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Short communication

Activity of bacteriocins synthesized by *Bacillus thuringiensis* against *Staphylococcus aureus* isolates associated to bovine mastitis

José Eleazar Barboza-Corona [a], Norma de la Fuente-Salcido [a,b], Nayeli Alva-Murillo [c], Alejandra Ochoa-Zarzosa [c], Joel E. López-Meza [c,*]

[a] *Universidad de Guanajuato, Campus Irapuato-Salamanca, División Ciencias de la Vida, Departamento de Ingeniería en Alimentos, Irapuato, Guanajuato, 36500, México, México*
[b] *Universidad Autónoma de Coahuila, Escuela de Ciencias Biológicas, Torreón, Coahuila, 27440, México, México*
[c] *Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo, Apdo. Postal 53, Administración Chapultepec, C.P. 58262, Morelia, Michoacán, México, México*

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ABSTRACT

Antimicrobial therapy is a useful tool to control bovine mastitis caused by *Staphylococcus aureus*, as consequence an increase in staphylococci resistant cases has been registered. Alternative strategies are desirable and bacteriocins represent attractive control agents to prevent bovine mastitis. The aim of this work was to evaluate the activity of five bacteriocins synthesized by *Bacillus thuringiensis* against *S. aureus* isolates associated to bovine mastitis. Fifty *S. aureus* isolates were recovered from milk composite samples of 26 Holstein lactating cows from one herd during September 2007 to February 2008 in México and susceptibility of those isolates to 12 antibiotics and 5 bacteriocins from *B. thuringiensis* was evaluated. *S. aureus* isolates were mainly resistant to penicillin (92%), dicloxacillin (86%), ampicillin (74%) and erythromycin (74%); whereas susceptibility to gentamicin, trimethoprim and tetracycline was detected at, respectively, 92%, 88%, and 72%. All *S. aureus* isolates showed susceptibility to the five bacteriocins synthesized by *B. thuringiensis*, mainly to morricin 269 and kurstacin 287 followed by kenyacin 404, entomocin 420 and tolworthcin 524. Our results showed that *S. aureus* isolates had differences in the antimicrobial resistance patterns and were susceptible to bacteriocins produced by *B. thuringiensis*, which could be useful as an alternative method to control bovine mastitis.

1. Introduction

Bovine mastitis is a major disease affecting the dairy industry that results in economic loses and decreases animal health (Ruegg, 2003). *Staphylococcus aureus* is the most predominant contagious pathogen responsible for clinical and subclinical infections in lactating cows (Kerro-Dego et al., 2002). Antimicrobial therapy has been a valuable tool for controlling mastitis and as a consequence an increase in the frequency of staphylococci resistance has been recorded (Myllys et al., 1998; Ochoa-Zarzosa et al., 2008). Thus, alternative methods for controlling the bovine mastitis are required. One of these alternatives could be the use of bacteriocins.

Bacteriocins are natural peptides synthesized and secreted by bacteria, which inhibit the growth of both closely related (Jack et al., 1995) and nonrelated species (De la Fuente-Salcido et al., 2008). Several bacteriocins have been proposed as potential alternatives to mastitis control. Nisin, from *Lactococcus lactis*, has activity against mastitis pathogens and has been formulated into some commercially available products (Ryan et al., 1998; Wu

* Corresponding author. Tel.: +52 443 295 8029; fax: +52 443 295 8029.
E-mail address: elmeza@zeus.umich.mx (J.E. López-Meza).

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et al., 2007). Also, bacteriocins produced by *S. aureus*, *S. epidermidis* and *Streptococcus gallolyticus* have been tested against strains of both *S. aureus* and *Streptococcus* species isolated from bovine mastitis (Varella Coelho et al., 2007; Pieterse et al., 2008).

