APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1985, p. 538–542
0099-2240/85/030538-05$02.00/0

Vol. 49, No. 3

Survey of Antimicrobial Resistance in Lactic Streptococci†

PAULO K. ORBERG‡ AND WILLIAM E. SANDINE*

Department of Microbiology, Oregon State University, Corvallis, Oregon 97331-3804

Received 26 July 1984/Accepted 4 December 1984

A total of 26 strains of *Streptococcus cremoris* and 12 strains of *Streptococcus lactis* were challenged with 18 antimicrobial agents and with nisin in the Bauer-Kirby disk susceptibility test. All strains were susceptible to ampicillin, bacitracin, cephalothin, chloramphenicol, chlortetracycline, erythromycin, penicillin G, tetracycline, and vancomycin. All strains were resistant to trimethoprim, and almost all strains were resistant to sulfathiazole. Variability in resistance to gentamicin, kanamycin, lincomycin, nafcillin, neomycin, nisin, rifampin, and streptomycin was observed. MICs of these substances for the less susceptible strains were determined, and high-level resistance factors could not be detected, except in the case of nisin. *S. lactis* ATCC 7962 was resistant to at least 40-fold-higher concentrations of nisin (>64 μg/ml) than most other strains tested. This strain was a potent nisin producer.

Group N streptococci are essential for the production of fermented dairy foods. Our knowledge of the genetics of these bacteria, especially as related to their plasmids, has progressed steadily in recent years (23). All naturally occurring lactic streptococcal strains examined to date contain one or more plasmid species; plasmid-carried genes confer on these bacteria some of their most important physiological properties, such as fast lactose fermentation or milk protein hydrolysis. However, in spite of extensive studies on lactic streptococcal plasmids, reports on the presence of antimicrobial resistance determinants in these bacteria are extremely scarce (11, 23). As a result, few endogenous selectable markers are presently available for genetic studies of group N streptococci, in particular for the construction of plasmid cloning vehicles.

Lactic streptococci are not ordinarily exposed to antimicrobial agents except for the presence, in milk, of residues of antibiotics used in mastitis therapy. That high-level antimicrobial resistance is not common in these bacteria has clearly been indicated by the work of Cogan (7), Lipinska (21), and Reinbold and Reddy (26). However, given the large plasmid complements of these bacteria and given the fact that known, previous exposure to antimicrobial agents is not a requisite for the presence of resistance determinants (14, 25, 30), it would be reasonable to expect that such determinants might still be found in group N streptococci. In this study we employed a large number of strains and the combined use of disk tests and MIC determinations to reexamine this question.

In this paper we report the examination of 38 randomly selected lactic streptococcal strains for their susceptibility or resistance to all major classes of antimicrobial agents and one bacteriocin (nisin). As was recently shown by McKay and Baldwin (24), we found resistance to nisin to be a feasible selectable marker which is endogenous in these bacteria. High-level resistance, characteristic of the presence of specific determinants, could not be found for any of the antimicrobial agents examined, with the possible exceptions of sulfathiazole and trimethoprim. The widely used strain *S. lactis* ATCC 7962 was shown to produce nisin.

MATERIALS AND METHODS

Bacterial strains. *S. lactis* ATCC 11454 was a gift from D. J. LeBlanc, National Institute for Dental Research, Bethesda, Md. *S. cremoris* ATCC 14365 was kindly provided by C. F. Gonzales, Microlife Genetics, Sarasota, Fla. *S. lactis* strains NCDO 497 and NCDO 1403 were obtained from the National Institute for Research in Dairying, Shinfield, Reading, England. Other lactic streptococci were from the culture collection maintained in this laboratory. The following strains were used in disk susceptibility tests: *S. cremoris* B1, C13, HP, M26, M45, R6, SK11G, SK11G-C, U134, 31N, 32, 104, 108, 108 M, 205, 224, 226, 227, 283, 284, 287, 289-C, 290 PC, 291, 292, and 378; and *S. lactis* O1, BA1, BA2, C2, C3, C10, C27, F2D2, ML8, ML8-R4, SLE, and ATCC 7962. All strains were challenged with all antimicrobial agents in the disk test. Culture maintenance was in M17 broth (32). (All M17 formulations used in this work contained 0.5% yeast extract.)

