JARQ 37(1), 53 – 57 (2003) http://www.jircas.affrc.go.jp

Molecular Characterization of a Lactococcal Plasmid Reducing the Growth Rate of Host Cells

Miho KOBAYASHI[1]*, Masaru NOMURA[1], Yasuhito FUJITA[1], Sadahiro OHMOMO[2] and Takashi OKAMOTO[1]

[1] Department of Animal Products Research, National Institute of Livestock and Grassland Science (Tsukuba, Ibaraki 305–0901, Japan)
[2] Japan International Research Center for Agricultural Sciences (Tsukuba, Ibaraki 305–8686, Japan)

Abstract

Lactococcus lactis subsp. *lactis* biovar. *diacetylactis* DRC1 carries more than 6 plasmids, including a 7.4 kb cryptic plasmid, which was designated as pDR1-1. The pDR1-1 plasmid was found to significantly affect the maximum specific growth rate (μ_{max}) of the host cells because of its limiting effect on growth. To investigate the properties of the limiting effect, the entire nucleotide sequence of pDR1-1 was determined. It consisted of 7412 bp, and 6 open reading frames (ORFs) were identified. The first ORF showed a high degree of similarity to a family of replication genes (*rep*) that are commonly found in lactococcal strains. The *rep* gene in pDR1-1was followed by a second ORF of unknown function. Directly downstream of the second ORF, a third ORF was found, that showed homology to the S subunit from type I restriction/modification systems. No significant similarity to the contents of the database was found for the other ORFs. PCR analysis was carried out in order to detect pDR1-1 in the other *L. lactis* strains. The μ_{max} of the pDR1-1-positive strain was the same as that of DRC1. These results suggest that the load of pDR1-1 (or pDR1-1-like plasmid) is a major factor influencing the μ_{max} of DRC1 because of its limiting effect on growth, an effect which is much more pronounced than that produced by the overall load of other coexisting plasmids.

Discipline: Biotechnology / Animal industry
Additional key words: *L. lactis*

Introduction

Lactococcal strains are used as starter organisms for dairy products. Such strains generally carry a number of different plasmids varying in size from approximately 2 kb to over 100 kb, some of which encode properties essential for the dairy industry and for metabolic functions such as lactose fermentation, proteolysis, diacetyl production and phage resistance. Lactococcal strains also carry many kinds of plasmids that encode non-essential or cryptic properties. A large number of studies have been conducted on the influence of the plasmid content on the cell growth of various microorganisms[3,5,8]. Some reports have shown that certain plasmids in microorganisms may cause a decrease of the μ_{max} of the host cells. This limiting effect of plasmids on the μ_{max} has been reported for a variety of host/plasmid combinations. This phenomenon was ascribed to the fact that plasmid replication and synthesis of plasmid gene products that load the cell metabolism, transport, biosynthesis, etc., may alter the growth parameters such as growth rate and yield constant. In the case of lactococci, there have been few reports on the influence of the plasmids on the cell growth rate. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* DRC1 carries more than 6 plasmids[11]. DRC1 wild-type strain grew more slowly than the plasmid-free derivative of DRC1. To identify the plasmid responsible for the limiting effect on host cell growth, the total plasmids extracted from DRC1 and the indicator plasmid were co-transformed into a plasmid-free strain. In this report, we describe the identification and characterization of a plasmid that limits the growth of DRC1.

Materials and methods

1. Bacterial strains and plasmids

The *L. lactis* strains and plasmids used in this study are listed in Table 1. *L. lactis* ssp. *lactis* bv. *diacetylactis* ATCC13675 and *L. lactis* DRC1 were obtained from the

*Corresponding author: fax +81–29–838–8606; e-mail mihokoba@affrc.go.jp
Received 8 November 2002; accepted 10 January 2003.

American Type Culture Collection (Manassas, VA, USA) and the National Institute for Research in Dairying (now the AFRC Institute of Food Research Council, Shinfield, UK), respectively, and the other strains were provided from our laboratory collection. Strain DRC1021 is a plasmid-free derivative of DRC1[7]. The *E. coli* strain XL1-Blue was used for cloning with pBluescriptII (Stratagene Cloning Systems, La Jolla, CA, USA). The plasmid p8Em1 contained both the erythromycin resistance (Em^r) gene from pAMβ1 and the multi-cloning site from pUC118 (obtained through Dr. Sasaki, Food Functionality Research Institute, Meiji Milk Products Co., Ltd., Tokyo, Japan).

