Bacteriocidal activity of *Lactobacillus plantarum* C-11

M. A. Daeschel*, M. C. McKenney and L. C. McDonald

Food Fermentation Laboratory, U. S. Department of Agriculture, Agricultural Research Service, and North Carolina Agricultural Research Service, Department of Food Science, North Carolina State University, Raleigh, NC 27695–7624, USA

Plantaricin A, a bacteriocin produced by a strain of Lactobacillus plantarum *isolated from a cucumber fermentation was shown to be bacteriocidal, degradable by proteases, heat-stable (>30 min at 100°C), non-dialyzable (>8000 MW), and active from pH 4·0 to 6·5. Plantaricin A was bacteriocidal toward some species of the four genera of lactic acid bacteria:* Lactobacillus, Pediococcus, Leuconostoc *and* Streptococcus. *Plantaricin A was not effective against other Gram-positive or Gram-negative bacteria or yeasts. Mutants of* L. plantarum *lacking bacteriocin activity isolated from chemostat cultures had identical plasmid profiles to that of the bacteriocin-producing parental strain.*

Introduction

Lactobacillus plantarum is commonly associated with plant material (Buchanan and Gibbons 1974) and has industrial application as a starter culture for the fermentation of vegetables (Fleming et al. 1985), silage (Beck 1978), and sausage products (Bacus and Brown 1985). In vegetable fermentations such as pickles where the fresh produce (cucumbers) are not aseptically handled, it is difficult to obtain a pure culture fermentation using starter cultures. This is primarily because of the competing natural lactic acid bacteria present. We have previously proposed using bacteriocin-producing strains of lactic acid bacteria as cultures to inhibit the competing natural flora (Daeschel and Fleming 1984). Using such strains in conjunction with other control measures during the fermentation process may promote consistent fermentations of high quality

Bacteriocinogenicity is the ability of bacteria to synthesize and release into their environment proteins which are toxic to other bacteria. Bacteriocin production is a feature common to many bacteria both gram positive and gram negative. Detailed reviews of basic research concerning bacteriocins are available (Reeves 1965, Hardy 1975, Konisky 1982). The lactic acid bacteria, widely used in the fermentation and preservation of foods and beverages have been recognized to produce a number of different bacteriocins which have been the subject of recent review by Klaenhammer (1988) Bacteriocins have been characterized from *Lactobacillus* acidophilus (Barefoot and Klaenhammer 1983), *Lactobacillus helveticus* (Upreti and Hinsdill 1973, 1975, Joerger and Klaenhammer 1986), *Lactobacillus fermentum* (de Klerk and Smit 1967). *Lactobacillus sake* (Schillinger and Lucke 1989) and an unspeciated homofermentative *Lactobacillus* (McCormick and Savage 1983). Our initial report (Daeschel et al. 1986) was the first to describe a bacteriocin

*Corresponding author at: Department of Food Science and Technology, Oregon State University, Corvallis, OR 97331, USA

from *Lactobacillus plantarum*. Recently, a bacteriocin (plantaricin B) has been demonstrated to be produced by *L. plantarum* NCDO 1193 (West and Warner 1988). In this paper we identify and characterize a bacteriocin produced by a strain of *L. plantarum*.

Materials and Methods

Bacterial cultures

Lactobacillus plantarum isolates were obtained from natural cucumber fermentations where starter cultures were not used. One strain, designated C-11, exhibited inhibitory activity toward other lactic acid bacteria. The strains of lactic acid bacteria tested for sensitivity to strain C-11 were obtained from the culture collection maintained by this laboratory. Identity of the C-11 strain was determined by sugar fermentation patterns (API Analytab Products, Plainview, NY), morphology, terminal pH and CO_2 production. *Lactobacillus plantarum* NCDO 965 was used as a sensitive indicator strain. *Escherichia coli* V517 was used as a source of reference plasmids (Macrina et al. 1982), and was propagated in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C. Lactic acid bacteria were propagated and maintained in MRS broth (Difco). Overnight cultures (16–20 h) were used as inocula for growth inhibition studies and for cell-free broth preparations.

Demonstration of inhibitory activity

Sensitivity to the inhibitor produced by strain C-11 was determined by using the agar spot test as described previously (Daeschel and Klaenhammer 1985). Sensitivity was also determined by the well diffusion method (Tagg et al. 1976) with cell-free broth preparations. Tests were conducted under aerobic and anaerobic atmospheres (Coy anaerobic glovebox, <3 ppm O_2 Ann Arbor, MI).

