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Calcium ion-dependent entry of the membrane-containing bacteriophage PM2 into its *Pseudoalteromonas* host

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ABSTRACT

Marine bacteriophage PM2 infects gram-negative *Pseudoalteromonas* species and is currently the only assigned member of the *Corticoviridae* family. The icosahedral protein shell covers an internal protein-rich phage membrane that encloses the highly supercoiled dsDNA genome. In this study we investigated PM2 entry into the host. Our results indicate that PM2 adsorption to the host is dependent on the intracellular ATP concentration, while genome penetration through the cytoplasmic membrane depends on the presence of millimolar concentrations of calcium ions in the medium. In the absence of Ca^{2+} the infection is arrested at the entry stage but can be rescued by the addition of Ca^{2+}. Interestingly, PM2 entry induces abrupt cell lysis if the host outer membrane is not stabilized by divalent cations. Experimental data described in this study in combination with results obtained previously allowed us to propose a sequential model describing the entry of bacteriophage PM2 into the host cells.

Introduction

Marine bacteriophage PM2 is the only known member of the *Corticoviridae* family, although prophages related to PM2 commonly reside in the genomes of aquatic bacteria (Bamford and Bamford, 2006; Krupovič and Bamford, 2007). PM2 was isolated from the coastal sea waters of Chile (Espejo and Canelo, 1968b) and infects gram-negative marine *Pseudoalteromonas* species (Espejo and Canelo, 1968a; Kivelä et al., 1999). The structure of the icosahedral PM2 virion has been determined by cryo-EM and 3D image reconstruction to a resolution of 8.4 Å (Huiskonen et al., 2004). Moreover, the entire PM2 virion has been crystallized and the structure solved by X-ray crystallography to ~7 Å resolution. An atomic model for all the ordered protein components of the PM2 virion (P1, P2, P3, and P6) has been built. In addition, high-resolution structures of the trimeric major capsid protein P2 and the pentameric receptor binding protein P1 were determined (Abrescia et al., 2008).

PM2 is a tailless bacteriophage with an inner membrane and is the first bacterial virus for which lipid components were shown to be a part of a virion (Camerini-Otero and Franklin, 1972). The outermost layer of the virion is an icosahedral protein shell composed of the major capsid protein P2, organized on a $T=21$ lattice, and the pentameric receptor-binding protein P1 occupying the vertices of the icosahedral capsid (Abrescia et al., 2008; Kivelä et al., 2002). The topology of the major capsid protein P2 as well as the overall organization of the PM2 icosahedral protein shell is very similar to those of other internal membrane-containing viruses infecting diverse bacterial, archaeal and eukaryotic hosts (Abrescia et al., 2008; Krupovič and Bamford, 2008). Underneath the protein shell resides the protein-rich membrane vesicle, designated as the lipid core (LC; Kivelä et al., 2002), which encloses the highly supercoiled circular double stranded (ds)DNA genome of ~10 kb (Gray et al., 1971; Männistö et al., 1999). The viral membrane contains eight PM2-encoded protein species (P3–P10), while the lipids are selectively incorporated from the host cytoplasmic membrane (CM; Laurinavičius et al., 2007).

Replication of the PM2 genome takes place in association with the host CM via a rolling-circle mechanism and is initiated by the phage-encoded replication initiation protein P12 (Brewer, 1978b; Männistö et al., 1999). The twenty-one predicted open reading frames are organized into three operons (two early and one late) that are regulated by phage-encoded transcription factors (Männistö et al., 2003). The assembly of the PM2 virion is also likely to take place in association with the host CM. The mechanism of PM2 genome encapsidation is not known. However, using temperature-sensitive PM2 mutants, virus-sized membrane vesicles have been observed to line up along the CM (Brewer, 1978a). Moreover, a putative NTPase P9, which is homologous to genome packaging ATPases of other inner-membrane containing viruses, such as PRD1 (Strömsten et al., 2005), was shown to be a part of the PM2 LC (Kivelä et al., 2002). These findings suggest that the PM2 genome is packaged into the preformed empty procapsids, as is the case for other structurally similar viruses (Strömsten et al., 2005).

