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SHORT COMMUNICATIONS

Antibiotic Resistance of Natural Isolates of Ruminococci and Properties of Plasmid pRA37 Encoding Erythromycin Tolerance of *Ruminococcus albus* 37

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Anaerobic bacteria of the genus *Ruminococcus* are permanent rumen inhabitants; they are actively involved in cellulose degradation and, thereby, contribute to the organic carbon flow through the food chain of terrestrial communities. These bacteria have a beneficial effect on the efficiency of feed utilization by ruminants, which are able to utilize the cellulose content of forage only by half, although it is the major nutrient ensuring their energy requirements [1]. Natural isolates of ruminococci differ with respect to their cellulolytic, adhesive, and other properties including the activities of endo-1,4-β-glucanase, cellobiohydrolase, β-glucosidase, and xylanase [2, 3]. In spite of the great importance of ruminococci, they are as yet insufficiently studied, mainly owing to the difficulties encountered in the cultivation of these strict anaerobes. Only for one cryptic plasmid from *Ruminococcus albus* AR67 was the structure completely established [4]. Thus, ruminococci are less studied genetically than other anaerobic inhabitants of the digestive tract [5]. This paper presents data on the antibiotic resistance of 27 natural isolates of ruminococci; on the genetic localization of appropriate determinants; on the ability of plasmids to encode other physiological properties, such as the activities of cellulolytic enzymes and xylanase; and the results of restriction analysis of plasmid pRA37 from strain *R. albus* 37 encoding its erythromycin resistance.

Anaerobic cellulolytic bacteria were isolated from rumina of cows which had been fed an antibiotic-free diet in the Kaluga and Voronezh oblasts. Samples of rumen contents were withdrawn through a fistula or with the help of a gastric probe and inoculated into liquid Hungate medium [6] containing filter paper strips. Pure cultures were obtained using agar medium of Scott and Dehority [7] supplemented with 2% of soluble cellulose sulfate (Serva, Germany) by the roll tube culture method [8]. According to the total of their properties, all the isolates producing succinic acid and forming yellow colonies were identified as *R. flavefaciens*, whereas other isolates were assigned to species *R. albus* [9].

Plasmid DNA was isolated from bacterial cells by the method of alkaline lysis with some modifications [10]; activities of cellulolytic enzymes and xylanase were determined by conventional methods [11].

It was found that 20 out of 21 *R. flavefaciens* strains and 5 out of 6 *R. albus* strains were able to grow in media containing 10 μg/ml of certain antibiotics (table). Thus, from 80 to 95% of strains isolated from animals fed antibiotic-free diet were resistant to antibiotics; 37% of strains were tolerant to high concentrations of antibiotics (up to 50 μg/ml); several strains exhibited multiple antibiotic resistance. The spectrum of antibiotics, to which ruminococci were resistant, was sufficiently wide.

To determine whether chromosomal or plasmid genes encoded antibiotic resistance in ruminococci, the medium for cultivation of the resistant strains was supplemented with either acridine orange or ethidium bromide, which are known to promote plasmid elimination from cells [12]; the cultures were exposed to light and then plated onto agar medium of Scott and Dehority containing cellulose sulfate. In each variant, 20 clones were isolated and their antibiotic resistance was compared with that revealed previously.

Out of 20 *R. flavefaciens* strains treated with plasmid-eliminating agents, 2 strains, RF50 and RF52, lost the resistance to tetracycline and erythromycin, respectively; out of 4 *R. albus* strains examined, strains RA37 and RA44 lost their resistance to erythromycin. This suggested plasmid localization of the appropriate determinants. It should be noted that plasmid localization of genes determining tetracycline and erythromycin resistance in ruminococci has already been reported [13].

To verify plasmid localization of the determinants of antibiotic resistance, we undertook the isolation of plasmid DNA from the parent strains (RA37, RA44, RF50, and RF52) and their antibiotic-susceptible derivatives (RA37 Em^S, RA44 Em^S, RF50 Tc^S RF52 Em^S) obtained after the treatment of parent strains with acridine orange or ethidium bromide. Plasmid DNA (with electrophoretic mobility corresponding to a molecular size of about 6 kb) was revealed only in the parent

