Eubacterium uniforme sp. nov. and Eubacterium xylanophilum sp. nov., Fiber-Digesting Bacteria from the Rumina of Sheep Fed Corn Stover

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A study was made of gram-positive nonsporforming strains isolated from the rumina of sheep fed corn stover with or without different amounts of corn grain. Two groups of xylanolytic, non-cellulosytic strains were found. A total of 13 strains fermented a variety of carbon sources and produced formic, acetic, and lactic acids and ethanol from xylan. The name proposed for this group of strains is Eubacterium uniforine. The type strain of this species is strain X6C58 (= ATCC 35992). Three strains utilized only cellobiose and xylan of the carbohydrate energy sources tested and produced formic, acetic, and butyric acids from xylan. The name proposed for this group of strains is Eubacterium xylanophilum; the type strain is strain X6C58 (= ATCC 35991).

Several Eubacterium species have been isolated from rumina, including Eubacterium helwigiae (5, 11), Eubacterium cellulosolvens (3, 10, 15), Eubacterium ruminantium (1), and strains assigned to this genus but not described yet (2).

We investigated the effect of increasing the proportions of corn grain supplements added to a basal diet of corn stover on the numbers and types of fiber-digesting bacteria in the rumen cellulosolvens (2). The basal medium contained the following components (in grams per liter): K2HPO4, 0.225; KH2PO4, 0.225; NaCl, 0.45; (NH4)2SO4, 0.45; CaCl2 (anhydrous), 0.045; MgSO4 · 7H2O, 0.09; NaHCO3, 6.37; cysteine hydrochloride · H2O, 0.25; Na2S · 9H2O, 0.25; and indigo carmine, 0.005. This medium also contained 400 ml of rumen fluid from sheep fed alfalfa hay (centrifuged at 1,500 × g for 30 min) and was used with a gas phase containing 98% CO2 and 2% H2. Strains were maintained on agar (15 g/liter) slopes in which the concentration of the mineral nutrients was doubled and which contained 15 g of xylan (catalog no. 95590; Fluka A.G., Buchs, Switzerland) per liter.

Motility was determined with growth from agar slopes containing 5 g of xylan per liter. Fermentation of carbohydrate energy sources (concentration, 5 g/liter) was tested in poorly buffered liquid medium containing one-tenth of the usual concentration of bicarbonate with a gas phase consisting of 10% CO2, 88% N2, and 2% H2. Fermentation products were determined in liquid medium containing 20 g of xylan per liter. For guanine-plus-cytosine (G+C) content determinations, glucose (5 g/liter) was used as the sole energy source for strains of Eubacterium uniforme and cellobiose (4 g/liter) was used for strains of Eubacterium xylanophilum.

For additional tests the energy source used was xylan (10 g/liter) unless otherwise specified. For production of gas, catalase, and hydrogen sulfide the medium contained 10 g of peptone (a tryptic digest of casein; E. Merck AG, Darmstadt, West Germany) per liter. For indole, α-methylindole, and acetylmethylcarbinol determinations the medium contained 5 g of glucose per liter in addition to 10 g of xylan per liter; Casitone (Difco Laboratories, Detroit, Mich.) was omitted. Gelatin liquefaction was tested in medium containing 2 g of cellobiose per liter and 120 g of gelatin per liter. Sodium ß-lactate (1.6 g/liter) was the sole energy source added to the medium used to determine the ability to utilize lactate.

Identification tests were done in duplicate as previously described (14). Formic acid, acetic acid ß- and ß-lactic acids, succinic acid, and ethanol were determined quantitatively by using enzymic methods. Volatile acids were determined in duplicate by gas-liquid chromatography. Caproic acid was used as an internal standard in samples for analysis. Since caproic acid was added to all samples, its production during carbohydrate fermentation could not be measured directly. However, a comparison of the peak heights of caproic acid in the chromatograms of the samples with the peak heights obtained with a standard solution analyzed under the same

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TABLE 1. Origins of strains of two xylan-fermenting Eubacterium species isolated from the rumina of sheep fed corn stover plus a protein-mineral mixture without or with different corn grits supplements

