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ORIGINAL ARTICLE

Purification and partial characterization of a bacteriocin produced by *Eikenella corrodens*

A.C.M. Apolônio[1], M.A.R. Carvalho[1], M.P. Bemquerer[2], M.M. Santoro[2], S.Q. Pinto[1], J.S. Oliveira[2], K.V. Santos[1] and L.M. Farias[1]

1 Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
2 Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

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bacteriocin, corrodecin, *Eikenella corrodens*, oral microbiota, periodontal disease.

Correspondence
Luiz de Macêdo Farias, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, C.P. 486, 30161-970 Belo Horizonte, MG, Brazil.
E-mail: macedo@icb.ufmg.br

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Abstract

Aims: The purpose of this study was to purify and characterize a bacteriocin produced by *Eikenella corrodens* A32E2.

Methods and Results: *Peptostreptococcus anaerobius* ATCC27337 was used as indicator strain in antagonistic assays for bacteriocin-producing *E. corrodens* A32E2. Protein extraction was influenced by pH and buffer composition. The protein was active in the pH range 6–8. Inhibitory activity was lost by both heating and treatment with proteolytic enzymes and decreased with organic solvents. The substance is rather unstable but maintains 100% of its activity after being exposed to acetone and when stored at −70°C. The antagonistic substance was first precipitated by ammonium sulfate and further partially purified by Mono-Q FPLC and C-18 HPLC. Mass spectrometry analysis showed that the molecular mass was 23 625 Da, and the sequence obtained for the N-terminus was: Met-Asn-Phe-Asp-Glu-Lys-Val-Gly-Lys-Val-X-Phe-Lys-Val-Gly-Asp.

Conclusions: The evidence presented in this study supports the idea that an antagonistic substance produced by *E. corrodens* A32E2 isolated from a periodontal diseased site is a novel bacteriocin, which we designate corrodecin.

Significance and Impact of the Study: We anticipated that corrodecin might play an important role at the periodontal site. This compound could also be attractive in biotechnological applications as an interesting tool for oral ecosystem control.

Introduction

Many different strategies are currently being explored to identify new antimicrobial agents, and the area of antibacterial peptides is under intensive investigation. Among the most promising antibacterial peptides are the bacteriocins (Sparo *et al.* 2006).

Bacteria of different taxa that inhabit different habitats produce antimicrobial substances that are active against other bacteria (Riosen *et al.* 2004), including oral cavity bacteria (Lima *et al.* 2002). Bacteriocins produced by lactic acid bacteria and colicins are among the better studied and well characterized ones, both concerning their structure and function and their mechanism of action (Cotter *et al.* 2005; Cascales *et al.* 2007). Because these antagonistic products exhibited a wide spectrum of activity against oral and nonoral Gram-negative and Gram-positive bacteria, it is likely that they possess clinical and microbiological relevance in the oral ecosystem. Bacteriocin production has been described for some putative oral pathogens, such as *Streptococcus* spp. (Kelstrup and Gibbons 1969), *Actinobacillus* (*Aggregatibacter*) *actinomycetemcomitans* (Hammond and Lillard 1987; Stevens *et al.* 1987; Miranda *et al.* 1996; Lima *et al.* 2002), *Prevotella nigrescens* (Kaewsrichan *et al.* 2004) and *Fusobacterium nucleatum* (Oliveira *et al.* 1998). Nevertheless, despite the many virulence factors exhibited by *E. corrodens*, such as lipopolysaccharides, proteins of the

outer membrane, adhesins and the exopolysaccharide layer (Chen and Wilson 1992), antagonistic substances produced by this bacterium have only recently been reported (Apolônio *et al.* 2007).

The present study was focused on purifying and partially characterizing a bacteriocin produced by *E. corrodens* strain A32E2, which was isolated from a patient with chronic periodontitis.

