrid is indicated by the triangle on the right. The sequence on the left is for the mRNA-like DNA strand (complementary to the one sequenced). The start region of *xyl* mRNA is indicated by the triangle.

DNA was redigested with *Eco*RI. The 366-bp *xyl* DNA was isolated from a polyacrylamide gel and ligated with the pDH32 DNA obtained as described above to yield pWH418 (Fig. 4). Since the *lacZ* gene in pDH32 is preceded by stop codons in all three reading frames (D. Henner, personal communication), the *xyl* translation initiated on the 366-bp *xyl* DNA should be terminated and translation of *lacZ* should be independent of *xyl* translation.

The procedure for integrating this construct is also outlined in Fig. 4. pWH418 cannot replicate in *B. subtilis*. The constructed gene fusion was preceded by a *cat* gene serving as a selective marker for *B. subtilis*. Furthermore, it was flanked by back and front sequences from the *B. subtilis amy* gene serving as targets for recombination. pWH418 was linearized with *Pst*I and transformed into *B. subtilis* 168 protoplasts. The transformants were selected for chloramphenicol resistance at 5 mg/liter. Starting from 4×10^4 cells

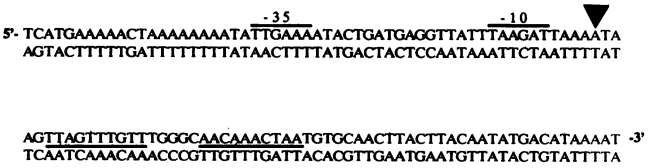

FIG. 3. Nucleotide sequence of *xyl* promoter. The sequence of the *xyl* regulatory region is taken from reference 37. The mRNA start site is indicated by the triangle. The −10 and −35 regions of the *xyl* promoter are indicated. These regions agree with those deduced previously from the nucleotide sequence (37). Sequences with palindromic symmetry (underlined) are located downstream of the *xyl* promoter.

containing 80% protoplast, 16 chloramphenicol-resistant colonies were obtained. Only two of these were *amy* mutants, whereas *B. subtilis* 168 and 14 of the candidates had the *amy*$^+$ genotype. The two *amy* mutant candidates grew as white colonies on X-Gal minimal medium supplemented with 2% glucose and as blue colonies on X-Gal minimal medium containing 2% xylose. The recombination events were analyzed by Southern hybridization of restricted chromosomal DNA from the two candidates. Figure 4 shows the restriction fragments expected from the digestion of chromosomal DNA with *Sac*II and *Eco*RV. The radioactive probes were

