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Membranes of five-fold alamethicin-resistant *Staphylococcus aureus, Enterococcus faecalis* and *Bacillus cereus* show decreased interactions with alamethicin due to changes in membrane fluidity and surface charge

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Abstract - The aim of this study was to understand development of resistance to alamethicin (a model barrel stave pore forming antimicrobial peptide) by investigating changes in phospholipid profile, fatty acid side chain analysis and extent of alamethicin insertion in biomimetic membrane prepared form wild type strains and five folds alamethicin resistant variants of *Staphylococcus aureus* NCDC 110, *Enterococcus faecalis* NCDC 114 and *Bacillus cereus* NCDC 66. The wild type strains NCDC 110, 114, 66, were sensitive to alamethicin with IC_{50} 5.5, 3.25 and 2.0 µg/ml respectively. Wild type strains were cultured in the presence of alamethicin to select resistant variants with IC_{50} 29.0, 17.0 and 9.5 µg/ml respectively. The phospholipid profile analysis revealed increase in amino-group containing phospholipids to amino-group lacking phospholipids ratio between wild-type and resistant variant in *S. aureus* and *B. cereus* but decreased in *E. faecalis*. Predominant fatty acids in all strains were composed of even number of carbons. Linoleic acid was detected only in resistant strain of *B. cereus*. As indicated by saturated-to-unsaturated fatty acids ratio, the membrane from *S. aureus* and *E. faecalis* became more rigid, whereas, in *B. cereus* it became more fluid. Using a colorimetric *in vitro* assay, a decrease in alamethicin insertion in the biomimetic membrane could be observed upon acquisition of resistance. The membranes of five-fold alamethicin-resistant *S. aureus, E. faecalis* and *B. cereus* revealed changes in membrane fluidity and surface charge upon acquisition of resistance to alamethicin.

Key words: alamethicin; antimicrobial peptide; phospholipids; fatty acids; resistance.

INTRODUCTION

The continuous use of antibiotics has resulted in multi-resistant bacterial strains all over the world and as expected, antibiotic resistance issue has been a major public health concern (Powledge, 2004). Consequently, there is an urgent need to search for alternatives to synthetic antibiotics. The discovery of diverse population of non-toxic, non-immunogenic and potent antimicrobial peptides (AMPs), as an essential component of anti-infective defence mechanisms in mammals, amphibians, insects, plants, fungi and bacteria offer effective alternative candidates against bacteria, fungi, viruses and protozoa resistant to synthetic drugs (Hancock and Chapple, 1999; Zasloff, 2002). Different modes of actions for these AMPs have been suggested and identified, such as pore forming, inhibition of cell-wall, nucleic acid or protein synthesis etc, (Zasloff, 2002; Brogden, 2005). Bacteriocins produced by lactic acid bacteria are generally considered to be safe and therefore, their use as natural food preservatives has been under investigation. Nisin, an AMP produced by *Lactococcus lactis*, has been granted generally regarded as safe (GRAS) status for certain applications by Food and Drug Administration (1988). It forms voltage dependent ion-permeable channels following barrel-stave manner. Other AMPs such as YGNGV motif containing bacteriocins (also known as pediocin-like or class IIa bacteriocins) from Lactic acid bacteria and alamethicin from fungi *Trichoderma viridae* also forms voltage dependent pores in barrel stave manner (North *et al.,* 1994; Moll *et al.,* 1997; Yang *et al.,* 2001; Sood and Sinha, 2003). However, as expected, resistance against Barrel-Stave Pore Forming Antimicrobial Peptides (BSPF-AMPs) such as nisin and YGNGV-motif containing bacteriocins has been reported (Davis and Adams, 1994; Ming and Daeschel, 1995; Gravesen *et al.,* 2001; Vadyvaloo *et al.,* 2002). Since these bacteriocins follow the same mechanism for their action i.e. barrel-stave type pore formation, therefore, it may be possible that the resistance among these is also developed through a similar mechanism.

Understanding of the mechanism of resistance development will facilitate effective AMP design against resistant varieties. Currently, the mechanism for development of resistance remains poorly understood. Changes in membrane fluidity and surface charge have been reported upon acquisition of intermediate (2-10 fold) and high level (> 100

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fold) resistance by *Listeria*, *Leuconostoc* or *Weissella* strains, or *Enterococcus faecium*. In addition, σ^{54} or σ^{54} dependent genes, specifically phosphotransferase system in *Listeria monocytogenes,* and σ^{54}, glycerophosphoryl diester phosphodiesterase and a protein with putative phosphodiesterase function in *Enterococcus faecalis* has been correlated with development of high level resistance (Gravesen *et al.,* 2001; Hechard *et al.,* 2001; Segolene *et al.,* 2007). Alterations in cell wall or requirement for divalent cations have also been reported for high level nisin resistance in *L. monocytognes*. However, there is no report that the changes associated with the development of resistant actually cause decrease in AMP-target membrane interaction *in vitro* upon acquisition of resistance. In addition, there is scarce information available in other pathogenic species such as *Bacillus cereus, Staphylococcus aureus* and *E. faecalis* and there is no report for development of resistance among microbes against the thoroughly studied model BSPF-AMP, alamethicin. *Bacillus* cereus is responsible for economic losses due to spoilage of pasteurized milk and cream. It is also implicated in food-borne delayed onset of diarrheal syndrome characterized by symptoms of enterotoxicosis, abdominal cramps and rectal tenesmus, and a rapid onset of emetic syndrome with nausea, vomiting and occasionally diarrhoea. *Enterococcus faecalis* is used as an indicator of fecal contamination of food and clinically it has become a serious nosocomially transmitted pathogen. *Staphylococcus aureus* is a well known bacterial pathogen which causes food borne illnesses and nosocomial infection in humans. It is a major etiological agent in ruminant mastitis.

BSPF-AMPs act on the cell membrane after diffusion through cell wall. Events involved are i) initial electrostatic binding and aggregation of monomers on the membrane surface to form a bundle, ii) insertion of the bundle in the cell membrane and iii) stabilization of pore in lipid bilayer through recruitment of more monomers or optimization of hydrophobic interactions (Sansom, 1993; Beven *et al.,* 1999; Yang *et al.,* 2001; Sood and Sinha, 2003). The aim of the present study was to elucidate the mechanism of resistance development through comparison of each of these steps between wild-type and its stable resistant variant. Therefore, we used three different Gram positive sensitive (wild-type) strains namely *Staphylococcus aureus* NCDC 110, *Enterococcus faecalis* NCDC 114 and *Bacillus cereus* NCDC 66 and their stable resistant variants against alamethicin to investigate each of these steps.