Bacillus thuringiensis is a naturally occurring Gram-positive spore-forming soil bacterium routinely used worldwide in insect biocontrol; however, diverse bacteriocins from this bacterium have been reported (Kamoun et al., 2005; Gray et al., 2006). Recently, five new *B. thuringiensis* bacteriocins (morricin 269, kurstacin 287, kenyacin 404, entomocin 420, and tolworthcin 524) from Mexican strains were reported (Barboza-Corona et al., 2007; De la Fuente-Salcido et al., 2008). In spite of their potential applied use, *B. thuringiensis* bacteriocins have not been evaluated against bovine mastitis pathogens. In this work, we demonstrated that each one of the bacteriocins tested showed inhibitory action against *S. aureus* isolates.

2. Materials and methods

2.1. S. aureus isolates

A total of 26 Holstein cows kept in one herd were analyzed through September 2007 to February 2008 in Morelia, Michoacán, México. Subclinical mastitis was determined by California mastitis test and 50 *S. aureus* isolates were collected. *S. aureus* isolates were obtained from raw milk composite aliquots (200 μl) distributed on Blood agar plates (37 °C, 24 h; Bioxon, México). *S. aureus* isolates were identified by standard biochemical tests and corroborate by amplifying the gene *nuc* encoding staphylococcal thermostable nuclease (Brakstad et al., 1992). Isolates were identified by the letters STA (*S. aureus*) followed by an arabic number.

2.2. Antimicrobial susceptibility of S. aureus isolates

S. aureus isolates were evaluated for antibiotic susceptibility with the disk diffusion method on Mueller-Hinton (MH) agar plates (Bioxon, México). The following Gram-positive multi discs (Bio-Rad, México) were used: ampicillin, 10 μg (≤28); cephalotin, 30 μg (≤14); cefotaxime, 30 μg (≤14); ceftazidime, 30 μg (≤14); cefuroxime, 30 μg (≤14); dicloxacillin, 1 μg (≤10); erythromycin, 15 μg (≤13); gentamicin, 10 μg (≤12); pefloxacin, 5 μg (≤14); penicillin, 10 U (≤28); tetracycline, 30 μg (≤14) and trimethoprim, 25 μg (≤10). In parenthesis are indicated the diameters (mm) of inhibition zones used to consider an isolate as resistant, according to the manufacturer's instructions. A MH agar plate without antimicrobials was used as negative control for each isolate. Plates were incubated at 37 °C for 24 h.

2.3. B. thuringiensis strains and production of partially purified bacteriocins

The five *B. thuringiensis* strains used as bacteriocin producers are listed in Table 1. *B. cereus* 183 (International Entomopathogenic *Bacillus* Centre, Institut Pasteur, Paris, France) was employed as an indicator bacterium to determine the activity of bacteriocins before testing against *S. aureus* isolates. To produce bacteriocin, bacterial strains were cultured overnight in TSB (Tryptic soy broth, Bioxon, México) at 28 °C with 180 rpm. An aliquot of 5 ml ($\sim 1 \times 10^9$ cells/ml) was mixed with 45 ml of fresh TSB and incubated overnight during the time of the highest bacteriocin synthesis as previously determined (Barboza-Corona et al., 2007). Cultures were centrifuged and supernatant was filtered through a 0.2-mm filter. Cell-free supernatants were concentrated with ammonium sulfate to 80% saturation at 4 °C and precipitated proteins were pelleted by centrifugation at 16,000 × *g* for 30 min at 4 °C, resuspended in 100 mM phosphate buffer (pH 7.0) and dialyzed overnight against the same buffer using a mini-dialysis kit with a 1 kDa cut-off (Amersham Biosciences). Activity of each strain was corroborated with both the fluorogenic (De la Fuente-Salcido et al., 2007) and the well-diffusion methods using *B. cereus* 183 as indicator bacterium (Barboza-Corona et al., 2007).

2.4. Antimicrobial activity of bacteriocins from B. thuringiensis against isolates of S. aureus

The modified well-diffusion method was used to assess antibacterial activity of partially purified bacteriocins against *S. aureus* isolates. Bacteria were cultivated overnight at 37 °C in Luria broth (LB, Difco), and 100 μl ($\sim 1 \times 10^9$ cell/ml) of each isolate were mixed with 15 ml of TSB with warm soft agar 0.7% (w/v) and plated. Wells, 8 mm in diameter, were dug into the agar and kept for 2 h at 37 °C. Then, 100 μl of partially purified bacteriocin

Table 1
Properties of bacteriocins produced by Mexican strains of *Bacillus thuringiensis*[a] used in this study.

