Partial Characterization of a Bacteriocin (Thermocin) from *Bacillus stearothersophilus* RS93

By R. J. SHARP, A. H. A. BINGHAM, M. J. COMER
AND A. ATKINSON

Microbiological Research Establishment, Porton, Salisbury SP4 0JG

(Received 4 September 1978; revised 23 December 1978)

INTRODUCTION

The inhibition of bacteria by closely related species has long been observed (Gratia, 1925), and bacteriocins have been reported from a wide range of genera (see, for example, Hardy, 1975; Holland, 1975). Evidence for bacteriocins produced by the thermophile *Bacillus stearothersophilus* has previously been presented (Shafia, 1966; Yule & Barridge, 1976).

This paper describes the production and some properties of a bacteriocin produced by *B. stearothersophilus* RS93. The bacteriocin, termed thermocin 93, inhibited the growth of all thermophilic bacilli examined but had no effect on mesophilic bacilli.

METHODS

Organisms. *Bacillus stearothersophilus* strain RS93 was isolated from the plant hot water supply ($65 \pm 5^\circ\text{C}$) at the Microbiological Research Establishment, Porton. The strain was catalase- and oxidase-positive; nitrate and nitrite were not reduced; and starch, casein and gelatin were not digested. The strain was tolerant to NaCl (5%, w/v) and fermented glycerol, mannitol, glucose, mannose, fructose, sorbitol and trehalose with the production of acid. The optimum growth temperature was between 60 and 65°C ; growth did not occur at 37 or 70°C .

Bacillus caldolyticus Heinen & Heinen 1972, obtained from Dr W. Heinen, University of Nijmegen, The Netherlands, was used to assay the bacteriocin. Other strains of thermophilic bacilli tested were obtained from the following sources: *B. caldovelox* and *B. caldotenax* from Dr Heinen, The Netherlands; *B. stearothersophilus* 4st and *B. stearothersophilus* 10 from Dr N. Welker, North Western University, Ill., U.S.A.; *B. stearothersophilus* RS44 isolated from sandy soil from Jean-De Monts, France; *B. stearothersophilus* 240 isolated from chalky soil in the Salisbury area; *B. stearothersophilus* 262 isolated from an Icelandic hot-water spring.

Growth media. Liquid medium for the growth of the thermophiles was that described by Sargeant *et al.* (1971). Mesophiles were grown in peptone broth. Plating medium was Tryptone Soya Bean Agar (TSBA) (Oxoid CM131).

Thermocin production. Thermocin 93 was produced by inoculating 400 ml medium in a 2 l Erlenmeyer flask from a fresh 18 h plate culture of *B. stearothersophilus* RS93. The flask was shaken at 200 rev. min^{-1} in a Gallenkamp Orbital Incubator at 60°C for 18 h. Bacteria were removed by centrifuging (13000 g , 15 min, 4°C) and the supernatant was assayed for thermocin activity using *B. caldolyticus* as indicator.

Thermocin assay. TSBA plates (4 mm deep) were seeded with 1 ml of a 6 h broth culture of *B. caldolyticus* (prepared by inoculating an overnight plate culture into 10 ml broth) and then dried at 37°C for 1 h. Wells (8 mm diam.) were cut into the plates and 0.1 ml of *B. stearothersophilus* RS93 culture supernatant was added to each. The plates were incubated at 60°C for 18 h and then the zone of inhibition around each well was measured.

Activity range. The activity range of thermocin 93 was determined using 6 h cultures of each test organism. TSBA plates were seeded with the appropriate organism, dried for 1 h and 8 mm diam. wells were cut into the plates. To each well was added 0.1 ml of *B. stearothersophilus* RS93 culture supernatant. Plates were incubated at the optimum growth temperature of the organism being tested for sensitivity to thermocin 93.

Molecular weight determination by gel filtration. Tenfold concentrated thermocin 93 (2 ml), obtained by rotary film evaporation at 70°C for 2 h, was centrifuged at 10000 g for 15 min. An equal volume of 0.05 M-

Table 1. Activity of thermocin 93 on thermophiles and mesophiles

Cultures were grown for 6 h and 1 ml was used to seed a TSBA plate. Wells (8 mm diam.) were cut into the plates and 0.1 ml of *B. stearothermophilus* RS93 culture supernatant was added. Zones of growth inhibition were recorded.

Organism	Incubation temp. (°C)	Inhibitory zone diam. (mm)
<i>Bacillus stearothermophilus</i> RS93	60	0
<i>B. stearothermophilus</i> NCA1503	60	22
<i>B. stearothermophilus</i> ATCC 12016	60	20
<i>B. stearothermophilus</i> 240	60	18
<i>B. stearothermophilus</i> 262	60	21
<i>B. stearothermophilus</i> 10	60	24
<i>B. stearothermophilus</i> 4st	60	23
<i>B. stearothermophilus</i> RS44	60	16
<i>B. caldolyticus</i>	60	22
<i>B. caldovelox</i>	60	22
<i>B. caldotenax</i>	60	22
<i>B. amyloliquefaciens</i> RUB500	37	0
<i>B. subtilis</i> 168	37	0
<i>B. subtilis</i> ATCC 10783	37	10
<i>B. cereus</i> 569H/9	37	0
<i>B. megaterium</i> KM	37	0
<i>B. globigii</i> NCIB 8056	37	0
<i>B. coagulans</i> ATCC 8038	37	0
<i>B. coagulans</i> ATCC 12245	37	0
<i>Escherichia coli</i> K12 W3110	37	0
<i>Proteus vulgaris</i> ATCC 13315	37	0
<i>Pseudomonas aeruginosa</i> NCTC 6750	37	0
<i>Staphylococcus aureus</i> NCTC 8530	37	0
<i>Salmonella typhimurium</i> ATCC 23564	37	0
<i>Rhodotorula glutinis</i> ATCC 4054	30	0

