Degradation of Xylan by Bacterial Plant Pathogens

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ABSTRACT

Sixty-three plant pathogenic bacteria (41 species) and seven saprophytes (three species) were tested for their capacity to degrade xylan with simultaneous release of reducing sugars into the culture medium. Of these only *Achromobacter* sp., *Pseudomonas acidovorans*, *P. apii*, *P. flectans*, and *Erwinia quercina* were positive. With *Achromobacter* strains, significant amounts of reducing sugars accumulated during growth on 1.0% xylan. Xylose was the principal monosaccharide remaining in culture filtrates of *Achromobacter* strains. Other degradation products included arabionose, galactose, mannose, glucose, di- and trisaccharides. Xylanase activities of 1.3 - 2.8 μmoles reducing sugar/min/mg protein were detected in culture filtrates of *Achromobacter*. Xylanase was not detected, either as an extracellular or intracellular enzyme, in *P. phaseolicola* when corn cob xylan was used as a growth substrate.

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Additional key words: Pseudomonas phaseolicola, enzymes, nutrition.

Achromobacter sp. interacts with *Pseudomonas phaseolicola* resulting in an increase in disease severity of halo blight of bean (7). Growth studies using bean cell wall components as a carbon source revealed that *Achromobacter* utilized a greater variety of hemicellulose substrates in vitro than did *P. phaseolicola* (7). A further examination of hemicellulose utilization indicated that *Achromobacter* degraded xylan and released reducing sugars whereas *P. phaseolicola* did not. The findings prompted the speculation that *Achromobacter* may in part contribute to disease severity by its greater capacity to degrade cell walls. Accordingly, Strobel (12) and Hancock (1) have reported xylanase systems in plant pathogenic fungi and suggested that production of degradative enzymes may be important in pathogenicity.

Although xylanases that degrade plant cell wall xylan are reported for insects (13), fungi (2, 4, 10, 14), and saprophytic bacteria (3, 9), we found no reports of xylanase production by plant pathogenic bacteria. Since xylanase production was a major character distinguishing *Achromobacter* from *P. phaseolicola*, a study was made of the nature of xylan degradation by *Achromobacter*. Seventy strains of bacteria representing 44 species were included in the study to determine whether xylanase production is common among plant pathogenic bacteria.

MATERIALS AND METHODS.—*Degradation and utilization of xylan.*—The capacity of *Achromobacter* sp. and *P. phaseolicola* to hydrolyze and utilize xylan was examined in culture with xylan as the sole carbon source. Two hundred-mg portions of xylan (corn cob) were separately sterilized in 250 ml Klett flasks by dry autoclaving 12 min at 121 C, 85 kg-force/mm^2. Twenty ml of sterilized standard mineral base (SMB) (11) were added to the xylan to make a suspension of 1%. Sterile yeast extract was added at 0.01% because at that concn it does not permit growth, but supplies growth factors which might be needed. Inoculum was prepared from 18-h-old cultures of *P. phaseolicola* strains HB 1b, 20, 23, 26, 31, 33, and 36, and *Achromobacter* strains AB 1a, 4b, and 6a grown on YDCP medium (5). Each flask was inoculated with 8×10^8 cells. Four additional flasks without yeast extract were inoculated with *P. phaseolicola* strains HB 26 and 36 and *Achromobacter* strains AB 4b and 6a. Uninoculated flasks were included as a control for contamination, two with and two without yeast extract. Flasks were shaken continuously (rotary action, 90 cycles/min) at 27 C. Duplicate 0.5 ml samples were removed at 0, 24, 41, 96, and 144 h; one for protein determination as an estimate of bacterial growth (6) and the other for reducing sugar determination (8). The pH of the culture flasks was determined at 0 and 144 h.

In a duplicate experiment to insure that absence of growth was not a factor in the lack of xylan hydrolysis, 0.01 M succinate was included as an additional carbon source since it was not a repressor with β-glucosidase (D. C. Hildebrand, *personal communication*). Growth was followed by measuring turbidity with a Klett-Summerson colorimeter and converting turbidity readings into cells/ml from standard curves prepared previously for each bacterium. One-half ml samples were removed at 72 h and tested for reducing sugar content.

