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Genes Controlling Xylan Utilization by *Bacillus subtilis*

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Eight mutants of *Bacillus subtilis* deficient in xylan utilization were isolated and characterized genetically and biochemically. Each mutant was obtained independently after nitrosoguanidine mutagenesis. All of the analyzed mutations were shown to be linked. Reciprocal transformation crosses revealed the existence of two genes controlling xylan utilization which have been designated *xynA* and *xynB*. Available data have indicated that these two genes code for two xylan-degrading enzymes existing in the wild-type strains, an extracellular β-xylanase (*xynA*) and a cell-associated β-xylosidase (*xynB*).

Bacillus subtilis is able to metabolize xylan. This carbohydrate is found associated with cellulose in plant cell walls and can represent up to one-third of the total sugar content of plant biomass (20). Xylan is a polymer consisting of a β-1,4-linked xylose backbone with branches formed by xylose, other pentoses, hexoses, and uronic acids. Xylan and related compounds are generically designated hemicelluloses.

Microbial degradation of xylan is similar to starch degradation. In both cases, the long chains of the polymer can not be transported inside the cell, being first degraded to oligosaccharides by extracellular carbohydrases. Although no genetic data concerning xylan utilization by *Bacillus* spp. are available, several genes related to starch utilization have been described. Structural gene *amyE*, which codes for α-amylase, and regulatory gene *amyR*, which controls the rate of α-amylase synthesis, are linked (21, 22). There is a third gene, *amyB*, whose mutation causes hyperproduction of α-amylase (14). This *amyB* gene seems to be involved in a general mechanism controlling exocellular enzymes since it has been found to be allelic to *sacU*^{h} and *pap*, which were identified as mutations, unlinked to the other *amy* genes, causing hyperproduction of levansucrase and protease (10, 16, 24).

The ability to metabolize xylan has been reported in different species of bacteria, yeasts, and molds, although in none of these instances have the genes responsible for such ability been identified. Two xylan-degrading enzymes, a xylobiase and a xylose-producing endoxylanase, have been purified and characterized in *Aspergillus niger* (4). Two other enzymes of similar characteristics, described as β-xylosidase and endo-1,4-β-xylanase, respectively, have been found in the yeast *Cryptococcus albidus* (1, 12). An endo-acting xylanase from *B. subtilis* has been investigated (18, 19), and a similar enzyme has been purified from *Bacillus* sp. (W. M. Fogarty and O. P. Ward, Biochem. Soc. Trans. 532nd Meet., Dublin, 1973, vol. 1, p. 260).

This paper reports the isolation and characterization of mutants of *B. subtilis* deficient in xylan utilization.

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MATERIALS AND METHODS

Bacterial strains. The main strains used in this study are listed in Table 1. Appropriate genotypes were constructed by transduction.

Media and growth conditions. Minimal medium was prepared as described by Spizizen (15). Different carbon sources were utilized. Glucose, xylose, and cellobiose were used at a final concentration of 0.5%, and xylan was used at 0.25%. For solid medium, agar was added at a concentration of 2%. Minimal medium was supplemented as required with amino acids and bases at concentrations of 100 and 50 μg/ml, respectively. Tryptose blood agar base (Difco Laboratories, Detroit, Mich.) was used as solid nutritive medium, and Penassay broth (Difco) was used as liquid nutritive medium. GMI and GMII media, used to obtain competent cells for transformation experiments, were prepared as described by Yasbin et al. (23). Liquid cultures were aerated by shaking. All cultures were incubated at 37°C.

Chemicals. Wood xylan was purchased from United States Biochemical Corp. (Cleveland, Ohio). To eliminate soluble contaminating sugars, xylan was suspended in water, autoclaved, and then washed several times with sterile water before addition of the insoluble fraction to the medium. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) and *p*-nitrophenyl-β-D-xylopyranoside were purchased from Sigma Chemical Co., St. Louis, Mo.

