Biochimica et Biophysica Acta 1848 (2015) 1367–1375

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Increased membrane surface positive charge and altered membrane fluidity leads to cationic antimicrobial peptide resistance in *Enterococcus faecalis*

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ARTICLE INFO

Article history:
Received 17 August 2014
Received in revised form 21 February 2015
Accepted 9 March 2015
Available online 14 March 2015

Keywords:
Antimicrobial peptide (AMP)
Cell-penetrating peptide (CPP)
Phospholipid vesicle
Membrane lipid
Drug resistance

ABSTRACT

To understand the role of cell membrane phospholipids during resistance development to cationic antimicrobial peptides (CAMPs) in *Enterococcus faecalis*, gradual dose-dependent single exposure pediocin-resistant (Ped^r) mutants of *E. faecalis* (Efv2.1, Efv3.1, Efv3.2, Efv4.1, Efv4.2, Efv5.1, Efv5.2 and Efv5.3), conferring simultaneous resistance to other CAMPs, selected in previous study were characterized for cell membrane phospholipid headgroups and fatty acid composition. The involvement of phospholipids in resistance acquisition was confirmed by in vitro colorimetric assay using PDA (polydiacetylene)-biomimetic membranes. Estimation of ratio of amino-containing phospholipids to amino-lacking phospholipids suggests that phospholipids in cell membrane of Ped^r mutants loose anionic character. At moderate level of resistance, the cell-membrane becomes neutralized while at further higher level of resistance, the cell-surface acquired positive charge. Increased expression of *mprF* gene (responsible for lysinylation of phospholipids) was also observed on acquiring resistance to pediocin in Ped^r *E. faecalis*. Decreased level of branched chain fatty acids in Ped^r mutants might have contributed in enhancing rigidification of cell membrane and contributing towards resistance. The interaction of pediocin with PDA-biomimetic membranes prepared from wild-type and Ped^r mutants was monitored by measuring percent colorimetric response (%CR). Increased %CR of pediocin against PDA-biomimetic membranes prepared from Ped^r mutants confirmed that cell membrane phospholipids are involved in the interactions of pore formation by CAMPs. There was a direct linear relationship between percent colorimetric response and IC_{50} of CAMPs for wild-type and Ped^r mutants. This relationship further reveals that in vitro colorimetric assay can be used effectively for quantification of resistance to CAMPs.

1. Introduction

Enterococcus faecalis, a ubiquitous commensal of mammalian gastrointestinal flora, is a leading cause of nosocomial infections and a growing public health concern. On some occasions, the commensal relationship with the host is disrupted causing serious diseases like endocarditis, urosepsis, meningitis, etc. [1]. Due to its innate and acquired resistance to most clinically used antibiotics, identification of new alternatives for treatment of *E. faecalis* is a high priority [2]. The discovery of diverse population of non-toxic, non-immunogenic and potent cationic antimicrobial peptides (CAMPs), as an essential component of anti-infective defense mechanisms in mammals, amphibians, insects, plants, fungi and bacteria offers effective alternative candidates against bacteria, fungi, viruses and protozoa resistant to synthetic drugs. In higher organisms, CAMPs such as α-defensins (HNP-1), cathelicidin, thrombocidin, cathepsin G, etc., act on cell membrane of target to serve as a first defense against invading harmful micro-organisms. Lactic acid bacterial CAMPs, known as bacteriocins like nisin, pediocin, etc., are also cell membrane active usually against Gram positive species with low G + C content such as *E. faecalis*. Among bacteriocins, pediocin-like bacteriocins (36–48 residues) are by far the most investigated being *Listeria* active. Pediocins contain two structural regions, a conserved N-terminal and a less conserved C-terminal region determining the target specificity and thus inhibitory spectrum [3].

The CAMPs used in the present study include pediocin, nisin and alamethicin. Although all these are CAMPs, their net charge differs. At pH approximately 7, the net charges of pediocin (Accession No. ACO56765.1), nisin (Accession No. AAA88606.1) and alamethicin [4] are 3.5, 4.2 and −1, respectively, which may contribute to the difference in their antimicrobial activity, in spite of having the same mechanism of action. The mechanism of action of CAMPs involves electrostatic interaction with the negatively charged bacterial cell surface followed by penetration into the lipid bilayer. The penetration of the pediocin into

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http://dx.doi.org/10.1016/j.bbamem.2015.03.007
0005-2736/

the cell membrane causes disruption in its homeostasis that is associated with a leakage of intracellular contents from the cytoplasm leading to bacterial cell death [5]. Emergence of CAMPs non-susceptibility has been described in *Staphylococcus aureus*, *Listeria monocytogenes*, *Mycobacterium tuberculosis* and enterococci. Several genes have been associated with this phenomenon and include: i) genes encoding enzymes involved in alteration of cell-wall in *dlt* operon responsible for D-alanylation of teichoic acids in *E. faecalis* [6], *L. monocytogenes* [7], and *Streptococcus* [8], ii) genes encoding enzyme IIAB subunit of a mannose-specific phosphotransferase system [7], and iii) genes encoding enzymes involved in cell membrane phospholipid metabolism: *mprF* in *L. monocytogenes* [9], *S. aureus* [10], *M. tuberculosis* [11], *B. anthracis* [12] and enterococci; *cls* (cardiolipin synthase) in *S. aureus* [13] and enterococci [14], *pgsA* in *S. aureus* [13] and *B. subtilis* [15]. Many studies have been carried out to analyze the changes in cell membrane composition viz., cell-membrane surface charge and cell-membrane fluidity, upon resistance acquisition. Cardiolipin and phosphatidylglycerol in *L. monocytogenes* are normally negatively charged, whereas the lysinylated forms of these two phospholipids bear a net positive charge [16]. *mprF* is involved in the increased lysinylation of phospholipids which lead bacterial surface to bear a net positive charge, thus imparting resistance to CAMPs in *S. aureus* [10], *L. monocytogenes* [9], *M. tuberculosis* [11], *B. anthracis* [12] and *E. faecalis* [17]. In addition, membrane fatty acid composition also plays an important role in resistance development as it decides the fluidity of membrane, which further correlates with degree of penetration by CAMPs in the membrane [18–21].

Recently, we selected the panel of Ped^r mutants of *E. faecalis*, which displayed collinear cross-resistance to other CAMPs, such as HNP-1, nisin and alamethicin, along with this we observed the neutralization of cell-wall surface charge, which had been thought to impose the permeability barrier to CAMPs to reach periplasmic space [22]. In this paper, we elucidate the role of head group and fatty acyl chain of phospholipids in the cell membrane of this panel of gradual Ped^r mutants of *E. faecalis*, which previously displayed the gradual changes in cell-wall and mannose PTS expression. The role of phospholipids head group and fatty acyl chain in resistance acquisition was further validated by the use of a polydiacetylene-based colorimetric assay.

2. Material and methods

2.1. Bacterial strains, media and pediocin PA-1 resistant mutants of *E. faecalis* NCDC 114

E. faecalis NCDC 114 was used as reference organism and gradual dose-dependent pediocin PA-1 (Sigma-Aldrich Pvt. Ltd.) resistant mutants of *E. faecalis* viz., Efm2.1, Efm3.1, Efm3.2, Efm4.1, Efm4.2, Efm5.1, Efm5.2 and Efm5.3 were generated by pediocin PA-1 exposure in the previous study [22]. Nutrient broth was used for the culturing of *E. faecalis* NCDC 114 and Ped^r mutants of *E. faecalis*.

