CURRENT MICROBIOLOGY Vol. 43 (2001), pp. 293–298
DOI: 10.1007/s002840010304

Current Microbiology
An International Journal

Identification of a Broad-Specificity Xylosidase/Arabinosidase Important for Xylooligosaccharide Fermentation by the Ruminal Anaerobe *Selenomonas ruminantium* GA192

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Received: 12 January 2001 / Accepted: 5 March 2001

Abstract. Strains of *Selenomonas ruminantium* vary considerably in their capacity to ferment xylooligosaccharides. This ability ranges from strain GA192, which completely utilized xylose through xylotetraose and was able to ferment considerable quantities of larger oligosaccharides, to strain HD4, which used only the simple sugars present in the hydrolysate. The ability of *S. ruminantium* GA192 to utilize xylooligosaccharides was correlated with the presence of xylosidase and arabinosidase activities. The production of these activities appears to be regulated in response to carbon source used for growth. Both arabinosidase and xylosidase were induced by growth on xylose or xylooligosaccharides, but no activity was detected in glucose-or arabinose-grown cultures. A genetic locus from *S. ruminantium* GA192 was cloned into *Escherichia coli* JM83 that produced both xylosidase and arabinosidase activities. Analyses of crude extracts from the *E. coli* clone and *S. ruminantium* GA192 by using native polyacrylamide gel electrophoresis and methylumbelliferyl substrates indicated that a single protein was responsible for both activities. The enzyme expressed in *E. coli* was capable of degrading xylooligosaccharides derived from xylan. DNA sequencing of the locus demonstrated the presence of an open reading frame that encodes for a protein of 61,174 molecular weight.

Digestion of feed material by ruminants is carried out by the complex microflora that inhabit the rumen. However, a major component of plant material, hemicellulose, is poorly degraded by this ruminal microflora. Such inefficiency results in monetary loss for the animal producer and increased production of waste material by the animal. Hemicellulose is composed primarily of xylan, a heteropolysaccharide with a β-(1, 4)-linked xylose backbone and substituted with side groups of arabinose, glucose, galactose, and glucuronic acid. In addition, the xylose moieties of the backbone can be acetylated. As a result, xylan degradation requires the interaction of several enzymatic activities, including xylanase, xylosidase, and arabinosidase. The end products of this degradation include xylose, arabinose, and methyl-glucuronic acid containing oligosaccharides [5, 7]. These oligosaccharides can be utilized by several species of xylanolytic ruminal bacteria and *Selenomonas ruminantium*, a non-xylanolytic species [3, 4]. Previous results have demonstrated that coculturing of *S. ruminantium* and xylanolytic ruminal microorganisms could reduce the accumulation of xylooligosaccharides in the medium and in some cases lead to increased xylan degradation [14]. Further investigation in our laboratories has demonstrated that strains of *S. ruminantium* vary in their ability to utilize xylooligosaccharides. This ability ranged from strain GA192, which completely utilized xylose through xylotetraose and was able to ferment considerable quantities of larger oligosaccharides, to strain HD4, which used only the simple sugars present in the hydrolysate (xylose and arabinose) [4]. The ability of *S. ruminantium* strains to utilize xylooligosaccharides was correlated with the presence of xylosidase and arabinosidase activ-

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ities (measured by hydrolysis of appropriate *p*-nitrophenyl glycosides) [4]. The objective of this report was to examine the enzymes and genes that may be important in the utilization of xylooligosaccharides by *S. ruminantium* GA192.

Materials and Methods

Organisms and media. *Selenomonas ruminantium* GA192 was grown anaerobically on routine growth medium [RGM, 7], a complex yeast extract-trypticase-salts medium containing 0.2% energy source. *Escherichia coli* strains JM83, BL21, and clones were grown on LB medium or LB medium supplemented with ampicillin (75 μg/ml, LBA) for selection of plasmids. For induction of T7 promoter in plasmid pET-21(+), IPTG was added to a final concentration of 1 mM. Xylooligosaccharides were prepared as described previously [3].

