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ORIGINAL PAPER

Andrimid production at low temperature by a psychrotolerant *Serratia proteamaculans* strain

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Abstract Andrimid, a known non-ribosomal pseudo-peptide antibiotic, was isolated from a psychrotolerant *Serratia proteamaculans* strain. The antibiotic peptide was produced at low temperature (8 °C) in a 7.5 l BIOFLO 101 bioreactor under batch culture mode. Andrimid activity from *S. proteamaculans* culture was only detected at 25 °C and below and potent antibacterial activity was revealed against both, pathogenic and non-pathogenic bacteria. Minimal inhibitory concentration values determined by microdilution experiments varied in the range between 0.01 and 0.78 μg/ml. Antimicrobial purification and structure elucidation were carried out by LC-MS/MS and $^{1}H/^{13}C$ NMR approaches. The effects on the ultrastructure of sensitive *Escherichia coli* 35,218 cells were observed by transmission electron microscopy at different inhibition stages. This work demonstrated the significance of bioprospection from cold environments through the screening of microorganisms with ability to produce cold-active biomolecules of biotechnological interest. *S. proteamaculans* 136 was revealed as a novel microbial source for andrimid production at low temperatures, showing biotechnological potential to be applied in cryopreservation, food or cosmetic industries against pathogenic bacteria.

Keywords Andrimid · Psychrotolerant · Serratia proteamaculans · Antimicrobial · Pseudo-peptide

Introduction

Research and development of new antibiotics represent a constant worldwide challenge, influenced by key factors such as a frequent and inappropriate use of antibiotics in addition to a detriment in pharmaceutical companies involved in drug discovery (Wenzel 2004; Spizek et al. 2010).

Due to the negative consumer perceptions about synthetic preservatives, attention is shifting towards alternatives perceived as natural by consumers (Smith-Palmer et al. 1998). This strategy has triggered for instance the search for natural antimicrobial substances from new environmental sources, in order to preserve processed foods by inhibiting pathogens associated with food-borne illness (Zahner and Fielder 1995; Davies and Webb 1998). Most antibiotics have emerged from programs that screened natural sources (Gootz 1990) by using microbiological tools such as the isolation of new microorganisms, modification of well-known microbial producers or metabolic engineering of fermentation processes (Souza et al. 1982).

An alternative receiving special attention involves the search for active metabolites in samples directly exposed to non-conventional environments, as in the case of extremophile microorganisms. The ability to achieve precise activity adaptation in a specific bio-process, such as antimicrobial activity, could contribute to a significant improvement in the field of biotechnology. At present, antagonistic properties of cold-living organisms have not been investigated as extensively as those from mesophiles (O'Brien et al. 2004). Differently from inhibitors produced by mesophile organisms, antimicrobials working at low temperatures confer a

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competitive advantage to the producing microorganisms during their growth cycle (O'Brien et al. 2004). Such cold-active antimicrobial compounds may be exploitable for biotechnological applications including chilled food preservation and cosmetic or pharmaceutical industries. Evidences of this fact are studies based on a bacteriocin produced by a thermophilic *Bacillus* strain (Kabuki et al. 2007); a thermo-labile β-lactamase produced by a psychrophilic *Psychrobacter immobilis* strain (Feller et al. 1997), several archaea strains producing archaeocins (halocins and sulfolobicins) (Aravalli et al. 1998; Prangishvili et al. 2000), and a recently described psychrophilic and psychrotolerant microbial species able to produce active antimicrobial compounds at low temperatures (O'Brien et al. 2004; Sánchez et al. 2009).

In previous works we have focused on the isolation of psychrotolerant and psychrophilic bacteria from sub-antarctic environments with capability to produce highly active antimicrobial compounds against relevant human pathogens such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella enterica* ser. Newport, *Salmonella. enterica* ser. Typhimurium and clinical isolates of verotoxin (*stx 1*, *stx 2*)-producer *Escherichia coli* O157:H7 strains. During this screening program a psychrotolerant *S. proteamaculans* strain, with ability to produce a low-molecular-weight antimicrobial, was isolated from a soil sample from the *Isla de los Estados* (Ushuaia) (Sánchez et al. 2009). The antimicrobial compound was temporally named *Serraticin A* prior to its scaled-up production and purification (Sánchez et al. 2010).

The present work describes MS/MS and NMR studies confirming the antimicrobial identity as andrimid, a known hybrid pseudo-peptide whose production was firstly reported in an intracellular brown *Planthopper* symbiont, *Nilaparvata lugens*, later described as an *Enterobacter* sp. strain isolated in Thailand (Fredenhagen et al. 1987).

The andrimid mode of action was unknown for several years. Freiberg et al. (2004) identified the target of this compound as the multimeric bacterial enzyme acetyl-CoA carboxylase (ACC). The C-terminal methylsuccinimide moiety is the active pharmacophore and blocks the carboxyltransferase subunit of bacterial acetyl-CoA carboxylase by mimicking the normal N-carboxybiotinyl substrate moiety. Since the enzyme catalyzes the initial step during fatty acid biosynthesis, its blockage causes the death of sensitive cells. Liu et al. (2008) described that andrimid producer strains (*P. agglomerans*) encode an andrimid-resistant form of the acetyl-CoA carboxyltransferase *β*-subunit, thus revealing the resistance mechanism by which producer strain does not inhibits itself during antibiotic production.

