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Isolation and Characterization of a New *Sarcina*-Like Cellulolytic Bacterium from an Anaerobic, Mesophilic, Cattle-Waste Digester

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ABSTRACT

Anaerobic digesters contain a variety of anaerobic cellulolytic bacteria but the presence of cellulolytic Sarcina *has been undetected in this or other habitats. The present paper describes the isolation and characteristics of a cellulolytic* Sarcina *species from an anaerobic, mesophilic cattle-waste digester. It produces lactate as a major product.*

INTRODUCTION

The vital role that anaerobic cellulolytic microorganisms play in breaking down polymeric cellulosic substrates into volatile fatty acids to be acted upon by a consortium of bacteria to produce CH_4 and CO_2 in anaerobic digestion, or to produce volatile fatty acids in the rumen, and thus bring about organic-carbon recycling in nature, is well documented (Hungate, 1966; Hobson *et al.*, 1974,1981; Ljungdahl & Eriksson, 1985). Production of commercially-vendable products like acids, solvents, liquid fuels, by these anaerobes has been, and is, of great interest too (Zeikus, 1980; Hobson *et al.*, 1984).

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However, strictly anaerobic cellulolytic species are not very many. Among cellulolytic cocci, there are only two major names, *Ruminococcus albus* and *R. flavefaciens*, and in the present paper we describe the characteristics of a new cellulolytic sarcina species which is sporulating.

METHODS

The methods and media for isolation were as described previously (Sharma & Hobson, 1985*a*) using the strictly anaerobic technique of Hungate (1950). Tests, except where specified, were done anaerobically and followed, in general, the media and methods of Holdeman *et al.* (1977).

The strains were isolated from dilution cultures of samples from a 150 liter, mesophilic, anaerobic digester which had been running for some years on dairy cattle waste, as described previously (Sharma & Hobson, 1985*a*). The substrate used for the isolation of these cultures was cellulose powder (C numbers). Fermentation and other tests were done as described by Sharma & Hobson (1985*a*). PY medium and the media based on this are peptone yeast medium described by Holdeman *et al.* (1977).

Salt tolerance was tested in PYG and nutrient broth containing glucose with various NaCl concentrations, mainly 6·5 and 7·0%.

Sporulation was fostered and detected using the sporulation medium developed during the course of this investigation (Sharma & Hobson, 1985*b*), and the presence of dipicolinic acid (pyridine-2,6-dicarboxylic acid) was checked by the method of Janssen *et al.* (1958).

Absence of sulphate reduction and desulfoviridin was determined by the method of Postgate (1959) and also by the method of Sharma and Hobson (1987).

Antigen preparation for serological testing was done both by the autoclave extraction method of Rantz and Randall (1955) as well as by the classic extraction method of Lancefield (1933). Slide agglutination tests were performed by placing four separate loopfuls of sterile saline on marked slides, followed by loopfuls of a test antiserum (either for M, N or D Group Streptococci from Burroughs Wellcome or the ones prepared in the Department for *S. bovis*, *R. albus* or *R. flavefaciens*). A loopful of antigen was added to two of the saline antiserum pools and mixed to give a uniform suspension. Agglutination was observed against controls of the saline antiserum mixture. If the suspension dried up before the results were obtained the tests were repeated. Antigen(s) were also cross-checked with capillary precipitation tests in glass capillary tubes of about 1 mm diameter, cut off square, wrapped and sterilized by autoclaving. A capillary was dipped in the antiserum to draw up a column of liquid about 1 cm long and the tube

held vertically and wiped on the outer surface. The tube was then dipped in the antigen extract until a further 1 cm of liquid was drawn up and the tube again wiped (any tubes with air bubbles were discarded). The tube was kept horizontal after mixing the contents by tilting and examined in bright light against a dark background. A strongly positive reaction gave a precipitate in 5 min, a weakly positive reaction took longer. If no precipitate appeared in 30 min the reaction was taken as negative, as recommended (Facklam & Wilkinson, 1981).

G + C mol% ratios were determined as described previously (Sharma & Hobson, 1985*a*) except that partially purified DNA was used as described by Hill (1968) and Gibson and Ogden (1979) employing isopropanol precipitation of DNA as one of the purifying steps. It has been suggested (Owen & Lapage, 1976) that the assay should be done on partially purified DNA. G + C mol% ratios were determined from the melting temperature (Tm) using Deley's equation (Deley, 1970).

RESULTS AND DISCUSSION

Strains C 456, C 457 and C 466 were obtained from 10^{-4} dilutions of digester contents. Morphological and fermentation-product tests were done on all isolates and strain C 456 was characterized extensively.

