Biotechnology Letters Vol 12 No 8 575-580 (1990)
Received as revised 26th June

Screening of *Bifidobacterium* strains for Bacteriocin production

J. MEGHROUS, P. EULOGE, A.M. JUNELLES, J. BALLONGUE and H. PETITDEMANGE*

Laboratoire de chimie biologique 1, Université de Nancy 1, Boite Postale 239, 54506- VANDŒUVRE LES NANCY-Cedex, FRANCE

SUMMARY

Thirteen strains of bifidobacteria (*Bifidobacterium*) were examined for bacteriocin production. One strain among these produced bacteriocin which is active against lactic streptococci strains and against other species of *Bifidobacterium* , *Clostridium* and *Lactobacillus*, but not against Gram-negative bacteria such as *Pseudomonas*, *Klebsiella* , *Serratia* and *Escherichia coli.*

Bacteriocin activity was inhibited by proteolytic enzymes, but not by heating 15 min or30 min at 100°C, and it was resistant for 15 min at 121°C and active at pH between 2 to 10.

INTRODUCTION

Bacteriocins are proteins or protein complexes produced by certain strains of bacteria and are usually active against some species closely related to the producer bacterium (Tagg et al., 1976; Govan, 1986). Their antimicrobial activities have long been known and recognized as important in food fermentations, food preservation and intestinal ecology. They have been classified by their narrow activity spectrum, diffusibility, and sensitivity to proteolytic enzymes (Ivanovics, 1962; Bradley, 1967) and their protein nature distinguishes them from most of the other known antibiotics.

Bacteriocins produced by lactic acid bacteria have been extensively studied in group N-streptococci and lactobacilli (Geis et al., 1983; Joerger and Klaenhammer, 1986).

Antibiotic-producing streptococci were first described by Whitehead and Riddet (1933). They identified one producer as *Lactococcus lactis subsp. cremoris* and showed that the active inhibitory substance was protein in nature. Mattick and Hirsh (1947) isolated inhibitor-producing strains of *Lactococcus lactis subsp. lactis* from milk and starters. The inhibitor substance of the strains was termed nisin . Bacteriocin production by *Bifidobacterium* is the subject of this present study.

MATERIALS AND METHODS

1-Strains and media

The identity and origin of the strains used in this study are given in table 1.

Table 1. List of strains used

Strains	Number	Code	Source
B. commercial strain	3	1, 2, 3	our collection
B. animalis	1	25527	ATCC
B. bifidum	1	29521	ATCC
B. breve	2	15700, 15698	ATCC
B. infantis	3	15697, 15702, 25962	ATCC
B. longum a		15707	ATCC
B. longum b		15708	ATCC
B. thermophilum		25525	ATCC
I. lactis subsp. lactis	1	11454	ATCC
	5	1, 2, 3, 5, 26	our collection
I. lactis subsp. cremoris	4	7, 10, 11, 12	our collection
I. lactis subsp. diacetylactis	1	124	CNRZ
Mesophilic lactic streptococci*	4	EII3, EII4, EII6, EII10	our collection
S. salivarius subsp. thermophilus	11	S.T.1, S 8, S 13, S 21, S 23, S 25, S 27, S 30, S 32, S 39, S40	our collection
L. acidophilus	3	L 1, L 2, L 3	our collection
C. butyricum		3044	Institut Pasteur
C. tyrobutyricum		510	CNRZ
C. perfringens		C.p	our collection
Escherichia coli		E.c.	our collection
Klebsiella oxytoca		501	our collection
Pseudomonas fluorescens		601	our collection
Serratia marcescens		532	our collection

* Strains not identified

Abreviations

I. : Lactococcus
S.: Streptococcus
L.: Lactobacillus
B.: Bifidobacterium
C.: Clostridium

a/ Strains

Bifidobacteria and *Lactococcus lactis* ATCC 11454 strains were obtained from the American Type Culture Collection (ATCC). Other

Bifidobacterium strains were isolated from the dairy product market and identified by the method of Scardovi (Scardovi, 1986b). Lactic cultures used were obtained from our laboratory collection. *Clostridium tyrobutyricum* and *Lactococcus lactis subsp. diacetylactis* CNRZ 124 were furnished by CNRZ (Centre National de la Recherche Zootechnique). *Clostridium butyricum* strain was furnished by the Institut Pasteur.

b/ Culture media

All lactic cultures were maintained as frozen stocks at - 20°C in litmus milk. Before experimental tests, cultures were propagated overnight in broth media.