Andrimid production was also reported in other mesophilic strains like *Pseudomonas fluorescens* (Needham et al. 1994) isolated in Alaska, *Vibrio* sp. (Oclarit et al. 1994; Long et al. 2005) from Southern California, *Pantoea agglomerans* (Jin et al. 2006) from upstate New York, and *Vibrio coralliilyticus* (Wietz et al. 2010, 2011) with two producing strains from distant geographical regions and sources. Thus, cosmopolitan occurrence of andrimid production was related to horizontal gene transfer by Fischbach (2009).

However, despite the importance of andrimid as a cryoprotectant compound of potential industrial value, no reports on its lab-scale production by any microorganism at low temperature have appeared. This work represents the first evidence of andrimid production at low temperatures by a specific psychrotolerant strain of *S. proteamaculans* isolated from sub-antarctic regions.

Materials and methods

Bacterial strains and culture media

Antibacterial activity was determined according to the critical dilution method (Mayr-Harting Mayr-Harting et al. 1972) against culture collection strains obtained from ATCC Rockville MD (USA), DSMZ Braunschweig, (Germany) and a psychrophilic strain of *P. fluorescens* previously isolated in our laboratory, as indicator strains (Table 1). Gram positive *Lactobacillus* and *Leuconostoc* strains were kindly provided by Dr. Savino, UNT. Phytopathogenic *Pseudomonas syringae* and endosymbiotic *Rhizobium* sp. were obtained from Agroindustrial Experimental Station "Obispo Colombres (EEAOC)".

Antimicrobial activity was expressed either as antibiotic arbitrary units per milliliter (AU/ml) or micrograms per milliliter (μg/ml) in non-purified or purified aliquots, respectively. Minimal inhibitory concentration (MIC) of andrimid against different microorganisms was determined by the two-fold serial dilution assay (Yaron et al. 2003). Luria-Bertani (LB) and M9-G (in g/l: $CaCl_2$, 0.12; NaCl, 0.5; NH_4Cl, 1; Na_2HPO_4, 12.8; KH_2PO_4, 3; $MgSO_4{\cdot}7H_2O$, 0.5 and glucose, 2) media were used, and MIC was considered as the lowest concentration that showed no turbidity increments (OD_{600}nm) after 48 h of incubation.

Temperature effects on andrimid production

Erlemeyer flasks containing LB broth (10 ml) were inoculated with 10 % v/v of an overnight culture containing $\sim 10^7$ cells/ml of *S. proteamaculans* 136. Flasks were incubated at 4, 8, 15, 20, 25, 30, 37 and 45 °C during 7 days in an orbital shaker at 200 rev/min. Cells were removed by centrifugation at 8,000×*g* and supernatants were assayed for andrimid activity by the critical dilution

Table 1 Bacterial strains used in this study

Strains	Source/reference
Lactobacillus brevis	Savino et al. (2012)
Leuconostoc mesenteroides	
Micrococcus luteus	ATCC 15307
Staphylococcus epidermidis	ATCC14990
Bacillus subtilis 168	BGSC[a] 1A1
Enterococcus faecium	CCM[b] 7167
Escherichia coli	ATCC 35218
Escherichia coli	ATCC 25922
Escherichia coli O157:H7 (stx1, stx2)	Sanchez et al. (2009)
Salmonella enterica ser. Newport	ATCC 27869
Citrobacter freundii	ATCC 14135
Enterobacter cloacae	ATCC 13047
Pseudomonas fluorescens	Sanchez et al. (2009)
Pseudomonas syringae	EEAOC
Rhizobium sp.	EEAOC
Serratia proteamaculans 136 (andrimid producer)	Sanchez et al. (2009)

EEAOC Estación Experimental Agroindustrial "Obispo Colombres"

[a] Bacillus Genetic Stock Center

[b] Czech collection microorganisms

method (Mayr-Harting et al. 1972) at 37 °C against *S. enterica* ser. Newport ATCC 27869 as indicator strain.

Production and purification

Antimicrobial production was carried out in a 7.5-l stirred tank bioreactor (BioFlo 110 Modular Benchtop Fermentor/Bioreactor; New Brunswick Scientific; Edison, NJ; USA) with a working volume of 6.5 l (LB broth) and a 10 % (v/v) inoculum ($\sim 10^7$ cells/ml). Agitation consisted in two 6-flat bladed Rushton-type impellers (52 mm), operating at 200 rev/min. Culture pH was controlled at 7.0 by automatic addition of 0.1 M HCl, while temperature was maintained at 8 °C throughout cultivation and air was injected at 0.5 v.v.m.

After 50 h of incubation, agitation and aeration were increased to 250 rev/min and 2 v.v.m. respectively, in order to stimulate foam formation 2 h before andrimid recovery. Thus, 2 l of a two-fold concentrated fraction (1,600 AU/ml) were obtained. Immediately afterwards, cells were removed from the foam by centrifugation at 8,000×*g* for 20 min and cell-free supernatants were maintained at 4 °C until purification.

During the purification scheme the concentrated cell-free fraction was subjected to three consecutive purification steps involving activated-charcoal (Sigma Chemical Co.) absorption, C18-solid phase extraction (SPE) (Varian, Inc.; Palo Alto, CA; USA) and semi-preparative RP-HPLC (C18 μBondapak 125Å pore size, 10 μ granulometry), as previously described (Sánchez et al. 2010). Antimicrobial purity was on-line checked by a photo-diode array (PDA) detector coupled to a Waters 2795 Alliance HT® HPLC system in a 200–400 nm range.