2.2. Effect of divalent cation (Mg^{2+}) on pediocin resistance

Effect of divalent cation (Mg^{2+}) on wild-type and Ped^r mutants of *E. faecalis* was studied as described by Sakayori et al. [21]. Cells of wild-type and Ped^r mutants of *E. faecalis* were grown in nutrient broth. The cells were harvested by centrifugation at 5000 × *g* for 15 min, washed with MES-buffered saline (0.1 M, pH 6.5) and then resuspended at a cell density of approximately 5×10^8 cells ml^{-1} (O.D. = 1.0) in different tubes with MES buffer (50 mM, pH 6.5) containing divalent cations (10 mM), divalent cations plus EDTA (20 mM) to chelate divalent cations and MES buffer only, separately. Pediocin (5 μg/ml) was added to all the cell suspension, except to the control. The cell suspensions were incubated at 37 °C for 20 min and then plated onto 10 ml nutrient agar and incubated at 37 °C for 24 h. The colonies were counted after incubation and results were reported as number of colonies appeared with same dilution (up to 10^8) in different combinations.

2.3. Analysis of phospholipids

The bacterial cells were harvested, washed, and resuspended in physiological saline. Total cellular lipids were extracted by the method of Bligh and Dyer [23]. The lipid extract was dissolved in chloroform and stored at −20 °C. Total lipid extracts of bacterial cells were resolved using chloroform:methanol:glacial acetic acid (35:10:4, [vol/vol]) as a mobile phase in 1 dimensional thin-layer chromatography (1D-TLC) [24], visualized using iodine vapors, and identified using specific stains, viz., ammonium molybdate for phospholipids and ninhydrin for amino group-containing phospholipids (ACPs). For 2D-TLC, the plate was lowered in the tank containing first solvent system consisting of chloroform:methanol:glacial acetic acid (35:10:4, [vol/vol]) to run mobile phase in first dimension in the vertical orientation. After drying the plate, second solvent system consisting of chloroform:water:methanol:glacial acetic acid:acetone (45:4:8:9:16, [vol/vol]) was used to run the mobile phase in second dimension in the horizontal orientation and visualized using ammonium molybdate reagent. The Alpha Imager gel documentation system was used for the quantification of phospholipids separated in TLC. The pixel areas were determined for each of the standards, and the concentrations of unknown phospholipid spots were determined.

2.4. Detection of L-lysine in membrane phospholipids of wild-type and Ped^r mutants of *E. faecalis*

Spots showing the presence of amino-containing phospholipids were scrapped from the TLC plate and acid hydrolyzed in 6 M HCl containing 0.5% phenol at 100 °C for 18 h. The hydrolyzed samples were then subjected to reverse phase-high performance liquid chromatography (RP-HPLC) on a C18 Spherisorb R column (ODS2 4.6 × 250 mm, 300 Å pore size) using Waters32 HPLC system, 515 HPLC pump, and 2487 dual λ absorbance detector. The conditions for the gradient elution were as follows: solvent B (100% acetonitrile) against solvent A (0.1% TFA in HPLC water), flow rate of 1 ml per minute, 0–2 min, 100% solvent A; 2–8 min, 85% solvent A and 15% solvent B; 8–11 min, 75% solvent A and 25% solvent B; 11–15 min, 100% solvent A. The peaks observed were compared with standard L-lysine obtained from Sigma-Aldrich Pvt. Ltd.

2.5. RNA isolation, cDNA synthesis, and RT-PCR

RT-PCR was performed on wild-type and Ped^r mutants of *E. faecalis* for expression analysis of *mprF* gene encoding multiple peptide resistance factors responsible for L-lysinylation of membrane phosphatidylglycerol, using 16S rRNA as house-keeping gene. Cells in exponential growth (OD_{600} ~ 0.5) were harvested by centrifugation and RNA isolation was performed as described by Shepard and Gilmore [25]. To remove remnants of DNA, RNA was treated with RNase-free DNase I (Fermentas Life Sciences). cDNA was synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences). RT-PCR was carried out using primers designed during the present study (Table 1).

Table 1
Primers designed using annotated genome sequence of *E. faecalis* V583.

Gene name	Primer Sequence (5′ to 3′)	Amplified fragment (bp)
mprF	Forward: TATGCGTTTCAAGATACACG Reverse: AGCGAAACACTTTGATGTCT	152
16S rRNA	Forward: GGTGGAGCATGTGGTTTAAT Reverse: CCATTGTAGCACGTGTGTAG	301

2.6. Fatty acid analysis

Total lipid extracts of bacterial cells were transesterified to obtain fatty acid methyl esters (FAMEs) for further analysis by gas chromatography. In brief, lipid extract was transesterified using methanol:hydrochloric acid:chloroform (10:1:1 [vol/vol/vol]) at 90 °C for 60 min. The extraction of FAMEs was carried out twice using distilled water (1 ml) and hexane:chloroform (4:1 [vol/vol]). Both aliquots were pooled and allowed to evaporate to 100 μl. The FAMEs were analyzed on a Michro 9100 gas chromatograph using a Supelcowax (Sigma-Aldrich Pvt. Ltd.)-fused silica capillary column (60-m by 0.32-mm by 0.25-m film thickness). Nitrogen gas was used as a carrier gas at a constant flow rate of 1 ml min^{-1}. The initial oven temperature was kept at 100 °C, and an isothermal time of 2 min was allowed. Then, a ramp rate of 5 °C min^{-1} was used to increase the temperature to 220 °C, and an isothermal time of 14 min was allowed. The injector temperature was kept at 230 °C and the flame ionization detector (FID) temperature at 240 °C. One microliter of sample containing FAME(s) was injected into the column through the polysilicone diaphragm, and the detector response was recorded.

2.7. Preparation of PDA-based biomimetic membranes

The PDA/lipid biomimetic membranes were prepared using the slightly modified method described by Kolusheva et al. [26]. In brief, total lipid extracts (3 mg, from the wild-type strain and Ped^r mutants) and 0.83 mg of 10,12-tricosadiynoic acid (Sigma-Aldrich Pvt. Ltd.) were hydrated by adding 2 ml of triple-distilled water and sonicated at 70 °C (or above the melting temperature [Tm] of the phospholipids used) for 5 to 6 min. The vesicle solution was then cooled at room temperature (RT) for 30 min and then kept overnight at 4 °C. The next day, after transfering to RT for 30 min, the vesicle solution was polymerized using UV irradiation (254 nm) for 45 s, with the resulting solutions exhibiting an intense blue appearance.

2.8. In vitro colorimetric estimation of biomimetic membrane penetration by CAMPs

Samples were prepared by adding CAMPs viz., pediocin, nisin (Sigma-Aldrich Pvt. Ltd.) and alamethicin (Sigma-Aldrich Pvt. Ltd.) to 0.03-ml vesicle solutions at 0.2 mM total lipid concentration and 2 mM Tris (pH 8.5). The pH of all components was maintained at 8.5. Following addition of the CAMPs, the solutions were diluted to 1 ml and the absorbance of the solutions was taken at 500 nm and 640 nm. The blue-to-red color transition in vesicle solutions was quantified by calculating the percent colorimetric response (%CR) as follows: $\%CR = (PB_0 - PB_I) / PB_0 \times 100$, where $PB_0 = A_{640} / (A_{640} + A_{500})$ for the control and $PB_I = A_{640} / (A_{640} + A_{500})$ for samples (PB_0 is the blue/red ratio of the control sample, and PB_I is the value obtained for the vesicle solution after colorimetric transition occurs). To study the effect of Mg^{2+} ions on percent colorimetric response, Mg^{2+} ions (10 mM) were added in the presence or absence of 20 mM EDTA before addition of pediocin.

