Turk J Vet Anim Sci
28 (2004) 257-263

Research Article

Antibiotic Resistance and Plasmid DNA Contents of *Streptococcus thermophilus* Strains Isolated from Turkish Yogurts

Belma ASLIM, Yavuz BEYATLI
Gazi University, Faculty of Arts and Science, Department of Biology, Ankara - TURKEY

Received: 16.08.2001

Abstract: Thirty-four *Streptococcus thermophilus* strains were isolated from yogurt samples obtained from various villages in different regions of Turkey. These strains were examined for their antibiotic resistance patterns and plasmid carriage. The susceptibility of the isolates to 8 antibiotics (vancomycin, tetracycline, chloramphenicol, erythromycin, cephalothin, ampicillin-sulbactam, gentamicin and penicillin G) was determined by the disk diffusion method. Most strains of *S. thermophilus* were resistant to gentamicin (79%) and penicillin G (64%) and susceptible to chloramphenicol (94%) and tetracycline (88%).

In plasmid DNA analyses, it was determined that 7 strains did not contain any plasmid DNA, while other strains included plasmid DNA ranging from 1 to 5. The molecular weights of these plasmids were found to be in various sizes from 1.88 to 19.89 kb. An interaction was observed between the resistance to antibiotics and the occurrence of plasmids in some *S. thermophilus* strains; for instance, some strains carrying only one or no plasmid were observed to be susceptible to most antibiotics. The resistance to most antibiotics was determined in 3 strains (B55.S, B56.S, B58.S) containing 5 plasmid DNAs. However, no correlation was observed between the resistance to antibiotics and the occurrence of plasmids in some strains.

Key Words: *Streptococcus thermophilus*, plasmid DNA, antibiotic resistance

Türk Yoğurtlarından İzole Edilen *Streptoccus thermophilus* Suşlarının Plazmid DNA İçerikleri ve Antibiyotik Dirençliliği

Özet: Türkiye'deki farklı köy ve kasabalardan toplanan yoğurt örneklerinden 34 suş *Streptococcus thermophilus* olarak teşhis edilmiştir. 34 *S. thermophilus* suşunun antibiyotik dirençlilikleri ve plasmid içerikleri incelenmiştir. Sekiz antibiyotiğe (vankomisin, tetrasiklin, kloramfenikol, eritromisin, sefalotin, sulbaktam ampisilin, gentamisin, ve penisilin G) suşların dirençliliği disk difüzyon metoduna göre incelenmistir. Bir çok *S. thermophilus* suşunun gentamisin (% 79) ve penisilin G'ye (% 64) dirençlilik gösterdiği belirlenirken, suşların % 88 oranında tetrasikline, % 94 oranında da kloramfenikole duyarlılık gösterdiği tespit edilmiştir.

Yapılan plazmid DNA analizlerinde 7 suşun plazmid DNA içermediği belirlenmiştir. Plazmid DNA'sı belirlenen suşlarda plazmid DNA sayısının 1 ve 5 arasında, molekül ağırlıklarının ise 1,88-19,89 kb arasında olduğu tespit edilmiştir. *S. thermophilus*'un bazı suşlarında antibiyotik dirençlilik ve plazmid içeriği arasında bir etkileşimin olduğu gözlenmiştir. Bir veya hiç plazmide sahip olmayan bazı suşların bir çok antibiyotiğe duyarlı oldukları belirlenmiştir. Beş plazmid DNA'ya sahip 3 suşun da bir çok antibiyotiğe dirençli olduğu tespit edilmiştir. Bunun yanısıra bazı suşlarda plazmid DNA içerikleri ile antibiyotik dirençlilikleri arasında korelasyon belirlenememiştir.