Chemical hydrolysis

A 3 mg/ml antibiotic suspension was heat-dried before hydrolysis by adding 200 μl of 0.5 N NaOH and autoclaved at 121 °C for 15 min. After hydrolysis, the sample was neutralized by adding 200 μl of 0.5 N HCl. In the same way, acid hydrolysis was performed by adding 200 μl of 6 N HCl under a nitrogen atmosphere, and then heated at 155 °C for 20 min. Alkali and acid-hydrolysed samples were completely dried under nitrogen atmosphere before being dissolved in 100 μl of *n*-propanol. An aliquot (10 μl) of hydrolysed samples was analysed by TLC on silica gel plates (20 cm × 20 cm Merck, Kieselgel 60 F; 0.25 mm). Leucine, valine, lysine, methionine, cysteine, proline, glutamine and phenylalanine (Sigma Chemical Co.) were used as standards in aqueous solution (10 mg/ml) without further purification. Chromatography was carried out with ethanol-ammonium hydroxide mixture (7:3, v/v) as developing solvents, and free amino acids were revealed by using a 0.2 % (w/v) ninhydrin (Merck 6762) reagent aqueous solution.

MS/MS and NMR analysis

The molecular mass of the antimicrobial compound was determined on a Hybrid Quadrupole-TOF LC/MS/MS Mass Spectrometer using a QSTAR® hybrid pulsar-*i* instrument (Applied Biosystems, Foster City, CA; USA). Prior to, and in-sequence with MS, the sample was separated on RP (C18)-HPLC Kromasil column (Eka, Sweden). Twenty microliters of sample ($\sim$0.05 mg/ml) were injected in a linear gradient of water/acetonitrile at a starting point of 80 % water. The gradient was allowed to proceed for 30 min to a final amount of 10 % water. The electrospray (ESI)

TurboIonSpray™ source was set to positive ion mode with a source voltage of +5,500 V. The quadrupole system was adjusted to scan between *m/z* 50–3,000 in TOF-MS mode, whereas for product-ion mode (*i.e.* MS/MS) a range of *m/z* 50–2,000 was chosen. The *m/z* value of individual precursor ions was automatically selected in the information dependent acquisition (IDA) software feature for fragmentation and was collided under argon pressure using rolling collision energies ranging from 12 to 60 eV (*i.e.* collision induced dissociation, CID).

NMR data were obtained in DMSO-d6 in a BRUKER® Avance II 300: ^{1}H NMR (300.13 MHz) and ^{13}C NMR (75.47 MHz). Complete assignments of signals were accomplished with the aid of 1D (1H, 13C BB and DEPT) and 2D (HH-COSY, HSQC-Ed DEPT and HMBC) experiments using the standard BRUKER® software.

Transmission electron microscopy

Andrimid effect into the ultrastructure of sensitive strain was evaluated by using transmission electron microscopy (TEM) after treating *E. coli* ATCC 35218 with andrimid at MIC (Sánchez et al. 2010), during different incubation times (60, 120 and 240 min) on LB broth medium. Before and after treatment, a culture aliquot was reserved to determine viable-cell counts. Then, the sensitive strain was recovered by centrifugation 10,000×*g* during 10 min, and washed twice with sterile saline solution in order to remove remaining cell debris. Cells were fixed with 3 % (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) during 2 h and post-fixed with osmium tetroxide staining during 1 h before gradual dehydration in a graded acetone or ethanol series (30, 50, 70, 95 and 100 % v/v) steps. Then, they were included in Spurr's low-viscosity resin. Ultramicrotome thin sections were mounted on copper grids and contrasted with uranyl acetate and lead citrate (Venable and Coggeshall, 1965). Observations were performed on a Zeiss EM 109 electron microscope (Zeiss, Jena, Germany).

Results

The new andrimid-producer strain was previously isolated from soil samples collected during a sampling expedition in *Isla de los Estados*, Ushuaia, Argentina. It was already identified as *S. proteamaculans* 136 by using a polyphasic approach involving 16S and *gyrB* gene sequence analysis, microscopic and macroscopic morphology and physiological tests (Sánchez et al. 2009).

Effects of temperature on andrimid production

Antimicrobial production was evaluated in LB medium at different incubation temperatures after 7 days of incubation. The psychrotolerant *S. proteamaculans* producer strain showed to be able to regularly produce the antimicrobial molecule at 800 AU/ml in the 4–20 °C range. However andrimid activity titers of 400 UA/ml were also observed at 25 °C. Higher temperatures affected andrimid production, being undetectable at 30 °C, despite growth was observed. The highest temperature assayed (45 °C) affected both, growth and antimicrobial production.

Antimicrobial production

For purification and chemical characterization purposes, the antimicrobial was produced in a 7.5-l stirred tank bioreactor. Antimicrobial activity became detectable during early stationary growth phase at 200 AU/ml at 30 h of cultivation, reaching a maximum of 800 AU/ml after 40 h of incubation at 8 °C. The purified antimicrobial from this culture showed a sharp absorbance peak by PDA detection with a maximum at $\lambda = 298$ nm (see additional data in Online Resource 1). The pure product was lyophilized, thus yielding 30 mg of antimicrobial from 6.5 l of bioreactor culture.

Minimal inhibitory concentration

Purified andrimid fractions produced at low temperature (8 °C) by *S. proteamaculans* 136 showed a broad inhibition spectrum. Minimal inhibitory concentration (MIC) corresponded to the lowest concentration showing no turbidity increments (OD = 600 nm) in sensitive cultures after 48 h of incubation at the optimal growth temperature for each tested strain. MICs against several bacterial species were determined (Table 2) as described above from a stock solution of purified andrimid (100, 20 and 1 μg/ml).

Indicator strains surprisingly showed to be more susceptible when LB medium was used for their growth, and Gram negative bacteria appeared to be inhibited at lower MICs than Gram positive ones (Table 2). Growth inhibition at low temperature (4 °C) was determined by including a psychrophilic strain of *P. fluorescens*, showing that *S. proteamaculans* 136 andrimid retained 100 % activity at 4 °C and remained as active as to inhibit *P. fluorescens* growth at low temperatures.