2. Media and culture conditions

The *L. lactis* strains were grown in a TYG medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, 1% glucose and 1% sodium succinate; pH 6.8) at 30°C. pGKV21 and the recombinant plasmid pBE1 were stably maintained in DRC1021 without the use of antibiotics at 30°C. *E. coli* was grown in a Luria-Bertani (LB) medium[14] at 37°C. Ampicillin (Ap) (50 μg mL^{-1}) was used to supplement the media for the selection of Ap resistance. For the selection of transformants containing pGKV21 or recombinant plasmid, TYG agar plates (1.5% agar) supplemented with erythromycin (Em) (5 μg mL^{-1}) (TYG-E) were used.

3. Plasmid preparation and modification

Lactococcal plasmid DNA was isolated and purified by the method described by Anderson and McKay[2]. *E. coli* plasmid DNA was isolated following the alkaline lysis procedure described by Sambrook[14].

4. Transformations

E. coli was transformed according to the $CaCl_2$ method described by Sambrook[14]. The transformants were selected on LB agar supplemented with Ap. *L. lactis* was transformed by electroporation with a 2 mm gap electroporation cuvette as described by Holo and Nes[9] using a Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, CA, USA). pGKV21 was encoded with an Em^r gene and used as an indicator plasmid for indirect selection, because transformants of DRC1 plasmids (except for the lactose plasmid) could hardly be discriminated from other cells. In the case of co-transformation, an electroporation mixture was prepared from 40 μL of competent DRC1021 cells, pGKV21 (10 ng) and total plasmids (100 ng) from DRC1. Transformants were selected on a TYG-E agar plate and then studied for the determination of the plasmid patterns and growth rate. Curing of indicator plasmid was performed by subculturing at 39°C, and Em-sensitive cells were selected.

5. Analysis of growth

Cell growth of the *L. lactis* strains was analyzed at 30°C. Overnight cultures grown in TYG medium at 30°C were diluted 1000-fold in 11-mm-diameter glass tubes in 10 mL of the same medium. The cultures were incubated statically at 30°C, and bacterial growth was monitored at 1 h intervals by measuring the turbidity with a Bausch & Lomb Model 21 spectrophotometer (Bausch & Lomb, NY, USA) at 620 nm. Viable cell counts were performed by plating 100 μL samples with appropriate dilution in saline on TYG agar. All the plates were then incubated at 30°C. The μ_{max} in the batch culture was

Table 1. *L. lactis* strains and plasmids

Strains and plasmids	Characteristics	References or sources
Strain		
DRC1	Wild-type	18, 4
DRC1021	Plasmid-free derivative of DRC1	7
DRC11	DRC1021 harboring pDR1-1	This study
DRC117	DRC1021 harboring pBE1	This study
ATCC13675	Wild-type	American Type Culture Collection
NIAI N7	Wild-type	Lab. collection
Plasmid		
pGKV21	*E. coli*, *B. subtillis*, *L. lactis* shuttle vector, Em^r, Cm^r	19
p8Em1	4.3 kb vector, source of erythromycin resistance gene	10
pDR1-1	7.4 kb cryptic plasmid from *L. lactis* subsp. *lactis* biovar. *diacetylactis* DRC1	This study
pBE1	Em^r gene from p8Em1 fused to 3.0 kb *Bgl*II-*Eco*RI fragment of pDR1-1	This study

Em^r, resistance to erythromycin; Cm^r, resistance to chloramphenicol.

determined from the plots with an increase in viable cell counts. Each value for the μ_{max} was expressed as the mean of 3 separate experiments. The generation time (T) was calculated by using the equation: $\mu_{max} = \ln 2/T$. Statistical analysis was performed on the data using the Student's t-test, and statistical significance was recognized at $P < 0.05$.

6. Amplification of the fragment from pDR1-1 by PCR

Polymerase chain reaction (PCR) analysis was carried out in order to detect pDR1-1 in the other *L. lactis* strains. The template DNA for PCR was prepared by scraping bacterial colonies from a plate after overnight growth and suspension in 20 μL sterile distilled water. Two μL of the suspension were used as the source of the template in PCR. The following PCR primers were used: SES1 (5'-GGTGGAACACCAAGTACATCGAACTCTG-3') and SC15c (5'-CTACACTGCCTTTAGAGATATTCAGTTG-3'), both of which corresponded to sequences in the *orf1* in pDR1-1 (Fig. 2). We expected that the length of the amplified fragment would be about 970 bp for the DRC1 strain.

7. DNA sequence analysis

Plasmid pDR1-1 (7.4 kb) was cloned into the *Hinc*II site of pBluescriptII and deleted unidirectionally by using a kilo-sequencing deletion kit (Takara Shuzo, Otsu, Japan). Nested deletion clones were sequenced as described by Sanger et al.[15] using a *Taq* dye-primer cycle sequencing kit and an Applied Biosystems 373A automated DNA sequencer (Applied Biosystems, CA, USA).