Anahtar Sözcükler: *Streptococcus thermophilus*, plazmid DNA, antibiyotik dirençilik

Introduction

Streptococcus thermophilus, a non-N Streptococcus, is a common dairy starter and an economically important microorganism. It is used extensively in the production of yogurts, cheeses and some fermented dairy products, and strain improvement through genetic manipulation has considerable potential. In these fermentations, the thermophilic streptococci are co-cultured with *Lactobacillus bulgaricus*, *L. helveticus, L. lactis* and Propionibacterium (1). Along with the metabolic activities of these bacteria commonly used as starters, they are required to be resistant to antibiotics and phages. Antibiotics, which are used for the treatment of milk-giving animals and especially in mastitis therapy, pass into milk. Even low concentrations of antibiotics in milk inhibit acid development and cause poor quality cheese and fermented milk. Furthermore, they cause large economic losses. (2-4). High sensitivity to most of the antibiotics

employed in mastitis therapy has been reported both in *S. thermophilus* and in *L. bulgaricus* used for yogurt production (4).

To apply recombinant DNA technology to *S. thermophilus*, a suitable cloning vector is required. An ideal plasmid for use in the development of an *S. thermophilus* cloning vector should be stably maintained in *S. thermophilus,* be small, be present in high copy number, and contain unique restriction enzyme sites for the insertion of selectable markers and for cloning purposes (5). The plasmids discovered in different strains have large spectra; some of these carry genes responsible for a variety of functions such as sugar metabolism, proteolysis, citrate fermentation and bacteriocin production as well as resistance to bacteriophage, inorganic ions and antibiotics (1,6).

Miteva et al. (7) reported that the isolation of plasmid and chromosomal DNA of lactic acid bacteria is difficult, and that these plasmid DNAs play a prominent role in recombinant DNA technology. They determined a plasmid in *S. thermophilus* strain 38, the weight of which is 3.4 MDa. This strain showed resistance to only 2 out of 12 antibiotics. The same researchers reported that the antibiotic resistance is coded in some plasmids. In addition, they pointed out that the resistance was also coded in chromosomal genes (7).

Lykova (8) reported that 21 Lactobacillus strains in normal microflora resistant to antibiotics could be widely used as probiotics.

This study aimed to determine the plasmid DNA carriage and antibiotic resistance of natural *S. thermophilus* isolated from Turkish yogurt. The plasmids identified in this study may be improved for use in cloning vectors. Moreover, it was thought that natural strains that are resistant to antibiotics and have stable plasmid DNA might be used in industry for high quality production. Antibiotic resistant strains may be selected for probiotic use in the human gastrointestinal system as a barrier to infections.

Materials and Methods

Isolation and identification

Yogurt samples were obtained from villages. The samples were serially diluted from 10^{-1} to 10^{-7} and the dilutions to 10^{-6} and 10^{-7} were plated on Neutral Red Chalk Lactose agar medium. The plates were incubated at 40-41 ºC for 24-48 h. Streptococcus strains were isolated from the Neutral Red Chalk Lactose agar. The bacteria were identified as *S. thermophilus* based on characteristics as described in the identification tests (9,10).

Elliker broth was used as liquid medium. The bacterial strains were stored frozen at –70 ºC in 25% glycerol broth to supply a stable inoculum for this study.

Plasmid DNA isolation

Plasmid DNAs were isolated according to the mini-prep isolation technique (11). They were electrophoresed through 0.7% agarose gels in a vertical slab chamber, at a setting of 100 V (3 V/cm) for 4 h in Tris-borate-EDTA buffer (89 mM Tris/ 89 mM Boric acid/ 2.5 mM EDTA, pH 8.2). The gels were stained with 0.5 µg/ml ethidium bromide and visualized by UV light (254 nm) (12). As a molecular marker DNA, super coiled DNA ladder (2.067-16.210 kb) was used and the molecular weights of the plasmid DNAs were calculated in kilobases (13).

Antibiotic susceptibility test

Antibiotic susceptibility tests of *S. thermophilus* isolates were performed using the disk diffusion method following NCCLS standards. Active broth cultures of isolates were swabbed onto plates of M17 agar and allowed to dry. Antibiotic disks were placed on the agar and plates were incubated for 24 h at 40-41 ºC (14-16).