Analysis of andrimid by chemical hydrolysis

In order to determine whether the molecule contained amino acid moieties, the pure antimicrobial was analysed by alkaline and acid hydrolyses. Both hydrolytic products were evaluated by TLC, revealing the presence of a few amino acids. That was expected taking into account its low molecular weight (479.2) which would be limited to no more than 4 or 5 amino acids. The TLC Rf analysis after

Table 2 MICs of sensitive strains

Sensitive strain	Growth temperature (°C)	MIC (μg/ml) in test medium	
		LB	M9-G[a]
Lactobacillus brevis	30	0.12	0.39
Leuconostoc mesenteroides	30	0.12	0.39
Micrococcus luteus	37	0.09	0.39
Staphylococcus epidermidis	37	0.09	0.09
Bacillus subtilis 168	30	DAP	0.06
Enterococcus faecium	37	0.50	0.78
Escherichia coli 25922	37	0.09	0.30
Escherichia coli O157:H7 (stx1, stx2)	37	DAP	0.04
Citrobacter freundii	37	0.06	0.15
Enterobacter cloacae	37	0.09	0.19
Pseudomonas fluorescens	4	0.07	0.12
Pseudomonas syringae	25	0.09	0.19
Rhizobium sp.	30	0.06	0.15

Inoculum:10^5 CFU/ml

DAP, Data already published in Sanchez et al. 2010

[a] M9-G, M9-Glucose

acid and alkaline hydrolysis showed signals that matched with phenylalanine (Online Resource 2; Column 8; Rf = 0.86) and either, valine (Online Resource 2; Column 2; Rf = 0.76) or methionine (Online Resource 2; Column 4; Rf = 0.79) whilst no signals were detected either after alkaline hydrolysis (Online Resource 2; Columns 11 and 12) or in non-hydrolyzed control samples (Online Resource 2; Column 13).

Structure elucidation: mass and NMR studies

Antimicrobial MS/MS data were analyzed by deconvolution, taking into account the natural abundance isotopes, using SIRIUS software. In order to determine the parent sum formula, *de novo* fragmentation-pattern reconstruction algorithms were used in the antimicrobial molecule under study (Böcker et al. 2009). From MS/MS experiments of the parental ion (479.2 m/z), six product ions were observed with 121, 195, 226, 239, 268, 343 m/z showing significant differences in the relative abundance of each ion type (Fig. 1). In order to obtain a fragmentation pattern useful to state the antimicrobial compound sum formula, mass data in addition to natural distribution of isotopes with their relative intensities in $[M + H]^+$ form were evaluated under SIRIUS software using an efficient algorithm to obtain a list of possible sum formula (Neumann and Böcker 2010).

In silico sum formula determination is the main step for identification of unknown metabolites and also for the reduction of possible structures to a convenient set. The presumed sum formulas obtained from MS/MS analysis are listed in Table 3.

The results were correlated with the data obtained from mass analysis using SIRIUS software to complete molecule structure elucidation (Fig. 2). The purified antimicrobial compound showed a white powder appearance with a slight pinkish shade. It was poorly soluble in D_2O and CD_3CN, whilst complete dissolution was achieved in DMSO-d6, reaching a concentration of 20 mg/ml. NMR studies for 27 ^{13}C and 33 ^{1}H signals were detected (Fig. 2; Online Resource 3).

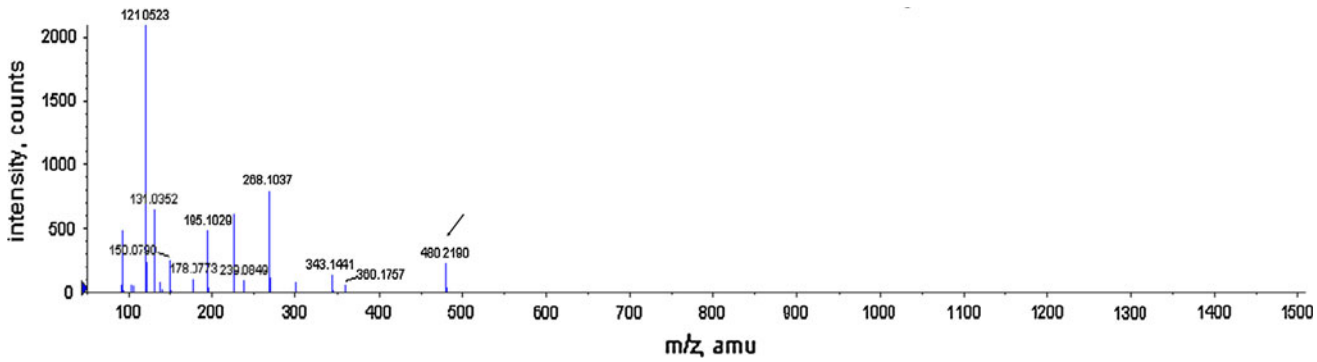

Fig. 1 MS/MS analysis of 480.2 m/z ion. The relative abundances of product ions were used under an algorithm-based process (deconvolution) to reverse the convolution effects of recorded data

Table 3 Possible sum formula obtained by mass deconvolution data using SIRIUS software