Cloning and DNA sequencing protocols. The gene(s) encoding for xylosidase and arabinosidase activity were cloned by using genomic DNA isolated from *S. ruminantium* GA192 by the method of Saito and Miura [11]. The DNA was partially digested with *Sau*3A and ligated into *Bam*HI-digested and dephosphorylated pUC18. *E. coli* JM83 clones expressing arabinosidase or xylosidase activity were screened on LB-ampicillin plates containing 5 μg/ml 5-methylumbelliferyl-α-L-arabinofuranoside or -β-D-xyloside, respectively. The colonies were examined under ultraviolet light for fluorescence, indicative of arabinosidase or xylosidase activity. For overexpression of the cloned gene in *E. coli*, the gene was ligated into the expression vector pET-21(+) (Novagen, Madison, WI) and introduced into *E. coli* BL21. Induction of the T7 promoter with IPTG was carried out according to the manufacturer's instructions. The cloned gene was sequenced by using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions and analyzed with an Applied Biosystems Model 370A automatic sequencer. DNA sequence analyses were carried out using the Lasergene software (DNASTAR, Inc., Madison, Wisconsin). Similarity analyses were carried out with the Advanced Blast Program of GenBank (NCBI, NIH, Washington, District of Columbia). The *S. ruminantium xsa* gene sequence has been deposited in the GenBank database under accession number AF040720.

Enzyme and protein analyses. *E. coli* clones were grown on LBA to mid-logarithmic phase and recovered by centrifugation. The cells were suspended in 50 mM sodium phosphate buffer (pH 6.8) containing 0.1 mM DTT and broken with one passage through a French Pressure Cell (16,000 psi). The suspension was centrifuged at 30,000 *g*, 4°C, for 30 min. The supernatant fluid (crude extract) was recovered and used for enzymatic assays. *S. ruminantium* was grown on RGM containing the appropriate carbon source and was treated as described above. Arabinosidase and xylosidase activities were measured by using *ρ*-nitrophenyl-α-L-arabinofuranoside and *ρ*-nitrophenyl-β-D-xyloside (Sigma Chemical Co., St. Louis, Missouri), respectively, as substrates. One unit of activity is described as the amount of enzyme that catalyzes the release of 1 μmole of *ρ*-nitrophenol in 1 h at 37°C, measured at 405 nm. Protein concentrations were estimated by the dye-binding assay of Bradford [2] by using the commercial Bio-Rad Laboratories (Richmond, California) reagent with Fraction V of bovine serum albumin as the standard. Proteins in crude extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli [9], with a 4% acrylamide stacking gel and a 10% acrylamide resolving gel. For detection of enzyme activity, proteins were analyzed by PAGE with native gels in the absence of SDS. Gels were washed with 50 mM sodium phosphate, pH 6.8, then incubated in the same buffer containing either 5-methylumbelliferyl-α-L-arabinofuranoside or -β-D-xyloside. Bands of enzymatic activity were detected by exposure to ultraviolet light (265 nm).

Table 1. Xylosidase and arabinosidase activity in crude extracts from *S. ruminantium* GA192 grown on various carbon sources[a]

Growth substrate	Xylosidase (U/mg)	Arabinosidase (U/mg)
Glucose	ND[b]	ND
Xylose	15.7	1.8
Arabinose	ND	ND
Xylooligosaccharides	13.5	1.3

[a]Values are averages of at least duplicate experiments. Standard deviations (pooled for all experiments) for xylosidase and arabinosidase assays were 1.30 and 0.12 units/mg protein, respectively.
[b]ND, not detectable.

Degradation of natural substrates by cloned arabinosidases. The cloned xylosidase and arabinosidase activities expressed in *E. coli* JM83 were tested with the natural substrates wheat arabinoxylan (WAX), oatspelt xylan (OX), and corn fiber xylan (CFX), each at 10 mg/ml in the reaction mix, in the presence or absence of purified cloned xylanase from *P. ruminicola* 23 (0.5 U/reaction) [12]. Products of incubations were analyzed by thin-layer chromatography (TLC) on Whatman silica gel plates (K-5; 250 μM particles) developed with acetonitrile–water (9:1, vol/vol). Products were visualized with N-(1-naphthyl) ethylenediamine spray and heating at 100°C [1].