Cell-free broth preparations

Inhibitor preparations were made in several ways, depending on the particular experiment in which they were used. In all preparations the growth medium was MRS broth. Cells were removed by centrifugation. Cell-free broths were concentrated by dialysis (Spectraphor tubing, #1, 6000–8000 MW cutoff)

against polyethylene glycol MW 8000 (Sigma Chemical Company, St Louis, MO). Sensitivity of the inhibitor to proteolysis was tested by treating preparations with 1 mg ml^{-1} of *Streptomyces griseus* XIV protease (Sigma) for 1 h at 37°C. Inhibitor activity in cell-free broth preparations was assayed using the method described by Reeves (1965) as modified by Reuckert (1979). Twofold serial dilutions of the supernatant were made with fresh medium. Tubes containing 5 ml were subjected to boiling for 6 min to kill remaining cells and each tube was inoculated (1%) with an overnight culture of the indicator strain (diluted to an O. D. of 0·1 at 650 nm). Tubes were incubated at 30°C for 16 h and the optical density determined at 650 nm. To test the possibility of the inhibitor being hydrogen peroxide, some broths were treated with catalase 340–400 units ml^{-1} (Sigma) for 1 h at 37°C.

Plasmid isolation and purification

Plasmid isolation was achieved using the general method of Klaenhammer (1984) with the following modifications: (1) the pH of the 25% (w/v) sucrose in 50 mM Tris-HCl, 5 mM Na_2 EDTA was raised from pH 7·5 to 8·0, (2) 100 μl of lysozyme (20 mg ml^{-1}) in 50 mM tris-HCl, 5 mM Na_2 EDTA, pH 8·0, was added to the cell suspension and incubated for 30 min at 37°C, (3) 10N NaOH was added until the pH of the lysates approximated 12·2.

Electrophoresis was conducted on 0·5% agarose, horizontal slab gels in tris acetate buffer (Maniatis et al. 1982) at pH 8·0, using a constant voltage of 75 V for 1 h and 45 min. The purified plasmids of *E. coli* V517 described by Macrina et al. (1982) were used as standards for molecular weight approximations. Chemostat cultures were established with strain C-11 using methodology as described previously (Daeschel and Klaenhammer 1985) with the purpose of exerting environmental pressures on the culture which would favor plasmid loss. Chemostat cultures were maintained under a limiting glucose concentration (3mM) and a temperature higher (43°C) than optimum (39–40°C) for the strain. To screen for loss of bacteriocin producing ability from chemostat cultures, cells were spread plated onto MRS agar (approximately 10^2 colonies per plate). When colonies first became visible, the plates were overlaid with 8 ml of MRS soft agar containing a sensitive indicator culture (10^6 CFU ml^{-1}). After incubation for 16–24 h

at 30°C, clear zones around colonies indicated bacteriocin producing activity.

Results and Discussion

The identity of strain C-11 was determined to be *L. plantarum* based upon comparisons with known strains of *L. plantarum* and published descriptions of *L. plantarum* (Buchanan and Gibbons 1974). Of 60 strains of lactic acid bacteria representing 23 species, approximately half displayed sensitivity to the inhibitor produced in *L. plantarum* C-11 (Table 1). There did not appear to be any set pattern as to what species were or were not inhibited. Of 26 strains of *L. plantarum* tested, 15 displayed sensitivity to plantaricin A. There was no difference in the sensitivity pattern among heterofermentative, as compared to homofermentative, strains of bacteria. Sensitivity to the

inhibitor was displayed from species of the four genera of lactic acid bacteria, but was variable within each genus and species. In comparison, inhibitors identified as bacteriocins from *L. acidophilus* (Barefoot and Klaenhammer 1983, Klaenhammer 1988) tend to inhibit only other members of the Lactobacilliaceae, whereas a bacteriocin described from *P. pentosaceus* (Daeschel and Klaenhammer 1985) was inhibitory to all gram-positive bacteria tested. The inhibitor in the present study was not active against bacteria other than lactic acid bacteria or against yeast (data not shown).

