Introduction of the Bacteroides ruminicola Xylanase Gene into the Bacteroides thetaiotaomicron Chromosome for Production of Xylanase Activity

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The xylanase gene from the ruminal bacterium Bacteroides ruminicola 23 is highly expressed in colonic Bacteroides species when carried on plasmid pVAL-RX. In order to stabilize xylanase expression in the absence of antibiotic selection, the xylanase gene was introduced into the chromosome of Bacteroides thetaiotaomicron 5482 by using suicide vector pVAL-7. Xylanase activity in the resulting strain, B. thetaiotaomicron BTX, was about 30% of that observed in B. thetaiotaomicron 5482 containing the xylanase gene on pVAL-RX. The data obtained from continuous culture experiments using antibiotic-free medium showed that expression of xylanase activity in strain BTX was extremely stable, with no demonstrated loss of the inserted xylanase gene over 60 generations, with dilution rates from 0.42 to 0.03 h⁻¹. In contrast, the plasmid-borne xylanase gene was almost completely lost by 60 generations in the absence of antibiotic selection. Incubation of strain BTX with oatspelt xylan resulted in the degradation of more than 40% of the xylan to soluble xylooligomers. The stability of xylanase expression in B. thetaiotaomicron BTX suggests that this microorganism might be suitable for introduction into the rumen and increased xylan degradation.

Digestion in ruminants is accomplished by a complex population of microorganisms that inhabit the rumina of these animals. This symbiotic relationship enables ruminants to utilize feedstuffs, such as cellulose and hemicellulose (xylans), that are not digestible by other mammals. The efficiency of animal production is largely dependent upon the efficiency of processes conducted by the ruminal microorganisms. These processes, however, are inherently inefficient, with energy losses occurring in the conversion of carbohydrate to microbial cells, volatile fatty acids, gases (CO₂ and CH₄), and heat. Thus, there is considerable interest in manipulating ruminal fermentation to optimize digestion in the rumen. Methods to accomplish this include altering the physical form of the feed, addition of buffering agents to diets, and inclusion of ionophores in rations. More recently, the use of molecular biological techniques to genetically manipulate the organisms has been suggested as a possible approach (for recent reviews, see references 5, 8, and 16).

One means by which ruminal fermentation can be manipulated is to alter the digestion of polysaccharides in the rumen. Plant polysaccharides such as starch and pectin can be extensively digested, whereas hemicelluloses or xylans are degraded much less extensively (20). Enhancement of the ability of certain ruminal bacteria to degrade xylans could improve digestion in the rumen. Bacteroides ruminicola strains are among the predominant organisms in the rumen, and many strains are xylanolytic (4). In addition, the first gene cloned from B. ruminicola encoded for xylanase (22). However, little has been achieved towards establishing genetic systems for this organism in order to use the cloned xylanase gene for increased xylanase expression.

The xylanase gene from B. ruminicola, interestingly, could be transferred to and highly expressed in the colonic Bacteroides species B. fragilis and B. uniformis (23), which are nonxylanolytic. These species make up about 20% of the human colonic microflora, indicative of their ability to compete for limited nutrients in the colonic ecosystem. On the basis of these criteria and the availability of genetic systems, it was decided that a Bacteroides sp. of human origin might be a suitable candidate as a test organism for overproduction of xylanase activity and increased xylan degradation in the rumen. We now report on the insertion of the B. ruminicola xylanase gene into the chromosome of Bacteroides thetaiotaomicron and production of high levels of xylanase activity by this genetically modified strain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Strains and plasmids used in this study are shown in Table 1. B. thetaiotaomicron 5482 (ATCC 29148) and plasmids pVAL-1, pVAL-7, R751, and pA818 were obtained from Abigail A. Salyers, University of Illinois, Urbana. For conjugation experiments, B. thetaiotaomicron was grown anaerobically on brain heart infusion (BHI; BBL Microbiology Systems, Cockeysville, Md.) medium supplemented with hemin (2.5 μg/ml) and cysteine (500 μg/ml) at 37°C in an atmosphere of 80% N₂-20% CO₂. Bacteroides transconjugants were selected on BHI agar plates containing clindamycin (5 μg/ml) and gentamicin (200 μg/ml) in an anaerobic chamber with an atmosphere of 75% N₂-20% CO₂-5% H₂. Escherichia coli was grown on LB medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter) or LB supplemented with ampicillin (50 μg/ml) or trimethoprim (200 μg/ml) for maintenance of plasmids. For continuous culture experiments, B. thetaiotaomicron was grown on routine growth medium (RGM) (9), a complex yeast extract-Trypticase-salt medium.

