Novel Plasmids Responsible for the Cellulose Utilizing Ability of *Ruminococcus albus*

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Abstract Two novel plasmids were isolated from *Ruminococcus albus* and named as pRAB and pRAC. Both were isolated from the bacterium acclimated on an anaerobic medium containing ball-milled cellulose (BMC) as a main carbon source. When the bacterium was acclimated on cellobiose containing medium, pRAB (7.4 kbp) remarkably decreased while pRAC (15.3 kbp) remained, suggesting that pRAB requires BMC as a selection pressure. The *Pst*I site on the HI fragment of pRAC prepared from BMC containing culture broth was cleaved by the restriction enzyme with more difficulty, compared with the site of pRAC prepared from cellobiose containing culture broth. These data suggest that both plasmids are responsible for the cellulose utilization ability of *R. albus*.

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Ruminococcus albus, a rumen anaerobe, is known to be a potent cellulolytic bacterium dominant in the rumen of cattle. The cow supplies us with many economically important materials (such as meat, milk and leather) through bioconversion of inexpensive lignocellulosic feedstuff, mainly grass. One of the growth-limiting factors of cattle is considered to be the lignocellulose degrading rate of rumen microorganisms. We have previously studied the cellulolytic properties of *R. albus*, *in vitro*¹⁻⁴⁾, and its cellulases⁵⁻⁷⁾. In addition, we also investigated the degradation of lignin related compounds⁸⁻¹²⁾ and cellulose¹³⁻¹⁶⁾ by the other rumen anaerobes. We studied the enhancement of lignocellulose degradation using gene technology such as cell fusion¹⁷⁻²²⁾ and gene cloning²³⁻²⁵⁾. During the course of our research, we have often noticed the sudden loss of the cellulolytic activity of *R. albus* depending on cultivation conditions such as the

presence or absence of insoluble cellulose in the medium. This suggested the presence of plasmids in *R. albus* associated with cellulose degradation. CHAMPION *et al.*²⁶⁾ and ASMUNDSON and KELLY²⁷⁾ previously reported the presence of some cryptic plasmids. However, up to now little is known about *R. albus* plasmids. This study describes the isolation and characterization of two plasmids in *R. albus*.

Materials and Methods

R. albus F-40, isolated from bovine rumen and donated from Tohoku University, was anaerobically cultivated in a synthetic medium containing 1% ball-milled cellulose (BMC medium), 1% cellobiose (cellobiose medium) or 1% glucose (glucose medium) at pH 6.8 and 37°C and denoted as *R. albus* F-40 B, *R. albus* F-40 C and *R. albus* F-40 G, respectively. The host strain *Escherichia coli* HB 101 and a

plasmid pACYC 184 were used. Transformants were cultivated in Luria-Bertani (LB) broth containing antibiotics at 37°C, pH 7.5 under aeration. Plasmids of *R. albus* were isolated by means of the method used by BIRNBOIM and DOLY²⁸⁾. The final crude preparations were treated with RNase (25 µg/ml), extracted once with phenol-chloroform (1:1) and twice with diethylether before precipitation with two volumes of ethanol to obtain the final preparation. Relative concentrations of the isolated plasmids were determined by measuring the density of the bands on the photo-film with a scanning densitometer (DUAL-WAVE LENGTH TLC SCANNER CS-910, SHIMADZU, Kyoto). The isolated plasmids were cleaved with some restriction enzymes for mapping the restriction enzyme sites and ligated into the plasmid pACYC 184 at 16°C for 30 min. The chimera plasmids constructed were used for transforming competent *E. coli* cells. Restriction endonucleases and other manipulation enzymes were purchased from Boehringer Mannheim (Tokyo) and Takara Shuzo (Kyoto). The separation and molecular size estimation of plasmids and DNA fragments were performed by electrophoresis with agarose gel (0.7%). All these gene manipulations were performed according to the methods described by MANIATIS *et al.*²⁹⁾.

Results and Discussion

Growth of R. albus on BMC, cellobiose or glucose.