Degradation of xylan by other bacteria.—A range of plant pathogenic and saprophytic bacteria were tested for their capacity to hydrolyze xylan. Xylan concn of 0.3% was used since preliminary tests showed that *Achromobacter* strains produced measurable levels of reducing sugars from xylan at that amount. An SMB-xylan suspension was prepared as previously described, but without yeast extract since this interfered with the detection of small amounts of reducing sugar. In place of yeast, the amino acids glutamine, alanine, serine, asparagine, and aspartic acid (DL forms) were Millipore-filtered and added to give a final concn of 0.01% of each amino acid. These were selected on the basis of nutritional data (D. C. Sands, *unpublished data*) which showed that all of the isolates tested could utilize one or more of these substrates as a single carbon source in SMB medium. Inoculum was taken from YDCP slants containing cultures of the bacteria listed in Table 1. Inoculated media were shaken for 77 h at 27 C and tested for reducing sugars as previously described.

Comparative utilization of xylan.—Bacteria which were positive in the previous survey were tested to quantitatively compare their capacity to hydrolyze xylan. A xylan-SMB-amino acid medium was prepared in 50-ml Erlenmeyer flasks with a xylan concn of 0.1%. Flasks were inoculated with 8.8×10^8 cells of *P. acidovorans*, *P. apii*, *P. flectans*, or *Achromobacter* strain AB 1a, shaken

TABLE 1. Phytopathogenic and saprophytic bacteria tested for capacity to release reducing sugars from xylan

No.[a]	Bacterium	Source and strain[b]
1.	*Achromobacter* sp.	UCBPP AB 1a
2.	*Achromobacter* sp.	UCBPP AB 4b
3.	*Achromobacter* sp.	UCBPP AB 5a
4.	*Achromobacter* sp.	UCBPP AB 6a
5.	*Achromobacter* sp.	UCBPP AB 12
6.	*Agrobacterium tumefaciens*	UCBPP CG 46
7.	*A. tumefaciens*	UCBPP CG 1
8.	*A. tumefaciens*	UCBPP CG 14
9.	*Erwinia amylovora*	UCBPP FB 1a
10	*E. carotovora*	UCBPP EC
11.	*E. quercina*	UCBPP AcC
12.	*Hydrogenomonas pantotropha*	UCBPP 350
13.	*Pseudomonas acidovorans*	UCBPP 14
14.	*P. aeruginosa*	UCBPP
15.	*P. angulata*	UCBPP 1238
16.	*P. apii*	UCBPP
17.	*P. aptata*	ICPB PA-122
18.	*P. cepacia*	CUPP 63-87
19.	*P. cichorii*	NCPB 907
20.	*P. cichorii*	NCPB 1512
21.	*P. coronafaciens*	NCPB 1348
22.	*P. flectans*	UCBPP
23.	*P. fluorescens*	UCBPP 192
24.	*P. garcae*	UCBPP
25.	*P. glycinea*	NCPB 1245
26.	*P. glycinea*	UMPP R1
27.	*P. helianthi*	NCPB 1229
28.	*P. hibicicula*	UCBPP
29.	*P. lachrymans*	NCPB 467
30.	*P. lachrymans*	NCPB 1436
31.	*P. maltophilia*	UCBPP
32.	*P. marginalis*	NCPB 247
33.	*P. marginalis*	UCBPP SR
34.	*P. marginata*	NCPB 316
35.	*P. mellea*	NCPB 280
36.	*P. mori*	NCPB 1445
37.	*P. morsprunorum*	NCPB 560
38.	*P. passiflorae*	ICPB PP-111
39.	*P. phaseolicola*	UCBPP HB 1b
40.	*P. phaseolicola*	UCBPP HB 8
41.	*P. phaseolicola*	UCBPP HB 19
42.	*P. phaseolicola*	UCBPP HB 20
43.	*P. phaseolicola*	UCBPP HB 2b
44.	*P. phaseolicola*	UCBPP HB 31
45.	*P. phaseolicola*	UCBPP HB 33
46.	*P. phaseolicola*	UCBPP HB 36
47.	*P. pisi*	NCPB 1652
48.	*P. polycolor*	UCBPP
49.	*P. primulae*	UCBPP
50.	*P. putida*	UCBPP 90
51.	*P. rubrilineans*	NCPB 359
52.	*P. savastanoi*	UCDPP HCS-2
53.	*P. savastanoi*	UCDPP 200
54.	*P. sesami*	UCBPP
55.	*P. solanacearum*	UFCo SFR-1
56.	*P. solanacearum*	UFCo SFR-2
57.	*P. solanacearum*	UFCo R-B 139
58.	*P. solanacearum*	UFCo R-D 100
59.	*P. syringae*	UCBPP S1
60.	*P. syringae*	UCBPP S6
61.	*P. syringae*	UCBPP S8
62.	*P. syringae*	UCBPP S9
63.	*P. syringae*	UCBPP S21
64.	*P. syringae*	UCBPP S40
65.	*P. tomato*	UCBPP
66.	*Xanthomonas incanae*	UCBPP X1
67.	*X. phaseoli*	UCDPP XP 2a
68.	*X. phaseoli*	UCDPP XP 2b
69.	*X. phaseoli*	UCDPP XP 4
70.	*X. phaseoli*	UCDPP XP 14