<table>
<thead>
<tr>
<th>Amt of supplement (g/kg of stover)</th>
<th>Short, straight, rod-shaped strains</th>
<th>Cocccoid rod-shaped strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>X3A56, X4A56, X2A58, X3C39, X5D39, X2E39</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>X3D34</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>X2D54</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>X2E38</td>
<td></td>
</tr>
<tr>
<td>210</td>
<td>X3C56</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>X4D63</td>
<td></td>
</tr>
<tr>
<td>270</td>
<td>X10C60</td>
<td></td>
</tr>
<tr>
<td>330</td>
<td>X8E33</td>
<td></td>
</tr>
<tr>
<td>360</td>
<td>X6C58†</td>
<td></td>
</tr>
<tr>
<td>390†</td>
<td>X5D62</td>
<td></td>
</tr>
</tbody>
</table>

* The protein-mineral mixture contained casein (46%), fishmeal (41%), and a mineral-trace element mixture (12.5%). Supplements were fed as pellets containing corn grits (94%), casein (1.6%), fishmeal (2.3%) and the mineral-trace element mixture (1.6%). All diets contained 13.8% crude protein and had the same mineral content.

† The final two digits of each strain designation indicate the animal from which the strain was isolated.

Table of the results of mixed analysis of variance indicating that no significant concentrations of caproic acid were produced by any of the strains.

To determine G+C content, cells from 17- to 24-h cultures were harvested by centrifugation and lysed with dodecyl sulfate (7). The deoxyribonucleic acid (DNA) was isolated and purified (8), and the G+C content was estimated from the ratio of absorbance at 245 nm to absorbance at 270 nm (12); DNAs from Clostridium perfringens, Escherichia coli, Micrococcus lysodeikticus, calf thymus, and salmon sperm (all from Sigma Chemical Co., St. Louis, Mo.) were used as calibration standards. Separate results (10 in total) were calculated from readings made against each standard by using DNA isolated on two separate occasions from each of the strains examined.

RESULTS AND DISCUSSION

Both groups of bacteria are anaerobic, nonsporeforming rods that stain gram positive; these organisms do not produce as major fermentation products either propionic acid alone, lactic acid alone, succinic acid (in the presence of CO2) and lactic acid with small amounts of acetic or formic acid, or acetic acid and lactic acid (more acetic acid than lactic acid) with or without formic acid. Therefore, they are members of the genus Eubacterium Prévot (5). Their characteristics do not conform with those of any previously described species.

Eubacterium uniforme sp. nov. Eubacterium uniforme (u. ni.for.me. L. neut. adj. uniforme uniform, denoting unusual uniformity among strains) cells are obligately anaerobic and nonmotile. The majority of cells from 16-h cultures on xylan agar stain gram positive. Most cells are short rods (cocccoid forms are present) and have rounded or somewhat blunt ends (Fig. 1). They occur singly, in pairs, and sometimes in short chains. The cells are usually about 0.4 μm wide, but some are wider (up to 0.6 μm), and the length varies from 0.6 to 1.5 μm. No differences in cell size occur on xylan or cellulose agar medium. No spores are produced.

After incubation for 3 days on films of xylan (3%) agar medium in roll bottles, surface colonies appear mucoid and round with smooth to wavy or irregular edges. Colony diameters vary from 2 to 8 mm. The colonies are white and show a strong bluish-green iridescence when they are viewed obliquely in transmitted light. Clearings surrounding the colonies and resulting from xylan solubilization in the opaque medium vary from 15 to 25 mm in diameter. The degradation of xylan within the clearings is complete in the vicinity of the colonies. Submerged colonies are lens shaped.

None of seven strains tested (strains X3C39T [T = type strain], X3C56, X10C60, X3D34, X4D63, X5E38, and X2E39) requires rumen fluid or carbon dioxide-bicarbonate for growth. All strains grow well in medium which lacks added reducing agent (sulfide and cysteine) but which is nevertheless reduced with respect to resazurin. Injection of 5 ml of sterile air into 30-ml bottles containing either 4 or 10 ml of medium results in at least partial oxidation of the medium, as indicated by the color of the resazurin indicator, and causes complete inhibition of growth. Although six of the seven strains grow at 45°C (strain X2E39 does not grow at this temperature), the pH of the cultures is lowered less at 45°C than at 38°C. No strain grows at 22°C.