Lactic streptococci were normally grown in lactic broth (Elliker et al., 1956). Lactic lactobacilli were grown in MRS broth (De Man et al., 1960), Reinforced Clostidia Medium (RCM, oxoid, London), Nutrient Agar and TPY (Mitsuoka, 1984) were used to hold clostridia, *Escherichia Coli* and bifidobacteria strains respectively. Solid medium was obtained for addition of agar (15 g/l). Solid medium used for bifidobacteria strains was BL Agar (Scardovi, 1986a). R_2A (Difco) was the solid medium used to propagate enterobacteria strains.

2- Detection and assay of inhibitory production

Cultures were screened for inhibitor production by the well method.

An indicator lawn on Elliker agar surface was prepared by inoculating 10 µl of an overnight culture of indicator strain (about 1. 10^8 cells/ ml), after it was mixed by gently inverting the petri dish containing the seeded agar. After 30 mn at room temperature, wells were then cut, and samples of test organism were placed into the wells (40 µl/well). Plates were placed at 4°C for 6 h to allow diffusion of the inhibitory substance. Incubation proceeded overnight at the appropriate temperature for growth of the indicator strain. Clear zones surrounding the wells were measured as an indicator of bacteriocin activity.

3- Effects of heat treatment and proteolytic enzymes

A sample of inhibitor substance was assessed for thermostability and enzymatic effects on activity.

2 ml of filtrate preparation was boiled for 15 mn and 30 mn, cooled and assayed for activity. Another sample was autoclaved at 121°C for 15 mn, cooled and assayed for activity.

To determine the sensitivity of bacteriocins to proteolytic enzymes, the filtrate preparation of bacteriocin was mixed with equal volume of the enzymes solutions and incubated at 37°C for 1 h; for control, bacteriocin without added enzyme was treated in the same manner as the test preparation. The enzymes used (trypsin, pepsin and pronase) were dissolved in 4 mM phosphate buffer, pH 7, at a concentration of 1, 2 and 5 mg/ml.

4- Effect of catalase

Crude beef liver catalase (Boeringer) was dissolved in 0,05 M phosphate buffer, pH 7, to give final solutions containing 1, 2 and 5 mg of enzyme per ml and sterilized by filtration. This preparation was diluted in phosphate buffer as required.

5- Effect of pH on antimicrobial activity.

The filtrates were adjusted to pH 2, 4, using phosphoric acid and to pH 7, 10, using sterile 3N NaOH.

RESULTS AND DISCUSSION

The inhibitory effects of *Bifidobacterium* cultures against different test organisms are shown in table 2. Thirteen *Bifidobacterium* strains were found to produce inhibitory substance after well test on Elliker agar plates. One strain of *Bifidobacterium* (1) showed larger activity spectrum than other strains of *Bifidobacterium* .

The inhibitor substance of the producer organism was active against six *Lactococcus lactis subsp. lactis* strains ; four *Lactococcus lactis subsp. cremoris* strains; one *Lactococcus lactis subsp. diacetylactis* strain; ten *Streptococcus salivarius subsp. thermophilus* strains, wich were isolated from the same habitat that *Bifidobacterium* strain; three *Lactobacillus acidophilus* strains; one *Clostidium tyrobutyricum* strain; one *Clostridium butyricum* strain and four mesophilic lactic streptococci strains.

No inhibition was observed with a variety of other Gram-positive or Gram-negative organisms. These included some strains of *Streptococcus salivarius subsp. thermophilus* (S.T.1), isolated from others habitat that *Bifidobacterium* strains, *Escherichia coli* and enterobacter such as *Pseudomonas, Klebsiella* and *Serratia* .

Effect of different factors on inhibitory activity

This activity could have been due to either hydrogen peroxide, acids, or bacteriocin

1- pH values

Filtered supernatants of *Bifidobacterium* strains were adjusted to different pH values(2, 4, 7 and 10). Inhibitory activities were not altered. These results show that the inhibition was not due to lactic acid.

2- Effect of catalase

hydrogen peroxide was not the inhibitory factor, because the inhibitory effect was not destroyed by the different concentrations of the enzyme used.

3- Thermostability

The inhibitory activity persisted after different heating treatments. The activity of inhibitory substance was not destroyed by heating at 100°C after 15 and 30 min. or by autoclaving at 121°C for 15 min.