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PM2 is a virulent phage. It has been shown that for cell lysis and progeny release PM2 employs a system, not previously described for other bacterial viruses (Krupovič et al., 2007a). A duet of phage-encoded membrane-perturbing proteins P17 and P18 is required for cell lysis to occur. Moreover, it was proposed, that unlike other dsDNA bacteriophages studied, PM2 relies on the cellular lytic factor (CLF) to digest the peptidoglycan layer. At the onset of lysis P17 makes holes in the CM, through which the CLF and P18 are released. CLF cleaves the peptidoglycan and P18 facilitates the disintegration of the host outer membrane (OM) which is stabilized by divalent cations present in the marine environment (Krupovič et al., 2007a). At the end of the infection cycle ~300 virions per one infected cell are released (Kivelä et al., 1999).

Although PM2 is structurally similar to the internal membrane-containing dsDNA tectiviruses PRD1 and Bam35 (Krupovič and Bamford, 2008), it uses a fundamentally different strategy to enter into the host cell. In the case of bacteriophage PRD1, receptor recognition and binding triggers structural rearrangements at one of the capsid vertices leading to the transformation of the spherical membrane into a tube-like structure, which serves as a channel for genome delivery (Bamford et al., 1995). The empty capsid remains attached to the cell surface resembling the entry of tailed dsDNA viruses of the order Caudovirales (Grahn et al., 2002; for a review see Poranen et al., 2002). PM2 infection starts with the recognition of the susceptible cell and subsequent binding of the virion to the receptor present on the cell surface via the pentameric receptor-binding protein P1. Despite considerable efforts to identify the PM2 receptor, it remains unknown (Kivelä et al., 2004). A high resolution X-ray structure of the receptor-binding protein P1 revealed that it is structurally similar to the Ca^{2+}-dependent carbohydrate-binding proteins from marine bacterium *Saccharophagus degradans*, suggesting that lipopolysaccharide (LPS) might serve as a receptor during PM2 entry (Abrescia et al., 2008; Kivelä et al., 2008). However, in contrast to other dsDNA bacteriophages, it was demonstrated that after PM2 adsorption the protein capsid (composed of P1 and P2) dissociates and the lipid core is exposed to the OM of the host cell. Moreover, the PM2 membrane, unlike in bacteriophage PRD1, does not form the tubular structure, instead it was proposed to fuse with the OM (Kivelä et al., 2004).

Due to the low permeability of the OM of gram-negative bacteria to lipophilic compounds, such as tetraphenylphosphonium (TPP^+) and the ionophoric antibiotic gramicidin D (GD), dissection of the virus-induced effects on the OM during the virus entry is possible (Daugelavičius et al., 2005; Kivelä et al., 2004). The transient permeability (~2 min) of the OM to GD observed during PM2 entry was suggested to signify the fusion between viral membrane and the OM of the host. It was proposed that PM2 membrane contributes a membrane "patch" devoid of LPS and therefore allows GD to reach the periplasm and depolarize the CM (Kivelä et al., 2004). Recently a PM2 mutant devoid of protein P10 was reported to be unable to induce entry-associated effects on the OM of the host cells. Accordingly, it was suggested that viral integral membrane protein P10 is involved in viral membrane fusion with the OM (Kivelä et al., 2008). By following the changes in membrane voltage and K^+ fluxes across the CM it was determined that PM2 genome penetration through the CM starts shortly after adsorption and lasts for approximately 2 min (Kivelä et al., 2004).

In addition, based on structural data, the highly supercoiled circular PM2 genome essentially exerts no pressure on the viral membrane (Abrescia et al., 2008), i.e. there is no internal pressure in the PM2 capsid to drive the initial injection of the viral genome into the host cell as was shown to be the case in many other bacteriophage systems (e.g., Sao-Jose et al., 2007). In accordance, it has been shown that insertion of at least 500 bp of additional DNA into PM2 genome has no effect on phage stability or infectivity (Krupovič et al., 2006). These observations suggest that PM2 uses a completely different genome delivery mechanism than that utilized by other dsDNA phages.

In the present study we demonstrate that the marine virus PM2 enters into the host cell in a unique, Ca^{2+}-dependent manner. We provide evidence that the presence of a PM2 receptor on the host cell surface is dependent on the intracellular ATP concentration, but not on the proton-motive force of the host cell. The role of divalent cations during the course of PM2 infection was also investigated. Finally, we present a model of bacteriophage PM2 entry into the host cells supported by experimental evidence reported here and obtained previously.

