Genetic Analysis of a Locus on the Bacteroides ovatus Chromosome Which Contains Xylan Utilization Genes

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Bacteroides ovatus, a gram-negative obligate anaerobe found in the human colon, can utilize xylan as a sole source of carbohydrate. Previously, a 3.8-kbp segment of B. ovatus chromosomal DNA, which contained genes encoding a xylanase (xyll) and a bifunctional xylosidase-arabinosidase (xsa), was cloned, and expression of the two genes was studied in Escherichia coli (T. Whitehead and R. Hespell, J. Bacteriol. 172:2408-2412, 1990). In the present study, we have used segments of the cloned region to construct insertional disruptions in the B. ovatus chromosomal locus containing these two genes. Analysis of these insertional mutants demonstrated that (i) xyll and xsa are probably part of the same operon, with xyll upstream of xsa, (ii) the true B. ovatus promoter was not cloned on the 3.5-kbp DNA fragment which expressed xylanase and xylosidase in E. coli, (iii) there is at least one gene upstream of xyll which could encode an arabinosidase, and (iv) xylosidase rather than xylanase may be a rate-limiting step in xylan utilization. Insertional mutations in the xyll-xsa locus reduced the rate of growth on xylan, but the concentration of residual sugars at the end of growth was the same as that with the wild type. Thus, a slower rate of growth on xylan was not accompanied by less extensive digestion of xylan. Mutants in which xyll had been disrupted still expressed some xylanase activity. This second activity was associated with membranes and produced xylose from xylan, whereas the xyll gene product partitioned primarily with the soluble fraction and produced xylulose from xylan.

Plant cell wall polysaccharides from the host's diet are probably a major source of carbohydrate for bacteria that colonize the intestinal tracts of humans and other mammals. Xylan, a polymer consisting of a β-(1,4)-linked xylose backbone with arabinose and glucuronic acid branches, is one of the most abundant polysaccharides in plant cell walls and may thus be an important source of carbohydrate for at least some intestinal bacteria. Xylan is also an important source of carbohydrate for bacteria which colonize the rumen of cattle and sheep. Despite the importance of xylan-degrading bacteria in these habitats, little is known about the xylan utilization pathways of obligate anaerobes. Xylan-utilizing bacteria have been isolated from the human colon and from the rumen of cattle. Some of the xylan-degrading enzymes produced by these obligate anaerobes have been characterized biochemically (5, 8, 12). However, to assess the importance of a particular enzyme for growth on xylan, genetic studies must be done. In particular, it is necessary to disrupt the gene encoding the enzyme so that the effect of losing the enzyme on the organism's ability to utilize xylan can be assessed.

Bacteroides is currently the only genus of gram-negative anaerobes which can be manipulated genetically. Some Bacteroides species can utilize xylan as a sole source of carbohydrate (13) and may thus serve as models for genetic analysis of xylan utilization by gram-negative anaerobes. The best-studied xylan-degrading Bacteroides species is Bacteroides ovatus (12, 20). Recently, Whitehead and Hespell (20) cloned two B. ovatus xylan utilization genes, one of which encoded a xylanase and one of which encoded a bifunctional xylosidase-arabinosidase. Since these genes were adjacent to each other on the B. ovatus chromosome, it was possible that they were part of the same operon. Whitehead and Hespell (20) found that the two genes were expressed independently of each other in Escherichia coli, but this finding did not rule out the operon hypothesis because promoters that drive expression in E. coli may not be the promoters that are being used in B. ovatus. Another question which remained unanswered was the question of whether the xylanase and xylosidase-arabinosidase encoded by the cloned genes were the only xylan-degrading enzymes produced by B. ovatus or whether there were additional xylanases and xylosidases. In this report, we present evidence that the xylanase and xylosidase-arabinosidase are in an operon and that a gene encoding an arabinosidase may also be part of this operon. Additionally, we show that the xylosidase-arabinosidase, rather than the xylanase, is probably a rate-limiting step in xylan utilization. Finally, we demonstrate that the cloned xylanase is not the only xylanase produced by B. ovatus. For this reason, we have designated the cloned xylanase gene xyll. Since the evidence for a second xylosidase is not as strong, we have designated the cloned xylosidase-arabinosidase gene xsa.

