Full Length Research Paper

Detection of bacteriocins in *Zymomonas mobilis* and RAPD fingerprinting of the producer strains

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Six strains of *Zymomonas mobilis* were evaluated in relation to the ability of producing bacteriocin against Gram-negative and Gram-positive bacteria, including three strains of *Escherichia coli,* one strain of *Salmonella enteritidis* and one strain of *Staphylococcus aureus and Streptococcus faecalis.* Two culture media and temperatures of 30 and 37°C were effective in the expression of the inhibitory activity and there was no involvement of bacteriofages or acid compounds in this activity. The bacteriocin was characterized as a thermosensitive protein released into the culture medium. The kinetics of bacteriocin production showed that the inhibitory activity was detected during the late logarithmic phase and maintained during the stationary phase. The fingerprinting pattern of the producer strains showed no polymorphism employing the sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) technique; however using randomly amplified polymorphic DNA (RAPD), the differentiation of the six strains in different degrees of similarity, was found.

Key words: *Zymomonas mobilis*, bacteriocin, RAPD.

INTRODUCTION

Zymomonas mobilis is a Gram-negative bacterium exhibiting antagonist activity against other bacteria responsible for enterical diseases as *Salmonella enteritidis, Shigella dysenteriae and Escherichia coli* (Ernandes and Garcia-Cruz, 2009; Gonçalves de Lima et al., 1972). Paula Gomes (1959) suggested the employment of *Z. mobilis* culture as a therapeutic agent for several infections, including enterocolitis and cystitis, and remission of symptoms has been observed. Even though the wide knowledge about the antagonist activity of *Z. mobilis,* the mechanism of this activity remains undetermined.

Some experiments have been carried out to verify if peroxide, lactic acid or antibiotics (Gonçalves de Lima et al., 1972) were associated with the inhibitory activity; however, they were unsuccessful. Bacteriocin is also another compound that is able to inhibit other bacteria (Montealbán-Lopez et al., 2011; Line et al., 2008; Wirawan et al., 2007; Tagg et al., 1976) and the production of this compound could be the answer for the inhibition of unrelated bacteria. Bacteriocin production has been analyzed in several Gram-positive and Gram-negative bacteria; however, no studies have been performed in *Z. mobilis*. To our knowledge only one strain of *Z. mobilis* was reported as bacteriocin producer (Tan and Rogers, 2000).

In this paper we report the bacteriocin production by six strains of *Z. mobilis*. Inhibitory activity was detected against *S. enteritidis, Streptococcus faecalis, Staphylococcus*

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aureus and pathogenic and non-pat characterized as a secreted protein in the supernatant hogenic strains of *E. coli*. Bacteriocin was and thermosensitive, and it was synthesized as a growth-dependent product (Karthikeyan and Santhosh, 2009). The producer strains showed a uniform SDS-PAGE pattern, whereas RAPD fingerprinting differentiated all strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Six strains of *Z. mobilis* (CP_4, Z1-86A, Z1-86B, Z1-87, Z1-88 and Z2-88) (Falcao de Morais et al., 1993) were used in the present study. The strains were routinely grown in Solid state dye lasers (SSDL) medium (Swings and de Ley, 1977) containing (per litter) 20 g glucose and 5 g yeast extract. Bacteriocin production was examined in Schreder agar medium (20 g glucose, 2 g yeast extract, 1 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 15 g/L agar) and SSDL agar medium. *E. coli* K12, *E. coli* BH57, *E. coli* ATCC 9637, *S. enteritidis, S. aureus* and *S. faecalis*, obtained from the collection of the Antibiotics Department, UFPE, Brazil, were grown in nutrient agar (NA) medium and were used as sensitive strains.

Detection of bacteriocin production

The detection was performed according to Azevedo and Costa (1973). *Z. mobilis* strains were grown overnight in SSDL medium and aliquots of 20 μl were inoculated in agar media (SSDL or Schreder). The plates were incubated during 24 h at 30°C or 37°C during 24 h and then treated with chloroform. Warmed semi solid nutrient agar (NA) medium (5 ml), previously inoculated with the sensitive strain (10^6 CFU/ml), was used to overlay the producer strains and the plates were incubated 24 h at 37°C. The inhibition was observed by the halo formation around the *Z. mobilis* colonies.