Disk susceptibility tests. Disk tests were done by the Bauer-Kirby method (3), according to the recommendations of Acar (1) and Barry (2). Cultures were grown in M17 broth for 4.5 h at 30°C (ca. 5.0×10^7 to 2.0×10^8 CFU/ml) and used for inoculation of agar plates. After disk application, plates were incubated at 30°C for 20 h, and inhibition zone diameters were measured. Chloramphenicol and rifampin disks were from BBL Microbiology Systems. Other commercially obtained disks were from Difco Laboratories. Sulfathiazole or trimethoprim disks contained 300 or 5 μg, respectively. Other disk potencies are given in Fig. 1 and 2. Purified nisin (ca. 40,000 U/mg) was purchased from Aplin & Barrett Ltd., Beaminster, Dorset, England. Nisin-containing disks were prepared (27) by spotting 20 μl of nisin solution (6.4 mg/ml in sterile distilled water) onto sterile filter paper disks of ca. 6.5-mm diameter (Difco). Bacitracin, chlortetracycline, sulfathiazole, trimethoprim, and vancomycin disk tests were on Mueller-Hinton agar (Difco); all other disk tests were on M17 agar.

MIC determinations. For MIC determinations (2, 34), a fresh overnight culture in M17 broth was diluted with normal saline (1:10), and ca. 1 μl of this suspension was used to inoculate the surface of M17 agar containing the test antimicrobial agent. Presence or absence of growth was recorded

* Corresponding author.

† Technical paper no. 7192 of the Oregon Agricultural Experiment Station.

‡ Present address: Laboratory of Tumor Virus Genetics, Dana-Farber Cancer Institute, Boston, MA 02115.