MATERIALS AND METHODS

Strains and growth media. B. ovatus 0038 was obtained from the culture collection of the Virginia Polytechnic Anaerobe Laboratory, Blacksburg. Mutants of B. ovatus which had disruptions in the xyll-xsa locus were constructed by conjugation (see below). E. coli DH5aMCR or E. coli HB101 was the donor in these matings. A spontaneous

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rifampin-resistant mutant of \textit{B. ovatus} 0038 (\textit{B. ovatus} V975 [20]) was used as the recipient. For cloning steps, \textit{E. coli} strains were grown in Luria broth (LB). For matings, the \textit{E. coli} donor was grown in LB broth and the \textit{B. ovatus} recipient was grown on Trypticase (BBL, Cockeysville, Md.)-yeast extract-glucose (TYG) medium (9). Transconjugants were selected on TYG medium containing gentamicin (200 \mu g/ml) and tetracycline (10 \mu g/ml).

To test gene disruption mutants of \textit{B. ovatus} for the ability to grow on xylan, glucose, or arabinose and to obtain cell extracts for enzyme assays, \textit{B. ovatus} strains were grown in the RGM medium of Hespell et al. (8). This medium contains Trypticase, yeast extract, salts, and short-chain fatty acids as well as the carbohydrate which serves as the carbon and energy source. In experiments in which xylan was the carbon source, a hot water-soluble fraction of oatspelt xylan prepared as described by Hespell and O'Bryan (7) and added to the medium. The final concentration of carbohydrate in the medium was 0.2%. RGM medium containing 0.2% oatspelt xylan was also used in experiments to assess the extent to which xylan was degraded by the disruption mutants. Growth of bacteria in these media was monitored by measuring optical density at 660 nm. In experiments in which insertional mutants were tested for growth in different media, tetracycline was added to the media (final concentrations of 5 \mu g/ml).

\textbf{Construction of the disruption mutants.} Disruption mutants were constructed by cloning \textit{B. ovatus} DNA from the \textit{xyl1-xsa} locus into pBT-2, a plasmid which replicates in \textit{E. coli} and is mobilized from \textit{E. coli} to \textit{B. ovatus} by R751 but which was unable to replicate in \textit{B. ovatus} (18). pBT-2 contains pJRD215, a low-copy-number IncQ plasmid which contains a kanamycin resistance gene that is expressed in \textit{E. coli}, a tetracycline resistance gene that is expressed in \textit{Bacteroides} species, and the \(\alpha\)-complementing segment of \textit{lacZ} which contains a multiple cloning site. Mobilization of plasmids from \textit{E. coli} to \textit{B. ovatus} and construction of gene disruptions in \textit{B. ovatus} have been described previously (19). The ratio of \textit{E. coli} to \textit{B. ovatus} in these matings was 1:5.