2.9. Calcein leakage assay

Calcein leakage assay was performed as described by Jin et al. [27]. Total cellular lipids were extracted from the wild-type and Ped^r mutants of *E. faecalis* by the method of Bligh and Dyer [25]. Briefly, large unilamellar vesicles (LUVs) were prepared by hydrating the dry lipid films in calcein containing buffer (50 mM HEPES, 100 mM NaCl, 0.3 mM EDTA and 80 mM calcein [Sigma-Aldrich Pvt. Ltd.], pH 7.4) at a concentration of 1 mg/ml. After freezing-thawing the LUV mixture for five cycles, the mixture was extruded ten times through a 0.1 μm pore size membrane resulting in 100 nm diameter LUVs. Calcein-loaded LUVs were separated from free calcein by size-exclusion chromatography using Sephadex G-50 column. Calcein leakage from the calcein-loaded LUVs was monitored by measuring the time-dependent increase in the fluorescence of calcein for 30 min (excitation at 490 nm and emission at 520 nm). Assays were performed in triplicate in a 96-well plate. A 180 μl aliquot of the calcein-loaded LUV suspension was added to each well followed by the addition of a 20 μl aliquot of pediocin (5 μg/ml). A negative control omitted peptide, while positive control involved addition of 20 μl of 10% triton X-100 in place of the pediocin. The peptide induced leakage was calculated by the following equation:

$$\%\ \text{Calcein leakage} = 100\ \text{X}\ (F - F_0) / (F_t - F_0)$$

where F is the fluorescence intensity induced by the peptide, F_0 is the fluorescence of intact vesicles and F_t represents the intensity at 100% leakage.

3. Results

3.1. Action of divalent cation (Mg^{2+}) on wild-type and Ped^r mutants of *E. faecalis*

Effect of pediocin (5 μg/ml), pediocin plus Mg^{2+} (10 mM) and pediocin plus Mg^{2+} plus EDTA (20 mM) on viability of wild-type and Ped^r mutants of *E. faecalis* is shown in Fig. 1. Viability of wild-type *E. faecalis* was considerably reduced on its treatment with pediocin. The viability was partially restored when Mg^{2+} was added together with pediocin. This indicated that Mg^{2+} reduced the lethality of pediocin. This was also confirmed by the observation that when EDTA was also included with pediocin and Mg^{2+}, the lethality of pediocin persisted. However, lethality of pediocin towards Ped^r mutants was <50% and was almost similar in all Ped^r mutants. There was no effect of Mg^{2+} or Mg^{2+} plus EDTA in reversal lethality caused by pediocin.

3.2. Changes in membrane phospholipid composition

Phospholipids from the wild-type and Ped^r mutants of *E. faecalis* were extracted and separated on TLC. The separated phospholipids were detected with Dittmer–Lester reagent and ninhydrin reagent (Fig. 2). Phospholipids extracted from Ef, Efm2.1, Efm3.1, Efm3.2, Efm4.1 and Efm4.2 were resolved into five spots and phospholipids extracted from Efm5.1, Efm5.2 and Efm5.3 were resolved into six spots, when exposed to Dittmer–Lester reagent. Moreover, when separated phospholipids were exposed to ninhydrin reagent, only one spot in case of Ef, Efm2.1, Efm3.1, Efm3.2, Efm4.1 and Efm4.2 and two spots in case of Efm5.1, Efm5.2 and Efm5.3 were detected.

Phospholipids extracted from the wild-type and Ped^r mutants could be categorized into two groups based on the spots detected; one is amino-lacking phospholipids group (ALP), including cardiolipin (CL)

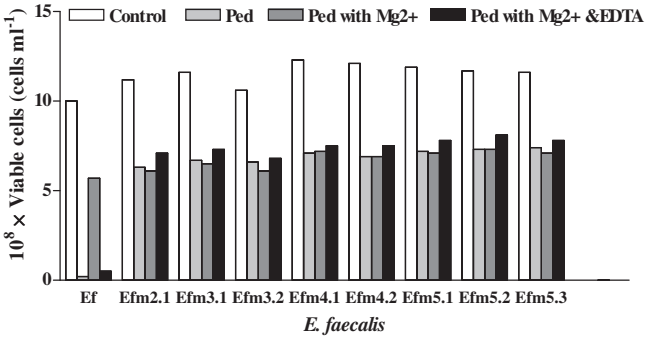

Fig. 1. Effect of Mg^{2+} on pediocin resistance of wild-type strain and Ped^r mutants of *E. faecalis*.

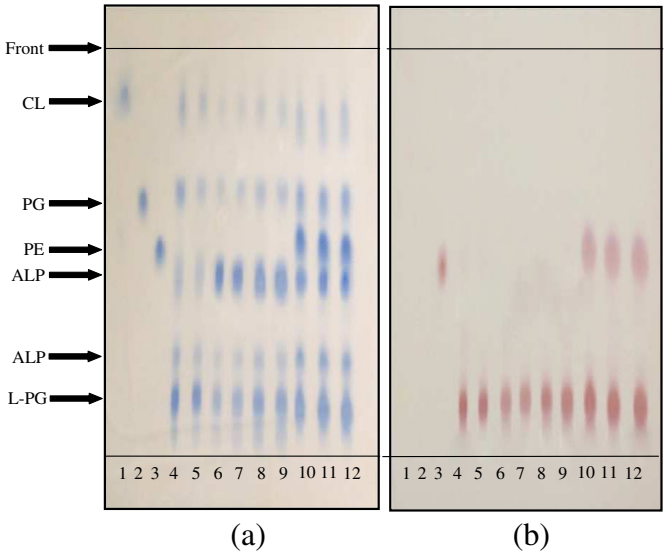

Fig. 2. TLC analysis of phospholipids extracted from wild-type and Ped^r mutants of *E. faecalis* visualized with ammonium molybdate (a) and ninhydrin (b) reagents. Lanes: 1, CL from bovine heart; 2, PG from egg yolk; 3, PE from porcine liver; followed by phospholipids extracted from *E. faecalis*, 4, Ef; 5, Efm2.1; 6 Efm3.1; 7, Efm3.2; 8, Efm4.1; 9, Efm4.2; 10, Efm5.1; 11, Efm5.2; 12, and Efm5.3.