MATERIALS AND METHODS

Bacterial strains and determination of IC_{50}. Three gram positive bacteria viz, *Staphylococcus aureus* NCDC 110, *Enterococcus faecalis* NCDC 114 and *Bacillus cereus* NCDC 66 obtained from National Collection of Dairy Cultures were grown in nutrient broth at 37 °C. The IC_{50} was determined using broth assay (Cabo *et al.,* 1999). Briefly, 995 µl of sterile nutrient broth was taken in sterile Eppendorf tubes. To first tube, 5 µl of alamethicin (5 mg/ml) solution was added. To rest of the tubes, 5 µl of alamethicin (5 mg/ml) was added after double dilutions. To each of the tubes 5 µl of test culture containing 1-5 x 10^5 CFU/ml was added. A positive control was setup with broth and test culture and a negative control with broth alone. All the tubes were mixed well and incubated at 37 °C overnight. Optical density (OD) of the culture was taken at 600 nm with negative control as blank. From OD data, dose response of inhibition was calculated using the formula:

$$I = 1-(A_m/A_o)$$

where, A_m is OD of the culture at different concentrations of alamethicin and A_o is OD of the positive control. IC_{50} was determined as the concentration of the alamethicin resulting in 50% inhibition of the test culture.

Selection of resistant variants. Resistant mutants were selected using the method described by Rekhif *et al* (1994). Sensitive culture containing 1-5 x 10^5 CFU/ml was added to nutrient broth containing alamethicin and incubated at 37 °C. The broth culture was serially diluted and plated on nutrient agar without alamethicin. The plates were incubated at 37 °C for 24 h. Five colonies were randomly selected, propagated in nutrient broth for 10 successive subcultures without alamethicin and IC_{50} was determined to assess resistance development.

Determination of growth curves for sensitive strains and resistant variants. Briefly, to 13 tubes of 5 ml sterile nutrient broth, 5 µl of overnight grown cultures of sensitive strains and resistant variants were added and incubated at 37 °C till the end of experiment. Optical densities of the cultures were recorded at 600 nm from 0 to12 h with 1 h interval using fresh media as blank.

Analysis of phospholipids. The analysis of phospholipids was done as follows. The wild-type or resistant strain was inoculated at 1% level in nutrient broth and incubated at 37 °C overnight. The cells were harvested through centrifugation, washed and resuspended in 50 ml of physiological saline. Total cellular lipids were extracted by the method of Bligh and Dyer (1959). Individual lipids were resolved using thin layer chromatography (TLC) for identification and quantification (Katz *et al*., 1999). Briefly TLC plates were prepared by using silica gel G. The plates were allowed to dry for 15 min and activated at 110 °C for 1 h in an oven before sample application. Known quantities of phospholipid standards and lipid extracts from sensitive strain and resistant mutant were dissolved in chloroform: methanol (2:1 v/v) to give a final concentration of 5-10 µg/µl. With a micropipette, 4 µl of each standard was spotted on the plate in each lane. Ten µl of mixture containing a mixture of standard phospholipids was also spotted in a separate lane. Similarly, extracted lipids from sensitive strain and resistant mutant were spotted in separate lanes. All the spots were allowed to dry before developing the TLC plates. Spotted plates were developed by mobile phase solvent consisting of chloroform: methanol: distilled water: glacial acetic acid (65:25:4:1 v/v). Different classes of resolved lipids were visualized using specific stains viz. iodine vapours for general lipids, ammonium molybdate for phospholipids and ninhydrin for amino-group containing phospholipids (ACP). The various classes of lipids were identified in comparison with standard lipids.

The identified phospholipids were quantified by estimating the phosphorus content in the resolved spots on TLC plates by the method of Kahovcova and Odivac (1969). Charring chromatographic plates with sulphuric acid releases phosphorous which upon reacts with Hahn and Luckhaus' reagent and gives blue colour. Colour developed can be estimated by taking absorbance at 700 nm and amount of phosphorous determined from standard curve. Multiplying the amount of phosphorous with a factor 25 gives the amount

of phospholipids in each spot. Briefly, to 5 numbers of test tubes 0.2, 0.4, 0.6, 0.8 , 1.0 ml of standard phosphorous solution (working solution containing 16 µg/ml phosphorous) was added and volume made to 4 ml with distilled water. One tube with 4 ml distilled water alone was set as blank. To these tubes 1 ml of Hahn and Luckhaus' reagent was added and heated in a boiling water bath for 30 min. The tubes were then cooled and absorbance recorded at 700 nm using spectrophotometer against blank. The amount of phosphorous and absorbance were plotted on a graph to get the standard curve. Spots on TLC plates were initially identified with iodine vapours and positions of spots were marked. Iodine was allowed to evaporate. The plate is then sprayed with 50% sulphuric acid and kept in an oven set at 180 °C for 1 h. Equal amounts of the charred spots were then scrapped in to test tubes. Silica gel without lipids was also scrapped to set up blank. To these tubes 4 ml of distilled water was added, mixed well and 1 ml Hans-Lukaus reagent was added. All the tubes were then heated in a boiling water bath for 30 min. The tubes were cooled and centrifuged at 2000 rpm for 2 min to sediment silica. The absorbance of the blue colour developed was taken at 700 nm. From the standard curve, the amount of phosphorous in each spot was calculated.

Analysis of fatty acid composition. Total cellular lipid extract from sensitive and resistant variant was transesterified to get fatty acid methyl esters (FAMEs) and analysed using gas liquid chromatography (Sakayori *et al.,* 2003). Briefly, 5-10 mg of total lipid extract from sensitive or resistant variant was dissolved in 1 ml of chloroform in Teflon coated screw capped tubes and 3 ml of methanol:hydrochloric acid:chloroform (10:1:1 v/v) solution was added. The tubes were kept in an oven set at 90 °C for 60 min. Then the tubes were taken out and cooled to room temperature. One ml of distilled water was added to the transesterified mixture in the tubes followed by 3 ml of hexane:chloroform (4:1 v/v) solution. The upper hexane phase was collected and extraction was repeated once with hexane: chloroform (4:1 v/v) solution. Both the aliquots were pooled and allowed to evaporate. The residue was weighed and then dissolved in known volume of chloroform to give final concentrations of FAMEs in the range of 10-100 µg/µl. Fatty acid methyl esters were analyzed on MICHRO 9100 Gas chromatograph with supelcowax-fused silica capillary column of 60 m x 0.32 mm x 0.25 µm film thickness. Initial oven temperature was kept at 150 °C and an isothermal time of 2 min was allowed. Then a ramp rate of 5 °C/min was used to bring the temperature to 220 °C and an isothermal time of 18 min was allowed. Injector temperature was kept at 230 °C and flame ionization detector at 240 °C. Nitrogen was used as carrier gas and flow rate was maintained at 30 ml/min. The profile of fatty acid composition included data on the retention time, response, area and height, equivalent chain length values, peak name, and percentage of each fatty acid. Identification of FAMEs from sensitive or resistant variant was carried out by comparing retention time range of standard FAMEs used. Quantification of identified FAMEs was done by analyzing peak height and peak area.