Table 2 : Screening of *Bifidobacterium* strains for antimicrobial activity

Test organisms		Indicator strains													
		L.l.l. (6)	L.l.c. (4)	L.l.d.	M. S. (4)	S. T. (10)	L. a. (3)	C. b.	C. tb.	C. p.	K. o.	P. f.	S. m.	E. c.	S.T.1
B.	1	+	+	+	+	+	+	+	+	+	−	−	−	−	−
B.	2	−	−	−	−	+	+	−	−	+	−	−	−	−	−
B.	3	−	−	−	−	+	+	−	−	−	−	−	−	−	−
B. bifidum	29521	−	−	−	−	+	+	−	−	−	−	−	−	−	−
B. breve	15700	−	−	−	−	+	+	−	−	+	−	−	−	−	−
B. breve	15698	−	−	−	−	+	+	−	−	+	−	−	−	−	−
B. infantis	15697	−	−	−	−	+	+	−	−	−	−	−	−	−	−
B. infantis	15702	−	−	−	−	+	+	−	−	−	−	−	−	−	−
B. infantis	25962	−	−	−	−	+	+	−	+	−	−	−	−	−	−
B. thermophilus	25525	−	−	−	−	+	+	−	−	−	−	−	−	−	−
B. longum	15707	−	−	−	−	+	+	−	−	−	−	−	−	−	−
B. longum	15708	−	−	−	−	+	+	−	−	−	−	−	−	−	−
B. animalis	25527	−	−	−	−	+	+	−	−	−	−	−	−	−	−

The number of the strains tested is indicated in parentheses

B. : *Bifidobacterium*
L.l.l. : *Lactococcus lactis subsp. lactis*
L.l.c. : *Lactococcus lactis subsp. cremoris*
L.l.d. : *Lactococcus lactis subsp. diacetylactis*
M. S. : mesophilic streptococci
S. T. : *Streptococcus salivarius subsp. thermophilus*
L. a. : *Lactobacillus acidophilus*
C. b. : *Clostridium butyricum*
C. tb. : *Clostridium tyrobutyricum*
C. p. : *Clostridium perfringens*
K. o. : *Klebsiella oxytoca*
P. f. : *Pseudomonas fluorescens*
S. m. : *Serratia marscecens*
E. c : *Escherichia coli*
S.T.1 : *Streptococcus salivarius subsp. thermophilus* 1

Samples of filtered supernatant stored at -20°C showed loss of activity for up to 2 week at pH 4, 7 and 10 ; filtrate supernatant was completely inactive after 1 week at 4°C.

4- Enzyme sensitivity

The sensitivity of bacteriocin to a variety of proteolytic enzymes was determined. Treatment with proteases destroyed activity indicating that the antimicrobial compounds could be heatstable proteins. The properties of these inhibitory activities allow us to classify them among the bacteriocins (Davey and Richardson, 1981; Geis et al., 1983; Hanna et al., 1978).

CONCLUSION

The results show for the first time that the bifidobacteria are able to produce antimicrobial substances which have the properties of bacteriocins, that is :
proteic nature of the molecule which is heatstable, active at pH between 2 to 10 and against other Gram-positive species such as other species of bifidobacteria, lactobacilli, clostridia strains and some streptococci strains.

Acknowledgements : The authors wish to thank Mr. J. PERRIN Saint-Hubert-Industrie Laitière for his support

REFERENCES

Bradley, D.E., 1967. Bacteriol. reviews. **31**: 230-314
Davey, G.P. and Richardson, R.C. 1981. Appl. Environ. Microbiol. **41** : 84-89
De Man, J.C., M. Rogosa and M.E. Sharp. 1960. J. Appl. Bacteriol. **23** : 130-135
Elliker, P.R., A. Anderson, and G.H. Hannesen 1956. J. Dairy Sci. **39** : 1611-1612
Geis, A., J. Singh, and M. Teuber. 1983. Appl. Environ. Microbiol. **45** : 205-221
Hanna, K., A.Y. Sirpa , A.L. Helvi and Matti, A. 1978. J. Dairy Sci. **36** : 20-34
Govan, J.R.W. 1986. J. Infect. Dis. Suppl. **49** : 31-37
Ivanovics,G. 1962. Bacteriol. reviews. **26** : 108-118
Joerger, M.C., and T.R. Klaenhammer. 1986. Appl. Environ. Microbiol. **167** : 439-446
Mattick, A.T.R., and A. Hirsh. 1947. Lancet. **ii** : 5-12
Mitsuoka T., 1984. Bifidobacterium Microflora. **3** : 11-28
Scardovi V. 1986a. Bergey's manual. **2** : 1418
Scardovi V. 1986b. Bergey's manual. **2** : 1423
Tagg, J.R., A.S. Dajani and L.W. Wannamaker. 1976. Bacteriol. Reviews
Whitehead, H.R. and W. Riddet. 1933. J. Agr. **46** : 225-229