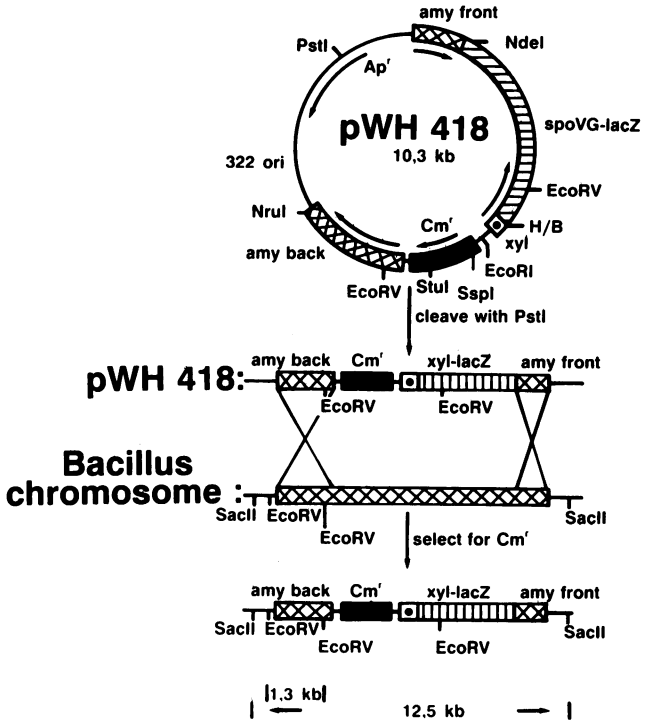

FIG. 4. Construction of single-copy transcriptional *xyl-lacZ* fusion. pWH418 is a pDH32 derivative constructed as described in the text. H/B denotes a filled-in *Hin*dIII site ligated to a filled-in *Bam*HI site. Symbols: ☒, *amy* front and back sequences used for integration; ■, *cat* gene used for selection; ⊡, xylose isomerase-coding region; ▨, *spoVG-lacZ* indicator gene fusion. The restriction sites used to derive DNA probes for Southern and dot blot hybridizations are indicated (see text for details). In the middle part of the figure, the recombination events leading to the chromosomal construction shown at the bottom are indicated. The sizes of DNA fragments expected from *Sac*II and *Eco*RV digests which hybridize with an *amy*-back DNA probe are indicated.

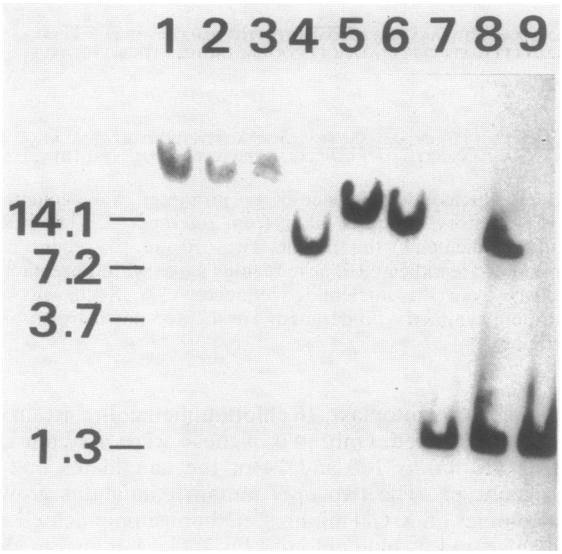

FIG. 5. Southern blot analysis of chromosomal *xyl-lacZ* fusion. An autoradiograph of the analysis with the *amy*-back probe described in the text is shown. Lanes 1, 2, and 3 contain nondigested chromosomal DNA from *B. subtilis* 168, the candidate called 6, and the candidate called 512, respectively. Lanes 4 to 6 contain *Sac*II-digested chromosomal DNA from the same strains. *B. subtilis* 168 (lane 4) shows the wild-type 8-kbp *amy* sequence (33), *B. subtilis* 6 (lane 5) shows a DNA greater than 14 kbp, and *B. subtilis* 512 (lane 6) shows the expected 12.5-kbp *Sac*II fragment. In lanes 7, 8, and 9, *Eco*RV-digested DNA samples from the same strains were analyzed. Strains 168 (lane 7) and 512 (lane 9) show the expected 1.3-kbp *Eco*RV fragment, while strain 6 (lane 8) shows an additional fragment of about 8 kbp. The numbers on the left denote the positions of the marker DNA fragments in kilobase pairs.

derived from an *Eco*RV-*Nde*I double digestion of pDH32 yielding a 1,845-bp DNA fragment from the *lacZ* gene (*lacZ* probe) and an *Eco*RV-*Nru*I double digestion of pDH32 yielding a 1,318-bp DNA fragment from the *amy* back region (*amy* probe) (Fig. 4). The results obtained with the *amy* probe are shown in Fig. 5. The 8-kbp *Sac*II fragment in *B. subtilis* 168 (Fig. 5, lane 4) was replaced by higher-molecular-weight fragments in the two recombinants (Fig. 5). The expected size was 12.5 kbp (Fig. 4), which was found for one of the candidates, designated *B. subtilis* 512 (Fig. 5, lane 6); the other candidate was longer (Fig. 5, lane 5). The *lacZ* probe was silent for *B. subtilis* 168 DNA and yielded identical results for the two candidates (data not shown). The *Eco*RV digest yielded a 1.3-kbp band in strain 168 DNA, two bands of 1.3 and 7.2 kbp for one candidate, and the expected single 1.3-kbp band for strain 512 DNA. This last digest was not analyzed with the *lacZ* probe. The results with both the *Sac*II and the *Eco*RV digests of strain 512 DNA agree with those expected from the double recombination event indicated in Fig. 4. The other candidate showed results expected for a Campbell-type recombination resulting from a circularization of the plasmid followed by a single recombination event at the *amy* back sequence (33). The latter candidate was not used for further experiments.