R. albus F-40B was inoculated into the medium containing BMC, cellobiose or glucose as a main carbon source and anaerobically cultivated for 2 days at 37°C. The extents of the bacterial growth were measured by the turbidity of the culture broth at 660 nm (Table 1, column B), showing that the bacterium grew well on the three carbon sources. *R. albus* F-40C grew rapidly on cellobiose and glucose but slowly on BMC, suggesting that the bacterium partly lost the ability to digest insoluble cellulose (BMC) after adaptation to

the soluble cellobiose substrate (Table 1, column C). *R. albus* F-40G grew in the glucose medium rapidly, grew in the cellobiose medium more slowly than *R. albus* F-40C and grew in the BMC medium much more slowly than *R. albus* F-40B, revealing that the organism partly lost the ability to utilize BMC and had a slightly reduced ability to utilize cellobiose (Table 1, column G).

Isolation of plasmids from R. albus.

Plasmid samples B, C and G were prepared from the fresh cells of *R. albus* F-40 B, F-40 C and F-40 G, respectively. Since only plasmids

Table 1. Growth* of *Ruminococcus albus* on various carbon sources during 1 day cultivation at 37°C and pH 6.8.

Main carbon source	<i>Ruminococcus albus</i> F-40		
	B**	C**	G**
BMC	0.530***	0.250	0.050
Cellobiose	2.000	2.300	1.050
Glucose	2.400	2.400	2.200

* Turbidity of whole culture broth at 660 nm

** Names of strains of *R. albus* F-40

*** Turbidity of supernatant only of the settled BMC-culture broth

Fig. 1. Agarose-gel electrophoretograms of plasmid samples B, C and G cleaved by *Bam*HI.

Lane M: λ DNA cleaved with *Hind* III (molecular size standards)

Lane B: plasmid sample B cleaved by *Bam*HI

Lane C: plasmid sample C cleaved by *Bam*HI

Lane G: plasmid sample G cleaved by *Bam*HI

Fig. 2. Restriction maps of pRAB and pRAC.

Fig. 3. Agarose-gel electrophoretograms of plasmid samples B, C and G cleaved by *Pst* I

Lane M: λ DNA cleaved with *Hind* III (molecular size standards)

Lane B: plasmid sample B cleaved by *Pst* I

Lane C: plasmid sample C cleaved by *Pst* I

Lane G: plasmid sample G cleaved by *Pst* I

with a linear form appeared as sharp electrophoretic bands on the agar gel, all the plasmid samples prepared in this experiment were cleaved by restriction enzymes such as *Bam* H I or *Hin* d III to make them linear before electrophoresis (Fig. 1). In the plasmid sample B, 3 bands with molecular sizes of 11.8, 7.4 and 3.5 kilobase pairs (kbp) of nucleotides were obtained (Fig. 1-B) when it was treated with

Bam H I. In the hydrolysates of sample B by *Eco* R I, one spread band with a molecular size larger than 23 kbp, which is known to be a closed circular plasmid DNA, and one sharp *Eco* R I-band of 15.3 kbp which is a linear plasmid DNA were detected. Considering these data, we concluded that two plasmids of 7.4 and 15.3 kbp were contained in sample B and denoted as pRAB and pRAC, respectively. In the plasmid sample C, two *Bam* H I-bands of 11.8 and 3.5 kbp were mainly found and a 7.4 kbp band was just detectable indicating that both plasmids pRAB and pRAC were present but the content of plasmid pRAB was considerably reduced. In the plasmid sample G, three bands having a molecular size and intensity identical to those from sample C were found on the electrophoretogram after being treated with *Bam* H I. Restriction mapping of the plasmid samples B, C and G was carried out using *Bam* H I, *Pvu* II, *Hin* d III, *Eco* R I and *Pst* I restriction enzymes. In all cases the electrophoretic patterns for each enzyme were identical in the three plasmid samples revealing that the same two plasmids, pRAB and pRAC, are present in all the plasmid samples. Restriction maps are shown in Fig. 2.

Properties of two plasmids from R. albus.

As described above, pRAB was significantly

decreased when *R. albus* was acclimated on cellobiose or glucose. *R. albus* F-40 C and F-40 G required a long lag time to detect their cellulose-utilizing ability; growth only began when they were incubated in the BMC medium at 37°C for 2 and 3 days, respectively. Thereafter, the bacterium was repeatedly transferred into fresh BMC medium and gradually, the cellulose-digesting rate was recovered normally. The reacclimated cells of *R. albus* F-40 C and F-40 G on BMC exhibited an increased level of pRAB. These results suggest that pRAB can be amplified in the presence of cellulose as a main carbon source and it is responsible for the cellulose digesting ability of *R. albus*. pRAB also requires cellulose as a selection pressure.