Partial characterization of the bacteriocins

Presence of phages

As inhibitory zones could be caused by phages, the presence of these organisms were investigated by transferring small areas (3 mm^2) of the halo to 1.5 ml of liquid SSDL medium, macerated and incubated 1 h at room temperature. 100 μl of this suspension were mixed with 100 μl of the sensitive strain (10^6 CFU/ml), followed by addition of warmed semi solid medium, and they were poured into plates. The plates were incubated 24 h at 37°C and were examined for inhibitory zone formation (Lewus et al., 1991).

Bacteriocin in the supernatant

Z. mobilis was grown 24 h in 15 ml of liquid SSDL medium, centrifuged and the supernatant recovered was neutralized with 0.1 M NaOH and was filtered with a Millipore membrane 0.22 μm. Small paper disks were soaked into the filtrate (30 μl) and were placed over a plate previously plated with the sensitive strain (10^6 CFU/ml). The plates were incubated 24 h at 37°C (Martinis et al., 2003). Tests were also conducted with the filtrate without the step of neutralization.

Thermostability and sensitivity to protease

Both tests were performed according to Lewus et al. (1991). 100 μl of the supernatant were incubated 15 min at 45, 60, 80 and 100°C, followed by the bacteriocin test. To test the sensitivity to protease, the supernatant was treated with proteinase K (10 mg/ml) and the mixture was incubated 90 min at 30°C, followed by the bacteriocin test.

Time course of bacteriocin production

Z. mobilis Z1-87, freshly grown in SSDL medium was inoculated into Schreder medium (250 ml) and samples were taken in 3 h intervals totalizing 48 h (Lewus et al., 1991). The samples were examined for optical density (A_{600}) and tested for bacteriocin production employing *E. coli* K12 as sensitive strain.

DNA extraction

Total DNA of *Z. mobilis* was extracted using standard procedures (Sambrook et al., 1989). Briefly, 5 ml of culture was washed with total antioxidant status (TAS) buffer (50 mM Tris-HCl pH 8, 50 mM ethylenediaminetetraacetic acid (EDTA) and 150 mM NaCl) and was resuspended in the same buffer. The suspension was treated with proteinase K (150 μg/ml) and 2% sodium dodecyl sulfate (SDS), and was incubated 1 h at 50°C. Proteins were removed with phenol, and phenol + chloroform and DNA was precipitated with ethanol and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA). DNA concentration was estimated by comparison with known concentrations of lambda DNA in standard agarose gel electrophoresis.

RAPD fingerprinting

PCR amplification was carried out in a final volume of 25 μl, employing 30 to 50 ng of DNA and the following primers (www.operon.com): OPQ4, OPR1, OPR4, OPA8, OPA9, OPX17 and OPX19. The reaction mixture contained the appropriated buffer, 100 μM dNTPs, 3 mM $MgCl_2$ and *Taq* polymerase (2 U). The cycling conditions were 1 × 94°C/3 min, 40 × (94°C/1 min, 37°C/1 min, 72°C/1 min). The amplification products were separated by agarose gel electrophoresis and visualized after ethidium bromide staining (Gonçalves and Rosato, 2000). The RAPD bands were analyzed using qualitative information (presence or absence of bands). Similarity matrix was generated using the unweighted pair group with arithmetic mean (UPGMA) with the Sj coefficient (NTSYS-PC, Applied Biostatistics, Inc.).