The cloned fragments used to make the gene disruptions are shown in Fig. 1. To make mutants \(\Omega H\) and \(\Omega K\), the 3.8-kbp \textit{EcoRI} fragment from pOX1 (20) was isolated from a low-melting-point agarose gel and blunted. pBT-2 was digested with \textit{BamH}I, blunted, and treated with calf alkaline phosphatase (11). The \textit{EcoRI} fragment from pOX1 was then ligated into the blunted \textit{BamH}I site of pBT-2. The desired orientation placed the pOX1 fragment between a unique \textit{HindIII} site and a unique \textit{KpnI} site in the multiple cloning site of pBT-2 so that the \textit{HindIII} site in the pOX1 fragment (shown to the left of the fragment in Fig. 1) was closest to the pBT2- \textit{KpnI} site and the \textit{KpnI} site in the fragment from pOX1 (shown to the right in Fig. 1) was closest to the pBT-2 \textit{HindIII} site. This clone was designated pBOX1. Digesting pBOX1 with \textit{HindIII} and religating the ends produced a plasmid (pBOX\_H) which contained only the 0.8-kbp \textit{EcoRI}-\textit{HindIII} fragment from pOX1. This plasmid was mobilized into \textit{B. ovatus}, with selection for tetracycline resistance to produce the mutant designated \textit{B. ovatus} \(\Omega H\). Southern blot analysis (not shown) confirmed that this mutant contained a chromosomal insertion in which pBT-2 was flanked by duplicate copies of the 0.8-kbp \textit{EcoRI}-\textit{HindIII} segment. Southern blot analysis and other DNA manipulations were done as described by Maniatis et al. (11). pBOX1 was also digested with \textit{KpnI} and religated to produce pBOX\_H and pBOX\_K, a plasmid which contained only the 0.5-kbp \textit{KpnI-EcoRI} fragment from pOX1 (Fig. 1). This plasmid was used to construct mutant \textit{B. ovatus} \(\Omega K\). To produce mutant \textit{B. ovatus} \(\Omega H, \Omega K, \Omega 20C,\) and \(\Omega K\). The actual insertions, which were made by a single-crossover homologous recombination event, would consist of two copies of the segment shown, flanking the delivery vector pBT-2. The approximate locations of \textit{xsa} (the gene encoding a bifunctional xylosidase-arabinosidase) and \textit{xyl1} (the gene encoding xylanase I) are shown above the restriction map. Dashed lines indicate uncertainty about the exact endpoints of the genes. The arrows indicate the direction of transcription suggested by the results shown in Table 1. The gene upstream of \textit{xyl1}, indicated by ?, is either a gene encoding an arabinosidase or a gene encoding a protein regulating arabinosidase expression. Abbreviations: \textit{RI}, \textit{EcoRI}; \textit{H}, \textit{HindIII}; \textit{Pv}, \textit{PvuI}; \textit{K}, \textit{KpnI}.

To produce \textit{B. ovatus} \(\Omega 20C\), the 2.5-kbp \textit{HindIII-KpnI} fragment was isolated from a low-melting-point agarose gel and digested with \textit{Sau3A}. The resulting fragments were ligated into \textit{BamH}I-digested pBT-2, and colonies containing cloned inserts were identified as white colonies on 5-bromo-4-chloro-3-indolyl-\(\beta\)-galactopyranoside (X-Gal) plates. The white colonies were picked into LB in mixtures containing five to six colonies each, and five of these mixtures were used as donors in matings with \textit{B. ovatus} V975. Only one insertion mutant was obtained in these matings, and this mutant was designated \textit{B. ovatus} \(\Omega 20C\). The location of the insertion (Fig. 1) was determined by Southern blot analysis of various restriction digests of \textit{B. ovatus} \(\Omega 20C\) chromosomal DNA, using the \(^{32}\text{P}\)-labeled \textit{HindIII-KpnI} fragment as a probe. The fragment responsible for the insertion was estimated from these blots to be about 0.4 kbp in size.

\textbf{Xylanase, xylosidase, and arabinosidase assays.} Cell extracts of wild-type \textit{B. ovatus} and the various disruption mutants were prepared from washed and resuspended bacteria, using a French pressure cell as described previously (20). In some experiments, the cell extract was fractionated into soluble and membrane fractions by ultracentrifugation (20). The membrane fraction was resuspended in 50 mM sodium phosphate buffer–1 mM dithiothreitol (pH 6.8), a portion was removed for assay, and the remainder was repelleted by ultracentrifugation. Assays for xylanase (by the orcinol method) and/or xylosidase and arabinosidase (using \(p\)-nitrophenyl substrates) were done as described previously (6, 8).