The expression of β-galactosidase in *B. subtilis* 512 was measured in different media (Table 4). *B. subtilis* 168 expressed less than 1 U of β-galactosidase, defining the background of the determination. *B. subtilis* 512 expressed only 13 U in minimal medium, indicating that transcription of the *xyl-lacZ* fusion was very low. The same result was found when the minimal medium was supplemented with glucose or succinate. In the presence of xylose, however, the expression of β-galactosidase increased 100-fold. It is thus concluded that xylose induces transcription from the *xyl* promoter. In the presence of xylose and glucose, the expression of *lacZ* was reduced to about one-third of the fully induced level. Identical results were obtained in minimal medium supplemented with 0.5% succinate as an independent carbon source in addition to the various sugars. We conclude that glucose can repress *xyl* expression partially. Basically the same results were found when *B. subtilis* 512 was transformed with pWH331, indicating that this plasmid does not contain DNA sequences interfering with the regulation of the *xyl* promoter. On the contrary, when pWH416 (Fig. 1) containing the *xyl* regulatory sequences was transformed into *B. subtilis* 512, the expression of β-galactosidase was derepressed in minimal medium to about 25% of the fully induced level. The induction with xylose was much less efficient in that situation, yielding about 60% of the fully induced expression without a competing plasmid. Roughly 25% expression was also found in the presence of glucose and glucose plus xylose. These results demonstrate clearly that multiple copies of the *Eco*RI-*Hin*dIII fragment containing the *xyl* regulatory DNA interfere with the regulation of the single-copy *xyl-lacZ* fusion. Therefore, *trans*-acting elements have to be involved in this regulation, and their target sequences must be contained in the 366-bp DNA fragment.

Although the *xyl-lacZ* fusion on pWH418 is supposed to be a transcriptional fusion (D. Henner, personal communication), we verified that the regulatory effects described above indeed occur at the level of transcription by measuring the amounts of *lacZ* and *cat* mRNAs present in *B. subtilis* 512 grown under different conditions. For that purpose, the strain was grown in minimal medium supplemented with either 2% glucose or 2% xylose. Total RNA was prepared from both cultures, and a dilution series containing equal amounts of RNA from both preparations was dotted on

TABLE 4. β-Galactosidase expression from single-copy *xyl-lacZ* fusion

B. subtilis strain	Medium[a]	β-Galactosidase activity[b] after growth in medium with:			
		No supplement	2% Xylose	2% Glucose	2% Xylose and 2% glucose
168	MM	<1	<1	<1	<1
512	MM	13 ± 1	1,500 ± 47	10 ± 1	530 ± 16
	MMS	25 ± 3	1,850 ± 50	7 ± 1	520 ± 7
512(pWH331)	MM	19 ± 4	1,300 ± 75	10 ± 0.5	420 ± 12
512(pWH416)	MM	320 ± 100	720 ± 180	200 ± 40	230 ± 50

[a] Measurements were done in minimal medium containing 0.05% yeast extract (MM) or in minimal medium containing 0.05% yeast extract and 0.5% succinate (MMS).

[b] Values are means ± standard deviations from three independent measurements. β-Galactosidase activities are given in units as defined by Miller (25).

