Inhibition of *Clostridium tyrobutyricum* by Bacteriocin-Like Substances Produced by Lactic Acid Bacteria

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ABSTRACT

Lactic acid bacteria were selected for their inhibitory activity against *Clostridium tyrobutyricum* under conditions that eliminate the effects of lactic acid and hydrogen peroxide. Four strains were isolated belonging to the species *Lactococcus lactis* ssp. *lactis*. The sensitivity of the inhibitory substances to pronase and trypsine indicates that they are proteins or peptides different from nisin. Their resistance to phospholipase D indicates that they are also different from lactostrepcin. The inhibitory substances are produced during the exponential phase of growth. Their activity is bactericidal and directed toward some strains of *Clostridium tyrobutyricum*, *Lactobacillus helveticus*, and *Streptococcus thermophilus*, but strains used as dairy starters, *Lactobacillus lactis*, *Streptococcus thermophilus*, and *Propionibacterium shermanii*, are not all affected by the inhibition.

(Key words: *Clostridium tyrobutyricum*, lactic acid bacteria, inhibition, bacteriocin)

Abbreviation key: MMRS = modified MRS medium, RCM = reinforced clostridial agar medium.

INTRODUCTION

Lactic acid bacteria exert an antagonistic action on foodborne pathogens and spoilage microorganisms because they produce lactic acid, hydrogen peroxide, and substances endowed with antibiotic activity.

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Lactococcus lactis ssp. *lactis* strains produce nisin, the only antibiotic-like substance used as a food preservative in many countries (14). Nisin consists of several closely related peptides that contain unusual amino acids (12). Recently, it was shown that the activity of nisin was dependent on the membrane potential (16, 19).

Lactic acid bacteria also produce bacteriocins. The criteria for identification of a substance as a bacteriocin are a proteinaceous nature, a bactericidal action, and an activity restricted to closely related species (20). *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *diacetylactis* produce bacteriocins (9) such as lactostrepcins (17). *Lactococcus lactis* ssp. *cremoris* produces diplococcin (7, 22).

Little is known about the bacteriocins of lactobacilli and pediococci. Nevertheless, it is known that some strains of *Lactobacillus acidophilus*, *Lactobacillus fermenti*, *Lactobacillus helveticus*, and *Lactobacillus plantarum* produce bacteriocins (1, 2, 8, 15, 21). Recently, plasmid-associated bacteriocins were found in the genus *Pediococcus* (6, 10, 11, 18).

Our aim was to use this ability of lactic acid bacteria to produce bacteriocins to prevent failures in cheese making due to the growth of *Clostridium tyrobutyricum* in milk. These sporulating bacteria contaminate milk via silage and provide a late swelling during the ripening of Emmental type cheese. Several preventive methods have been suggested, but none is totally satisfactory. Hirsch et al. (13) and Bergère et al. (3) selected lactic acid bacteria intended for the inhibition of *C. tyrobutyricum* in cheese. The inhibitory substances were, respectively, a heat and acid labile substance and an uncharacterized substance. Their trials to inhibit *C. tyrobutyricum* growth repetitively during the ripening of the cheese were not successful. In this report, we present the results of another selection of lactic acid bacteria inhibiting *C. tyrobutyricum* by proteinaceous inhibitory agents.

MATERIALS AND METHODS

Bacterial Strains and Culture Media

Inhibitory lactic acid bacteria were isolated from fresh milk samples obtained from local dairy farms on modified MRS medium (MMRS); the modification consisted in the omission of diammonium citrate, which was shown in a preliminary experiment to be inhibitory toward *C. tyrobutyricum*. The sources of test strains and of media are presented in Table 1.

Clostridium tyrobutyricum 9L5 and 11L8 were identified by Popoff (Institut Pasteur, Paris, France). The *C. tyrobutyricum* spores were obtained in tryptone glucose extract broth (4) as described by Bourgeois et al. (5). The *C. tyrobutyricum* was also cultivated on reinforced clostridial agar medium (RCM).

Strain Identification

Lactic acid bacteria were identified by Gram stain, catalase test, growth at 15 and 45°C, and carbohydrate fermentation patterns.

Detection and Assay of Inhibitory Substances

Milk samples were screened for the presence of strains of lactic acid bacteria producing substances inhibitory toward *C. tyrobutyricum*. Modified MRS agar plates were inoculated with a decimal dilution of milk samples in a physiological solution composed of .85% NaCl and .1% tryptone (Difco, Detroit, MI). Plates were then overlaid with 4 ml of MRS soft agar (MMRS broth added with .7% agar) and were incubated at 30°C for 30 h. Plates showing 10 to 100 colonies were then overlaid with RCM inoculated with 10^6 spores/ml of *C. tyrobutyricum* and examined for inhibition areas after anaerobic incubation for 48 h at 37°C. Colonies surrounded by a clear halo were picked up for further testing.