DNA and mating procedures. Plasmids were isolated as previously described (12). B. thetaiotaomicron genomic DNA was isolated as described by Saito and Miura (13).
TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Phenotype*</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td>E. coli HB101</td>
<td>Str&lt;sup&gt;a&lt;/sup&gt; RecA Gen&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>B. thetaiotaomicron 5482</td>
<td>Te&lt;sup&gt;c&lt;/sup&gt; Ce&lt;sup&gt;c&lt;/sup&gt; Gen&lt;sup&gt;c&lt;/sup&gt;</td>
<td>VPI&lt;sup&gt;a&lt;/sup&gt; Anaerobe Laboratory</td>
</tr>
<tr>
<td>B. ruminicola 23</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>pRX1</td>
<td>Amp&lt;sup&gt;d&lt;/sup&gt; Xyl&lt;sup&gt;e&lt;/sup&gt;</td>
<td>22</td>
</tr>
<tr>
<td>pVAL-1</td>
<td>Te&lt;sup&gt;f&lt;/sup&gt; Amp&lt;sup&gt;f&lt;/sup&gt; Cm&lt;sup&gt;f&lt;/sup&gt; Ce&lt;sup&gt;f&lt;/sup&gt; Mob&lt;sup&gt;f&lt;/sup&gt; Rep&lt;sup&gt;f&lt;/sup&gt;</td>
<td>19</td>
</tr>
<tr>
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<td>17</td>
</tr>
<tr>
<td>pVAL-RX</td>
<td>Te&lt;sup&gt;f&lt;/sup&gt; Amp&lt;sup&gt;f&lt;/sup&gt; Cm&lt;sup&gt;f&lt;/sup&gt; Ce&lt;sup&gt;f&lt;/sup&gt; Mob&lt;sup&gt;f&lt;/sup&gt; Rep&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>This study</td>
</tr>
<tr>
<td>R751</td>
<td>IncB(eta) Tp&lt;sup&gt;f&lt;/sup&gt; Tra&lt;sup&gt;f&lt;/sup&gt;</td>
<td>15</td>
</tr>
</tbody>
</table>

* Abbreviations used: r, resistant; s, sensitive; Rec, E. coli recombination mutant; Str, streptomycin; Gen, gentamicin; Te, tetracycline; Ce, clindamycin; Amp, ampicillin; Xyl, contains xylanase gene; Cm, chloramphenicol; Mob, able to be mobilized by a conjugative element; Rep, able to replicate in Bacteroides species; Tp, trimethoprim; Tra, ability to self-transfer; Inc, plasmid incompatibility group.

** Restrictions and Endonucleases.** Endonucleases were used according to manufacturer's instructions. Preparation of competent E. coli by CaCl<sub>2</sub> and transformation were carried out as previously described (12).

Bacterial matings were performed essentially as described by Guthrie and Salyers (6). Briefly, the donor (E. coli HB101) and recipient (B. thetaiotaomicron) were grown to an optical density at 660 nm of 0.2. Samples of each culture (0.2 ml of donor and 1.0 ml of recipient) were combined, and the cells were pelleted by centrifugation. The pellet was suspended in 0.2 ml of BHI, and the cell suspension was spotted onto a 0.45-µm-pore nitrocellulose filter (HAWP; Millipore Co., Bedford, Mass.) placed on a BHI agar plate. The conjugation was carried out aerobically at 37°C for 18 h. The cells were suspended by vortexing the filter in 3 ml of BHI, and the resuspended cells anaerobically on BHI agar plates containing clindamycin and gentamicin. The conjugation frequency was based on the total viable recipient cells at the end of the mating period.

