Biotechnology Letters **26:** 115–119, 2004.

Purification and characterization of a new peptide antibiotic produced by a thermotolerant *Bacillus licheniformis* strain

Sónia Mendo[1,*], Nuno André Faustino[1], Ana Cristina Sarmento[1], Francisco Amado[2] & Arthur J.G. Moir[3]

[1]*Departamento de Biologia, Centro de Biologia Celular, Universidade de Aveiro, 3810-193 Aveiro, Portugal*
[2]*Departamento de Química, Universidade de Aveiro, 3810-193 Aveiro, Portugal*
[3]*Krebs Institute, University of Sheffield, Western Bank, Sheffield S10 2TN, UK*
[*]*Author for correspondence (Fax: +351 234 426408; E-mail: smendo@bio.ua.pt)*

Received 28 May 2003; Revisions requested 11 June 2003; Revisions received 10 November 2003; Accepted 11 November 2003

Key words: peptide antibiotics, peptide synthetases, thermotolerant Bacillus

Abstract

A *Bacillus licheniformis* strain, I89, isolated from a hot spring environment in the Azores, Portugal, strongly inhibited growth of Gram-positive bacteria. It produced a peptide antibiotic at 50 °C. The antibiotic was purified and biochemically characterized. It was highly resistant to several proteolytic enzymes. Additionally, it retained its antimicrobial activity after incubation at pH values between 3.5 and 8; it was thermostable, retaining about 85% and 20% of its activity after 6 h at 50 °C and 100 °C, respectively. Its molecular mass determined by mass spectrometry was 3249.7 Da.

Introduction

Bacteria with new mechanisms of antimicrobial resistance are continuously emerging. Consequently, in addition to the engineered drugs, screening for new natural drugs from new sources, such as microbes from extreme ecological niches, is of major importance for the pharmaceutical industry (Jacob & Zasloff 1994).

Several natural products, and their semi-synthetic analogs, active only against Gram-positive bacteria, are in clinical development, as for instance the streptogramins which completed phase III of clinical trials in 1999 (Hancock & Chapple 1999).

The genus *Bacillus* has been widely used in the fermentation industry for the production of antibiotics as well as several extracellular enzymes. Secondary metabolites have a diverse chemical structure and biological activities and are produced only by some species of a genus, usually as families of closely related compounds (Stachelhaus *et al.* 1995) which, in the case of peptide antibiotics, may differ from each other by one or, at most, a few amino acid residues (Katz & Demain 1977).

Here we report the purification and characterization of a new peptide antibiotic produced by a new isolated *Bacillus licheniformis* (Mendo *et al.* 2000). Resistance to proteolytic enzymes, thermostability and pH stability of the antibiotic were investigated. Additionally, the molecular mass and partial amino acid sequence of the antibiotic were determined.

Materials and methods

Bacterial strains

Bacterial strains used for A89 activity spectrum determination are listed in Table 1.

Culture of Bacillus licheniformis *I89 and antibiotic production*

B. licheniformis I89, identified by the BIOLOG Micrologg 4.01 C system, was grown as described by

Table 1. Activity spectrum of antibiotic A89.

Microorganism	Sensitivity
Bacillus subtilis ATCC 14593	++
Micrococcus luteus ATCC 9341	++
Staphylococcus aureus ATCC 6538	++
Staphylococcus aureus (hospital isolate)	+
Escherichia coli ATCC 13762	–
Escherichia coli (hospital isolate)	–
Pseudomonas spp. (hospital isolate)	–
Saccharomyces cerevisiae	–
Candida albicans	–

(+) Growth inhibited (+: the inhibition zone is comparable to that obtained with 0.5 μg bacitracin ml^{-1}; ++: the inhibition zone is comparable to that obtained with 1–2.5 μg bacitracin ml^{-1}); (–) no growth inhibition.

Mendo *et al.* (2000). Antibiotic production medium contained 10 g tryptone l^{-1}, 5 g yeast extract l^{-1}, 9.9 g NaCl l^{-1}, pH adjusted to 6.5. Antibiotic production occurred in 500 ml shake-flasks at 50 °C and 250 rpm.

Activity spectrum and assessment of antimicrobial activity

The activity spectrum of the antibiotic, A89, was determined by a modification of the agar diffusion method described by Haavik & Thomassen (1973). Throughout the work, its activity was routinely tested against *Micrococcus luteus* ATCC 9341.

Antibiotic concentration

The culture supernatant was clarified by centrifugation and then extracted twice with 1/10 of the culture volume of 1-butanol in each extraction. The butanol layer was removed and frozen at −70 °C in 500 μl aliquots.

