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Divalent Cations (Mg^{2+}, Ca^{2+}) Protect Bacterial Outer Membrane Damage by Polymyxin B

(Kation Divalen (Mg^{2+}, Ca^{2+}) Melindungi Membran Luar Bakteria daripada Kemusnahan oleh Polymyxin B)

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ABSTRACT

Polymyxin B interacts with divalent cations by displacing cations from their binding sites in the lipopolysaccharide (LPS) molecules. It leads to the disorganization of the outer membrane component of the Gram negative bacteria, which releases LPS component from bacterial surface causing severe membrane leakage and finally cell death. In this paper, the reversible activity of PMB was investigated in the presence of access divalent cations such as Mg^{2+} or Ca^{2+}. Membrane damage and cell disruption were monitored by detecting leakage of the outer membrane and cytoplasmic enzyme marker (β-lactamase and β-galactosidase), release of LPS component (KDO) from bacterial cells and bacterial survival. With the presence of divalent cations, leakage of enzyme markers and LPS released was significantly reduced when bacteria were exposed to PMB. The survival curve also significantly increased. The inhibitory of damage caused by PMB also depended on the type of divalent cation present. Ca^{2+} has been shown to be more effective in protecting the bacteria cells than Mg^{2+}. This is because Ca^{2+} appears more frequently as a constituent of the structural components of the bacteria. In conclusion, the presence of higher concentration of divalent cation particularly with Ca^{2+} inhibits PMB activity and maintained bacterial survival.

Keywords: Calcium; divalent cation; E.coli*; magnesium; polymyxin B*

ABSTRAK

Polymixin B (PMB) bertindak pada dinding sel bakteria dengan mengeluarkan kation divalen daripada tapak ikatannya di lipopolisakarida (LPS). Keadaan ini akan mengakibatkan komponen dinding bakteria gram negatif terganggu kestabilannya serta menyebabkan komponen dinding sel khasnya LPS terkeluar dari permukaan sel bakteria, seterusnya mengakibatkan kebocoran pada dinding sel dan akhirnya bakteria akan mati. Dalam penyelidikan ini proses berbalik daripada kesan di atas telah dikaji. Perencatan aktiviti PMB telah diuji dengan kehadiran kation seperti Mg^{2+} atau Ca^{2+}yang berlebihan dalam persekitaran bakteria. Kerosakan pada dinding dan kemusnahan sel dipantau dengan menggunakan penanda enzim sitoplasma seperti β-laktamase dan β-galaktosidase serta pembebasan LPS yang diuji daripada komponennya seperti (KDO) dan kemandirian bakteria. Dengan kepekatan kation divalen ditingkatkan, didapati berlakunya penurunan kerosakan daripada dinding sel bakteria akibat daripada tindakan PMB. Lengkuk kemandirian bakteria juga didapati telah meningkat. Kesan perencatan kerosakan pada bakteria oleh PMB juga banyak bergantung kepada jenis kation divalen. Ca^{2+} telah menunjukan kesan yang baik dalam melindungi bakteria daripada PMB berbanding dengan Mg^{2+}. Ini adalah kerana Ca^{2+} merupakan bahan penting di dalam komponen struktur dinding sel bakteria. Kesimpulannya, kehadiran kepekatan kation yang tinggi khasnya Ca^{2+} boleh menyebabkan aktiviti PMB terrencat seterusnya mengekalkan kemandirian bakteria.

Kata kunci: E.coli*; kalsium; kation divalen; magnesium; polymyxin B*

INTRODUCTION

The bacterial cell wall contains many types of cations including Mg^{2+}, Ca^{2+}, Na^{+}, and K^{+}. These ions are responsible for various bacterial activities, including enzyme actions, metabolic regulation and maintaining the integrity of the outer layers. Mg^{2+} and Ca^{2+} ions particularly, play an important role in preserving the stability of the outer structures (Athena et al. 2003; Ferrero et al. 2007; Peshenko et al. 2007). The outer most layer of the outer membrane in Gram-negative bacteria is the lipopolysaccharide (LPS). Individually, these molecules are negatively charged. Divalent cations help to stabilize and maintain the integrity of the outer membrane by binding in between adjacent LPS molecules. It binds to the negative charged lipid molecules, neutralizing the repulsion forces and grasping the LPS molecules together (Katowsky et al. 1991; Raetz et al. 2007).