Materials and methods

Bacterial strains and media

The bacteriocin-producing strain used in this study was *E. corrodens* A32E2, which was isolated from a human subject with chronic periodontitis (Collection of the Laboratory of Oral Microbiology and Anaerobe, Universidade Federal de Minas Gerais). This strain was selected because of its high antagonistic action towards all previously tested indicator strains (Apolônio *et al.* 2007). Micro-organism identification was performed using biochemical and physiological methods (Holt *et al.* 1994) and was confirmed by polymerase chain reaction (Slots *et al.* 1995). *Peptostreptococcus anaerobius* ATCC 27337 was used as an indicator strain.

The strains were stored at −80°C in brain heart infusion (BHI) (Difco) with 25% (v/v) glycerol. Before each experiment, the micro-organisms were subcultured twice in tryptic soy agar (Difco) supplemented with 5% (v/v) horse blood, 0·05 mg ml^{-1} hemin and 0·01 mg ml^{-1} menadione (referred to as TSA-S) at 37°C for 48 h. Tryptic soy agar supplemented with 0·1% (p/v) L-cistine, 0·05 mg ml^{-1} hemin and 0·01 mg ml^{-1} menadione, pH 7·2 (referred to as TSA) was used for bacteriocin production; BHI supplemented with 0·5% yeast extract, 0·05 mg ml^{-1} hemin and 0·01 mg ml^{-1} menadione (referred to as BHI-S) was used for the indicator strain; and BHI-S added with 0·7% granulated agar (Difco) was used as soft agar.

Determination of bacteriocin activity

Bacteriocin activity was assayed by the agar diffusion method, and the bacteriocin titre was determined by serial dilution, as previously described (Mayr-Harting *et al.* 1972; Batdorj *et al.* 2006). Aliquots of filtrate extracts were serially diluted twofold with sterilized ultra-pure water in microtitration plates (Micrower Plate 96F; Greiner Bio-one, Frickenhausen, Germany). Petri dishes with TSA were overlaid with soft agar (4 ml) after addition of 200 μl of a 24-h culture of *P. anaerobius* ATCC 27337. After drying for 10 min, 10 μl of each diluted sample was spotted onto the plates. Plates were incubated at 37°C for 24–48 h under anaerobic conditions, and the titre was defined as 2^n, where n is the reciprocal of the highest dilution that resulted in inhibition of the indicator strain. Thus, the arbitrary unit (AU) of antibacterial activity per millilitre (AU ml^{-1}) was defined as $2^n \times 1000\ \mu l \times v\ \mu l^{-1}$, where v is the volume of bacteriocin used in the test.

Determination of protein concentration

The protein concentration was determined according to Bradford (1976). Bovine serum albumin was used as a standard.

Influence of buffers and pH values on protein extraction

To assay the influence of buffers and pH values on partially purified bacteriocin, a 24-h culture of *E. corrodens* A32E2 incubated at 37°C in an anaerobic chamber (85% N_2, 10% H_2 and 5% CO_2; Forma Scientific Company, Marietta, OH, USA) was scraped and resuspended in 10 mmol l^{-1} acetate buffer pH 4·0 and 5·0, 10 mmol l^{-1} citrate buffer pH 6·0 and 10 mmol l^{-1} Tris–HCl pH 7·2 and 8·0. The cell suspension was sonicated (Branson Sonifier 450; VWR Scientific, Danburg, USA) for 15 min, with 15 cycles of 60 s at 40 W in an ice bath. Cellular disruption was verified under an optic microscope after being stained by the Gram method. The extract was then centrifuged at 10 000 ***g*** for 30 min at 4°C, and the supernatant was precipitated with ammonium sulfate (Labsynth, São Paulo, Brazil) at three increasing concentration ranges (0–50, 50–75 and 75–100%), respectively, referred to as C-50, C-75 and C-100, with constant stirring for 2 h at 4°C. After centrifugation at 10 000 ***g*** for 30 min, the precipitates were dissolved in 5 ml of the extraction buffer and dialysed against the same buffer at 1 mmol l^{-1} for 24 h. The obtained fractions were assayed for antagonistic activity as described above.