Antibiotic disks including vancomycin (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), cephalothin (30 µg), ampicillin-sulbactam (20 µg), gentamicin (10 µg) and penicillin G (10 U) were used in this study (Oxoid).

Results

Sixty-six strains were isolated from yogurt samples obtained from various villages in different regions of Turkey. Thirty-four of these isolates were identified as *S. thermophilus.*

The 34 *S. thermophilus* strains were assayed for susceptibility to 8 antibiotics (vancomycin, tetracycline, chloramphenicol, erythromycin, cephalothin, ampicillin-sulbactam, gentamicin and penicillin G). Most strains were resistant to gentamicin (79%) and penicillin G (64%) and susceptible to chloramphenicol (94%) and

tetracycline (88%) (Table 1). B56.S, B55.S, B48.S, B40.S, B36.S, B35.S, B34.S, B25.S, B16.S, S4.S, S3.S and S2.S strains showed resistance to most of the antibiotics. B44.S, B43.S, B27.S, B26.S, B20.S and B12.S strains were susceptible to many antibiotics.

In a survey of 34 *S. thermophilus* strains, 7 strains were found not to harbor plasmid DNA. Most of these strains contained plasmid species varying in size from 1.88 to 19.89 kb (Table 2). It was found that the molecular weights of some of these plasmid DNAs (B40.S

Table 1. The antibiotic susceptibility tests and plasmid numbers of *S. thermophilus* strains.

Strains	Tested Antibiotics								Number of plasmids
	Van	Amp	Pen G	Ceph	Ery	Gent	Tet	Chl	
B58.S	++	+	-	+	-	-	++	+	5
B56.S	++	-	-	-	-	-	++	-	5
B55.S	-	-	-	+	-	+	-	++	5
B49.S	-	+	-	+	+	-	++	++	4
B48.S	-	-	-	-	-	-	+	+	2
B46.S	++	++	-	+	-	-	++	+	2
B45.S	++	++	-	++	+	+	++	++	-
B44.S	++	++	++	++	++	-	++	++	-
B43.S	++	++	++	++	++	-	++	++	1
B40.S	++	-	-	-	-	-	++	++	3
B36.S	++	-	-	-	-	-	++	+	1
B35.S	+	-	-	-	-	-	++	-	3
B34.S	++	-	-	-	-	-	-	++	3
B32.S	-	++	+	++	+	-	++	++	-
B30.S	++	++	-	-	-	-	++	++	-
B27.S	++	++	++	++	++	-	++	++	-
B26.S	++	++	++	+	++	-	++	++	-
B25.S	+	-	-	-	-	-	++	+	2
B24.S	+	-	-	-	++	-	++	+	1
B23.S	-	-	+	+	+	-	+	+	3
B22.S	++	++	+	+	+	-	++	++	1
B20.S	++	++	++	++	++	++	++	++	1
B19.S	-	++	-	+	-	-	+	++	2
B17.S	-	-	-	+	+	+	-	+	2
B16.S	-	-	-	+	+	-	-	++	1
B12.S	++	++	++	++	+	-	++	++	-
B11.S	+	-	-	-	+	-	+	+	2
B10.S	++	++	-	-	+	-	++	++	2
B8.S	-	-	+	+	+	+	+	++	2
S23.S	-	-	+	+	+	-	+	+	1
S4.S	-	-	-	-	+	+	-	++	3
S3.S	-	-	+	-	-	-	-	+	1
S2.S	+	-	-	-	-	-	++	++	1
S1.S	-	-	-	+	+	+	+	++	3

++ Susceptible + Intermediate Susceptible - Resistant

Table 2. Number of plasmids and molecular weights in *S. thermophilus* strains.