Strain	Bacteriocin	Molecular weight[b]	T_{50} (°C)[c]	Highest pH stability[d]
B. thuringiensis subsp. *morrisoni* (LBIT 269)	Morricin 269	~10 kDa	65.1	5–9
B. thuringiensis subsp. *kurstaki* (LBIT 287)	Kurstacin 287	~10 kDa	67.5	5–9
B. thuringiensis subsp. *kenyae* (LBIT 404)	Kenyacin 404	~10 kDa	124.5	5–11
B. thuringiensis subsp. *entomocidus* (LBIT 420)	Entomocin 420	~10 kDa	120.3	5–11
B. thuringiensis subsp. *tolworthi* (LBIT 524)	Tolworthcin 524	~10 kDa	123.2	5–11

[a] Strains used in this study are held at Bioinsectides laboratory, CINVESTAV Campus Guanajuato, México. Information to elaborate this table was obtained from Barboza-Corona et al. (2007).
[b] Molecular weight was estimated by SDS-PAGE.
[c] Temperature required to obtain 50% inhibition in the well-diffusion assay using *Bacillus cereus* 183 as indicator bacterium.
[d] Highest inhibitory activity to *B. cereus* was obtained at pH 5.

Table 2
Inhibitory activity (U[a]) of partial purified bacteriocins from *B. thuringiensis* against *S. aureus* isolates determined by the well-diffusion method[b,c].

S. aureus isolates	Morricin 269	Kurstacin 287	Bacteriocins Kenyacin 404	Entomocin 420	Tolworthcin 524
STA 50	204	365	204	82	45
STA 51	151	269	104	45	63
STA 52	104	233	177	104	126
STA 53	151	233	151	28	28
STA 54	126	**45**	82	63	233
STA 55	151	233	151	82	45
STA 56	233	233	126	45	45
STA 57	204	233	204	104	63
STA 58	330	402	365	82	82
STA 59	151	104	126	63	63
STA 60	204	233	204	45	28
STA 61	177	233	104	28	28
STA 62	104	176	104	82	28
STA 63	126	264	151	104	28
STA 64	177	233	126	126	28
STA 65	126	176	104	104	28
STA 66	151	204	151	82	28
STA 67	63	204	126	82	28
STA 68	104	204	126	104	28
STA 69	126	233	126	104	45
STA 70	402	402	296	296	330
STA 71	296	402	264	63	82
STA 72	365	441	264	296	296
STA 73	233	330	151	63	45
STA 74	104	233	296	63	63
STA 75	204	177	151	45	28
STA 76	***441***	441	264	233	296
STA 77	296	365	233	**28**	63
STA 78	296	296	330	45	63
STA 79	**28**	82	126	45	45
STA 80	296	***481***	264	63	63
STA 81	***441***	***481***	441	402	441
STA 82	330	82	296	233	104
STA 83	104	126	151	45	45
STA 84	126	151	126	126	28
STA 85	***441***	296	402	402	441
STA 86	296	402	330	330	365
STA 87	296	365	296	63	45
STA 88	151	63	104	63	45
STA 89	330	330	204	63	63
STA 90	264	204	***481***	***441***	***481***
STA 91	330	441	**63**	45	63
STA 92	296	402	264	63	63
STA 93	204	233	204	63	28
STA 94	151	204	104	45	**21**
STA 95	291	269	296	63	63
STA 96	264	441	177	63	204
STA 97	402	233	330	365	151
STA 98	330	330	233	45	82
STA 99	126	264	177	45	45

[a] One unit is defined as 1 mm^2 of the zone of inhibition as determined by the well-diffusion method (see text). Data are the average of triplicate assays.
[b] In all cases, *S. aureus* isolates were grown on LB medium and TSB plus agar was used in well-diffusion assays.
[c] Number in bold indicates the *S. aureus* isolate with the lowest susceptibility to bacteriocins tested in the column. Number in bold and italics indicates the *S. aureus* isolates with the highest susceptibility to bacteriocins tested in the same column.

