

Identification of a Lactose Utilization and Copper Resistance Plasmid in *Lactococcus lactis* subsp. *lactis* MCL64

Mustafa AKÇELİK

Ankara University, Faculty of Agriculture, Department of Food Engineering,
Dışkapı, 06110, Ankara - TURKEY

Received: 04.03.2001

Abstract: Lactose negative and copper sensitive (Lac^-/Cu^s) mutants of *Lactococcus lactis* subsp. *lactis* MCL64 were isolated after extended incubation periods at 32°C and 37°C. A 30.0 kb plasmid was absent in all Lac^-/Cu^s mutants. These results showed that lactose metabolism and copper resistance were linked to the 30.0 kb plasmid in strain MCL64. Using MCL64 as a donor, the 30.0 kb plasmid transferred into Lac^-/Cu^s recipient strains P81 and MCL64-90 with the frequencies of 4.1×10^{-5} – 8.2×10^{-3} per donor cell, respectively. All transconjugants were found to be of a Lac^+/Cu^r phenotype.

Key Words: *L. lactis* subsp. *lactis*, lactose utilization, copper resistance, plasmid

Lactococcus lactis subsp. *lactis* MCL64 Suşunda Laktoz Metabolizması ve Bakır Dirençlilik Plazmidinin Tanımlanması

Özet: *Lactococcus lactis* subsp. *lactis* MCL64 suşunda laktoz negatif ve bakır duyarlı (Lac^-/Cu^s) mutantlar, 32°C ve 37°C de uzatılmış inkübasyon süreleri sonunda seçildi. Tüm Lac^-/Cu^s mutantlarda 30,0 kb büyüklükteki plazmidin bulunmadığı saptandı. Bu sonuçlar, MCL64 suşunda laktoz metabolizması ve bakır dirençliliğinin 30,0 kb büyüklükteki plazmid ile ilişkili olduğunu gösterdi. 30,0 kb büyüklükteki plazmid, MCL64 verici suşundan Lac^-/Cu^s P81 ve MCL64-90 alıcı suşlarına sırasıyla, $4,1 \times 10^{-5}$ ve $8,2 \times 10^{-3}$ sıklıkla aktarıldı. Oluşan transkonjugantlar Lac^+/Cu^r fenotipte saptandı.

Anahtar Sözcükler: *L. lactis* subsp. *lactis*, laktoz kullanımı, bakır dirençlilik, plazmid

Introduction

Lactococcus lactis is the main bacterium used as a starter in cheese, sour cream, buttermilk and other fermented milk products, and its rapid growth in milk is essential for yielding an appropriate rate of lactic acid production, optimum curd formation, the required flavor, and texture development in the final products and for the inhibition of undesirable microorganisms (1,2). Very often, these characteristics, as well as other relevant properties such as bacteriophage and inorganic salt resistance or bacteriocin production, are encoded by plasmid DNA (3,4,5). Owing to concentrated efforts in several laboratories, the lactococci became accessible to manipulation by recombinant DNA technologies. However, the success in the strain improvement applications are still limited partly because most of the cloning vectors developed to date utilize antibiotic

resistance as the basis for selection and screening. Such easily selectable markers are a valuable laboratory tool; however, antibiotic-resistant starter cultures will probably not be permissible in food fermentation systems due to the possibility of transmission of this property to indigenous human microorganisms (6,7). Recently, several selection markers that are potentially useful for the construction of food-grade cloning vectors have been described; examples of these include nisin resistance (Nis^r) (8,9) and cadmium resistance (Cd^r) (10). There is need for more investigations on the lactococcal resistance markers, other than antibiotic markers, to construct food-grade vectors for starter strains.

In this study, a 30.0 kb plasmid encoding lactose fermentation and conjugal transfer abilities, and copper (Cu) resistance in *L. lactis* subsp. *lactis* MCL64 was identified.

Materials and Methods

Bacterial strains and culture conditions

Table 1 lists the strains used in this study. Lactococcal cultures were routinely grown in M17 medium (11) at 32°C with lactose being replaced by glucose (GM17) when necessary. Bacteriophage and culture stocks were stored in broth containing 15% glycerol at -40°C. Antibiotics were used in the following concentrations: Streptomycin (Str), 200 µg/ml and kanamycin (km) 70 µg/ml.

Resistance tests

Copper resistance was determined by the method of Novick and Roth (12) and M17 agar and M17 Broth (11) were used for colony-forming ability and serial tube dilution assays. Tests were conducted at 32°C.