Strains	Number of plasmids	Size of plasmids (kb)
B58.S	5	3.32, 5.82, 11.46, 17.88, 19.89
B56.S	5	5.47, 9.56, 11.48, 14.19, 17.17
B55.S	5	2.83, 5.70, 9.59, 14.19, 17.17
B49.S	4	4.20, 7.05, 9.70, 14.68
B48.S	2	3.32, 6.54
B46.S	2	1.88, 2.49
B45.S	-	-
B44.S	-	-
B43.S	1	5.04
B40.S	3	6.33, 11.65, 18.75
B36.S	1	14.51
B35.S	3	6.33, 11.65, 18.75
B34.S	3	3.61, 5.81, 10.83
B32.S	-	-
B30.S	-	-
B27.S	-	-
B26.S	-	-
B25.S	2	1.99, 3.69
B24.S	1	4.89
B23.S	3	3.67, 6.71, 12.80
B22.S	1	3.61
B20.S	1	6.07
B19.S	2	6.12, 6.66
B17.S	2	2.44, 3.73
B16.S	1	3.66
B12.S	-	-
B11.S	2	1.91, 3.34
B10.S	2	2.06, 3.58
B8.S	2	2.29, 3.36
S23.S	1	4.89
S4.S	3	5.47, 9.56, 11.48
S3.S	1	3.36
S2.S	1	3.61
S1.S	3	5.47, 9.56, 17.17

and B35.S; B22.S and S2.S) were similar in size. B56.S and B55.S strains had 5 plasmids, 2 of which were similar, while the others were dissimilar. It was observed that all the strains had generally small plasmid DNAs. Only 3 strains (B58.S, B56.S and B55.S) harbored 5 plasmid DNAs. The plasmid DNA contents of B34.S and S2.S are shown in Figure 1 and the plasmid DNA contents of B8.S and S3.S are exhibited in Figure 2.

In our study, some strains having no plasmid DNA (B45.S, B44.S, B32.S, B30.S, B27.S, B26.S and B12.S) were susceptible to most of the antibiotics. On the other hand, B30.S was resistant to some of the antibiotics although it did not contain plasmid DNA. It was observed that most strains containing plasmid were resistant to antibiotics but also showed susceptibility to some antibiotics as well. B 55.S and B 56.S, which contained 5 plasmid DNAs showed resistance to most antibiotics. B40.S and B35.S, which had similar plasmid DNA content, were also alike in their resistance to antibiotics; however, S2.S and B22.S, which had similar plasmid

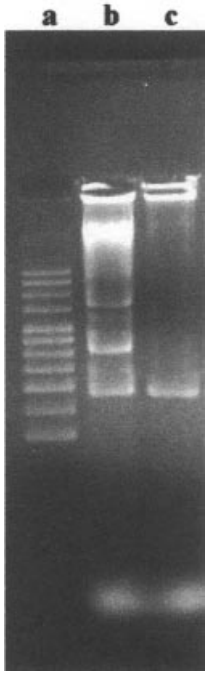

Figure 1. a. Supercoiled DNA ladder (16.210, 14.174, 12.138, 10.102, 8.066, 7.048, 6.030, 5.012, 3.990, 2.972, 2.067 kb); b. B34.S strain (10.83, 5.81, 3.61 kb); c. S2S strain (3.61 kb)

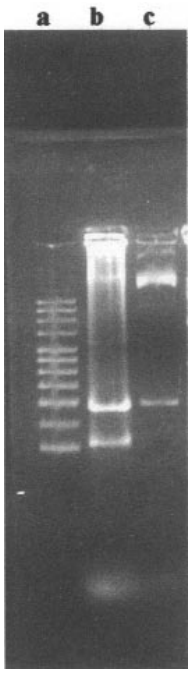

Figure 2. a. Supercoiled DNA ladder; b. B8.S strain (3.36, 2.29 kb); c. S3.S strain (3.36 kb)

content, were different in terms of antibiotic resistance. Some strains having only one and no plasmid were observed to be susceptible to most antibiotics (B12.S, B20.S, B22.S, B26.S, B27.S, B30.S, B43.S, B44.S and B45.S). While in some strains there was a correlation between the resistance to antibiotics and the occurrence of plasmids, in some strains no correlation was observed.

