Polymyxin B Induces Lysis of Marine Pseudoalteromonads

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Polymyxin B (PMB) is a cationic antibiotic that interacts with the envelopes of gram-negative bacterial cells. The therapeutic use of PMB was abandoned for a long time due to its undesirable side effects; however, the spread of resistance to currently used antibiotics has forced the reevaluation of PMB for clinical use. Previous studies have used enteric bacteria to examine the mode of PMB action, resulting in a somewhat limited understanding of this process. This study examined the effects of PMB on marine pseudoalteromonads and demonstrates that the frequently accepted view that “what is true for Escherichia coli is true for all bacteria” does not hold true. We show here that in contrast to the growth inhibition observed for enteric bacteria, PMB induces lysis of pseudoalteromonads, which is not prevented by high concentrations of divalent cations. Furthermore, we demonstrate that a high membrane voltage is required for the interaction of PMB with the cytoplasmic membranes of pseudoalteromonads, further elucidating the mechanisms by which PMB interacts with the cell envelopes of those gram-negative bacteria.

Polymyxin B (PMB) is a cationic antibiotic that interacts with envelopes of gram-negative bacteria (21). It has been shown that at low concentrations PMB compromises the barrier of the outer membrane (OM) to lipophilic molecules, such as ionophoric antibiotics, while at higher concentrations it also depolarizes the cytoplasmic membrane (CM) by forming ion-permeable pores (12). When Escherichia coli cells are treated with high concentrations of PMB, periplasmic as well as cytoplasmic proteins are released to the medium (6, 17, 18); however, PMB-mediated cell lysis has not been reported.

Although a number of reported side effects have prevented the intensive use of PMB to treat bacterial infections (43, 46), PMB has recently been the subject of several studies (8, 11, 22, 23, 29) due to the need to respond to increasing antibiotic resistance. PMB has several cell-damaging properties: (i) it disturbs the surface charge, lipid composition, and structure of the membranes; (ii) it dissipates the K+ gradient on the CM; and (iii) it depolarizes the CM (12).

The permeability of the OM to lipophilic compounds is one of the main factors controlling bacterial sensitivity to PMB (12). Since PMB is bulkier than the inorganic divalent cations it displaces, the packing order of lipopolysaccharide (LPS) is altered in the presence of PMB. This results in increased permeability of the OM to a variety of molecules and also facilitates the uptake of PMB (“self-promoted” uptake) (26, 45). However, this can be prevented by increasing the divalent cation concentration in the medium. At high Mg2+ concentrations, increased amounts of PMB are needed to permeabilize the OM to lipophilic compounds. Under these conditions, the depolarizing activity of PMB becomes considerably weakened. It has been further demonstrated that at a 40 mM concentration of Mg2+, the self-promoted entry of PMB into E. coli cells is prevented, though PMB’s ability to bind to the OM surface is not affected (12). Additional studies have reported that a 20 mM concentration of Ca2+ or Mg2+ abolishes the antibacterial effects of PMB on both E. coli and Pseudomonas aeruginosa cells (7). Finally, it has been reported that the binding of PMB to the acidic phospholipids is also sensitive to the charge-screening effect of high ionic strength (36, 47).

Our laboratory has a history of studying lipid-containing bacterial viruses. One such virus is anicosahedral double-stranded DNA marine bacteriophage, PM2 (1, 20), infecting Pseudoalteromonas espejiana BAL-31 cells (19) as well as the closely related Pseudoalteromonas sp. strain ER72M2 (32). Pseudoalteromonads (previously classified as pseudomonads) are strictly aerobic, polarly flagellated, rod-shaped, heterotrophic gram-negative bacteria that are common inhabitants of the open sea and coastal waters and are frequently associated with the surfaces of eukaryotic organisms (2, 25, 35). Consequently, the ionic composition of standard SB broth, which is used for propagation of marine pseudoalteromonads, is close to that of seawater, and it is rich in divalent cations (approximately 10 mM CaCl2 and 50 mM MgSO4). During studies on the lifecycle and lysis system of bacteriophage PM2 (33), we have observed that these cells are highly sensitive to PMB and that treatment with this antibiotic causes rapid cell lysis.