Bacteriocin purification

The active fraction obtained by precipitation with ammonium sulfate (C-50) using 10 mmol l^{-1} Tris–HCl, pH 8·0 was called partially purified bacteriocin (PPB) and was used for characterization studies. The PPB was submitted to ion-exchange chromatography on a fast protein liquid chromatography (FPLC) system equipped with a Mono-Q HR 5/5 (Pharmacia, Uppsala, Sweden) column, equilibrated with 10 mmol l^{-1} Tris–HCl buffer (pH 8·0) and eluted with the same buffer, followed by a gradient from 0 to 1 mol l^{-1} NaCl in Tris–HCl buffer, pH 8·0. Aliquots (1·5 ml) were collected, lyophilized and assayed for antagonistic activity. The active aliquots were individually submitted to reverse phase chromatography on a high protein liquid chromatography (HPLC) system equipped with a C18 (Pharmacia) column (300 × 4·6 mm). Mobile phases A and B were 0·1% trifluoroacetic acid (TFA) and

50% aqueous acetonitrile solution containing 0·1% TFA, respectively. Aliquots were collected, concentrated and assayed as previously described. Elution profiles were monitored at 280 nm using both FPLC and HPLC analysis. Active fractions were freeze-dried.

Effect of different treatments on bacteriocin activity

The residual bacteriocin activity in PPB samples was determined after each treatment as previously described. Assays were carried out twice. The following treatments were applied.

pH variations
The activity of bacteriocin at different pH values was estimated after dilution and storage of PPB for 24 h at 37°C in buffer solutions (1 : 1, v/v) ranging from pH 3 to pH 11. Controls were PPB without buffer, PPB with sterile water (1 : 1 v/v) and buffers without PPB. The following buffers were employed: 0·1 mol l^{-1} citrate for pH 3·0 and pH 6·0; 0·1 mol l^{-1} acetate for pH 4·0 and 5·0; and 0·1 mol l^{-1} Tris–HCl for pH 7·2, 8·0, 9·0, 10·0 and 11·0.

Temperature
The effect of temperature was evaluated by incubating PPB for 15 min up to 24 h at 5, 20, 25, 37, 45, 70 and 100°C and for 60 days at –5, –10, –20, –40 and −70°C. Residual activity was then assayed against the indicator strain.

Enzymes
Incubations with proteolytic enzymes were performed for 4 h at 37°C in a 1 : 1 (v/v) diluted solution of PPB with one of the following enzyme solutions at 1 mg ml^{-1}: proteinase K in 20 mmol l^{-1} Tris–HCl, pH 7·2; trypsin in 20 mmol l^{-1} Tris–HCl, pH 8·0; α-chymotrypsin in 20 mmol l^{-1} Tris–HCl, pH 8·0; or papain in 50 mmol l^{-1} phosphate buffer, pH 7·0. Before use, all enzymes (Sigma, St Louis, MO, USA) were sterilized through a 0·22-μm filter. Untreated samples, buffer alone and enzyme solutions alone were used as controls.

Organic solvents
The effect of some organic solvents was evaluated after incubation for 1 h at 25°C in PPB diluted at a 1 : 1 ratio with the following aqueous solutions (10% by volume): acetone, butanol, ethanol, methanol and chloroform. As a control, untreated samples of PPB and aqueous solvent solutions alone were employed.

Mass spectrometry

ESI-Q-TOF (electrospray-quadrupole-time of flight) mass spectrometry analyses were carried out using a Q-TOF Micro™ (Micromass, Manchester, UK) equipped with an electrospray ionization source operated in positive or negative ion mode. Capillary voltage was 3·0–3·5 kV and sample cone voltages were 40–60 V. Samples were diluted in 50% acetonitrile/0·1% TFA in Milli-Q® (Millipore, Billerica, MA) water and directly injected in the spectrometer by using a syringe pump with flow rates of 5–10 μl min^{-1}. Data were analysed using MassLynx® 3·5 software (Micromass).