The criteria for establishing an inhibitory substance as a bacteriocin have been proposed for gram-positive bacteria (Tagg et al. 1976). Essentially two attributes must be documented: (1) the inhibitor displays a bacteriocidal mode of

Table 1. Bacterial strains and their sensitivity to plantaricin A as determined by the agar spot test

| Species	Number of strains	Number sensitive
Lactobillus plantarum	26	15
Lactobillus brevis	2	1
Lactobillus cellobiosus	3	0
Lactobillus acidophilus	1	1
Lactobillus hilgardii	1	0
Lactobillus fermentum	1	0
Lactobillus casei	1	0
Lactobillus salvarius	1	0
Lactobillus lactis	1	0
Lactobillus helveticus	1	0
Lactobillus buchneri	1	1
Lactobillus viridescens	1	1
Lactobillus coryneformis	1	1
Lactobillus xylosus	1	1
Leuconostoc mesenteroides	3	0
Leuconostoc lactis	1	1
Leuconostoc dextranicum	1	0
Leuconostoc paramesenteroides	1	1
Pediococcus acidilactici	4	3
Pediococcus pentosaceus	5	4
Streptococcus cremoris	1	0
Streptococcus lactis	1	0
Streptococcus faecalis	1	1
	60	32
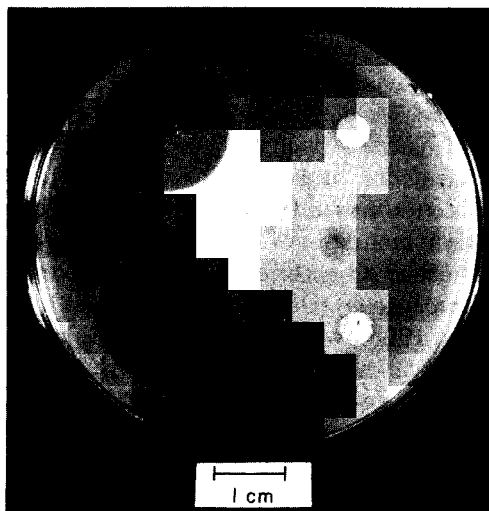
Fig. 1. *L. plantarum* C-11 cell free culture broth concentrated approx. 50× in dialysis membrane tubing with a molecular weight cutoff range of 6000–8000. Concentrate (50 µl) was applied to wells in agar and allowed to diffuse for for 1 h prior to overlaying with *L. plantarum* 965. Left = untreated concentrate; Right = concentrate treated with 1 mg ml^{-1} protease for 1 h at 37°C.

action, and (2) it is proteinaceous in nature.

Figure 1 shows that the inhibitor from strain C-11 was rendered inactive when treated with a nonspecific proteolytic enzyme preparation. This data suggested that the inhibitor was proteinaceous in nature and had a molecular weight greater than 6000 since it was retained in the dialysis tubing.

Lactic acid bacteria, by virtue of the acids they produce, can inhibit many other bacteria. In order to distinguish between the inhibitory effects of acid and other antagonistic compounds, one must eliminate either the acid or the bacteriocin. This can be achieved by dialyzing the culture supernatants to remove the acid or by treating the preparation with proteolytic enzymes to degrade the bacteriocins, Figure 1 demonstrates that

both of these experimental approaches were successful in this study. Figure 2 shows the inactivation of the inhibitor by protease, as evidenced by the growth of *L. plantarum* 965 when added to the cell-free preparations. This graph also portrays a bacteriocidal mode of action of the inhibitor as shown by the death of *L. plantarum* 965 in cell-free preparations not treated with protease. *Lactobacillus plantarum* C-11 was able to grow in cell-free preparations treated with or without protease presumably because it is immune to its own product (plantaricin A). In addition, the growth of *L. plantarum* C-11 in both types of cell-free preparations indicated the preparations contained sufficient nutrients.

The lactic acid bacteria have the ability to produce hydrogen peroxide, which can be inhibitory toward other bacteria. Treatment of *L. plantarum* bacteriocin preparations with catalase did not reduce or enhance activity (data not shown). The presence or absence of oxygen did not appear to have an effect on either the
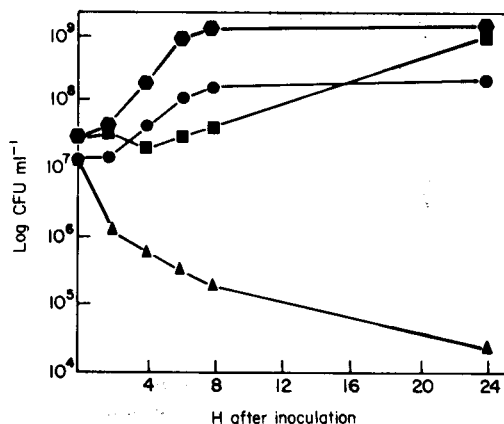

Fig. 2. Growth of *L. plantarum* 965 and *L. plantarum* C-11 after inoculation into cell-free broth preparations in which *L. plantarum* C-11 was cultivated. ▲ = 965 in untreated broth; ● = 965 in protease broth; ■ = C-11 in untreated broth; ◖ = C-11 in protease treated broth.

production of the bacteriocin or the activity of the bacteriocin preparations (data not shown).