Mutagenesis. Nitrosoguanidine mutagenesis was carried out as described by Ruiz-Vázquez et al. (13), with minor modifications. Immediately after the muta-

TABLE 1. List of *B. subtilis* strains

Strain	Genotype	Source
BRC	*lys-3 cysA14*	T. Henkin
1A294[a]	*guaB2 trpC2 metB7 purH1*	J. Heinze and E. Freeze
QB944[a]	*trpC2 cysA14 purA16*	R. Dedonder
QB928[a]	*trpC2 aroI906 dal-1 purB33*	R. Dedonder
QB934[a]	*trpC2 glyB133 metC3 tre-12*	R. Dedonder
QB943[a]	*trpC2 pyrD ilvA1 thyA thyB*	R. Dedonder
QB922[a]	*trpC2 gltA292*	R. Dedonder
QB935[a]	*trpC2 aroD120 lys-1*	R. Dedonder
QB936[a]	*trpC2 leuA8 aroG932 ald*	R. Dedonder
QB917[a]	*trpC2 hisA1 thr-5*	R. Dedonder
QB123[a]	*trpC2 sacA321 ctrA1*	R. Dedonder
R1	*lys-3 cysA14 purB33*	This study
R2	*xyn-1 trpC2*	This study
R3	*xyn-1 trpC2 cysA14*	This study
R4	*xyn-2 trpC2 cysA14*	This study
R5	*xyn-2 trpC2*	This study
R6	*xyn-3 trpC2 cysA14*	This study
R7	*xyn-3 trpC2*	This study
R8	*xyn-4 lys-3*	This study
R9	*xyn-5 lys-3*	This study
R10	*xyn-6 lys-3*	This study
R11	*xyn-7 lys-3*	This study
R12	*guaB2 lys-3 cysA14*	This study
R21	*xyn-8 lys-3*	This study
R22	*xyn-5 lys-3 cysA14*	This study
R25	*xyn-7 lys-3 cysA14*	This study
R30	*xyn-8 lys-3 cysA14*	This study
R32	*xyn-6 lys-3 cysA14*	This study

[a] These strains were from the mapping kit.

gen was washed out, the cell suspension was split into several aliquots which were resuspended in Penassay broth medium. These cultures were incubated for 10 h to allow the expression of the induced mutations. Only one mutant was kept from each culture to assure the independent origin of all recovered mutants.

Transduction. Transduction experiments were performed with the generalized phage PBS1 (17). Donor and recipient strains were grown by the method of Young et al. (25). Transduction lysates were prepared by three successive passages through the appropriate donor strain. Phage stocks were titrated on *B. subtilis* 168 as described by Ivánovics and Csiszár (8). A mapping kit constructed by R. Dedonder (University of Paris) was used for mapping the *xyn* mutations (Table 1).

Transformation. Transformation was carried out according to Boylan et al. (2). Donor DNA was prepared by a modification of the method of Gryczan et al. (5).

Enzyme assays. β-Xylanase activity was determined by measuring the rate of reducing sugar liberation from xylan. The assay mixture, which contained 0.5 ml of supernatant culture and 5 mg of insoluble xylan in 4.5 ml of 0.1 M phosphate buffer (pH 6.5) was incubated at 42°C. The reaction was started with the addition of the culture supernatant. Reducing sugars were measured by the dinitrosalicylic acid method (11). One unit of β-xylanase was defined as the amount of enzyme which liberates from xylan 1 μmol equivalent of xylose in 1 min. This procedure, although nonspecific for determination of β-xylanase, is standardly used for this purpose.

β-Xylosidase activity was determined by a modification of a method described for the assay of β-glucosidase (6). This method is based in the release of nitrophenol from a synthetic substrate. The reaction mixture, which contained 1 ml of permeabilized cells and 2 mg of *p*-nitrophenyl-β-D-xylopyranoside in 1 ml of 0.1 M phosphate buffer (pH 6.5), was incubated at 42°C. Reaction was terminated by addition of 1 ml of 1 M Na_2CO_3. The rate of nitrophenol liberation was measured in a spectrophotometer at 410 nm. One unit of β-xylosidase is defined as the amount of enzyme which produces an increase of 0.01 optical density unit in 1 min.