The products of xylanase I and the other xylanase activity were determined by paper chromatography with acetone-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{restriction_map}
\caption{Restriction map of the 3.8-kbp fragment cloned by Whitehead and Hespell, together with the sizes and locations of the cloned regions used to generate mutants \(\Omega H, \Omega H, \Omega 20C,\) and \(\Omega K\). The actual insertions, which were made by a single-crossover homologous recombination event, would consist of two copies of the segment shown, flanking the delivery vector pBT-2. The approximate locations of \textit{xsa} (the gene encoding a bifunctional xylosidase-arabinosidase) and \textit{xyl1} (the gene encoding xylanase I) are shown above the restriction map. Dashed lines indicate uncertainty about the exact endpoints of the genes. The arrows indicate the direction of transcription suggested by the results shown in Table 1. The gene upstream of \textit{xyl1}, indicated by ?, is either a gene encoding an arabinosidase or a gene encoding a protein regulating arabinosidase expression. Abbreviations: RI, EcoRI; H, HindIII; Pv, PvuI; K, KpnI.}
\end{figure}
butanol-water as the solvent (12) and by thin-layer chromatography with acetonitrile-water as the solvent (2, 20). In both cases, the reaction mixture contained 2 mg of oatspelt xylan, 50 mM sodium phosphate buffer (pH 6.8), 1 mM dithiothreitol, and enzyme. Control assays containing no enzyme or no xylan were also done. The final volume of the reaction mixture was 1 ml. The water-soluble fraction of oatspelt xylan was prepared as described previously (7). Reaction mixtures were incubated for 16 h at 37°C. A portion of the mixture (200 μl) was then removed and added to 600 μl of ice-cold 5% acetic acid in 95% ethanol. This mixture was allowed to stand on ice for 30 min and then centrifuged at 16,000 × g (4°C) for 30 min. The supernatant fluid was lyophilized, resuspended in 20 μl of water, and spotted on the chromatograms. Products of the cloned xylanase (XyII) were determined by using cell extracts of E. coli JM83 carrying pHX10, a clone which expressed XyII but not the arabinosidase-xylosidase (20). The extract added to the reaction mixture contained 400 μg of protein and 5 U of xylanase activity. The products of the other xylanase were determined by using resuspended membranes from wild-type B. ovatus (120 μg of protein, 4.5 U of xylanase activity) or B. ovatus ΔXK, a mutant which did not express either xylL or xsa (240 μg of protein, 2.6 U of xylanase activity). A small amount of xylosidase activity was still detectable in the membrane suspensions from ΔXK and the wild type.

**Extent of xylan digestion by intact bacteria.** Wild-type B. ovatus or the disruption mutant B. ovatus ΔXK was inoculated into RGM-xylan medium (8) and sampled at intervals during growth. Unfractionated samples were assayed for xylan by the orcinol procedure with xylose as the standard (5, 14), for neutral sugars by the gas-liquid chromatography procedure of Albersheim et al. (1) as modified by Stack (17), and for uronic acids by the procedure of Scott (15). Although Trypticase, yeast extract, and the bacterial cells themselves contained polysaccharides, the contribution of these sources to the carbohydrate analyses was negligible compared with the contribution of xylan (data not shown). In addition, samples were fractionated into high- and low-molecular-weight components on the basis of their solubility in an acidic-alcohol solution as described by Coen and Dehority (4). Culture samples were combined with 3 volumes of ice-cold 5% acetic acid in 95% ethanol. The mixture was allowed to stand for 30 min on ice and was then centrifuged at 16,000 × g for 30 min at 4°C. The supernatant fluid was removed, and the pellet was resuspended in the original volume of water. The fractionated samples were assayed for xylan components as described above for the unfractionated samples. Thin-layer chromatography, done as described above, was used to determine the composition of the low-molecular-weight fraction.

**RESULTS AND DISCUSSION**

**Effect of gene disruptions outside xylL and xsa.** Two gene disruptions, ΔH and ΔIK (Fig. 1), were made in the B. ovatus chromosome outside the region known to encode xylL and xsa. These disruptions would affect growth on xylan or xylanase, xylosidase, and arabinosidase activities only if other xylan utilization genes were in the regions interrupted by the insertions and were part of the same operon as xylL and/or xsa. Whitehead and Hespell (20) had not detected expression of genes other than xylL and xsa from the cloned region in E. coli. However, it was possible that other xylan utilization genes that were linked to xylL and xsa had not been detected either because the entire gene had not been cloned or because no fortuitous E. coli promoter caused expression of the gene in E. coli.