Results

PM2 adsorption correlates with the intracellular ATP concentration

It has been observed that PM2 does not irreversibly adsorb if the host cells are not aerated. This observation in conjunction with the fact that it was not possible to isolate the receptor (Kivelä et al., 2004) implies that PM2 receptor is a metastable structure. In order to find out whether the presence of the receptor on the surface of the host is dependent on the energetic state of the cells we used a range of poisons affecting different cellular processes associated with transformation and storing of energy (Fig. 1A). The energy inside bacterial cells can be stored in two forms: (i) intracellular pool of ATP, and (ii) H^+ electrochemical gradient across the CM also known as the

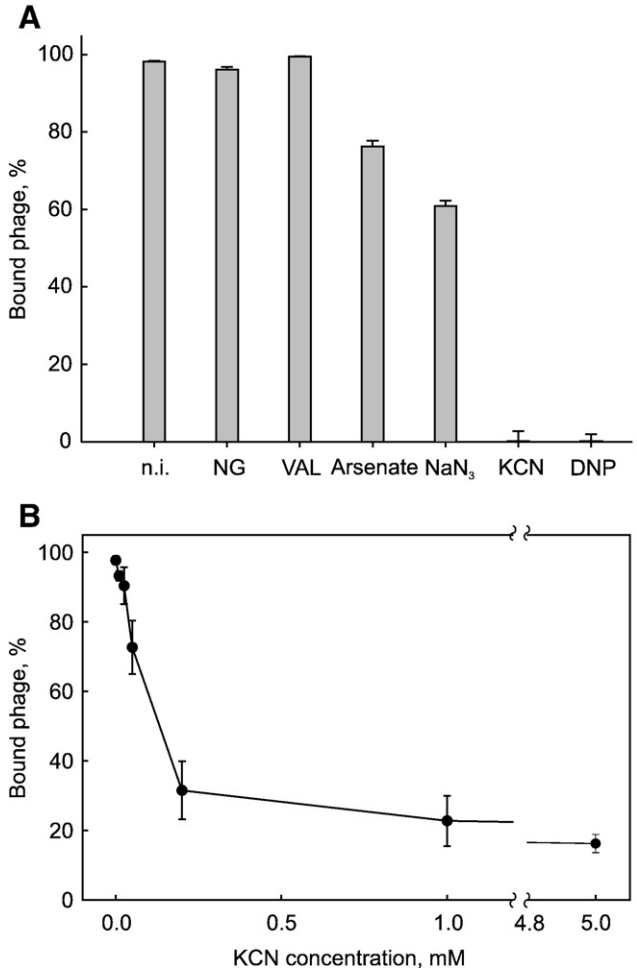

Fig. 1. Effect of energy poisons on PM2 adsorption. (A) The percentage of bound PM2 particles to ER72M2 cells treated with energy poisons. N.i.—adsorption to non-treated ER72M2 cells. ER72M2 cells were incubated with aeration for 5 min at 28 °C with Nigericin (NG; 24 μg/ml), KCN (20 mM), sodium arsenate (20 mM), NaN_3 (20 mM), dinitrophenol (DNP; 1 mM), or valinomycin (VAL; 12 μg/ml) prior to infection with PM2 (MOI of 0.2). After 15 min of incubation, the number of unadsorbed particles was determined. (B) Concentration-dependent KCN effect on PM2 adsorption. The assay was carried out like in A, except that KCN concentration was varied. The error bars in A and B represent standard errors.

proton motive force (PMF). The PMF consists of two components, the membrane voltage ($\Delta\Psi$) and the pH gradient (ΔpH; Mitchell et al., 1979). In actively respiring cells the ATP is mainly produced via oxidative phosphorylation and therefore dissipation of the PMF has an immediate effect on the intracellular ATP concentration.