In the process of determining the restriction sites of pRAC, *Pst* I did not effectively cleave pRAC, especially that which was isolated from plasmid sample B. The plasmid cleaved at two sites by *Pst* I and, represented by the positions of molecular sizes 13.7 and 1.6 kbp, was present at the highest relative concentration in plasmid sample B compared to sample C and G as shown in Fig. 3. The concentrations of the hydrolysis products with molecular sizes of 10.2 and 3.5 kbp were relatively lower in sample B. The *Pst* I site on the H1 fragment of pRAC seems to be cleaved with the most difficulty (data not shown). These results suggest that the high cellulolytic activity of *R. albus* may be related to the modification of pRAC.

For detecting additional functions of pRAB and pRAC, both plasmids from plasmid sample B were completely cleaved by *Bam* HI. The DNA fragments thus obtained were ligated into pACYC 184 conferring chloramphenicol resistance. With the resulting chimera plasmids, *E. coli* HB 101 was transformed, from which a chimera plasmid containing pRAB and two plasmids containing either B1 or B2 fragment from pRAC were isolated. Three transformants each harboring a chimera plas-

mid with individual *Bam* HI fragments of pRAB or pRAC were isolated and plated on an LB-agar medium. Detection of CMCase activity of the colonies was examined by the CMC-containing agar method³⁰. A series of similar experiments was repeated with plasmid sample B using *Pst* I or *Hin* d III. However, no CMCase could be detected in any transformant. This indicates that (a) neither pRAB nor pRAC encodes CMCase or (b) transformants could not express CMCase activity.

In conclusion, we isolated 2 novel plasmids from *R. albus*, pRAB and pRAC, encoding functions for the cellulose utilization ability of *R. albus*. These cellulose utilization functions are clearly important for an adequate *R. albus* growth on cellulosic resources. This report is the first which describes the characterization of plasmids occurring in *R. albus*.

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References

- 1) TAYA, M., Y. ITO, K. OHMIYA, T. KOBAYASHI and S. SHIMIZU, *J. Ferment. Technol.*, **57**: 178-185. 1970.
- 2) TAYA, M., K. OHMIYA, T. KOBAYASHI and S. SHIMIZU, *J. Ferment. Technol.*, **58**: 463-469. 1980.
- 3) TAYA, M., K. HONMA, K. OHMIYA, T. KOBAYASHI and S. SHIMIZU, *J. Chem. Eng.*, **14**: 330-335. 1981.
- 4) OHMIYA, K., K. NOKURA and S. SHIMIZU, *J. Ferment. Technol.*, **61**: 25-30. 1983.
- 5) OHMIYA, K., M. SHIMIZU, M. TAYA and S. SHIMIZU, *J. Bacteriol.*, **150**: 407-409. 1982.
- 6) OHMIYA, K., M. SHIRAI and S. SHIMIZU, *J. Bacteriol.*, **161**: 432-434. 1985.
- 7) OHMIYA, K., K. MAEDA and S. SHIMIZU, *Carbohydrate Research*, **166**: 145-155. 1987.
- 8) CHEN, W., K. OHMIYA, S. SHIMIZU and H. KAWAKAMI, *Appl. Environ. Microbiol.*, **49**: 211-216. 1985.
- 9) CHEN, W., K. SUPANWONG, K. OHMIYA, S. SHIMIZU and H. KAWAKAMI, *Appl. Environ. Microbiol.*, **50**: 1451-1456. 1985.
- 10) OHMIYA, K., M. TAKEUCHI, W. CHEN, S. SHIMIZU and H. KAWAKAMI, *Appl. Microbiol. Biotechnol.*, **23**: 274-279. 1986.
- 11) CHEN, W., K. OHMIYA, S. SHIMIZU and H. KAWAKAMI,

