The properties of forms of *Ruminococcus flavefaciens* which differ in their ability to degrade cotton cellulose

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1. SUMMARY

Two mutant strains of *Ruminococcus flavefaciens* strain 007 that differ in their ability to hydrolyse cotton fibres have been shown also to differ in their cell-surface topology, in that the cotton degrading form possessed larger and more protuberant cell surface structures. The strains had similar CMCase, cellobiosidase and β-glucosidase activities. The results indicate the importance of cell-surface properties in cotton degradation by *R. flavefaciens*.

2. INTRODUCTION

Some strains of the anaerobic rumen bacterium *Ruminococcus flavefaciens* extensively degrade cotton fibres [1,2], an archetypal form of cellulose used as a reference substrate for certain cellulases [3]. On first isolation *R. flavefaciens* strain 007 caused losses in weight of cotton fibres of up to 80% [2]. Single-colony re-isolates of strain 007 lost around 75 to 85% of this activity during prolonged cultivation on cellobiose. Conversely, cultivation on cotton (but not cultivation on straw) restored degradative activity to approximately that of the wild-type strain. The pattern of these changes suggests that they result from mutations [4]. The form with high cotton degrading activity (007C) was only slightly more active than the form with weak cotton-degrading activity (007S) in its ability to degrade filter paper and avicel, and the two forms differed little in their ability to degrade cereal straws [4]. Some further properties of the mutant strains 007C and 007S are described here.

3. MATERIALS AND METHODS

3.1. Bacteria

Unless stated otherwise, cultures of 007C and 007S were maintained anaerobically with cotton or cellobiose as substrate respectively, as previously described [4].

3.2. Enzyme assays and zymograms

Cultures were grown on 300 mg barley straw in 90 ml medium M2 [5] without soluble substrates as described in [4]. After incubation for 5 days at
38°C, the cultures were shaken using a vortex mixer for 3 min to detach adherent cells. The straw was allowed to settle out and the liquid fraction containing the suspended cells was decanted off and centrifuged at 5000 × g for 10 min at 4°C. The cell pellet was resuspended in 7.2 ml sodium phosphate buffer (0.05 M, pH 6.8) containing 0.01% (w/v) dithiothreitol and disrupted by ultrasonic disintegration for 3 min. The resulting cell extracts were immediately assayed for carboxymethylcellulase (CMCase) by measuring the sugar released on incubation with CMC, and for p-nitrophenyl (PNP)-glucosidase and PNP-cellobiosidase activities by the methods referred to by Flint et al. [6]. Protein was determined using the Bio-Rad assay system (Bio-Rad Laboratories Ltd., Watford, Herts., U.K.) after boiling the samples in 0.5 M NaOH to release intracellular proteins. Zymograms were prepared from cell pellets, and visualised after electrophoresis using polyacrylamide gels containing 0.1% CMC, essentially as previously described [7].

### 3.3. Colonisation

Cultures were grown for 16 to 18 h in medium M2 with 0.25% (w/v) cellobiose as sole energy source, and the colonisation of cotton, avicel and straw during 45 min exposure to these substrates was determined by an optical density (OD<sub>650</sub>) method [8]. The colonising biomass was calculated by (separate) calibration of the culture OD<sub>650</sub> of the two mutant strains against cell dry weight, determined by recovering cells from 100 ml of medium by filtration through 0.22 μm membrane filters, washing with water and drying at 80°C.

### 3.4. Scanning electron microscopy (SEM)

Cells were fixed with glutaraldehyde and post-fixed with OsO<sub>4</sub> [9]. After critical point drying, the cells were coated with platinum and observed in a Jeol 1200 XB TEM Scan.