Bacteria lacking these divalent cations are found to be more sensitive to certain chemicals and outer membrane (OM) disorganizing agents. Divalent cations can be

removed from their binding sites by some weak acids such as EDTA (Leive 1974) and Tris buffer (Irvin et al. 1981). EDTA is a chelator which interferes with the stability of the OM by chelating the cations and removing them from their binding sites. As a result, LPS is released from the bacterial OM and the damaged membrane is a less effective barrier to solutes thus increasing its permeability.

Polymyxin is an example of cyclic cationic antibiotics which appears to target and displace divalent cations from the LPS thus disorganizing the bacterial membrane. In the presence of excess cations however, a significant decrease in antibacterial activity and increase in MIC has been noted (Hanckock et al. 1991). Despite this, little is known regarding the role of these cations.

The objective of this study was to determine the inhibition activity of PMB with presence of divalent cations. It was carried out by measuring the release of membrane enzyme markers such as β-lactamase (outer membrane marker) and β−galactosidase (cytoplasm membrane marker) (Sahalan & Dixon 2008), LPS component i.e. KDO (Kawasaki et al. 2007) and bacterial survival curve.

This study led to a better understanding in the roles of cations in maintaining the stabilization of the bacterial membrane and bacterial survival.

Methods and Materials

Bacteria

Escherichia coli K12 3300 with plasmid Pbr322 was used. The plasmid induces high production of β-lactamase and β-galactosidase. This makes the detection of outer and inner membrane damage easier. The culture was maintained on nutrient agar containing 50 μg/mL of Ampicillin, subcultured into fresh media every four weeks and was stored at 4°C.

Cations

The cations used in the experiments were the $MgCl_2$ and $CaCl_2$ salts.

Bacterial growth and study of bacterial leakage due to the presence of polymyxin B and divalent cations

The early log phase of *E. coli* was collected after centrifugation of 3500 rpm for 10 min. The bacteria was washed twice in saline and resuspended in phosphate buffer (pH7.4, 0.01 mM). The concentration of the bacteria was adjusted to 0.5 at 650 nm.

The culture was divided into four portions, one was added with 5 μg/mL of PMB and another was without PMB. The other two cultures were added with 5 μg/mL PMB and 0.01 mM or 0.05 mM of the divalent cation solution ($MgCl_2$ or $CaCl_2$).

All suspensions were incubated in the orbital waterbath at 37°C. Samples were collected after 30 min of incubation and centrifuged at 10000 g. The supernatants were collected and analysed for β-lactamase and β-galactosidase or stored at -20°C.

β-lactamase assay

β-lactamase (EC 3.5.2.6) was assayed using chromogenic cephalosporin nitrocefin assay (O'Callaghan et al. 1972). Samples (50 μL) containing β-lactamase were diluted in 0.95 mL of 10 mM sodium phosphate buffer (pH7.0) and transferred into a 3 mL cuvette. The cuvette was placed in a 37°C water bath. Two milliliters of nitrocefin (50 μg/mL) were added in the cuvette and immediately read with a spectrophotometer. The initial absorbance at 482 nm was recorded and the cuvette was left in the spectrophotometer for 1 min, followed by another reading and the difference in the absorbance was applied according to O'Callaghan et al. (1972).