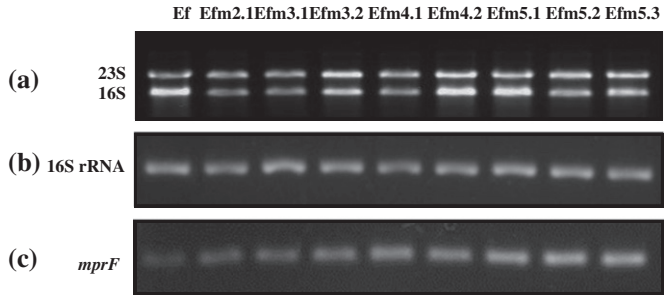

Fig. 3. Agarose gel electrophoresis of the (a) total RNA isolated, (b) PCR product of 16S rRNA obtained from the RT-PCR of total RNA as a house-keeping gene, and (c) PCR product of *mprF* obtained from the RT-PCR of total RNA, from the wild-type and Ped^r mutants of *E. faecalis*.

and phosphatidylglycerols (PG) and another is amino-containing phospholipids (ACP) group, which includes phosphatidyl-ethanolamine (PE) and lysyl-phosphatidylglycerol (L-PG). Presence of L-PG was confirmed by HPLC analysis of the spot detected as ACP, which will be discussed later.

By comparing the mobilities of the phospholipids on TLC with those of standards, we identified two spots with *Rf* values of 0.73 and 0.51 as CL and PG, respectively in case of wild-type and all Ped^r mutants. In the highly-resistant mutants (Efm5.1, Efm5.2, and Efm5.3), one additional spot with *Rf* value of 0.41 was identified as PE. Remaining three spots (in case of wild-type and all Ped^r mutants) could not be identified. Out of these three spots, two with *Rf* values of 0.37 and 0.20 were tentatively designated as ALP as these were reactive with Dittmer–Lester reagent only, but not with ninhydrin reagent and one with *Rf* value of 0.12 was designated as ACP as it was reactive with both Dittmer–Lester and ninhydrin reagent. The quantitative analysis of phospholipids stained by Dittmer–Lester reagent was carried out using densitometry (Alpha Imager). So, increased ACP/ALP ratio in the Ped^r mutants suggests their contribution in pediocin resistance (Table 2). Phospholipid profiling by 2D-TLC (data not shown) showed the appearance of three additional spots in case of Efm5.1, Efm5.2 and Efm5.3 when compared with the wild-type and rest of the Ped^r mutants, which is to be taken forward for further investigation.

Furthermore, expression analysis of *mprF* gene in wild-type and Ped^r mutants of *E. faecalis* revealed that *mprF* gene is expressed in wild-type as well as in Ped^r mutants of *E. faecalis*. However, the intensity of band increased with increase in degree of resistance (Fig. 3). This supports the presence of lysyl-group in the phospholipid content of both wild-type and Ped^r mutants of *E. faecalis*.

ACP spot with *Rf* value of 0.12 from wild-type and all Ped^r mutants of *E. faecalis* (Fig. 2) was hydrolyzed and then analyzed on RP-HPLC and compared with elution profile of L-lysine hydrochloride (Sigma-Aldrich Pvt. Ltd.), where retention time was found similar (data not shown). This suggests that spot with *Rf* value 0.12 might be L-lysylphosphatidylglycerol.

3.3. Changes in membrane fatty acid composition

The fatty acid profile of the wild-type and all the Ped^r mutants were identified by comparing the retention time (Rt) of fatty acid methyl ester peaks with the Rt of known bacterial acid methyl esters (Sigma-Aldrich Pvt. Ltd.). The relative quantity of each fatty acid identified was determined on the basis of percentile peak area (Table 3).

The relative quantity of saturated fatty acids, unsaturated fatty acids, hydroxy fatty acids and branched chain fatty acids is shown in Fig. 4a. It is clear from the figure that the level of branched chain fatty acids in all Ped^r mutants of *E. faecalis* was significantly lower than that present in wild-type *E. faecalis*. The ratio of saturated to unsaturated fatty acids in Efm2.1, Efm3.1 and Efm3.2 was not significantly different from that present in wild-type strain of *E. faecalis*. However, the saturated to unsaturated fatty acid ratio increased significantly in Efm4.1, Efm4.2, Efm5.1, Efm5.2 and Efm5.3, as compared to wild-type strain (Fig. 4b). The increased saturated to unsaturated fatty acid ratio leads to decreased fluidity of the cell membrane, which prevents the insertion of pediocin into phospholipid bilayer.

The total hydroxy fatty acids in all Ped^r mutants of *E. faecalis* were in general higher than wild-type strain. The increase in total hydroxy fatty acids and decrease in branched chain fatty acids contribute to the rigidity of the membrane, making membrane rigid enough to be penetrated by pediocin (Fig. 4a).

Table 2
Phospholipid composition of wild-type (Ef) and Ped^r mutants (Efm2.1, Efm3.1, Efm3.2, Efm4.1, Efm4.2, Efm5.1, Efm5.2 and Efm5.3) of *E. faecalis*.

Phospholipids	% of total phospholipids[a]								
	Ef	Efm2.1	Efm3.1	Efm3.2	Efm4.1	Efm4.2	Efm5.1	Efm5.2	Efm5.3
L-PG	19.91 ± 2.46	22.84 ± 2.09	23.43 ± 2.63	23.55 ± 2.02	24.91 ± 1.40	25.31 ± 1.24	23.82 ± 1.19	23.93 ± 1.63	21.51 ± 3.85
ALP	20.04 ± 0.89	18.99 ± 0.53	18.09 ± 1.61	17.97 ± 1.53	17.60 ± 0.89	17.77 ± 0.94	15.47 ± 0.53	13.70 ± 0.96	15.94 ± 0.93
ALP	19.77 ± 0.72	19.52 ± 0.74	23.40 ± 1.66	24.35 ± 1.84	13.11 ± 2.00	23.27 ± 1.87	16.83 ± 2.29	17.32 ± 0.63	19.06 ± 0.85
PE	nd	nd	nd	nd	nd	nd	17.40 ± 0.28	17.59 ± 0.11	16.38 ± 2.32
PG	20.86 ± 1.26	19.36 ± 1.60	17.37 ± 0.42	17.54 ± 0.93	17.58 ± 1.16	16.96 ± 0.49	13.93 ± 0.75	14.31 ± 1.40	15.18 ± 0.39
CL	19.74 ± 1.17	19.33 ± 0.58	17.73 ± 1.65	16.73 ± 0.40	17.03 ± 1.75	16.77 ± 1.68	12.56 ± 1.19	13.18 ± 0.59	12.00 ± 1.41

nd = not detected.
[a] Experiments were conducted in triplicate and their mean ± SD are presented.

Table 3
Peak areas of different fatty acids identified from total cellular lipids extracted from wild-type (Ef) and Ped^r mutants (Efm2.1, Efm3.1, Efm3.2, Efm4.1, Efm4.2, Efm5.1, Efm5.2 and Efm5.3) of *E. faecalis*.