Position	Sumformula	Monoisotopic mass (Da)	Deviation	Score
1	$C_{27}H_{33}N_3O_5$	479.242022	+0.000387	80.4
2	$C_{27}H_{34}N_3OPS$	479.216023	+0.000387	50.9
3	$C_{27}H_{25}N_7O_2$	479.206975	+0.000384	38.6
4	$C_{27}H_{33}N_3OS_2$	479.206505	−0.000383	23.2
5	$C_{22}H_{42}NO_2PS$	479.211525	−0.000382	19.9

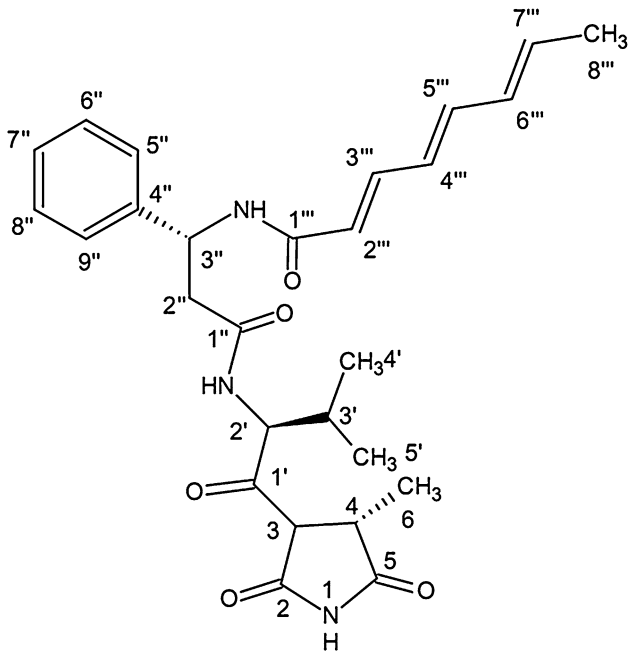

Fig. 2 Deduced antimicrobial structure and simulated spectra from NMR data analysis by ACD Labs software package (Advanced Chemistry Development Inc., Toronto, Ontario, Canada)

Antimicrobial effects over a sensitive strain

Figure 3 shows sensitive *E. coli* ATCC 35218 cells after MIC andrimid treatment during the exponential growth phase on LB broth medium, which resulted in clear morphological changes compared to the untreated control (Fig. 3a). After 1 h of adding the antimicrobial, the presence of early membrane disruption in the sensitive bacterial surface was observed, thus leading to the loss of cytoplasmic content (Fig. 3b). After 2 h of treatment, several cells with damaged membranes and electron-clear zones were observed (Fig. 3c). In addition, optical density measurements and cell counts throughout cultivation were close to the inoculum size, revealing that the antibiotic would be extremely toxic at MIC concentrations. After 4 h of incubation, *E. coli* ATCC 35218 cell membrane showed to be extensively damaged. A significant loss of cytoplasmic cell content was additionally observed in the form of electron-clear zones (Fig. 3d).

Discussion

Temperature-dependent production of andrimid by *S. proteamaculans* 136 was observed at shake flask scale after 7 days of incubation, cultivation time was selected based on previous time course data of andrimid production (Sanchez et al. Sánchez et al. 2010).

Production of secondary metabolites in a temperature-dependent mode was previously reported in *S. marscecens.* Production of the red-pigment *prodigiosin* (Yu 1979; Williams and Quadri 1980; Thomson et al. 2000) and the biosurfactant *serrawettin* (Matsuyama et al. 1986, 1995) were reported at 30 °C but not at 37 °C. Oppositely, temperature-dependent bacteriostatic activity affecting *S. marscecens* itself and other bacterial species was detected at 37 °C but not at 30 °C (Tanaka et al. 2004). Unfortunately, there are not available data about the production of these biomolecules at low temperatures. Recently, Pesciaroli et al. (2012) reported the isolation of three *S. proteamaculans* strains from the White Sea, Russia, along with two *P. agglomerans* strains, showing a wide range of growth temperature (0–40 °C) suggesting that such ubiquitous microorganisms share a common habitat in the environment and horizontal genetic transfer could be possible among them.

The elucidated structure obtained from NMR data, for the antimicrobial produced by *S. proteamaculans* 136 strain (Fig. 2) would be in agreement with those ^{1}H and ^{13}C NMR data reported by Needham et al. (1994).

Andrimid was firstly described by Fredenhagen et al. (1987) as an antimicrobial compound isolated from an intracellular symbiont of *N. lugens* (brown planthopper). This first andrimid structure description, in association to the specific activity against *Xanthomonas campestris* showed structural similarity to the antimicrobial of *S. proteamaculans* 136 herein described.

Previous works also described andrimid activity on a diverse group of Gram-negative bacterial strains. Needham et al. (1994) described by NMR a group of molecules structurally similar to andrimid (moiramids A, B and C) isolated from a marine *P. fluorescens* strain. Concurrently, Oclarit et al. (1994) reported andrimid production by the strain M22-1 belonging to *Vibrio* sp. in association with marine sponges (*Hyatella* sp.). Later, Long et al. (2005) reported its production by *Vibrio cholerae* strains, whilst Jin et al. (2006) and Wietz et al. (2010) have recently reported andrimid production by *P. agglomerans* and *Vibrio coralliilyticus* strains, respectively.