Cells were permeabilized with toluene as follows. The cultures were washed twice with 0.1 M phosphate buffer (pH 6.5) and finally suspended in one-third of the initial volume. A few drops of toluene were added to the tubes containing the cells and mixed by vigorous vortexing. The tubes were incubated at 37°C for 30 min, after which the toluene was evaporated by blowing air into the tubes.

RESULTS

Isolation of Xyn⁻ mutants. Wild-type strains of *B. subtilis* were able to grow on minimal medium with xylan as sole carbon source. Plates of this medium were white and opaque because of the insolubility of the long chains of the polymer. After 2 days of incubation, the colonies grown on xylan plates appeared surrounded

TABLE 2. Two-factor transduction crosses involving *xyn* and *purB* markers

Recipient genotype[a]	Donor genotype[a]	Cotransfer		% Recombination
		$Pur^+ Xyn^-/Pur^+$	$Xyn^+ Pur^-/Xyn^+$	
purB33 (R1)	*xyn-1* (R3)	207/482		57
	xyn-2 (R4)	236/450		48
	xyn-3 (R6)	201/360		44
	xyn-4 (R8)	185/400		54
	xyn-5 (R22)	110/508		78
	xyn-6 (R32)	107/238		55
	xyn-7 (R25)	108/340		68
	xyn-8 (R30)	149/529		72
xyn-1	*purB33* (QB928)		109/198	45
xyn-2			49/97	50
xyn-3			92/150	39
xyn-4			128/200	36
xyn-5			107/200	47
xyn-6			89/200	55
xyn-7			218/495	56

[a] Only the relevant markers are shown. Pur^+ was selected in crosses in which the recipient strain was *purB33*. Xyn^+ was selected in crosses in which the recipient strains were *xyn-1* to *xyn-7*.

by a clarified halo, owing to the degradation of xylan.

Two wild-type strains were mutagenized with nitrosoguanidine. To assure the selection of mutants specifically affected in xylan degradation but normal for xylose metabolism, the survivors of the mutagenic treatment were first plated on minimal medium with xylose as carbon source. Colonies grown on this medium were replicated onto xylan plates. Two phenotypically distinct kinds of mutants were observed among the clones tested. Mutants of the first class did not grow on xylan plates, but they were able to form a halo surrounding the inoculum. The second class of mutants gave some growth on xylan plates, although no halo could be observed surrounding the growing biomass. Seven mutants belonging to the first class (mutations designated *xyn-1* to *xyn-7*) and one mutant belonging to the second class (mutation *xyn-8*) were isolated. Both classes of mutants appeared among the survivors of the mutagenic treatment at a frequency of about 10^{-3}.

Mapping by transduction. The mutation *xyn-7* was randomly chosen as the first one to be mapped. Transduction crosses involving this mutation and markers covering the entire chromosome were carried out, and it was found that *xyn-7* was linked to *purB33*. Linkage of all the other *xyn* mutations to *purB33* was investigated and found to exist. These results are presented in Table 2. The mean recombination value between the different *xyn* mutations and *purB33* was around 50%. According to Henner and Hoch (7), this value corresponds to 1.3% of the total chromosome length.

Two additional markers located in the vicinity of *purB33* were used for a more precise mapping. Mutation *guaB2* is on the left side of *purB33* and tightly linked to it. Mutation *tre-12* is on the right side of *purB33*. Recombination values of these two markers with *purB33* are 8% (D. H. Dean, D. M. Ellis, and M. J. Kaebling, 1982, The *Bacillus* Genetic Stock Center, *Catalog of Strains*, 2nd ed, Columbus, Ohio; this study) and 52% (10; this study).

Table 3 shows recombination values obtained from crosses involving *guaB2* and the different *xyn* mutations. As could be expected from the close linkage between *purB33* and *guaB2*, recombination values resulting from this experiment were similar to those represented in Table 2.

