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NOTES

Bacteriocin-Producing Strain of *Lactococcus lactis* subsp. *diacitilactis* S50

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***Lactococcus lactis* subsp. *diacitilactis* S50 produces a bacteriocin, designated bacteriocin S50, which has a narrow antibacterial spectrum. It was active only against *Lactococcus* species, including a nisin producer exhibiting a bactericidal effect. The activity of bacteriocin S50 was sensitive to proteases. It retained antimicrobial activity after being heated to 100°C for up to 60 min and in the pH range 2 to 11.**

Antagonism by lactic acid bacteria in fermented food has been associated with the major end products of their metabolism, such as lactic acid, acetic acid, and hydrogen peroxide (9). Bacterial antagonism via production of low-molecular-weight compounds, termed bacteriocins, also has been identified in lactic acid bacteria (5).

Nisin is the most thoroughly characterized bacteriocin among the antimicrobial proteins produced by *Lactococcus lactis* (7). It is known that nisin has a bactericidal effect on gram-positive bacteria, including sporeforming *Clostridium* and *Bacillus* spp. In contrast to nisin, the activity of diplococcin (bacteriocin produced by *L. cremoris* 346) is restricted to dairy lactococci. It was found that the genetic determination of diplococcin production and immunity in *L. cremoris* 346 was associated with a large conjugative plasmid (2). Bacteriocins are also produced by other lactococci, and they can be distinguished by their spectrum of activity and biochemical characteristics (for a review, see reference 10). This study describes the characterization of a bacteriocinlike compound, termed bacteriocin S50, produced by *L. lactis* subsp. *diacitilactis* S50.

For bacteriocin detection, soft GM17 agar (0.7%) containing the indicator strain, *L. lactis* subsp. *cremoris* NS1, was overlaid on GM17 plates (13). Wells were made in the lawn of hardened soft agar; aliquots (50 μl) of supernatants of overnight cultures (16 h) were poured into the wells. The plates were incubated overnight at 30°C. The appearance of a clear zone of inhibition around the well was taken as a positive signal for bacteriocin production. One arbitrary unit of bacteriocin was defined as the reciprocal of the highest dilution yielding a zone of growth inhibition on the indicator lawn.

The antimicrobial activity of bacteriocin S50 was confirmed by using pH-neutralized, catalase-treated culture supernatants of *L. lactis* subsp. *diacitilactis* S50 to eliminate a possible inhibitory effect of either hydrogen peroxide or lactic acid on indicator strain growth. In addition, a supernatant of indicator strain *L. lactis* subsp. *cremoris* NS1 was used in the same experiment as a negative control (Fig. 1). The inhibitory spectra of lactococcal bacteriocins, other than nisin, produced by different species and strains of lactococci were described before (3, 6, 11). Bacteriocin S50 has a relatively narrow antibacterial spectrum. Only species relatively closely related to the producer were inhibited (*L. lactis* subsp. *lactis* NCDO712 and C10, *L. lactis* subsp. *cremoris* CH-1, and *L. lactis* subsp. *diacitilactis* NCDO176, NCDO823, and ATCC 13675). Interestingly, bacteriocin S50 inhibits the growth of a nisin producer (*L. lactis* subsp. *lactis* NP45). In this respect, it is similar to the bacteriocin type VII produced by *L. lactis* subsp. *diacitilactis* (4). It had no effect on other gram-positive bacteria (*Lactobacillus casei* 161, *Lactobacillus acidophilus* ATCC 4356, *Bacillus subtilis*, *Staphylococcus aureus* ATCC 25923, group A or B streptococci, and *Enterococcus* sp.). Several gram-negative bacteria (*Escherichia coli* C600, *Salmonella typhimurium* LT2, and *Pseudomonas* sp.) and a yeast (*Saccharomyces cerevisiae* 3918-KF) were also tested, but no effect was observed. Bacteriocin S50 exhibited a bactericidal effect on the sensitive cells, as did many of the lactococcal and lactobacillus bacteriocins described so far (1, 4, 14).