Fatty acids[a]	% of total fatty acids								
	Ef	Efm2.1	Efm3.1	Efm3.2	Efm4.1	Efm4.2	Efm5.1	Efm5.2	Efm5.3
C11:0	nd[b]	nd	nd	nd	nd	nd	nd	nd	nd
C12:0	nd	nd	nd	nd	nd	nd	nd	nd	nd
C13:0	nd	nd	nd	nd	nd	nd	nd	nd	nd
C14:0	0.103 ± 0.012	nd	nd	nd	nd	nd	9.360 ± 1.069	nd	0.440 ± 0.135
C15:0	0.412 ± 0.052	nd	1.748 ± 0.182	nd	0.798 ± 0.360	nd	3.449 ± 1.584	nd	2.988 ± 0.975
C16:0	nd	nd	0.634 ± 0.063	nd	4.984 ± 1.352	4.995 ± 0.633	1.436 ± 0.695	6.641 ± 1.261	17.632 ± 1.914
C17:0	0.063 ± 0.019	18.537 ± 1.583	4.282 ± 0.557	9.190 ± 0.415	9.554 ± 1.577	11.452 ± 1.791	nd	4.276 ± 0.467	23.209 ± 2.279
C18:0	0.691 ± 0.124	nd	2.404 ± 0.515	18.692 ± 1.501	4.571 ± 0.168	2.385 ± 0.685	2.011 ± 1.119	20.503 ± 0.688	nd
C19:0	14.474 ± 0.882	nd	nd	nd	11.487 ± 2.069	1.288 ± 0.393	1.819 ± 1.181	nd	0.607 ± 0.297
C20:0	0.262 ± 0.139	25.477 ± 1.599	24.970 ± 1.931	3.698 ± 0.546	3.128 ± 0.976	6.457 ± 1.403	nd	0.189 ± 0.014	nd
C16:1^9	0.459 ± 0.050	nd	7.434 ± 0.320	2.559 ± 0.577	nd	nd	2.722 ± 0.501	0.437 ± 0.237	4.142 ± 1.093
cis-C18:1^9	1.120 ± 0.138	nd	nd	2.543 ± 0.475	2.874 ± 0.683	nd	1.578 ± 0.615	nd	1.644 ± 0.451
trans-C18:1^9	0.799 ± 0.067	17.971 ± 1.016	0.833 ± 0.168	0.767 ± 0.049	nd	4.033 ± 1.358	0.429 ± 0.169	0.232 ± 0.063	0.532 ± 0.123
cis-C18:2^{9,12}	7.764 ± 0.692	nd	7.362 ± 0.615	8.519 ± 0.203	6.221 ± 0.926	2.891 ± 0.856	1.953 ± 1.336	9.395 ± 0.594	4.633 ± 1.960
2OH-C10:0	0.042 ± 0.008	nd	nd	nd	nd	nd	nd	nd	nd
2OH-C12:0	32.144 ± 1.071	0.002 ± 0.003	21.131 ± 1.039	nd	nd	21.130 ± 1.498	0.816 ± 0.184	nd	10.507 ± 1.497
3OH-C12:0	1.750 ± 0.186	16.076 ± 0.884	2.047 ± 0.082	5.881 ± 0.161	0.947 ± 0.687	0.944 ± 0.795	11.233 ± 0.892	2.506 ± 0.513	14.354 ± 1.335
2OH:C14:0	2.363 ± 0.384	16.567 ± 2.132	1.448 ± 0.244	16.339 ± 1.366	35.900 ± 0.861	2.664 ± 1.144	0.777 ± 0.218	12.156 ± 0.739	11.943 ± 1.214
3OH-C14:0	0.064 ± 0.009	4.692 ± 1.422	13.043 ± 1.911	17.611 ± 1.301	3.563 ± 0.543	26.231 ± 1.349	51.306 ± 0.897	9.102 ± 0.169	nd
2OH-C16:0	0.059 ± 0.017	nd	3.315 ± 0.378	nd	nd	0.269 ± 0.114	nd	14.049 ± 0.985	nd
i-C15:0	2.988 ± 0.215	0.694 ± 0.128	nd	nd	0.329 ± 0.225	nd	2.102 ± 0.924	nd	3.851 ± 0.714
i-C16:0	3.020 ± 0.202	0.001 ± 0.001	0.872 ± 0.114	nd	1.646 ± 0.564	8.914 ± 1.976	0.054 ± 0.033	nd	nd
i-C17:0	5.126 ± 0.495	nd	nd	2.208 ± 0.729	2.130 ± 1.370	nd	1.688 ± 0.463	nd	nd
a-C15:0	10.420 ± 0.711	nd	1.480 ± 0.247	nd	1.031 ± 0.956	nd	1.484 ± 0.591	2.185 ± 0.147	nd
C17:0 Δ^9	0.819 ± 0.058	nd	7.111 ± 2.174	nd	10.580 ± 0.442	5.690 ± 0.081	0.576 ± 0.303	10.828 ± 1.607	nd
C19:0 Δ^9	15.009 ± 0.998	nd	nd	11.989 ± 1.064	0.289 ± 0.204	0.745 ± 0.091	5.343 ± 2.837	7.587 ± 0.595	3.441 ± 0.473

Experiments were conducted in triplicate and their mean ± SD are presented.
[a] Dodecanoate (C12:0); tetradecanoate (C14:0); pentadecanoate (C15:0); hexadecanoate (C16:0); heptadecanoate (C17:0); octadecanoate (C18:0); nonadecanoate (C19:0); eicosanoate (C20:0); cis-9-hexadecenoate (C16:1^9); cis-9-octadecenoate (C18:1^9); cis-9,12-octadecadienoate (C18:2^{9,12}); 2-hydroxydodecanoate (2OH-C12:0); 3-hydroxydodecanoate (3OH-C12:0); 3-hydroxytetradecanoate (3OH-C14:0); 2-hydroxyhexadecanoate (2OH-C16:0); 13-methyltetradecanoate (i-C15:0); 14-methylpentadecanoate (i-C16:0); 15-methylhexadecanoate (i-C17:0); 12-methyltetradecanoate (a-C15:0); cis-9,10-methylenehexadecanoate (C17:0 Δ^9); cis-9,10-methyleneoctadecanoate (C19:0 Δ^9).
[b] nd: not detected.

Therefore, in case of Efm2.1, Efm3.1 and Efm3.2 mutants, although there was not significant change observed in the saturated to unsaturated fatty acids ratio, the increase in total hydroxy fatty acids and decrease in branched chain fatty acids contributed to the decreased fluidity of membrane. However, in case of Efm4.1, Efm4.2, Efm5.1, Efm5.2 and Efm5.3 mutants, increased saturated to unsaturated fatty acids ratio further added on to the increased rigidity of the membrane making them highly resistant.

3.4. In vitro assessment of resistance in wild-type and Ped^r mutants of E. faecalis to CAMPs

The percent colorimetric response (%CR) of CAMPs viz., pediocin, nisin and alamethicin against PDA/lipid biomimetic membranes prepared from lipids extracted from wild-type and all the Ped^r mutants of *E. faecalis* was determined. The %CR of pediocin, nisin and alamethicin was found to be higher in case of all Ped^r mutants of *E. faecalis* as compared to the wild-type strain (Fig. 5), indicating lesser penetration of these AMPs in PDA/lipid biomimetic membranes prepared from lipids extracted from Ped^r mutants. The %CRs of pediocin, nisin and alamethicin against the biomimetic membranes prepared from the lipids of wild-type strain and all the Ped^r mutants were found to be significantly different ($F(8,18) = 229.6$, $P < 0.001$), ($F(8,18) = 295.5$, $P < 0.001$) and ($F(8,18) = 339.4$, $P < 0.001$), respectively. Thus, the %CR increased with the increase in degree of resistance.

An inverse relationship was observed between the percent colorimetric response of pediocin and percent inhibition of wild-type and Ped^r mutants of *E. faecalis* by pediocin (Fig. 6), while a linear relationship was observed between percent colorimetric response and the IC_{50} of pediocin ($R^2 = 0.996$) against the wild-type and Ped^r mutants of *E. faecalis* (Fig. 7). With increased inhibitory concentration (IC_{50}) of pediocin and decreased percent inhibition, the %CR increased, which can be related to increased degree of resistance. Fig. 8 shows that there was reduction in percent colorimetric response upon addition of Mg^{2+} which could not be avoided even after chelating Mg^{2+} with EDTA in all the strains.