SDS-PAGE

Aliquots of 1.5 ml of *Z. mobilis,* freshly grown in SSDL medium, were centrifuged and the recovered cells were washed in TE buffer, centrifuged again and resuspendend in 300 μl of the same buffer. Subsequently, the sample buffer (10 mM Tris-HCl pH 8.0, 20% glycerol, 10% SDS, 5% β-mercaptoethanol and 0.02% bromophenol blue) was added and the mixture was incubated 3 min at 100°C. After centrifugation, aliquots of the supernatant were loaded into the polyacrylamide gel (10 and 4% total acrylamide for the running and stacking gels, respectively). Protein bands were visualized with Coomassie brilliant blue R-250 staining (Vauterin et al., 1991).
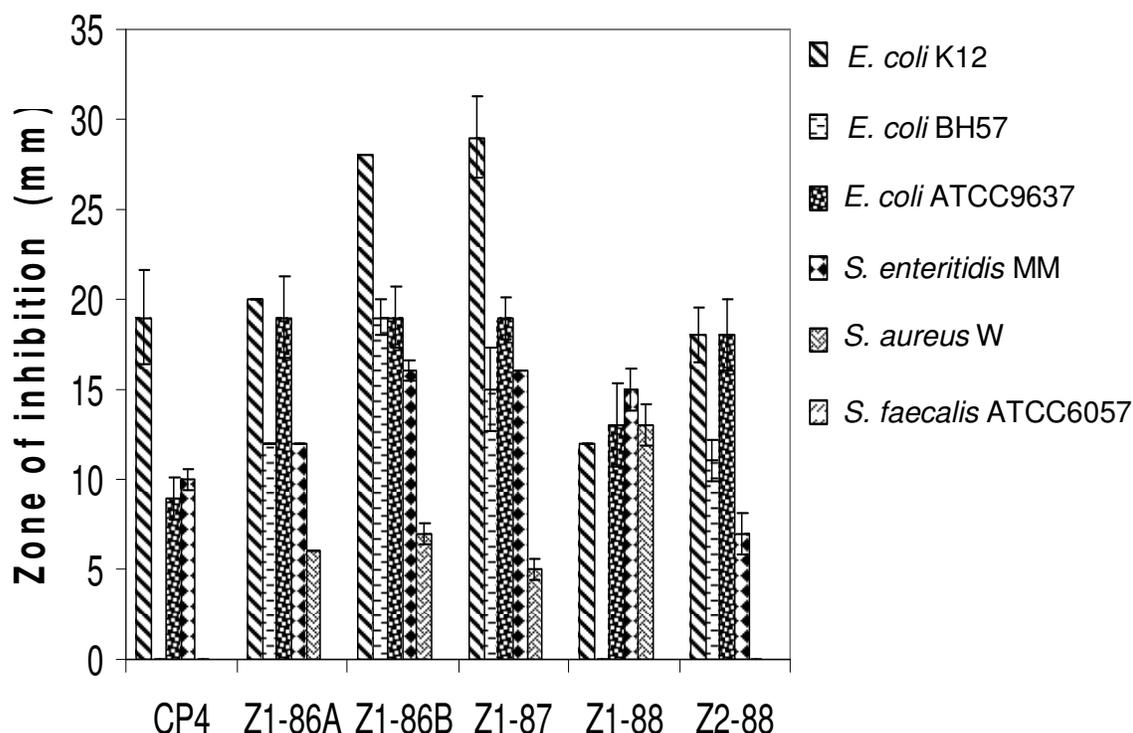
Figure 1. Size of the inhibition halo (mm) produced by different strains of *Z. mobilis* using *S. enteritidis, S. aureus, S. faecalis* and three strains of *E. coli*, as sensitive strains. Each error bar represents the mean and standard deviation of separate experiments.

Statistical analysis

Student's *t* test was performed to determine statistical significance.