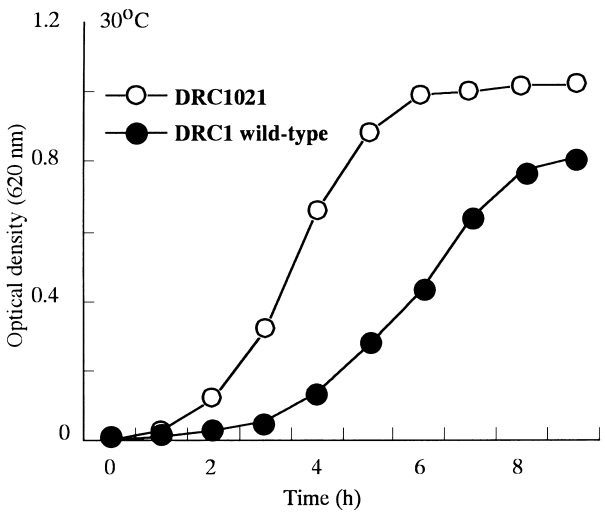

Fig. 1. Growth of DRC1 wild-type and a plasmid-free derivative

Results and discussion

1. Limiting effects of plasmids on μ_{max}

We compared the growth of the DRC1 wild-type and DRC1021, the plasmid-free derivative of DRC1. DRC1 was found to grow more slowly than DRC1021 (Fig. 1). To identify a plasmid that limits the growth of DRC1, total plasmid was prepared from DRC1 and transformed into DRC1021 by electroporation. Since plasmids from DRC1 have no available selective marker (except for the lactose plasmid), Em^r-plasmid pGKV21 was co-transformed. Transformants with Em^r were selected and their growth was compared with that of DRC1021. Three transformants which grew more slowly than DRC1021 were obtained. All 3 carried pGKV21 as well as a 7.4 kb cryptic plasmid, which had been identified as pDR1-1. DRC11 was derived from the transformant by curing of pGKV21. The μ_{max} and the generation times of DRC1, DRC11 containing only plasmid pDR1-1 and DRC1021 were determined (Table 2). The growth rate of DRC11 decreased by 27% compared to that of DRC1021. On the other hand, no significant difference was detected between the μ_{max} of DRC11 and that of DRC1, although the latter contained more than 6 additional plasmids, such as the lactose-fermenting plasmid (50 kb)[11] and the citrate-utilizing plasmid (8.3 kb)[12]. These results suggest that the load of pDR1-1 is a major factor influencing the μ_{max} of DRC1 because of its limiting effect on growth, an effect which was much more pronounced than that produced by the overall burden of other coexisting plasmids.

2. DNA sequence analysis of pDR1-1

The entire nucleotide sequence of pDR1-1 was submitted to GenBank and assigned the Accession No. AB079381. It consisted of 7412 bp with an overall G+C content of 33.7%. Six open reading frames (ORFs) were identified (Fig. 2). The nucleotide sequence of ORFs and

Table 2. Specific growth rate (μ_{max}) of DRC1 and variants

L. lactis strain	Presence of pDR1-1[a)]	μ_{max}[b)] (h^{-1})
DRC1 wild-type	+	0.853 (± 0.044)
DRC11	+	0.830 (± 0.071)
DRC1021	–	1.14 (± 0.032)[c)]
DRC117	–	1.12 (± 0.077)[c)]

a): pDR1-1 was detected by PCR. +, PCR products were obtained with pDR1-1 specific primers; –, no PCR products were obtained.
b): Values are means of 3 trials (± S.D.).
c): Significantly different from the value of the DRC1 wild-type ($P < 0.05$).