In order to understand the nature of the connection between the presence of the PM2 receptor and the energetic state of the *Pseudoalteromonas* cells, we first used compounds that collapse the PMF (Fig. 1A). The treatment of ER72M2 cells with the proton ionophore dinitrophenol (DNP) rendered them incapable of binding PM2 particles. The same effect was observed when bacteria were pre-incubated with potassium cyanide, which blocks the respiration by inhibiting terminal oxidase and consequently dissipates the PMF. KCN inhibited PM2 adsorption in a concentration-dependent manner (Fig. 1B). About 95% of phage particles were bound to the cell surface 15 min post infection (p.i.) at normal infection conditions, whereas the presence of KCN reduced PM2 binding to the host cell by ~70% even at micromolar (200 μM) concentrations.

Since both agents affecting the PMF had a drastic effect on PM2 adsorption we decided to elucidate whether the presence of the receptor on the cell surface is preferentially dependent on a particular component (ΔpH versus $\Delta\psi$) of the PMF. For that, we used different agents specifically affecting either ΔpH or $\Delta\psi$. Treatment of ER72M2 cells with nigericin (NG), which dissipates ΔpH exchanging K^+ for H^+, had no apparent effect on PM2 adsorption (Fig. 1A). Then the effect of valinomycin (VAL) was tested. Valinomycin transports K^+ down its electrochemical gradient and dissipates the $\Delta\psi$ in the presence of high extracellular K^+ concentrations. Therefore, to test the effect of the $\Delta\psi$ on the course of PM2 adsorption, the K^+ concentration in the SB medium was increased (from 10 mM to 300 mM) prior to the addition of VAL. Surprisingly, VAL, similarly to NG, had no detectable effect on PM2 binding to the host cells.

As mentioned above, collapse of the PMF also affects the intracellular pool of ATP. To ascertain whether the presence of the receptor is dependent on the PMF or the pool of ATP, the effects of arsenate and azide were examined. Arsenate reduces the intracellular ATP pool via arsenolysis reaction, while azide decreases ATP concentrations due to the inhibition of the terminal oxidase and/or membrane H^+-ATPase. We have demonstrated previously that azide and arsenate decreased the intracellular ATP concentration of ER72M2 cells by ~55% and ~26%, respectively, but had no prominent effect on $\Delta\psi$ (Krupovič et al., 2007a). Adsorption assays revealed that treatment of ER72M2 cells with azide or arsenate decreased PM2 binding by 40% and 24%, respectively (Fig. 1A). It should be noted that previous studies also indicate that KCN not only dissipates the $\Delta\psi$, but also completely depletes the intracellular ATP (Krupovič et al., 2007a). Accordingly, the inability of KCN-treated ER72M2 cells to bind PM2 particles (Fig. 1A) is likely associated with the drop in ATP concentration rather than collapse of PMF. The presence of the receptor for PM2 on ER72M2 cells therefore seems to be dependent on the intracellular ATP concentration.

The course of PM2 infection depends on the concentration of divalent cations

The marine bacteriophage PM2 propagates in an environment containing high concentrations of magnesium (~50 mM) and calcium (~10 mM) ions. In the laboratory, PM2 is cultured in SB medium, which in ionic composition mimics the sea water. SB is a rich medium containing small amounts of divalent cations, negligible when compared to the added 10 mM Ca^{2+} and 50 mM Mg^{2+}. Therefore, when saying that the medium was devoid of divalent cations, we mean that calcium and magnesium ions were not added.

We checked the effect of Mg^{2+} and Ca^{2+} on the PM2 adsorption. Data presented in Fig. 2A illustrate the considerable difference in the kinetics

