

Selection of transformants of *Escherichia coli* containing cellulase gene from *Ruminococcus albus* isolated from rumen of crossbred steers

M Chandrasekharaiah*, A Thulasi and K T Sampath

National Institute of Animal Nutrition and Physiology, Hosur Road, Adugodi, Bangalore 560 030, India

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Ruminococcus albus, considered best fibrolytic bacterium was isolated and characterized from the rumen of crossbred steers. It was found as wrinkled white colonies, slightly elevated with a slightly undulated margin, no surface spreading with the absence of liquefaction and a zone of hydrolysis. The cells were gram positive single cocci or diplococci. *R. albus* DNA was a high molecular weight DNA and it had just moved out of the well during electrophoresis. A genomic library of the Hind III fragments of *R. albus* DNA in pBR322 was constructed in *Escherichia coli*. Four clones were obtained with cellulase activity.

Keywords: *Ruminococcus albus*, cellulase genes, transformants, *Escherichia coli*, rumen

IPC Codes: Int. Cl.⁷ C 12 N 15/10, 15/11

Introduction

Cellulose is one of the most abundant polymers on earth and is the chief structural component of plant cell-wall polysaccharides. It is the major contributor to the global carbon cycle and its biodegradation supports a large number of specialist organisms. Research on cellulose biodegradation in the rumen includes studies on the physiological characteristics of the cellulolytic organisms and their biochemical properties¹. The arrangement of cellulose is variable and complex in the plant material. The degree of crystallinity, surface area and pore structure have variable effects on the rate and extent of biodegradation of cellulose by different classes of cellulolytic microbes. Resistance to degradation is related to the degree of order within the molecule, with celluloses of high crystallinity index showing the lowest rates of degradation in the rumen².

The digestion of celluloses or fibre is about 35-65% in the rumen. An increase in the fibrolysis by another 10-15% would serve to increase the production performance of the animal that will be beneficial to the farmers in India, where the livestock primarily depend on the high fibrous crop residues. This can be done by isolating the cellulase gene from cellulolytic organisms and cloning them in non-cellulolytic

organisms. The recombinant organisms could then be studied for altered cellulose utilization. The present study was undertaken to prepare a genomic library of the Hind III fragments of *Ruminococcus albus* DNA in *Escherichia coli* and screen the recombinants for cellulase activity.

Materials and Methods

Animals, Collection of Rumen Digesta

Fistulated steers (wt, 350 kg) maintained on straw and concentrate diet, gave rumen digesta, which was collected in sterile bottles flushed with carbon dioxide and was used as a source of inoculum. Anaerobic dilution solution (ADS) was prepared and used to obtain serial dilution of the rumen digesta. For the cultivation of cellulolytic bacteria, 0.1 ml of the diluted contents from 10⁻⁸ dilution was used. The rumen fluid was also collected, centrifuged, flushed with carbon dioxide and used as a component of the medium.

Media Used

ADS

The composition of ADS³ was as follows: (i) Mineral solution I-0.3% dipotassium hydrogen phosphate, 15.0 ml; (ii) Mineral solution II-0.3% potassium hydrogen phosphate+0.6% ammonium sulphate+0.6% sodium chloride+0.6% magnesium sulphate+0.06% calcium chloride, 15.0 ml; cysteine hydrochloride, 0.05%; sodium carbonate, 0.3%; resazurin, 0.001%; and distilled water, to make upto 100 ml.

*Author for correspondence:

Tel: 91-80-25711304, 25711164; Fax: 91-80-25711420

E-mail: root@nainpbng.kar.nic.in

RGCA

Modified Rumen Glucose Cellobiose Agar (RGCA) Medium⁴ used for the isolation of the cellulolytic bacteria was as follows: clarified rumen fluid, 20.0%; glucose, 0.0248 g; cellobiose, 0.0248 g; ammonium sulphate, 0.1 g; agar, 2.0 g/100 ml; mineral solutions I and II, 15% each; haemin, 50 mg; vitamin K₁, 0.1 mg; L-cysteine hydrochloride, 0.5 mg; resazurin, 0.1 mg/100 ml; distilled water, to make upto 100 ml.

The media was prepared and dispensed into roll tubes and then autoclaved at 15 psi for 45 min. The diluted rumen contents were inoculated into the roll tubes^{5,6} at 45°C and the agar was solidified. The tubes were then incubated at 39°C in a Memmert ICP 500 programmable incubator. The colonies with a zone of hydrolysis were then picked up using a long platinum loop and inoculated into the rumen glucose cellobiose broth kept in sterile bottles. The bottles were crimp sealed and incubated at 39°C. The various isolates were identified using the standard microbiological techniques.