Plasmid curing and detection of lactose negative (Lac⁻) mutants.

Plasmid cured derivatives were obtained by using the extended incubation method described by Sinha (13). Lactose-utilizing colonies (Lac⁺) were differentiated from lactose non-utilizing colonies (Lac⁻) on Elliker agar (14) containing 1% lactose as the main source of carbohydrate with 0.004% bromocresol purple used as indicator (13). On such test plates, Lac⁺ colonies were yellow, while Lac⁻ colonies were white. Total viable cells were also determined on the same kind of plates.

Plasmid analysis

The lysis procedure of Anderson and McKay (15) was used to isolate plasmid DNA from lactococcal strains. Plasmid sizes were estimated on 0.7% agarose gels by comparing their relative mobility to commercial ccc DNA markers containing plasmid species of 16.2, 14.2, 12.1, 10.1, 8.1, 7.0, 6.0, 5.0, 4.0, 3.0 and 2.1 kb (Bathesda Research Laboratories, Product No: 5622SA, USA).

Conjugation

Donor and recipients were combined in ratios of 1:2 to 1:5 and matings were performed on the surface of bromocresol purple lactose indicator agar (13). For MCL64 X P81 matings, streptomycin (200 µg/ml) and kanamycin (70 µg/ml) were added to the selection medium. Lac⁺ transconjugants were purified by replating three times on the same medium and subcultured to M17 broth and M17 agar for copper resistance tests and plasmid screening (16).

Results and Discussion

The appearance of lactose negative (Lac⁻) mutants in M17 broth after extended incubation of MCL64 cultures at 22°C, 32°C and 37°C is shown in Table 2. No Lac⁻ mutant was determined at 22°C after 24 h, 96 h and 168 h incubation periods. The number of Lac⁻ mutants increased with the length of incubation at 32°C and 37°C,

Strains	Phenotype ^a	Comments and References
<i>L. lactis</i> subsp. <i>lactis</i>		
MCL64	Lac ⁺ , Cu ^r , Str ^s , Km ^s	Donor strain. This study
MCL64-90	Lac ⁻ , Cu ^s , Str ^s , Km ^s	Plasmid free recipient strain. This study
MCL64-121	Lac ⁻ , Cu ^s , Str ^s , Km ^s	One plasmid cured (30.0kb) derivative of strain MCL64. This study
MCLT36	Lac ⁺ , Cu ^r , Str ^s , Km ^s	Conjugant from MCL64 X MCL64-90 mating. This study
P81T20	Lac ⁺ , Cu ^r , Str ^r , Km ^r	Conjugant from MCL64 X P81 mating. This study
P81	Lac ⁻ , Cu ^s , Str ^r , Km ^r	(17)

Table 1. Bacterial strains used in this study.

^a Lac⁺/Lac⁻: Utilizes/does not utilize lactose
 Str^r/Str^s: Resistant/sensitive to streptomycin
 Km^r/Km^s: Resistant/sensitive to kanamycin
 Cu^r/Cu^s: Resistant/sensitive to copper

Table 2. Effect of extended incubation of *L. lactis* subsp. *lactis* MCL64 cultures at different incubation temperatures on the appearance of Lac⁻ colonies^a.

Strain	Incubation Temperature	Time of Incubation (h)	Total Viable Counts (cfu/ml)	No of Colonies Examined	No of Lac ⁻ Type Colonies
<i>L. lactis</i> subsp. <i>lactis</i> MCL64	22°C	24	4.6x10 ⁹	412	0
		96	8.1x10 ⁸	425	0
		168	5.3x10 ⁶	425	0
	32°C	24	8.8x10 ⁹	386	17
		96	6.9x10 ⁴	303	188
		168	6.2x10 ²	265	265
	37°C	24	2.7x10 ⁶	330	15
		96	4.2x10 ⁴	300	183
		168	4.7x10 ²	158	158

^a The data reported are the average of two experiments

Donor	Recipient	Representative Transconjugants	Lac ⁺ /Cu ^r Transconjugants ^a	
			Frequency (Per donor cell)	Plasmid Content ^b (kb)
MCL64	MCL64-90	MCLT36	8.2x10 ⁻³	30.0
MCL64	P81	P81T20	4.1x10 ⁻⁵	30.0

Table 3. Conjugal transfer of lactose fermentation ability and copper resistance from *L. lactis* subsp. *lactis* MCL64 to plasmid free recipient strains.