Partial purification of the cloned xylosidase/arabinosidase activities from *E. coli* BL21. The xylosidase/arabinosidase enzyme from the induced *E. coli* BL21 clone was partially purified from a crude extract prepared as described above. The crude extract was subjected to ultracentrifugation at 165,000 *g* for 1 h at 4°C. The supernatant fluid (cytoplasmic fraction) and membrane pellet were recovered and assayed for activity. The membrane pellet was suspended in buffer and subjected to a second ultracentrifugation step. The two cytoplasmic fractions were combined and contained more than 90% of the original enzyme activities. The cytoplasmic fraction was then subjected to ammonium sulfate precipitation at 4°C. The material precipitating at between 50% and 75% of saturation was found to contain more than 75% of the original enzymatic activities, while removing approximately 75% of the original protein. This partially purified material was used for inhibition assays with xylose and arabinose.

Inhibition assays. Effects of addition of the end products arabinose and xylose on xylosidase and arabinosidase activities were determined by adding increasing amount of the sugar to the enzyme mixtures described above. Partially purified enzyme, buffer, and sugar were combined, incubated at 37°C for 1 min, then combined with the remaining reaction components also heated to 37°C. Results are presented as percentage of control activities with no sugar added to the reaction mix.

Results and Discussion

Regulation of expression of xylosidase and arabinosidase activities was demonstrated by growth of *S. ruminantium* GA192 on different carbon sources [Table 1, ref. 4]. No detectable activity was found following growth on glu-

cose or arabinose; however, both xylosidase and arabinosidase activities were induced when the microorganism was grown on either xylose or xylooligosaccharides. A similar pattern of expression was also found with *S. ruminantium* strains D, GA31, and H18 [4], whereas other strains such as HD4, HD1, and W-21 produced no enzymatic activities and could not use xylooligosaccharides for growth [4].

Cloning of the genes encoding for xylosidase and arabinosidase activities from strain GA192 was carried out by preparing a genomic library in pUC18 and introduction of the recombinant plasmids into *E. coli* JM83, since *E. coli* has no detectable xylosidase or arabinosidase activities [13]. Screening on methylumbelliferyl-linked substrates resulted in clones that produced fluorescence under UV light from the xylosidase substrate. The cloning was confirmed by streaking of the original colonies on media containing the xylosidase substrate and detection of enzymatic activity by UV light screening. Because of the previous discovery in our laboratory of a bifunctional xylosidase/arabinosidase in *Bacteroides ovatus* [13], *E. coli* clones were also tested for arabinosidase activity by streaking the colony out on media containing the methylumbelliferyl-arabinofuranoside substrate. The clones were found to fluoresce under UV light, indicating the presence of arabinosidase activity in addition to the xylosidase activity. Plasmid DNA was isolated from one of the *E. coli* clones, and the plasmid was termed pSRA1. The plasmid contained a 2.7-kb genomic DNA insert. When a crude extract was prepared from the JM83/pSRA1 clone, xylosidase and arabinosidase activities were assayed and found to be 13 U/mg and 1.6 U/mg, respectively.

Plasmid pSRA1 DNA was used for sequencing of the 2.7-kb insert. Sequence analysis revealed the presence of a 1617-bp open reading frame that encoded a 61,136 Dalton protein. This open reading frame ended just upstream of an *Eco*RI restriction site. When the pSRA1 plasmid was digested with *Eco*RI, a 1,700-bp fragment could be recovered and ligated again with pUC18. Transformation of JM83 with the new plasmid resulted in expression of both xylosidase and arabinosidase activities, confirming the presence of the gene(s) for these activities on the fragment containing the 1617-bp open reading frame. Since only one open reading frame was evident, the data suggested that both activities were associated with a single protein.

Crude extracts were prepared from both the *E. coli* JM83/pSRA1 and *S. ruminantium* GA192 (grown on xylose), and aliquots of each were subjected to native gel electrophoresis, followed by incubation with methylumbelliferyl-xyloside or methylumbelliferyl-arabinofuranoside. The results of viewing under UV light are shown in Fig. 1. Both enzymatic activities in each crude extract migrated to the same location in the gel, supporting the conclusion that a single protein was associated with both activities in strain GA192 and the *E. coli* clone. Also, there appeared to be only one band of activity for each in GA192, suggesting that only one gene was responsible for xylosidase and arabinosidase activities under induced conditions.