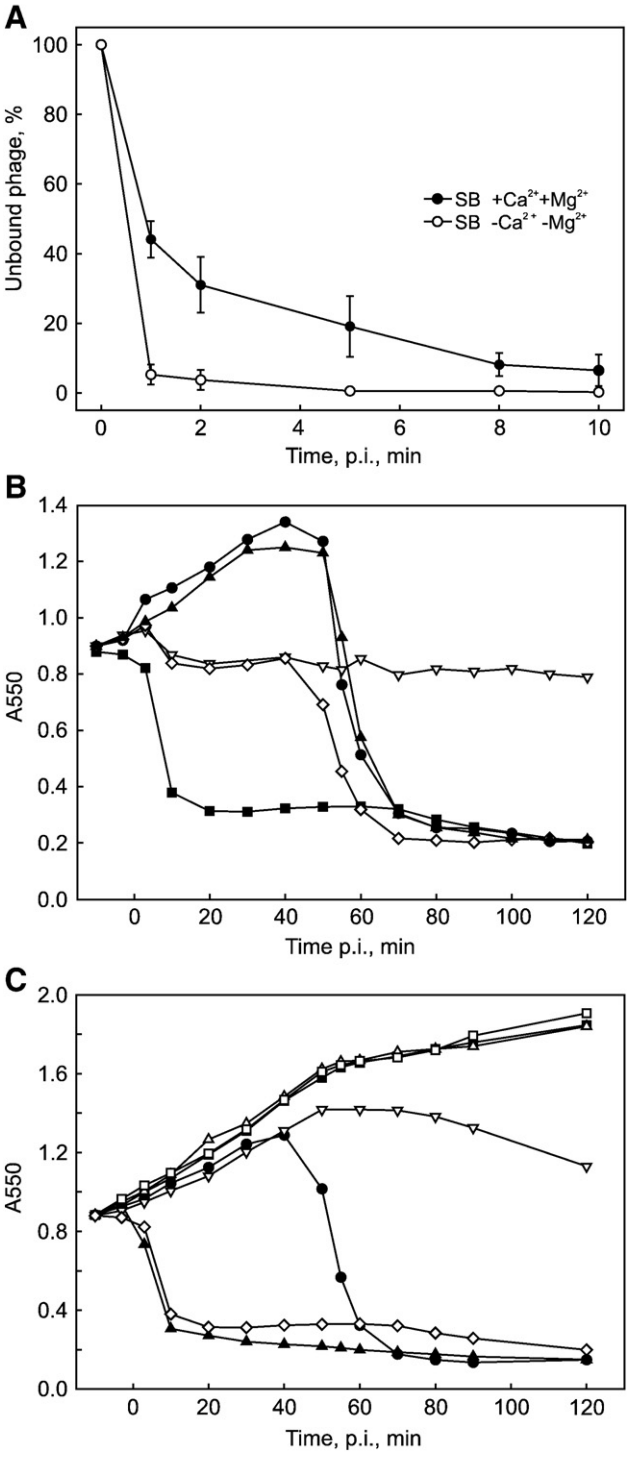

Fig. 2. Effect of divalent cations on PM2 infection. (A) Kinetics of PM2 adsorption to ER72M2 cells in SB with or without Ca^{2+} and Mg^{2+}. ER72M2 cells were infected with PM2 using an MOI of 0.2 in the presence (50 mM Mg^{2+}, 10 mM Ca^{2+}; filled circles) or absence (open circles) of divalent cations. At different time points post infection (p.i.) the number of unbound virus particles was determined. (B) Effect of different Ca^{2+} concentrations on PM2 infection in the absence of Mg^{2+}. ER72M2 cells were infected with PM2 using an MOI of 10 and culture turbidity was followed ($\lambda = 550$). Legend key: normal infection- filled circles; 0 mM Ca^{2+}—open triangles; 2.5 mM Ca^{2+}—filled squares; 5 mM Ca^{2+}—open diamonds; 10 mM Ca^{2+}—filled triangles. (C) ER72M2 cells were infected with *wt* or mutant PM2 using an MOI of 10. Culture turbidity was followed ($\lambda = 550$). Filled squares—uninfected ER72M2 cells; filled circles—PM2 infection in SB medium; filled triangles—*wt* PM2 infection in the presence of 2.5 mM Ca^{2+}; open triangles facing up—*sus2* infection in SB; open squares—*sus2* infection, 2.5 mM Ca^{2+}; open triangles facing down—M85 infection in SB; open diamonds—M85 infection, 2.5 mM Ca^{2+}. The number of *sus2* particles was estimated from the protein amount of the 1× purified virus preparation as compared to the *wt* PM2 (5×10^{12} PFU/mg of protein).