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Strains	Antibiotics and their concentrations in the medium (μg/ml)											
	tetracycline		ampicillin		erythromycin		streptomycin		rifampicin		chloramphenicol	
	10	50	10	50	10	50	10	50	10	50	10	50
R. flavefaciens RF3	+	–	–	–	+	–	+	–	–	–	–	–
R. flavefaciens RF6	+	–	–	–	+	–	–	–	–	–	–	–
R. flavefaciens RF7	+	–	–	–	–	–	+	–	+	–	+	–
R. flavefaciens RF8	+	–	–	–	–	–	+	–	–	–	–	–
R. flavefaciens RF9	+	–	–	–	–	–	+	–	–	–	–	–
R. flavefaciens RF10	–	–	–	–	–	–	–	–	–	–	+	+
R. flavefaciens RF11	–	–	–	–	+	+	–	–	+	–	+	–
R. albus RA16	+	–	–	–	+	–	–	–	+	–	–	–
R. flavefaciens RF18	–	–	–	–	–	–	+	–	+	–	–	–
R. albus RA19	+	–	–	–	+	–	+	+	–	–	–	–
R. flavefaciens RF23	–	–	–	–	+	–	–	–	+	–	–	–
R. flavefaciens RF24	–	–	+	–	+	–	–	–	–	–	+	–
R. albus RA27	+	–	–	–	+	+	–	–	–	–	+	–
R. flavefaciens RF31	–	–	–	–	–	–	–	–	–	–	–	–
R. flavefaciens RF34	+	–	–	–	–	–	–	–	–	–	–	–
R. flavefaciens RF35	–	–	+	–	–	–	–	–	–	–	+	+
R. flavefaciens RF36	–	–	–	–	–	–	+	+	–	–	–	–
R. albus RA37	–	–	–	–	+	+	–	–	–	–	–	–
R. flavefaciens RF41	–	–	–	–	–	–	–	–	–	–	+	–
R. flavefaciens RF43	+	–	–	–	+	–	+	–	+	–	–	–
R. albus RA44	–	–	–	–	+	+	+	–	–	–	–	–
R. flavefaciens RF48	–	–	–	–	+	–	–	–	+	–	–	–
R. albus RA49	–	–	–	–	–	–	–	–	–	–	–	–
R. flavefaciens RF50	+	+	–	–	–	–	–	–	–	–	–	–
R. flavefaciens RF52	–	–	–	–	+	+	–	–	–	–	+	–
R. flavefaciens RF55	+	–	–	–	–	–	+	+	–	–	–	–
R. flavefaciens RF58	+	–	–	–	+	–	–	–	–	–	+	–

Note: "+" and "–" stand for "growth" and "no growth," respectively.

strains but not in their antibiotic-susceptible derivatives (data not shown). These results supported our supposition that antibiotic resistance in these strains is encoded by plasmids.

Detailed investigation was performed with strain *R. albus* RA37, which was described earlier as a highly active producer of cellulolytic enzymes [3]. This strain bears a single plasmid pRA37, which seems to encode the cell resistance to erythromycin. By means of digestion with restriction endonucleases, the plasmid size of 5.7 kb was determined and the physical map of plasmid pRA37 was constructed (Fig. 1). Plasmid DNA was found to have single sites for the restriction endonucleases *Eco*32I and *Hin*dIII and two sites for *Pvu*II; no recognition sites for the restriction endonucleases *Bgl*II, *Pst*I, or *Sma*I were revealed.

Since plasmids may encode not only antibiotic resistance of bacteria, but may also carry genes determining metabolic functions in the cells, we examined activities of the cellulose complex enzymes and xylanase in the strains that had lost antibiotic resistance. Methods for the determination of these enzyme activities were described earlier [3]. It was found that plasmid elimination produced no effect on the activities of endo-1,4-β-glucanase, cellobiohydrolase, and xylanase (Fig. 2). This suggests chromosomal localization of the appropriate genes. A more complicated pattern was observed with β-glucosidase activity, which was virtually absent in all four strains of ruminococci that had