***In vitro* estimation of alamethicin insertion in the membrane.** A colorimetric assay developed by Kolusheva *et al.* (2000) was used to quantify alamethicin interaction with biomimetic membrane prepared from total cellular lipid extract. Briefly, pure phospholipid-polydiacetylene biomimetic membrane vesicle solution was prepared by sonication of the aqueous mixture of phospholipid and 10-12 tricosadiynoic acid (4:6 mole ratio) at 70 °C, followed by slow cooling overnight and subsequent irradiation at 254 nm. Similarly, biomimetic membrane vesicle solution was prepared by mixing total cellular lipid extract and 10-12 tricosadiynoic acid at 1:1 mole ratio. The vesicles exhibited intense blue colour. Samples were prepared by adding alamethicin to vesicles solution to obtain 1 mM vesicles and 2 mM Tris (pH 8.5). The solution was diluted 5 times and spectrum was acquired. The colorimetric response was calculated as:

$$CR = (PB_0 - PB_1) / PB_0$$

where PB_0 and PB_1 is proportion of blue without and with alamethicin respectively, and:

$$PB = A_{blue} / (A_{blue} + A_{red})$$

where A is the absorbance for either red component (500 nm) or blue component (640 nm).

RESULTS

Selection of alamethicin-resistant variants

Sensitivity of three susceptible wild type gram positive strains to alamethicin was determined by broth assay and expressed as IC_{50}. From dose response inhibition curves, IC_{50} of wild type *S. aureus* NCDC 110, *E. faecalis* NCDC 114 and *B. cereus* NCDC 66 were determined as 5.5 µg/ml, 3.25 µg/ml and 2 µg/ml respectively (Fig. 1A, 1B and 1C). These three sensitive wild type strains were selected for enrichment of resistant mutants with reduced sensitivity. In the presence of 5 times of alamethicin IC_{50} i.e. 27.5 µg/ml, *S. aureus* NCDC 110 showed visible growth in broth only after 5 days. *Enterococcus faecalis* NCDC 114 showed no visible growth for 3 days with 9 times of alamethicin IC_{50} i.e. 30.0 µg/ml. However, after 5 days, some colonies could be obtained upon plating. *Bacillus cereus* NCDC 66 showed no visible growth with 10 times of IC_{50} i.e. 20.0 µg/ml. However, visible growth could be obtained after 3 days at 5 times of IC_{50}. Each of the mutants of *S. aureus* NCDC 110, *E. faecalis* NCDC 114 and *B. cereus* NCDC 66 after 10 successive subculturing without alamethicin showed IC_{50} of 29, 17 and 9.5 µg/ml respectively (Fig. 1A, 1B and 1C) and were approximately 5 times less sensitive as compared to its wild-type strain.

Determination of growth curves of alamethicin sensitive and resistant variants

The growth of the sensitive strain of *S. aureus* NCDC 110 showed a lag phase of 2 h, log phase of 3 to 5 h and finally reached stationary phase at around 6 to 7 h. Sensitive strain of *E. faecalis* NCDC 114 revealed a lag phase of about 3 h followed by a log phase that continued up to 9 h and followed by stationary phase. Whereas *B. cereus* NCDC 66 showed a lag phase of 2 h, log phase of 4 h and finally reached stationary phase at around 6 to 7 h. However, in all the three resistant variants lag phase was extended by 2 h and log phase was slightly reduced when compared to sensitive strains. We found no significant difference in stationary phase of sensitive and resistant variants (Figure. 2A, 2B and 2C).

Comparison of fatty acid and phospholipid composition between wild-type and resistant variants

There were significant differences in composition of fatty acids between wild-type and respective resistant strain.