^a Lac⁺: Metabolizes lactose; Cu^r: Copper resistant; kb: kilobase

^b Twenty-five transconjugants for each conjugation experiment were used to examine the plasmid content.

and 100% of examined colonies were found to be Lac⁻ after 168 h at 32°C and 37°C. The results indicated that cellular metabolites released in to the medium by growth may have prompted the appearance of Lac⁻ mutants.

To determine the copper resistance (Cu^r), *L. lactis* subsp. *lactis* MCL64 plated on M17 agar containing 0.1M CuSO₄ was used. Wild type strain MCL64 was found to be Cu^r. However, all Lac⁻ mutants of strain MCL64 were also copper sensitive (Cu^s) (data not shown). In order to characterize genetic determinants of lactose fermentation ability (Lac⁺) and Cu^r in MCL64, plasmid profiles of the wild type strain and its representative Lac⁻/Cu^s mutant MCL64-121 were examined (Figure). Wild type strain MCL64 carried 9 plasmids, with molecular sizes of 45.8, 30.0, 28.8, 22.5, 16.7, 12.3, 8.4, 4.1 and 2.6 kb. On

the other hand, Lac⁻/Cu^s mutant strain MCL64-121 had lost the 30.0 kb plasmid only. These results strongly suggested that associated lactose utilization and copper resistance in *L. lactis* subsp. *lactis* MCL64 are encoded by the 30.0 kb plasmid. In a second experiment, *L. lactis* subsp. *lactis* MCL64 was used as the donor, and conjugated with two plasmid free and Lac⁻/Cu^s recipient strains. Transfer of lactose fermentation ability/Cu marker to the recipient strains *L. lactis* subsp. *lactis* P81 and MCL64-90 was observed at the frequencies of 4.1x10⁻⁵ and 8.2x10⁻³ per donor cell, respectively. Fifty Lac⁺/Cu^r transconjugants were plasmid extracted, and all contained the same 30.0kb plasmid (Table 3). Conjugation results clearly confirmed that the Lac⁺/Cu^r genes were encoded by the 30.0kb plasmid DNA in *L. lactis* subsp. *lactis* MCL64.

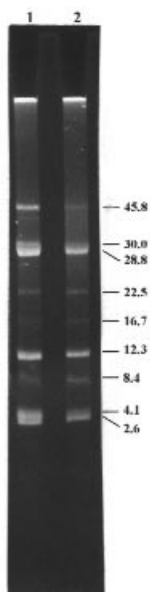


Figure. Plasmid profiles of Lac⁺/Cu^I wild type strain MCL64 (lane 1) and its Lac⁻/Cu^S mutant MCL64-121 (lane 2).

Conjugation has proven to be a useful gene transfer system in furthering the genetic analysis of plasmid encoded traits in lactococci, including lactose fermentation ability, bacteriocin production, bacteriophage and inorganic salt resistance, and proteolytic activity (17,18,19,20,21). In addition, conjugation has been exploited as a food-grade mechanism for the construction of industrial starter cultures with recombinant vector systems (22,23). The lactose fermentation/copper resistance plasmid, determined in this study, is a suitable food-grade vector because of its conjugal ability and Lac⁺/Cu^I selection capacity. Lac⁺/Cu^I markers offer a powerful selection advantage to recombinant strains in the absence of antibiotic resistance markers which are unsafe for food systems.