The disruption in mutant ΔIK reduced the growth rate on xylan from 1 to 2 h per doubling to more than 4 h per doubling (Table 1). Despite the slower growth rate of mutant ΔIK on xylan, it eventually reached the same optical density as did the wild type. This was not due to loss of the insertion. For one thing, tetracycline (5 μg/ml) was included in the medium to select against loss of the insertion by homologous recombination. For another, reinoculation of ΔIK, which had reached its maximum optical density into a fresh tube of xylan medium, produced the same slow-growth profile. Revertants should have grown as rapidly on xylan as did the wild type when reinoculated into fresh medium. The effect of the ΔIK insertion on growth was specific to xylan because the ΔIK mutant grew as rapidly as did the wild type in medium containing glucose or arabinose as the carbohydrate source. This result also confirms that addition of tetracycline to media in which insertion mutants were grown did not affect growth rates.

Mutant ΔIK also had different enzyme specific activities than did the wild type. Xylanase and xylosidase specific activities were substantially reduced (Table 1). The fact that both xylanase and xylosidase activities were affected by a disruption which did not interrupt either of these genes indicated that the ΔIK insertion produced a polar effect on expression of both xylL and xsa, i.e., that xylL and xsa were part of the same operon. In contrast to the ΔIK disruption, the ΔH disruption did not affect growth on xylan. Specific activities of xylosidase and arabinosidase in cell extracts were similar to those of the wild type (Table 1). In the set of experiments shown in Table 1, the xylanase specific activity in extracts from ΔH was slightly lower than the wild-type activity, although it was much higher than the xylanase activity in extracts from ΔIK. In other experiments, however, xylanase activity in extracts from ΔH was the same as in the wild type. The fact that ΔH was essentially the same as the wild type with respect to growth on xylan and activities of xylan-degrading enzymes indicates that there was no gene downstream of xsa which was essential for xylan utilization.

**Effect of disruptions in xylL and xsa.** To determine which gene in the cloned region was responsible for the difference in growth rate between mutant ΔIK and the wild type,
insertions Ω20C and ΩHK were constructed. Insertion Ω20C lay completely within the xyll gene and should have disrupted this gene. An effect on expression of xsa would also be seen if the Ω20C insertion was having a polar effect. In fact, mutant Ω20C exhibited decreased xylosidase specific activity as well as decreased xylanase activity, as expected if xyll was upstream of xsa in the same operon. Mutant Ω20C exhibited the same slow growth rate on xylan as did the insertion mutant ΩK (Table 1).

To determine whether loss of xyll expression or loss of xsa expression, or both, was responsible for the decreased rate of growth on xylan, we needed a mutant which expressed xyll but not xsa. Initially, we had hoped to obtain an insertion lying within the xsa gene by using the random Sau3A mutagenesis approach which produced Ω20C. However, no insertion within the xsa gene was obtained by this method, presumably because of the small size of the Sau3A fragments in this region. In our experience, it is virtually impossible to obtain a gene disruption in Bacteroides species unless the cloned region is at least 400 bp in size. Thus, to construct a disruption that left xyll intact but eliminated expression of xsa, we made a disruption by using the 2.5-kbp HindIII-KpnI fragment (Fig. 1). Insertion of this fragment into the B. ovatus chromosome would produce a duplication of the integrated region, flanking the vector DNA. Such an insertion would leave the xyll gene intact but would prevent expression of the xsa gene and of the second copy of xyll by interrupting transcription of the operon.

As expected, the xylanase specific activity of this insertion mutant was comparable to that of the wild type, but the xylosidase activity was still decreased to the low level seen in mutants ΩK and Ω20C (Table 1). Mutant ΩHK still had the slow growth rate on xylan associated with the upstream insertions. This result indicated that loss of xsa expression and not loss of xyll expression was responsible for the slow growth on xylan. We consistently observed that the xylanase and arabinosidase activities in cell extracts from mutant ΩHK were about 20 to 30% higher than those in cell extracts from the wild type, but we do not have an explanation for this observation. It is not due to a fortuitous promoter in pBT-2 driving expression of the second copy of the cloned segment, since this would have resulted in an increase in expression of xsa as well as xyll.