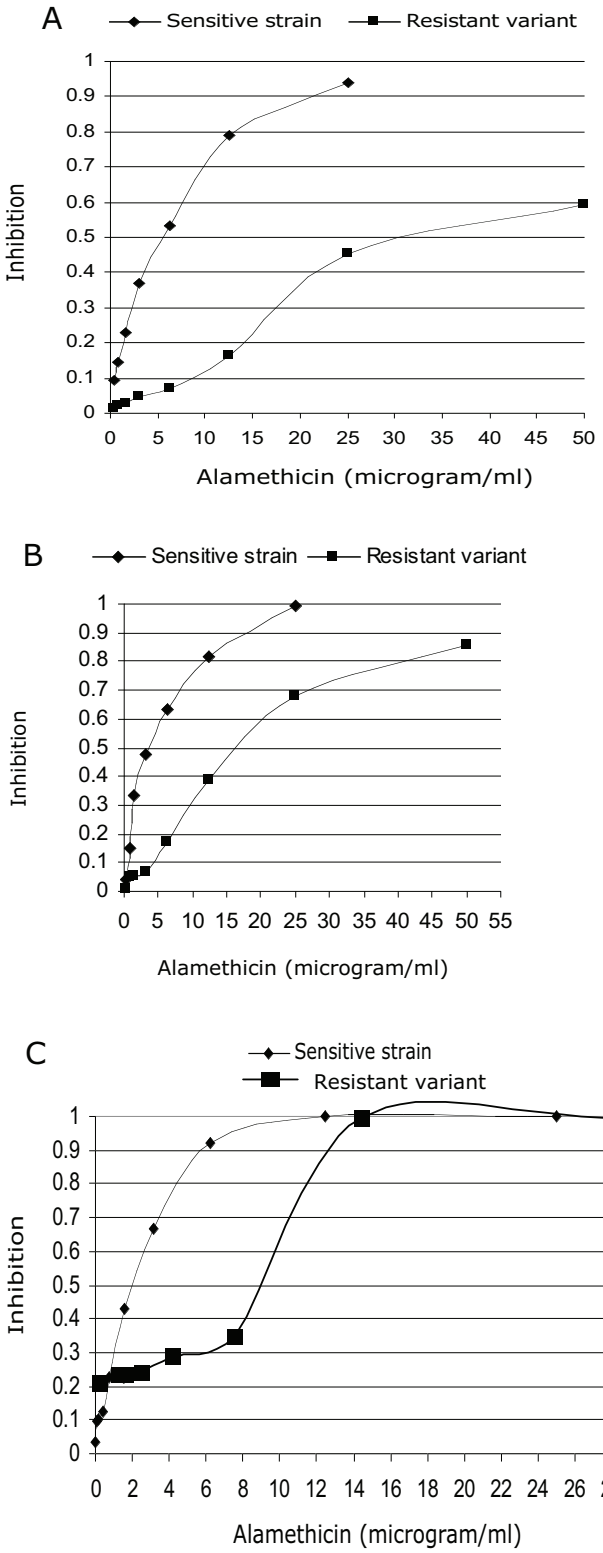

FIG. 1 - Dose-Response inhibition curves for alamethicin sensitive and resistant variant of *Staphylococcus aureus* (A), *Enterococcus faecalis* (B) and *Bacillus cereus* (C) cultured in presence of various concentrations of alamethicin for overnight at 37 °C.

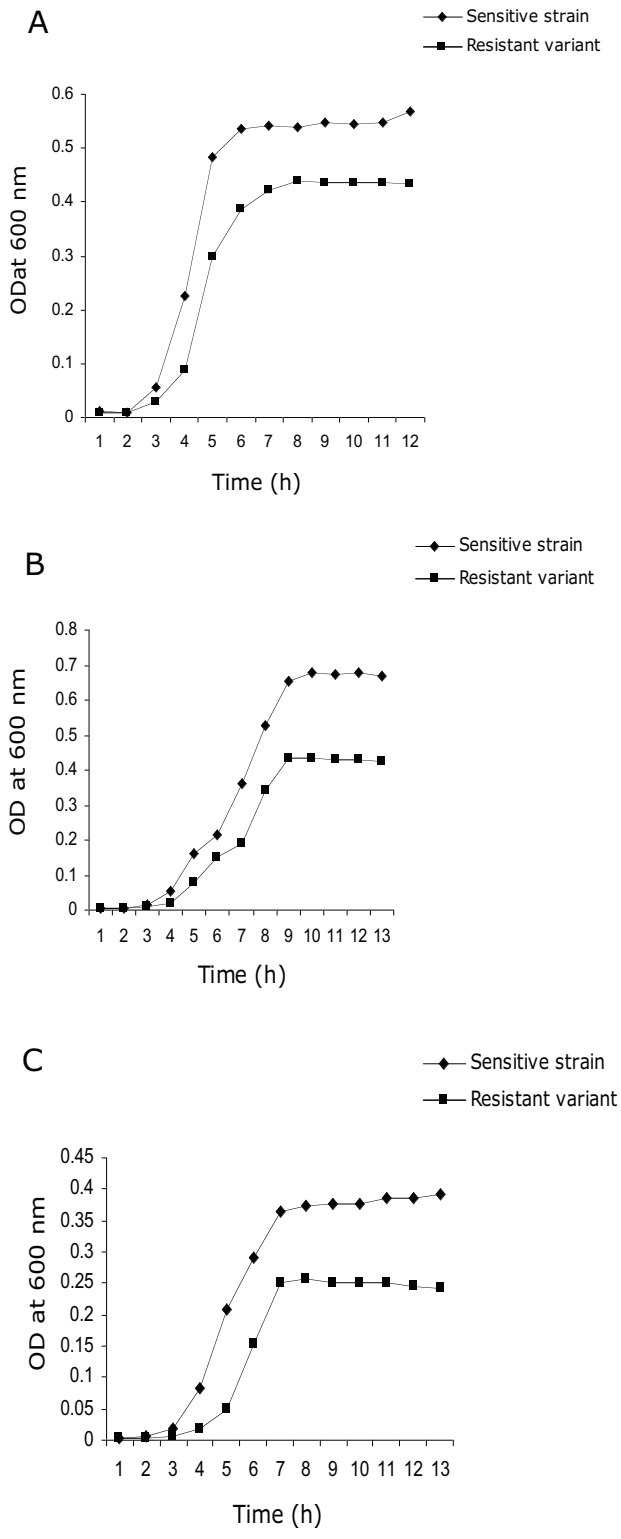

FIG. 2 - Growth curves of alamethicin sensitive and resistant variant of *Staphylococcus aureus* (A), *Enterococcus faecalis* (B) and *Bacillus cereus* (C).

Predominant fatty acids in all strains were composed of even number of carbon (Fig. 3). Linoleic acid (C18:2) was detected only in resistant strain of *B. cereus*. As indicated by saturated-to-unsaturated fatty acids ratio (Table 1), the membranes of *S. aureus* and *E. faecalis* became more rigid, whereas, in *B. cereus* it became more fluid. These results indicate that the saturated-to-unsaturated fatty acids ratio and consequently membrane fluidity correlates with development of resistance against alamethicin in these strains. The membranes of wild-type strains were composed mainly

