A novel antimicrobial activity of a *Paenibacillus polymyxa* strain isolated from regional fermented sausages

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M. PIURI, C. SANCHEZ-RIVAS AND S.M. RUZAL. 1998. A strain isolated from Argentinean regional fermented sausages was found to produce and secrete a compound that inhibited growth of *Lactobacillus* strains used as indicators. It was characterized as *Paenibacillus polymyxa* (P13). The antimicrobial activity, named polyxin, was obtained from culture supernatant fluid of late stationary phase and was inhibitory to actively growing cells. It was effective against a wide range of Gram-positive and Gram-negative bacterial species tested including food-borne pathogens. Bacteriocin-like properties such as proteinaceous nature (sensitive to proteases), insensitivity to organic solvents and chelators, stability to heat (up to 10 min at 90 °C), and acidic pH but instability in alkaline conditions, were determined. A molecular mass of 10 kDa was estimated by molecular gel filtration.

INTRODUCTION

The Gram-positive spore forming bacterium *Paenibacillus polymyxa* is widely distributed in the environment as well as in food products (Pirttijarvi *et al*. 1996). Several small peptide antibiotics, for example Polymyxin B, are produced by this micro-organism (Kajimura and Kaneda 1997). In this paper, the isolation from Argentinean regional fermented sausages of a strain that produces a new antimicrobial activity is described. The inhibitory spectrum was determined against Gram-positive and Gram-negative organisms, including the food-borne pathogens *Bacillus cereus* and *Escherichia coli*. Physiological and biochemical characterization of the bacteriocin-like activity were performed.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strains listed in Table 1 are laboratory stocks obtained from different sources and kept frozen with 15% (v/v) glycerol at – 20 °C.

Bacillus cereus 6A1 obtained from the *Bacillus* Genetic Stock Centre (Ohio State University, USA) was used as indicator strain in all antimicrobial activity assays unless otherwise indicated.

Organisms were grown in the appropriate medium (BHI,

Table 1 Antimicrobial activity spectrum of polyxin

Indicator organism (strain)	Inhibition (halo)*
Bacillus cereus (6A1)	+ (8·2)
Bacillus licheniformis (ATCC 10716)	+ (5·5)
Bacillus sphaericus (MR4)	+ (5)
Bacillus thuringiensis israeliensis (4Q2)	+ (8·5)
Bacillus subtilis (168)	+ (2·4)
Paenibacillus larvae (ATCC 9545)	+
Paenibacillus pulvifaciens (CCM 38)	+
Lactobacillus casei (ATCC 393)	+
Lactobacillus plantarum (ATCC 14917)	+
Micrococcus luteus	+ (9·6)
Staphylococcus aureus	–
Pseudomonas aeruginosa	–
Escherichia coli (C600)	+ (4·8)
Klebsiella pneumoniae	+ (6·3)
Proteus vulgaris	+ (21)
Serratia marcescens	+ (20)
Salmonella newport	–

*Diameter of the inhibition halo in mm around an isolated colony is reported for the strains in which it was determined.

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LB, MRS, MYPGP) according to the physiological requirements of each as described in Balows *et al.* (1992).

Isolation of P13 strain

Fermented sausages (2·5–5 g) were cut into thin slices, resuspended in peptone water 0·1% (w/v), and serial decimal dilutions plated on appropriate media (MRS and BHI agar) for colony counts. Plates of about 300 isolated colonies were replica-plated to the same medium with or without catalase (40 U ml^{-1}) and incubated for 18 h at 37 °C. Antimicrobial producers were revealed by seeding the indicator strain (*Lactobacillus plantarum* ATCC 14917) in 10 ml of molten BHI agar and overlaying on the replicated plates. Following incubation, colonies surrounded by inhibition zones were recovered from the master plate. A strain isolated in such conditions was designated P13, cultured in BHI broth (Oxoid) at 30 °C and further characterized.

Species level determination of P13 strain

The isolate was characterized using the API20 multitest system (BioMerieux, Marcy l'Etoile, France) and complementary biochemical determinations as described (Sneath 1986; Balows *et al.* 1992; Heyndrickx *et al.* 1996).