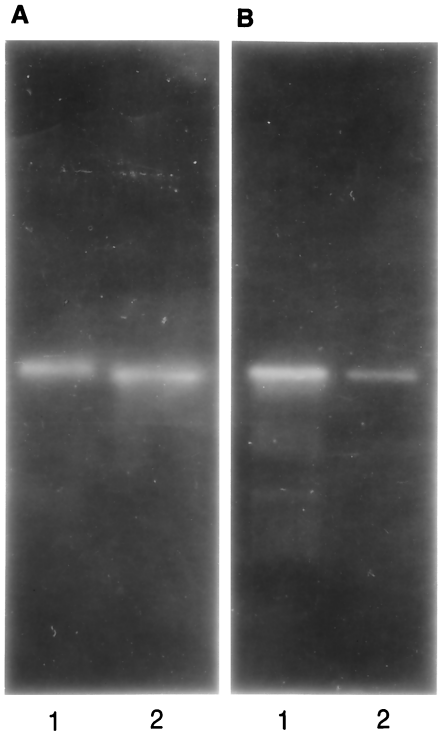

Fig. 1. Native gel electrophoretic analysis of arabinosidase and xylosidase activities in crude extracts from *S. ruminantium* GA192 and *E. coli* clone JM83/pSRA1. The crude extracts were prepared as described in Materials and Methods. Following electrophoresis, the gels were incubated in the presence of methylumbelliferyl-xyloside (Panel A) or methylumbelliferyl-arabinofuranoside (Panel B) and examined under UV light. Lane 1, *S. ruminantium* GA192 crude extract; lane 2, *E. coli* JM83/pSRA1 crude extract.

The protein sequence of the cloned gene, termed *Xsa*, was compared with other sequences in GenBank by using the Advanced Blast search function. Significant similarity (>65% identity) was found between the GA192 Xsa protein and the sequences to xylosidases from *Bacillus subtilis*, *Bacillus pumilus*, and *Bacillus* sp. KK-1 (data not shown). There was also less sequence similarity (<50%) to other reported xylosidases/arabinosidases from *Azospirillum irakense*, *Butyrivibrio fibrisolvens*, and *Bacillus subtilis*, as well as to the bifunctional arabinosidase/arabinosidase from *Bacteroides ovatus*.

Since the enzymatic activities of both enzymes are determined with artificial substrates, it is of great impor-