Our results confirm the cosmopolitan occurrence of andrimid production in distant affiliated strains (Mansson

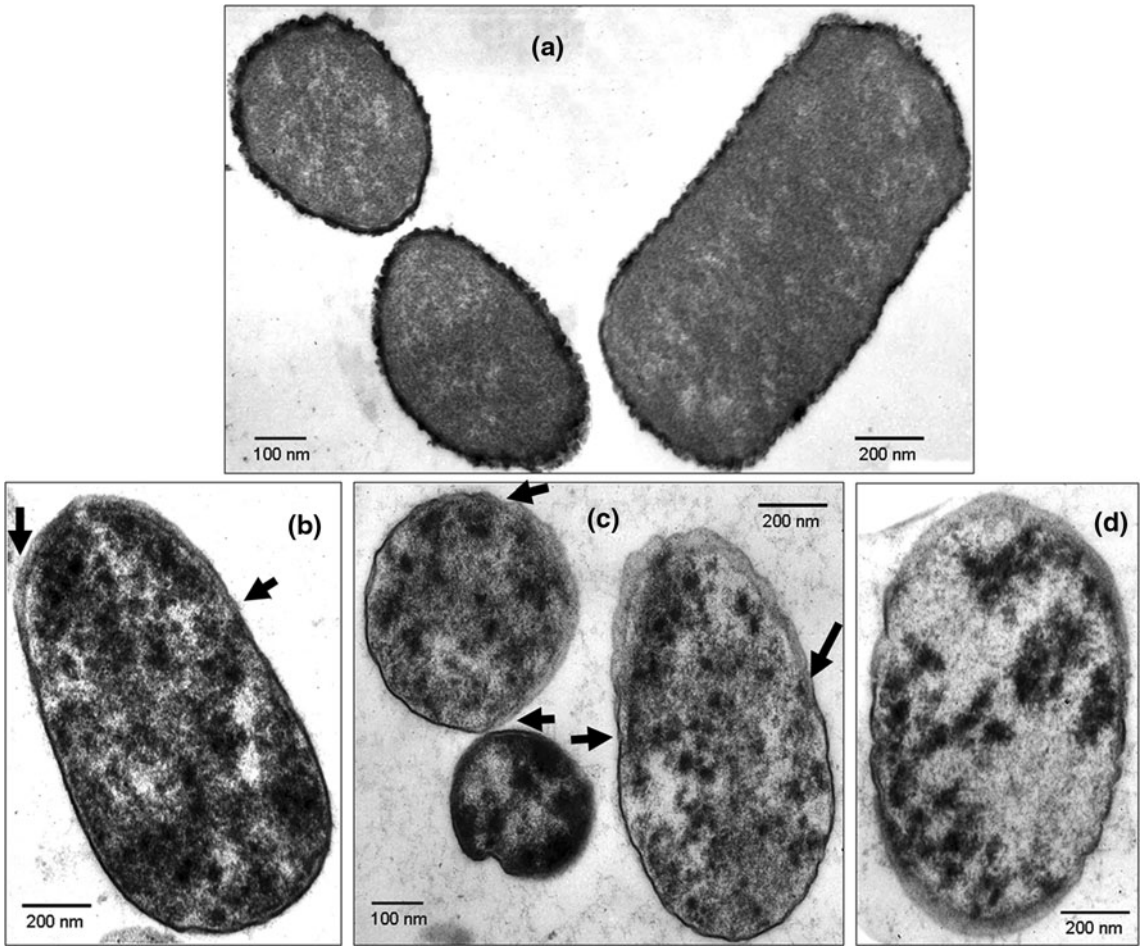

Fig. 3 Transmission electron micrograph showing morphological changes of *E. coli* ATCC 35218 cells after andrimid addition during exponential growth phase (30,000× magnifications) in LB medium. **a** Control without andrimid treatment. **b** After 60 min of treatment, showing morphological changes in bacterial surface (*area between arrows*). **c** After 120 min, displaying loss of cytoplasmic content (electron-clear zones) and membrane disruption (*area between arrows*). **d** After 240 min, with most of cytoplasmic content and cell membrane integrity affected respect to the untreated control

et al. 2011). The work, also highlights andrimid production and antimicrobial activity at low temperatures and contribute to expand the broad inhibition spectrum beyond the limits previously reported (Singh et al. 1997; Wietz et al. 2010). It involves the addition of *M. luteus, Lact. brevis, Leuc. mesenteroides, Staph. epidermidis, S. aureus* and *Enterococcus faecium* as Gram-positive sensitive strains and *P. aeruginosa*, *Sh. flexneri, Sh. sonnei, L. monocytogenes, Cit. freundii, Enterobacter cloacae, S. enterica* and the shiga-toxin producer *E. coli* O157:H7 as Gram-negative sensitive strains, under mesophile conditions. Interestingly, low temperature activity against *P. fluorescens* was also observed. In addition, inhibition of *P. syringae* (plant pathogen) and *Rhizobium* sp. (endosymbiotic bacterium) indicates its potential application as agroceutical for plant pathogen biocontrol.

Andrimid antimicrobial activity is related to fatty acid biosynthesis interference involving its two terminal subunits, pyrrolidinedione ring group and fatty acid side chain (Pohlmann et al. 2005). Consistent with this report, both subunits were also identified in the andrimid produced by *S. proteamaculans* 136 in the present work. The MS/MS fragmentation patterns were in agreement with those described by Fredenhagen et al. (1987), since we showed that daughter ions with m/z of 121 and 195 correlated with the pyrrolidinedione ring and the fatty acid tail, respectively.

Andrimid mode of action was described by Freiberg et al. (2004) as interfering with biosynthesis of fatty acid membrane by inhibiting the carboxyltransferases of bacterial ACC. In agreement, critical effects of andrimid produced by *S. proteamaculans* 136 were observed over sensitive bacterial cell membranes by TEM. These effects might be likely related to the mode of action, however further studies will corroborate this hypothesis.