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- J. Ferment. Technol., **65** : 223-226. 1987.
- 12) CHEN, W., K. OHMIYA, S. SHIMIZU and H. KAWAKAMI, Appl. Environ. Microbiol., **54** : 1254-1257. 1988.
- 13) SUKHUMAVASI, J., K. OHMIYA, M. SUWANA-ADTH and S. SHIMIZU, J. Ferment. Technol., **62** : 545-550. 1984.
- 14) SUKHUMAVASI, J., K. OHMIYA, S. SHIMIZU and K. UENO, Intern. J. Syst. Bacteriol., **38** : 179-182. 1988.
- 15) SUKHUMAVASI, J., K. OHMIYA, M. SUWANA-ADTH and S. SHIMIZU, J. Sci. Soc. Thailand, **15** : 109-120. 1989.
- 16) FUJINO, T., J. SUKHUMAVASI, K. OHMIYA and S. SHIMIZU, J. Bacteriol., **171** : 4076-4079. 1989.
- 17) TAYA, M., K. OHMIYA, T. KOBAYASHI and S. SHIMIZU, J. Ferment. Technol., **61** : 197-199. 1983.
- 18) CHEN, W., K. OHMIYA, S. SHIMIZU and H. KAWAKAMI, Appl. Environ. Microbiol., **52** : 612-616. 1986.
- 19) CHEN, W., K. OHMIYA and S. SHIMIZU, Appl. Environ. Microbiol., **53** : 542-548. 1987.
- 20) CHEN, W., M. OHMORI, K. OHMIYA and S. SHIMIZU, J. Ferment. Technol., **66** : 341-346. 1988.
- 21) CHEN, W., K. OHMIYA and S. SHIMIZU, Appl. Environ. Microbiol., **54** : 2300-2304. 1988.
- 22) CHEN, W., K. NAGASHIMA, T. KAJINO, K. OHMIYA and S. SHIMIZU, Appl. Environ. Microbiol., **54** : 1249-1253. 1988.
- 23) OHMIYA, K., K. NAGASHIMA, T. KAJINO, E. GOTO, A. TSUKADA and S. SHIMIZU, Appl. Environ. Microbiol., **54** : 1511-1515. 1988.
- 24) OHMIYA, K., T. FUJINO, J. SUKHUMAVASI and S. SHIMIZU, J. Bacteriol., Appl. Environ. Microbiol., **55** : 2399-2402. 1989.
- 25) OHMIYA, K., T. KAJINO, A. KATO and S. SHIMIZU, J. Bacteriol., **171** : 6771-6775. 1989.
- 26) CHAMPION, K.M., C.T. HELASZEK and B.A. WHITE, Can J. Microbiol., **34** : 1109-1115. 1988.
- 27) ASMUNDSON, R.V. and W.J. KELLY, Curr. Microbiol., **16** : 97-100. 1987.
- 28) BIRNBIOM, H.C. and J. DOLY, Nucleic Acids Res., **7** : 1513-1523. 1976.
- 29) MANIATIS, T., E.F. FRITSCH and J. SAMBROOK, Molecular cloning : a laboratory manual. Cold Spring Harbor Laboratory. Cold Spring. Harbor. N.Y. 1982.
- 30) TEATHER, R.M. and P.J. WOOD, Appl. Environ. Microbiol., **43** : 777-780. 1982.

Ruminococcus albus のセルロース資化能に 関与する新奇なプラスミド

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ルーメン由来の嫌気性セルロース分解細菌 *Ruminococcus albus* から, Birnboim-Doly 法により 2 種のプラスミドを単離した。これらを pRAB および pRAC と命名し, 各々の制限酵素地図を作成した。両プラスミドはボールミルで磨砕したセルロース (BMC), セロビオースあるいはグルコースを主炭素源とするいずれの培地に *R. albus* を馴養した場合にも単離された。pRAB (分子量 7.4 kbp) はセロビオースあるいはグルコースを主炭素源とする培地に馴養した場合に著しく減少した。このとき, *R. albus* のセルロース分解能はほとんど消失していた。したがって, pRAB は *R. albus* のセルロース分解能を支配していると推定した。

pRAC は BMC を主炭素源とする培地に馴養した場合の方がグルコースやセロビオースを主炭素源とした場合に比べ *Pst* I で著しく分解され難くなっていたことから, pRAC も *R. albus* のセルロース分解能に関与しているものと考えている。また, pRAC を *Hind* III あるいは *Bam* HI で完全分解したのち, pACYC 184 に組みこみ, これを用いて大腸菌を形質転換し, CMCase 活性を測定したが, いずれの場合にもこの活性を検出できなかった。

以上の結果より, *R. albus* から単離された 2 種類のプラスミドは *R. albus* のセルロース分解能に著しく関与していることが判明した。

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