RESULTS AND DISCUSSION

Activity of *Z. mobilis* against other strains

The six strains of *Z. mobilis* analyzed in the present study showed inhibitory activity, in different degrees, against the Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *S. enteritidis*) bacteria used as a sensitive strains. The pattern of bacteriocin activity varied among the strains as well the size of the inhibitories zones depending on the sensitive strain (Figure 1). The Z1-86B and Z1-87 strains displayed a very similar pattern of inhibition against all the sensitive strains analyzed yielding the largest inhibitory zones of 28 and 30 mm (P < 0,05), respectively, against *E. coli* K12 and thus considered as the best bacteriocin producers. Four strains (Z1-86A, Z1-86B, Z1-87 and Z1-88) showed activity, in variable ranges (from 5 to 13 mm) against the Gram-positive *S. aureus,* indicating the wide spectrum of activity of the bacteriocin. In the other side, *S. faecalis* was completely resistant. Similarly, Tan and Rogers (2000) reported inhibition of *E. coli* K12 and *Lactobacillus*

lactis subsp. *lactis* by a strain of *Z. mobilis*. Although, bacteriocins produced by Gram-negative bacteria display in general restricted range of action to the closest species, several reports have demonstrated the ability of certain strains to inhibit non-related organisms (Miranda et al., 1993).

Effect of culture medium and temperature

Growth conditions have been revealed as important factors on bacteriocin production (Bromberg et al., 2006; Drider et al., 2006; Joerger, 2003) and therefore two media (SSDL and Schreder) were evaluated as well temperatures of 30 and 37°C. The results (Figure 2) showed that bacteriocin was produced in both media and the Schreder medium favored slightly the production at both temperatures. The best bacteriocin producer, in all conditions tested, was again the strain Z1-87. Why the bacteriocin production is affected by growth conditions is unclear in most cases and it should be related to the biosynthetic mechanism of the particular category of bacteriocin. Glycerol for example, is a precursor in the biosynthesis of many compounds and it has been associated to antibiotics production by *Erwinia carotovora* (Axelrood et al., 1998). However in most cases, the
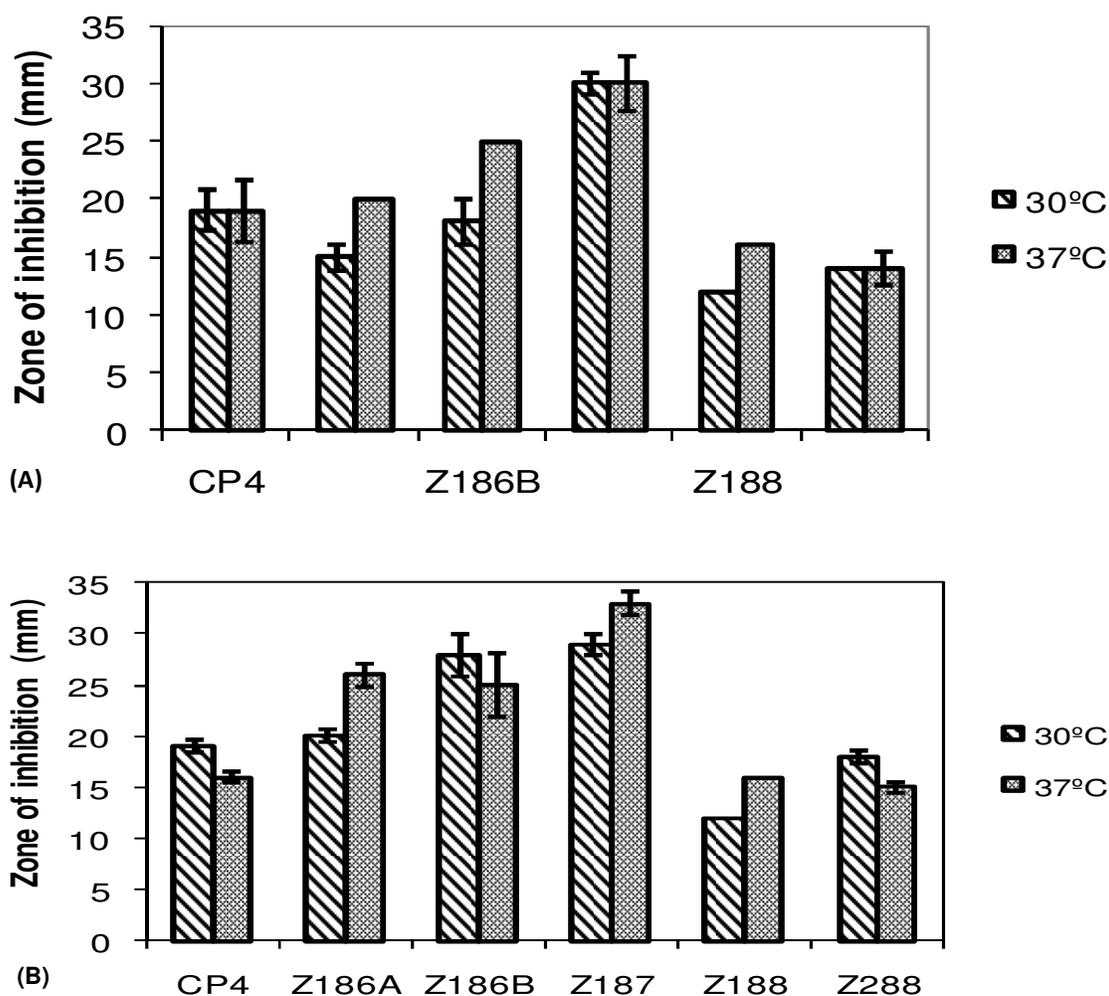
Figure 2. Size of the inhibition halo (mm) produced by different strains of *Z. mobilis*, in SSDL (A) and Schreder (B) media and temperatures of 30 and 37°C, using *E. coli* K12 as sensitive strain. Each error bar represents the mean and standard deviation of separate experiments.