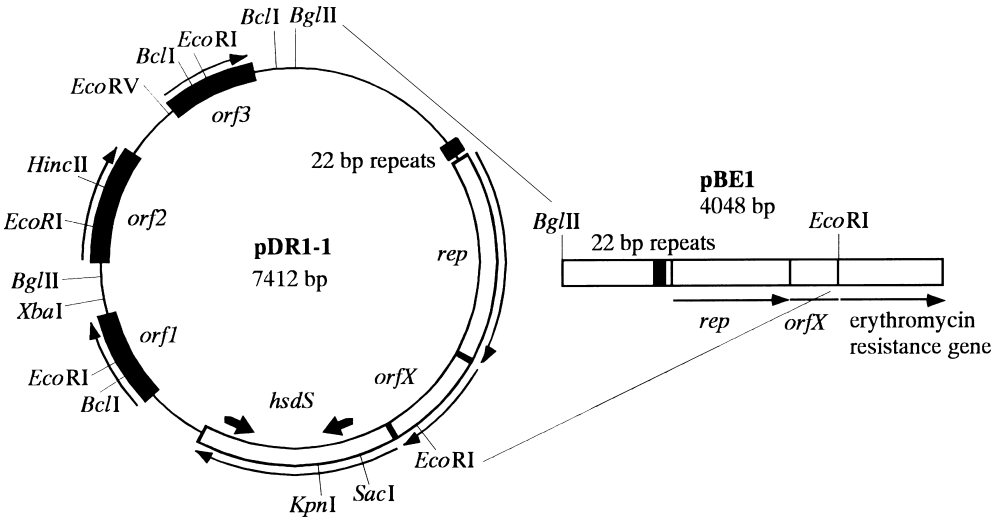

Fig. 2. Physical and genetic maps of pDR1-1 and the recombinant plasmid pBE1

Thin arrows indicate ORFs. The 22 bp repeats refer to the putative replication origin (*ori*) preceding the replication genes (*rep*). Open boxes indicate the *rep-hsdS* operon-like structure. Filled boxes indicate ORFs that encode unknown property. The rightward- and the leftward-pointing thick arrows indicate the relative positions of the PCR primers SES1 and SC15c, respectively.

their deduced amino acid sequences were subjected to BLAST[1] and FASTA[13] net searches of the databases. The first ORF showed a high degree of similarity to the family of replication genes (*rep*) that are commonly found in lactococci and that encode the replication-initiation protein of theta (θ) replicating plasmids. Similarity to the *repB* gene of pNZ4000 (AF036485) showed the highest level with an 81.0% identity. The *rep* was preceded by a putative replication origin (*ori*) that consisted of three 22 bp repeats and one truncated repeat, followed by a second ORF (*orfX*), which had been sometimes identified as an adjacent ORF of *rep* from lactococcal plasmids[16,17]. Directly downstream of *orfX*, we found a third ORF (*orf1*) that showed high levels of homology to the S subunit from type I restriction/modification systems (*hsdS*)[6]. The *rep*, *orfX* and *hsdS* genes are organized in an operon-like structure, with the last 2 codons of *rep* overlapping the first 2 codons of *orfX* and the last 4 codon sequences of *orfX* overlapping the first 4 of *hsdS*. No significant similarity to the contents of the database was found for the other ORFs (*orf2*, *orf3* and *orf4*).

3. Effect of the replicon of pDR1-1 on its host cell growth rate

A replication origin and a *rep* of pDR1-1 were encoded in the *Bgl*II-*Eco*RI fragment (Fig. 2). This fragment with a 1.1 kb p8Em1-derived Em^r gene was used to construct the recombinant plasmid pBE1. To study the effect of the replicon of pDR1-1 on the μ_{max}, this plasmid was used to transform DRC1021, generating DRC117 (Table 1). After continuous growth for 100 generations, pBE1 could be stably maintained in DRC117 without selective pressure. No significant difference was detected between the μ_{max} of DRC117 and that of DRC1021 (Table 2). The copy number of pBE1 was almost the same as that of pDR1-1 (data not shown). These results suggest that the replication system and copy number of pDR1-1 were not responsible for the decrease in the μ_{max}. We suggest that growth rate control occurs in domains other than the *Bgl*II-*Eco*RI fragment.

