

Evaluation of Bacteriocin Resistance in *Staphylococcus aureus* against the Bacteriocin Complex Secreted by *Bacillus subtilis* LFB112

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Abstract: In order to pre-evaluate the possibility of bacteriocin resistance when utilizing a bacteriocin complex against pathogenic bacteria, *Staphylococcus aureus* was used to develop a resistant variant by stepwise method. The spontaneous frequency of *S. aureus* resistance to bacteriocin complex was about 10^{-5} . The stability of resistant variant was confirmed by hundred successive transfers without bacteriocin complex stress. The growth rate of resistant variant was similar as that of its original strain. Interestingly in co-culture system, even inoculated with 90% of resistant variant, the resistant variant was cleared out by wild strain in eight consecutive transfers. When treated with either antibiotics or nisin, the resistant variant was more susceptible than the wild strain to all tested antimicrobial agents except chloramphenicol. However, only the tetracycline and chloramphenicol tested groups showed statistically significant increase and decrease of antibacterial potency ($p < 0.05$), respectively. The cellular membrane fatty acid composition of resistant variant was similar to that of wild strain when cultured without bacteriocin complex suggested that the alterations of membrane fatty acid composition in resistant variants might not be the primary defense against bacteriocin. It is proposed that the bacteriocin complex can be used in animal disease preventions and therapeutics as an alternative or complement to traditional antibiotics.

Key words: Bacteriocin-resistance, *Staphylococcus aureus*, fitness cost, fatty acid composition, antibiotic alternative, China

INTRODUCTION

In recent decades, the dramatic rise in antibiotic resistant pathogens has renewed efforts to identify, develop and redesign antibiotics. It is already obvious that the current known antibiotics and possible derivatives will sooner or later lose their efficiency (Dubin *et al.*, 2005). Therefore, the search for novel antimicrobial agents is of great value.

Bacteriocins, a category of antimicrobial bactericidal peptides, ribosomally synthesized by bacteria, exhibited a high bactericidal potency in almost all species of pathogens and a low toxicity to human and animals (Abdi-Ali *et al.*, 2004; Mota-Meira *et al.*, 2000). Thus, they represent potential alternatives or complements to conventional antibiotics in the treatment of infections (Asaduzzaman and Sonomoto, 2009; Hammami *et al.*, 2010; Parisien *et al.*, 2008). According to Klaenhammer (1993), Bacteriocins produced by gram-positive bacteria

can be divided into three main classes based on their chemical and genetic properties. The first class is referred to as lantibiotics and nisin was the most studied one. The second class includes small heat stable bacteriocins such as pediocin PA-1. The 3rd group comprises large heat labile bacteriocins such as helveticin J.

However, the bacteriocin resistant variants were generated experimentally in many laboratories. In fear of bacteriocins following the same step of antibiotics, researchers became more and more concerned with bacteriocin resistance. Previous data showed that the frequency of spontaneous bacteriocin resistance varied randomly from 10^{-9} - 10^{-2} (Gravesen *et al.*, 2002; Guinane *et al.*, 2006). Frequently, the acquisition of bacteriocin resistant variants increased their fitness costs despite an exception in some cases. Bacteriocin resistance was reported to associate with the reduced expression of the mpt operon (Opsata *et al.*, 2010), the positive charges on the cell wall and the composition of fatty acids and

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phospholipids in the cell membrane (Jasniewski *et al.*, 2008; Vadyvaloo *et al.*, 2004a). All these studies were mainly focused on the food bio-preservatives while few were concerned on the risk of bacteriocin resistance when bacteriocins were used in animal disease treatment (Kirkup, 2006). We previously reported that *Bacillus subtilis* LFB112 produced a bacteriocin-like substance with a broad inhibitory spectrum of both gram-positive and negative pathogenic bacteria including *Escherichia coli*, *Salmonella pullorum*, *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Clostridium perfringens*, *Micrococcus luteus*, *Streptococcus bovis* and *Staphylococcus aureus* (Xie *et al.*, 2009). This antimicrobial substance presented a potential application in producing a bacteriocin veterinary medicine. Further investigation demonstrated that this bacteriocin-like substance was a complex composed of a bacteriocin of 47 amino acid residues (AIKLVQSPN GNFAASF VLDGT KWIFKSKY YDSSKGYW VGIYEVW D RK) and several other bacteriocin-like substances (data unpublished). In this study, trials were conducted to investigate the bacteriocin resistance against the bacteriocin complex with the use of *Staphylococcus aureus* as the pathogenic bacterium.