Results of crosses involving *tre-12* and seven *xyn* mutations are shown in Table 4. Because this experiment was carried out by selecting for Xyn^+ transductants, mutation *xyn-8* was excluded from the cross, as it does not prevent growth on xylan plates. The transduction map resulting from the crosses which have been described is presented in Fig. 1.

Mapping by transformation. Reciprocal transformation crosses involving the different *xyn* mutations were carried out to give a more detailed genetic map. The recipient strains harbored a second marker (Z) used as a reference for the determination of the recombination index which has been defined by the method of Lacks et al. (9). Donor strains carried the wild-type allele corresponding to the second marker (Z^+). Xyn^+ and Z^+ transformants were selected independently. For each cross, the ratio Xyn^+/Z^+ obtained after using DNA from a *xyn* mutant was normalized to the value obtained when the

TABLE 3. Two-factor crosses involving *xyn* and *guaB* markers[a]

Recipient genotype[a]	Donor genotype	Cotransfer Gua$^+$ Xyn$^-$/Gua$^+$	% Recombination
guaB2 (R12)	*xyn-1* (R3)	160/364	56
	xyn-2 (R4)	53/86	38
	xyn-3 (R6)	158/335	53
	xyn-4 (R8)	227/405	44
	xyn-5 (R22)	78/219	64
	xyn-6 (R32)	52/208	75
	xyn-7 (R25)	59/133	56
	xyn-8 (R30)	56/131	57
guaB2 (1A294)	*xyn-8* (R30)	26/100	74

[a] Only the relevants markers are shown. Selection was for Gua$^+$.

DNA came from a wild-type strain. Results obtained in these experiments are presented in Table 5.

Recombination indices obtained from *xyn-8* indicated that this mutation clearly belonged to a different gene than the others. Reciprocal transformation among *xyn-1*, *xyn-3*, *xyn-4*, *xyn-5*, and *xyn-7* mutants gave low recombination indexes. Such low frequencies suggest that these mutations are different alleles of the same gene. *xyn-2* and *xyn-6* mutants showed low recombination frequencies with each other and higher values with all the other mutations. They might represent a third gene. Figure 2 shows the map resulting from the recombination indices determined in these experiments. The gene represented by mutation *xyn-8* is designated *xynA*; the gene represented by *xyn-7* and the other mutations closely linked to it are designated *xynB*.

Xylan-degrading enzymes in wild-type strains. Two xylan hydrolytic activities could be differentiated in wild-type strains of *B. subtilis*: a 1,4-β-xylanase activity, which degraded long chains of xylan to oligosaccharides, and a β-xylosidase (xylobiase) activity, which further hydrolyzed these oligosaccharides to xylose.

The 1,4-β-xylanase activity was assumed to be extracellular, as polysaccharide-degrading enzymes generally are. The effect of different carbon sources (glucose, xylose, cellobiose, and xylan) on the production of this enzyme was investigated. Cells growing with glucose or xylose as the carbon source did not produce activity, whereas cellobiose and xylan appeared to induce identical levels of activity. These results are consistent with those of Forgarty and Ward (Biochem. Soc. Trans. 532nd Meet.) for *Bacillus* sp. Time course production of xylanase by a wild-type strain growing either in xylan or cellobiose was studied. The highest values of specific activity were similar in the two media, and in both cases, the peak of activity was attained after the cultures reached the stationary phase of growth. Because of the Xyn$^-$ phenotype of the mutants studied in this work, cellobiose was found to be a suitable carbon source for cultures to be used in enzyme assays.