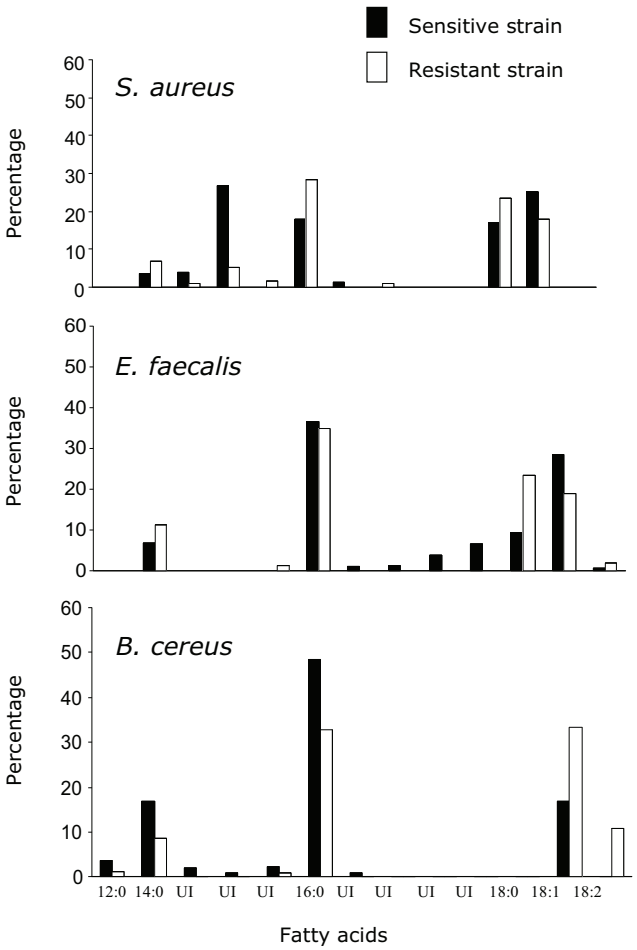

FIG. 3 - Fatty acids composition of wild type and resistant strains of *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus cereus*. 12:0: Lauric acid; 14:0: Myristic acid; 16:0: Palmitic acid; 18:0: Stearic acid; 18:1: Oleic acid; 18:2: Linoleic acid; UI: Un identified.

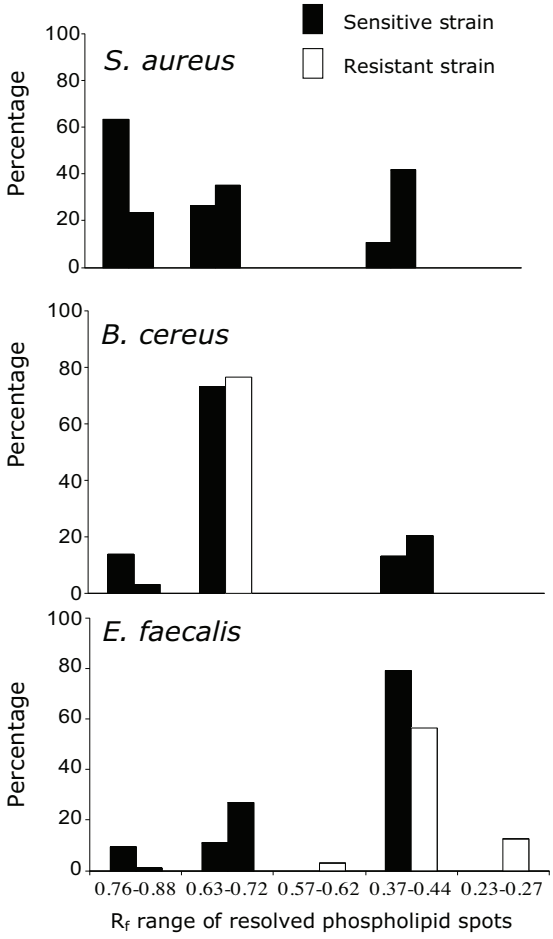

FIG. 4 - Phospholipids spots of wild type and resistant strains of *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus cereus* resolved by thin layer chromatography. R_f: Retardation factor.

TABLE 1 - Fatty acid composition of wild-type and alamethicin resistant (Ala^r) mutants resolved using SUPELCOWAX 10 column

Retention time range (min after solvent peak)	Fatty acid type	Fatty acid (%)					
		Staphylococcus aureus		*Enterococcus faecalis*		*Bacillus cereus*	
		Wild-type	Resistant	Wild-type	Resistant	Wild-type	Resistant
3.33-3.50	C12:0	nd	nd	nd	nd	3.62 ± 0.07	1.12 ±0.22
4.04-4.10	UI	nd	nd	nd	nd	nd	nd
5.59-5.99	C14:0	3.65 ± 0.17	6.95 ± 0.44	6.83 ± 0.67	11.22 ± 0.82	16.88 ± 0.70	8.56 ± 0.62
6.33-6.41	UI	4.01 ± 0.32	w	nd	nd	2.12 ± 0.18	nd
6.57-6.85	UI	26.64 ± 0.38	5.34 ± .027	nd	nd	w	nd
7.17-7.33	UI	nd	w	nd	w	2.41 ± 0.24	w
8.70-9.20	C16:0	18.05 ± 0.54	28.31 ± 2.43	36.66 ± 0.59	34.79 ± 2.10	48.26 ± 1.54	32.66 ± 1.00
9.39-9.74	UI	w	nd	w	nd	w	nd
10.62-11.80	UI	nd	w	w	nd	nd	nd
12.20-12.40	UI	nd	nd	3.83 ± 0.42	nd	nd	nd
12.70-12.92	UI	nd	nd	6.61 ± 0.33	nd	nd	nd
12.98-13.58	C18:0	16.80 ± 0.55	23.53 ± 0.19	9.34 ± 0.78	23.49 ± 1.22	nd	nd
13.60-13.92	C18:1	25.10 ± 2.25	17.95 ± 2.24	28.51 ± 1.23	18.86 ± 1.32	16.80 ± 1.43	33.31 ± 1.30
13.99-14.42	C18:2	nd	1.95 ± 0.11	w	w	nd	10.73 ± 0.56
Saturated to Unsaturated ratio		1.53	2.95	1.85	3.68	4.09	0.98

UI: un identified; nd: not detected; w: weak signal (< 2% peak area).
Experiments were conducted in triplicates and their mean ± SD are presented.

TABLE 2 - Phospholipid composition of wild-type and alamethicin resistant (Ala^r) mutants

Rf range	Phospholipid (Phosphorous %)					
	Staphylococcus aureus		*Enterococcus faecalis*		*Bacillus cereus*	
	Wild-type	Resistant	Wild-type	Resistant	Wild-type	Resistant
0.76-0.88	64.10 ± 1.10 (ALP)	25.15 ± 0.20 (ALP)	43.06 ± 0.15 (ALP)	33.27 ± 0.12 (ALP)	7.30 ± 0.40 (ALP)	11.16 ± 0.18 (ALP)
0.63-0.72	25.55 ± 0.13 (ALP)	35.09 ± 0.24 (ACP)	24.24 ± 0.11 (ALP)	14.50 ± 0.52 (ALP)	77.12 ± 0.30 (ACP)	69.08 ± 0.35 (ACP)
0.57-0.62	nd	nd	nd	3.44 ± 0.20 (ALP)	nd	nd
0.37-0.44	10.08 ± 0.12 (ACP)	39.25 ± 0.23 (ACP)	32.45 ± 0.29 (ACP)	34.02 ± 0.65 (ACP)	14.61 ± 0.27 (ACP)	19.41 ± 0.25 (ACP)
0.23-0.27	nd	nd	nd	13.27 ± 0.29 (ACP)	nd	nd
ACP-to-ALP ratio	0.11	2.95	0.48	0.92	12.56	7.92