Antibiotic purification

After lyophilisation the samples were re-hydrated and centrifuged. The pellet formed was solubilized in 80% (v/v) acetonitrile, 0.1% (v/v) trifluoracetic acid and applied to a C18 column in an HPLC. Elution was achieved by an acetonitrile (0–80% v/v) gradient at 0.8 ml min^{-1}. Detection was at 220 nm. Fractions were collected and assayed for antimicrobial activity, as previously described (Haavik & Thomassen 1973).

Analysis of the 1-butanol crude solution was also performed by FPLC using a Superdex Peptide HR 10/30 column (Amersham Pharmacia Biotech). Purification was carried out using an isocratic elution of 30% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic. Detection was at 280 nm. Fractions were assayed for antibiotic activity. Commercial bacitracin (Sigma) was used as control.

Mass spectrometry

Electrospray mass spectra were acquired with a Q-TOF 2 (Micromass, Manchester). Data acquisition was carried out with a Micromass MassLynkx 3.4 data system.

Peptide sequence analysis of A89

N-Terminal sequencing of purified A89 was performed on a 476A protein sequencer (Applied Biosystems).

Antibiotic characterisation: stability to proteases

Antibiotic stability to proteases was assessed by incubation with aspartic (cardosin A and cardosin B) and serine proteinases (α-chymotrypsin, trypsin and endoproteinase Glu-C). Incubation buffers were as follows: sodium citrate/phosphate buffer pH 5, for aspartic proteinases; 80 mM Tris/HCl, 100 mM $CaCl_2$, pH 7.8, for α-chymotrypsin; 50 mM Tris/HCl, 20 mM $CaCl_2$, pH 8.5 for trypsin; and 50 mM NaH_2PO_4 buffer, pH 7.8 for endoproteinase Glu-C. The 1-butanol extract was lyophilised and the residue dissolved in ultra-pure water which was centrifuged, the pellet freeze-dried and re-solubilised in the appropriate buffer prior to incubation. One μg enzyme was added to 100 μl A89 and reaction proceeded overnight at 37 °C, at 300 rpm. Antibiotic activity was assessed as previously described and compared to that of the native peptide. Plates containing only the protease in appropriate buffer were used as controls.

Stability to pH

pH stability of A89 was determined as a measurement of retained activity after incubation at a given pH. Samples were prepared as described above, solubilised in 100 mM sodium citrate/phosphate buffer and incubated at 4 °C for 16–18 h. Antibiotic activity was measured as previously described. pH solutions, without A89, were used as control.

Thermal resistance

Thermal resistance of A89 was assessed by incubation at different temperatures (between 37 °C and 100 °C) for 15 min to 6 h. A89 stability to sterilisation processes was assessed by autoclave.

Results

A89 activity spectrum and purification

B. licheniformis I89 produces a compound (A89) that showed anti-microbial activity against several Gram-positive bacteria, as shown in Table 1.

The antibiotic was purified either by HPLC (analytical purposes) or by FPLC (Figure 1A, B) retaining its activity against all the tested strains after the purification procedures (see Table 1). Both the isolated material from the HPLC column and from the FPLC column were re-analysed by HPLC, confirming the purity of the compound. After lyophilisation, purified A89 was a creamy amorphous powder, soluble in organic solvents such as butanol and acetonitrile and also in aqueous acidic solutions.

Antibiotic characterisation

Bacitracin is a widely known peptide antibiotic naturally produced by *B. licheniformis* ATCC 10716 and other *B. subtilis* strains. In the present work, naturally synthesised bacitracin, and also commercial bacitracin were used as controls. Thin layer chromatography revealed that bacitracin has a retention factor (Rf) value higher (0.68) than that of A89 (0.44) suggesting that the latter is different from bacitracin. These results were confirmed by comparison of the inhibition zones produced after placing the thin layer over a plate inoculated with *M. luteus*. Additionally, co-elution of bacitracin and A89 in RP-HPLC revealed that A89 has a higher retention time than that of bacitracin, indicating that it is more hydrophobic than the latter and also commercial bacitracin (data not shown).

A89 molecular weight determined by ESI-mass spectrometry was 3249.7 Da validating the results previously obtained by gradient SDS-PAGE that suggested a molecular weight of 3–4 kDa (data not shown). The partial amino acid sequence of A89 was determined by Edman degradation (see Table 2).