preparations obtained as described above was added to each well and incubated for 12 h at 4 °C to allow diffusion of samples, followed by an additional incubation at 28 °C or 37 °C for 1 day before diameters of inhibition zones were measured (Table 2). The minimum detectable zone measured for analytic purposes was 1 mm^2 beyond the well diameter. Assays were repeated in triplicate and the average result was recorded. For our purposes, we defined one unit (U) of bacteriocin activity as equal to 1 mm^2 of growth inhibition zone of the indicator bacterium (Delgado et al., 2005; Barboza-Corona et al., 2007).

3. Results

3.1. Collect of S. aureus isolates

In a regional program for the study of mastitis, 26 Holstein cows maintained in one herd were analyzed through September 2007 to February 2008 in Morelia, Michoacán, México. Incidence of subclinical mastitis in the herd during this period was 51%. Fifty *S. aureus* isolates were obtained from these cows and identified according to biochemical properties and PCR amplification of the gene

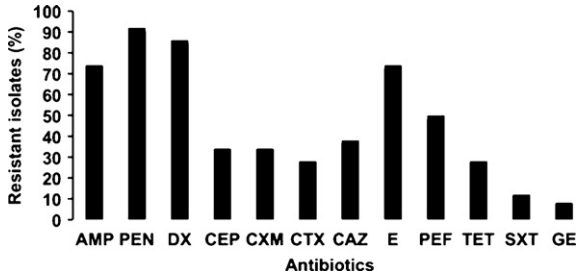

Fig. 1. Phenotypic antimicrobial resistance of *Staphylococcus aureus* isolated from cows with mastitis. AMP, ampicillin; CAZ, ceftazidime; DX, dicloxacillin; PEN, penicillin; CEP, cephalotin; CTX, cefotaxime; CXM, cefuroxime; E, erythromycin; PEF, pefloxacin; TET, tetracycline; SXT, trimethoprim; GE, gentamicin.

nuc. Otherwise, the environmental pathogens responsible of mastitis, *Escherichia coli* and *Klebsiella pneumoniae*, were also isolated but were not included in this study.

3.2. Antimicrobial susceptibility of S. aureus isolates

In general, the *S. aureus* isolates showed a variable susceptibility behavior towards the 12 antimicrobials tested. All *S. aureus* isolates were resistant to two or more antimicrobials, except STA 71, STA 89, STA 90 and STA 91 isolates. The 50 *S. aureus* isolates were mainly resistant to penicillin (92%), dicloxacillin (86%), ampicillin (74%) and erythromycin (74%). In the other hand, some isolates were resistant to gentamicin (8%), trimethoprim (12%) and tetracycline (28%) (Fig. 1). Isolates STA 51 and STA 59 showed resistance to all antibiotic tested, whereas isolates STA 71, STA 90 and STA 91 were susceptible to all antibiotics.

3.3. Inhibitory activity of bacteriocins from B. thuringiensis against S. aureus isolates

The sensitivity of 50 *S. aureus* isolates to five different bacteriocins from *B. thuringiensis* (Table 1) was evaluated by assays performed by well-diffusion method. All *S. aureus* isolates showed susceptibility to the bacteriocins synthesized from *B. thuringiensis* (Table 2). Morricin 269 and kurstacin 287 showed the highest activities followed by kenyacin 404, entomocin 420 and tolworthcin 524. *S. aureus* isolates STA 70, STA 72, STA 76, STA 81, STA 86 and STA 90 showed greater inhibition zones to bacteriocins tested. Alternatively, *S. aureus* isolates STA 79, STA 83, STA 84 and STA 88 showed the lowest sensitivity to bacteriocins (Table 2). No correlation among sensitivity to antibiotics and bacteriocins was detected.