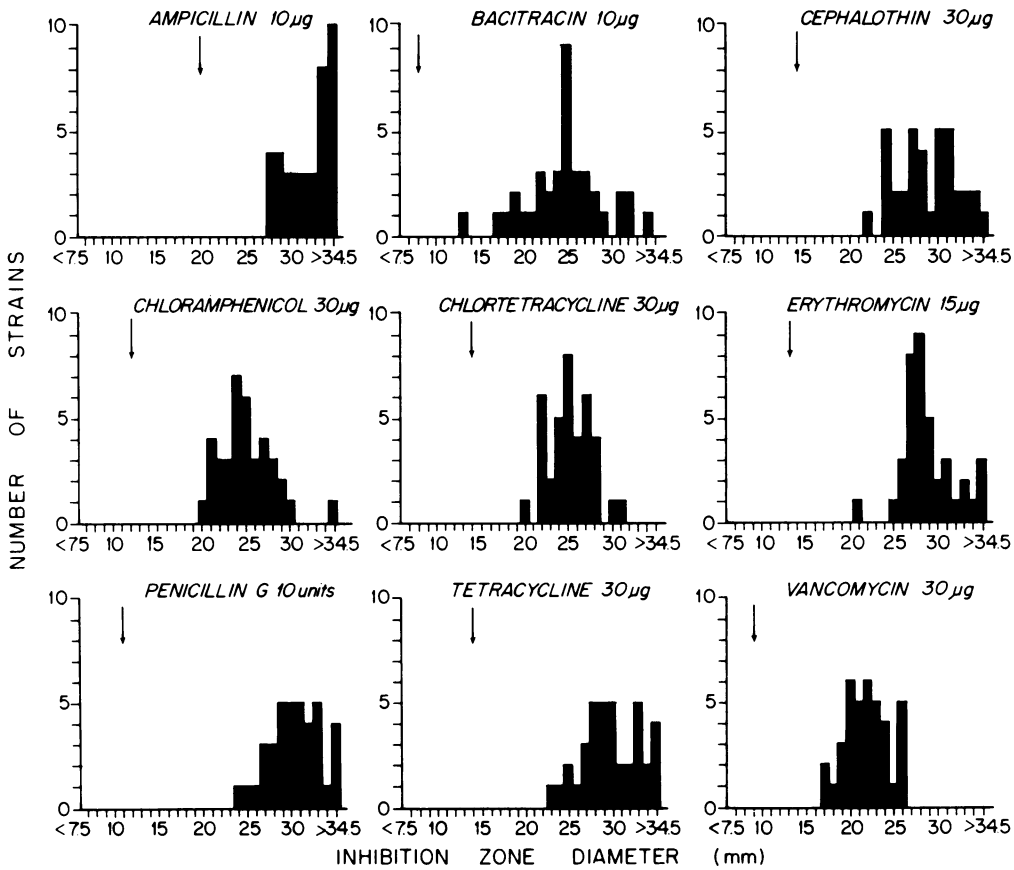

FIG. 1. Distributions of inhibition zone diameters observed in disk tests. Grouped in this figure are those antimicrobial agents for which all strains had inhibition zones larger than the maximum exhibited by clinically resistant bacteria (arrows).

after 24 h of incubation at 30°C. With the exception of nisin, all antimicrobial agents were from Sigma Chemical Co.

Bacteriocin production tests. The method of Kékessy and Piguet (19) and glucose-M17 agar plates were used. Plates were inoculated with ca. 1 μl of a fresh culture of the strain to be tested as producer, incubated at 30°C for 20 h, inverted, and inoculated with the indicator strain. Results were read after a second 20-h period at 30°C.

Tests of identity between the bacteriocin produced by *S. lactis* ATCC 7962 and nisin (produced by *S. lactis* NCDO 497) were performed essentially as described by Gasson (15). These two strains, as well as *S. cremoris* B1 (indicator strain and negative control for bacteriocin production), were grown in M17 broth (with glucose instead of lactose, since 7962 ferments lactose slowly [9]). Cultures were prepared from 0.2% inocula and incubated at 30°C for 10 h. Cells were removed by centrifugation, and the supernatants were sterilized by membrane filtration (0.2-μm pore size). Filtrates were divided in three parts: A (untreated), B (pH adjusted to 2.0 with HCl), and C (pH adjusted to 8.0 with NaOH). Fractions B and C were boiled for 30 min and cooled, and their pHs were adjusted to 5.8. Four-hour-old cultures (ca. 7.0×10^7 CFU/ml) of all three strains were used to prepare lawns on glucose-M17 agar. After drying, the lawns were spotted with ca. 1 μl of each fraction (A, B, or C). All three strains were tested both as bacteriocin producers and as indicators. Presence or absence of inhibition was recorded after 20 h of incubation at 30°C. The same protocol was used for testing bacteriocin sensitivity to proteolytic digestion. Culture filtrates had their pHs adjusted to 7.5, and α-chymotrypsin or trypsin (both from Sigma) were added (5.0 mg/ml final concentration). After 2 h of incubation at 25°C, the filtrates were spotted onto lawns.

RESULTS

Disk susceptibility tests. The distributions of inhibition zone diameters observed with the strains tested are shown in Fig. 1 and 2. In both figures, the vertical arrows indicate the maximum inhibition zone diameter exhibited by bacteria considered clinically resistant to the given antimicrobial agent. (This information, when available, was compiled from reference 3 or from materials published by BBL Microbiology Systems.) Clearly, two types of situations were observed. The first type was represented by the distributions shown in Fig. 1; all strains tested had inhibition zones significantly larger than the maximum accepted as compatible with resistance. No further tests were conducted with the antimicrobial agents grouped in Fig. 1.

A second type of situation, displayed in Fig. 2, was discernible; the more resistant strains showed inhibition zone diameters equal to, or smaller than, the maximum diameter observed with clinically resistant strains (arrows). In other words, as a first approximation, the disk test indicated the presence of strains resistant to one or more of these substances. These strains were then subjected to the more stringent test of MIC determination (see below).