Discussion

Lim et al. (17) found that some lactic acid bacteria *(Lactobacillus casei, L. acidophilus, S. thermophilus* and *L. bulgaricus),* which are commonly used as starters, were susceptible to penicillin G, bacitracine, erythromycin, and vancomycin. They were resistant to gentamicin, and streptomycin, and all strains of *L. casei* were resistant to vancomycin. Other researchers pointed out that all of the L group streptococci were resistant to tetracycline; however, some isolates were resistant to chloramphenicol, erythromycin and gentamicin, and most isolates were susceptible to penicillin and cephalothin (18). Charteris et al. (19) reported that the natural resistance of lactobacilli to a wide range of clinically important antibiotics may enable the development of antibiotic/probiotic combination therapies for such conditions as diarrhea, female urogenital tract infection, and infective endocarditis. The susceptibility of 21 strains of normal microflora to 25 antibiotics was tested. It was shown that *L. acidophilus* (probiotic "Acidlact") was resistant to metronidazole only. It was concluded that the use of stable antibiotic-resistant strains of normal microflora was favorable as an addition to antibiotic therapy (20).

Extra-genomic DNA in the form of plasmids has been found in many species of lactic acid bacteria. Some of these plasmids have important characteristics such as drug resistance, metabolic functions, a restriction system or phage resistance. Industrially important thermophilic starters for milk fermentation have been reported to carry plasmid DNA. Some plasmids are important for industrial applications (21).

According to these results, in some strains the resistance to some antibiotics may be under the control of plasmid DNAs; however, the resistance to some antibiotics may be coded by chromosomal genes, and

different plasmids caused resistance to different antibiotics.

Penicillins are inactivated by β-lactamases (penicillinases) produced by many Gram-positive and Gram-negative bacteria. The enzyme is coded by chromosomal or plasmid genes (22).

Some researchers have reported that the resistant strains to various antibiotics were located in a high copy number and transferable plasmid DNAs and the plasmids of these strains can be developed as cloning vectors for use in Gram-positive bacteria used in recombinant DNA technology because of their traits (23-25).

Somkuti and Steinberg (12) identified plasmid DNA whose weight varied from 2.2 to 7.15 Kb in 13 out of 35 *S. thermophilus* strains.

Axelsson et al. (26) reported that in some lactic acid bacteria erythromycin resistance was coded by plasmid DNAs that could be used as a cloning vector. The coding to erythromycin and fucidin plasmids was found in the lactic acid bacteria whose molecular weights were 26.5 and 60 kb (27).

Miteva et al. (7) found that in a study conducted with *S. thermophilus, L. helveticus* and *L. bulgaricus,* the resistance to antibiotics was not coded by plasmid DNAs, and they claimed that the genes of resistance were in the chromosomal DNA part.

To conclude, most of the natural isolates carried low molecule weight plasmid DNA. Plasmid DNAs of these strains may be used in recombinant DNA technology. Strains that showed resistance to most of the antibiotics tested may also be used in the industrial process. This result suggests that *S. thermophilus* strains naturally resistant to antibiotics are favorable for use as probiotics or starters.