chemical nature and structure of the bacteriocin is unknown and therefore many growth media are tested in order to increase the bacteriocin production.

Characterization of the bacteriocin produced

Several compounds, other than bacteriocin, are able to inhibit bacterial growth of related or unrelated bacteria, displaying haloes of clear zones around the producer strain. Among these compounds, lactic acid and peroxides have been tested previously in *Z. mobilis* (Falcão de Morais et al., 1993). The bacterium promotes a high decrease in the pH of the culture medium, and therefore, the acidic and neutralized supernatants of *Zymomonas* strains were tested using *E. coli* K12 as sensitive strain. All strains yielded the expected haloes

sizes (14 to 15 mm) indicating that the pH was not causing the inhibition. Phage-like components have also been considered as inhibitory agent since lytic plaques similarly to the inhibition zones are also produced when phages are released from the bacteria. Tests conducted as suggested (Lewus et al., 1991) revealed no evidence of phages.

Other characteristics of the bacteriocin produced, such as the sensitivity to proteases and thermostability were also evaluated. The results showed that the bacteriocin was degraded by proteinase K lacking inhibitory activity afterwards and that the bacteriocin was stable up to 15 min at 80°C loosing completely the activity when incubated at 100°C.

Finally, it was observed that inhibitory compound was released into culture medium and the bacterial cells alive were not necessary to halo formation.
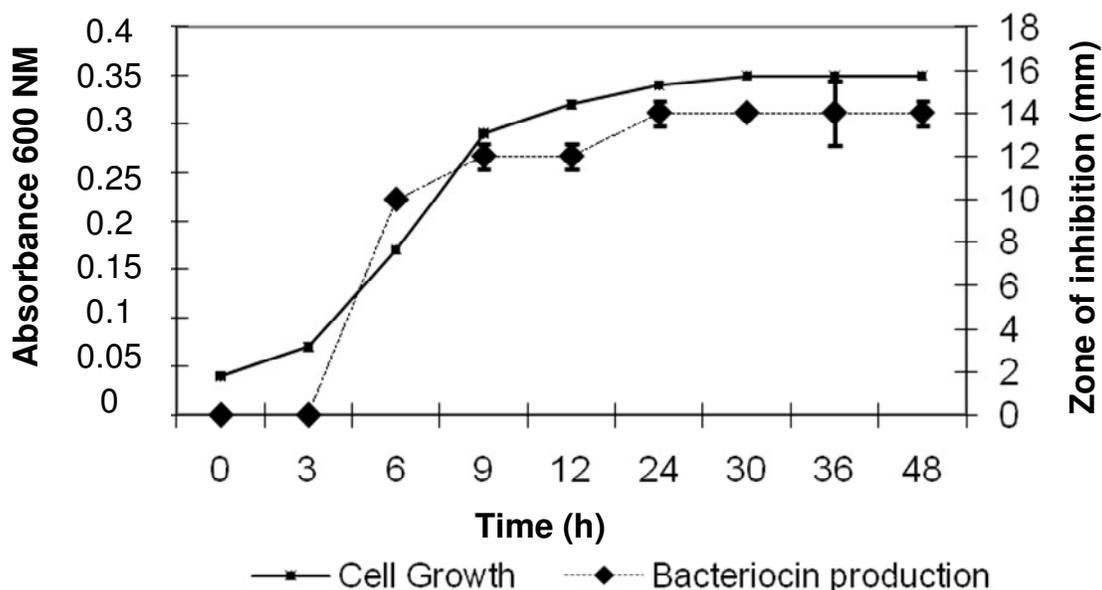
Figure 3. Growth curve of *Z. mobilis* Z1-87 (Schreder medium at 37ºC) and bacteriocin production using *E. coli* K12 as sensitive strain. Each error bar represents the mean and standard deviation of separate experiments.