Figure 3 provides information as to the heat stability and pH-dependent activity of the inhibitor. There was essentially no effect on activity by heating 100°C for 30 min, or by pH adjustment from 4 to 6·5. The figure indicates the potency of uncon-centrated broth preparations. It was not until the preparations were diluted to less than 1% of the original that the indicator strain was no longer inhibited.

The production of bacteriocins has been reported to occur at various stages in the cell growth cycle. A bacteriocin from *Streptococcus zymogenes* (Davie and Brock 1966) was shown to be maximally produced after 90 min of growth with a rapid loss in activity during late logarith-mic growth (220 min). The loss in activity was attributed to the appearance of an inactivator which was shown to be a teichoic acid. In the present study, plan-taricin A was observed to be maximally accumulated during the mid log phase of
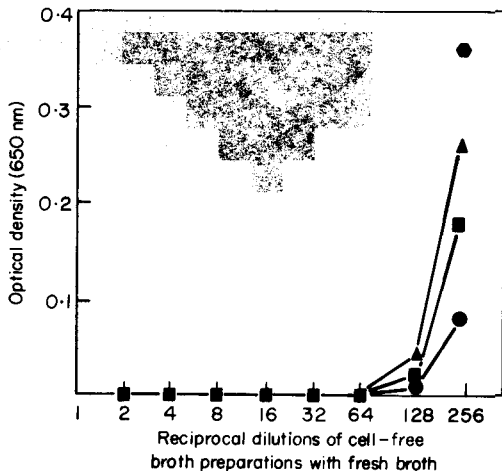
Fig. 3. Inhibition of *L. plantarum* 965 16 h after inoculation into cell-free broth prep-arations in which *L. plantarum* C-11 had been grown. Broth preparations were treated after removal of cells by: ▲ = boiling for 30 min, followed by sterile filtration; ■ = pH adjusted from 4.0 to 6.5, followed by sterile filtration; ● = sterile filtration only; ● = control, fresh broth.

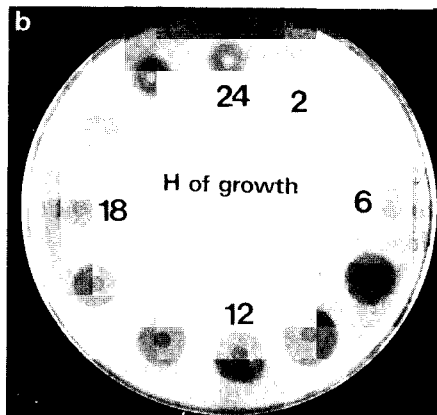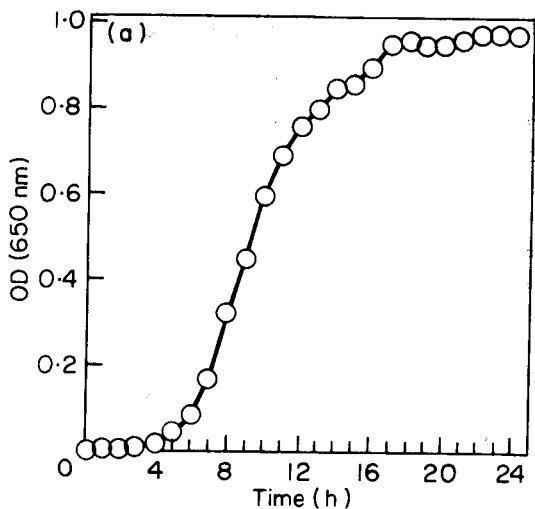growth (Fig. 4), with a decrease in activity thereafter. The loss of activity