K₂HPO₄ was added to 0.75 ml of the supernatant and then loaded on to a Sephadex G-75 column (85 × 1.5 cm) pre-equilibrated with 25 mM-potassium phosphate, pH 6.8, containing 50 mM-KCl. The column was precalibrated with chymotrypsinogen (24 500), myoglobin (17 000), lysozyme (14 400) and cytochrome *c* (12 500). Fractions (1 ml) were collected at a flow rate of 8 ml h⁻¹ and assayed for activity as above using Nunc bioassay plates (no. 1015; Nunc U.K., Stafford, ST16 2JU).

RESULTS AND DISCUSSION

The impure extracellular preparation of thermocin 93 from shake flask cultures gave a 22 mm diam. zone of growth inhibition of *B. caldolyticus*. It inhibited the growth of a wide range of thermophilic bacilli, but no inhibition of mesophilic bacilli or other mesophiles was observed (Table 1). The thermocin had no inhibitory effect on the producer strain, *B. stearothermophilus* RS93.

Gel filtration of thermocin 93 on a precalibrated Sephadex G-75 column indicated a mol. wt of 13 500. Thermocin 93 therefore appears to be smaller than the thermocin isolated from *B. stearothermophilus* NU10 which was estimated to have a mol. wt of 20 000 (Yule & Barridge, 1976). No evidence was found for the presence of plasmid DNA in the producing organism using [³H]thymidine and CsCl-ethidium bromide density gradient centrifugation analysis (Le Hégarat & Anagnostopoulos, 1977). However, recently improved methods for the detection of covalently closed circular DNA molecules from bacilli (A. H. A. Bingham, C. J. Bruton & A. Atkinson, unpublished), using agarose-gel electrophoresis of cleared lysates, have shown that strain RS93 does contain a plasmid, although only small amounts of plasmid DNA could be detected in the cleared lysates. The amount of covalently closed circular plasmid DNA in cleared lysates was probably too low to be detected in CsCl-ethidium bromide density gradients.

Physical and genetic characterization of the plasmid (pT93) is in progress and at the present time it is not known whether the bacteriocin function is coded for on pT93.

Ammonium sulphate precipitated thermocin 93 between 70 and 95 % of saturation at 4 °C. DNAase and RNAase (up to 250 µg ml⁻¹) did not affect thermocin activity following 4 h incubation at 37 °C. Pronase at 50 µg ml⁻¹ reduced thermocin 93 activity to 13 % of the original after 4 h incubation at 37 °C and pH 7.8, and at 250 µg ml⁻¹ it reduced activity to 1 % under the same conditions. Similar effects were noted with chymotrypsin and trypsin.

It appears, therefore, that thermocin 93 is a small protein, but the possibility of the molecule being a glyco- or lipo-protein cannot be excluded. Thermocin 93 displays remarkable thermostability: rotary evaporation at 70 °C for 2 h showed no loss in activity, whereas the half-life of the activity at 75 °C was about 80 min.

Optimum activity of thermocin 93 occurred at pH 7.0; adjustment of the pH to 4.1 or 9.4 resulted in an apparent 70 % loss of activity. This effect was reversible since when the material was returned to pH 7.0 full activity was recovered.

The mode of action of this small, heat-stable protein is not yet understood. Experiments designed to demonstrate lysis of thermophilic bacilli by thermocin 93 have yielded variable results. It is possible that this molecule functions by affecting bacterial wall/membrane structure or function without directly promoting lysis.

REFERENCES

- GRATIA, A. (1925). Sur un remarquable exemple d'antagonisme entre deux souches de colibacille. *Comptes rendus des séances de la Société de biologie* **93**, 1040-1041.
- HARDY, K. E. (1975). Colicinogeny and related phenomena. *Bacteriological Reviews* **39**, 464-515.
- HEINEN, U. J. & HEINEN, W. (1972). Characteristics and properties of a caldo-active bacterium producing extracellular enzymes and two related strains. *Archiv für Mikrobiologie* **82**, 1-23.
- HOLLAND, I. B. (1975). Physiology of colicin action. *Advances in Microbial Physiology* **12**, 55-139.
- LE HÉGARAT, J. C. & ANAGNOSTOPOULOS, C. (1977). Detection and characterisation of naturally occurring plasmids in *Bacillus subtilis*. *Molecular and General Genetics* **157**, 167-174.
- SARGEANT, K., EAST, D. N., WHITAKER, A. R. & ELSWORTH, R. (1971). Production of *Bacillus stearothermophilus* NCA 1503 for glyceraldehyde-3-phosphate dehydrogenase. *Journal of General Microbiology* **65**, iii.
- SHAFIA, F. (1966). Thermocins of *Bacillus stearothermophilus*. *Journal of Bacteriology* **92**, 524-525.
- YULE, R. & BARRIDGE, B. D. (1976). Isolation and characterisation of a bacteriocin produced by *Bacillus stearothermophilus* strain NU-10. *Canadian Journal of Microbiology* **22**, 1743-1750.