The OMs of gram-negative bacteria are permeable to small metabolites and inorganic ions (K+, H+). However, LPS, which makes up the outer leaflet of the OM, forms a barrier to lipophilic compounds, including tetraphenylphosphonium (TPP+) and phenyldicarbaundecaborane (PCB-) ions or the ionophoric antibiotic gramicidin D (GD). Conversely, the CM is impermeable to inorganic ions but allows the translocation of lipophilic compounds (39). We have recently developed a potentiometric method to monitor changes in the physiology of bacteriophage-infected cells (16, 33). In our current study, we extended the application of this electrochemical technique in order to more fully elucidate the mechanisms of PMB-induced physiological effects. Here we report that in the presence of...
high concentrations of divalent cations, PMB interacts with the OMs as well as the CMs of marine pseudoalteromonads. Furthermore, disruption of the CM with PMB results in the lysis of bacterial cells, which potentially occurs as a consequence of the release of autolytic factors residing in the cytosol. This lytic effect that we describe for _Pseudoalteromonas_ species has not been observed for enteric bacteria. Finally, we analyzed the role of cell energetics in the interactions of PMB with the envelopes of pseudoalteromonads. Collectively, the results presented in this study further elucidate the mechanisms by which PMB interacts with the cell envelopes of certain aquatic gram-negative bacteria.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** _Pseudoalteromonas_ sp. strain ER72M2 (32) and _Pseudoalteromonas_ sp. strain A25 (31), as well as _Pseudoalteromonas espejiana_ BAL-31 (19), were cultured in SB broth at 28°C. SB broth (32) contained 8 g nutrient broth (Difco), 26 g NaCl, 12 g MgSO_4 · 7H_2O, 1.5 g CaCl_2 · 2H_2O, and 0.7 g KC1 per liter of water. When appropriate, MgSO_4 and/or CaCl_2 was not included in the SB broth. In such cases the broth is referred to as “depleted” of either or both of these divalent cations. However, residual amounts of these divalent cations originating from the nutrient broth were present in the medium.

All experiments were performed with exponentially growing cells, obtained by diluting an overnight culture 10-fold (resulting in an _A_550 of ~0.35) into an appropriate fresh medium and continuing the incubation until the _A_550 reached ~0.9. Then the bacterial culture was divided into individual flasks, and the experiments were performed while the culture turbidity was measured (λ, 550 nm) using a Selecta Chromic digital spectrophotometer (J. P. Selecta). _Escherichia coli_ K-12 HMS174 cells (Novagen) were propagated in Luria-Bertani broth (44) at 37°C. All experiments were done in triplicate, and representative regression curves are presented. The results presented in this study are intended to be qualitative rather than quantitative. Either a phenomenon (such as lysis) could be detected or it could not. Minor variations were not used to draw conclusions.

**Reagents.** PMB, polymyxin B nonapetide (PMBN), GD, nigericin (NG), Na_2HAsO_4, and CHCl_3 were purchased from Sigma, while KCN was obtained from Fluka.

**MIC determinations.** The epilometer agar diffusion gradient test (Etest; AB Biodisk, Solna, Sweden) was performed according to the manufacturer’s instruction by inoculating ER72M2 cells onto SB agar plates containing different amounts of divalent cations. The inoculated plates were dried for 30 min; then the plastic Etest strips containing a continuous exponential gradient (in the range of 0.064 to 1,024 μg/ml) of PMB were applied to the agar surface. Plates were incubated for ~50 h at 28°C, and MICs were determined by the interpret of the inhibition zone with the graded Etest strip.

**Electrochemical measurements.** _Pseudoalteromonas_ sp. strain ER72M2 cells were grown to a density of ∼6 × 10^9 CFU/ml (A_550 ~1.0), collected by centrifugation (Sorvall GSA rotor, 8,500 rpm, 25 min, 4°C), resuspended in fresh SB broth (not supplemented with MgSO_4 and CaCl_2) to obtain ∼1/65 of the original volume, and kept on ice until use (for a maximum of 6 h). The concentrated cell suspension was added to 30 ml of SB broth in a thermostated (28°C) vessel and kept on ice until use (for a maximum of 6 h). The concentrated cell suspension was added to 30 ml of SB broth in a thermostated (28°C) vessel and kept on ice until use (for a maximum of 6 h). The concentrated cell suspension was added to 30 ml of SB broth in a thermostated (28°C) vessel and kept on ice until use (for a maximum of 6 h). The concentrated cell suspension was added to 30 ml of SB broth in a thermostated (28°C) vessel and kept on ice until use (for a maximum of 6 h).