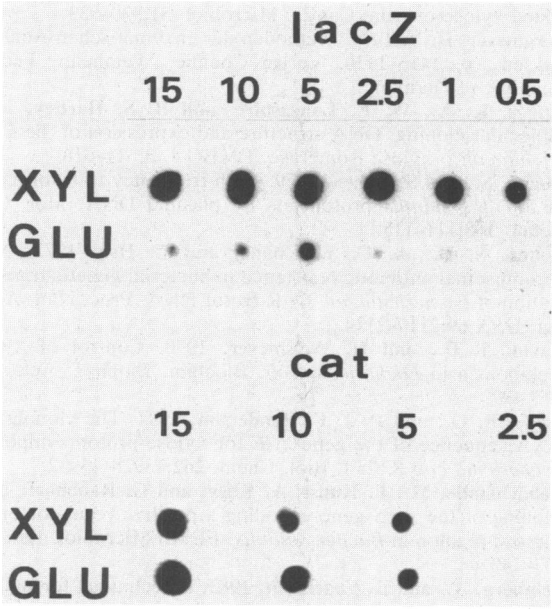

FIG. 6. Dot blot analysis of the amounts of *cat* and *xyl* mRNAs. Autoradiographs of RNA dots with a *lacZ* probe (top) and a *cat* probe (bottom) are shown. The dots from left to right contain successive dilutions of total RNA prepared from *B. subtilis* 512 grown in glucose (GLU)- and xylose (XYL)-supplemented minimal media. The numbers above each filter indicate the amount in micrograms of RNA applied in the respective dots. The specific activities were 3×10^8 cpm/pmol for the *lacZ* probe and 0.6×10^8 cpm/pmol for the *cat* probe. The filters were exposed for 17 h for the *lacZ* probe and for 12 days for the *cat* probe.

nitrocellulose filters and hybridized with either the *lacZ* probe described above or a *cat* probe derived as a 723-bp DNA after the digestion of pDH32 with *Ssp*I and *Stu*I (33) (Fig. 6). The *cat* mRNA was present in roughly equal amounts in *B. subtilis* 512 regardless of the presence of glucose or xylose in the growth medium. This control indicates that both RNA preparations were of the same quality, since no effect of sugar supplementation on *cat* transcription was anticipated. The same RNA samples probed for *lacZ* transcription yielded quite different amounts of that mRNA. When the cells were grown in glucose, the amount of *lacZ* mRNA was reduced about fivefold compared with the *cat* mRNA. In the presence of xylose, the *lacZ* mRNA was clearly induced, and its amount exceeded that of the *cat* mRNA severalfold. The specific activities of the probes are given in the legend to Fig. 6 and were used to estimate the amount of mRNA. Compared with the noninduced state, the greatest dilution shown in Fig. 6 hybridized at least 10 times stronger than did the highest amount of glucose-grown RNA. This result indicates that induction on the mRNA level could be as high as 300-fold. Considering that this value is only a rough estimation, it is fair to conclude that the induction of β-galactosidase activity (Table 4) and *lacZ* mRNA are both about 100-fold. It is thus quite clear that the expression of the *xyl* genes in *B. subtilis* is regulated at the level of transcription.

DISCUSSION

The xylose isomerase and xylulokinase genes from *B. subtilis* have been recently cloned and sequenced (37). In this paper, we describe the regulation of expression of the *xyl* genes in *B. subtilis* W23 and 168. The initial determinations of xylose isomerase activities in both strains indicated clearly that the expression of xylose isomerase is regulated. For both *B. subtilis* strains, induction occurred in the presence of xylose in the culture medium. In addition, for strain 168 and its derivative, BR151, the expression of xylose isomerase was repressed by 2% glucose. This effect was not observed with strain W23.