of PM2 adsorption in the presence and absence of these divalent cations. Surprisingly, PM2 adsorption to the host cells was significantly more rapid if the medium was not supplemented with calcium and magnesium ions, with nearly all (95%) added viruses adsorbed to the cells after 1 min p.i. The adsorption rate constants calculated at this time point were 1.46×10^{-9} ml/min and 1.74×10^{-10} ml/min in the absence and presence of divalent cations, respectively. It has been shown previously that calcium ions are needed for PM2 virion stability (Kivelä et al., 1999). The stability of PM2 virions during the period of adsorption assay was therefore monitored and found to be unaffected. We next tested the effect of different Ca^{2+} and Mg^{2+} concentrations on PM2 infection. When Mg^{2+} ions (50 mM) were not included in the infection medium, no effect on PM2 propagation was observed as can be judged from unaltered growth curve and normal phage progeny yield (Fig. 2B, Table 1). However, variation in calcium ion concentration in the absence of Mg^{2+} had a remarkable effect on the course of PM2 infection. By testing a range of Ca^{2+} concentrations, we determined three threshold conditions with most prominent consequences in terms of the growth of PM2-infected ER72M2 cells (Fig. 2B). In the absence of Ca^{2+} ions the adsorption of PM2 was not compromised (Fig. 2A), but further steps of infection cycle were affected as the infected cells neither grew nor lysed throughout the course of experiment (Fig. 2B). The cells infected in the medium devoid of both divalent cations were very unstable and sensitive to mechanical stress (e.g., stirring; see below). If the medium contained 2.5 mM of Ca^{2+} instantaneous and complete lysis of ER72M2 cells was observed shortly after the phage addition. When the medium contained 5 mM Ca^{2+} PM2 infected cells did not grow but lysed at the same time as those infected in normal SB medium and the number of released viruses was of the same order of magnitude as in the presence of both divalent cations (Table 1).

To ascertain that the lysis of the ER72M2 cells early in the infection is a PM2 entry-specific event, we used PM2 mutants defective in either entry or lysis steps. PM2 nonsense mutant *sus2* is deficient in structural protein P10, which is dispensable for virion assembly, but is essential for the entry step following adsorption, i.e. fusion of the viral membrane with the OM of the host (Kivelä et al., 2008). When ER72M2 cells were infected with *sus2* mutant in normal SB medium cells continued to grow with no signs of decrease in culture turbidity throughout the experiment (Fig. 2C). Differently from the infection with the *wt* PM2, when SB medium contained 2.5 mM calcium but no magnesium ions, *sus2*-infected cells did not lyse at the beginning of infection. This indicates that binding of the viral particle to the host cell surface *per se* was not the cause of cell lysis under such growth conditions. Mutant M85 has a LacZ′-Mu(*Not*I) transposon inserted in the PM2 gene coding for protein P18, which is involved in the lysis of PM2-infected cells at the end of the phage reproduction cycle (Krupovič et al., 2007a). Infection of ER72M2 cells with M85 mutant in SB containing 2.5 mM Ca^{2+} and no Mg^{2+} resulted in immediate cell lysis, although in normal SB medium cells infected with this mutant did not lyse at the time of normal lysis (Fig. 2C). This indicates that virus entry-related lysis in the presence of low Ca^{2+} concentrations and the progeny release-associated disruption of the host envelope at the end of the replication cycle are two distinct phenomena mediated by different factors.

Table 1
PM2 progeny production in the SB medium containing different concentrations of divalent cations.

Concentrations of divalent cations in SB medium	PM2 titer in the supernatant[a], PFU/ml
10 mM Ca^{2+}, 50 mM Mg^{2+}	1.7×10^{11}
0 mM Ca^{2+}, 0 mM Mg^{2+}	4.0×10^{6}
2.5 mM Ca^{2+}, 0 mM Mg^{2+}	3.1×10^{10}
5 mM Ca^{2+}, 0 mM Mg^{2+}	1.4×10^{11}
10 mM Ca^{2+}, 0 mM Mg^{2+}	1.3×10^{11}
0 mM Ca^{2+}, 2.5 mM Mg^{2+}	1.7×10^{7}
2.5 mM Ca^{2+}, 50 mM Mg^{2+}	1.6×10^{11}
2.5 mM Ca^{2+}, 50 mM Ba^{2+}	1.2×10^{11}
0 mM Ca^{2+}, 50 mM Mg^{2+}	1.7×10^{9}

[a] For experimental details see Materials and methods.