**Evidence for the existence of a gene upstream of xyll in the same operon.** Our finding that the insertion in mutant ΩK reduced expression of xyll and xsa, despite the fact that the segment of DNA used to make this insertion did not overlap xyll, indicated that there was at least one gene upstream of xyll in the same operon and that the insertion in ΩK was producing a polar effect on the downstream genes. The possible identity of the putative gene upstream of xyll was suggested by our finding that the xylan-inducible arabinosidase activity of insertion mutant Ω20C was the same as that of the wild type, whereas arabinosidase activity was reduced twofold in mutant ΩK (Table 1). Thus, the gene upstream of xyll encodes either a xylan-inducible arabinosidase or a protein that affects expression of the arabinosidase activity. Whatever the identity of the gene product, its loss was not responsible for the slow growth of ΩK because Ω20C exhibited the same slow-growth phenotype.

Whitehead and Hespell (20) had observed that xylan was not the only carbohydrate which induced expression of arabinosidase activity. Growth on arabinose also led to production of a high level of arabinosidase activity. To determine whether the arabinosidases induced during growth on arabinose were the same enzymes as the ones induced during growth on xylan, we grew the wild type and mutant ΩK on arabinose and measured the arabinosidase specific activity in cell extracts. The arabinosidase activities in the two extracts were identical (data not shown). Thus, the arabinosidase which is affected by the ΩK insertion appears to be induced specifically during growth on xylan. There is at least one other arabinosidase produced by B. ovatus during growth on xylan, because the ΩK mutant still produced some arabinosidase activity.

**Evidence that the true Bacteroides promoter was not on the cloned 3.8-kbp EcoRI fragment.** The phenotypes of mutants ΩK, Ω20C, and ΩHK were all consistent with the hypothesis that the gene upstream of xyll, xyll itself, and xsa are part of the same operon and are not in separate transcriptional units. The promoter operator region of this operon would be located outside the DNA segment cloned by Whitehead and Hespell (20). The fact that Whitehead and Hespell (20) detected expression of xyll and xsa from the cloned region in E. coli indicates that the promoters recognized in E. coli are not operational in Bacteroides species and are not the true Bacteroides promoters. Thus, analysis based on expression of Bacteroides genes in E. coli should not be used to locate Bacteroides promoters.

**Characterization of a second xylanase from B. ovatus.** Insertion mutants ΩK and Ω20C had lower xylanase specific activity than did the wild type, but some xylanase activity was still detectable in cell extracts from these mutants. This result suggested that there was at least one other xylanase beside the one encoded by xyll. When cell extracts from wild-type B. ovatus were ultracentrifuged to pellet membranes, most (70 to 80%) of the xylanase activity remained in the soluble fraction. Resuspending these membranes in phosphate buffer and recentrifuging them resulted in solubilization of over half of the activity that had partitioned with the membranes in the original centrifugation step. When cell extracts from mutant ΩK were fractionated under the same conditions, only 20 to 30% of the xylanase activity was in the soluble fraction. Approximately 30 to 40% of the activity which pelleted with the membrane was removed in the second buffer wash. Thus, the xylanase activity in mutant ΩK appeared to be loosely associated with membranes, whereas the xylanase due to xyll, which accounted for most of the xylanase in the wild type, appeared to be a soluble enzyme.

Although the xylosidase activity in mutants ΩK, Ω20C, and ΩHK was very low, activity was still detectable. This finding might indicate that there is a second xylosidase beside the one encoded by xsa. The residual low level of xylosidase activity fractionated primarily in the soluble fraction (90 to 95% after the first centrifugation step). This activity was so low that no further characterization was attempted. We cannot rule out the possibility that this low level of residual p-nitrophenyl-b-D-xyloside hydrolyzing activity in cell extracts was a side reaction of the remaining xylanase. The fact that the xylosidase activity did not fractionate the same way as did the xylanase activity in extracts from mutant ΩK provides indirect evidence that the xylosidase activity is not due to a xylanase. Nonetheless, since the evidence for a second xylosidase is not as strong as the evidence for a second xylanase, we decided not to designate xsa as xsaI.