β-galactosidase assay

The β-galactosidase was assayed by its ability to liberate the O-nitrophenol from O-nitrophenol-beta-D-galactopyranoside (ONPG) (Miller 1972). Samples of 50 μL, was diluted with 0.95 mL of 10 mM sodium phosphate buffer (pH 7.4) and preincubated for 5 min at 37°C. The ONPG (0.1 M) was diluted in 100 mM of sodium phosphate buffer solution and 4 mL of the freshly prepared ONPG solution was added to the sample. The mixture was immediately incubated in 37°C waterbath for 15 to 30 min or until yellow colour appeared. The reaction was stopped by adding 5 mL of 0.5 M of sodium carbonate and left at room temperature for 5 to 10 min. The optical density was measured against a reagent blank at 420 nm in a spectrophotometer. The ONP liberated from ONPG is a measure of β-galactosidase in the sample.

Assay of LPS released by the semi-carbazide method

LPS in a sample is quantified by the measurement of its KDO (2-keto-3-deoxyoctuloconate) (Batley et al. 1985). Samples following treatment with polymyxins or untreated were mixed with 1 mL of 0.125 M of sulphuric acid and heated in boiling waterbath for 8 min. The hydrolysed sample (0.5 mL), was neutralized with 0.05 mL of 2.5 M of NaOH and vortexed before adding 0.5 mL of semi-carbazide reagent (1% w/v). The mixture was again vortexed and heated at 60°C for 30 min. When cooled at room temperature, 1.5 mL of water was added followed by 0.5 mL of chloroform. After homogenizing, the solution was centrifuged to separate the phases. The upper aqueous layer was retained and measured with spectrophotometer at 250 nm absorbance against a reagent blank.

Bacterial survivability

E. coli was treated with divalent cation i.e. Mg^{2+} or Ca^{2} (0.01 and 0.05 mM) after exposure to PMB. Some small amount of the culture (0.1 mL) was collected and dropped

on to a Nutrient agar. The culture was spread with sterile L-shaped glass rod and incubated at 37°C. After 18 to 20 h the bacterial colonies which appear on the plate were counted and recorded.

Results

PMB (5 mg/ml) induced bacterial membrane leakage which triggered the released of the outer and inner membrane markers, i.e. β-lactamase and β-galactosidase (Figures 1 and 2). However, the leakage was inhibited by the addition of Mg^{2+} and Ca^{2+} to the culture. For instance in Figure 1, β-lactamase leakage was reduced from 0.18 μmol to 0.081 μmol when 0.01 mM of Mg^{2+} was added into the culture. This was also observed with similar concentration of Ca^{2+}, where only 0.045 μmol of β-lactamase was released.

PMB damaged of the inner membrane was also observed with the presence of these divalent cations. Figure 2 shows that β-galactosidase, an inner membrane enzyme marker, was significantly reduced in the presences

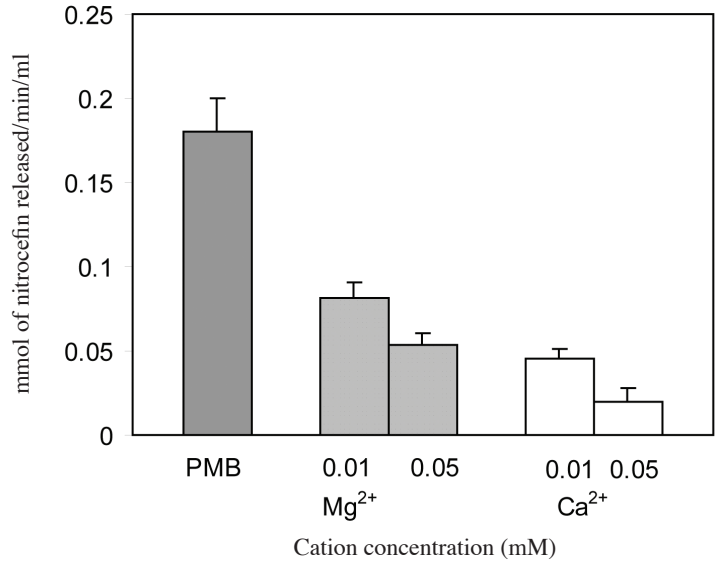

FIGURE 1. The effect of various concentration of Mg^{2+} and Ca^{2+} on the leakage of periplasmic β-lactamase in the presence of PMB *E. coli* K12 was incubated at 37°C. The bacteria was exposed to 5 μg/ml PMB. Some of bacteria portion were with 0.01 or 0.05 mM of Mg^{2+} or Ca^{2+} OR without the divalent cation treatment. Supernatants were collected, processed and quantified for β-lactamase