Susceptibility tests were also performed with disks containing sulfathiazole or trimethoprim. In both instances, Mueller-Hinton agar was used as the test medium, since it is practically free of interfering substances. Only three strains

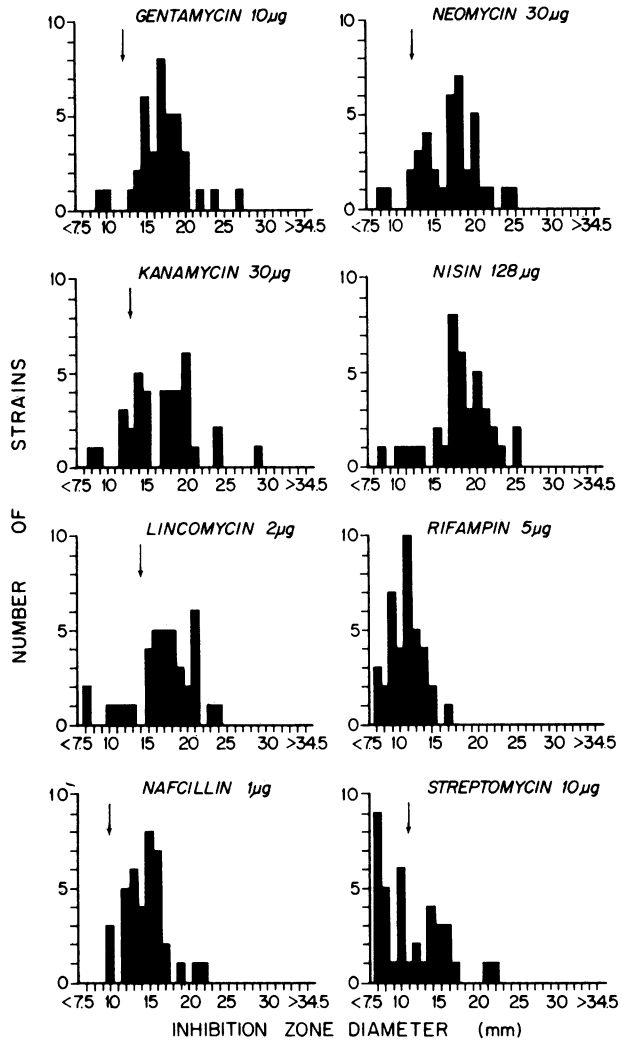

FIG. 2. Distributions of inhibition zone diameters observed in disk tests. Grouped in this figure are those antimicrobial agents for which some strains had inhibition zones smaller than the maximum exhibited by clinically resistant bacteria (arrows) (values were not available for rifampin or nisin).

(*S. lactis* BA1, BA2, and C10) showed a zone of complete inhibition around a disk with sulfathiazole. None of the strains were inhibited by trimethoprim.

MIC determinations. Strains showing inhibition zone diameters indicative of resistance to one or more antimicrobial agents were used in MIC determinations. The following strains were tested in this manner: *S. cremoris* B1, HP, R6, SK11G, U134, 31N, 108, 224, 226, 290 PC, and 291; and *S. lactis* BA2, C2, C10, C27, ML8, SLE, and ATCC 7962. The results of these determinations are shown in Table 1. This table includes, when available, the MICs of the same substances for *Streptococcus* strains known to carry resistance factors. Comparison of the highest experimental MICs with the modal experimental MICs, or with these values from the literature, indicated that the strains examined did not carry determinants for high-level resistance to any of these antimicrobial agents, with the exception of nisin. *S. lactis* ATCC 7962 was resistant to at least 40-fold-higher concentrations of nisin than most other strains tested.

Bacteriocin production tests. Since *S. lactis* ATCC 7962 was clearly much more resistant to nisin than any of the other strains, it was logical to test whether it might be a nisin producer. Bacteriocin production by *S. lactis* 7962 and by three reference nisin producers was tested against five indicator strains, including 7962 itself. The results confirmed that 7962 was resistant to nisin and demonstrated that this strain was a potent bacteriocin producer (Table 2). The sensitivities of the indicator strains to this bacteriocin paralleled their sensitivities to nisin. For both bacteriocins, *S. lactis* C10 showed the smallest inhibition zones, whereas *S. cremoris* U134 showed the largest.