Time course of bacteriocin production

Bacteriocin production, measured as the size of the inhibition halo, was followed during the growth of *Z. mobilis* Z1-87 in Schreder medium (Figure 3). The results revealed that the production of the compound began during logarithmic phase (6 h after inoculation), reached the maximum after 24 h and afterwards, the halo size remained constant. Correlation between bacteriocin production and growth phase seems to be commonly found (Biswas et al., 1991). Although, additional studies are necessary, it is likely that bacteriocin production could be under a quorum sensing regulation (Lewus et al., 1991). This regulation requires the presence of a diffusible signal (autoinducer) for cellular communication within a bacterial population and the signal is usually produced at high cellular density. The signal is able to regulate several bacterial characteristics, such as virulence, secondary metabolism, symbiosis, biofilm formation and others. It would be interesting to verify if the auto-inducers, usually a homoserine lactone or analogs, are present in the cultures of *Z. mobilis* and how bacteriocin production is regulated.

Bacteriocin production was detected in the six strains of *Z. mobilis* against some Gram-positive and Gram-negative bacteria, including pathogenic and non-pathogenic *E. coli* strains. Some of the strains tested as sensitive strains are considered causal agents of many intestinal disorders and it is conceivable that the therapeutic effect of *Z. mobilis* administered orally

(Falcão de Morais et al., 1993) might be due to the bacteriocin inhibiting *E. coli* or *Salmonella*. However, other factors as the competitive effect promoting balance of intestinal flora should not be neglected. Interestingly, it was observed that the bacteriocin production by *Z. mobilis* was growth phase dependent in agreement with several other reports, which described this mode of regulation. Also, it was demonstrated that the bacteriocin is a protein, fulfilling the designation as a bacteriocin.