At present, we were not able to in silico identify andrimid biosynthetic genes in *S. proteamaculans* 568

(genome completely sequenced) by using conserved regions of specific enzymes belonging to the biosynthetic cluster of *P. agglomerans* (AY192157) and *anrF* of *E. cloacae* (AY633625) available in databases. Biosynthetic gene clusters are often horizontally transferred because small molecules confer a selective advantage to the host, like antibiotic resistance genes (Holden et al. 2004), which are transmitted by specific genetic elements such as pathogenicity islands (Hacker et al. 1997) or plasmids (Szczepanowski et al. 2005). Further studies will help to evaluate the presence of the andrimid biosynthetic gene cluster in the psychrotolerant *S. proteamaculans* 136 strain.

The findings herein reported give evidence of the potential for low-temperature andrimid production by the selected psychrotolerant strain *S. proteamaculans* isolated from sub-Antarctic environments. It represents a valuable alternative to the conventional antibiotic producers described so far, and a novel and promising source for antibacterial compounds. This work highlights the importance of exploring environmental biodiversity for the discovery of biologically active natural products.

Furthermore, the possibility to maintain full antibacterial activity at low temperatures implies an additional benefit that has gained attention in the latter years, particularly when antibiotic activity is required for some applications e.g., cryopreservation, cold chain maintaining, cosmetic or food industry (O'Brien et al. 2004). The direct pursuit for active metabolites under specific conditions (in samples from appropriate environments) has turned into an expanding area of research which led to the study of extremophile microorganisms (Antranikian et al. 2005). These microorganisms constitute a potential source of unexplored natural products, varying from the conventional ones, in key aspects such as chemical structure and physiological activities (Hugenholtz and Pace 1996).

References

Antranikian G, Vorgias CE, Bertoldo C (2005) Extreme environments as a resource for microorganisms and novel biocatalysts. Adv Biochem Eng Biotechnol 96:219–262

Aravalli RN, She Q, Garrett RA (1998) Archaea and the new age of microorganisms. Trends Ecol Evol 13:190–194

Böcker S, Letzel MC, Liptak Z, Pervukhin A (2009) SIRIUS: decomposing isotope patterns for metabolite identification. Bioinformatics 25:218

Davies J, Webb V (1998) Antibiotic resistance in bacteria. In: Krause RM (ed) Emerging infections. Biomedical research reports San Diego. Academic Press, CA

Feller G, Zekhnini Z, Lamotte-Brasseur J, Gerday C (1997) Enzymes from cold-adapted microorganisms the class C ß-lactamase from the antarctic psychrophile *Psychrobacter immobilis* A5. Eur J Biochem 244:186–191

Fischbach MA (2009) Antibiotics from microbes: converging to kill. Curr Opin Microbiol 12:520–527

Fredenhagen A, Tamure SY, Kenny PTM, Komura H, Naya Y, Nakanishi K (1987) Andrimid, a new peptide antibiotic produced by an intracellular bacterial symbiont isolated from a brown planthopper. J Am Chem Soc 109:4409–4411

Freiberg C, Brunner N, Schiffer G, Lampe T, Pohlmann J, Brands M, Raabe M, Häbich D, Ziegelbauer K (2004) Identification and characterization of the first class of potent bacterial acetyl-CoA carboxylase inhibitors with antibacterial activity. J Biol Chem 279:26066–26073

Gootz TD (1990) Discovery and development of new antimicrobial agents. Clin Microbiol Rev 1:13–31

Hacker J, Blum-Oehler G, Mühldorfer I, Tschäpe H (1997) Pathogenicity islands of virulent bacteria: structure, function and impact on microbiol evolution. Mol Microbiol 23:1089–1097

Holden MTG, Feil EJ, Lindsay JA, Peacock SJ, Day NP, Enright MC, Foster TJ, Moore CE, Hurst L, Atkin RA, Barron A, Bason N, Bentley SD, Chillingworth C, Chillingworth T, Churcher C, Clark L, Corton C, Cronin A, Doggett J, Dowd L, Feltwell T, Hance Z, Harris B, Hauser H, Holyroyd S, Jagels K, James KD, Lennard N, Line A, Mayes R, Moule S, Mungall K, Ormond D, Quail MA, Raddinowitsch E, Rutherford K, Sanders M, Sharp S, Simmonds M, Stevens K, Whitehead S, Barrell BG, Parkhill J (2004) Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci USA 101:9786–9791

Hugenholtz P, Pace NR (1996) Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. Trends Biotechnol 14:190–197

Jin M, Fischbach MA, Clardy J (2006) A biosynthetic gene cluster for the acetyl-CoA carboxylase inhibitor andrimid. J Am Chem Soc 128:10660–10661

Kabuki T, Uenishi H, Watanabe M, Seto Y, Nakajima H (2007) Characterization of a bacteriocin, Thermophilin 1277, produced by *Streptococcus thermophilus* SBT1277. J Appl Microbiol 102:971–980

Liu XY, Fortin PD, Walsh CT (2008) Andrimid producers encode an acetyl-CoA carboxyltransferase subunit resistant to the action of the antibiotic. Proc Natl Acad Sci USA 105:13321–13326

Long RA, Rowley DC, Zamora E, Liu JY, Bartlett DH, Azam F (2005) Antagonistic interactions among marine bacteria impede the proliferation of *Vibrio cholerae*. Appl Environ Microbiol 71:8531–8536

Mansson M, Gram L, Larsen TO (2011) Production of bioactive secondary metabolites by marine Vibrionaceae. Marine drugs 9:1440–1468

Matsuyama T, Murakami T, Fujita M, Fujita S, Yano I (1986) Extracellular vesicle formation and biosurfactant production by *Serratia marcescens*. J Gen Microbiol 132:865–875