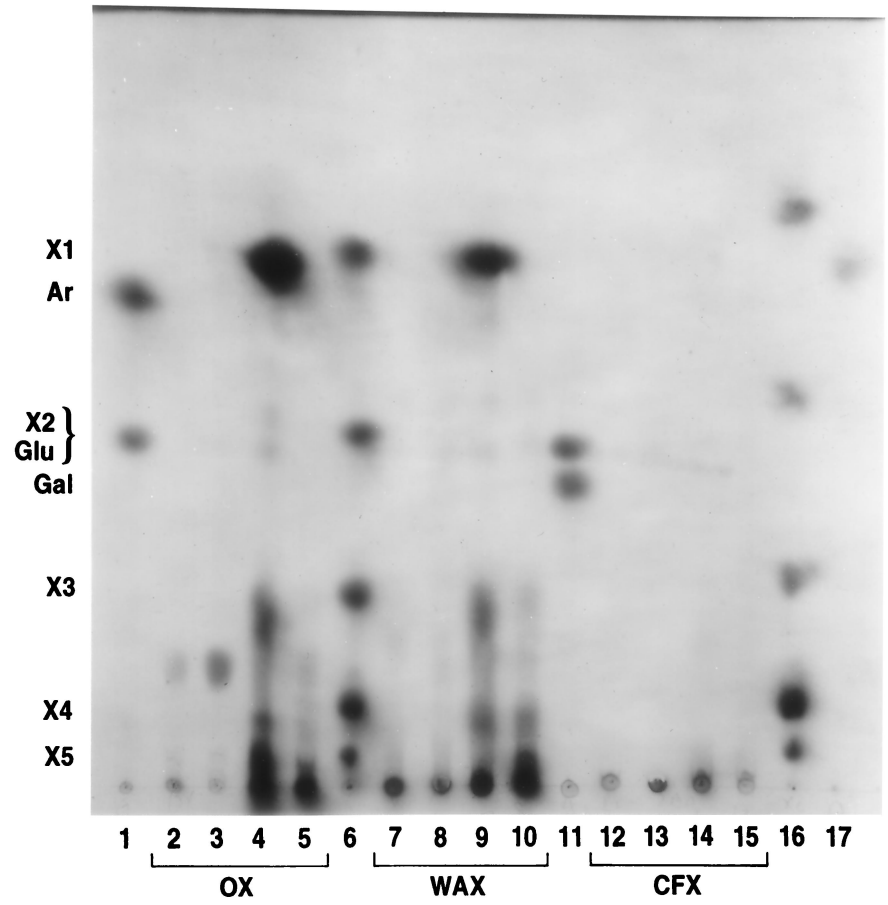

Fig. 2. Thin-layer chromatography analysis of xylan degradation by cloned *S. ruminantium* GA192 xylosidase/arabinosidase with partially purified *P. ruminicola* 23 xylanase. Xylosidase/arabinosidase in crude extract of *E. coli* JM83/pSRA1 was incubated (24 h, 37°C) with 1 mg of oatspelt xylan (OX), wheat arabinoxylan (WAX), or corn fiber xylan (CFX) in the presence or absence of partially purified xylanase from *P. ruminicola* 23. Lanes 2, 7, 12, no addition; lanes 3, 8, 13, xylosidase/arabinosidase; lanes 4, 9, 14, xylosidase/arabinosidase + xylanase; lanes 5, 10, 15, xylanase. Lanes 6 and 16, xylooligosaccharide standards (X1, X2, X3, X4, X5), which are indicated on the left side. Lane 1, arabinose (Ar) and glucose (Glu) standards. Lane 11, glucose (Glu) and galactose (Gal) standards. Lane 17, arabinose (AR) standard.

tance to determine whether the activities will actually degrade natural substrates. Therefore, crude extracts from JM83/pSRA1 were combined with oatspelt xylan, wheat arabinoxylan, and corn fiber xylan in the presence or absence of partially purified xylanase cloned from *Prevotella ruminicola* 23 [12], added to produce xylooligosaccharides. Products were analyzed by thin-layer chromatography, and the results are shown in Fig. 2. The results and previous data confirm that the *P. ruminicola* xylanase alone produced only products greater than X3 (lanes 5, 10, 15). Also, the xylanase was not active against the corn fiber xylan (lane 15). The Xsa protein was not capable of degrading the intact xylans (lanes 3, 8, 13). The combination of xylanase and Xsa produced large amounts of xylose (X1) and smaller amounts of arabinose (partially hidden by the large xylose spots) from oatspelt xylan and wheat arabinoxylan (lanes 4 and 9). These results indicate that the Xsa protein was capable of acting on natural xylooligomers derived from xylan substrates, which allow the GA192 bacterium to break down and utilize xylooligosaccharides for growth.

In order to produce larger amounts of and further characterize the Xsa protein, the cloned gene was inserted into the *E. coli* expression vector pET-21 (Petsral) and introduced into *E. coli* strain BL21. The expression promoter was induced with IPTG, and aliquots of BL21/pETSRA1 cells were removed at various time points for enzymatic assays and SDS-PAGE analyses. Enzymatic activity increased over time, with a final specific activity in crude extracts after 120 min for xylosidase of 585 U/mg and arabinosidase of 46 U/mg. The concomitant increase in the amount of Xsa protein is shown in Fig. 3, and the molecular weight of the Xsa protein correlates well with that predicted by the deduced protein sequence.