The 16S rRNA was partially sequenced (538 bp; PE Applied Biosystems MicroSeq™, Quick Search, MIDI Labs Inc., Newark, USA) and compared using sequence alignment software with sequences obtained from different databases (MicroSeq, BLAST, Genebank, EMBL and Ribosomal Database Project).

Antimicrobial production

Polyxin activity was monitored during the growth cycle of P13 strain in different media. Optical density at 600 nm, viable counts and spores (heat treated counts after 70 °C for 10 min) were recorded and supernatant fluids tested for activity on indicator bacterial lawns. Growth and production of polyxin were tested in DSM (Schaeffer *et al.* 1965), Minimal medium (Spizizen 1958) and BHI buffered (at pH 6) or unbuffered.

Polyxin crude preparation

Strain P13 was cultivated in BHI broth buffered with KPO_4 (0·1 mol l^{-1}, pH 6) for 72 h at 30 °C. After centrifugation, the supernatant culture fluid was filter sterilized (0·45 μm pore size) and precipitated with 10% (w/v) TCA overnight at 4 °C. Pellets were resuspended in KPO_4 buffer (0·1 mol l^{-1}) and adjusted to pH 6 with NaOH 1 mol l^{-1}. Total protein concentration was determined according to Lowry *et al.* (1951). Specific activity was 10 AU mg^{-1} for culture supernatant fluid and 120 AU mg^{-1} for polyxin crude preparation.

Antimicrobial activity detection and assay

BHI agar plates containing 0·15% (w/v) starch were seeded with 10^7 cells of the indicator strain; 5–10 μl of a serially diluted polyxin preparation were then spotted on the plates and incubated at the appropriate temperature. Activity was determined by the highest dilution giving a halo. Arbitrary Units (AU) ml^{-1} were defined as the product of the reciprocal of the greatest dilution that showed a zone of inhibition multiplied by the volume dilution of the spot.

Treatments with proteolytic enzymes, organic solvents, chelators, heat and pH on antimicrobial activity

Crude preparation (100 μl samples) were submitted to the different treatments (Table 2) and incubated for 90 min at 37 °C. For heat treatment, the preparation was heated for

Table 2 Effects of different treatments on the antimicrobial activity of polyxin

Treatment	Activity*
None	+
Pronase E (40 U ml^{-1})	−
Proteinase K (0·2 mg ml^{-1})	−
Trypsin (1 mg ml^{-1})	−
Ethyl ether†	+
Chloroform†	+
Toluene†	+
Acetone†	+
Ethyl alcohol†	+
Methyl alcohol†	+
EDTA (0·01 ml l^{-1})	+
Na citrate (0·1 mol l^{-1})	+
pH 2 to 8	+
pH 9 to 10	−
Incubation (10 min) at:	
60 °C	+
90 °C	+
100 °C	±‡

*Activity was determined by the presence of an inhibition halo on the indicator strain. The crude preparation tested had a concentration of 1600 AU ml^{-1}.
†A 10% (v/v) concentration was used. Activity was assayed after precipitation with 10% (v/v) TCA to avoid inhibition due to the presence of the organic solvent.
‡ ± indicates partial activity recovered.

different time periods at 60, 90 or 100 °C. For pH stability, the preparation was incubated in KPO_4 adjusted to different pH values with HCl or NaOH. Antimicrobial activity was determined before and after the different treatments.

Susceptibility range

The different indicator strains listed in Table 1 were tested by overlaying plates containing isolated colonies of P13 pregrown at 30 °C for 48 h to ensure late stationary phase, with 10 ml BHI molten agar seeded with 10^6 cells of the listed strains. Diameters of the inhibition zones were scored and reported.

Mode of action

Exponentially or stationary growing cultures of the indicator strains in BHI broth were exposed to 400 AU ml^{-1} of crude polyxin preparation. At various times, the O.D. 600 nm and/or viable counts were determined.