MATERIALS AND METHODS

Bacterial strains and culture conditions: *Bacillus subtilis* LFB112, the producer of bacteriocin complex was isolated from a Chinese herb by predecessors in the group. The optimized conditions to produce bacteriocin in a 50 L automatic fermentation system (GUJS-50, Zhenjiang East Biotech Equipment and Technology) were 30°C, stirring at 180 rounds per minute, ventilating vigorously with filtrated sterile air and using a Landy medium (Landy *et al.*, 1948).

Staphylococcus aureus IVDC C56005 (here-in-after referred to as SA), obtained from China Veterinary Culture Collection Center and grown aerobically at 37°C in nutrient broth/agar was the indicator bacterium used in the bacteriocin activity determination.

Resistant *Staphylococcus aureus* (here-in-after referred to as RSA), developed from SA was grown under the same condition as SA.

Bacteriocin complex preparation: All the bacteriocin complex used in this study were produced in a batch using *B. subtilis* LFB112 cultivated in a 50 L automatic fermentation system (GUJS-50, Zhenjiang East Biotech Equipment and Technology) for 20 h. The culture was harvested and filtered using a nanometer-filter system (Nanjing Kaimi Science and Technology) after the cells

were removed by a ceramic filter system (Nanjing Kaimi Science and Technology). The concentrated liquor with bacteriocins was processed to fine powder by spray-drying forming the final bacteriocin complex. The product was kept in stock at -20°C throughout the experiments.

Antimicrobial activity determination: The antimicrobial activity of bacteriocin complex was determined using the microdilution method described by Naghmouchi *et al.* (2007) replacing nutrient agar as culture media and SA as indicator bacterium. Antimicrobial activity was expressed in arbitrary units per milliliter (AU mL⁻¹), using the formula (1000/125)×(1/D) where, D was the highest dilution causing inhibition of SA. The Minimum Inhibitory Concentration (MIC) was the lowest concentration of bacteriocin complex giving complete inhibition of growth in SA (optical density equal to that of blank). The microdilution assay was done in triplicate.

Bacteriocin resistance frequency determination: The spontaneous bacteriocin resistance frequency was determined referring to the procedure proposed by Watanabe *et al.* (1989). Briefly, a 10 µL portion of each 10-fold serial diluted SA culture (fresh grown on its early stationary phase) was spotted onto nutrient agar with bacteriocin complex at 4 times MIC. Plates seeded SA were incubated at 37°C for 20 h. The values of colony forming unit per milliliter (cfu mL⁻¹) were calculated from colony count giving up to 30 colonies per spot (in most cases were 10-20). The number of viable count in SA culture was checked simultaneously by standard plate count method. The resistance frequency was calculated using the formula: fr = cfur/cfus where, fr was resistance frequency, cfur was cfu mL⁻¹ when SA grown in plates with bacteriocin complex and cfus was cfu mL⁻¹ when SA grown in plates without bacteriocin complex. This test was done in triplicate and the resistance frequency was determined by the average of two independent experiments.

Bacteriocin-resistant variant generation and stability determination: resistant *S. aureus* was generated from SA by stepwise process (Naghmouchi *et al.*, 2007) with 0.5, 1, 2, 4, 8 and 16×MIC bacteriocin complex in nutrient broth in series transfer cultures at 37°C. The purity and identity of each transfer was confirmed by streak plate method. The strain overcoming bacteriocin complex stress at 16×MIC was regarded as RSA in this study and cultured routinely in nutrient broth containing bacteriocin complex at 16×MIC. The stability of the resistant phenotype was tested by hundred generations of exponential growth without bacteriocin complex.