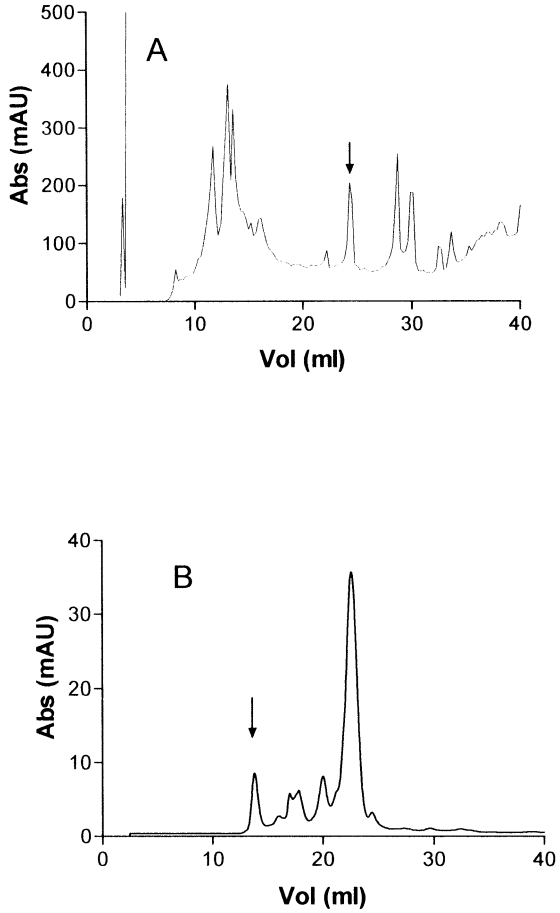

Fig. 1. Elution profiles of A89. (A) From RP-HPLC, at 220 nm, after solubilisation in acetonitrile acidified with trifluoroacetic acid. Purification was carried out using a gradient of 0–80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. Arrow indicates antimicrobial activity of the collected fractions, tested against *M. luteus*. (B) Elution profile of A89 in size exclusion chromatography by FPLC, at 280 nm, after solubilisation in acetonitrile acidified with trifluoroacetic acid. Purification was carried out using an isocratic elution of 30% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. Arrow indicates antimicrobial activity of the collected fractions, tested against *M. luteus*.

Stability to proteases

A89 was resistant to proteolytic degradation since none of proteases tested decreased its anti-microbial activity.

pH stability

A89 was stable from pH 3.5 to 8 with no decrease in antimicrobial activity detectable after 18 h.

Table 2. Primary structure of antibiotic A89 determined by Edman degradation on an automated protein sequencer.

Residue number	Amino acid
1	X
2	X
3	X
4	Tyr
5	Asn
6	Thr
7	Lys
8	Lys
9	X
10	Lys
11	Pro
12	Lys
13	Leu
14	Leu
15	Leu/Pro

X corresponds to non-identified residues.

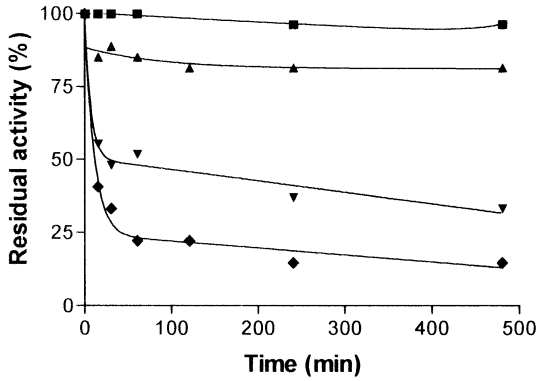

Fig. 2. Effect of temperature on A89 stability. Incubation temperatures were: ■, 37 °C; ▲, 50 °C; ▼, 75 °C; ♦, 100 °C. After incubation, samples were allowed to cool to 4 °C for 12 h, irreversible loss of activity was measured by bioassay and compared to a control sample.

Thermal stability

Thermal stability of A89 was assessed from 37 °C to 100 °C (Figure 2). Most of the irreversible inactivation of A89 occurred during the first 15 min of incubation. After this initial stage no significant loss of activity was detected. In addition, there was no significant loss of activity after 12 months of storage at 4 °C.

Discussion

Although several of the non-ribosomal synthesized antimicrobial peptides have been known for decades, many others with antibiotic activity have been described only recently. These peptides offer a potentially rich source of novel antimicrobials. The modification of known existing peptides (and presumably also isolation of novel peptides from nature and modification of these) and the use of identified structures as templates for chemical synthesis and diversity are areas currently under investigation (Hancock & Chapple 1999).