Matsuyama T, Bhasin A, Harshey RM (1995) Mutational analysis of flagellum-independent surface spreading of *Serratia marcescens* 274 on a low-agar medium. J Bacteriol 177:987–991

Mayr-Harting A, Hedges A, Berkeley RCW (1972) Methods for studying bacteriocins. In: Norris JR, Ribbons DW (eds) Methods in microbiology 7a. Academic Press, London, pp 315–422

Needham J, Kelly MT, Ishige M, Andersen RJ (1994) Andrimid and moiramides A-C, metabolites produced in culture by a marine isolate of the bacterium *Pseudomonas fluorescens*: structure elucidation and biosynthesis. J Org Chem 59:2058–2063

Neumann S, Böcker S (2010) Computational mass spectrometry for metabolomics Identification of metabolites and small molecules. Anal Bioanal Chem 398:2779–2788

O'Brien A, Sharp R, Russell N, Roller S (2004) Antarctic bacteria inhibit growth of food-borne microorganisms at low temperatures. FEMS Microbiol Ecol 48:157–167

Oclarit JM, Okada H, Ohta S, Kaminura K, Yamaoka Y, Lizuka T, Miyashiro S, Ikegami S (1994) Anti-bacillus substance in the

marine sponge, *Hyatella* species, produced by an associated *Vibrio* species bacterium. Microbios 78:7–16

Pesciaroli C, Cupini F, Selbmann L, Barghini P (2012) Temperature preferences of bacteria isolated from seawater collected in Kandalaksha Bay, White Sea, Russia. Polar Biol 35:435–445

Pohlmann J, Lampe T, Shimada M, Nell PG, Pernerstorfer J, Svenstrup N, Brunner NA, Schiffer G, Freiberg C (2005) Pyrrolidinedione derivatives as antibacterial agents with a novel mode of action. Bioorg Med Chem Lett 15:1189–1192

Prangishvili D, Holz I, Stieger E, Nickell S, Kristjansson JK, Zillig W (2000) Sulfolobicins, specific proteinaceous toxins produced by strains of the extremophilic archaeal genus Sulfolobus. J Bacteriol 182:2985–2988

Sánchez LA, Gomez FF, Delgado OD (2009) Cold-adapted microorganisms as a source of new antimicrobials. Extremophiles 13:111–120

Sánchez LA, Hedström M, Delgado M, Delgado OD (2010) Production, purification and characterization of Serraticin A, a novel cold-active antimicrobial produced by *Serratia proteamaculans* 136. J Appl Microbiol 109:936–945

Savino JM, Sanchez LA, Saguir FM, Manca de Nadra MC (2012) Lactic acid bacteria isolated from apples are able to catabolise arginine. World J Microbiol Biotechnol 28:1003–1012

Singh MP, Menendez AT, Petersen PJ, Ding WD, Maiese WM, Greenstein M (1997) Biological and mechanistic activities of phenazine antibiotics produced by culture LL-14I352. J Antibiot 50:785–787

Smith-Palmer A, Stewart J, Fyfe L (1998) Antimicrobial properties of plant essential oils and essences against five important foodborne pathogens. Lett Appl Microbiol 26:118–122

Souza NJ, Ganguli BN, Reden J (1982) Strategies in the discovery of drugs from natural sources. Annu Rep Med Chem 17:301–310

Spizek J, Novotna J, Rezanka T, Demain AL (2010) Do we need new antibiotics? The search for new targets and new compounds. J Ind Microbiol Biotechnol 37:1241–1248

Szczepanowski R, Braun S, Riedel V, Schneiker S, Krahn I, Puhler A, Schlüter A (2005) The 120 592 bp IncF plasmid pRSB107 isolated from a sewage-treatment plant encodes nine different antibiotic-resistance determinants, two iron-acquisition systems and other putative virulence-associated functions. Microbiology 151:1095–1111

Tanaka Y, Yuasa J, Baba M, Tanikawa T, Nakagawa Y, Matsuyama T (2004) Temperature-dependent bacteriostatic activity of *Serratia marcescens*. Microbes Environ 19:236–240

Thomson NR, Crow MA, Crow SJ, McGowan A, Salmond GPC (2000) Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. Mol Microbiol 36:539–556

Venable JH, Coggeshall R (1965) A simplified lead citrate stain for electron microscopy. J Cell Biol 25:407–408

Wenzel RP (2004) The antibiotic pipeline challenges, costs, and values. New Engl J Med 351:523–526

Wietz M, Mansson M, Gotfredsen CH, Larsen TO, Gram L (2010) Antibacterial compounds from marine Vibrionaceae isolated on a global expedition. Mar Drugs 8:2946–2960

Wietz M, Månsson M, Gram L (2011) Chitin stimulates production of the antibiotic andrimid in a *Vibrio coralliilyticus* strain. Environ Microbiol Rep 3(5):559–564

Williams RP, Quadri SMH (1980) The pigment of *Serratia*. In: von Gravenitz A, Rubin SJ (eds) The genus Serratia. CRC Press, Boca Raton, Fla, pp 31–75

Yaron S, Rydlo T, Shachar D, Mor A (2003) Activity of dermaseptin K4–S4 against foodborne pathogens. Peptides 24:1815–1821

Yu VL (1979) Serratia marcescens, historical perspective and clinical review. New Engl J Med 300:887–893

Zahner H, Fielder HP (1995) The need for new antibiotics: possible ways forward In: Hunter PA, Darby GK, Russell NJ (eds) Fifty years of antimicrobials: past perspectives and future trends. Cambridge SGM Symp, vol. 53. Cambridge University Press, pp 67–83