Determination of N-terminal amino acid sequences

Protein sequence was determined by Edman degradation using a Shimadzu PPSQ-21A automated protein sequencer (Shimadzu, Kyoto, Japan) coupled to reversed phase separation of PTH-amino acids on a WAKOSIL-PTH (4·6 × 250 mm) column (Wako, Osaka, Japan) at 1 ml min^{-1} with detection at 235 nm.

Sequence comparisons

The amino acid sequences of the various peptides/proteins were compared with the sequences of other related proteins in the Swiss-Prot/TrEMBL databases using FASTA 3 and BLAST.

Results

Influence of buffers and pH values on protein extraction

The fractions obtained with acetate buffer were inactive against the indicator strain *P. anaerobius* ATCC 27337 at both pH 4 and pH 5. With the other buffers, only the C-50 fraction exhibited antagonistic activity. At pH 6·0, no activity was observed with acetate buffer (data not shown), but the C-50 fraction obtained with citrate buffer showed a specific inhibitory activity of 27 AU mg^{-1} protein. Similar values were obtained for Tris–HCl buffer at pH 7·2, 20 AU mg^{-1} protein. However, optimal experimental conditions were observed at pH 8·0, in which the protein showed a specific activity of 114 AU mg^{-1} protein.

Effect of different treatments on bacteriocin activity

The PPB was active in pH range 6–8 until 6 h of incubation. The inhibitory activity of the extract was lost either by heating at temperatures higher than 45°C for 15 min or by treatment with all the proteolytic enzymes tested (Table 1). Bacteriocin activity could be preserved by long-term storage at −70°C (>60 days). Among the organic solvents assayed, treatment of the PPB extract with a 10% aqueous acetone solution (by volume) did not give rise to loss of bacteriocin activity, but activity was significantly reduced by treatment with alcohols or chloroform (Table 1).

Table 1 Properties of the partially purified bacteriocin (PPB)

Treatment*	Bacteriocin activity (AU mL^{-1})
None (PPB)	25 600
Trypsin	0†
A-Chymotrypsin	0
Proteinase K	0
Papain	0
Acetone	25 600
Butanol	100
Ethanol	400
Methanol	400
Chloroform	400
100°C/15 min	0
70°C/15 min	0
45°C/4 h	100
37°C/24 h	100
25°C/24 h	1600
20°C/24 h	1600
5°C/24 h	1600
−5°C/14 days	100
−10°C/30 days	100
−20°C/30 days	100
−40°C/60 days	400
−70°C/60 days	25 600
pH 6/6 h	100
pH 7/6 h	100
pH 8/6 h	100
pH 9/15 min	100
pH 10/15 min	100

*Enzymes were used at 1 mg mL^{-1}; organic solvents were used at 10% by volume.
†The inhibitory activity was totally lost.

Bacteriocin purification

The antimicrobial substance produced by *E. corrodens* A32E2 was precipitated with 50% ammonium sulfate (0–50%). When PPB was applied to an anion-exchange Mono-Q column, the protein peak eluted at nearly 120 mmol l^{-1} NaCl (fractions 13–16) contained the antimicrobial activity (Fig. 1).

Additional studies showed that, when active fractions from the Mono-Q column were pooled and applied to a C-18 reverse phase chromatography column, the activity of bacteriocin was retained at a fraction that eluted with 58·5% acetonitrile. This observation suggests that the bacteriocin is a hydrophobic protein, which is common among this class of antagonistic substances.

Mass spectrometry

Mass spectrometry analysis revealed that the molecular mass of the purified protein was 23 625 Da (Fig. 2).

Determination of the amino acid sequence

N-terminal sequencing of the 23 625 Da protein yielded the following sequence: Met-Asn-Phe-Asp-Glu-Lys-Val-Gly-Lys-Val-X-Phe-Lys-Val-Gly-Asp. The 'X' probably represents a lysine residue or a modified amino acid. BLAST searches using different protein databases did not yield any significantly similar matches.