[a]Xylan-reduction by selected members of this list is referred to in Fig. 3.

[b]CUPP = Department of Plant Pathology, Cornell University, Ithaca, New York 14860, J. W. Lorbeer; ICPB = International Collection of Phytopathogenic Bacteria, Department of Bacteriology, University of California, Davis 95616, M. P. Starr; NCPB = National Collection of Phytopathogenic Bacteria, Plant Pathology Laboratory, Harpenden, Hertfordshire, England, R. A. Lelliott; UCBPP = University of California, Department of Plant Pathology, Berkeley 94720, M. N. Schroth; UCDPP = Department of Plant Pathology, University of California, Davis 95616, R. G. Grogan; UFCo = United Fruit Company, La Lima, Honduras, I. W. Buddenhagen; UMPP = Department of Plant Pathology, University of Minnesota, St. Paul 55104, B. W. Kennedy.

continuously, and reducing sugar content was measured at 1, 4, 7, and 23 days. To determine the amount of xylan utilized by each bacterium, the remaining xylan in the flasks was filtered (Whatman No. 1 filter paper), washed two times with distilled water, and hydrolyzed in sealed tubes with 2 ml 2.0 N trifluoroacetic acid (TFA) at 121 C for 2 h. The hydrolysate was evaporated to dryness at 45 C under partial vacuum, redissolved in 10 ml distilled water, and a 0.5-ml sample was analyzed for the presence of reducing sugars (8). As standard, a 10.5-mg sample of xylan was carried through the procedure. Assuming that under identical conditions all xylan samples were hydrolyzed equally, xylan remaining in the sample flasks (χ) was calculated by using the proportion:

$$\chi = \frac{\text{(mg reducing sugar in sample)}}{\text{(mg reducing sugar in standard)}} \times \text{(mg in xylan standard)}$$

The amount of xylan remaining after bacterial degradation as determined by the equation was subtracted from the initial weight of the xylan substrate to determine the xylan utilized by the bacteria in each case.

Reducing sugars released into the culture filtrate were qualitatively and quantitatively estimated using paper and gas chromatography procedures (7).