** Southern blot analysis.** Chromosomal DNA from B. thetaiotaomicron was digested, electrophoresed through agarose gels in 89 mM Tris-68 mM phosphoric acid-2 mM EDTA, and stained with ethidium bromide. For hybridization with biotinylated probes, the DNA was transferred to nitrocellulose by the method of Southern (18). A gel-purified DNA fragment containing the B. ruminicola xylanase gene was labeled by using a random-primed DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.) and [11-biotin]dUTP according to the manufacturer's instructions. Hybridization and staining with strepavidin-biotin-alkaline phosphatase were carried out with a DNA detection kit (Bethesda Research Laboratories, Gaithersburg, Md.) according to the manufacturer's instructions.

** Continuous culture experiments.** For continuous culture experiments, B. thetaiotaomicron strains were grown anaerobically (100% CO<sub>2</sub> atmosphere) at various dilution rates under glucose limitation in a Multigen model F1000 benchtop fermentor with a 350-ml chemostat growth vessel (New Brunswick Scientific Co., Edison, N.J.). Medium was delivered to the culture vessel with a Rainin Rabbit peristaltic pump (Rainin Rabbit, Woburn, Mass.). For determination of cell numbers, aliquots were removed, diluted, and plated on RGM-glucose agar. To determine the number of colonies that possessed antibiotic resistance or xylanase activity, 100 colonies were picked onto RGM agar, RGM agar containing clindamycin, and RGM agar containing 2 mg of Remazol brilliant blue-xylan (RBB-xylan) per ml. Clearing zones around the colonies were indicative of xylanase activity (22). The number of colonies resistant to clindamycin or producing xylanase activity was expressed as a percentage of the number growing on RGM agar. This value represented the proportion of the population which carried the plasmid or chromosomal insert. The data from these experiments were then applied to previously published mathematical models for analyzing the stability of the plasmid or insert (3).

** Protein and enzyme analyses.** B. thetaiotaomicron cells were centrifuged at 10,000 x g at 4°C for 10 min and washed once with 50 mM sodium phosphate (pH 7.0)—1 mM dithiothreitol. Cells were suspended in 0.2 ml of washed cells in the same buffer and broken by single passage through a French pressure cell at 12,000 lb/in<sup>2</sup>. The broken cell suspension was centrifuged at 30,000 x g, 4°C, for 20 min. The supernatant fluid (crude extract) was recovered and assayed for xylanase activity and protein concentration.

Xylanase activity was determined by measuring the release of soluble sugars from oatspelt xylan (2 mg/ml) with orcinol following acid-alcohol precipitation as described previously (9), using xylose as the standard. One unit of enzyme activity was described as the amount of enzyme that catalyzed formation of 1 µmol of xylose equivalents in 1 h at 37°C. Protein concentration was estimated by the dye-binding assay of Bradford (2) with the commercial Bio-Rad (Bio-Rad Laboratories, Richmond, Calif.) reagent with fraction V of bovine serum albumin used as the standard.

** Materials.** Restriction endonucleases, T4 DNA ligase, 1-kb DNA ladder, and [11-biotin]dUTP were purchased from Bethesda Research Laboratories. Oatspelt xylan, RBB-xylan, and orcinol were obtained from Sigma Chemical Co., St. Louis, Mo. Nitrocellulose BA-85 (0.45 µm pore size) was purchased from Schleicher & Schuell, Keene, N.H. All other reagents were of reagent grade or better.

** RESULTS**

** Expression of the xylanase gene.** The xylanase gene cloned from B. ruminicola 23 was previously subcloned into the E. coli-Bacteroides shuttle vector pVAL-1 and termed pVAL-RX (23). Plasmids pVAL-1 and pVAL-RX were conjugated from E. coli HB101 into B. thetaiotaomicron 5482 by using the mobilization plasmid R751, and transconjugants were selected for clindamycin resistance. Crude extracts were prepared from B. thetaiotaomicron (pVAL-1) and B. thetaiotaomicron (pVAL-RX) and assayed for xylanase activity. Low background activity was found with pVAL-1, but high activity was detected with pVAL-RX (Table 2). This activity was comparable with those previously found with B. fragilis and B. uniformis containing pVAL-RX (23).