Monitoring the production of bacteriocin S50 during the growth of *L. lactis* subsp. *diacitilactis* S50 in GM17 showed that it was continuously produced, but the highest production was observed after 8 h of incubation (Fig. 2). Bacteriocin S50 belongs to the heat-stable group of bacteriocins, such as nisin or bacteriocins produced by lactobacilli (8, 12). It possesses antimicrobial activity after heating for up to 60 min at 100°C.

Bacteriocin S50 retains its activity in the pH range 2 through 11. However, antimicrobial activity was gradually lost at pH 12. Namely, bacteriocin S50 kept the activity for up to 30 min, when the pH of the assay suspension was adjusted to 12. After that, the activity was lost irreversibly, and it could not be regained upon lowering the pH to 7. Similar activity was observed when nisin was tested. There was no detectable inhibition when the supernatant of a nonproducer was tested in parallel experiments, excluding the effect of pH alone. On the contrary, many lactococcal bacteriocins are active over a pH range of 4 to 6.5. More-

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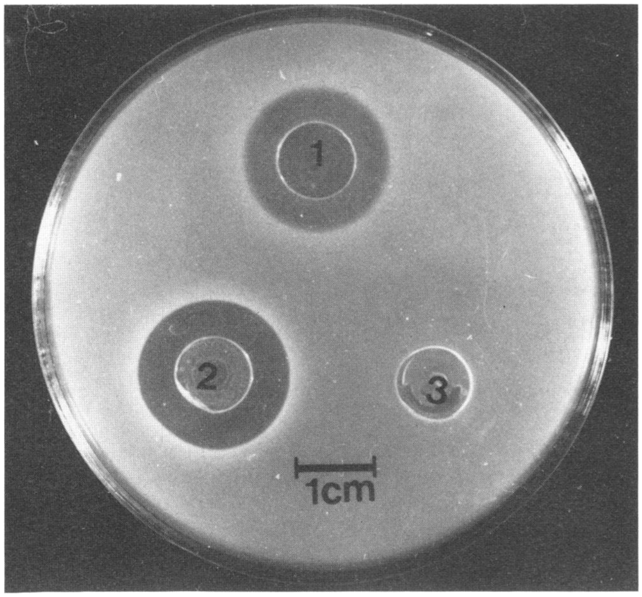

FIG. 1. Agar plate indicating bacteriocin activity. 1, *L. lactis* subsp. *diacitilactis* S50; 2, *L. lactis* subsp. *lactis* NP45; 3, *L. lactis* subsp. *cremoris* NS1.

over, antimicrobial activity of lactostrepcins is completely lost when the pH is raised to 7 (11).

The sensitivity of bacteriocin S50 to various enzymes (proteolytic enzymes, α-amylase, lysozyme, catalase, DNase I, and RNase A) has been tested by enzymatic treatment of the filtered supernatant (0.45-μm Millipore filter) of an overnight culture. Incubation of reaction mixtures containing 1 and 0.5 mg/ml and of controls (buffers without enzymes) was for 1 h at 37°C. Retention of bacteriocin activity in treated samples was assayed as described above. Similar experiments were also carried out with supernatants from an overnight culture of the nisin-producing strain *L. lactis* subsp. *lactis* NP45. Treatment of bacteriocin S50 with various proteolytic enzymes (pepsin, trypsin, α-hymotrypsin, pronase E, and proteinase K) resulted in the loss of its antibacterial activity. However, trypsin, pepsin, and proteinase K had no effect on nisin. These experiments suggested that bacteriocin S50 contains a proteinaceous moiety. Lysozyme, α-amylase, catalase, DNase I, and RNase A had no effect on either bacteriocin or nisin (Table 1).