### 4. RESULTS AND DISCUSSION

When cultures were grown for 18 h with cellobiose as substrate, examination by Gram staining [4] and by SEM showed that 007C formed chains of up to 60 cells, whereas 007S existed mainly as diplococci and short chains. Cells of 007C possessed surface protrusions that were much more prominent than those of 007S (Fig. 1a,b). This finding is interesting in view of the observation [10] that several species of cellulolytic bacteria possess protuberant surface structures immunogenically related to the cellulosomes of *Clostridium thermocellum*, but that such structures are absent from non-cellulolytic bacteria. The presence of these structures was shown to be influenced by the culture conditions; the present observation sug-

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**Fig. 1.** SEM and Gram stain (inset) appearance of (a) 007S and (b) 007C. The cultures were grown for 18 h on medium M2 [5] with cellobiose as sole carbohydrate; the culture of 007C had previously been maintained on medium M2 with cotton as sole carbohydrate. Bar = 1 μm.
Table 1

Carboxymethylcellulase, PNP-cellobiosidase and PNP-glucosidase activity associated with cells of 007C and 007S grown on straw

<table>
<thead>
<tr>
<th>Strain</th>
<th>007C</th>
<th>007S</th>
</tr>
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<tbody>
<tr>
<td>CMCase</td>
<td>0.25 ± 0.05</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>PNP-glucosidase</td>
<td>1.6 ± 0.2</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>PNP-cellobiosidase</td>
<td>0.5 ± 0.2</td>
<td>0.6 ± 0.3</td>
</tr>
</tbody>
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\[ \text{a } \mu\text{mol glucose released (h}^{-1}\text{ mg protein}^{-1}) \]

\[ \text{b } \mu\text{mol PNP released (h}^{-1}\text{ mg protein}^{-1}) \]

\[ \text{The average amount of protein present in the cell extracts was 392 } \mu\text{g ml}^{-1} \text{ (007C) and 375 } \mu\text{g ml}^{-1} \text{ (007S)} \]

suggests that the presence of such structures may sometimes be correlated with the ability to degrade a particular form of cellulose.

Cells of 007C and 007S grown on barley straw showed similar PNP-cellobiosidase, PNP-glucosidase and carboxymethyl cellulase activities (Table 1). Analysis of the culture supernatants (data not shown) also revealed no major differences between the cultures. Zymograms of 007C, 007S and strain 17 [6] CMCases revealed the presence in each strain of 4 apparently identical major activity bands (data not shown). Both endo- and endwise acting enzymes have been found [11] among the cellulases of the type strain (FD1) but the presence of cellobiohydrolases, components of the cellulase systems of soft-rot fungi [12] essential for the degradation of cotton and some other celluloses, has not been demonstrated in ruminococci; indeed their role in anaerobic bacterial cellulolysis has yet to be verified [13]. Since cellobiohydrolases may differ in their substrate stereospecificity [14], not all such enzymes may be active against PNP-cellobiosidase. Furthermore, PNP-cellobiosidase activity may be due partly to endoglucanases, \( \beta \)-glucosidases and cellobextrinases. Despite the similarity of the enzymes of 007S and 007C in these tests, it remains possible that significant differences exist in the individual enzyme components or in the organisation of their cellulases.

Measurement of the colonisation of cotton fibres, avicel and barley straw showed that the average proportion of the biomass that adhered to the substrates tended to be highest with 007C, but the differences between 007C and 007S were not statistically significant when cotton or avicel were substrates. Colonisation of pure celluloses was greater than that of straw, and colonisation of straw was enhanced by pretreatment with NH4. The colonising biomass (\( \mu \)g cells/mg substrate ± S.D.) of 007C and 007S was as follows, with values for 007C quoted first. Cotton, 5.8 ± 1.0 and 4.0 ± 1.0; avicel 5.7 ± 0.6 and 4.9 ± 0.5; untreated barley straw (BS) 3.5 ± 0.6, and 1.9 ± 0.7; NH4 treated BS 5.6 ± 1.3 and 3.3 ± 0.6. It is very unlikely from these findings that the differences in the cotton degrading activity of these cultures rests primarily on their colonising ability. A study of adherence and cellulolysis in strains of Ruminococcus albus [7] showed that although cellulolytic strains tended on average to adhere more readily to cellulose than non-cellulolytic strains, the most actively cellulolytic strains were not necessarily the most adherent, stressing the importance of events following initial colonisation/adhesion.

Hofsten [15] has suggested that the effectiveness of some bacterial cellulases depends on contact between the cell surface and the substrate, resulting in the alignment of the enzyme systems along the cellulose molecules. The present results suggest that the high cotton degrading activity of 007C is accompanied by the presence of protruberant cell surface structures, but that it is probably events after initial colonisation of the substrate that are critical in determining the ability to degrade cotton.

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REFERENCES