To map the transcription start of the *xyl* operon, we constructed a *xyl-cat* fusion gene on a multicopy plasmid. In this fusion, the putative *xyl* promoter (37) directed the expression of CAT in BR151. The expression of CAT from this fusion was identical to that from *B. subtilis* BR151 and W23, indicating that a promoter utilized by each strain is located on this DNA and exhibits similar activities in both strains. The regulation of CAT expression from this fusion was not very pronounced; however, the highest expression was found in the presence of xylose. Glucose, either alone or with xylose, reduced the expression of CAT by roughly threefold. We assume that regulatory factors may be titrated by the *xyl* sequence on the multicopy plasmid, resulting in the observed small residual regulation of expression. Owing to the poor regulation in the multicopy state, this construction was only used to map the promoter; regulation is being further investigated by the use of a single-copy fusion gene.

The S1 map established a *xyl* promoter sequence utilized in strain BR151. The sequence agrees with that suggested previously on the basis of sequence homology (37). The promoter sequence agrees well with the consensus sequence established for σ^{43} recognition (26). On the basis of CAT expression from the *xyl-cat* fusion on pWH416, we estimate that the *xyl* promoter is strong (27). The resolution of the S1 map was not sufficient to specify the start nucleotide precisely; however, the mRNA start falls most probably within the AAT sequence (Fig. 2 and 3). No other bands were detected in the S1 map, indicating that a single *xyl* promoter is present on the 366-bp DNA fragment.

To characterize the regulation of *xyl* expression in greater detail, a *xyl-lacZ* fusion gene was constructed by using an integration vector (33). pDH32 contains a *spoVG-lacZ* fusion preceded by stop codons in all three reading frames (D. Henner, personal communication). Thus, the *xyl-lacZ* fusion should monitor regulatory effects at the level of transcription. The α-amylase gene sequences on pDH32 were used to establish a single copy of the *xyl-lacZ* fusion in the chromosome of *B. subtilis* 168, replacing part of the *amy* gene. Southern analysis proved the success of this experiment. The resulting strain contained, with respect to the *xyl* regulatory sequence, one copy of its own *xyl* operon and one additional copy of the 366-bp *xyl* promoter region obtained from *B. subtilis* W23. Colonies of this strain on X-Gal indicator plates were white in the presence of glucose and blue in the presence of xylose.

The different β-galactosidase activities expressed in that strain when grown in different media established the regulation of the *xyl-lacZ* fusion very clearly. The induction by xylose was approximately 100-fold, and the repression by glucose in the presence of xylose led to expression of about 30% of the fully induced level. We thus conclude that the regulation of expression of the *xyl-lacZ* fusion is qualitatively the same as that of the xylose isomerase in strain 168. Quantitatively, for strain 168, glucose repression was greater on xylose isomerase expression than on the heterologous *xyl-lacZ* fusion. However, this regulation was functional with the nucleotide sequence from W23 in the background of 168.

The dot blot estimations of mRNA amounts confirmed the result that at least the induction of expression mediated by xylose occurs at the level of transcription. RNA from the same preparations contained identical amounts of *cat* mRNA when grown in glucose and xylose, while the *lacZ* mRNA was clearly induced by xylose. Although the quantitative estimation of relative mRNA levels was only approximate, the result seems to indicate that the 100-fold induction of expression seen for β-galactosidase is due to a 100-fold induction of transcription.

The *xyl* regulatory sequence on the multicopy plasmid pWH416 was used to study the derepression of the single-copy *xyl-lacZ* fusion. The results (Table 4) show that the expression of *lacZ* was induced to about 200 U in the absence of xylose and in the presence of glucose and glucose plus xylose. The derepression in the absence of xylose suggests that a xylose-dependent regulatory protein is titrated. This result indicates that xylose induction is mediated by a soluble factor in *trans*, most likely a repressor which can be inactivated by xylose and which is present only in small amount in the *B. subtilis* 168 cell. In the presence of xylose and the 366-bp DNA on pWH416, the *xyl* promoter was induced only by 60%. Further investigations are required to clarify the mechanisms mediating this effect.