β-Xylosidase activity was determined in stationary cultures grown on medium with cellobiose. Preliminary experiments were carried out to determine the localization of the activity. The enzyme was assayed in the supernatant of the culture, in intact cells, in cells which had been permeabilized with toluene, and in cell extracts of mechanically disintegrated cells which were prepared by vortexing cells in the presence of glass beads. The highest values were observed when intact or permeabilized cells were as-

TABLE 4. Two-factor crosses involving *xyn* and *tre* markers[a]

Recipient genotype[a]	Donor genotype	Cotransfer Xyn$^+$ Tre$^-$/Xyn$^+$	% Recombination
xyn-1 (R3)	*tre-12*(QB934)	0/105	>99
xyn-2 (R4)		9/200	95
xyn-3 (R6)		5/113	95
xyn-4 (R8)		0/57	>98
xyn-5 (R22)		7/193	96
xyn-6 (R32)		5/257	98
xyn-7 (R25)		8/478	98

[a] Only the relevant markers are shown. Selection was for Xyn$^+$.

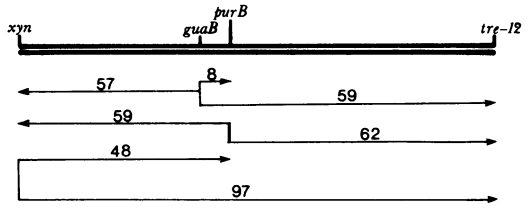

FIG. 1. Transductional map of the *xyn* region. Numbers indicate percentage of recombination. Arrows point to the unselected marker.

sayed. Lower values of activity (about one-half to one-third of that observed for permeabilized cells) were found for culture supernatants, and even lower values (about one-tenth of the activity observed for permeabilized cells) were found for the internal fluid of mechanically disintegrated cells. From these experiments, it was concluded that the activity of β-xylosidase is cell associated and probably membrane associated. In the characterization of the Xyn$^-$ mutants, permeabilized cells were used for the assay of this activity.

The two enzyme activities considered in this study, 1,4-β-xylanase and β-xylosidase, showed identical temperatures and pH optima, 42°C and 6.5.

Biochemical characterization of the Xyn$^-$ mutants. β-Xylanase and β-xylosidase activities were measured in the eight Xyn$^-$ mutants, as well as in the two parental strains, BRC and QB944, from which the mutants were derived; the results are presented in Table 6. *xyn-1*, *xyn-3*, *xyn-4*, *xyn-5*, *xyn-6*, and *xyn-7* mutants showed the same values of β-xylanase activity as did the two parental wild-type strains but reduced β-xylosidase activity, which indicated they were affected in the structural gene coding for this last enzyme. Strain R30 (*xyn-8*), previously characterized because of its inability to form halos in xylan plates, showed the opposite effect, almost no β-xylanase activity and a normal level of β-xylosidase, indicating that this mutant lacks a functional gene for β-xylanase. Finally, mutant R4 (*xyn-2*) showed a decrease for both enzyme activities. This mutation might affect a regulatory mechanism.

DISCUSSION

Genetic and biochemical data obtained from this study are mutually consistent and lead to the following conclusions. Two enzymes, a 1,4-β-xylanase and a β-xylosidase, are responsible for xylan utilization by *B. subtilis*. The structural genes coding for these two enzymes are tightly linked, being located in map position 50 of the *B. subtilis* chromosome, on the left side of *purB33*, distal to *tre-12*.

Mutations in *xyn-1*, *xyn-3*, *xyn-4*, *xyn-6*, and *xyn-7* cause deficiency in β-xylosidase production. These mutations are alleles of a same gene (designated *xynB*), as deduced from the very low recombination indices observed in the pairwise transformation crosses.

According to the results obtained in the transformation crosses, the mutation at *xyn-8* was the only representative of a gene which has been designated *xynA*. This mutation probably affected the structural gene for β-xylanase, since it caused the loss of this extracellular activity. This fact was earlier suggested by the initial phenotypic classification of the mutants by the criteria of halo formation and growth on xylan plates. Although the mutation in *xyn-8* is, among the mutations which have been studied, the only one affecting β-xylanase activity, it should be pointed out that this phenotype was observed to appear at a relatively high frequency. The residual growth of this mutant on xylan plates was initially interpreted as poor growth, owing to unrelated mutations generated during the mutagenic treatment, and thus only one representative of this class was picked for further characterization.