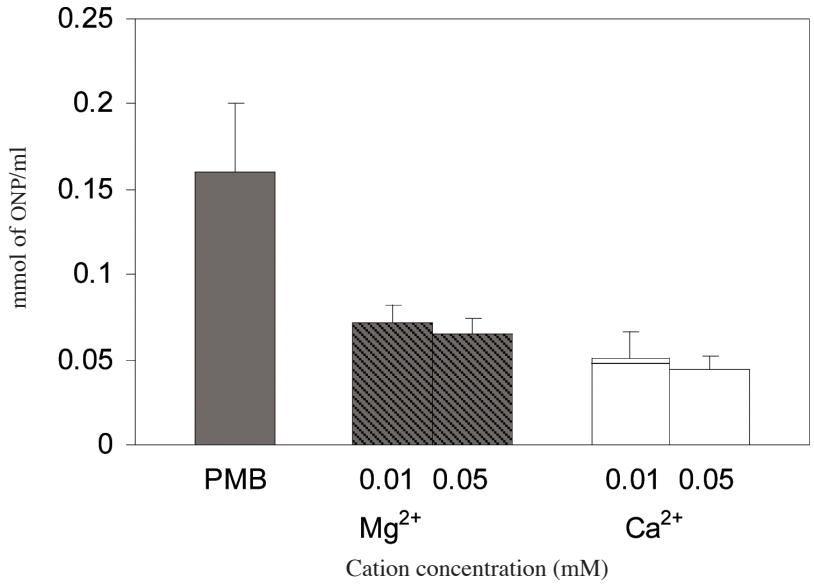

FIGURE 2. The effect of various concentration of Mg^{2+} and Ca^{2+} on leakage of cytoplasmic β-galactosidase in the presence of PMB *E.coli* K12 was incubated at 37°C of either PMB (5 μg/ml) with 0.1, 0.5 mM of Mg^{2+} or Ca^{2+}. The control culture was exposed to polymyxin B alone and represent as positive controls. Supernatants were collected, processed and quantified for β-galactosidase

of access Mg^{2+} and Ca^{2+} compared with PMB without the divalent cations.

Beside membrane leakage, PMB has also caused the shedding of LPS from the bacterial surface (Figure 3) where 70% of free LPS detected in the supernatant. However this was reduced to 32% in the presence of 0.01 mM of Mg^{2+} and 29% when the concentration was increased to 0.05 mM. Ca^{2+} treatment was more effective than Mg^{2+} at modulating the release of LPS. About 21% of LPS was released from the outer membrane of *E. coli* when treated with 0.01 mM of Ca^{2+} and 7.5% when the concentration was increased to 0.05 mM.

Bacterial survival curve (Figure 4) showed the significant protective effect of the divalent cations against PMB antibacterial activity. With 0.01 mM and 0.05 mM of Mg^{2+}, only 43% and 47% bacteria were able to survive. Ca^{2+} has showed higher ability to shield bacteria from PMB around 52% - 60%.

The percentage of bacterial survival was calculated according to:

$$\text{Survival Percentage} = \frac{\text{No. of colonies exposed to PMB and treated with divalent cation}}{\text{No. of colonies without PMB and divalent cation}} \times 100\%.$$

Overall, our results showed that Ca^{2+} has more profound effect than Mg^{2+} in reducing PMB damage.