Fingerprinting of the bacteriocin producer strains

SDS-PAGE was used to examine the whole-cell protein pattern of the six strains of *Z. mobilis*. All strains revealed identical protein bands and therefore no discrimination could be made. However the strains could be different-tiated by RAPD fingerprinting pattern. Preliminarily 13 primers were used however most of them yielded non-repetitive bands and thus they were disregarded. Seven primers were consistent and used to assess the genetic diversity among the six strains of *Z. mobilis*. A total of 50 bands were generated and 60% of these bands were polymorphic, present in one or five strains but not in all 6 strains. The fragments sized from 0.3 to 4.0 kb and differences in the intensity of bands were observed but they were not considered. The highest number of bands was obtained with the OPA9 primer, which yielded 10 bands whereas OPR1 amplified only 4 bands. In the other side, the OPA9 and OPX19 primers generated most
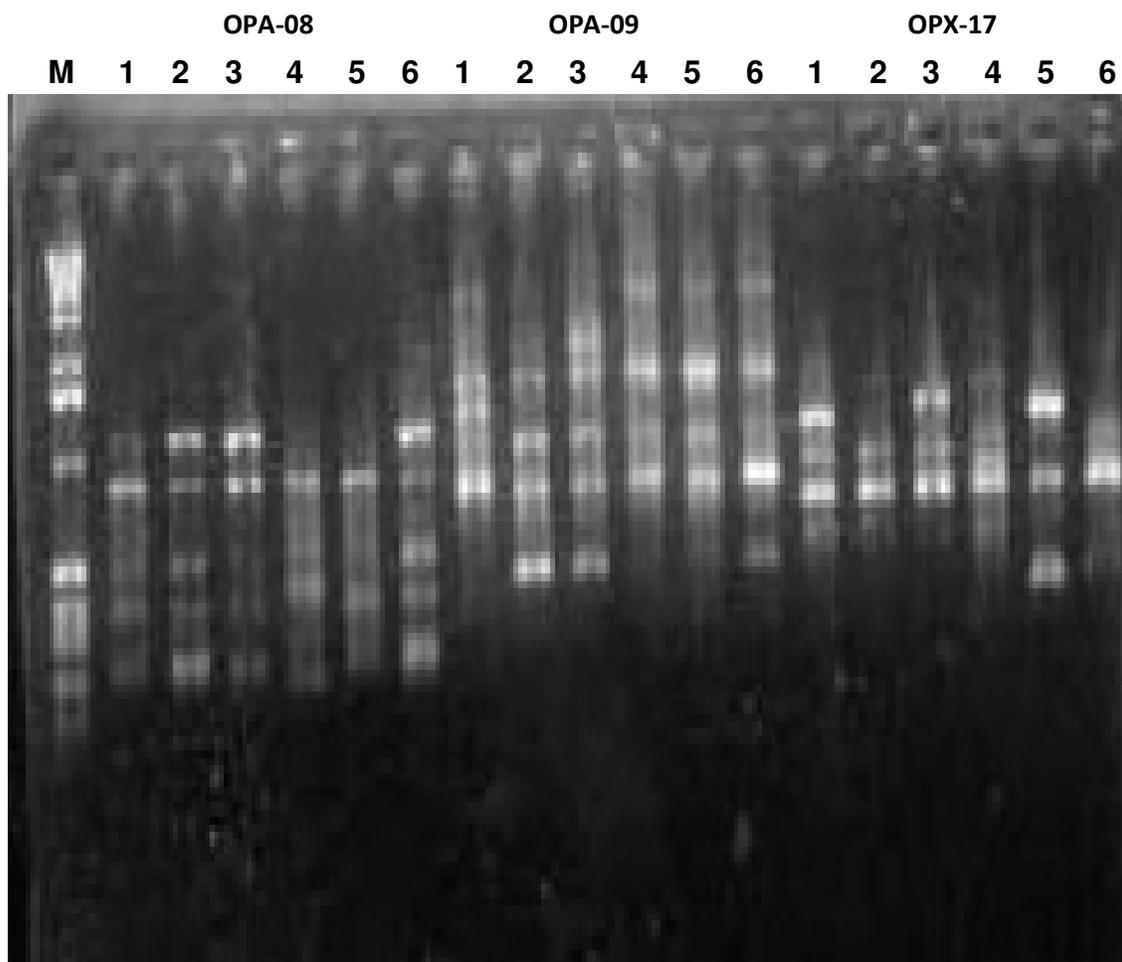
Figure 4. Electrophoretic analyses of DNA amplification products with primer OPA-08, OPA-09 and OPX-17. Lane M contains the molecular weight markers (1 kb). Lanes 1 through 6 correspond to the strains CP4, Z1-86A, Z1-86B, Z1-87, Z1-88 and Z2-88.

of the polymorphic bands and among the 9 bands generated by both primers 6 were polymorphic, making them useful for genetic diversity studies (Figure 4).