Fitness cost examination: The growth rate of RSA was comparatively studied with SA without bacteriocin complex selection. Nutrient broths inoculated with about 5×10^7 cfu mL⁻¹ SA or RSA, respectively (adjusted to the same OD values) were incubated aerobically at 37°C. Samples were collected at a 2 h intervals from 0-12 h. OD values were measured at 600 nm using an ultraviolet spectrophotometer (UV-8500, Techcomp) and viable bacteria were checked using Plate count method simultaneously for each sample. All the samples were checked in duplicate and the results were the average of three independent experiments.

Competitiveness examination: A competitive experiment of RSA and SA was performed. RSA and SA were mixed on their logarithmic phase at a cell number ratio of 1/9, 1/4, 1/1, 4/1 and 9/1 according to their OD value. Each co-culture system was started at a total cell number of about 5×10^7 cfu mL⁻¹ in the absence of bacteriocin complex in nutrient broth. Ten further successive transfers of the culture with 9/1 and 1/9 of RSA/SA were made to follow up the growth competitiveness of RSA in the co-cultured situation. All cultures were examined their susceptibility by bacteriocin complex at 16×MIC using Agar well diffusion method described below.

Antimicrobial susceptibility determination: The susceptibility of RSA to different antimicrobial agents was determined by agar well diffusion assay. Four oxford cups were assembled symmetrically in each plate which was seeded about 10^6 cfu RSA cells. Each cup was filled with 100 µL streptomycin sulphate, kanamycin sulphate, gentamicin sulphate, penicillin G sodium, ampicillin sodium, chloramphenicol, tetracycline, vancomycin hydrochloride, nisin and the bacteriocin complex, respectively. The concentrations were: antibiotics 0.01 mol L⁻¹, nisin 1.25 g mL⁻¹ and bacteriocin complex 64 AU mL⁻¹.

The plates were incubated aerobically at 37°C for 18-20 h. Diameters of the inhibition zone were recorded to determine the susceptibility of RSA to the antimicrobial agents. SA was tested as a comparison with the identical procedure. This test was done in duplicate and the results were the average of three independent experiments.

Bacteriocin complex decomposition: About 10 mL bacteriocin complex was prepared at 16×MIC using sterilized nutrient broth or normal saline prior to mixing with ca. 1.0×10^9 cfu RSA which was prepared with 11068 g centrifugation from fresh RSA culture. The bacteriocin complex (diluted by nutrient broth and normal saline) without RSA were made as control groups. Both 2

controls and two treatment mixtures were incubated at 37°C with vigorous shaking. Samples were collected from each control and treatment group at 2 h intervals from 0-8 h. Antimicrobial activity of the mixture was examined using Agar well diffusion method described above post RSA removing by centrifugation at 11068 g.

Membrane fatty acids composition analysis: RSA was grown in nutrient broth with or without bacteriocin complex, respectively and SA was grown without bacteriocin complex. Cells were collected on their early stationary phase by an 11068 g centrifugation. The pellets were washed three times with normal saline and lyophilized at -50°C. Cellular fatty acids were converted to methyl esters by acetyl chloride/methanol (1/9, volume in volume) solution using tightly jam-packed tubes with water bath at 80°C for 2 h. The fatty acid methyl esters were extracted by toluene and analyzed using an Hp 6890 series system (Hewlett Packard) equipped with a DB-23 capillary column (60m×0.25mm; J and W scientific). The temperature inside the column was initially maintained at 180°C for 10 min and then gradually increased to 250°C at a rate of 10°C min⁻¹ and finally held at 250°C for 6 min. All samples were analyzed in triplicate and the result was the average of two independent experiments.

Statistical analysis: All the data acquired was statistically analyzed using SPSS Version 12.0. The significant difference between SA and RSA was compared using one-way ANOVA program. For all analysis, difference was considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Antimicrobial activity of bacteriocin complex: The antimicrobial activity of bacteriocin complex in the spraying powder product was 1024 AU g⁻¹ and the MIC was 7.8 mg mL⁻¹.