Strain C 456 and others were Gram-variable, but frankly Gram-positively staining, cocci, mainly in bundles and packets, with some diplococci. Refractile spores could be seen. Extracellular slime, presumably polysaccharide in nature, was formed. C 456 gave cream or lighter coloured, 0·5 mm flat to raised colonies in glucose roll tubes. Larger colonies (2 mm) were seen which had a transparent outer rim and growth from larger colonies tended to flow as a slime down the culture tube. C 457 was similar, while C466 colonies tended to be bigger (2–4 mm) with a distinct outer slime layer. On medium 2 (Sharma & Hobson, 1985*a*), these colonies were more yellow to orange than those of other isolates. Colonies on cellulose roll tubes were similar, and colony size increased somewhat in mixed culture (see later). On egg yolk agar medium all colonies were cream coloured, raised, 1–2·5 mm and mucoid with a tendency to form a rugged margin. An inoculum from the sporulation medium gave white, raised colonies with C 456, but C 457 and C 466 gave creamy, 0·5–2 mm, raised colonies, with an entire margin. On blood agar all colonies were mucoid, glistening, 0·5–3 mm, with umbonate center and some rugged margins in bigger colonies. On aerobic nutrient agar C 456 formed cream, convex, pin point to 0·5 mm colonies with a rugged margin to larger colonies. C 456 was initially anaerobic, but became capable of aerobic growth after long-term

subculture. All three isolates gave a uniform turbidity in PYG broth or other liquid culture, which finally sedimented. The sediment was gelatinous and difficult to break up. Repeated transfers showed that they would grow well in the dilution liquid without added sugars, though the growth lysed within a week, and so required quicker transfers.

In PYG medium C 456 formed (all values in parentheses are mM concentrations detected in duplicate cultures) propionate (1·1), acetate (0·3), ethanol (0·8), lactate (38·5), succinate (5·1). C 457 formed acetate and ethanol (1·1 each), lactate (33·1), succinate (5·1), and C 466 formed acetate (5·7), ethanol (3·0), butyrate (2·7), lactate (34·5), succinate (3·1). All showed the presence of formate and C 457 formed traces of propionate, otherwise no other acid products up to caproate were found. Carbon dioxide was the only gas formed in cultures grown under 90% N_2, 10% CO_2.

Isolate C 456 fermented cellobiose, dextrin, fructose, glucose, lactose, maltose, mannose, melibiose, ribose, salicin, sucrose, and (weakly) erythritol, xylose, xylan. Aesculin was hydrolysed, milk formed an acid clot, haemolysis was negative to weakly α and there was growth in bile and Tween media. A slight, mucoid growth was seen in starch roll tubes, but no detectable degradation of starch took place. C 457 and C 466 grew in bile, and all three isolates grew in 6·5% NaCl, while C 457 and C 466 also grew in 7% NaCl. All grew at pH 9·6 and C 456, C 457 grew in methylene blue.

Acetyl methyl carbinol, indole and urease were not produced and nitrate was not reduced. Arabinose, adonitol, dulcitol, inositol, inulin, mannitol, melezitose, raffinose, rhamnose, sorbitol, trehalose were not fermented. Gelatin was not hydrolysed and the isolates were catalase negative.

C 456 did not grow at 20 or 50°C, but had a growth optimum between 35 and 45°C. There was some growth at pH 4·5 and 5·0 and growth increased with increasing pH to 8·0 and there was growth at pH 9·6. Terminal pH in sugar media was about 4–4·5. All three isolates survived 75 and 80°C for 10 min. Dipicolinic acid was detected in sporulating cultures.

None of the isolates gave antigens reacting with the commercial streptococcal Group M, N, D antisera. These antisera were selected for the following reasons, Group M: *Streptococcus pyogenes* could be an aerial contaminant. Group N: *Streptococcus cremoris* are associated with cattle and Group D: *S. bovis*, *faecium*, *faecalis* are common rumen inhabitants. The lack of reactions suggested that the isolates were not these species. They gave slight agglutination with antisera to *S. bovis*, *R. flavefaciens* and *R. albus* prepared in the Department.