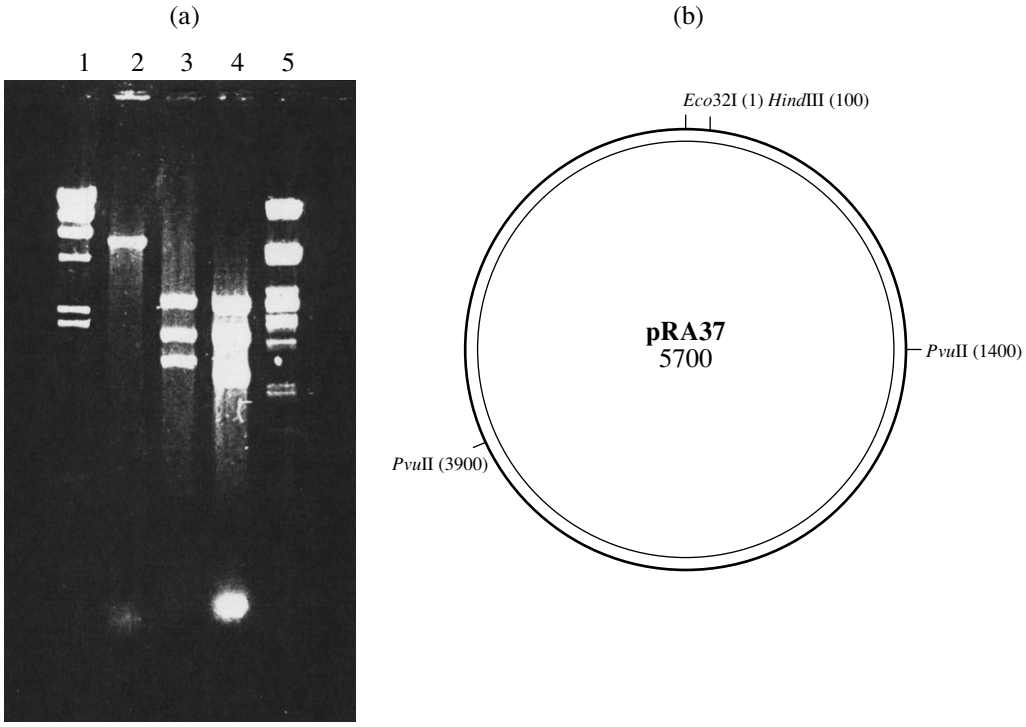

Fig. 1. Structure of plasmid pRA37 from *R. albus*. (a) Electrophoresis of pRA37 DNA digested by combinations of restriction endonucleases: (*2*) *Eco*RI and *Hin*dIII; (*3*) *Eco*32I and *Pvu*II; (*4*) *Eco*32I, *Pvu*II, and *Hin*dIII. Markers of the restriction fragment size were digests of bacteriophage λ DNA by endonucleases (*1*) *Hin*dIII and (*5*) *Pst*I. (b) Physical map of plasmid pRA37.