Based on the qualitative analysis of the bands generated by the 7 randomic primers, a dendrogram (Figure 5) was constructed. Two major groups were formed: the first included four strains (CP4, Z1-86A, Z1-86B, Z1-87 and Z2-88), whereas the second group included Z1-88. Both groups showed similarity of 65%. No relationships were observed between the group formation and year or geographic region. Indeed, the two strains, Z1-86A and Z1-86B, were collected at the same time in the same region and the differences between both strains were related to flocculation ability during growth in liquid medium (Falcão de Morais, personal communication). The differences observed among all strains analyzed confirm the variable pattern observed in the bacteriocin production.

Conclusion

Conclusively, bacteriocin production by *Z. mobilis* was fully reported for the first time. Its inhibitory action was tested against some Gram-positive and Gram-negative bacterium, and inhibition in different degrees was found. All producers' strains were differentiated by RAPD fingerprinting. Substantial efforts are required to identify the gene encoding the bacteriocin and to determine its chemical structure. Also, additional studies have to be performed with the bacterial free extract to verify if it is also able to control enteric diseases and others infections as the bacteria alive.
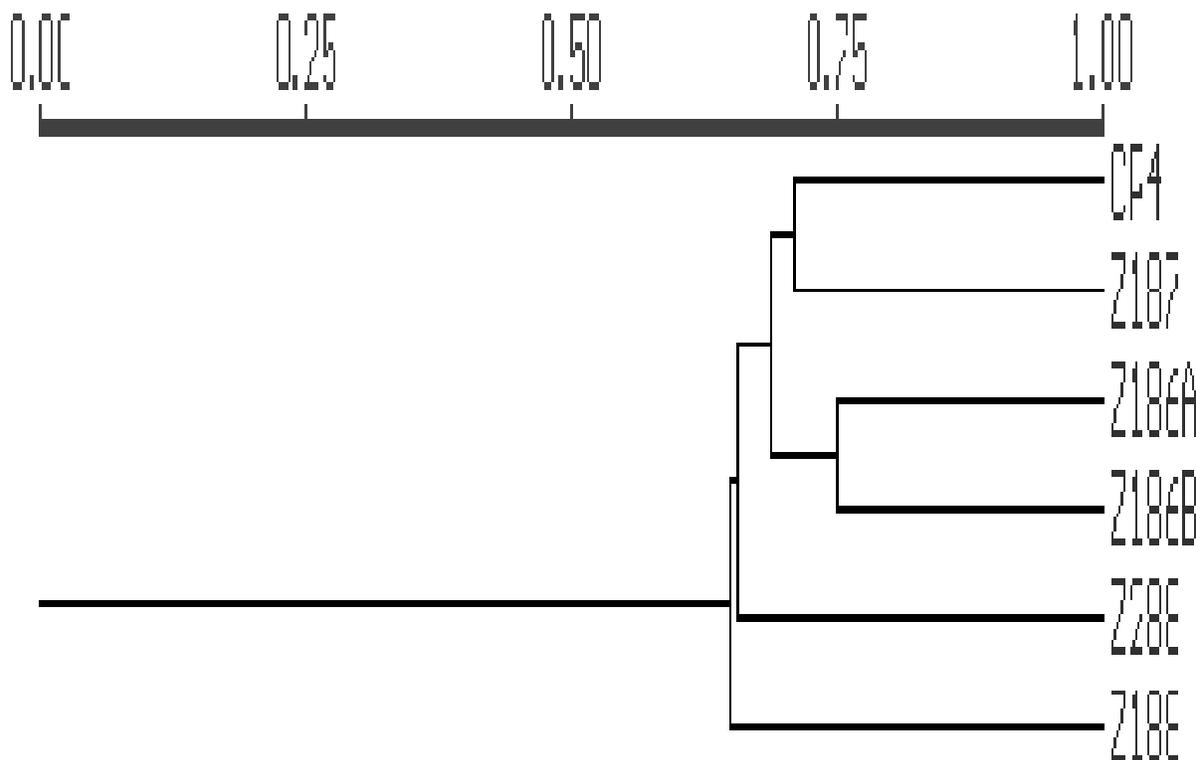
Figure 5. RAPD dendrogram obtained by the similarity matrix generated using UPGMA (Sj coefficient).

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