** Insertion of the xylanase gene into the B. thetaiotaomicron chromosome.** For insertion into the chromosome, the suicide vector pVAL-7 was used. This vector is a derivative of pVAL-1 from which a 1.5-kb EcoRV fragment has been
TABLE 2. Expression of xylanase activity in various strains

<table>
<thead>
<tr>
<th>Organism a</th>
<th>Plasmid</th>
<th>Xylanase activity b (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. ruminicola (glucose)</td>
<td>pVAL-1</td>
<td>0.03</td>
</tr>
<tr>
<td>B. ruminicola (xylan)</td>
<td>pVAL-1</td>
<td>0.89</td>
</tr>
<tr>
<td>B. thetaiotaomicron 5482</td>
<td>pVAL-RX</td>
<td>48.0</td>
</tr>
<tr>
<td>B. thetaiotaomicron BTX</td>
<td>pVAL-RX</td>
<td>15.3</td>
</tr>
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</table>

a Bacteroides strains were grown on RGM plus glucose and clindamycin when appropriate, except B. ruminicola, which was also grown with oatspelt xylan.
b Results are averages of at least two experiments.

RGM plus glucose, and xylanase activity was determined (Table 2). B. thetaiotaomicron BTX demonstrated high xylanase specific activity of 15 U/mg, which is about 500-fold greater than B. ruminicola grown on the same medium (Table 2). The activity was about threefold lower than that observed with the xylanase gene on the multicopy plasmid pVAL-RX (Table 2).

In order to demonstrate that the xylanase gene was inserted into the chromosome of B. thetaiotaomicron BTX, DNA was isolated from strains 5482 and BTX. There was no evidence of plasmid DNA in either strain. The chromosomal DNA was digested with BamHI and SalI, electrophoresed through an agarose gel, and transferred to nitrocellulose. The DNA was then probed with the BamHI-SalI fragment from pRX1 containing the xylanase gene. The chromosomal DNA from B. thetaiotaomicron BTX contained a hybridizing band (Fig. 2, lane 8) that corresponds to the BamHI-SalI band from pRX1 (lane 6). No hybridization was observed with DNA from B. thetaiotaomicron 5482 (lane 7). These data indicate that the xylanase gene has been introduced into the chromosome of B. thetaiotaomicron BTX.

Stability of the xylanase gene in B. thetaiotaomicron BTX and B. thetaiotaomicron 5482(pVAL-RX). The stability of the xylanase character in genetically altered B. thetaiotaomicron strains was studied in continuous culture. The cells were grown under glucose limitation in the absence of clindamycin for approximately 60 generations at each dilution rate. The results are shown in Fig. 3. At dilution rates (D) of 0.42, 0.10, and 0.03 h⁻¹, representing generation times of 1.7, 6.9, and 23 h, respectively, there was no detectable loss of xylanase or clindamycin resistance phenotypes over 60 generations. Significantly, when B. thetaiotaomicron 5482(pVAL-RX) was grown under similar conditions (D = 0.33 h⁻¹; generation time, 2.1 h) the plasmid was almost completely lost by 60 generations (Fig. 3). Aliquots of B. thetaiotaomicron BTX were removed from each of these continuous cultures, and xylanase activity was determined in crude extracts. No significant difference in xylanase activity between cultures could be detected (data not shown).

Degradation of xylan by B. thetaiotaomicron BTX. B. thetaiotaomicron BTX and 5482 were incubated with oatspelt xylan to determine the extent of degradation of the xylan to acid-alcohol soluble xylooligomers. Both strains were inoculated into RGM medium containing 0.2% oatspelt xylan with or without 0.05% glucose. Both inocula were from glucose-grown cultures. The cells were incubated at 37°C over a 168-h period, and the amount of solubilized sugars was determined at various time points. The results of these incubations are shown in Fig. 4. B. thetaiotaomicron BTX, but not 5482, was able to degrade xylan in the presence or
absence of added glucose. The rate of digestion of the xylan was greater with the added glucose, and the total cell numbers were higher in these cultures (data not shown). B. thetaiotaomicron BTX was able to degrade 40% of the xylan to soluble products within 48 h with the addition of a small amount of glucose. There was no measurable loss of total xylose equivalents, indicating the organism was not utilizing the degradation products.

**DISCUSSION**

Improvement in the efficiency of digestion in the rumen should result in increased animal production and lower feed costs. Genetically altering organisms that can inhabit the rumen for increased degradation of feed components is certainly a viable approach to accomplish this objective. However, attempts to introduce organisms into the rumen, such as E. coli, have not been successful (21). In addition, the genetic systems needed for manipulation of ruminal bacteria have not yet been developed. We proposed, therefore, to use an organism for increased production of the degradative enzyme xylanase that was naturally adapted for survival in anaerobic environments.