Recovery of xylanase from culture filtrates.—Flasks were inoculated with *P. phaseolicola* and *Achromobacter* sp. and tested for the presence of reducing sugars as previously described. At the peak of reducing sugar release, insoluble xylan was removed by filtration, the filtrate was cooled to 0 C, Millipore-filtered (0.22 μ) and dialyzed 24 h against a 0.01 M phosphate buffer, pH 7.0 at 4 C. The buffer was changed twice. The xylan substrate was prepared by dissolving 20 mg xylan in 1 ml of 4% KOH, adjusted to pH 7.0 with HCl, and diluted to 20 ml with 0.1 M phosphate buffer at pH 7.0 to make a final xylan concn of 0.1%. A reducing sugar test did not reveal

any breakdown of xylan. The assay mixture consisted of 2 ml enzyme solution and 4 ml of xylan in substrate solution. The mixture was incubated at 30 C and 0.5 ml was removed at intervals from 0-162 h and tested for the presence of reducing sugars. For controls, 0.5 ml of each dialyzed sample was tested in absence of the substrate. In addition, xylan was tested for autolytic breakdown using the reducing sugar test. Activity was related to mg protein as determined by Lowry et al. (6). One unit of activity was defined as 1 μmole reducing sugar released/minute (1). Since fungal xylanases are known to have lower pH optima (1, 14), the procedure was repeated using 0.01 M acetate buffer (pH 5.0) and activity was compared to that detected when using phosphate buffer at pH 7.0.

RESULTS.—*Degradation and utilization of xylan.*—*Achromobacter* strains AB 1a and AB 4b released reducing sugars from xylan in a mineral medium supplemented with 0.01% yeast extract at the average rate of 15 μg/ml/h between 24 and 96 h (Fig. 1). After 96 h a rapid decrease in reducing sugars occurred, probably because the bacteria consumed the degradation products. Total protein had increased (representing bacterial growth) to 389 and 464 μg/ml for strains AB 1a and AB 4b, respectively. *Achromobacter* strain AB 6a released reducing sugars at approximately the same rate, but did not utilize the free sugars after 96 h. This strain also produced less growth (protein increase of 233 μg/ml). The seven strains of *P. phaseolicola* did not release detectable reducing sugars into the medium and very slight growth occurred in these cultures (average protein increase of 24 μg/ml). The pH varied little during the experiment; it remained between 6.8 - 7.1 in both *Achromobacter* and *P. phaseolicola* cultures.

When yeast extract (0.01%) was omitted from the medium, *Achromobacter* strains AB 1a and 6a did not grow and reducing sugars did not accumulate, in contrast to *Achromobacter* strain AB 4b which released reducing sugars at 15 μg/ml/h after a 48-h lag phase. Xylanase activity again was not detected in cultures of *P. phaseolicola*. The addition of 0.01 M succinate to the medium enabled *P. phaseolicola* to grow to the extent of $7\text{-}8 \times 10^8$ cells/ml, but only traces of reducing sugars were detected (Fig. 2). The amount of reducing sugars released from xylan by *Achromobacter* in the presence of succinate was the same as when yeast extract was added as a growth factor.

Degradation of xylan by other bacteria.—Only 9 of 70 bacteria tested produced measurable quantities of reducing sugars from xylan in 77 h (Fig. 3). These were *Achromobacter* strains AB 1a, 4b, 5a, 6a, and 12, *P. acidovorans*, *P. apii*, *P. flectans*, and *E. quercina*.

Comparative utilization of xylan.—With an initial xylan concn of 0.1%, little reducing sugar accumulated in culture filtrates of *Erwinia* and *Pseudomonas* strains after 48 h (Table 2). In *Achromobacter* cultures, reducing sugars accumulated at the rate of 0.78 μg/ml/h, which was 1/20 the rate observed when 10 times the concn of xylan was used, as in the previous experiments. Xylan (10.6 mg) was completely utilized in 23 days by *Achromobacter* strain AB 1a and to a lesser extent by *P. acidovorans* and *P. flectans* (Table 3).