We tested whether the 7962 bacteriocin could be distinguished from nisin on the basis of sensitivity to boiling at pH 2.0 or 8.0. *S. lactis* NCDO 497 (a reference nisin producer) and *S. cremoris* B1 (a nisin-sensitive organism) were used as controls. The capacity of 7962 or 497 culture filtrates to inhibit *S. cremoris* B1 was retained after boiling at pH 2.0 but destroyed by boiling at pH 8.0, as expected for nisin (17). None of the three filtrates from *S. cremoris* B1 inhibited any of the three strains. Nisin-containing 497 filtrates inhibited none of the two *S. lactis* strains; the same was true for the 7962 filtrates, showing that *S. lactis* NCDO 497 was insensitive to the 7962 bacteriocin. Like nisin, this bacteriocin was inactivated by α-chymotrypsin but not by trypsin. These results indicated that *S. lactis* ATCC 7962 produced nisin.

TABLE 1. MICs of selected antimicrobial agents for *Streptococcus* strains[a]

Substance	Experimental MIC (μg/ml)		MIC (μg/ml) for R-factor-carrying strains[b]
	Mode(s)	Highest	
Gentamicin	4.0	80	>2,000 (5, 8)
Kanamycin	8.0, 16	160	>2,000 (5, 8)
Lincomycin	1.0	8.0	>1,000 (20)
Nafcillin	1.0	2.0	
Neomycin	16, 64	160	>2,000 (5)
Nisin	1.6[c]	>64[d]	
Rifampin	32	64	
Streptomycin	8.0, 64	160	>2,000 (5)

[a] Comparison of MICs determined in this work with those reported for strains known to carry resistance factors (R factors).
[b] Numbers in parentheses indicate references.
[c] Smallest concentration tested.
[d] Poor solubility prevented testing of higher concentrations.

DISCUSSION

In his review (23), McKay pointed out the scarcity of known selectable markers in the lactic streptococci and addressed the need for studies directed at uncovering such

TABLE 2. Bacteriocin production by *S. lactis* strains[a]

Producer[b]	Inhibition zone diam (mm) with the following indicator strain:				
	S. lactis		*S. cremoris*		
	7962	C10	14365	B1	U134
S. lactis ATCC 7962	0	13	16	15	21
S. lactis ATCC 11454	0	8	11	10	17
S. lactis NCDO 497	0	6	10	10	14
S. lactis NCDO 1403	0	8	10	10	13

[a] Kékessy-Piguet method (19).
[b] All five indicators were also tested as producers; among these five strains, *S. lactis* ATCC 7962 was the only one to inhibit any other strain.

markers for use in genetic manipulations of these bacteria. The present work directly meets that need.

In this study, at least one representative of every major class of antimicrobial agent was used to challenge 38 randomly selected strains of lactic streptococci. More than one antimicrobial agent of a given structural class was included in case different mechanisms of inactivation exist (e.g., kanamycin and neomycin, which might be inactivated by different enzyme specificities).

Although the probability of finding antimicrobial resistance determinants in lactic streptococci is clearly much lower than in isolates from clinical or animal sources (33), it certainly cannot a priori be considered vanishingly small. The lactic streptococci carry, as a rule, large plasmid complements (6, 23); all of the strains tested in this work contained at least two plasmid species (data not shown). Furthermore, resistant determinants have often been found in bacteria not previously exposed to antimicrobial agents disseminated by humans (14, 25, 30).

In view of these premises, it is somewhat surprising that resistance factors have not been readily found in lactic streptococci (23). The results of the present work support the hypothesis that such factors might indeed be rare in these strains, with the possible exception of determinants for resistance to sulfathiazole or trimethoprim. Our findings on the scarcity of resistance factors in groups N streptococci are corroborated by previous work by Cogan (7) and Lipinska (21), who found no evidence of antimicrobial resistance among lactic acid bacteria.

In a large survey which included 15 lactic streptococcal strains, Reinbold and Reddy (26) reported sensitivity of these strains to almost all of 30 antimicrobial agents, with the notable exceptions of colistin, nalidixic acid, polymixin B, and sulfonamides. Evidence of resistance was from disk tests only, and confirmatory MIC determinations were not performed. Similarly, Sozzi and Smiley (31) reported antimicrobial resistance in yogurt bacteria, but the level of resistance was not assessed by means of MIC tests.