*B. thetaiotaomicron* was chosen based on several criteria, including previous genetic work on this organism that indicated it would be amenable to the desired manipulation (6, 7, 17). Guthrie and Salyers (6) previously demonstrated that insertional inactivation of the *B. thetaiotaomicron* 5482 chondroitin lyase II gene did not adversely affect the organism. We decided to take advantage of that information and use the chondroitin lyase II gene as the target site for introduction of the xylanase gene into the chromosome. It was hoped this would increase stability of the xylanase gene in *B. thetaiotaomicron* in the absence of antibiotic selection. Introduction of the *B. ruminicola* xylanase gene into the *B.
the *Bacteroides* chromosome was accomplished by using suicide vector pVAL-7. The xylanase activity in *B. thetaiotaomicron* BTX was much greater than that observed in *B. ruminicola* (Table 2). Interestingly, the xylanase activity in strain BTX was only threefold lower than that observed in *B. thetaiotaomicron* 5482 containing the xylanase gene on a multicopy plasmid (pVAL-RX). This would suggest that the xylanase gene is highly expressed, even at a single-copy level. Xylanase activity in crude extracts from cells grown in continuous culture at various dilution rates was not significantly different.

In order to confirm our premise that introduction of the xylanase gene into the *B. thetaiotaomicron* chromosome should increase the stability of the gene in the absence of clindamycin compared with when the gene is carried on pVAL-RX, continuous cultures were established at various dilution rates. The stability of the chromosomal gene over the plasmid-born version is amply demonstrated by the data shown in Fig. 3. Even at the slowest dilution rate ($D = 0.03$ h$^{-1}$) when the effect of any increase in maintenance energy expenditures from addition of this character would be greatest, the xylanase gene was maintained. In contrast, the plasmid pVAL-RX was rapidly lost from *B. thetaiotaomicron* even at a relatively rapid growth rate, $D = 0.33$ h$^{-1}$. Cooper et al. (3) have developed a method for examining the stability of plasmid-containing microbial populations in continuous culture. In their model, the two important determinants of the plasmid stability are the segregational instability ($R$, the rate at which plasmid-free cells are generated) and the growth rate difference between plasmid-free and plasmid-containing cells ($du$). When the data from this experiment were fitted to the model (Fig. 3), values for $R$ and $du$ were estimated as 0.04 and 0.0014 h$^{-1}$, respectively. On this basis, the loss of the plasmid was apparently due to segregational instability (i.e., 4% per h) rather than the growth rate differences.

The ability of *B. thetaiotaomicron* BTX to degrade xylan was determined by incubation with oatspelt xylan. At least 40% of the xylan was digested to acid-alcohol soluble xylo-oligosaccharides by strain BTX (Fig. 4). The rate of degradation was increased by the addition of 0.05% glucose. Interestingly, there appeared to be no measurable use of the degradation products by the organism. However, these results suggest the *B. thetaiotaomicron* BTX may assist in the degradation of xylan in a more complex system such as the rumen.

The feasibility for using *B. thetaiotaomicron* strains as a mechanism for modification of ruminal xylan fermentation will require more experimentation. This species has many desirable characteristics when considering the introduction of a genetically modified organism into the rumen. These include its ability to grow on a wide variety of carbohydrate sources (10, 14) and effectively compete in nutrient-limited ecosystems such as the mammalian colon. Another important concern is the ability to detect an organism and determine the degree of establishment in the rumen ecosystem. *B. thetaiotaomicron* has advantages in this respect. First, *B. thetaiotaomicron* is aerotolerant. The use of less than strict anaerobic techniques can be helpful for selecting BTX isolates from ruminal contents. A selective plating procedure could include glucose minimal medium containing clindamycin and RBB-xylan. We are currently working on deleting or inactivating the clindamycin gene in the BTX strain for environmental release concerns, and therefore this selection technique would need to be modified. However, specific DNA probes have been developed for *B. thetaiotaomicron* (11) and could be used for directly determining the number of cells in ruminal samples. We are beginning to carry out coculture studies of *B. thetaiotaomicron* BTX with ruminal bacteria and to determine the extent of xylan degradation in these cultures.

**ACKNOWLEDGMENT**

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**REFERENCES**