The *xyl* operons in *E. coli* and *S. typhimurium* seem to be regulated by a xylose-dependent positive control mechanism (3, 32). The result described here, namely the increased expression of the *xyl-lacZ* fusion when titrated with *xyl* regulatory DNA in *trans*, suggests a negative regulation of the *xyl* operon in *B. subtilis*. This regulation may be mediated by a repressor protein, as has been described for penicillinase expression (16). Inspection of the nucleotide sequence (Fig. 3) revealed the presence of an element with palindromic symmetry typical for bacterial operators. A region with high sequence homology to this putative operator has recently been identified for the *xyl* regulatory sequence in *B. subtilis* 168 (S. Hastrup, personal communication). Experiments are currently under way to identify the regulatory elements.

The effect of glucose on gene expression in *B. subtilis* is well established (12, 28). Different mechanisms for glucose repression in this species have been discussed (10, 13, 20). The search for sequence homology with other glucose-repressed operons did not give any hint of a possible target in the *xyl* operon. It may also be possible that the repression observed with the W23 *xyl* promoter in BR151 is due to inducer exclusion of xylose by glucose. Further studies are in progress to define the molecular basis for the glucose effect.

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LITERATURE CITED

1. **Anagnostopoulos, C., and J. Spizizen.** 1960. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
2. **Band, L., D. G. Yansura, and D. J. Henner.** 1983. Construction of a vector for cloning promoters in *Bacillus subtilis*. Gene **26**: 313–315.
3. **Batt, C. A., M. S. Bodis, S. K. Picataggio, M. C. Claps, S. Jamas, and A. J. Sinskey.** 1985. Analysis of xylose operon regulation by Mud (Ap^r, lac) fusion: trans effect of plasmid coded xylose operon. Can. J. Microbiol. **31**:930–933.
4. **Bergmeyer, H.-V.** 1974. Methoden der enzymatischen Analyse, 3rd ed., p. 1416–1420. Verlag Chemie, Weinheim, Federal Republic of Germany.
5. **Briggs, K. A., W. E. Lancashire, and B. S. Hartley.** 1984. Molecular cloning, DNA structure and expression of the *Escherichia coli* D-xylose isomerase. EMBO J. **3**:611–616.
6. **Chang, S., and S. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168**:111–115.
7. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.
8. **David, J. D., and H. Weismeyer.** 1970. Control of xylose metabolism in *Escherichia coli*. Biochim. Biophys. Acta **201**: 497–499.
9. **Davis, E. O., and P. J. F. Henderson.** 1987. The cloning and DNA sequence of the gene *xylE* for xylose-proton symport in *Escherichia coli* K12. J. Biol. Chem. **262**:13928–13932.
10. **Débarbouillè, M., F. Kunst, A. Klier, and G. Rapoport.** 1987. Cloning of the *sacS* gene encoding a positive regulator of the sucrose regulon in *Bacillus subtilis*. FEMS Microbiol. Lett. **41**: 137–140.
11. **Feinberg, A., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
12. **Fisher, S. H., and B. Magasanik.** 1984. Isolation of *Bacillus subtilis* mutants pleiotropically insensitive to glucose catabolite repression. J. Bacteriol. **157**:942–944.
13. **Fujita, Y., and T. Fujita.** 1986. Identification and nucleotide sequence of the promoter region of the *Bacillus subtilis* gluconate operon. Nucleic Acids Res. **14**:1237–1252.
14. **Ghangas, G. S., and D. B. Wilson.** 1984. Isolation and characterization of the *Salmonella typhimurium* LT2 xylose regulon. J. Bacteriol. **157**:158–164.
15. **Hillen, W., R. D. Klein, and R. D. Wells.** 1981. Preparation of milligram amounts of 21 deoxyribonucleic acid restriction fragments. Biochemistry **20**:3748–3756.
16. **Himeno, T., T. Imanaka, and S. Aiba.** 1986. Nucleotide sequence of the penicillinase repressor gene *penI* of *Bacillus licheniformis* and regulation of *penP* and *penI* by the repressor. J. Bacteriol. **168**:1128–1132.
17. **Hollenberg, C. P., and M. Wilhelm.** 1987. New substrates for old organisms. Bio/Technology **1**:21–31.
18. **Igo, M. M., and R. Losick.** 1986. Regulation of a promoter that is utilized by minor forms of RNA polymerase holoenzyme in *Bacillus subtilis*. J. Mol. Biol. **191**:615–624.
19. **Klein, R. D., E. Selsing, and R. D. Wells.** 1980. A rapid microscale technique for isolation of recombinant plasmid DNA suitable for restriction enzyme analysis. Plasmid **3**:88–91.
20. **Mach, H., M. Hecker, and F. Mach.** 1984. Evidence for the presence of cyclic adenosine monophosphate in *Bacillus subtilis*. FEMS Microbiol. Lett. **22**:27–30.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Marcel, T., D. Drocourt, and G. Tiraby.** 1987. Cloning of the glucose isomerase (D-xylose isomerase) and xylulose kinase genes of *Streptomyces violaceoniger*. Mol. Gen. Genet. **208**: 121–126.
23. **Marsh, J. L., M. Erfle, and E. J. Wykes.** 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32**:481–485.
24. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavage. Methods Enzymol. **65**:499–560.
25. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Moran, C. P., N. Lang, S. F. J. LeGrice, G. Lee, M. Stephens, A. L. Sonenshein, J. Pero, and R. Losick.** 1982. Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol. Gen. Genet. **186**:339–346.