Discussion

Within the last several years, studies of bacteriocins have attracted significant attention because of their potential use in many fields, such as food preservatives (Chen and Hoover 2003). Concerning oral micro-organisms, many bacterial strains have been reported to produce bacteriocins or bacteriocin-like substances (Farias *et al.* 1994; Lima *et al.* 2002; Kaewsrichan *et al.* 2004). However, the production of these substances by *E. corrodens* has only recently been reported (Apolônio *et al.* 2007).

The results obtained with protein extraction using different buffers suggest that, even at low concentrations, medium pH affects the extraction efficiency, which is optimal at pH 8·0 with Tris–HCl. A higher specific bacteriocin activity was obtained with Tris–HCl buffer extraction at pH 8·0 (114 AU mg^{-1} protein) than at pH 7·2, which yielded 20 AU mg^{-1} protein.

It is accepted that the optimum pH value for bacteriocin production could be different from that required to guarantee the stability of the antagonistic compound (Bromberg *et al.* 2006). Despite the observation that bacteriocin production by *E. corrodens* strains was not statistically influenced by pH values (data not shown), it was observed that pH values near neutral were better for retaining protein stability (Table 1).

The bacteriocin studied was thermally labile as heating at temperatures higher than 45°C for 15 min vanished its inhibitory activity. Nevertheless, it exhibited activity at low temperatures and the protein maintains its initial activity when it was kept at −70°C. This property is quite interesting because bacteriocins are used for the preservation of foods and industrializing products. As described for entomocin 110 (Cherif *et al.* 2006), bacteriocin activity was not completely lost by organic solvents, which indicates that the activity does not require lipid moieties, as proposed for other bacteriocins (class IV bacteriocins). Acetone treatment did not affect the initial activity. The inhibitory activity of this antagonistic substance was totally lost after treatment with either proteinase K, papain, trypsin or α-chymotrypsin, thereby revealing its proteinaceous nature. Other bacteriocins, such as cerein 8A (Bizani *et al.* 2005), are also susceptible to these same enzymes.