Discussion

The relationships between bacterial cell leakage induced by PMB and the effect of divalent cations in bacterial revival were investigated. β-lactamase and β-galactosidase were markers used to detect outer membrane and cytoplasmic damage, respectively. The negative control without any treatment showed non significant or no released of the outer membrane and cytoplasmic markers at all. This signify that the membrane or bacterial cell wall to function normally. The positive control or *E. coli* with PMB have very high amount of both enzyme markers release into its supernatant (Figures 1 and 2). This showed the severity of damaged to the outer cell wall and inner cytoplasmic membrane of the bacteria. However with the addition of 0.01 mM of divalent cations (Mg^{2+}) to PMB (5 mg/ml) treated bacteria, this immediately reduced the β-lactamase leakage from 0.18 μmol to 0.081 μmol (Figure 1). When the concentration of Mg^{2+} was increased to 0.05 mM, the outer membrane leakage has also decreased further to only 0.054 μmol.

Ca^{2+} was observed to reduce even more outer membrane leakage for instance, 0.01 mM of Ca^{2+} was able to inhibit β-lactamase released from the culture to 0.045 μmol. If concentration was increased to 0.05 mM, only 0.02 μmol β-lactamase was detected in the supernatant.

The protective effect of cations was also observed in the bacteria cytoplasmic membrane. In Figure 2, the cytoplasmic membrane damaged was monitored by the released of β-galactosidase (measured with ONP/ml) from *E. coli* exposed to PMB with or without the presence of Mg^{2+} and Ca^{2+}. The 0.01 mM and 0.05 mM of Mg^{2+} reduced β-galactosidase from 0.16 mmol to 0.072 and 0.065 mmol of ONP/ml. The addition of Ca^{2+} to PMB treated bacteria (*E. coli*) resulted in 0.051 and 0.044 mmol of ONP/ml, respectively.

The LPS which has been the outer most component of the outer membrane plays a potent role particularly in maintaining cell wall stability. It would be interesting to study the fate of this molecule before and after presence of divalent cations in bacteria exposed to PMB. Figure 3

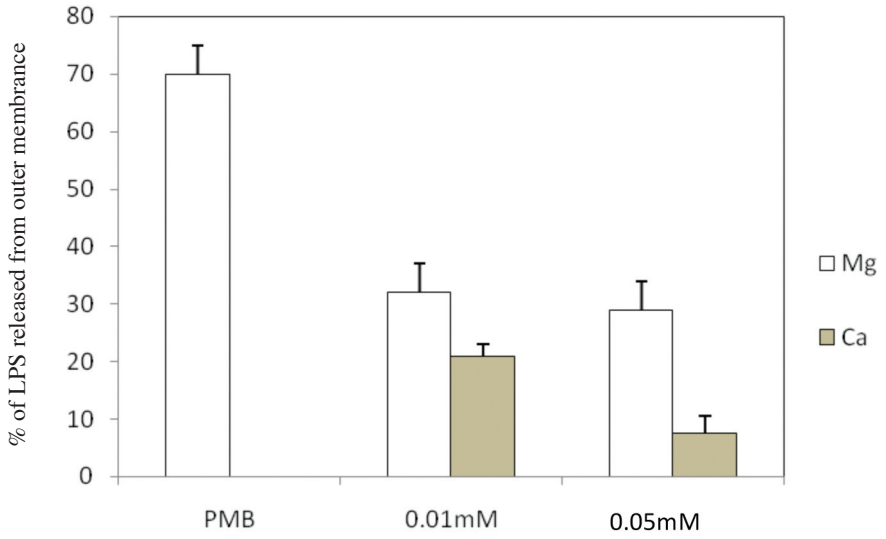

FIGURE 3. LPS released from *E. coli* exposed to PMB
LPS released from *E. coli* exposed to 5 μg/mL PMB in the presence or absence of Mg^{2+} or Ca^{2+}. *E. coli* was harvested during early log phase and the PMB was added to the culture at the same time as Mg^{+2} or Ca^{+2}. Samples were taken after an hour of incubation at 37°C