Molecular weight determination

Estimation of size was done by gel filtration on a Sephadex G-75 column (45 cm × 0·5 cm) equilibrated with KPO_4 0·1 mol l^{-1} pH 6·5. Molecular weight markers were used to calibrate the elution pattern: BSA (66 000 Da), DNAse I (31 000 Da), Lysozyme (14 300 Da) and Insulin (5800 Da) detected by absorbance at 280 nm. Crude preparation (1 mg) was passed through the column and the elution fractions obtained were concentrated with TCA 10% (w/v), resuspended in KPO_4 buffer (0·1 mol l^{-1} and adjusted to pH 6 with NaOH 1 mol l^{-1}) and assayed for antimicrobial activity.

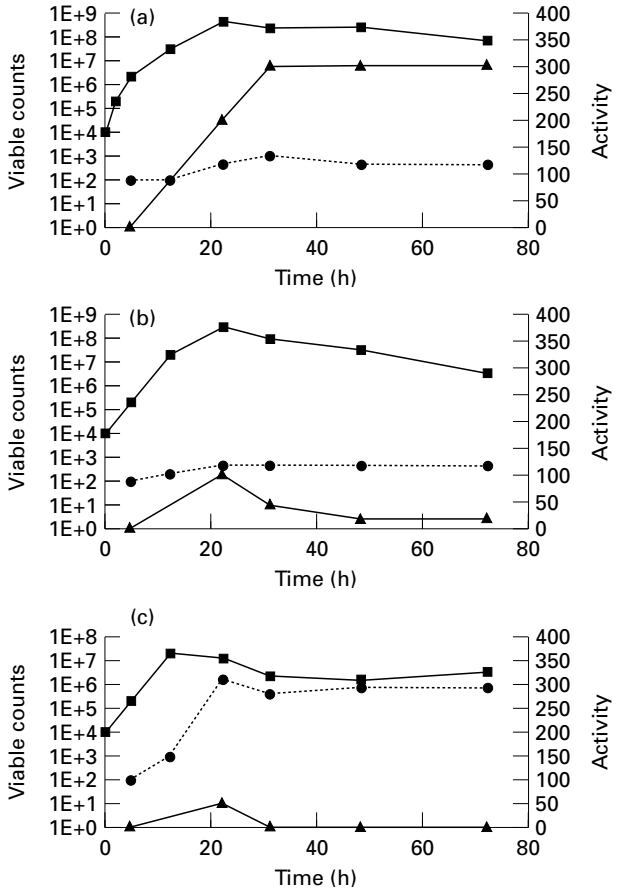

Fig. 1 Production of polyxin during growth and relation with sporulation. (a) BHI buffered at pH 6; (b) BHI; (c) DSM. (■), Viable counts (cfu ml^{-1}); (▲), activity (AU ml^{-1}); (●) spores (cfu ml^{-1}) after heat treatment at 70 °C for 10 min)

RESULTS

A *Paenibacillus polymyxa* isolated strain showed a secreted antimicrobial activity

Following the screening for antimicrobial activity-producing bacteria from Argentinean regional fermented sausages, a strain named P13 was isolated. P13 produced a large inhibition zone on the indicator strain *Lactobacillus plantarum*. It was a Gram-positive, rod-shaped, motile, facultatively anaerobic, catalase-positive, spore-forming organism producing spores within a swollen sporangium. It grew at 40 °C but not at 50 °C and produced acid from glucose, manitol, amigdaline, ribose, salicine, lactose, glycerol and trehalose but not from inositol, sorbitol, rhamnose, sucrose or melibiose. Casein and starch were hydrolysed.

Partial sequence alignment of 16S rRNA has been used successfully to determine species level as described in Rogall *et al*. (1990). From the partial 16S rRNA sequence alignment (538 bp), P13 strain resulted in 0·99% genetic distance (99·01% homology) with *Paenibacillus polymyxa* (BAC16SRR11(ddbj) and BPOL16SR(embl)) (Ash *et al*. 1993).

Its antimicrobial activity was secreted in the culture supernatant fluid and was insensitive to the addition of catalase, and it remained active in the absence of glucose in the growth medium that prevented acid production. The results eliminated either hydrogen peroxide or organic acids as responsible for the inhibitory activity, respectively.