To assess the production of inhibitory substances other than lactic acid or hydrogen peroxide, the inhibitory isolates were grown overnight in 10 ml of MRS broth; cells were removed by centrifugation at 8000 × *g* for 10 min at 4°C. The supernatant was dialyzed overnight against .01 *M* phosphate buffer pH 6.9 (dialysis tubing, molecular weight cutoff: 6000 to 8000, Union Carbide, Chicago, IL) and sterilized by filtration through a membrane filter, .2 μm porosity (Sartorius, Goettingen, Germany). Then 100-μl samples were poured into wells in a plate containing about 10^6 spores of *C. tyrobutyricum*/ml. After 48 h at 37°C, the distance from the margin of the well to the limit of growth of the indicator strain was measured with a caliper.

Enzyme Treatment

Aliquots of dialyzed extracts were incubated with .2 mg/ml pronase, trypsin, phospholipase D (Boehringer Manheim, Manheim, Germany), or catalase (Sigma, St. Louis, MO) at 37°C for 2 h, and the residual activity of the inhibitor was then assayed. Pronase and trypsin were used in Tris-HCl .05 *M*, pH 8.0, $CaCl_2$ .02 *M*, and phospholipase D in .01 *M* phosphate pH 6.9.

Heat Treatment

The inhibitory culture extracts (.5 ml) were heated in an Eppendorf tube (Eppendorf Geratebau, Hamburg, Germany) at 100°C for 20 min in a waterbath or at 120°C for 15 min in an autoclave and tested for residual activity.

pH Influence on Stability and on Activity

The inhibitory culture extracts were extensively dialyzed against the following buffers: .01 *M* acetate pH 4.0 and pH 5.0, .01 *M* phosphate pH 7.0, .01 *M* Tris-HCl pH 9.0, and .01 *M* glycine pH 11.0; the extracts were kept at that pH for 24 h, then again dialyzed against the phosphate buffer .01 *M* pH 6.9, and tested for residual activity.

Action Spectra

Spoilage microorganisms and starter microorganisms used in the dairy industry were tested for their sensitivity to inhibition by the agar well method. Tested strains were cultivated in the agar media indicated in Table 1. These media were inoculated with 1% of a 16-h culture. Inhibition areas whose width was greater than 1 mm were retained as positive.

Action of the Inhibitor

Nine milliliters of activated spores (75°C, 15 min) of *C. tyrobutyricum* CNRZ 608 were incubated in RCM broth in the presence of 1 ml of inhibitor in a tube sealed with paraffin. In the controls, 1 ml of trypsin-treated inhibitor was added. Growth and gas production were determined after anaerobic incubation for 4, 6, 8, 12, and 24 h. Gas formation was shown by the parting of the paraffin from the medium. A heat-shocked aliquot (75°C 15 min) was used for the spore count. An unheated one was used for the total count of vegetative cells and spores that germinated without thermal activation.

RESULTS AND DISCUSSION

Screening

From 40 raw milk samples, 33 strains, each issued from a different milk sample, showing an inhibitory activity toward *C. tyrobutyricum* CNRZ 608 were isolated by the overlay method, and their inhibitory capacity was then confirmed by the same method.

These strains were propagated in MRS broth overnight. The broths were tested by the well method after dialysis and sterilization as described.

Twenty-nine broths showed no inhibition, indicating that the inhibition observed on agar plate is due to lactic acid, hydrogen peroxide, or other dialyzable inhibitor or even that the inhibitor is produced in solid media but not in liquid media, as already shown for the inhibitor of some strains of *Lactobacillus acidophilus* (2). These strains were not studied further.

Four strains produced inhibitors in both solid and liquid media. After dialysis for neutralization and after treatment with catalase, the culture broth of these inhibitory strains remained active. This indicated that the inhibition

TABLE 1. Bacterial strains and media used in this study.