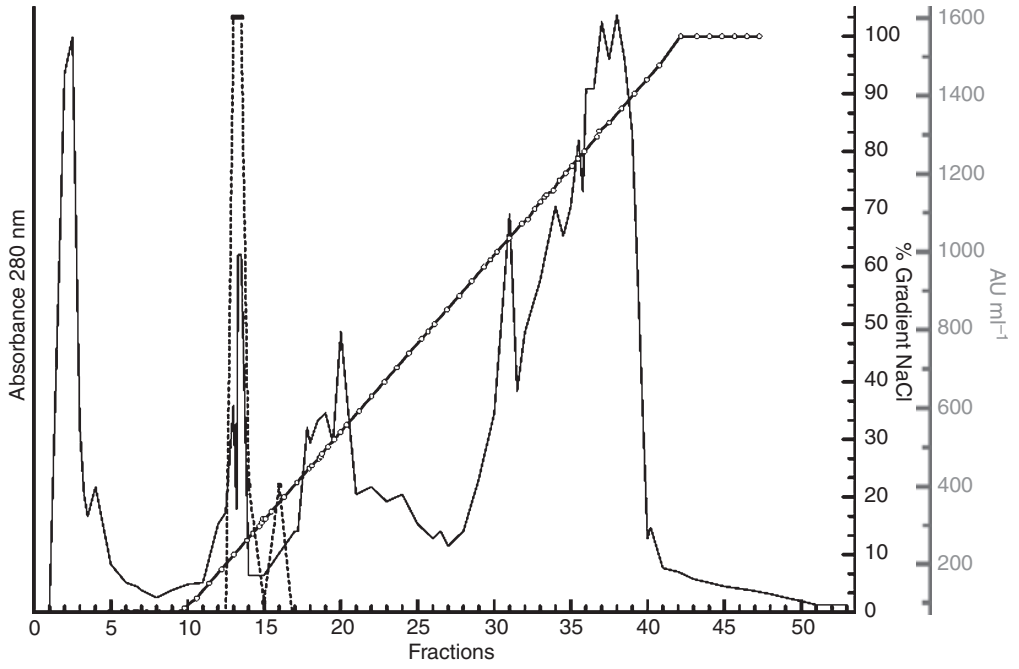

Figure 1 Mono-Q ion exchange – fast liquid chromatogram of the bacteriocin. (—) Absorbance 280 nm, (–) AU ml^{-1}, (·-·) %Gradient NaCl.

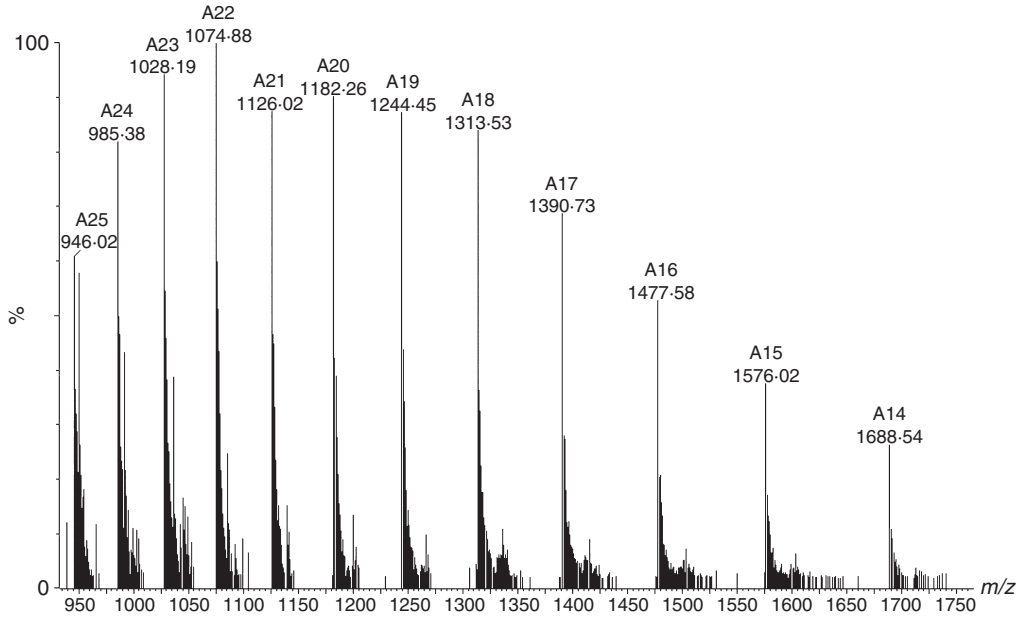

Figure 2 Mass spectrum of purified bacteriocin: relative intensity as a function of mass to charge ratio (*m/z*). The molecular mass of the protein was 23 625·00 ± 0·73 Da.

Several protocols and chromatographic methods have been proposed for the analytical purification of bacteriocins. Chromatographic methods, such as ion-exchange or size-exclusion, are usually applied after an initial concentration step by salt precipitation (Muriana and Klaenhammer 1991) or acid extraction (Yang *et al.* 1992). Our bacteriocin was purified by a sequential salt precipitation, followed by ion-exchange and reverse phase chromatography protocol. The elution pattern in a gel filtration column (Superose 12 FPLC; Pharmacia) suggested that the secreted protein formed high molecular weight aggregates because it was eluted in void (data not shown). The sub-