The distribution of TPP^- and PCB^-selective electrodes have been described previously (14, 15). The electrodes were connected to the electrode potential-amplifying system, based on an ultralow-input bias current operational amplifier (AD5493; Analog Devices). The amplifying system was connected to a computer through the data acquisition board (AD302; Data Translation, Inc.). The K^-selective electrode (Orion model 93-19) was from Thermo Inc. The

**RESULTS**

Lysis of marine pseudoalteromonads can be initiated by agents disrupting the integrity of the CM. When ER72M2 cells were treated with PMB at concentrations higher than 2.5 μg/ml, a sharp concentration-dependent decrease in the culture turbidity occurred (Fig. 1A). The same result was obtained by using chloroform (Fig. 1B), with a threshold concentration of 0.5% (vol/vol).

In order to distinguish which of the two PMB-caused effects (OM or CM disintegration) was responsible for the observed decrease in the culture turbidity, we tested whether the deacetylated PMB derivative PMBN would have a similar effect on ER72M2 cells. PMBN permeabilizes the OMs of gram-negative bacteria but has no effect on CM integrity (12, 17, 49). The Fig. 1A inset shows that PMBN has no prominent effect on
ER72M2 cell growth over the concentration range tested (up to 50 \(\mu\)g/ml). Furthermore, the addition of 1 mM EDTA to the cell suspension (in 50 mM Tris-HCl containing 450 mM NaCl [pH 8.0]) did not result in changes in cell suspension turbidity (data not shown).

To determine whether the lytic phenomenon observed was unique to ER72M2, two additional marine pseudoalteromonads were included in this study. Although the effects of PMB on the turbidities of \(P.\) espejiana BAL-31 and \(Pseudoalteromonas\) sp. strain A28 and \(E.\) coli (Fig. 2), they were not as intense as that observed for ER72M2 cells.

The efficiency of PMB-induced lysis is dependent on the presence of divalent cations. PMB destabilizes the OM by competing with \(Ca^{2+}\) and \(Mg^{2+}\) for binding sites in the negatively charged LPS layer of the OM (7, 37). Consequently, high concentrations of divalent cations antagonize the bactericidal action of PMB (12, 28). Since the decrease in the turbidity of the ER72M2 cell culture in SB medium was not complete (Fig. 1A), we tested whether the efficiency of lysis correlates with the presence of \(Ca^{2+}\) and \(Mg^{2+}\) in the growth medium by altering the divalent cation composition of SB broth (Fig. 3A).

We observed that when neither of the divalent cations was included in the growth medium, PMB addition resulted in a rapid and complete decrease in culture turbidity. A similar result was obtained when the cells were treated with PMB in a buffer containing 50 mM Tris-HCl and 450 mM NaCl (pH 8.0) (data not shown). Unexpectedly, in the presence of divalent cations, a temporary increase in the culture turbidity was observed (Fig. 3A), indicating shrinkage of the intracellular volume, characterized by higher refractivity of the cells. Shrinkage of PMB-treated cells was also confirmed by light microscopy (Fig. 3B). However, the initial increase in turbidity preceded a rapid decrease (Fig. 3A), although no complete lysis was observed. It should be noted that the cation concentration differences in SB medium had no effect on the growth of ER72M2 cells (data not shown).

The MICs of PMB were determined in SB medium in the absence and the presence of either one or both divalent cations. In all cases, the MICs were lower than 0.5 \(\mu\)g/ml. In the presence of both divalent cations, the MIC was determined to be 0.42 \(\pm\) 0.06 \(\mu\)g/ml, while in the absence of both divalent cations, the MIC was 0.33 \(\pm\) 0.07 \(\mu\)g/ml. In the presence of only \(Mg^{2+}\) or \(Ca^{2+}\), the MIC was determined to be 0.33 \(\pm\) 0.07 \(\mu\)g/ml or 0.11 \(\pm\) 0.01 \(\mu\)g/ml, respectively.