3.5. Pediocin-induced leakage of calcein from calcein-loaded LUVs

A comparison of the calcein leakage from the different calcein-loaded LUVs prepared from the lipids extracted from the wild-type (Ef) and Ped^r (Efm3.2 and Efm 4.2) mutants of *E. faecalis* is shown in Fig. 9. It is clear from the figure that the calcein leakage is approximately 1.5-fold reduced in case of Efm 3.2 and 3-fold in case of Efm 4.2, as compared to Ef, after 15 min of the addition of pediocin to calcein-loaded LUVs.

4. Discussion

In the present study, we found that there is neutralization of surface charge on membrane phospholipids and alterations in fatty acids composition in lipid bilayer in gradual Ped^r mutants of *E. faecalis*. This resulted in reduced membrane penetration and consequent inability to form pores in Ped^r mutants of *E. faecalis* by CAMPs tested viz. pediocin, nisin and alamethicin. This was validated by reduced penetration of pediocin, nisin and alamethicin, into PDA/lipid biomimetic membranes prepared from the phospholipids isolated from Ped^r mutants of *E. faecalis*. This established the fact that only membrane phospholipids decide the fate of CAMPs bactericidal action.

Development of in vitro colorimetric assays provides rapid and easy evaluation of interactions between pore-forming antimicrobial peptides and lipid bilayers. The lipid:polydiacetylene vesicles obtained upon

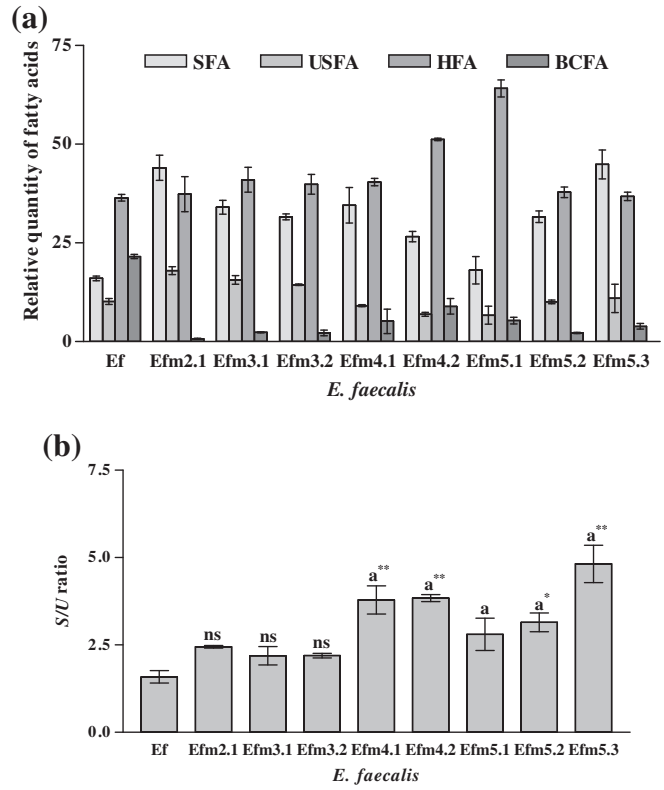

Fig. 4. (a) Comparison of relative quantity of different types of fatty acids present in total lipid extracts of wild-type and Ped^r mutants of *E. faecalis*. SFA: saturated fatty acids; USFA: unsaturated fatty acids; HFA: hydroxy fatty acids; BCFA: branched chain fatty acids. (b) comparison of saturated to unsaturated fatty acids ratio of the wild-type and Ped^r mutants of *E. faecalis*. The data are the means of three independent experiments, with error bars representing the SD. ns ($P > 0.05$), no significant difference from their respective Ef value, a ($P < 0.05$), a* ($P < 0.01$), a** ($P < 0.001$), and significantly different from their respective Ef values.

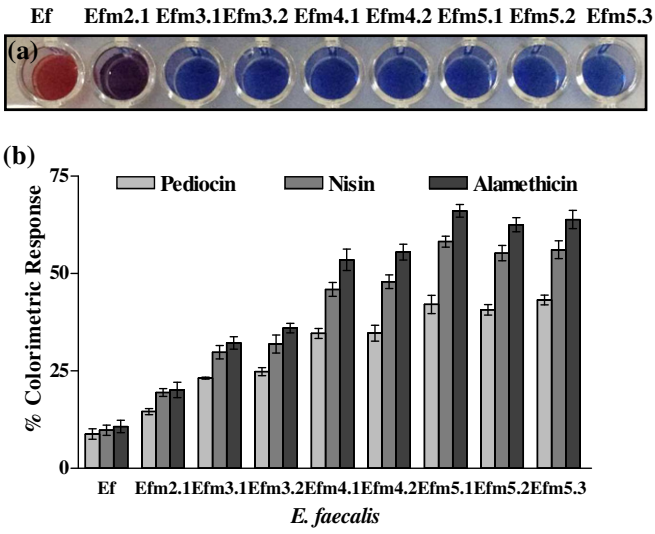

Fig. 5. (a) A portion of 96-well plate containing PDA/lipids biomimetic membranes prepared from wild-type and Ped^r mutants of *E. faecalis*, after the addition of pediocin, (b) comparison of percent colorimetric response of pediocin, nisin and alamethicin against PDA/lipid biomimetic membranes prepared from lipids extracted from wild-type and Ped^r mutants of *E. faecalis*. The data are the means of three independent experiments, with error bars representing the SD.