4. Discussion

In this study, we assessed the antimicrobial activities of bacteriocins from *B. thuringiensis* against *S. aureus* isolates from cases of bovine mastitis. *S. aureus* is one of the most frequently isolated pathogen causing clinical or subclinical mastitis. This bacterium is difficult to treat with antimicrobial therapy owing to an increase in the resistance frequency to antimicrobial agents (Sandholm et al., 1990; Owens et al., 1997; Kerro-Dego et al., 2002). Subclinical mastitis prevalence during this study was 51%, which was greater than a previous data shown in the same region (29%) (Ochoa-Zarzosa et al., 2008). These differences could be explained as the preceding study was carried out in different herds with heterogeneity in the cattle.

Antimicrobial susceptibility evaluation for mastitis pathogens is a valuable tool in mastitis control and should be performed in different dairy regions, since resistance patterns and diversity of isolates vary on a per-region basis. In this study, we detected that *S. aureus* isolates have a high level of resistance to penicillin class antibiotics and erythromycin (>74%) (Fig. 1). In the herd used for this study, penicillin class antibiotics have been frequently used for the control of mastitis; this could explain the high level of resistance to penicillin detected. Nevertheless, all isolates showed high sensitivity levels to gentamicin, and in less proportion to trimethoprim and tetracycline. These results are in agreement with previous studies performed in others herds of the region (López-Meza et al., 2006; Ochoa-Zarzosa et al., 2008).

Alternatively, bacteriocins from several origins have shown antibacterial activities against staphylococci, and are promising candidates for the treatment of *S. aureus* (Ryan et al., 1998; Capparelli et al., 2007). Recently, it has been demonstrated that only concentrated bacteriocins from *B. thuringiensis* obtained by induction assays showed inhibitory activity against a strain of *S. aureus* (ATCC 25923) (De la Fuente-Salcido et al., 2008). In the present study we have demonstrated that the five bacteriocins from *B. thuringiensis* produced by assays without induction (i.e. low bacteriocin concentration) had inhibitory activity to all *S. aureus* isolates tested by the well-diffusion assay (Table 2). This interesting observation suggests that all *S. aureus* isolates from cows with mastitis from Morelia, Michoacán, México showed susceptibility to the five bacteriocins of *B. thuringiensis,* confirming the potential applied use of those bacteriocins as an alternative to control mastitis where the etiological agent is *S. aureus*. It is important to note that further experiments including purified and normalized samples are required to do comparison assays between the five bacteriocins employed in this work. Although all *S. aureus* isolates were inhibited by the five bacteriocins tested, we did not detect any correlation between sensitivity to antibiotics and bacteriocins, probably because they might have different mechanisms of action (Chen and Hoover, 2003). Although differences in the inhibitory action against a large number of *S. aureus* isolates were found, bacteriocins could be gathered in two groups according to their activities. In the first and second groups we can include, respectively, morricin 269, kurstacin 287 and kenyacin 404, entomocin 420, tolworthcin 524. Bacteriocins from the first group showed the highest activity toward the *S. aureus* isolates followed by those of the second group. Interestingly this classification is similar to a previously reported work (Barboza-Corona et al., 2007) where bacteriocins were categorized in two groups based on distinctive biophysical, biochemical and antimicrobial properties, which suggest that bacteriocins from the same group could indeed, represent similar or bacteriocins with

high identity. Our results are in agreement with others studies that showed that bacteriocins from different bacteria (*L. lactis*, *S. aureus*, *S. epidermidis* and *gallolyticus*) have antibacterial activity against mastitis pathogens (Ryan et al., 1998; Varella Coelho et al., 2007; Wu et al., 2007; Pieterse et al., 2008).

In conclusion, we have demonstrated that *S. aureus* isolates from this work showed two important characteristics: differences in the resistance patterns to antibiotics and susceptibility to bacteriocins from *B. thuringiensis*. These antimicrobial peptides could be useful as an alternative method to control bovine mastitis. Currently, we are conducting experiments to hyperexpress bacteriocins from *B. thuringiensis* to use purified and concentrated bacteriocins for safety and applied use in mastitis control.

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