Xylose was the principal monosaccharide released by the enzymatic breakdown of xylan in all cultures except

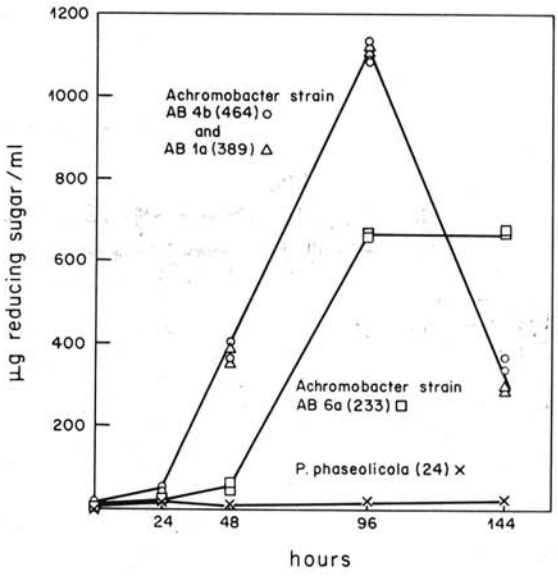

Fig. 1. Reducing sugars released from xylan into the culture medium by *Pseudomonas phaseolicola* (average of seven strains) and *Achromobacter* sp. Numbers in parentheses represent bacterial growth as measured by total μg protein.

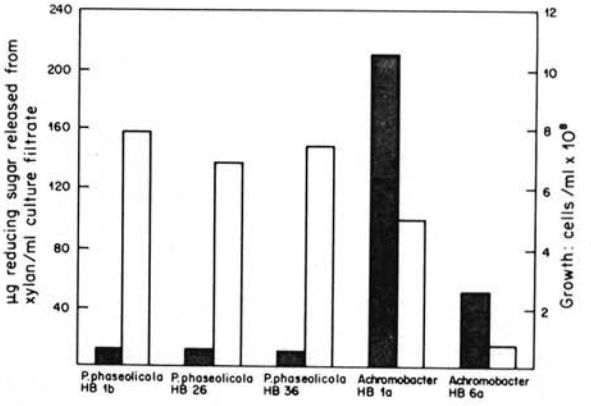

Fig. 2. Growth of bacteria and release of reducing sugars from xylan in a xylan mineral base-succinate medium. Clear bars indicate growth; solid bars indicate quantities of reducing sugars released.

those of *E. quercina*, in which large amounts of arabinose also accumulated. In culture filtrates of *Achromobacter* strain AB 1a, 71.4% of the total reducing sugar accumulated was xylose, 14.4% oligosaccharide, and the remaining 14.2% included arabinose, glucose, galactose, and mannose.

Recovery of xylanase from culture filtrates.—Xylanase activities of 1.3 - 2.8 units/mg protein were found in culture filtrates of *Achromobacter* strains, dialyzed and assayed in phosphate buffer at pH 7.0. The same filtrates exhibited very low activity when assayed in acetate buffer at pH 5.0. Xylanase was not detected in culture filtrates of *P. phaseolicola* or the controls. Insignificant activity (9.5×10^{-4} units/mg

TABLE 2. Reducing sugars released from xylan by bacteria grown on a mineral base medium supplemented with xylan and 0.05% amino acid solution

Bacterium	Reducing sugars detected (μg/ml)			
	24h	98 h	7 days	23 days
Pseudomonas acidovorans	7.0[b]	6.6	10.0	8.3
P. flectans	11.2	6.6	5.0	4.2
Erwinia quercina	12.0	11.2	11.6	14.9
Achromobacter (strain AB 1a)	14.9	88.0	102.9	101.3
Control[a]	2.5	3.3	2.5	1.7

[a]Uninoculated medium.
[b]Each value is the mean ot two determinations.

protein) was detected in sonicated cell suspensions of *P. phaseolicola*.