27. **Osburne, M. S., and R. J. Craig.** 1986. Activity of two strong promoters cloned into *Bacillus subtilis*. J. Gen. Microbiol. **132:** 565–568.
28. **Price, V. L., and J. A. Gallant.** 1983. The glucose effect in *Bacillus subtilis*. Eur. J. Biochem. **134:**105–107.
29. **Puyet, A., H. Sandoval, P. Lopez, A. Aguilar, J. F. Martin, and M. Espinosa.** 1987. A simple medium for rapid regeneration of *Bacillus subtilis* protoplasts transformed with plasmid DNA. FEMS Microbiol. Lett. **40:**1–5.
30. **Rodriguez, R. L., and R. C. Tait.** 1983. Recombinant DNA techniques: an introduction, p. 187–192. Addison-Wesley Publishing Co., Reading, Mass.
31. **Sekiguchi, J., N. Takada, and H. Okada.** 1975. Genes affecting the productivity of α-amylase in *Bacillus subtilis* Marburg. J. Bacteriol. **121:**688–694.
32. **Shamanna, D. K., and K. E. Sanderson.** 1979. Genetics and regulation of D-xylose utilization in *Salmonella typhimurium* LT2. J. Bacteriol. **139:**71–79.
33. **Shimotsu, H., and D. Henner.** 1986. Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. Gene **43:**85–94.
34. **Shimotsu, H., M. I. Kuroda, C. Yanofsky, and D. J. Henner.** 1986. Novel form of transcription attenuation regulates expression of the *Bacillus subtilis* tryptophan operon. J. Bacteriol. **166:** 461–471.
35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
36. **Wilhelm, M., and C. Hollenberg.** 1984. Selective cloning of *Bacillus subtilis* xylose isomerase and xylulokinase in *Escherichia coli* genes by IS5-mediated expression. EMBO J. **3:**2555–2560.
37. **Wilhelm, M., and C. P. Hollenberg.** 1985. Nucleotide sequence of the *Bacillus subtilis* xylose isomerase gene: extensive homology between the *Bacillus* and *E. coli* enzyme. Nucleic Acids Res. **13:**5717–5722.
38. **Williams, J. G., and P. J. Mason.** 1985. Hybridization in the analysis of RNA, p. 139–144. *In* B. D. Hames and S. J. Higgins (ed.), Nucleic acid hybridization. IRL Press, Oxford.