It was not dialysable through dialysing tubing of 12 kDa cut-off, implying that neither polymyxin (1280 Da) nor other very low molecular weight peptide antibiotics was responsible for the inhibitory effect.

The antimicrobial was inhibitory to several lactic acid bacterial (LAB) strains occurring naturally in the food source, and to other Gram-positive and Gram-negative organisms

(Table 1). This wide range of activity ruled out phages as the inhibitory factor; nevertheless, no plaques were observed on the *Bacillus* strains tested.

Strain P13 produced this antimicrobial factor as a secondary metabolite when it was grown in BHI broth buffered at pH 6 at 30 °C, with increasing activity during the late stationary phase of growth (30 h of incubation) (Fig. 1a). No activity was observed in unbuffered BHI (where pH increases to 7·5–8) (Fig. 1b), nor in other media such as Schaeffer sporulation (DSM, Fig. 1c) where sporulation efficiency was maximal (10%), or Spizizen minimal media (not shown). Activity was also absent during exponential growth. As sporulation is greatly reduced in BHI broth (less than 0·1%), expression of antimicrobial activity does not seem to be related to this differentiation process.

Effect of proteolytic enzymes, organic solvents, chelators, heat and pH on the antimicrobial activity

A crude preparation of the antimicrobial factor was tested for sensitivity (measured as the loss of inhibition zone in agar plate spot assays) to various proteolytic enzymes, organic solvents, chelators, heat and pH as described in Materials and Methods.

The antimicrobial activity was (see Table 2) insensitive to organic solvents, EDTA or sodium citrate but highly sensitive to protease treatments (Proteinase K, Pronase E or Trypsin) indicating the proteinaceous nature of this compound. It was heat stable after incubation at 90 °C for 10 min, and active over a wide range pH conditions (2–8), but it was inactivated by extreme alkaline conditions (pH 9–10).

This behaviour is reminiscent of bacteriocin-like substances (Jack *et al.* 1995). The wider spectrum of susceptible species make it an interesting new activity and was named polyxin.

Mode of action

To determine whether polyxin action was bactericidal or bacteriostatic, samples of crude preparation (400 AU ml^{-1}) were added to exponential or stationary phase cultures of indicator strains. A Gram-negative species (*Escherichia coli*) and two Gram-positives (a spore-forming and an asporogenic species) were chosen (*Bacillus cereus* and *Micrococcus luteus*). *Bacillus cereus* is a known food-borne pathogen (Naclerio *et al.* 1993). A bacteriolytic effect was observed in exponentially growing cultures of *B. cereus* and *M. luteus* (Fig. 2a–b), while a bacteriostatic effect was observed in *E. coli* (Fig. 2c). However, actively growing cells only are susceptible to polyxin as no lytic effect was observed when polyxin crude preparations were added to stationary phase cultures of both Gram-positive strains (not shown).