PMB-induced effects on \(Pseudoalteromonas\) sp. strain ER72M2 cell physiology. In order to more fully understand the mechanisms of PMB-induced physiological effects, we monitored simultaneously (Fig. 4), in real time, the extracellular concentrations of TPP\(^{+}\), PCB\(^{-}\), \(K^{+}\), and \(H^{+}\), while turbidity measurements were recorded with a delay of approximately 20 s. When added to the standard SB medium, ER72M2 cells spontaneously released some amount of intracellular \(K^{+}\). Following an initial acidification, a steady alkalinization of the culture medium occurred. intact cells weakly bound PCB\(^{-}\) and accumulated small amounts of TPP\(^{+}\) from the medium (Fig. 4A). In contrast, when \(Ca^{2+}\) and \(Mg^{2+}\) were not included in the medium, cellular membranes bound more PCB\(^{-}\), and significantly more TPP\(^{+}\) was accumulated by the cells in a \(\Delta\Psi\)-dependent manner (Fig. 4B), demonstrating the need for divalent cations to stabilize the OM. Addition of PMB to the cell suspension (Fig. 4C) in the medium devoid of divalent cations resulted in the instantaneous and complete leakage of intracellular \(K^{+}\), a decrease in culture turbidity, and an abrupt accumulation of PCB\(^{-}\) by the cell membranes. Furthermore, the extracellular concentration of TPP\(^{+}\) increased, reflecting the depolarization of the CM. Simultaneously, an intense al-

![Graph](http://aac.asm.org/)

**FIG. 2.** Effects of PMB on turbidities of cultures of marine bacteria (\(P.\) espejiana BAL-31 and \(Pseudoalteromonas\) sp. strain A28) and \(Escherichia coli\) HMS174. PMB was added at the time point indicated by the arrow to a final concentration of 50 \(\mu\)g/ml.

![Graph](http://aac.asm.org/)

**FIG. 3.** (A) Effects of divalent cations on the effectiveness of PMB-induced lysis of ER72M2 cells. Standard SB medium contains 10 mM CaCl\(_2\) and 50 mM MgSO\(_4\). Plus and minus signs indicate whether the ions were added or not (when they were added, the final concentrations were the same as in standard SB medium). PMB was added at the time indicated by the arrow to a final concentration of 50 \(\mu\)g/ml. (B) Demonstration of the shrinkage of the intracellular volume caused by PMB treatment. ER72M2 cells were treated with PMB (final concentration, 50 \(\mu\)g/ml) for 25 min and visualized under light microscopy. Bar, 20 \(\mu\)m.
FIG. 4. Electrochemical online registration of PMB-induced changes in the physiology of ER72M2 cells in different media. Shown are results for untreated (A and B) and PMB-treated (C and D) cells in the medium containing both divalent cations (A and D) or no divalent cations (B and C). Fluxes of TPP⁺ (black), PCB⁺ (blue), K⁺ (green), and H⁺ (yellow) were measured as described in Materials and Methods. $A_{550}$ (red) was measured by taking samples from the vessels. PMB was added at the time point indicated by the arrow to a final concentration of 50 $\mu$g/ml.
calization of the medium was observed, indicating the disappearance of the pH gradient on the CM (Fig. 4C).

When cells were PMB treated in the presence of either one (data not shown) or both (Fig. 4D) of the divalent cations, slightly different physiological profiles were obtained. Under these conditions, PMB-dependent effects on cell physiology can be divided into two stages, separated by approximately 5 min. During the primary stage, the rapid accumulation of PCB from the medium and an increase in the extracellular K concentration were observed. Concurrently, cells started to accumulate TPP from the medium in a ΔΨ-dependent manner. In contrast, the second stage was characterized by a rapid depolarization of the CM, which coincided with intense medium alkalization. Finally, an abrupt dissipation of the K gradient on the CM took place (Fig. 4D).

**Depolarization of the CM is not sufficient to initiate lysis of ER72M2 cells.** Classical experiments utilizing *Bacillus subtilis* have demonstrated that autolysis is rapidly induced with agents that dissipate either the electrical or the pH gradient across the CM (5, 30). The same phenomenon was observed in *Streptomyces griseus*, where GD or β-lactam antibiotics caused autolysis that occurred as a consequence of a drop in ΔΨ (42).