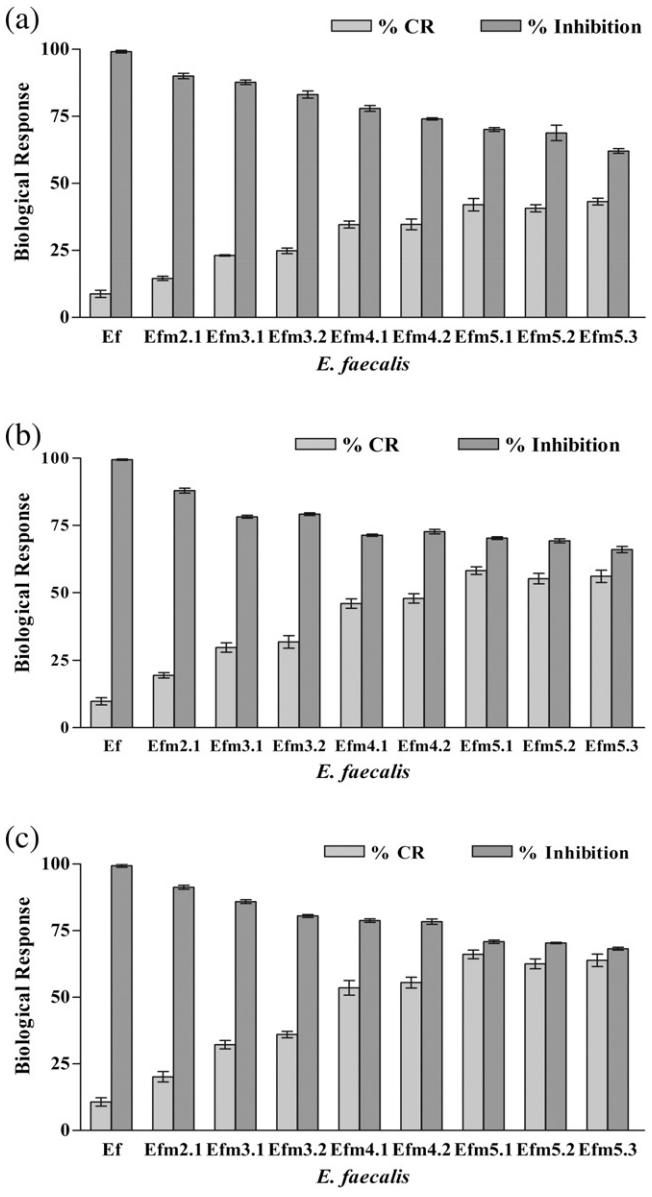

Fig. 6. Mapping of percent colorimetric response of (a) pediocin, (b) nisin, and (c) alamethicin against wild-type and Ped^r mutants of *E. faecalis* to percent inhibition of wild-type and Ped^r mutants of *E. faecalis* by pediocin, nisin and alamethicin, respectively.

polymerization reaction between diyne monomers and phospholipids results in lipid:PDA nanosomes which undergo specific blue to red transitions induced by interactions with antimicrobial peptides. A quantitative value for the extent of blue-to-red color transition is given by the colorimetric response (%CR). Molecules that preferably interact with and disrupt the lipid head group region were shown to induce more pronounced color transitions, while deeper penetration into the hydrophobic lipid core generally gave rise to more moderate blue-to-red transformations [26].

Stronger color change (%CR) has been observed when lipid/PDA vesicles were employed compared to pure PDA, which indicates that the colorimetric effects are biologically relevant rather than due to electrostatic binding of the peptides to the PDA interface [26]. The "effective" biologically meaningful colorimetric response, which is solely due to association of the examined peptide with the phospholipid domain, can be obtained through subtraction of the background CR acquired in the presence of pure PDA-vesicle solution, from the CR detected in

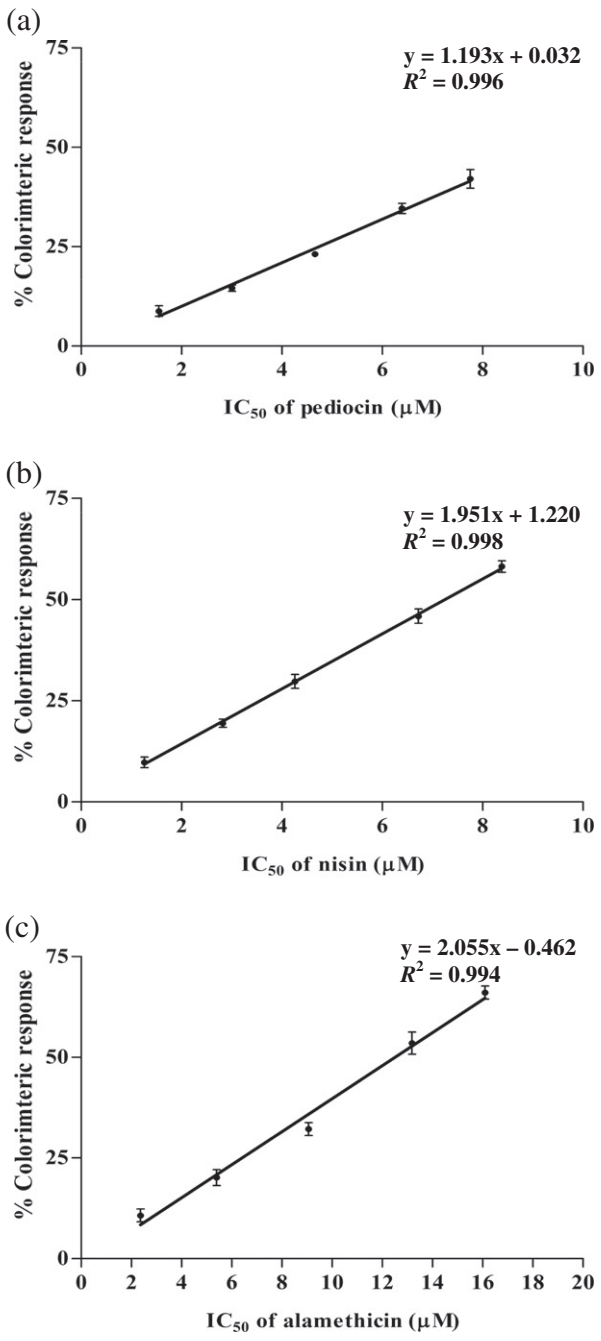

Fig. 7. Mapping of percent colorimetric response of (a) pediocin,(b) nisin and (c) alamethicin against wild-type and Ped^r mutants of *E. faecalis* to IC_{50} of pediocin, nisin and alamethicin for wild-type and Ped^r mutants of *E. faecalis*.

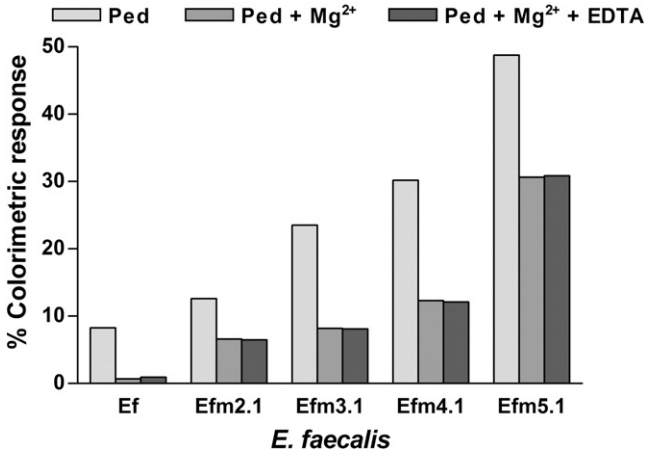

Fig. 8. Effect of Mg^{2+} on percent colorimetric response of pediocin against lipid-PDA vesicles prepared from lipids extracted from wild type (Ef) and Ped^r (Efm2.1, Efm3.1, Efm4.1 and Efm5.1) mutants of *E. faecalis*.

phospholipid-PDA vesicles [26]. The extent of colorimetric transitions is affected by size and charge of lipid head group, length and degree of unsaturation of fatty acids and presence of ions [28].

The %CR data revealed that pediocin, nisin and alamethicin penetration in phospholipid bilayer depends upon the composition of the phospholipids, i.e., ACP/ALP ratio and the fatty acyl chain of phospholipids in biomimetic membranes. In principle, the percent colorimetric response and interfacial lipid binding are directly related to each other because the mechanism of blue to red color transition in PDA assumes higher mobility of the pendant side-chains, induced through structural perturbations at the surface of lipid/PDA nanosomes [26]. Blue-to-red color transition in sensitive biomimetic membranes was because of the binding of CAMPs with polar head group at lipid–water interface, leading to stronger perturbations in pendant side chains of PDA, followed by penetration into the hydrophobic lipid core of fatty acyl chains. While no blue-to-red color transition in resistant biomimetic membranes may be attributed to the inability of CAMPs to bind to the bacterial membrane as the membrane surface is becoming less negative because of the increased ACP/ALP ratio in Ped^r mutants, and further penetration may be prohibited by the decreased membrane fluidity, which is mainly because of decrease in BCFA as observed in this study. Thus, fatty acyl chain and the polar head group of phospholipids may decide the colorimetric profile of CAMPs with sensitive and resistant biomimetic membranes. The resistance developed in bacterial cells was coupled to membrane fluidity and surface charge, and simultaneously, these principles could be applied for combating resistance against pore-forming antimicrobial peptides [29]. PDA vesicles incorporating a high percentage of phospholipid molecules could be used as biosensors for screening of antibacterial peptides [26]. Phospholipid/PDA mixed vesicles could be utilized as a model system for studying peptide–membrane interactions and interfacial membrane processes [26].