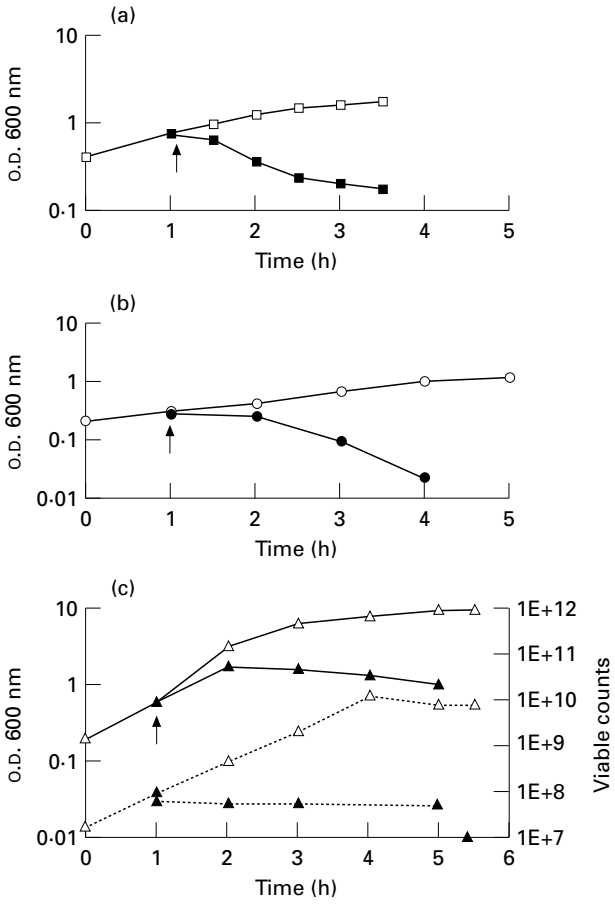

Fig. 2 Mode of action. (a) *Bacillus cereus* 6 A1; (b) *Micrococcus luteus*; (c) *Escherichia coli* C600. The arrow indicates the time where polyxin crude preparation (400 AU ml^{-1}) was added to the culture. Solid symbols are treated and empty symbols are non-treated. Solid lines are O.D. 600 nm and dashed lines are viable counts (cfu ml^{-1})

Polyxin is a 10 kDa protein

SDS-PAGE of the late stationary phase culture supernatant fluid of P13 revealed about 20 proteins with a molecular range from 8 to 120 kDa. Molecular weight estimation of this compound was therefore carried out by Sephadex G-75 gel filtration. Activity was found to elute in fractions corresponding to 10 kDa compared to molecular weight markers.

DISCUSSION

Antagonistic factors have attracted much attention for their potential use as food additives as well as for alternative biological control of diseases caused by micro-organisms. Bacteriocin and bacteriocin-like substances have been reported for several *Bacillus* (some reclassified as *Paenibacillus*) species (Jack *et al.* 1995). The finding of a new antimicrobial activity for *P. polymyxa*,

distinct from those already reported, is described here. This activity seems to be induced under non-sporulating conditions as a secondary metabolite. The same type of expression has been reported for cerein, a bacteriocin produced by *Bacillus cereus* (Naclerio *et al.* 1993), as well as for amoebolytic substances in *Bacillus licheniformis* (Galvez *et al.* 1993), indicating that the presence of these bacteriocin-like molecules is common to the genus *Bacillus* and would also be common to the new reclassified members of the *Paenibacillus* genus. However, special growth conditions are needed for the induction of their expression. Lack of sporulation would be a prerequisite for the synthesis of secondary metabolites as an alternative adaptive response to environmental changes, in this case, nutrient exhaustion. Although the sporulation process seems not to be required for the production of polyxin, we could not rule out the possibility of a common early regulatory pathway as reported for other antimicrobial compounds (Leifert *et al.* 1995). The maintenance of a pH value of 6 seems to be very important in the induction of the secondary metabolism as in unbufffered BHI medium, no activity was detected and cells began to die after 30 h of incubation (Fig. 1b).

Polyxin is a proteinaceous factor with a bacteriolytic effect on Gram-positive, but bacteriostatic effect on Gram-negative actively growing cells. Differences observed between the two classes of organisms would result directly from their cell wall structure.

Two major consequences arise from our findings. First, the occurrence of *P. polymyxa* strains often present in fermented food products would interfere with the ripening process through inhibition of LAB if the strains produced polyxin as observed in strain P13. Second, further characterization of this compound is necessary to establish its molecular structure more precisely for potential use in the control of food-borne pathogens such as *B. cereus* (over which it has a bacteriolytic effect). In conditions where LAB strains are absent such as in spices, cereals and meat products, the presence of polyxin as a food additive would be worthwhile, in view of its stability at a wide range of pH and temperature.

One remarkable characteristic of polyxin is that it was obtained from a food product. This fact is valuable for the classification of GRAS (Generally Recognized as Safe) products by the FDA (Food and Drugs Administration, USA). In the same way, preliminary results of toxicity on eukaryotic cells have shown no toxic effect on the viability of Vero cells when exposed to crude preparations of polyxin (data not shown).

Therefore, detailed biochemical and molecular studies are being carried out to analyse this antimicrobial compound further.

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