The work of Dobrzanski et al. (11) presently stands as the sole finding of plasmid-mediated resistance to antimicrobial agents (bacteriocins excluded) in mesophilic lactic streptococci. These authors reported transformation of *Bacillus subtilis* to kanamycin resistance by plasmid DNA from *S. lactis* (kanamycin MIC, 50 μg/ml). Such a low-level marker would seem inadequate for genetic experiments with lactic streptococci. Aminoglycoside-aminocyclitol antibiotics are not effectively uptaken by streptococci because of their nonoxidative metabolism (4, 10, 14, 28). In fact, media containing these substances are routinely used for the isolation of streptococci from clinical specimens (12). These bacteria can be resistant to levels of aminoglycoside-aminocyclitols of the order of 250 μg/ml in the absence of specific resistance factors (5). Our results with gentamicin, kanamycin, neomycin, and streptomycin are in complete agreement with these notions. It should be noted that in some strains at least, part of the low-level resistance to these antibiotics detected in this study might be due to the use of M17 agar (32) for susceptibility testing. Sinha has recently observed (29) that the resistance of lactic streptococci to aminoglycoside-aminocyclitol antibiotics increases in phosphate-buffered media.

Low susceptibility of lactic streptococci to sulfonamides was reported by Reinbold and Reddy (26). Sozzi and Smiley (31) found evidence of resistance to sulfonamides and trimethoprim in *S. thermophilus*. Our results support and extend these findings. Clearly, more work is needed to determine whether factors for resistance to these antimicrobial agents are present in these bacteria.

As shown by McKay and Baldwin (24), resistance to nisin is a suitable selectable marker for use in genetic studies of the lactic streptococci. Plasmid linkage of this marker has been strongly suggested by the results of many workers (15, 17, 23).

Bacteriocin production is common among lactic streptococci (16). Our results show that *S. lactis* ATCC 7962 produces nisin, a denomination that actually applies to several closely related polypeptides (17). As is the case with most nisin producers (15, 17), this strain ferments sucrose (13). The information presented here on nisin production by *S. lactis* ATCC 7962 should be a valuable addition to the knowledge on what is, from a physiological standpoint, the most thoroughly studied lactic streptococcal strain (9, 13, 18, 22).

ACKNOWLEDGMENT

This work was supported by a grant from Microlife Technics, Sarasota, Fla.

LITERATURE CITED

1. **Acar, J. F.** 1980. The disc susceptibility test, p. 24–54. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
2. **Barry, A. L.** 1980. Procedures for testing antibiotics in agar media: theoretical considerations, p. 1–23. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
3. **Barry, A. L., and C. Thornsberry.** 1980. Susceptibility testing: diffusion test procedures, p. 463–474. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
4. **Bryan, L. E.** 1982. Bacterial resistance and susceptibility to chemotherapeutic agents, p. 164–167. Cambridge University Press, London.
5. **Carlier, C., and P. Courvalin.** 1982. Resistance of streptococci to aminoglycoside-aminocyclitol antibiotics, p. 162–166. *In* D. Schlessinger (ed.), Microbiology—1982. American Society for Microbiology, Washington, D.C.
6. **Clewell, D. B.** 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45**:409–436.
7. **Cogan, T. M.** 1972. Susceptibility of cheese and yogurt starter bacteria to antibiotics. Appl. Microbiol. **23**:960–965.
8. **Courvalin, P., C. Carlier, and E. Collatz.** 1980. Plasmid-mediated resistance to aminocyclitol antibiotics in group D streptococci. J. Bacteriol. **143**:541–551.
9. **Crow, V. L., and T. D. Thomas.** 1984. Properties of a *Streptococcus lactis* strain that ferments lactose slowly. J. Bacteriol. **157**:28–34.
10. **Davies, J.** 1980. Aminoglycoside-aminocyclitol antibiotics and their modifying enzymes, p. 474–489. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
11. **Dobrzanski, W. T., J. Bardowski, W. Kozak, and J. Zajdel.** 1982. Lactostrepcins: bacteriocins of lactic streptococci, p. 225–229. *In* D. Schlessinger (ed.), Microbiology—1982. American Society for Microbiology, Washington, D.C.
12. **Eickhoff, T. C., and J. M. Ehret.** 1980. The use of antibiotics as tools in epidemiology and taxonomy, p. 490–505. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
13. **Farrow, J. A. E., and E. I. Garvie.** 1979. Strains of *Streptococcus lactis* which contain β-galactosidase. J. Dairy Res. **46**:121–125.
14. **Foster, T. J.** 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. **47**:361–409.