Isolation of Genomic DNA and Partial Digestion of DNA from *R. albus*

Genomic DNA was extracted from the primary cellulolytic bacterium, *R. albus*⁷. A loopful of culture was inoculated into 100 ml of RGC broth and incubated at 37°C for 48 hrs. The culture was centrifuged at 5000 rpm at 4°C for 30 min to pellet the cells. To the bacterial pellet, a minimum volume of Tris EDTA buffer pH 7.5 (10 mM Tris/1 mM EDTA) was added and the suspension was transferred to a micro-centrifuge tube. Lysozyme was then added to the cells (1 mg/ml) and the contents incubated at 37°C for 1 hr. Proteinase K (10 µl) was then added and the contents incubated at 37°C for 30 min. RNase (10 µl) was also added and the contents incubated overnight at 37°C. To this, an equal volume of phenol:chloroform (25:24) was added and centrifuged at 10,000 rpm for 10 min. The aqueous layer was then removed and this step was repeated twice. To the aqueous layer, chilled ethanol (3-6 vols) was added to precipitate the DNA, which was then washed with 70% ethanol and the pellet thus obtained was dried under vacuum. The DNA thus obtained was dissolved in TE buffer (pH 8.0). The purity and the quantity of DNA obtained from 100 ml of the cultures were determined. The DNA was run on 0.8% agarose gel with 1 X TAE buffer.

The reaction mixture for the restriction of the genomic DNA was: genomic DNA, 10; assay buffer, 2; Hind III, 3; and water, 5 µl. This mixture was then incubated for 2 hrs at 37°C. The reaction was stopped by the addition of EDTA and chilling the contents of the tube to 0°C.

Agarose Gel Electrophoresis

Agarose (0.24 g) was mixed with 30 ml of 1X TAE buffer. The mixture was heated to melt the contents, cooled and added ethidium bromide and then cast the gel.

The DNA (restricted/unrestricted) was then mixed with the gel-loading buffer (0.25 g each of bromophenol blue and xylene cyanol in 40% sucrose). A marker, λ Hind III digest, was also run alongside. The gel was placed in a submarine electrophoresis unit and run at 50 V for 3 hrs. The gel was then viewed under a UV-Transilluminator. Partially digested genomic DNA was loaded onto the wells in 0.6% low m p agarose gel and electrophoresis was done. The gel portion containing the 2-10 fragments was cut by viewing the gel under UV light. The DNA fragments were eluted from the gel⁸.

Ligation of the Various Hind III Fragments to pBR 322

The ligation mixture comprised *R. albus* DNA (fragments, 2-10 kb) partially digested with Hind III, pBR 322 digested with Hind III, Ligation buffer and DNA ligase. The mixture was incubated at 15°C for 12 hrs and then stored at 4°C.

Preparation of Competent Cells and their Transformation

The lyophilized *E. coli* HB101 cells were revived using LB broth. A loopful of the revived culture was inoculated in LB broth, grown overnight, centrifuged and chilled CaCl₂ was added to the pellet. The contents were then chilled for 45 min and used for transformation⁹. The ligated DNA mixture was added to the *E. coli* strain HB101 cells kept over ice. The cells were transferred to 42°C for 5 min and then LB medium was added to the tube. The tubes were rapidly transferred to 37°C for 1 hr. The contents of the tube were then plated in Petri dishes containing LB-ampicillin.

Screening of the Transformants

The colonies on the LB-ampicillin plates were screened by the Congo Red assay. Single colonies on the LB-amp plates were grown in replica plates. After the appearance of colonies, the plates were overlaid

with 1% agar containing carboxy methylcellulose. The plates were incubated and then flooded with Congo Red¹⁰ (1 mg/ml).