Species	Source[1]	Culture media
Bacillus spp. *B. cereus*	Our collection	Eugon (Biokar, Beauvais, France)
Clostridium spp.		
C. tyrobutyricum CNRZ608	INRA Jouy en Josas	RCM[2] (Merck, Darmstadt, Germany)
C. tyrobutyricum 9L5	Our collection	RCM (Merck, Darmstadt, Germany)
C. tyrobutyricum 11L8	Our collection	RCM (Merck, Darmstadt, Germany)
C. perfringens	Our collection	RCM (Merck, Darmstadt, Germany)
C. sporogenes	Our collection	RCM (Merck, Darmstadt, Germany)
Escherichia coli	Our collection	
E. coli	Our collection	L.B.
Lactobacillus spp.		
L. casei CNRZ 602	INRA Jouy en Josas	MRS Oxoid (Basingstoke, England)
L. helveticus A	Commercial starters	MRS
L. lactis N	Eurozyme	MRS
Propionibacterium spp.		
P. shermanii CNRZ 433	INRA Jouy en Josas	Van Niel
Pseudomonas spp.		
P. putida	SEAP Ploufragan	Mead base (Oxoid)
Salmonella spp.		
S. braenderup	Our collection	Eugon (Biokar, Beauvais, France)
S. typhimurium	Our collection	Eugon (Biokar)
Staphylococcus spp.		
S. aureus	Our collection	Eugon (Biokar)
Streptococcus spp.		
S. thermophilus TA061	Commercial starters	Elliker (Difco, Detroit, MI)
S. thermophilus B	Eurozyme	Elliker (Difco, Detroit, MI)
S. faecium	Our collection	Eugon (Biokar)

[1]INRA = Institut National de la Recherche Agronomique; SEAP = Station Expérimentale d'Aviculture de Ploufragan.

[2]RCM = Reinforced clostridial agar medium.

TABLE 2. Identity of inhibitory strains isolated and their effects on three *Clostridium tyrobutyricum* strains; (+) inhibition, (–) no inhibition.

Inhibitory strains	*C. tyrobutyricum* indicator strains		
	CNRZ608	9L5	11L8
Lactococcus lactis ssp. *lactis*			
ADRIA 85L044	+	+	–
ADRIA 85L045	+	+	+
ADRIA 85L046	+	+	–
ADRIA 85L030	+	+	+

was not due to lactic acid or to hydrogen peroxide.

These four isolates were identified and considered for the inhibition of two other *C. tyrobutyricum* strains, 9L5, which was a typical strain, and 11L8, which was atypical by its fermentation pattern: maltose inhibition, sucrose inhibition, and esculine hydrolysis inhibition. Results are presented in Table 2. The 9L5 strain was inhibited by all four *Lactococcus lactis* ssp. *lactis* strains; strain 11L8 was inhibited only by two *Lactococcus lactis* ssp. *lactis* strains. These results indicated that the inhibitors can be divided into two groups on the basis of their inhibitory spectra.

Properties of Inhibitors

With all four strains, which were active after culture extracts were dialyzed, the activity assayed by the agar well diffusion method was lost after treatment with pronase or trypsin. This suggests a proteinaceous nature, which is a characteristic of bacteriocins; this also indicates that the inhibitors are different from nisin, which is not inactivated by these enzymes (14).

For all four strains, the activity was intact after treatment with phospholipase D, which indicates that the inhibitors are also different from lactostrepcin (17).

Finally, the fact that extracellular extracts are active shows that this phenomenon is different from the inhibition of *C. tyrobutyricum* by lactic acid bacterial strains studied by Bergère et al. (3), which needed a contact between the cells of the two strains.

The inhibitors showed no detectable loss in activity when heated at 100°C for 20 min at pH 7.0 and lost 25 to 50% of their activity after 15 min at 121°C. For *Lactococcus lactis* ssp. *lactis* 30, inhibitor stability was tested at different pH. No activity loss was detected after exposure for 1 d at any pH between 4.0 and 11.0 at 4°C.

Growth and Bacteriocin Production: pH and Temperature Influence

For *Lactococcus lactis* ssp. *lactis* 30, maximum yields of inhibitor were obtained in exponential phase cultures. Production rate was not significantly dependent on the incubation temperature during the growth phase (Figure 1) nor on the medium pH between 5.0 and 7.0.

Bactericidal Action of the Bacteriocin

Figure 2 shows the results obtained by counting spores only (heat-shocked sample) and spores plus vegetative cells (nonshocked sample) during their incubation in presence of the dialyzed extract of *Lactococcus lactis* ssp. *lactis* 30 (assay) or in presence of the same but trypsin-treated and then inactive extract (control).

In the control, the total number of spores plus vegetative cells increased by 2 logs, which reflected the germination of activated spores and the growth of emerging cells; at the same time, the number of spores decreased by 1 log, which was the consequence of their germination.