DISCUSSION.—Xylanase activity does not appear to be a common character of plant pathogenic bacteria since only one *Achromobacter* sp. and four other species of 70 tested bacterial strains degraded xylan with the simultaneous release of reducing sugars. However, positive results might be obtained with some other source of xylan. Accordingly, other experiments showed that *P. phaseolicola* produced xylanase when cultured on bean cell walls (7). Xylanase activity has not previously been shown for plant pathogenic bacteria, or for *Achromobacter* spp. Although only insignificant xylanase activity was detected in culture filtrates of *P. phaseolicola* when cultured on corn cob xylan, indications of growth were sometimes observed with xylan as the sole carbon source using the replica plating technique (7). However, the apparent growth was probably caused by a carry-over of nutrients and cells, a problem inherent in the replica-plating technique. Little multiplication is sufficient to produce a visible patch; the same amount of growth would not be considered significant in liquid culture.

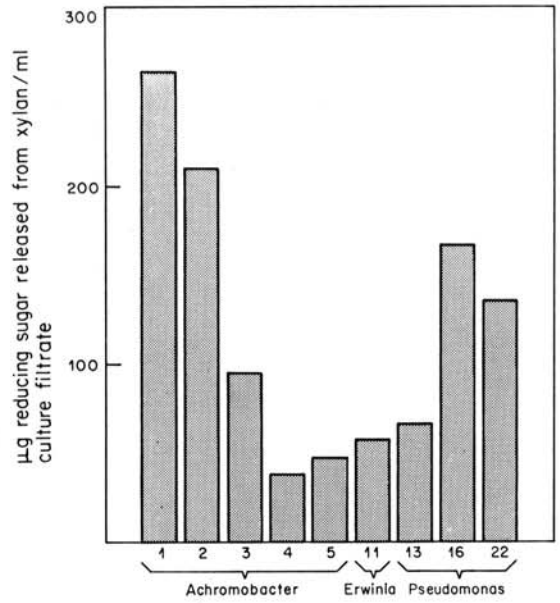

Fig. 3. Reducing sugars released from xylan in 77 h by bacteria grown on xylan mineral base medium supplemented with a 0.05% amino acid solution. Numbers below the graph are references to strains listed in Table 1.

TABLE 3. Degradation of xylan by bacteria

Bacterium	Initial xylan (mg)	Xylan solubilized[a] (mg)
Pseudomonas acidovorans	10.6	3.0
P. flectans	10.0	4.9
Erwinia quercina	10.0	0.3
Achromobacter (strain AB 1a)	10.6	10.6
Control[b]	10.2	0

[a]Measured 23 days after inoculation and calculated on basis of reducing sugars released from xylan standard after 2-h hydrolysis with trifluoroacetic acid. Each value is the mean of two determinations.
[b]Uninoculated xylan mineral base medium carried through the procedure with other samples.

Since the amount of reducing sugars measured in culture filtrates of *Achromobacter* strains represented the remainder after utilization for growth, the rates of reducing sugar release from xylan are greater than those observed. In cultures of *Achromobacter* strains AB 1a and AB 4b, the disappearance of reducing sugars in the medium after 96 h probably occurred because the rate of utilization of breakdown products exceeded their rate of hydrolysis from the polymer. In contrast, the accumulated reducing sugars did not decrease after 96 h in cultures of *Achromobacter* strain AB 6a. Since this strain also did not grow to the extent of the other two strains, and since all the *Achromobacter* strains readily utilized the monosaccharides present in xylan, the accumulated reducing sugars detected were probably not monosaccharides.

When bean plants were inoculated simultaneously with *Achromobacter* sp. and *P. phaseolicola*, growth of *P. phaseolicola* was stimulated (7). Whether this growth and the increased disease severity (7) are related to a nutritional association between the two bacteria and/or the added deleterious effect of *Achromobacter* on the host is not known. No evidence for cross-feeding was found using xylan as a substrate but these tests do not eliminate this possibility within plant tissues (7). *Achromobacter* contrasts with *P. phaseolicola* in its greater capacity to utilize crude bean cell wall materials and hemicellulose from other plant sources, to degrade xylan, and to utilize bean wall glucan and holocellulose. Investigation of hemicellulose-degrading enzymes of *Achromobacter* would be an interesting area to pursue in determining how the bacterium contributes to the disease complex.

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