Calcium ions are necessary during the early steps of PM2 infection

As mentioned above, PM2-infected cells lysed rapidly when the medium contained 2.5 mM Ca^{2+} (Fig. 2A). We therefore checked if this effect is calcium ion-specific, or whether it could also be obtained with other divalent cations. Therefore, calcium ions were substituted with magnesium ions. Interestingly, if the medium contained 2.5 mM Mg^{2+} infected cell behaved similarly to the ones in SB medium without divalent cations, neither grew nor lysed (Fig. 3A). The titer of

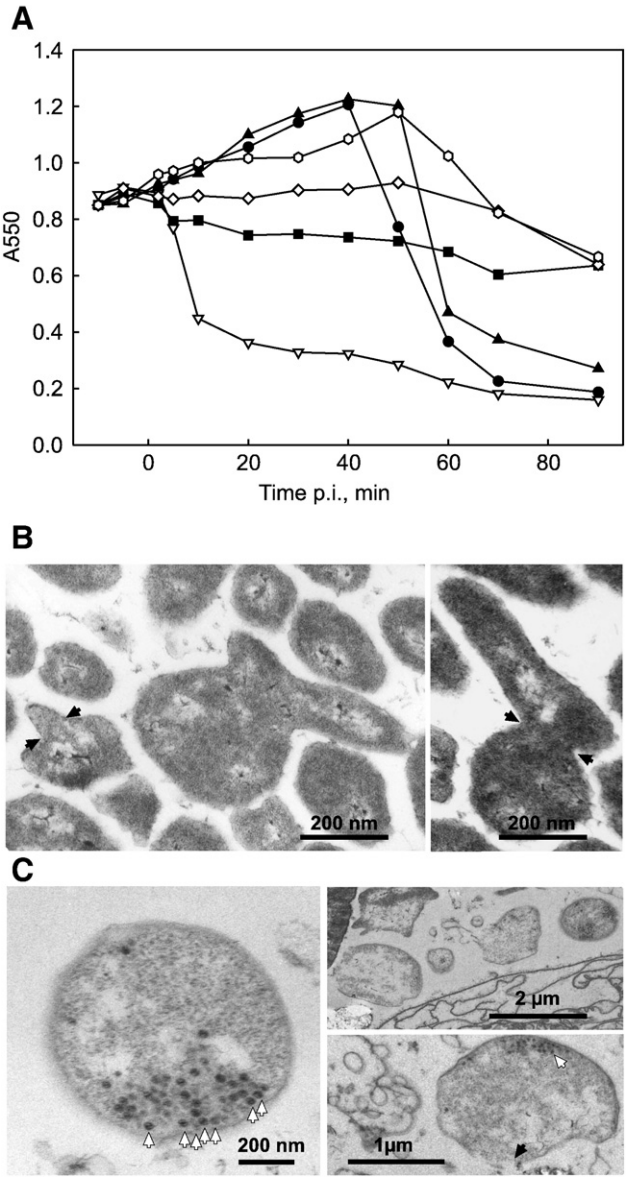

Fig. 3. Effect of divalent cations on the stability of the OM of PM2-infected cells. (A) PM2 infection in SB containing different concentrations of divalent cations. Filled circle—PM2 infection in SB; open triangles—2.5 mM Ca^{2+}, 0 mM Mg^{2+}; filled squares—0 mM Ca^{2+}, 2.5 mM Mg^{2+}; open diamonds—0 mM Ca^{2+}, 50 mM Mg^{2+}; filled triangles—2.5 mM Ca^{2+}, 50 mM Mg^{2+}; open circles—2.5 mM Ca^{2+}, 50 mM Ba^{2+}. (B–C) Thin section electron micrographs of PM2-infected ER72M2 cells. (B) Early lysis of PM2-infected cells in the presence of 2.5 mM Ca^{2+} and 0 mM Mg^{2+}, 3 min p.i. Black arrows point to the bases of the cytoplasmic membrane bulges externalized due to high intracellular osmotic pressure. (C) Lysis of PM2 infected cells in the presence of 2.5 mM Ca^{2+}, 50 mM Ba^{2+}. The two panels on the right depict PM2-infected cells undergoing lysis at the end of infection cycle (cells were fixed 80 min p.i.). White arrows point to intracellular PM2 particles, while black arrow points to the opening in the envelope.

the infected culture at 90 min p.i. did not indicate any virus production (Table 1).