In the assay, i.e, in the presence of the inhibitor, the numbers of spores plus vegetative cells were not very different and not very variable during incubation; the variations were probably not significant. After 48 h gas production had taken place in the control but not in the assay. From these results, it is possible to conclude that the inhibition acts during or after germination and not on resting spores.

TABLE 3. Sensitivity of various strains to bacteriocins of lactic acid bacteria, (+) inhibition, (–) no inhibition.

Species	Bacteriocin-producing strains isolated in this study			
	30	44	45	46
Lactobacillus casei CNRZ 602	–	–	–	–
Lactobacillus delbrueckii ssp. *lactis* N	–	–	–	–
Lactobacillus helveticus A	+	–	+	–
Streptococcus thermophilus TA061	+	–	+	–
Streptococcus thermophilus B	+	+	+	+
Propionibacterium shermanii CNRZ 433	–	–	–	–

Action Spectra

We extended the study of the inhibitory action of our selected lactic acid bacteria to other spoilage microorganisms and to dairy starters.

Among other spoilage microorganisms that were tested (*Bacillus cereus, Clostridium perfringens, Escherichia coli, Pseudomonas putida, Salmonella braenderup, Salmonella typhimurium, Staphylococcus aureus*), only the three *Clostridium tyrobutyricum* strains were inhibited.

Table 3 shows that lactic acid bacterial strains tested were generally resistant to all of the inhibitory extracts, except for strain B of *Streptococcus thermophilus*, which was sensitive to all extracts. *Lactobacillus helveticus* A and *Streptococcus thermophilus* TAO 61 were resistant to two of the four extracts, namely, those from strains 44 and 46. *Lactobacillus delbrueckii* ssp. *lactis* N was resistant to all of the extracts.

Lactococcus lactis ssp. *lactis* 44 and 46 could then be used with these resistant strains in Emmental production. These results open the way to the practical application of the inhibition phenomenon in dairies.

CONCLUSION

The aim of this work was to isolate lactic acid bacteria able to inhibit *C. tyrobutyricum* by means other than lactic acid and hydrogen peroxide and for use as an agent to prevent late swelling of cheese due to *C. tyrobutyricum*. Four strains of *Lactococcus lactis* ssp. *lactis* were isolated that had the desired property.

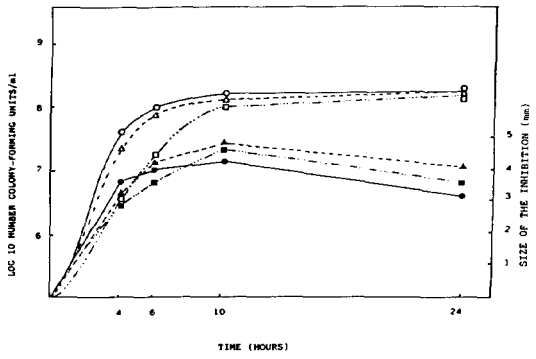

Figure 1. Production of inhibitory substances during the growth of *Lactococcus lactis* ssp. *lactis* 30 and influence of temperature. Growth at 30°C (○); 37°C (△); 42°C (□) and inhibitory substance production at 30°C (●); 37°C (▲); 42°C (■).

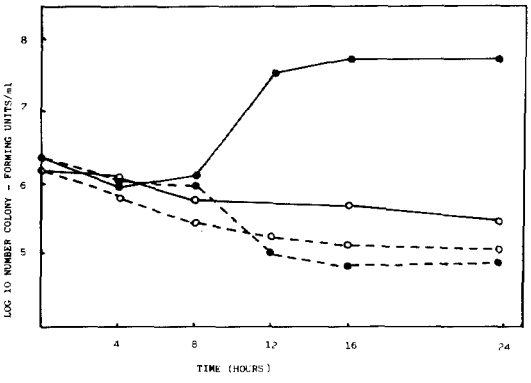

Figure 2. Bacterial activity of the bacteriocin of *Lactococcus lactis* ssp. *lactis* 30 toward *Clostridium tyrobutyricum* CNRZ 608: log viable spores number in the control (●- - -●) and in the assay (○- - -○); log viable spores plus vegetative cells number in the control (●—●) and in the assay (○—○).

They act by bactericidal molecules somewhat similar to bacteriocins but different from nisin and lactostrepcin. Their action is directed against *Clostridia* and also against some but not all starters of lactic acid bacteria. These strains can thus be used in cheese making. Biochemical and genetic studies of this inhibitor are in progress in our laboratory concurrently with cheese making trials.