It is interesting to point out the observed linkage of *xynA* and *xynB* in the *B. subtilis* chromosome. This clustering is characteristic of

TABLE 5. Analysis of recombination index

Donor genotype	Recipient genotype (Z marker)						
	xyn-1 cysA	*xyn-2 cysA*	*xyn-3 cysA*	*xyn-4 lys-3*	*xyn-5 cysA*	*xyn-6 cysA*	*xyn-7 cysA*
xyn-1	$<10^{-3}$	0.17	$<10^{-3}$	0.022	0.008	0.11	0.019
xyn-2	0.16	$<10^{-3}$	0.25	0.08	0.028	0.047	0.080
xyn-3	$<10^{-3}$	0.06	$<10^{-3}$	0.04	0.015	0.093	0.031
xyn-4	$<10^{-3}$	0.20	$<10^{-3}$		0.015	0.17	0.045
xyn-5	$<10^{-3}$	0.13	$<10^{-3}$		$<10^{-3}$	0.18	0.018
xyn-6	0.13	0.007	0.24		0.096	$<10^{-3}$	0.091
xyn-7	$<10^{-3}$	0.065	$<10^{-3}$		0.011	0.21	$<10^{-4}$
xyn-8	0.18	0.67	0.60	0.38	0.48	0.62	0.78
Wild type	1	1	1	1	1	1	1

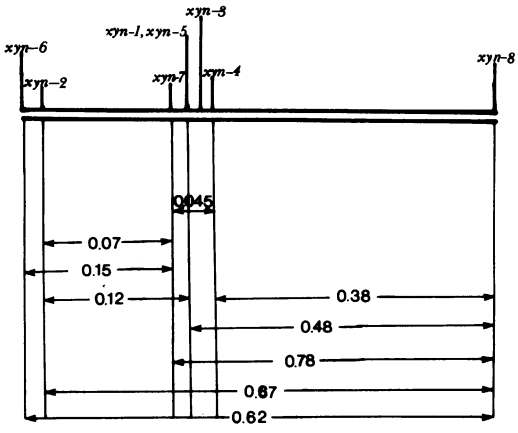

FIG. 2. Transformation map of the *xyn* region. Numbers indicate recombination indices between each pair of mutations.

TABLE 6. β-Xylanase and β-xylosidase activities in Xyn⁻ mutants and in wild-type strains of *B. subtilis*

Strain	Activity (U per 1.4×10^9 cells × ml^{-1} × min) of:	
	β-Xylanase[a]	β-Xylosidase
QB944 (wild type)	0.27	43.5
R3 (*xyn-1*)	0.27	10.4
R4 (*xyn-2*)	0.16	17.9
R6 (*xyn-3*)	0.39	13.8
BRC (wild type)	0.26	45.1
R8 (*xyn-4*)	0.38	25.8
R22 (*xyn-5*)	0.21	17.1
R32 (*xyn-6*)	0.36	15.3
R25 (*xyn-7*)	0.22	4.8
R30 (*xyn-8*)	0.03	48.5

[a] The reported values should correspond mainly to β-xylanase activity, although because of the nonspecificity of the assay used, a contribution from other enzyme activities can not be completely excluded.

functionally related genes in procaryotic organisms.

Results are less clear, however, for *xyn-2* and *xyn-6*. Because of the low recombination frequencies observed between the two mutations, it can be concluded that they are different alleles of the same gene. Whether this gene is allelic to *xyn-1* and the other mutations closely linked to it remains uncertain. Recombination indices between these two groups of mutations are around 0.1. This value, although high, still can be due to intragenic recombination. Two mutations affecting the tryptophan synthetase gene of *B. subtilis* showed a recombination index of 0.2 (3). Biochemical characterization of the *xyn-6* and *xyn-2* mutants does not allow one to distinguish between these possibilities. The characteristics of the *xyn-6* mutant are similar to those of the *xyn-1* mutant, whereas the *xyn-2* mutant showed different properties. This last mutant shows a 50% reduction, in relation to the values found for wild-type strains, for both enzyme activities.

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