Spontaneous resistance frequency of SA and stability of RSA: The spontaneous resistance frequency of SA was 9.3×10^{-6} . RSA was developed from SA under the progressive selection (Fig. 1) and the stability of its resistant phenotype was confirmed by hundred successive transfers without selection.

Fitness cost and competitiveness of RSA: The specific growth rate of RSA was similar to that of SA in both viable count and OD value (Fig. 2). In co-culture system, RSA multiplied much slower than SA and remained the minority on stationary phase even inoculated at a RSA/SA cell ratio of 9/1. Moreover, RSA was replaced by SA gradually in eight successive transfers following up.

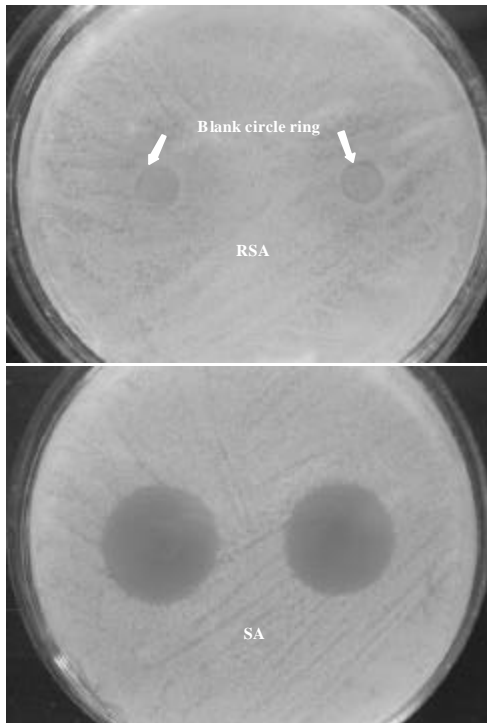


Fig. 1: Resistant *S. aureus* (RSA) was seeded ca. 10^6 cfu and challenged with 100 μ L bacteriocin complex at $16\times$ MIC using Agar well diffusion method. The blank circle ring on RSA was formed by the press of oxford cup. *S. aureus* (SA) was the comparison made with same procedure

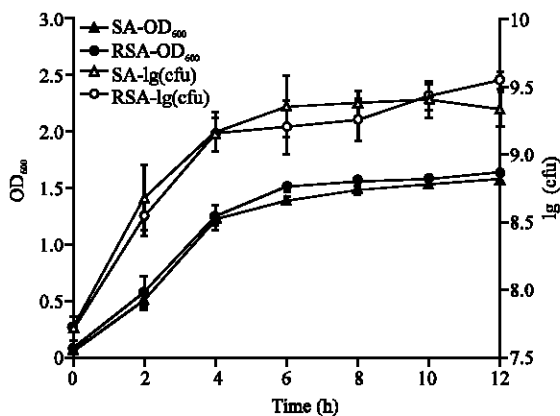


Fig. 2: The growth characteristics of *S. aureus* (SA) and Resistant *S. aureus* (RSA) cultured with vigorous shaking in nutrient broth at 37°C in the absence of bacteriocin complex

Antimicrobial susceptibility of RSA: SA was sensitive to all antimicrobial agents and RSA was completely resistant to the bacteriocin complex as presumed (Fig. 3). Seven out of eight antibiotics tested in this study improved their

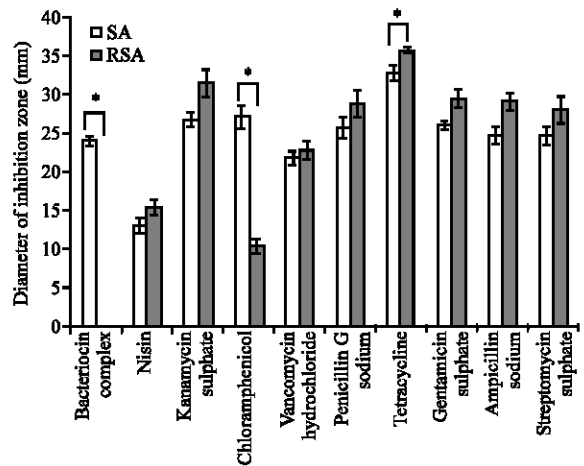


Fig. 3: Antimicrobial susceptibilities of *S. aureus* (SA) and Resistant *S. aureus* (RSA) checked with 100 μ L antibiotics (0.01 mol L^{-1}), nisin (1.25 g mL^{-1}), bacteriocin complex ($16\times$ MIC), respectively using Agar well diffusion method. *Significant difference ($p<0.05$) was presented between RSA and SA