4. Distribution of pDR1-1 in *L. lactis* strains

To investigate the distribution of pDR1-1, 24 *L. lac-*

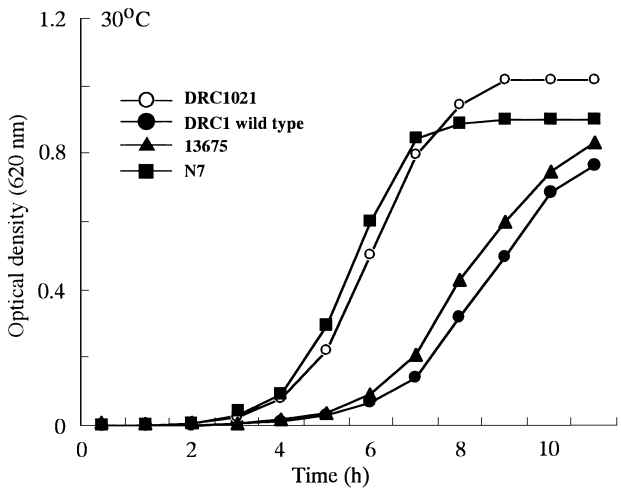

Fig. 3. Comparison of growth of *L. lactis* strains with or without pDR1-1

tis strains were examined by PCR screening with pDR1-1-specific primers, which were located within the *orf1* (data not shown). Specific DNA amplification was observed with *L. lactis* ATCC13675 and DRC1. The size of the amplified products was 972 bp, as predicted from the DNA sequence. The fragments were sequenced, and it was found that the 2 sequences were identical with each other. The growth curve of 13675 was in agreement with that of DRC1 (Fig. 3). On the other hand, *L. lactis* NIAI N7, which contained at least 2 plasmids (lactose plasmid and citrate plasmid), did not react with the PCR primers, suggesting that strain N7 does not have a pDR1-1. It grew faster than PCR-positive strains (Fig. 3). Therefore, we conclude that, of the plasmids typically carried by lactococci, pDR1-1 is most likely to be a maximum load and may also alter the μ_{max} as does DRC1. pDR1-1 encodes a homologous *hsdS* but we do not anticipate that the general HsdS affects growth parameters. Further studies should be conducted to locate the genes involved in the load of pDR1-1 on cell growth.

References

1. Altschul, S. F. et al. (1990) Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403–410.
2. Anderson, D. G. & McKay, L. L. (1983) Simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl. Environ. Microbiol.*, **46**, 549–552.
3. Caunt, P., Impoolsup, A. & Greenfield, P. F. (1989) The effect of oxygen limitation on stability of a recombinant plasmid in *Saccharomyces cerevisiae*. *Biotechnol. Lett.*, **11**, 5–10.
4. Collins, E. B. & Harvey, R. J. (1962) Failure in the production of citrate permease by *Streptococcus diacetylactis*. *J. Dairy Sci.*, **45**, 32–35.
5. Diaz Ricci, J. C. & Hernàndez, M. E. (2000) Plasmid effects on *Eschericha coli* metabolism. *Crit. Rev. Biotechnol.*, **20** (2), 79–108.
6. Forde, A. & Fitzgerald, G. F. (1999) Bacteriophage defense systems in lactic acid bacteria. *Antonie van Leeuwenhoek*, **76**, 89–113.
7. Fujita, Y. & Okamoto, T. (1999) Cloning and identification of the lactococcin A and M gene cluster from *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* DRC1. *JARQ*, **33**, 133–137.
8. Harington, A. et al. (1988) Stability during fermentation of a recombinant -amylase plasmid in *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.*, **27**, 521–527.
9. Holo, H. & Nes, I. F. (1989) High-frequency transformation by electroporation of *Lactococcus lactis* subsp. *cremoris* growing with glycine in osmotically stabilized media. *Appl. Environ. Microbiol.*, **55**, 3119–3123.
10. Ito, Y., Sasaki, Y. & Sasaki, T. (1992) Novel plasmid pBUL1 derived from *Lactobacillus* and Derivative thereof. PTC Patent WO 92/14825.
11. Kempler, G. M. & McKay, L. L. (1979) Characterization of plasmid deoxyribonucleic acid in *Streptococcus lactis* subsp. *diacetylactis*: Evidence for plasmid-linked citrate utilization. *Appl. Environ. Microbiol.*, **37**, 316–327.
12. Kempler, G. M. & McKay, L. L. (1981) Biochemistry and genetics of citrate utilization in *Streptococcus lactis* subsp. *diacetylactis*. *J. Dairy Sci.*, **64**, 1527–1539.
13. Pearson, W. R. & Lipman, D. J. (1988) Improved tools for biological sequence comparison. *PNAS,* USA, **85**, 2444–2448.
14. Sambrook, J., Fritsch, E. R. & Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
15. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *PNAS,* USA, **74**, 5463–5467.
16. Seegers, J. F. M. L., van Sinderen, D. & Fitzgerald, G. F. (2000) Molecular characterization of the lactococcal plasmid pCIS3: natural stacking of specificity subunits of a type I restriction/modification system in a single lactococcal strain. *Microbiology*, **146**, 435–443.
17. Seegers, J. F. M. L. et al. (1994) The majority of lactococcal plasmids carry a highly related replicon. *Microbiology*, **140**, 1291–1300.
18. Swartling, P. F. (1951) Biochemical and serological property of some citric acid fermenting streptococci from milk and dairy products. *J. Dairy Res.*, **18**, 256–267.
19. van der Vossen, J. M., Kok, J. & Venema, G. (1985) Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streptococcus lactis*. *Appl. Environ. Microbiol.*, **50**, 540-542.