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REFERENCES

1 Anderson, R. 1986. Inhibition of *Staphylococcus aureus* and spheroplasts of Gram-negative bacteria by an antagonistic compound produced by a strain of *Lactobacillus plantarum*. Int. J. Food Microbiol. 3:149.

2 Barefoot, S. R., and T. R. Klaenhammer. 1983. Detection and activity of Lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 45:1808.

3 Bergère, J. L., T. Sys, and L. Vassal. 1978. Bactéries lactiques susceptibles d'inhiber la croissance de *Clostridium tyrobutyricum* en culture et dans le fromage. Lait 575:215.

4 Bergère, J. L., and J. Hermier. 1965. Étude des facteurs contrôlant la croissance et la sporulation de *Clostridium tyrobutyricum*. Ann. Institut Pasteur 109:80.

5 Bourgeois, C. M., O. Le Parc, B. Abgrall, and J. J. Cleret. 1984. Membrane filtration of milk for counting spores of *Clostridium tyrobutyricum*. J. Dairy Sci. 67: 2493.

6 Daeschel, M. A., and T. R. Klaenhammer. 1985. Association of 13.6 megadalton plasmid in *Pediococcus pentosaceus* with bacteriocin activity. Appl. Environ. Microbiol. 50:1538.

7 Davey, G. P., and B. C. Richardson. 1981. Purification and some properties of Diplococcin from *Streptococcus cremoris* 346. Appl. Environ. Microbiol. 41:84.

8 Deklerk, H. C., and J. A. Smit. 1967. Properties of a *Lactobacillus fermenti* bacteriocin. J. Gen. Microbiol. 48:309.

9 Geis, A., J. Singh, and M. Teuber. 1983. Potential of lactic streptococci to produce bacteriocin. Appl. Environ. Microbiol. 45:205.

10 Gonzales, C. F., and B. S. Kunka. 1987. Plasmid-associated bacteriocin production and sucrose fermentation in *Pediococcus acidilactici*. Appl. Environ. Microbiol. 53:2534.

11 Graham, D. C., and L. L. McKay. 1985. Plasmid DNA in strains of *Pediococcus cerevisiae* and *Pediococcus pentosaceus*. Appl. Environ. Microbiol. 50:532.

12 Gross, E., and J. L. Morrel. 1970. The structure of nisin. J. Am. Chem. Soc. 93:4634.

13 Hirsch, A., M. McClintoch, and G. Mocquot. 1952. Observation on the influence of inhibitory substances produced by the lactobacilli of Gruyère cheese on the development of anaerobic sporeformers. J. Dairy Res. 19:179.

14 Hurst, A. 1983. Nisin and other inhibitory substances from lactic acid bacteria. Page 327 *in* Antimicrobials in food. A. L. Branen and P. M. Davidson, ed. Marcel Dekker, Inc., New York, NY.

15 Joerger, M. C., and T. R. Klaenhammer. 1986. Characterization and purification of Helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J. Bacteriol. 167:439.

16 Kordel, M., and H. G. Sahl. 1986. Susceptibility of bacterial eukaryotic and artificial membranes to the disruptive action of the cationic peptides Pep 5 and nisin. Fed. Eur. Microbiol. Soc. Microbiol. Lett. 34: 136.

17 Kozak, W., J. Bardowski, and W. T. Dobrzanski. 1978. Lactostrepcins-acid bacteriocins produced by lactic streptococci. J. Dairy Res. 45:247.

18 Pucci, M. S., E. R. Vedamuthu, B. S. Kunka, and P. A. Vandenbergh. 1988. Inhibition of *Listeria monocytogenes* by using Bacteriocin PA 1. produced by *Pediococcus acidilactici* PAC 1.0. Appl. Environ. Microbiol. 54:2319.

19 Ruth, E., and H. G. Sahl. 1985. Mode of action of peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrob. Agents Chemother. 27:841.

20 Tagg, J. R., A. S. Dajani, and L. W. Wannamaker. 1976. Bacteriocins of gram positive bacteria. Bacteriol. Rev. 40:722.

21 Upreti, G. C., and R. D. Hinsdill. 1973. Isolation and characterization of bacteriocin from a homofermentative *Lactobacillus*. Antimicrob. Agents Chemother. 4: 487.

22 Whitehead, H. R. 1933. A substance inhibiting bacterial growth produced by certain strains of lactic streptococci. Biochem. J. 27:1793.