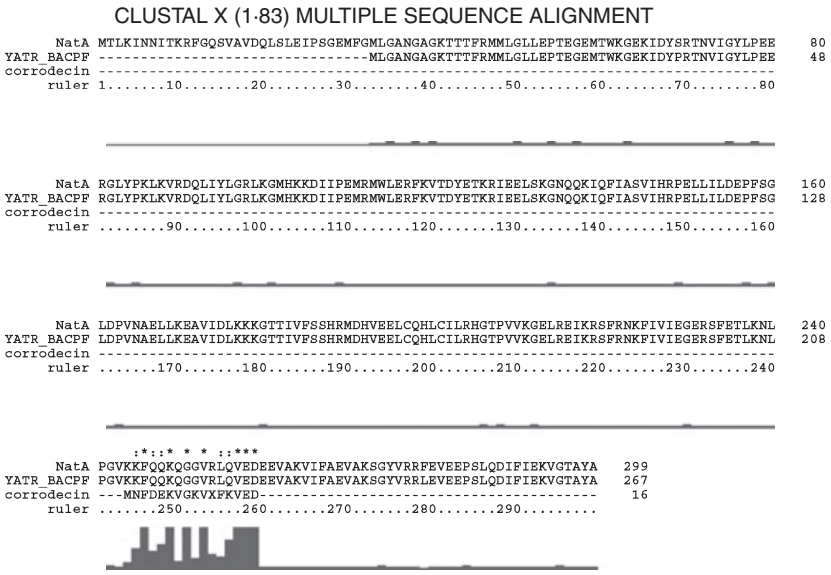

Figure 3 Sequence alignment of the 16 residues of corrodecin, with the deposited sequences of proteins from other two micro-organisms, in that order: NatA *Bacillus firmus* (AAC62423), YATR_BACPF Hypothetical ABC transporter ATP-binding protein *Bacillus pseudofirmus* (P26946), corrodecin.

stance was bound to the Mono-Q matrix, indicating that the protein is anionic. The elution pattern in the C-18 column at roughly 60% acetonitrile suggested that the secreted protein is quite hydrophobic. Because it is anionic, it is likely that its mode of action is different from class 1 and 2 bacteriocins, which are membrane-active peptides and proteins. Its high molecular mass (23 625 Da) suggested that it could be similar to colicins, which serve as the model for other Gram-negative bacteriocins.

N-terminal sequencing of the 23 625 Da protein identified 16 amino acids, of which 46·7% were hydrophobic, confirming the characteristic previously observed in reverse phase chromatography. These properties also seem to be greatly conserved among many other bacteriocins (Klaenhammer 1988). The sequence included both acidic and basic amino acids, which could explain the improved stability of the bacteriocin at neutral pH values, as previously described.

It is common to isolate a bacteriocin from several strains of the same species or from several species of the same genus (Callewaert *et al.* 1999), like pediocin AcH, which has been reported to be synthesized by different strains, namely *Pediococcus acidilactici* and *Lactobacillus plantarum* (Ennahar *et al.* 1996). Nevertheless, the N-terminal sequence of the bacteriocin isolated in our study was not significantly similar to any protein present in the sequence databases. Thus, we strongly suggest that the bacteriocin produced by *E. corrodens* A32E2 is a novel and naturally occurring one, which we have named corrodecin. However, it is noteworthy that the ClustalW multiple sequence alignment (Fig. 3) revealed a relative consensus among our 16-residue sequence with two other proteins (AAC62423 and P26946). These proteins have a region containing a conserved domain of the ABC putative ATPase, which is a subfamily of the ABC ATPase domain and is involved in drug resistance, nodulation, lipid transport, and bacteriocin and lantibiotic immunity.

In conclusion, the evidence presented in this study supports the hypothesis that *E. corrodens* A32E2 isolated from a diseased periodontal site produces a novel bacteriocin. More studies are necessary to better describe the protein and elucidate its mode of action and genetic characteristics. Nevertheless, we anticipate that a relationship between corrodecin production and the clinical periodontal status of patients is a reasonable possibility. It would also be attractive for biotechnological applications, such as eventual therapeutic alternatives for the control and prevention of periodontal disease in clinical dentistry.

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