ALP: amino group lacking phospholipid; ACP: amino group containing phospholipid; nd: not detected.
Experiments were conducted in triplicates and their mean values with standard deviation are presented along with phospholipid spots identified (given in brackets) using specific stains.

of three phospholipids (Fig. 4). By comparing the mobility of the phospholipids on TLC with those of standards and staining pattern, these were identified as either amino-group containing (ACP) or amino-group lacking phospholipids (ALP). The membranes from both wild-type and resistant strains of *B. cereus* mainly composed of ACP. In *S. aureus* phospholipid with R_f in the range of 0.63-0.72 (spot 2) acquired amino-group in its head group. Two additional phospholipids in *E. faecalis* viz., one ALP (spot 3) and one ACP (spot 5), appeared in resistant strains (Fig. 4). The ACP-to-ALP ratio between wild-type and resistant variant increased in *S. aureus*, *E.* faecalis and decreased in *B. cereus* (Table 2). These results suggest that composition level as well as change of head group differences in phospholipids contribute to development of alamethicin resistance.

Comparison of alamethicin-membrane interactions between wild-type and resistant variants

Effective colorimetric response % (ΔCR%) i.e. biologically meaningful colorimetric response was higher in case of vesicles prepared from pure phospholipids such as distearoyl phosphatidylethanolamine, dipalmitoyl phosphatidylglycerol and dimyristoyl phosphatidylglycerol (Fig. 5) as compared to vesicles prepared from total cellular lipid extracts (Fig. 6). The decrease in ΔCR% upon acquisition of resistance indicates that development of resistance among these Gram positive bacteria is due to decrease in membrane-alamethicin interactions.

DISCUSSION

BSPF-AMPs kill their target bacteria through formation of pores in the cell membrane after diffusion through cell wall into the periplasmic space. For pore formation by these BSPF-AMPs such as nisin (Moll *et al.,* 1997) YGNGV motif containing bacteriocins (Sood and Sinha, 2003) and alamethicin (Sansom, 1993; Yang *et al.,* 2001) at least 3 steps are required. These include i) initial electrostatic binding onto the membrane surface and threshold concentration dependent self association, ii) insertion of the self associated bundle into the cell membrane to form a pore and iii) the increase in pore size or stabilization of pore due to recruitment of more peptides. Disruption in any of these steps may lead to failure of pore formation thereby imparting resistance to AMP. The aim of the present study was

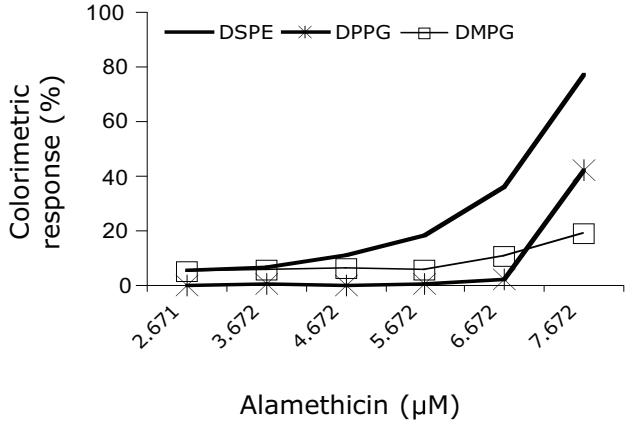

FIG. 5 - Colorimetric response of pure phospholipids-polydiacetylene biomimetic membrane vesicles (4:6 mole ratio) titrated with alamethicin. DSPE: Distearoyl phosphatidylglycerol; DPPG: Dipalmitoyl phosphatidylglycerol; DMPG: Dimyristoyl phosphatidylglycerol.

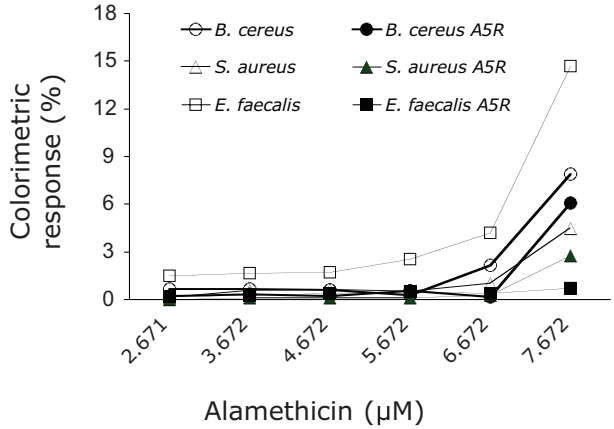

FIG. 6 - Colorimetric response of total cellular lipid extract-polydiacetylene membrane vesicles (1:1 mole ratio) of wild type and resistant strains of *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus cereus* titrated with alamethicin. A5R: alamethicin five fold resistant strain.

to elucidate the mechanism of resistance development through investigation of each of these three steps with three different gram positive pathogenic bacteria against the model BSPF-AMP, alamethicin. Five-fold stable resistant varieties of each of *S. aureus*, *E. faecalis* and *B. cereus* against alamethicin were selected and used for comparison with wild-type varieties. To the best of our knowledge this is the first report for alamethicin resistant varieties among *S. aureus*, *E. faecalis* and *B. cereus*.

Initial electrostatic binding depends upon the membrane surface charge which is determined by the ratio of zwitterionic phospholipids (a positively charged amino-group containing phospholipid i.e. ACP) with no net charge such as phosphatidyl ethanolamine and phosphatidyl choline to anionic phospholipids (a negatively charged amino-group lacking phospholipid i.e. ALP) with net negative charge such as phosphatidyl glycerol and cardiolipin (essential a double phospholipid). Alamethicin has been shown to exist in two states, an S (inactive conformation) state predominant at low concentration which changes to I state (active conformation) upon interaction with membrane at threshold concentration in sigmoidal manner (Chen *et al.,* 2002). We observed sigmoidal dependence for alamethicin insertion in the biomimetic membranes. We also observed a change in surface charge in each of three species as indicated by a change in ACP to ALP ratio between wild-type and resistant varieties. It increased significantly in *S. aureus* and *B. cereus* but decreased in *E. faecalis*. Sakayori *et al* (2003) reported an increase in ACP content in two mundticin KS resistant mutants of *E. faecium*. Similarly, specific changes including more zwitterionic phosphatidyl ethanolamine and less anionic phosphatidyl glycerol and cardiolipin has been reported in nisin-resistant strains of *L. monocytogenes* Scott A (Verheul *et al.,* 1997; Crandall and Montville, 1998). However, this is the first report for a change in phospholipid head groups such as decrease in ACP and appearance of two additional phospholipids of *E. faecalis*. These changes might contribute towards a decrease in the initial electrostatic binding. Consequently, an optimum electrostatic interaction along with sigmoidal dependence of alamethicin concentration is necessary to allow the self association of the peptide to form an active conformation aggregate, which gets inserted into the membrane to form a barrel type pore.