antibacterial potency to RSA and tetracycline demonstrated a significant ($p<0.05$) change when they were compared to that of SA. Unfortunately, chloramphenicol shrank the inhibition zone significantly ($p<0.05$) when the bacterium developed resistance of bacteriocin complex from SA to RSA.

Bacteriocin complex decomposition and membrane fatty acids composition: In the mixture of RSA and the bacteriocin complex, RSA did not attenuate the antimicrobial activity of bacteriocin complex either incubated in normal saline or nutrient broth.

The mass content of fatty acids was significant increased in C18:3 ($p<0.05$) and decreased in C20:0, 18:0 and 14:1 ($p<0.05$) in RSA cultured with bacteriocin complex selection as compared with RSA cultured without bacteriocin complex. However, the fatty acid constituent of SA and RSA cultured in the absence of bacteriocin complex appeared similar ($p>0.05$, Table 1).

Recently, superbugs with NDM-1 which was mostly found among *Escherichia coli* and *Klebsiella pneumonia* (Kumarasamy *et al.*, 2010) have killed several human lives and fatal cases continue to occur all over the world. The situation that the speed of developing novel antibiotics is much slower than the rate at which pathogens acquire resistance makes the development of alternative therapeutic strategies a burning necessity. Bacteriocins targeting the membrane with their observed diversity and proven effectiveness, provide a very promising approach worthy of development in animal disease control.

Table 1: Cellular fatty acids composition of *S. aureus* and its resistant variant grown in nutrient broth with or without bacteriocin complex (mg g⁻¹)

Bacteriocin complex	<i>S. aureus</i>		Resistant <i>S. aureus</i>	
	0 MIC	0 MIC	0 MIC	16 MIC
C14:0	0.181±0.037	0.199±0.080	0.124±0.001	
C14:1	1.438±0.164 ^{AB}	2.051±0.385 ^A	1.114±0.025 ^B	
C16:0	1.705±0.212	1.869±0.536	1.856±0.158	
C16:1	0.034±0.009	0.047±0.037	0.040±0.004	
C18:0	3.143±0.183 ^{AB}	3.959±0.583 ^A	2.402±0.104 ^B	
C18:1n9	0.419±0.061	0.338±0.088	0.263±0.009	
C18:2n6	0.126±0.032	0.083±0.016	0.044±0.021	
C18:3n3	0.106±0.026 ^A	0.115±0.025 ^A	0.288±0.019 ^B	
C20:0	4.420±0.240 ^A	4.608±0.381 ^A	2.550±0.072 ^B	
C20:1	0.279±0.046	0.271±0.042	0.140±0.001	
C22:0	0.068±0.028	0.049±0.035	0.128±0.108	

Cells of *S. aureus* (SA) and Resistant *S. aureus* (RSA) were harvested and lyophilized on their early stationary phase. Fatty acids were extracted and analyzed by converting to methyl esters with acetyl chloride/methanol (1/9 volume) solution using Gas chromatography method. Data were acquired from two independent experiments. Different superscript in the same line means significant difference (p<0.05)

However, more studies were required to ensure the safeties and low risks of bacterial resistance in the application of bacteriocins. In this study, bacteriocin resistance to the bacteriocin complex was observed occurring at a frequency of 9.3×10^{-6} in SA. RSA was generated and the stability of its resistant phenotype was confirmed by hundred successive generations without bacteriocin complex.

The result that the specific growth rate of RSA was similar to that of SA was consistent with results from Sakayori *et al.* (2003) but contrary to those that the resistant variant had fitness costs in reducing specific growth rate (Vadyvaloo *et al.*, 2004b).