Biochemical reactions of Eubacterium uniforme are listed in Table 2. In addition, the type strain and the six other
isolates tested liquefy gelatin. None produces indole, α-methylindole, hydrogen sulfide, or catalase. Nitrate is not reduced. Despite the fact that all strains produce acid from starch (weakly in the case of one strain), in all cases at least some starch remains unhydrolyzed, as indicated by reaction with iodine.

The major products of xylan fermentation are formic, acetic, and L-lactic acids and ethanol; some propionate is utilized (Table 3). The type strain and the six other strains all produce gas and acetylmethylcarbinol.

The G+C content of the DNA is 35.2 ± 2.04 mol% for type strain X3C39 and 34.9 ± 2.00 mol% for strain X10C60.

The type strain is strain X3C39 (= ATCC 35992), which was isolated from the rumen of a sheep.

The end products of fermentation of Eubacterium uniforme (formic, acetic, and lactic acids and ethanol) are similar to those of several other eubacteria. Eubacterium uniforme differs from Eubacterium formicigenes (in the G+C content of the DNA (which is 40 to 44 mol% for the latter species), the ability to ferment cellobiose, fructose (some strains of Eubacterium uniforme ferment fructose weakly), salicin, starch, and xylose, the production of acetylmethylcarbinol, and cell dimensions. It differs from Eubacterium eligens (6), which has a similar G+C content (36 mol%), in the ability to ferment L-arabinose, fructose (see above), and galactose, the production of acetylmethylcarbinol, motility, and cell dimensions. Eubacterium uniforme differs from Eubacterium aerofaciens (4) in cell morphology and that in the latter species ferments fructose (see above) and mannose and does not ferment arabinose and starch.

Bryant et al. (1, 2) described bacteria (groups +R-2 and +SR-gGXC) which they placed in the genus Eubacterium. No specific epithet was assigned. The characteristics that were determined conformed in many respects with those of Eubacterium uniforme; except that ethanol production was not detected and the strains of Bryant et al. did not ferment a xylan which was easily degraded by other species of rumen bacteria.

Eubacterium xylanophilum sp. nov. Eubacterium xylanophilum (xy. lan. o. phil'um. xylan xylan; Gr. part. philo loving; L. neut. adj. xylanophilum xylan loving) cells are obligately anaerobic, nonsporeforming, and motile, exhibiting a rapid corkscrew-like motion. Flagella are observed by transmission electron microscopy, but their points of insertion and their number (one or two per cell) have not been determined with certainty. After growing for 16 h on xylan agar medium, fewer than one-half of the cells stain gram positive. The cells are straight rods, or sometimes they are coccolid with rounded ends (Fig. 2). They occur singly, in pairs, and sometimes in short chains. The cell width varies from 0.4 to 0.6 μm, and the cell length varies from 0.5 to 2.0 μm (sometimes up to about 3 μm).

Surface colonies in roll bottles containing xylan (3%) agar medium incubated for 3 days are 2 to 4 mm wide, circular, entire, smooth, white, and iridescent in obliquely transmitted light. Submerged colonies are lenticular and 0.5 to 1 mm in diameter. Clearings due to solubilization of xylan surrounding surface colonies vary in diameter from 6 to 20 mm. Xylan is extensively degraded within the clearings.

Rumen fluid is not required for growth, but growth is slightly enhanced in the presence of carbon dioxide and bicarbonate. Growth is not inhibited by deletion of reducing agent provided that the medium remains reduced with respect to resazurin. Injection of 5 ml of sterile air into 30-ml bottles containing 4 or 10 ml of medium oxidizes (at least partially) the resazurin indicator and causes complete inhibition of growth. There is no growth at either 22 or 45°C.