References

1. Somkuti, G.A., Steinberg, D.H.: Distribution and analysis of plasmids of *Streptococcus thermophilus.* J. Ind. Microbiol., 1986; 1, 157-163.
2. Kosikowski, F.; Cheese and Fermented Milks. 2nd ed.; Edwards Broth. Inc. Ann. Arbor: Michigan, 1977.
3. Lewis, J.E.: Cheese Starters: Developments and application of the Lewis System. Elsever Applied Science Publishers: London. 1987.
4. Larsen, R.F., Anon, M.C.: Interaction of antibiotics and water activity on *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. J. Food Sci., 1989; 54: 922-923.
5. Herman, R.E., Mc Kay, L.L.: Isolation and partial characterization of plasmid DNA from *Streptococcus thermophilus*. Appl. Environ. Microbiol., 1985; 50: 1103-1106.
6. Mc Kay, L.L.: Functional properties of plasmids in lactic streptococci. Anton. Van. Leeuw. J. Microbiol. 1983; 43**,** 259-274.
7. Miteva, V., Stefanova, T.Z., Takova, T.Z, Grigorova, R.: Isolation and characterisation of plasmids from different strains of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Acta Microbiol. Bulg., 1991; 27**:** 3-8.
8. Lykova, E.A.: The antibacterial resistance of the strains making up the components of probiotic preparations. Zh. Microbial. Epidemiol. Immunobiol., 2000; 2: 63-66.
9. Buchanan, R.E., Gibbons, N.E.: Bergey's Manual of Determinative Bacteriology, Williams and Wilkins Co., Baltimore, 1974.
10. Holdeman, L.V., Cato, E.P., Moore, W.E.C.: Anaerobe Laboratory Manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 1975.
11. O'Sullivan, D.J., Klaenhammer, T.R.: Rapid mini prep isolation of high quantity plasmid DNA from Lactococcus and Lactobacillus spp. Appl. Environ. Microbiol., 1993; 59: 2730-2733.
12. Somkuti, G.A., Steinberg, D.H.: General method for plasmid DNA isolation from thermophilic lactic acid bacteria. J. Biotech.,1986; 3: 323-332.
13. Sambrook, J., Fidsch, E.F., Maniatis, T.: Molecular Cloning. 2nd ed., 1989.
14. Murray, P.R., Baron, E., Pfaller, M.A, Tenover, F.C., Yolken, R.H.: Manual of Clinical Microbiology (Antimicrobial susceptibility tests) 6th ed. ASM, Washington, DC, 1995.
15. Performance Standard for Antimicrobial Disk Susceptibility Tests; Approved Standard-Seventh Edition, January 2000, M2-A7, Vol. 20, No 1, NCCLS.
16. Çetin, E.T., Gürler, N.: Bakterilerin antibiyotiklere duyarlılık deneyinin yapılması. Kükem Derg., 1989; 12: 97-104.
17. Lim, K.S., Huh, C.S., Baek, Y.J.: Studies on the antimicrobial susceptibility of lactic acid bacteria in cultured milk products. Korean J. Dairy Sci., 1995; 17 (In Korean, Abstract in English).
18. Sippel, K., Dulffer-Sceitzer, B., Lammer, C.T.: The characteristic properties of streptococci of serological group. L. Zbl. Vet. Med. B., 1995; 42: 42-50.
19. Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K.: Antibiotic susceptibility of potentially probiotic Lactobacillus species. J. Food Prot., 1998; 61: 1636-1643.

20. Lykova, E.A.: Intestinal dysbacteriosis in antibacterial therapy and perspectives in treatment by antibiotic-resistant probiotics. Antibiot Khimioter., 2001; 46: 21-25.

21. Casey, M.G., Jimeno, J.: *Lactobacillus delbruckii* subsp. *lactis* plasmids. Net. Milk Dairy, 1989; 43: 279-286.

22. Hardy, K.: Aspects of Microbiology: Bacterial Plasmids, Thomas and Nelson Ltd., Hong Kong, 1981.

23. Levy, B.S.: Antibiotics use for growth promotion in animals. Ecologic and public health consequences. J. Food Protect., 1987; 50: 616-620.

24. Lin, J.H., Savage, D.C.: Genetic transformation of rifampicin resistance in *Lactobacillus acidophilus*. J. Gen. Microbiol., 1986; 132: 2107-2111.

25. Swenson, J.M., Facklam, R.R., Thornsberry, C.: Antimicrobial susceptibility of vancomycin resistant Leuconostoc, Pediococcus and Lactobacillus species. Antimicrob. Agents Chemoter., 1990; 34: 543-549.

26. Axelsson, L.T., Ahrne, S.E.I., Andersson, M.C., Stahl, S.R.: Identification and cloning of plasmid-endocoded erythromycin-resistance determinant form *Lactobacillus reuteri*. Plasmid, 1988; 20: 171-174.

27. Tyurin, M.V., Shenderov, B.A., Grigoryev, A.E., Zhukov, V.G., Navashin, S.M.: Transfer of various markers in Lactobacilli during plasmid DNA transformation and their joint cultivation. Antibiot. Chemioter., 1990; 35: 16-21.