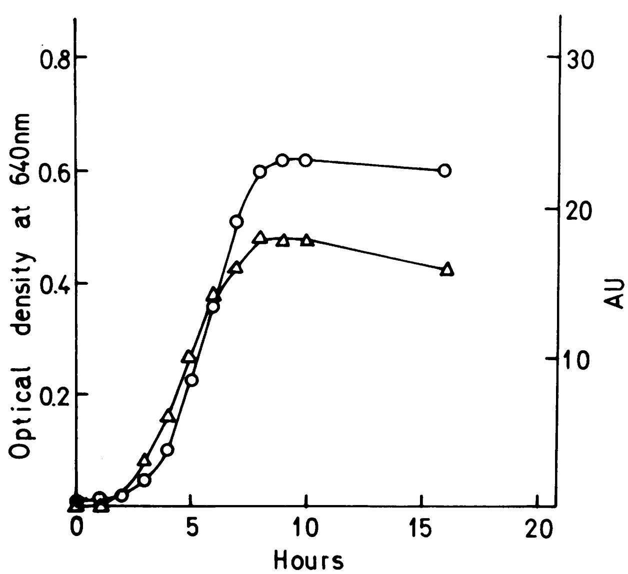

FIG. 2. Growth of *L. lactis* subsp. *diacitilactis* S50 (○) and bacteriocin production (△). AU, Arbitrary units.

TABLE 1. Effects of various enzymes on bacteriocin S50 and nisin activities

Enzyme	Residual activity (arbitrary units)	
	Bacteriocin S50	Nisin
None	16	64
Pepsin	0	64
Trypsin	0	64
α-Chymotrypsin	0	0
Pronase E	0	0
Proteinase K	0	64
α-Amylase	16	64
Lysozyme	16	64
Catalase	16	64
DNase I	16	64
RNase A	16	64

L. lactis subsp. *diacitilactis* S50 contains four plasmids. Plasmid curing resulted in loss of bacteriocin production and resistance simultaneously. Unfortunately, the loss of both characters could not be correlated with the loss of any of the plasmid species detected in this strain. Thus, the real location of these genes awaits further experimentation.

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REFERENCES

1. **Barefoot, S. F., and T. R. Klaenhammer.** 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. **45:**1808–1815.
2. **Davey, G. P.** 1984. Plasmid associated with diplococcin production in *Streptococcus cremoris*. Appl. Environ. Microbiol. **48:** 895–896.
3. **Davey, G. P., and L. E. Pearce.** 1982. Production of diplococcin by *Streptococcus cremoris* and its transfer to non-producing group N streptococci, p. 221–224. *In* D. Schlessinger (ed.), Microbiology—1982. American Society for Microbiology, Washington, D.C.
4. **Geis, A., J. Singh, and M. Teuber.** 1983. Potential of lactic streptococci to produce bacteriocin. Appl. Environ. Microbiol. **45:**205–211.
5. **Gilliland, S. E., and M. L. Speck.** 1977. Antagonistic action of *Lactobacillus acidophilus* towards intestinal and food borne pathogens in associative culture. J. Food Prot. **40:**820–823.
6. **Hurst, A.** 1978. Nisin: its preservative effect and function in growth cycle of the producer organism. Soc. Appl. Bacteriol. Symp. Ser. **7:**297–314.
7. **Hurst, A.** 1981. Nisin. Adv. Appl. Microbiol. **27:**85–123.
8. **Joerger, M. C., and T. R. Klaenhamer.** 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J. Bacteriol. **167:**439–446.
9. **Klaenhamer, T. R.** 1982. Microbiological considerations in

selection and preparation of lactobacillus strains for use as dietary adjuncts. J. Dairy Sci. **65**:1339–1349.

10. **Klaenhammer, T. R.** 1988. Bacteriocins of lactic acid bacteria. Biochimie **70**:337–349.
11. **Kozak, W., J. Bardowski, and W. T. Dobrzansky.** 1978. Lactostrepcins—acid bacteriocin produced by lactic streptococci. J. Dairy Res. **45**:247–257.
12. **Muriama, P. M., and T. R. Klaenhammer.** 1987. Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus* 88. Appl. Environ. Microbiol. **53**:553–560.
13. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807–813.
14. **Zajdel, J. K., P. Ceglowski, and W. T. Dobrzanski.** 1985. Mechanism of action of lactostrepcin 5, a bacteriocin produced by *Streptococcus cremoris* 202. Appl. Environ. Microbiol. **49**:969–974.