Peptide insertion was estimated using biomimetic membrane system. This membrane changes its absorption maximum upon insertion of the peptide. There is blue to red transition only if the peptide is inserted deep in the membrane rather than simple surface association (Charych *et al.,* 1993; Jonas *et al.,* 1999) i.e. if the protein or peptide interacts with the membrane on the surface only then the blue to red shift is not observed. Therefore this biomimetic membrane provided a suitable system to estimate the extent of insertion of the peptide. A supralinear dose response of alamethicin in membranes prepared from three pure phospholipids confirmed cooperative insertion of the peptide in pure phospholipid domains. Further, similar supralinear response with membranes prepared from total cellular lipid extract from the sensitive strain and its resistant varieties also confirms the cooperative insertion of the peptide in these biomimetic membranes. The decrease in alamethicin insertion in the membrane upon acquisition of resistance by each of the three species tested indicates that this step is also affected during pore formation. This is the first report of alamethicin-membrane interactions comparison between wild-type and resistant varieties.

Stabilization of pores depends upon hydrophobic interactions between the hydrophobic faces of pores and fatty acid side chains of phospholipids. There was a change in membrane fluidity as indicated by saturated-to-unsaturated fatty acids ratio in each of the species tested. The membranes of *S. aureus* and *E. faecalis* became more rigid, whereas, in *B. cereus* it became more fluid. Similar changes in membrane fluidity have been reported during development of resistance against other barrel-stave type pore forming antimicrobial peptides. A decrease in membrane fluidity upon development of resistance among *L. monocytogenes* against leucocin A and in *E. faecium* against mundticin KS (Sakayori *et al.,* 2003), two class IIa bacteriocins have been reported. They observed saturated (C12:0, C14:0, C16:0, C18:0, C19:0) and unsaturated fatty acids (C16:1and C18:1) as major composition of membrane fatty acids in mundticin KS sensitive and resistant mutants of *E. faecium* and also reported significant reduction in unsaturated fatty acids and increase in saturated fatty acids in the muditicin KS resistant mutants than in wild type strains of *E. faecium*. Decrease in membrane fluidity has also been reported in *L. monocytogenes* against nisin (Mazotta and Montville, 1997; Li *et al.,* 2002) and in *Leuconostoc* or *Weisella* strains against mesentericin 52A and 52B (Limonet *et al.,* 2002). There were significant increase in saturated fatty acids (10% more) and reduction in unsaturated fatty acids (6% less) of mesentericin 52A and 52B resistant mutants of *Leuconostoc* species making their membrane more rigid when compared to wild type strains. On the other hand Verheul *et al.* (1997) observed no difference in membrane fluidity between sensitive and resistant verities of *L. monocytogenes* against nisin. Further, use of the ratio of saturated fatty acids and unsaturated fatty acids as an index of membrane fluidity and subsequent correlation of membrane fluidity with acquisition of resistance has been reported by Chen *et al.* (1998). They reported that the saturation state of the phosphatidylglycerol acyl chains in vesicles had little effect on the binding affinity of pediocin PA-1 (an antimicrobial peptide) for the vesicle. However their fluorescence results indicated that the penetration of pediocin PA-1 into a bilayer of the saturated dimyristoyl-phosphatidylglycerol was deeper than into a bilayer of unsaturated dioleoyl-phosphatidylglycerol. The dioleoyl-phosphatidylglycerol is therefore thought to be less favourable for efficient membrane permeabilization due to its higher fluidity. A weaker insertion ability of AMPs into unsaturated phosphatidylglycerol could point to the role of increased amounts of unsaturated fatty acids and increased membrane fluidity in resistant strains. Similarly it has been observed that *Kluyveromyces lactis* mutant cells with reduced amphotericin B sensitivity have a higher unsaturated fatty acid-to-saturated fatty acid ratio than do wild type *K. lactic cells* (Younsi *et al*., 2000). Hence membrane fluidity could be an important contributing factor to class IIa bacteriocins and AMPs resistance by affecting the insertion into membranes and consequently the formation and stability of the pores. It appears that stable pores could be formed only with the optimum fluidity. Therefore either an increase or decrease in membrane fluidity could affect this step. The cell membrane fluidity is modulated throughout species-dependent mechanisms. Indubitably the ratio between saturated and unsaturated fatty acids is one of the multiple ways to modulate cell membrane fluidity. The cell membrane fluidity is also regulated by other important fatty acids like branched chain fatty acids. Higher levels of branched chain fatty acids impart increased membrane

fluidity. However, here we report that except for unidentified fatty acid resolved at 6.57 to 6.85 retention time range in wild type *S. aureus*, all unidentified fatty acids consisted of less than 7% and all identified fatty acids were of even number straight chain fatty acids. Therefore contribution of branched chain fatty acids to regulate membrane fluidity in these sensitive and resistant variants seems to less significant as compared to straight chain fatty acids. We could not able to ascertain branched chain fatty acid composition in sensitive and resistant variants due to unavailability of branched chain fatty acid methyl esters and lack of funding for the project. Further investigation is needed for identification and quantification of branched chain fatty acids. The present results correlate poor stability of pores among five fold resistant varieties of *S. aureus, E. faecalis* and *B. cereus* with change in membrane fluidity due to altered fatty acids of membrane phospholipids of resistant mutants.

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REFERENCES

Beven L., Helluin O., Molle G., Duclohier H., Wroblewski H. (1999). Correlation between anti-bacterial activity and pore sizes of two classes of voltage-dependent channel-forming peptides. Biochim. Biophys. Acta, 1421 (1): 53-63.

Bligh E.G., Dyer E.J. (1959). A Rapid method for total lipid extraction and purification. Can. J. Biochem. Physiol., 37: 911-917.

Brogden K.A. (2005). Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria. Nature Rev. Microbiol., 3: 238-250.