In order to assess the potential spread of RSA in natural populations of SA, RSA was competed with SA in co-culture systems. The dwindlement of RSA in all co-cultures illustrated that RSA was weaker than SA when they grew together. Furthermore, the replacement by SA in co-culture with 90% inoculum of RSA demonstrated that RSA would be eventually cleaned out by SA when bacteriocin complex was not present. When challenged with antimicrobial agents, RSA became more susceptible than SA to nisin and all tested antibiotics except chloramphenicol, however only the decrease of chloramphenicol antimicrobial potency and the increase of tetracycline antimicrobial potency were statistically significant (p<0.05). These observations displayed the controversial situation of inter-functions between bacterions and antibiotics in treatment of bacteriocin resistant bacteria. Duffes *et al.* (2000) reported that bacteriocin resistance did not confer any resistance to penicillin, amoxicillin, vancomycin, rifampin, kanamycin, chloramphenicol, erythromycin, gentamicin or tetracycline. On the contrary, Naghmouchi *et al.* (2007)

declared that all bacteriocin resistant variants were more resistant to ampicillin, chloramphenicol, vancomycin and kanamycin than that of its original strain. Actually, cross-resistance between bacteriocin and antibiotics or inter bacteriocins might be primarily decided by the physiological characteristics of resistant variants and the targets that antimicrobial agents act on.

The increased resistance to antibiotics may indicate that the compositional changes in the cell of variants selected by exposure to bacteriocin can also confer protection to other inhibitory substances such as antibiotics. By the same token, the resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes (Draper *et al.*, 2009). Cross-resistance of bacteria to a bacteriocin is an intrinsic characteristic and no clear correlation has been established between the bacteriocins and antibiotics. In order to investigate the possible mechanisms of RSA resistance, bacteriocin complex was incubated with RSA in normal saline solution or nutrient broth at 37°C. Both groups illustrated identically that RSA had no effect on the activity of bacteriocin complex.

In other words, the components of bacteriocin complex were not decomposed by intracellular or extracellular enzymes of RSA. Based on this precondition, we inferred that the reason why RSA resisted to the bacteriocin complex was that RSA escaped from the influence of the bacteriocin complex instead of diminishing its activity. Therefore, we analyzed the cellular fatty acid composition which was supposed to confer the resistance property of RSA. Surprisingly, there was no difference in fatty acid composition between RSA and SA when they were cultured in media without bacteriocin complex. Therefore, it suggested that the resistance of RSA was not due to the change of cellular membrane fatty acids. However, the increase of C18:3 and decrease of C20:0 were significant in RSA which was cultured with bacteriocin complex as compared to RSA and SA which were cultured without bacteriocin complex. These results illustrated that variation of membrane fatty acids was not the factor that induced RSA resistance but the outcome of additional bacteriocin complex.

The mechanism of resistance to bacteriocins has been correlated with changes in membrane fatty acid and phospholipid composition, cell wall structure and requirements for divalent cations. The increased proportion of saturated fatty acids in the bacteriocin-resistant variants should increase membrane rigidity rendering it less fluid and thereby impeding penetration by bacteriocin molecules (Naghmouchi *et al.*, 2007). On the other hand, an increase in short-acyl-chain and unsaturated phosphatidylglycerol in resistant strains

indicated greater fluidity of the cell membranes, thus also increasing the resistance of mutant varieties (Vadyvaloo *et al.*, 2004a). In the study, the fatty acid composition was similar between SA and RSA when cultured without bacteriocin complex but significantly different in some fatty acids of RSA when cultured with bacteriocin complex. The result was consistent with Limonet *et al.* (2002). These results suggested that the alterations of membrane fatty acid composition in resistant variants might not be the primary defense against bacteriocin.

CONCLUSION

RSA can not be spread out in reality due to its weaker competitiveness to SA when they grow together. The difference of fatty acid composition was induced by bacteriocin complex nevertheless, it was not the cause of resistance in RSA. Cross-resistance inter-bacteriocins or between bacteriocin and antibiotics is a characteristic that requires further studies of resistant strains. It is proposed that bacteriocins can be used in animal disease preventions and therapeutics as an alternative to antibiotics which were serious in high and/or multi-resistance of bacteria for the moment.

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