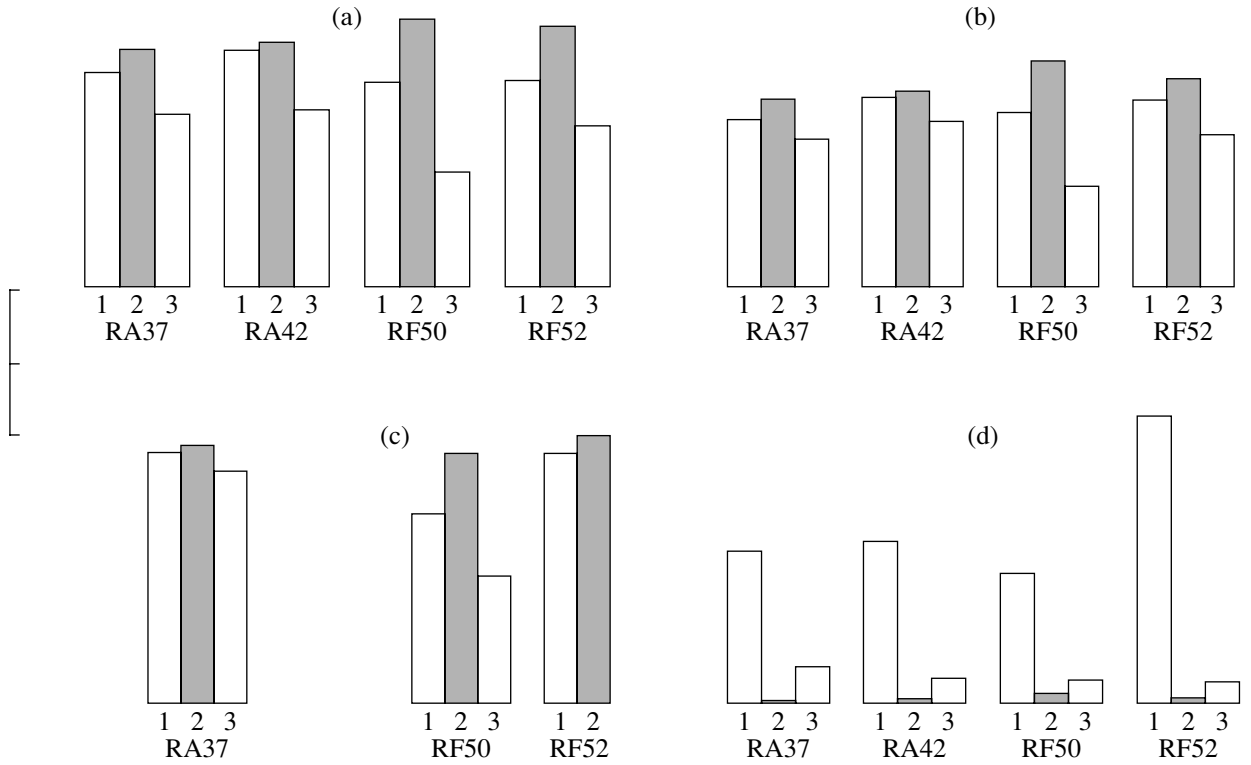

Fig. 2. Activities of (a) cellobiohydrolase; (b) endo-1,4-β-glucanase; (c) xylanase, and (d) β-glucosidase in *Ruminococcus* (1) parent strains, (2) strains treated with plasmid-eliminating agents, and (3) strains subjected to several passages on glucose-containing media. Scale bar denotes enzyme activity of 10 units/ml.

lost plasmids (Fig. 2). The most simple explanation for this fact is that the gene of β-glucosidase or of activating factors are localized in the small plasmids encoding antibiotic resistance. However, this assumption obviously needs additional substantiation. We also found that the β-glucosidase activity was highly susceptible to catabolite repression: it decreased several times after three passages of cells on medium in which cellulose, the sole energy source, was replaced by glucose (2%); in this case, the activities of endo-1,4-β-glucanase, cellobiohydrolase, and xylanase decreased only by 13–18% (Fig. 2).

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REFERENCES

1. Pivnyak, I.G. and Tarakanov, B.V., *Mikrobiologiya pishchevareniya zhvachnykh* (Microbiology of the Rumen), Moscow: Kolos, 1982.
2. Tarakanov, B.V. and Lavlinskii, D.Yu., Cellulolytic Bacteria of the Genus *Ruminococcus* from Bovine Rumen, *Mikrobiologiya*, 1998, vol. 67, pp. 518–521.
3. Tarakanov, B.V. and Lavlinskii, D.Yu., Activity of the Cellulase Complex Enzymes and Xylanase in Bacteria of the Genus *Ruminococcus*, *Mikrobiologiya*, 1999, vol. 68, pp. 312–317.
4. Ohara, H., Miyagi, T., Kaneichi, K., Karita, S., Kobayashi, Y., Kimura, T., Sakka, K., and Ohmiya, K., Structural Analysis of a New Cryptic Plasmid pAR67 Isolated from *Ruminococcus albus* AR67, *Plasmid*, 1998, vol. 39, pp. 84–88.
5. Aleshin, V.V., Semenova, E.V., Tarakanov, B.V., and Livshits, V.A., A Family of Shuttle Vectors for Lactic Acid Bacteria and Other Gram-Positive Bacteria Based on Plasmid pLF1311, *Mikrobiologiya*, 2000, vol. 69, pp. 75–80.
6. Hungate, R.E., The Anaerobic Mesophilic Cellulolytic Bacteria, *Bacteriol. Rev.*, 1950, vol. 14, pp. 1–50.
7. Scott, H.W. and Dehority, B.A., Vitamin Requirements of Several Cellulolytic Rumen Bacteria, *J. Bacteriol.*, 1965, vol. 89, p. 1169.
8. Hungate, R.E., A Roll Tube Method for Cultivation of Strict Anaerobes, *Methods Microbiol.*, 1969, vol. 38, pp. 117–132.
9. *Bergey's Manual of Determinative Bacteriology. Ninth Edition*, Holt, J. *et al*, Eds., Baltimore: Williams & Wilkins, 1994. Translated under the title *Opredelitel' bakterii Berdzhi*, Moscow: Mir, 1997.
10. Asmundsen, R.V. and Kelly, W.J., Isolation and Characterization of Plasmid DNA from *Ruminococcus*, *Curr. Microbiol.*, 1987, vol. 16, pp. 97–101.
11. Ismailova, D.Yu. and Loginova, L.G., The Effect of Certain Compounds on the Biosynthesis of Cellulase by the Thermotolerant Fungus *Aspergillus terreus* 17R, *Prikl. Biokhim. Mikrobiol.*, 1975, vol. 11, pp. 676–681.
12. Couturier, M., Bex, F., Bergquist, P.L., and Maas, W.K., Identification and Classification of Bacterial Plasmids, *Microbiol. Rev.*, 1988, vol. 52, pp. 375–395.
13. Champion, K.M., Helaszek, C.T., and White, B.A., Analysis of Antibiotic Susceptibility and Extrachromosomal DNA Content of *Ruminococcus albus* and *Ruminococcus flavefaciens*, *Can. J. Microbiol.*, 1988, vol. 34, pp. 1109–1115.