All three isolates showed moderate filter-strip disintegration (3 units) on an arbitrary scale as described previously (Sharma & Hobson, 1985*a*), and degraded ball-milled cotton to a greater extent in a mixture than individually. Isolates C 466 and C 456, which individually produced 3·5 and

1·7% loss in weight of cellulose powder, gave enhanced cellulolysis in mixed culture, but the experiment gave varying results when repeated so the numerical results are not presented. It has been reported (Wood *et al.*, 1982) that the cellulase of *R. albus* is cell wall-bound and of high molecular weight, but that it is released into the culture medium in forms varying in molecular weight with culture conditions. As cultural conditions were the same in repeat cultures in the present investigation, this does not explain the variable results. Growing the mixed culture in cellulose roll tubes the same amount of inoculum (0·5 each for the mixed culture as compared to 1·0 for individual culture) gave bigger colonies than the individual culture; this seems to suggest that there was mutual stimulation of the culture in utilizing the only available carbon source, cellulose, and that the components of the cellulase complex were produced by the cultures in variable amounts and showed some synergistic effect. Since simultaneous enzyme assays were not done, it is difficult to ascertain if the varying results were because of alterations in proportions of endo- and exoglucanases and cellobiase, though this looks probable. Synergistic action between variants of a strain of *Ruminococcus* which apparently had different effects on cellulose agar, in that either a clear zone, or a hazy zone or no zone was formed around colonies, has been reported (Leatherwood, 1969). Interactions of substances diffusing from colonies without zones or with hazy zones produced clear zones. The results were put forward in a hypothesis of cellulase being a multicomponent enzyme.

In spent cellulose medium, glucose, cellobiose, cellodextrins and xylose remained. The presence of glucose appears interesting in the sense that the other digester anaerobes, in general, did not produce it as a result of cellulose hydrolysis (Sharma, 1983; Sharma & Hobson, 1985*a*, 1986*a*). This test was done about 2 years after the isolation of the culture and the presence of released sugars from cellulose further suggests the cellulolytic nature of the cultures.

Since bacteriocin-like compounds showed variable effects (complete inhibition, stimulation or no effect) on the growth of representative cellulolytic bacterial populations of the anaerobic digester (Sharma & Hobson, 1986*b*), the effect of these compounds was also investigated on these cultures. The source of these compounds was the cell-free growth filtrate from *Clostridium butyricum* T 278, *C. sporogenes* C 249 and one *Clostridium* species not identified to a species level. The present isolates were unaffected by the compound(s).

The present isolates became aerotolerant. Some anaerobes like *Selenomonas*, *Peptococcus*, *Peptostreptococcus* and *Streptococcus* are known to become aerotolerant after repeated sub-culture (Holdeman *et al.*, 1977). However, the fermentative nature of metabolism suggests that oxygen is not the terminal electron acceptor.

The present isolates differ from *Gemmiger*, *Coprococcus* and *Ruminococcus* in growing without added carbohydrate, and since they are Gram-positive to Gram-variable they differ from the Gram-negative *Veillonella*, *Acidaminococcus* and *Megasphaera*. They differ from *Micrococcus*, *Paediococcus* and *Sporosarcina ureae* in being catalase-negative. They also differ from *Streptococcus*, *Peptococcus* and *Peptostreptococcus* in a number of characteristics. The G + C% ratio for the present isolates was 34·9.

In general morphology, spore formation and negative catalase reaction the isolates seem best classified as *Sarcina*. Some *Sarcina* have been reported to form small amounts of hydrogen (Canale-Parola, 1970), but these isolates did not. Knoll (1965) said that *Sarcina* do not ferment starch and mannitol, in conformity with these isolates. Isolate C 466 produced butyrate, and differentiation of *S. ventriculi* from *S. maxima* has been reported (Holt & Canale-Parola, 1967) in that the former produced ethanol and the latter butyrate, amongst other products. However, all three of the present isolates produced some ethanol but only C 466 produced some butyrate.

No isolate produced urease and this differentiates it from the aerobic *S. ureae*. C 456 differed from *S. ventriculi* in being of smaller size and in fermenting xylose and ribose but not melezitose and raffinose, in weakly fermenting erythritol, and in producing lactate as a major product. It is proposed that C 456 is representative of a new species and genus and a suggested name is *Lactosarcina cellulosolvens* ('Lacto' for lactic acid production, 'sarcina' (Latin) meaning packet or bundle) even if cellulolytic activity diminishes with time. This latter part of the name is in consonance with *Butyrivibrio fibrisolvens*, which shows variable cellulolytic activity (almost nil to 'some'; Krieg & Holt, 1984) and is still called *fibrisolvens*. *Cellulomonas fermentans* is called 'cellulolytic' because of 'some aptitude' for cellulose (Begnara *et al.*, 1985). Cellulolytic bacteria from the rumen tend to lose cellulolytic activity even when maintained on cellulosic substrate (Hobson, 1969) but are called 'cellulolytic'.

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