We next sought to determine whether dissipation of the ΔΨ using GD could induce lysis of ER72M2 cells. As discussed above, the OM is not permeable to this ionophoric antibiotic; therefore, we permeabilized the OM using a low concentration of PMB (2 μg/ml), which was determined to have no effect on either CM integrity or cellular growth (Fig. 5). As expected, PMB treatment caused accumulation of increased amounts of TPP and PCB from the medium. GD addition resulted in an abrupt leakage of intracellular K, binding of PCB to the cells, and a complete depolarization of the CM. However, these GD-dependent effects resulted in growth inhibition with no decrease in culture turbidity.

**PMB-triggered lysis is dependent on the ΔΨ.** Surprisingly, when an amount of PMB sufficient to trigger lysis was added to a suspension of GD-depolarized cells, no decrease in turbidity was detected (Fig. 5). We next investigated whether the resistance of GD-treated cells to PMB-induced lysis was due to the growth inhibition effect of GD. In order to address this, we treated ER72M2 cells with arsenate (20 mM) for 10 min prior to PMB addition. We have previously determined that such treatment completely inhibits the growth of ER72M2 cells but does not affect the membrane voltage (33). Addition of PMB (10 μg/ml) simultaneously triggered lysis of both arsenate-treated and untreated cells (data not shown).

It has been shown previously that the interaction of different cationic antimicrobial peptides with planar membrane bilayers is transmembrane voltage dependent (53). Consequently, we hypothesized that the interaction of PMB with ER72M2 cells might also rely on ΔΨ. To test this hypothesis, we examined the efficiency of PMB-induced lysis in the presence of agents dissipating or increasing the ΔΨ (Fig. 6). In order to depolarize the CM, we used KCN, which blocks respiration by inhibiting cytochrome c oxidase and completely dissipates the ΔΨ of ER72M2 cells (33). At each concentration tested, PMB-mediated lysis was delayed in the presence of KCN, starting about 20 min after PMB addition (Fig. 6A). To increase the ΔΨ, we used the ionophoric antibiotic NG. NG increases ΔΨ (Fig. 6B inset) by exchanging K with H according to their chemical gradients (38). When NG and PMB were added simulta-
neously, an immediate decrease in the culture turbidity was observed, irrespective of the PMB concentration (Fig. 6B). It should be noted that NG alone had no effect on culture turbidity (data not shown). These results indicate that an interaction between PMB and the CM, and consequently the induction of lysis, is dependent on the ΔΨ.

**DISCUSSION**

The emergence and spread of resistance against many of the currently employed antibacterial agents is an increasing problem for human and animal health worldwide (9). Membrane-active peptides such as polymyxins are promising options as the next generation of antibiotic and are therefore being reevaluated for their therapeutic potential (21, 24). Cationic peptides, such as PMBN, permeabilize the OM to lipophilic compounds. Consequently, a number of studies have touted the potential of these peptides to sensitize gram-negative bacteria to other antibiotics (40, 50, 51). Such an ability of PMB and PMBN is exploited not only in therapy but also in in vitro studies of cell susceptibility to various compounds (27, 51, 52). Previous studies analyzing the antimicrobial effects of PMB have focused predominantly on the effects against human pathogens, resulting in a limited understanding of the mode of action regulating PMB-dependent changes in bacterial cell physiology as well as the potency of this compound. In this study we investigated the effects of PMB on marine pseudoalteromonads, abundant microorganisms that occupy a completely different environmental niche from enteric bacteria. Our results show that the effects of PMB on marine bacteria deviate considerably from those observed for enterobacteria.

Studies with *E. coli* have indicated that PMB interaction with the cell envelope occurs in two major steps. The compound interacts first with the OM and then with the CM (12). Permeabilization of the CM is not a prerequisite for the antimicrobial activity of PMB, and bacteriostasis can be achieved with nondepolarizing concentrations (12, 41). In contrast, in the case of ER72M2 cells, concentrations permeabilizing the OM but not the CM (threshold concentration, 2.5 μg/ml) had no effect on bacterial growth in the liquid medium (Fig. 1A and 5). Furthermore, ER72M2 cells treated with low (OM-permeabilizing) concentrations of PMB supported infection with bacteriophage PM2, resulting in normal virus production and progeny release (33), indicating that *Pseudoalteromonas* cells with the PMB-permeabilized OM maintained metabolic activity. Interestingly, the MIC of PMB for ER72M2 cells grown on solid SB agar plates was determined to be lower than 0.5 μg/ml. However, it should be noted that local cell concentrations around the Etest strip were presumably lower than during experiments carried out in the liquid medium. Consequently, the antibiotic/cell ratios in the two experiments were different, i.e., in the former experiment the ratio was higher, leading to different amounts of bound PMB per cell.