It is evident from the polydiacetylene-based colorimetric assay and calcein leakage assay that phospholipid head group and fatty acyl chain play an important role in protecting the cells from CAMPs. The addition of Mg^{2+} ions significantly reduced the inhibitory activity of

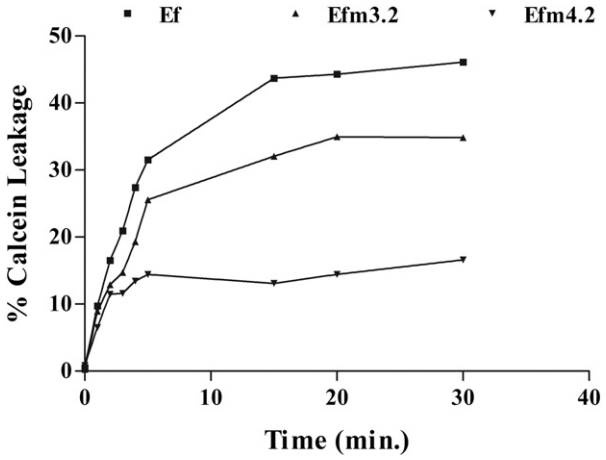

Fig. 9. Percent leakage of calcein from the different calcein-loaded LUVs prepared from the lipids extracted from the wild-type (Ef) and Ped^r (Efm3.2 and Efm 4.2) mutants of *E. faecalis* after the addition of pediocin (5 μg/ml).

pediocin on wild-type cells and the addition of EDTA restored this activity. Contrary to this, the inhibitory activities on Ped^r mutants were not affected by the addition of Mg^{2+} ions. These results suggest that Mg^{2+} ions protect wild-type cells against pediocin activity, by competing with the pediocin and rendering it unable to bind to the bacterial cell-surface. But there was no effect of Mg^{2+} in the Ped^r mutants, due to bacterial cell-surface charge alterations. Addition of Mg^{2+} ions before pediocin was able to screen pediocin interactions with lipid-PDA vesicles. However EDTA could not reverse this screening effect. The possibility that excess EDTA is also interacting with pediocin cannot be ruled out. Crandall and Montville [30] suggested that di- and trivalent cations might inhibit the electrostatic interactions between the positive charges on the nisin molecule and negatively charged phospholipid head groups. Our results were concomitant with the findings of Sakayori et al. [21] showing that Mg^{2+} ions protect wild-type cells against mundticin activity and that the effect of Mg^{2+} ions is suppressed in the Mun^r mutants. The impact of divalent cations on listericidal activity of divergicin M35 revealed that Mn^{2+} cations were able to reduce the binding of antimicrobial peptide to the *L. monocytogenes* cell membrane [31].

It is clear from the results that the amount of ACP is increasing with the degree of resistance, indicating that the cell-membrane is becoming less negative on being resistant to CAMPs. The increase in lysyl-PG can be correlated with the increased expression of *mprF*, but the appearance of PE is to be elucidated further. Several studies showed that *mprF* is sufficient for phospholipid lysinylation in *S. aureus* [10], *L. monocytogenes* [9], *M. tuberculosis* [11], *Bacillus anthracis* [12], and *E. faecalis* [17], which in turn increases the resistance against cationic antimicrobial peptides (CAMPs). Expression of *mprF* is upregulated by the Aps/GraRSX system in *S. aureus* in response to the presence of CAMPs. This Aps/GraRSX system constitutes a two-component system composed of a membrane inserted sensor histidine kinase (Aps/GraS), which gets activated on exposure to CAMPs and then activate the second component, a transcriptional response regulator, by phosphorylation resulting in the upregulation of *mprF* gene. Aps/GraRSX system also upregulates the genes involved in lysine biosynthesis [32]. Thus, regulation by Aps/GraRSX system might be a strategy to limit the energetic burden of lysine biosynthesis when CAMPs are not encountered and the levels of L-PG are sufficient for bacterial survival [32]. In Ped^r mutants, the increase in L-PG content along with the upregulation of *mprF* gene shows that Aps/GraRSX system might get activated. Further, appearance of PE in three Ped^r mutants is the subject of further investigation and the link for the appearance of PE in Ped^r mutants may be completed by expression analysis of the genes involved in PE biosynthesis. Genes encoding enzymes phosphatidylserine (PS) synthase and PS decarboxylase, which are responsible for synthesis of PE from PS in other genera are not found in *E. faecalis* through blastP analysis (unpublished data). Therefore, enzymes responsible for biosynthesis of PE in three Ped^r mutants need to be investigated.

The increased saturated to unsaturated fatty acid ratio in Efm4.1, Efm4.2, Efm5.1, Efm5.2 and Efm5.3, as compared to wild-type strain might lead to decreased fluidity of the cell membrane. Further, decrease in total branched chain fatty acids in all Ped^r mutants of *E. faecalis* also resulted in decreased membrane fluidity, which prevent the insertion of pediocin into phospholipid bilayer. The purpose of branched chain fatty acids in bacteria is to increase the fluidity of lipids as an alternative to double bonds, which are more liable to oxidation. Increase in cell membrane rigidity has been reported in the daptomycin-resistant clinical enterococcal strains [18] and nisin-resistant *L. monocytogenes* [19]. By contrast, an increase in membrane fluidity upon development of resistance among *L. monocytogenes* against leucocin A [20] and in *Enterococcus faecium* against mundticin KS [21], has been reported. Therefore, either an increase or decrease in membrane fluidity could affect the pore formation by bacteriocins in bacterial cell membrane. The present results correlate the inability of CAMPs to form pores among dose dependent Ped^r mutants of *E. faecalis* with change in membrane fluidity due to altered fatty acids composition of membrane phospholipids of Ped^r mutants.

From the outcome of the present study, it can be concluded that it is the cell-membrane of *E. faecalis* which modulate itself on resistance acquisition to pediocin. This was supported by reduced membrane surface negative charge as well as membrane fluidity. Conclusively, in vitro colorimetric assay with biomimetic membranes prepared from lipids extracted from wild-type and Ped^r mutants of *E. faecalis* revealed that only cell membrane components i.e., phospholipids and fatty acids are involved in interaction with CAMPs. Therefore, enzymes involved in biosynthesis of amino-containing phospholipids may be inhibited for tackling resistance to CAMPs. Alternatively, pediocin analogs may be designed for targeting altered phospholipids. In addition, in vitro colorimetric assay employing bio-mimetic membrane can be used for quantification of resistance to CAMPs.

Funding

None.

Conflict of interest

No conflicts of interest declared.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

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