The partial sequence of A89 obtained (Table 2) reveals that A89 is different from bacitracin A (Zimmer *et al.* 1979). The presence of lipids and carbohydrates has been detected in peptide antibiotics, such as lichenysin A (Yakimov 1995) and surfactin (Menkhaus *et al.* 1993), as well as thiazoline rings involving *N*-terminal residues of bacitracin (Zimmer *et al.* 1979), for example. It is well known that such elements interfere with peptide sequencing procedures and is our belief that the repetitive failure of the identification of several residues of A89 sequences was a consequence of the presence of one or several of these elements. According to these findings, the molecular mass determined by mass spectrometry was higher than that of bacitracin (1422.7 Da) and lychenisin (1034 Da) (Yakimov 1995).

Generally, any antimicrobial compound to be used in food or health industry should be stable. A89 was resistant to proteases which is the same as for other peptide antibiotics (Martin & Gutierrez 1995) which are resistant because of the presence of D-amino acids.

Bacitracin is readily hydrolysed by acid; under mild alkaline conditions the thiazoline ring of bacitracin is oxidised conferring maximum stability in solution in the pH range 5–6 (Zimmer *et al.* 1979). A89, however, remained active from pH 3.5 to 8 over 18 h.

Previous work (data not shown) showed that the amino acid sequence deduced from the ORF of two fragments involved in A89 synthesis has a high similarity with other biosynthetic genes coding for peptide synthetases, namely lychenisin (90%) and bacitracin (63%). In fact, the same motifs described by Kleinkauf & Von Dohren (1996) that are present in amino acid activation domains of peptide synthetases were identified in the nucleotide sequence of the fragments involved in antibiotic synthesis of A89. The biosynthesis of non-ribosomally synthesized peptides is directed

by large multienzyme complexes that have a modular structural organization and are thought to orderly link the amino acid residues of the final peptide (Kleinkauf & Von Döhren 1996, Challis *et al.* 2000). A model has been recently proposed by Challis *et al.* (2000) that allows for the prediction and identity of amino acids activated by non-ribosomal peptide synthetases based on adenylation domain nucleotide sequence. This information will be helpful in elucidating the complete amino acid composition of A89.

Acknowledgements

A.C. Sarmento and N.A. Faustino were supported by Fundação para a Ciência e Tecnologia (grants BPD-7183/2001 and BM-20979/1999).

References

Challis G, Ravel J, Townsend C (2000) Predictive structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. *Chem. Biol.* **7**: 211–224.

Haavik H, Thomassen S (1973) A bacitracin-negative mutant of *Bacillus licheniformis* which is able to sporulate. *J. Gen. Microbiol.* **76**: 451–454.

Hancock R, Chapple D (1999) Peptide antibiotics. *Antimicrob. Agents Chemother.* **43**: 1317–1323.

Jacob L, Zasloff M (1994) Potential therapeutic applications of magainins and other antimicrobial agents of animal origin. In: Boman HG, Marsh J, Goode JA, eds. *Antimicrobial Peptides*, Ciba Foundation Symposium. Chichester: J Willey & Sons, pp. 186.

Katz E, Demain A (1977) The peptide antibiotics of *Bacillus*: chemistry, biogenesis, and possible functions. *Bacteriol. Rev.* **41**: 449–474.

Kleinkauf H, Von Döhren H (1996) A nonribossomal system of peptide biosynthesis. *Eur. J. Biochem.* **236**: 335–351.

Martin JF, Gutierrez S (1995) Genes for beta-lactam antibiotic biosynthesis. *Antonie van Leeuwenhoek* **67**: 181–200.

Mendo S, Henriques I, Correia A, Duarte J (2000) Genetic characterization of a new thermotolerant *Bacillus licheniformis* strain. *Curr. Microbiol.* **40**: 137–139.

Menkhaus M, Ullrich C, Kluge B, Vater J, Vollenbroich D, Kamp RM (1993) Structural and functional organization of the surfactin synthetase multienzyme system. *J. Biol. Chem.* **268**: 7678–7684.

Stachelhaus T, Schneider A, Marahiel M (1995) Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. *Science* **269**: 69–72.

Yakimov M, Timmis K, Wray V, Fredeickson H (1995) Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. *Appl. Environ. Microbiol.* **61**: 1706–1713.

Zimmer TL, Froyshov O, Laland SG (1979) Peptide antibiotics. In: AH Rose ed. *Secondary Products of Metabolism*. New York: Academic Press, pp. 124–147.