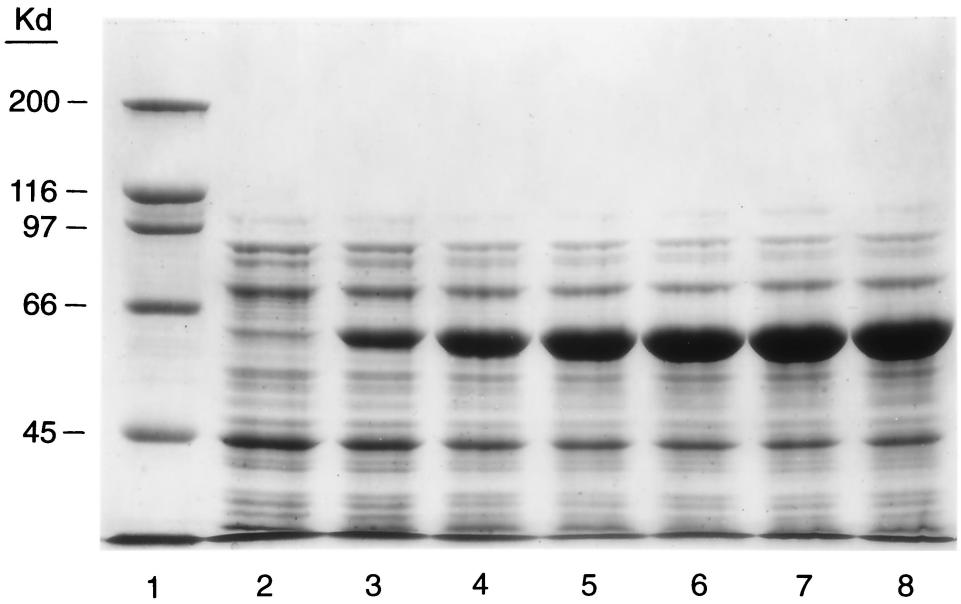

Fig. 3. SDS-PAGE analysis of crude extracts from *E. coli* BL21 containing the expression vector pETSRA1. Cells were induced at time 0 with 1 mM IPTG for overproduction of the Xsa protein, and samples were removed over time and processed as described in Material and Methods. Lanes 2–8 contain 10 μg of protein. Lane 1, molecular size markers (indicated on left-hand side in kilodaltons); lane 2, time 0; lane 3, 15 min; lane 4, 30 min; lane 5, 45 min; lane 6, 60 min; lane 7, 90 min; and lane 8, 120 min.

Table 2. Effects of addition of xylose on xylosidase and arabinosidase activities of the cloned GA192 Xsa protein partially purified from *E. coli* BL21/pETSRA1[a]

Xylose (mM)	Xylosidase (% of control)	Arabinosidase (% of control)
0	100	100
10	77	75
20	60	62
40	43	44

[a]Values are averages of at least duplicate experiments. Specific activities for xylosidase and arabinosidase activities of the control were 10.1 and 0.9 units/mg protein, respectively.

Table 3. Effects of addition of arabinose on xylosidase and arabinosidase activities of the cloned GA192 Xsa protein partially purified from *E. coli* BL21/pETSRA1[a]

Arabinose (mM)	Xylosidase (% of control)	Arabinosidase (% of control)
0	100	100
10	90	91
20	81	86
40	63	70
60	45	51
80	39	41

[a]Values are averages of at least duplicate experiments. Specific activities for xylosidase and arabinosidase activities of the control were 10.1 and 0.9 units/mg protein, respectively.

The question of how one protein can carry out two different enzymatic activities with two different substrates suggests that there may be more than one active site in the protein. We decided to approach this question by using the end products of the two reactions, xylose and arabinose, as potential inhibitors of the reactions with the artificial substrates. The Xsa protein overexpressed in BL21 was first partially purified, as described in Materials and Methods, then increasing amounts of arabinose or xylose were added to the enzymatic reaction mixtures to determine whether any inhibition of either activity could be detected. The results are shown in Tables 2 and 3. Addition of either arabinose or xylose was found to inhibit both the arabinosidase and xylosidase activities to similar degrees, with the xylose being more effective at lower concentrations. While not conclusive, these data suggest that both activities share a common active site, and the protein may possess broad specificity for substrates.

The isolation and characterization of the gene for xylosidase/arabinosidase activities from *S. ruminantium* GA192 lends credence to the importance of these activities for the breakdown and utilization of xylooligosaccharides by various other strains of *S. ruminantium* [4]. When the GA192 *xsa* gene was used as a probe against DNA from various *S. ruminantium* strains that could use

xylooligosaccharides for growth, several but not all produced a hybridizing band (data not shown). This suggests that the hybridizing strains may share a similar *xsa* gene, but the other strains may have a different system for growth on xylooligosaccharides. Variations in other genetic and enzymatic characteristics in *S. ruminantium* strains have been noted by other workers [6, 8, 10], including DNA-DNA homologies, DNA RFLP patterns, and lactate dehydrogenase activity. The presence or absence of the xylosidase/arabinosidase activities may prove useful for further identifying relationships between strains of *S. ruminantium*.

ACKNOWLEDGMENTS

The authors acknowledge the excellent technical assistance of Patricia J. O'Bryan, David A. Lee, and Rhonda L. Zeltwanger.

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