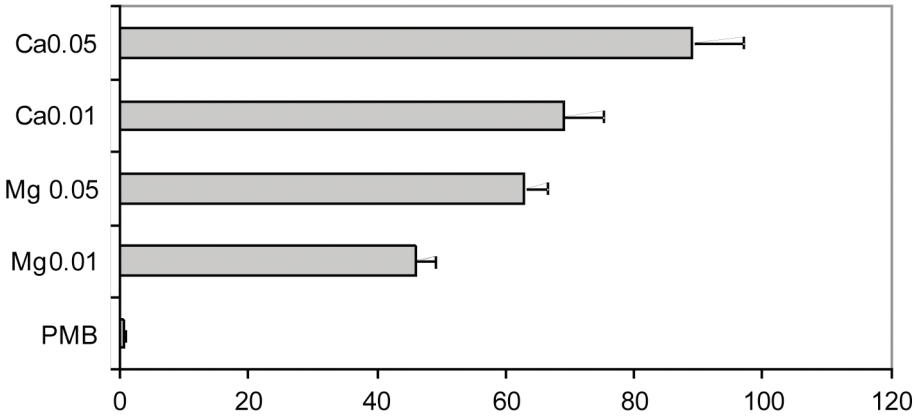

FIGURE 4. Bacterial survivability when exposed to PMB with or without divalent cation
After exposure to PMB and some portion of *E. coli* were treated with divalent cation. A 0.1 ml amount of the culture was dropped and spread on to a nutrient agar. The plates were incubated at 37°C for 18 to 20 h and bacterial colonies were counted and recorded

represents the effect of Mg^{2+} and Ca^{2+} on release of LPS from *E. coli* when exposed to PMB.

About 70% of the (KDO) LPS were released when *E. coli* was exposed to PMB alone. However, this was reduced to 32% with the presence of 0.01 mM of Mg^{2+} and 29% when the concentration was increased to 0.05 mM. Ca^{2+} treatment was more effective than Mg^{2+} at modulating the release of LPS. About 21% of LPS was released from the outer membrane of *E. coli* when treated with 0.01 mM of Ca^{2+} and 7.5% when Ca^{2+} was increased to 0.05 mM.

As a result from this experiment, Ca^{2+} inhibit PMB antibacterial activity better compared with Mg^{2+}. This characteristic showed by calcium is due to the attribute of particular cations suiting them to particular roles. Unlike Mg^{2+}, Ca^{2+} does not often feature as a co-factor of active proteins, but it appears more frequently as a constituent of the structural components of the cells (Verkhovskaya et al. 2011).

The bacteria survivability has also increased with divalent cation (Figure 4). This was shown with the increase of percentage of survivability in the presence of Ca^{2+} or Mg^{2+}. The survivability data correspond with β-lactamase, β-galactosidase and LPS results. As the membrane marker enzymes and LPS released were inhibited by the increasing concentration of divalent cation, more bacteria are able to survive the PMB antibacterial activity.

The interaction between PMB molecules and bacteria cell wall also depended on the concentration of the divalent cations. Divalent cations with the concentrations of 0.05 mM has prevented PMB damaging effect on the two membranes. This suggested that any excess of divalent cations within the bacteria environment could have strengthens the binding affinity to LPS. Due to this, the PMB would not be able to remove many divalent cations from LPS easily and therefore less membrane damaged was initiated.

However, the decreased activity of PMB may also be due to the binding of cations to PMB molecules itself. PMB is made up of peptide and may carries a positive charges (Vaara et al. 2008) although its hydrophobic tail is negatively charge (Sahalan & Dixon 2008). There is a tendency for a divalent cations to capture and bind to PMB. This complex formation may reduce the efficacy of PMB and should be the focused for future study.

CONCLUSION

As a conclusion, studying the effects of polymyxin and divalent cations in this experiment has yielded some information about competition between the two molecules. The experiments have clearly shown that divalent cations could prevent outer and inner membrane (cytoplasm membrane) damage by PMB. Furthermore, Ca^{2+} appears to be more effective than Mg^{2+} in deactivating PMB. The present investigation lends support to the functional role of divalent cations in the structure of the OM.

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