Fig. 4. (a) Growth of *L. plantarum* C-11 in MRS broth at 37°C. (b) Detection of bacteriocin activity during growth of *L. plantarum* C-11 in MRS broth using the well diffusion method with cell-free broth preparations taken over time.
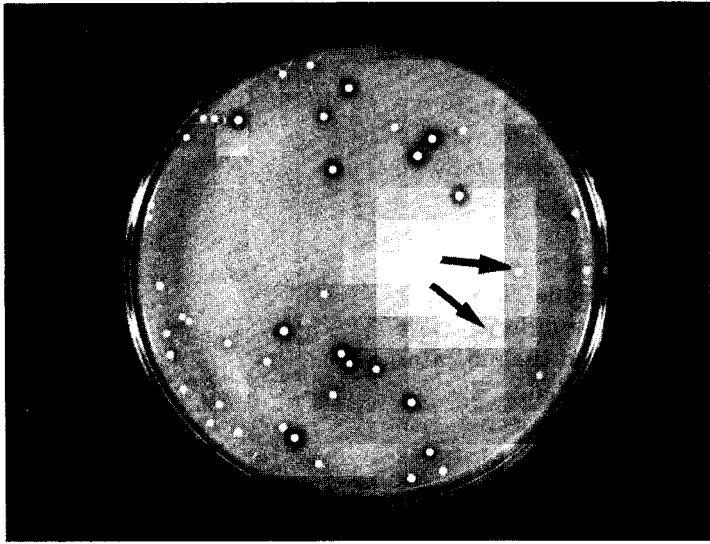
Fig. 5. Presumptive BAC+ and BAC− (arrow) colonies isolated from a chemostat culture.

may be the result of one or more factors. An inactivator may be produced as was demonstrated with *S. zymogenes* (Davie and Brock 1966). Studies with a bacteriocin from *Serratia marcescens* showed that an inactivator which appeared to be an extracellular protease (Foulds and Shemin 1969) was concomitantly produced. Alternatively, the bacteriocin may be unstable under the increasing acidic conditions that develop during the later stages of growth.

Plasmid involvement in the production of an immunity to bacteriocins has been documented in other lactic acid bacteria (Neve et al. 1984) *Lactobacillus plantarum* C-11 contains two cryptic native plasmids of approximately 43 and 6.5 MDa. Our approach was to subject the culture to various curing methodologies and then screen for variants that no longer produced the bacteriocin-like inhibitor (BAC-). We were able to isolate BAC-mutants from a chemostat culture of strain C-11 (Fig. 5). Plasmid analysis of BAC-isolates did not reveal any differences in plasmid content or size as compared to the BAC+ parental strain (Fig.
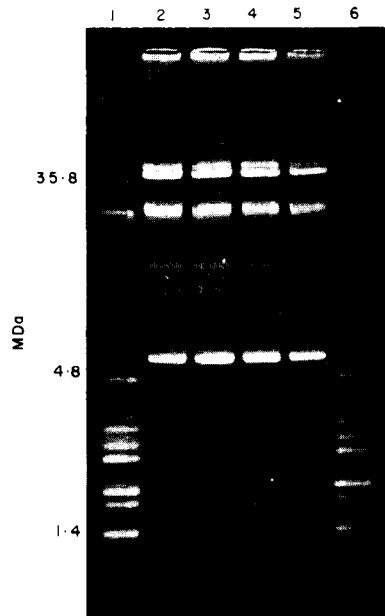
6). Attempts with other plasmid-curing

Fig. 6. Agarose gel electrophoretic patterns of plasmid DNA from *E. coli* V517 (lanes 1 + 6), *L. plantarum* C-11 (lane 2), *L. plantarum* C-11 bac-mutants (lanes 3, 4, 5). Plasmid DNA was not purified with CsCl-ethidium bromide density gradients, hence the presence of chromosal and open circular plasmid DNA.

agents using agents such as ethidium bromide, acridine orange and novobiocin were not successful in producing BAC-mutants.

The BAC-mutants isolated were not sensitive to the bacteriocin produced by the parent strain, suggesting the mutation responsible for the BAC-phenotype affected the gene for BAC production or for the secretion of the bacteriocin but did not affect immunity. Earlier studies in our laboratory with a bacteriocin-producing strain of *Pediococcus* indicated that both bacteriocin production and immunity genes were plasmid mediated, and mutants obtained with curing experiments were lacking both production and immunity properties (Daeschel and Klaenhammer 1985). Recently, evidence for a chromosomally determined bacteriocin produced by a strain of *L. helveticus* was obtained by experiments showing that when the strain was cured of its one detectable plasmid both bacteriocin and immunity were still expressed (Klaenhammer 1988). It appears that genetic determinants for bacteriocin production and immunity can be either plasmid or chromosomally mediated in the Lactobacilli. Current studies in our laboratory are focused on characterizing the biochemical properties of plantaricin A, as well as the practical application of this antagonistic activity in fermentation systems.

Acknowledgments

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