15. **Gasson, M. J.** 1984. Transfer of sucrose fermenting ability, nisin resistance and nisin production into *Streptococcus lactis* 712. FEMS Microbiol. Lett. **21**:7–10.
16. **Geis, A., J. Singh, and M. Teuber.** 1983. Potential of lactic streptococci to produce bacteriocin. Appl. Environ. Microbiol. **45**:205–211.
17. **Hurst, A.** 1981. Nisin. Adv. Appl. Microbiol. **27**:85–123.
18. **Kashket, E. R.** 1981. Proton motive force in growing *Streptococcus lactis* and *Staphylococcus aureus* cells under aerobic and anaerobic conditions. J. Bacteriol. **146**:377–384.
19. **Kékessy, D. A., and J. D. Piguet.** 1970. New method for detecting bacteriocin production. Appl. Microbiol. **20**:282–283.
20. **LeBlanc, D. J., and F. P. Hassell.** 1976. Transformation of *Streptococcus sanguis* Challis by plasmid deoxyribonucleic acid from *Streptococcus faecalis*. J. Bacteriol. **128**:347–355.
21. **Lipinska, E.** 1977. Nisin and its applications, p. 103–130. *In* M. Woodbine (ed.), Antibiotics and antibiosis in agriculture. Butterworths, London.
22. **Maloney, P. C., S. V. Ambudkar, J. Thomas, and L. Schiller.** 1984. Phosphate/hexose 6-phosphate antiport in *Streptococcus lactis*. J. Bacteriol. **158**:238–245.
23. **McKay, L. L.** 1983. Functional properties of plasmids in lactic streptococci. Antonie van Leeuwenhoek J. Microbiol. **49**:259–274.
24. **McKay, L. L., and K. A. Baldwin.** 1984. Conjugative 40-megadalton plasmid in *Streptococcus lactis* subsp. *diacetylactis* DRC3 is associated with resistance to nisin and bacteriophage. Appl. Environ. Microbiol. **47**:68–74.
25. **Novick, R.** 1980. Plasmids. Sci. Am. **243**:76–90.
26. **Reinbold, G. W., and M. S. Reddy.** 1974. Sensitivity or resistance of dairy starter and associated microorganisms to selected antibiotics. J. Milk Food Technol. **37**:517–521.
27. **Rippere, R. A.** 1980. Preparation and control of antibiotic susceptibility discs and other devices containing antibiotics, p. 549–572. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
28. **Rosenblatt, J. E.** 1980. Antimicrobial susceptibility testing of anaerobes, p. 114–134. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
29. **Sinha, R. P.** 1984. Effect of buffering media with phosphates on antibiotic resistance of lactic streptococci. Appl. Environ. Microbiol. **47**:1175–1177.
30. **Smith, D. H.** 1967. R factor infection of *Escherichia coli* lyophilized in 1946. J. Bacteriol. **94**:2071–2072.
31. **Sozzi, T., and M. B. Smiley.** 1980. Antibiotic resistances of yogurt starter cultures *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Appl. Environ. Microbiol. **40**:862–865.
32. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807–813.
33. **Vescovo, M., L. Morelli, and V. Bottazzi.** 1982. Drug resistance plasmids in *Lactobacillus acidophilus* and *Lactobacillus reuteri*. Appl. Environ. Microbiol. **43**:50–56.
34. **Washington, J. A., II, and V. L. Sutter.** 1980. Dilution susceptibility test: agar and macro-broth dilution procedures, p. 453–458. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.