In addition, it has been reported previously (7, 12) that 20 to 40 mM concentrations of Mg²⁺ or Ca²⁺ abolish the bactericidal effect of PMB on *E. coli* and *P. aeruginosa*. However, in our experiments utilizing pseudoalteromonads, the two phases, temporally separated by 5 min, were easily distinguishable in the presence of high concentrations of divalent cations in the culture medium when we were analyzing the interaction between PMB and the envelopes of ER72M2 cells (Fig. 4D). These data indicate that Ca²⁺ and Mg²⁺ influence the interaction of PMB with the envelopes of ER72M2 cells but are unable to block the detrimental effects of this antibiotic. During the primary stage, interaction of PMB with the OM, we measured increased permeability of the OM to lipophilic ions as well as some leakage of intracellular K⁺. Concurrently, a sharp temporary increase in culture turbidity was observed (Fig. 3A and 4D), indicating the increase in the refractivity index of the cell suspension due to shrinkage of the intracellular volume (Fig. 3B) as a response to the PMB-induced osmotic shock. These findings are in agreement with previous studies using *E. coli*, which indicated that PMB induced an osmotic shock by promoting phospholipid exchange between the inner leaflet of the OM and the outer leaflet of the CM without fusion of the two membranes (3, 4, 41).

During the second phase, characterized by an interaction between PMB and the CM, we registered depolarization of the CM, dissipation of the pH gradient, rapid efflux of intracellular K⁺, and, most surprisingly, decreased culture turbidity. The ability of PMB to form pores in the CM is well known (12). However, equilibration of ion concentrations across the CM is insufficient to induce cell lysis. We observed, however, using ER72M2 cells, that PMB induced cell lysis. Consequently, the peptidoglycan layer of the cell envelope must be digested. To our knowledge, PMB-mediated lysis of enterobacteria has never been reported.

Depolarization of the CM in gram-positive bacteria stimulates periplasmic autolysins (peptidoglycan-hydrolyzing enzymes) and subsequent cell lysis (30, 42). However, our results demonstrate that ER72M2 cells do not undergo lysis as a consequence of the dissipation of the ΔΨ, since treatment with either GD or KCN did not result in decreased culture turbidity (Fig. 5 and 6A). Our data indicate that the formation of fixed-size ion-permeable channels in the CM is not sufficient to cause lysis. In addition, by manipulating the ΔΨ with KCN or NG and monitoring the efficiency of lysis, we were able to show that interaction of PMB with the CM of ER72M2 cells is ΔΨ dependent (Fig. 6). We predict that this phenomenon is not restricted only to pseudoalteromonad cells but extends also to other gram-negative bacteria, including human pathogens.

Interestingly, marine *Pseudoalteromonas haloplanktis* cells (previously classified as pseudomonad B-16) are sensitive to lysis by surfactants, such as Triton X-100. Lysis can be prevented by the presence of cations, but not when the OM is removed (48). Similarly, surfactants induce lysis of *Enterococcus faecalis* (10) and *Streptococcus pneumoniae* cells (34), but only if autolytic enzymes are synthesized by the cells. Therefore, the potential exists that PMB, like surfactants, may have the capacity to induce lysis using a similar mechanism. We conclude that disruption of the CM with PMB or chloroform is a prerequisite for the lysis of marine pseudoalteromonads. Lysis of ER72M2 cells was most effective when the incubation medium lacked divalent cations (Fig. 3 and 4B); PMB addition resulted in instantaneous and complete clearance of the culture turbidity, accompanied by equilibration of the electrochemical gradients across the CM. Interestingly, almost identical effects were registered during the lysis of ER72M2 cells caused by bacteriophage PM2 infection (33). In contrast to other bacteriophage systems, a PM2-encoded lytic enzyme
could not be detected, suggesting that both PM2-induced lysis and PMB-induced lysis utilize the autolytic enzymes of the host. One mechanistic difference is that the PM2-encoded membrane-permeabilizing factor acts from the cytoplasmic side while PMB targets the periplasmic side of the CM.

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