We compared the products of xylanase I with those of the xylanase remaining in extracts from ΩK. To obtain xylanase I free of the other xylanase activity and xylosidase-arabinosidase activity, we used extracts from E. coli carrying pOX10, a plasmid that expressed xylanase I but not the
xylan. Mutant ΩK decreased the concentration of xylose and arabinose equivalents in the xylan more slowly than did the wild type, but the composition of the xylan remaining at the end of the incubation period was the same in both cases (Fig. 3).

Although xylose and arabinose are the main constituents of xylan, xylan also contains glucose and hexuronic acids. Interestingly, although the disappearance of xylose and arabinose during xylan digestion was slower by mutant ΩK than by the wild type, the rates of disappearance of glucose and hexuronic acid residues during incubations with the mutant and the wild type were more similar (Fig. 3). This result may indicate that removal of glucose and hexuronic acid residues from xylan is not simply a by-product of xylanase and xylosidase activities but is carried out by separate enzymes which are not affected by the ΩK insertion. There was a trace of galactose and mannose in the xylan preparation (about 2% of total carbohydrate). These residues were utilized little or not at all by both the wild type and the mutant (data not shown).

We also attempted to determine the composition of high- and low-molecular-weight fractions of residual xylan, which were differentiated on the basis of their solubility in acidic-alcohol solution. The concentration of sugars in the soluble low-molecular-weight fraction was too low to allow accurate quantitation of individual components by gas-liquid chromatography, although the sugar composition of this fraction appeared to be the same qualitatively as the composition of the high-molecular-weight fraction. Only xylose, as estimated by the orcinol reagent, was present in sufficient quantity to allow its accurate determination in the low-molecular-weight fraction. A comparison of high- and low-molecular-weight xylose equivalents in cultures of the wild type and ΩK is shown in Fig. 4.

During xylan digestion by the wild type, little low-molecular-weight carbohydrate accumulated. This was also true of mutant ΩK, but the level of low-molecular-weight xylose equivalents was higher than that exhibited by the wild type (Fig. 4). Thin-layer chromatographic analysis of the low-molecular-weight material produced during growth of mutant ΩK revealed xylobiose and higher oligomers of xylose but no xylose (data not shown). Thus, the greater accumulation of low-molecular-weight material during digestion of xylan by mutant ΩK was probably due to slower assimilation of xylan hydrolysis products rather than to loss of xylose produced by the membrane-associated xylan-degrading activity.

Implications for genetic engineering of polysaccharide-utilizing bacteria. Since bacterial digestion of xylan and other plant cell wall polysaccharides makes an important contribution to the nutrition of ruminant animals, there has been considerable interest in improving the efficiency of polysaccharide utilization by genetic engineering of rumen bacteria. Although B. ovatus is a human colonic bacterium, at least one xylan-degrading species of rumen bacteria, Prevotella (formerly Bacteroides) ruminicola, is in the same phylogenetic group (10, 16). Thus, results of our genetic study of B. ovatus xylan utilization may have implications for strategies used to improve xylan utilization by rumen bacteria such as P. ruminicola. To improve the speed and extent of xylan degradation by intact bacteria, it will be necessary to target genes which encode rate-limiting steps in the breakdown process. It has been commonly assumed that xylanase activity would be a rate-limiting step in xylan digestion. In keeping with this assumption, emphasis has
been placed on cloning and characterizing xylanase genes. Our results provide one example of a xylanase gene (xylI) which probably does not encode a rate-limiting step in xylan digestion. Rather, the decrease in growth rate appeared to be due to loss of xylosidase activity. We cannot rule out the possibility that the membrane-associated xylanase is rate limiting, but this possibility cannot be tested at the present time because the gene encoding the other xylanase has not been cloned.

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