Results and Discussion

In the RGCA medium, white and yellow colonies were obtained. Six wrinkled white colonies, taken for the study, were found to be slightly elevated, with no surface spreading and had a zone of hydrolysis. The cells were Gram positive and either single cocci or diplococci. In this study, only the diplococci were classified as *R. albus*¹¹. The isolate (pH, 5.2-5.7) was non-motile and capsulated with no spore formation. Cellulose, cellobiose lactose and glucose were fermented by these isolates. However, xylose, rhamnose, raffinose, salicin, maltose, trehalose, inulin, glycerol and starch were not used by these strains. The results obtained were in accordance with the fermentation characteristics of *Ruminococci*. All the isolates tested positive for the O.F. test. However, none of the isolates were tested positive for catalase test, gelatin liquefaction, nitrate reduction, methyl red test, hydrogen production test and Vogues Prauskauer test. These strains were obligate anaerobes, and were not proteolytic¹². The end products of fermentation were analyzed to characterize the various bacteria. In the case of these isolates, the end products were acetate, formate and lactate. These isolates were identified as *R. albus*, which were devoid of yellow pigments. *R. albus* is considered to be the most active bacteria involved in the plant cell wall digestion in the rumen¹³⁻²⁰.

The genomic DNA (3.9-4.2 mg/ml of the isolates), isolated from *R. albus*⁷, was confirmed by 0.8% agarose electrophoresis. It had just moved out of the well during electrophoresis. After partial digestion with the restriction endonuclease Hind III, streaky bands were obtained.

The vector pBR322 used in this study was restricted with Hind III (single site for this enzyme on this vector) to linearize it. *R. albus* DNA was inserted in the *tet*^r region of pBR322 and hence the clones containing the plasmid with the insert were sensitive to tetracycline. *E. coli* HB101 strain used was rec A⁻ and was of the genotype: *thi*-1, *hsdS*20 (*r*_B⁻, *m*_B⁻), *supE*44, *recA*13, *ara*-14, *leuB*6, *proA*2, *lacY*1 *rps* L20 (*Str*^r), *xyl*-5, *mtl*-1. *E. coli* cells were first made competent with chilled calcium chloride and the transformed with pBR322 containing 2-10 kb fragments. Thus, a genomic library comprising Hind

III fragments of *R. albus* DNA in pBR 322 was constructed in *E. coli* (Fig. 1).

The genomic library prepared by ligating the 2-10 kb fragments of *R. albus* DNA into the Hind III site of pBR 322 was screened for cellulase activity by Congo Red Assay¹⁰ based on the expression of the activity of the gene in the recombinants. Slots were made on the medium and each clone added to it. The plates were then incubated at 37°C and Congo Red added to the plates. After incubation again, the Congo Red was discarded and the various clones were observed for a zone of clearance. The recombinant clones did not take up the dye and showed a zone of clearance. After screening 2600 clones, 4 clones were found positive for the Congo Red Assay. Hence in this study, genomic DNA was extracted from *R. albus*, restricted with Hind III and a genomic library of the Hind III fragments in pBR322 was constructed in *E. coli*. Of the 2600 transformants screened for cellulase activity, 4 clones were obtained with cellulase activity. The plasmid was isolated from these clones and the size of insert was found to be 2.5 k_B. Further studies are going on to characterize cellulase gene and its expression.

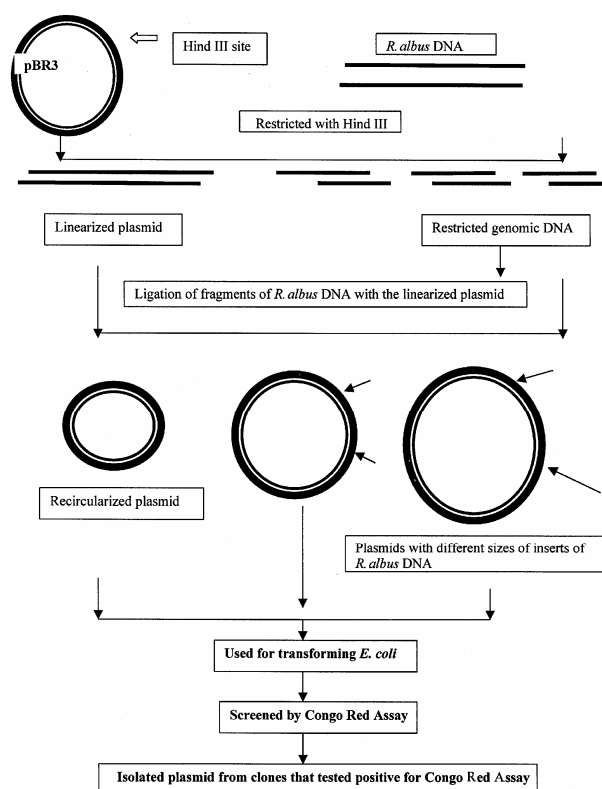


Fig. 1—Scheme of cloning in *E. coli*

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