Of the substrates which have been tested, only xylan and cellobiose are fermented when the inoculum for the tests is grown on xylan agar slopes (Table 2). However, when the inoculum is grown on cellobiose agar slopes, esculin is also (but poorly) fermented (pH drop of 0.2 to 0.3 U) by all three strains. The final pH in poorly buffered medium containing xylan or cellobiose does not drop below 5.6. These bacteria utilize cellobiose but not glucose or xylose, which suggests that they may utilize xylose derived from xylan. None of the strains produces indole or α-methylindole. The strains do not liquefy gelatin, reduce nitrate, produce catalase, or hydrolyze starch. Only strain X10D63 produces hydrogen sulfide.

The fermentation products are formic, acetic, and butyric acids (Table 3). Gas and acetylmethylcarbinol are also produced.

### TABLE 3. End products of xylan fermentation by two new species of Eubacterium isolated from rumina of sheep

<table>
<thead>
<tr>
<th>End product</th>
<th>Eubacterium uniforme (Strain X3C39)</th>
<th>Eubacterium xylanophilum (Strain X3C58)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic acid</td>
<td>+1.91 ± 0.26*</td>
<td>+2.00</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>+1.12 ± 0.38</td>
<td>+1.07</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>-0.16 ± 0.09</td>
<td>-0.16</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>-0.05 ± 0.03</td>
<td>-0.05</td>
</tr>
<tr>
<td>n-Valeric acid</td>
<td>-Trace</td>
<td>-0.01</td>
</tr>
<tr>
<td>a-Lactic acid</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-Lactic acid</td>
<td>+1.61 ± 0.56</td>
<td>+2.06</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>+1.59 ± 0.35</td>
<td>+1.15</td>
</tr>
</tbody>
</table>

*None of the strains produced or utilized significant amounts of isobutyric, 2-methylbutyric, isovaleric, or α-caproic acid.

*Uninoculated xylan medium contained the following components (in millimoles per 100 ml of medium): formic acid, 0.16; acetic acid, 4.89; propionic acid, 1.13; butyric acid, 0.49; α-valeric acid, 0.08; α-lactic acid, 0.06; L-lactic acid, 0.11; succinic acid, 0.06; ethanol, 0.04.

*Mean ± standard deviation. Positive values are amounts produced; negative values are amounts utilized.
The G+C content of the DNA is 39 mol% for the type strain X6C58 and for strain X10D63.

The type strain is strain X6C58 (= ATCC 35991), which was isolated from the rumen of a sheep.

_Eubacterium xylanophilum_ resembles _Eubacterium ramulus_ (9) in G+C content and, except for lactic acid production by the latter species, also in types of fermentation products. An important difference between the two species is that _Eubacterium ramulus_ ferments at least six more carbohydrates that _Eubacterium xylanophilum_. Whereas _Eubacterium ramulus_ cells occasionally have marked swellings, this does not occur in _Eubacterium xylanophilum_. Also, _Eubacterium ramulus_ is nonmotile.

_Eubacterium uniforme_ and _Eubacterium xylanophilum_ are not readily detected without selective media because they form only small proportions of the total bacterial population in ruminas; this contrasts with _Eubacterium cellulosolvens_, which digests cellulose but ferments xylan poorly, if at all.

In our previous study (13) no isolates of _Eubacterium ruminantium_ were found. This species constituted between 3 and 7% of the total isolates from a relatively nonspecific medium inoculated with ingesta from the rumina of animals that were fed a range of high-roughage diets (1). About one-half of these organisms fermented xylan. However, this species was not isolated from animals fed wheat straw or ladino clover (1). It is possible that _Eubacterium ruminantium_ produces only xylanases that are not readily released from the cells and that do not diffuse and produce clearings in xylan agar media. In that case we would not have detected it among the xylanolytic bacteria in our previous study (13), even if it were present in high numbers in the rumina.

_Eubacterium helwigiae_ has also been isolated from rumina (11) but was not found in the study (13) during which the strains described in this paper were isolated. It is not known whether _Eubacterium helwigiae_ plays any role in fiber digestion.

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LITERATURE CITED