Cabo M.L., Murado M.A., Gonzalez M.P., Pastoriza L. (1999). A method for bacteriocin quantification. J. Appl. Microbiol., 87 (6): 907-914.

Charych D.H., Nagy J.O., Waynespevak M.D., Bednarski (1993). Direct colorimetric detection of a receptor-ligand interaction by a polymerized bilayer assembly. Science, 261: 585-588.

Chen Y., Ludescher R.D., Montville T.J. (1998). Influence of lipid composition on pediocin PA-1 binding to phospholipid vesicles. Appl. Environ. Microbiol., 64: 3530-3532.

Chen F., Lee M., Hang H.W. (2002). Sigmoidal concentration dependence of antimicrobial peptide activities: A case study of alamethicin. Biophysics J., 82: 908-914.

Crandall A.D., Montville T.J. (1998). Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. Appl. Environ. Microbiol., 64 (1): 231-237.

Davis E.A., Adams M.R. (1994). Resistance of *Listeria monocytogenes* to the bacteriocin nisin. Int. J. Food Microbiol., 21: 341-347.

Food and Drug Administration (1988). Nisin preparation: Affirmation of GRAS status as a direct human food ingredient. Food and Drug Administration, Federal Register, 53: 11247.

Gravesen A., Ramnath M., Rechinger K.B., Anderesen N., Jansch L., Hechard Y., Hastings J., Knochel S. (2001). High level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes*. Microbiology, 148: 2361-2369.

Hancock R.E.W., Chapple D.S. (1999). Peptide antibiotics. Antimicrob. Agent Chemother., 43 (6): 1317-1323.

Hechard Y., Pelletier C., Cenatiempo Y., Free T. (2001). Analysis of σ^{54}-dependent genes in *Enterococcus faecalis*: a mannose PTS permease (EII^{Man}) is involved in sensitivity to a bacteriocin, mesentericin Y105. Microbiology, 147: 1575-1580.

Jonas U., Shah K., Norvez S., Charych D.H. (1999). Reversible color switching and unusual solution polymerization of hydrazide modified diacetylene lipids. J. Am. Chem. Soc., 121 (19): 4580–4588.

Kahovcova J., Odavic R. (1969). A simple method for the quantitative analysis of phospholipids separated by thin layer chromatography. J. Chromatog., 40: 90-96.

Katz S.S., Weinrauch Y., Munford R.S., Weiss E.P. (1999). Deacylation of PS in whole *E. coli* during destruction by cellular and extracellular components of a rabbit peritoneal inflammatory exudate. J. Biol. Chem., 274.

Kolusheva S., Shalal T., Jelinek R. (2000). Peptide-membrane interactions studied by a new phospholipid-polydiacetylene colorimetric assay. Biochemistry, 39: 15851-15859.

Li J., Chikindas, M.L., Ludescher R.D., Montville T.J. (2002). Temperature and surfactant induced membrane modifications that alter *Listeria monocytogenes* nisin sensitivity by different mechanisms. Appl. Environ. Microbiol., 68 (12): 5904-5910.

Limonet M., Revol-Junelles A.M., Milliere J.B. (2002). Variations in the membrane fatty acid composition of resistant or susceptible *Leuconostoc* or *Weissella* strains in presence or absence of Mesenterocin 52A and Mesenterocin 52B produced by *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52. Appl. Enrivon. Microbiol., 68: 2910-2916.

Mazzotta A.S., Montville T.J. (1997). Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10 °C and 30°C. J. Appl. Microbiol., 82: 32-38.

Ming X., Daeschel M.A. (1995). Correlation of cellular phospholipid content with nisin resistance of *Listeria monocytogenes*. Scott A. J. Food Prot., 58: 416-420.

Moll G.N., Clark J., Chan W.C., Bycroft B.W., Roberts G.C., Konings W.N., Driessen A.J. (1997). Role of transmembrane pH gradient and membrane binding in nisin pore formation. J. Bacteriol., 179 (1): 135-140.

North C.L., Franklin J.C., Bryant R.G., Cafiso D.S. (1994). Molecular flexibility demonstrated by paramagnetic enhancements on nuclear relaxation. Application to alamethicin: a voltage-gated peptide channel. Biophys. J., 67: 1861-1866.

Powledge T.M. (2004). New antibiotics-resistance is futile. PLoS Biology, 2 (2): 151-154.

Rekhif N., Atrih A., Lefebvre G. (1994). Characterization and partial purification of plantaricin LC74, a bacteriocin produced by *Lactobacillus plantarum* LC74. Biotech. Lett., 16 (8): 771-776.

Sakayori Y., Muramatsu M., Hanada S., Kamagata Y., Kawamoto S., Shima J. (2003). Characterization of

Enterococcus faecium mutants resistant to mundticin KS, a class IIa bacteriocin. Microbiology, 149 (10): 2901-2908.

Sansom M.S.P. (1993). Alamethicin and related peptaibols: model ion channels. EurBiophys. J., 22: 105-124.

Segolene C., Alain R., Yanick A., Herve P., Djamel D. (2007). Identification of new genes associated with intermediate resistance of *Enterococcus faecalis* to divercin V41, a pediocin-like bacteriocin. Microbiology, 153: 1609-1618.

Sood S.K., Sinha P.R. (2003). Analysis of structure of YGNGV motif containing bacteriocins: A model for pore formation. Indian J. Biotech., 2: 227-235.

Vadyvaloo V., Hastings J.W., Marthinus J., Van der Merwe, Rautenbach M. (2002). Members of class IIa bacteriocin-resistant *Listeria monocytogenes* cells contain increased levels of desaturated and short-acyl-chain phosphatidylglycerols. Appl. Environ. Microbiol., 68 (11): 5223-5230.

Verheul A., Russel N.J., Hof R.V.T., Rombouts M., Abee T. (1997). Modifications of membrane phospholipid composition in nisin-resistant *Listeria monocytogenes* Scott A. Appl. Environ. Microbiol., 63 (9): 3451-3457.

Yang L., Harroum T.A., Weiss T.M. (2001). Barrel stave model or Toroidal model? A case study on Melitin pores. Biophysics J., 81:1475-1485.

Younsi M.E., Ramananddraibe R.B., Donner M., Coulon J. (2000). Amphotericin B resistance and membrane fluidity in *Kluyveromyces lactis* strains. Antimicrob. Agents Chemother., 44: 1911-1916.

Zasloff M. (2002). Antimicrobial peptides of multicellular organisms. Nature, 415: 389-395.