References

1. Wang, H., Yu, W., Coolbear, T., O'Sullivan, D., McKay, L. L. A Deficiency in Aspartate Biosynthesis in *Lactococcus lactis* subsp. *lactis* C2 Causes Slow Milk Coagulation. *Appl. Environ. Microbiol.*, 64: 1673-1679, 1998.
2. Labrie, S., Moineau, S. Multiplex PCR for Detection and Identification of Lactococcal Bacteriophages. *Appl. Environ. Microbiol.*, 66: 987-994, 2000.
3. Mayo, B., Hardisson, C., Brana, A. Characterization of Wild Strains of *Lactococcus lactis* subsp. *lactis* Isolated from Cabrales Cheese. *J. Dairy Res.*, 57: 125-134, 1990.
4. Kok, J. Inducible Gene Expression and Environmentally Regulated Genes in Lactic Acid Bacteria. *A. van Leeuwen.*, 70: 129-145, 1996.
5. Akçelik, M. Plasmid Mediated Industrial Traits in *Lactococcus lactis* subsp. *lactis* LL140. *Milchwissenschaft*, 54: 603-606, 1999.
6. Klaenhammer, T.R. Development of Bacteriophage Resistant Strains of Lactic Acid Bacteria. *Biochem. Soc. Trans.* 19: 675-681, 1991.
7. Somkoti, G. A., Steinberg, D. H. Distribution of Plasmid-borne Stress Proteine Genes in *Streptococcus thermophilus* and other Lactic Acid Bacteria. *Current Microbiol.*, 38: 43-47, 1999.
8. Froseth, B. R., Herman, R., McKay, L.L. Cloning of Nisin Resistance Determinant and Replication Origin on 7.6 kb EcoRI Fragment of pNP40 from *Streptococcus lactis* subsp. *diacetylactis* DRC3. *Appl. Environ. Microbiol.*, 54: 2136-2139, 1988.
9. Liu, C., Duan, K., Dunn, N. W. Cloning and Sequence Analysis of a Plasmid Replication from *Lactococcus lactis* subsp. *cremoris* FG2. *J.Gen. Microbiol.*, 43: 75-80, 1997.
10. Farias, M. E., Grohmann, E., Espinosa, M. Expression of the mobM Gene of the Streptococcal Plasmid pMV158 in *Lactococcus lactis* subsp. *lactis*. *FEMS Microbiol. Lett.* 176: 403-410, 1999.
11. Terzaghi, B. E., Sandine, W. E. Improved Medium for Lactic Streptococci and their Bacteriophages. *Appl. Environ. Microbiol.* 29: 807-813, 1975.
12. Novick, R.P., Roth, C. Plasmid-Linked Resistance to Inorganic Salts in *Staphylococcus aureus*. *J. Bacteriol.* 95: 1335-1342, 1968.
13. Sinha, R. P. Effect of Growth Media and Extended Incubation on the Appearance of Lactose-Nonfermenting Variants in Lactococci. *J. Food. Protect.* 53: 583-587, 1990.
14. Anonymous. *Difco Manual*, Difco Lab. Detroit- Michigan, USA, 333p, 1984.
15. Anderson, D.G., McKay, L. L. A Simple and Rapid Method for Isolating Large Plasmid DNA from Lactic Streptococci. *Appl. Environ. Microbiol.*, 46: 549-552, 1983.
16. Jarvis, A. W. Conjugal Transfer of Plasmid-Encoded Insensitivity to Prolate- and Small Isometric-Headed Bacteriophages. *Appl. Environ. Microbiol.*, 54: 777-783, 1988.

17. Akçelik, M., Tunail, N. A 30kd Cell Wall Protein Produced by Plasmid DNA which Encodes Inhibition of Phage Adsorption in *Lactococcus lactis* subsp. *lactis* P25. *Milchwissenschaft*, 47; 215-217, 1992.
18. Lucey, M., Daly, C., Fitzgerald, G. F. Analysis of a Region from the Bacteriophage Resistance Plasmid pCI528 Involved its Conjugative Mobilization Between *Lactococcus* Strains. *Appl. Environ. Microbiol.* 175; 6002-6009, 1993.
19. Coakley, M., Fitzgerald, F. G., Ross, P. Application and Evaluation of the Phage Resistance and Bacteriocin-Encoding Plasmid pMRC01 for the Improvement of Dairy Starter Cultures. *Appl. Environ. Microbiol.*, 63; 1434-1440, 1997.
20. Akçelik, M. The Conjugal Plasmid pLL10236 Encodes Lactose Fermentation Ability, Restriction/Modification Activity, Bacteriocin Production and Immunity in *Lactococcus lactis* subsp. *lactis* LL102. *Food Microbiology*, 16; 487-494, 1999.
21. Guzman, L., Espinosa, M. The Mobilization Protein, MobM, of the Streptococcal Plasmid pMV158 Specifically Cleaves Supercoiled DNA at the Plasmid *oriT*. *J. Mol. Biol.*, 267; 688-702, 1999.
22. Teuber, M. Strategies for Genetic Modification in Lactic Acid Bacteria. *Food Biotechnol.*, 4; 537-546, 1990.
23. Scott, K. P., Mercer, D. K., Richardson, A. J., Melville, C. M., Glover, A. L., Flint, H. J. Chromosomal Integration of Green Fluorescent Protein Gene in Lactic Acid Bacteria and the Survival of Marked Strains in Human Gut Simulations. *FEMS Microbiol. Lett.*, 182; 23-27, 1999.