Anionic groups in LPS bind divalent cations with high affinity. The LPS layer of the OM can be disorganized and destabilized by removing divalent cations with chelating agents (Nikaido and Vaara, 1985). We therefore checked if early lysis of the infected cells is a result of insufficient stabilization of the cell envelope due to the lack of divalent cations. ER72M2 cells were infected with PM2 in the medium containing 50 mM Mg^{2+}. Again, similarly to infection in the presence of 2.5 mM Mg^{2+}, cells did not grow, and did not lyse at the time of normal lysis. Phage titers at the end of the experiment indicate that no progeny viruses were released (Table 1). Then we examined whether PM2 infection could be rescued if 2.5 mM Ca^{2+} is included in the medium containing 50 mM Mg^{2+}. Indeed, no lysis at the beginning of infection was observed, and at ~50 min p.i. cells started to lyse, resembling the characteristic PM2 growth curve in normal SB medium. The yield of the progeny viruses was of the same order of magnitude as during infection in normal SB medium (Table 1). Notably, magnesium ions could be substituted with other divalent cations, such as Ba^{2+}. If the SB medium contained 2.5 mM of calcium and 50 mM barium ions, no early lysis was observed and PM2 infected cells started to lyse at the time of normal lysis. However, at these conditions the turbidity of the infected ER72M2 cell culture remained high whereas the culture turbidity in normal SB medium decreased normally (Fig. 3A). EM pictures of the cells fixed 80 min p.i. illustrate that ER72M2 cells contain progeny PM2 particles or are lysed (Fig. 3C). Moreover, the titer of the sample at 90 min p.i. was the same as determined for infection in normal SB medium (Table 1). Most probably the decrease in the turbidity of PM2-infected ER72M2 cell was masked by aggregates visible in EM micrographs (not shown).

In order to determine the physiological response of ER72M2 cells to PM2 infection and to verify that the observed effects described above were PM2 entry-specific, we employed the recently developed potentiometric assay that allows following the entire PM2 infection cycle in real time (Krupovič et al., 2007a). We monitored the turbidity of PM2-infected cell culture as well as the concentrations of K^+ and dissolved oxygen in SB medium containing different concentrations of calcium ions. The cytoplasmic membrane of bacteria is impermeable to inorganic ions, such as K^+ or H^+ (Mitchell et al., 1979). Therefore, efflux of intracellular potassium ions indicates the increase in the CM permeability (Daugelavičius et al., 1997). The major oxygen consumption is due to respiration, which takes place at the bacterial CM (Babcock, 1999). Accordingly, it has been observed that even slight perturbations in the CM change the course of O_2 consumption (Votyakova et al., 1994). It has been demonstrated that at the end of the infection cycle, upon cell lysis mediated by different bacteriophages, cellular respiration ceases leading to a rapid increase in the concentration of dissolved oxygen in the medium (Daugelavičius et al., 2007; Krupovič et al., 2008).

The course of PM2 infection in SB containing 10 mM calcium but no magnesium ions did not differ from the one in normal SB medium. After phage addition to the cells no phage-induced changes in intracellular K^+ concentration were observed during the first 2 min of the infection (Fig. 4A). Note that a slight increase in extracellular K^+ concentration immediately after phage addition does not signify the increased permeability of CM, but results from the addition of potassium ions present in the PM2 agar-stock, as no such effect was observed during the measurements in a buffer with highly purified virus preparations (Kivelä et al., 2004). However, approximately 2 min p.i. partial leakage of intracellular K^+ was observed (Fig. 4A), most probably signifying the transport of the phage genome across the CM. Importantly, the genome-entry-related K^+ leakage ceased after 2 to 3 min (~5 min p.i.) with concomitant re-accumulation of K^+ (Fig. 4A). This indicates that the permeabilization of the CM was transient and its integrity was re-established. Changes in the physiology of ER72M2 cells associated with the release of PM2 progeny mediated by phage-encoded proteins P17 and P18 were described in detail previously (Krupovič et al., 2007a). Progeny

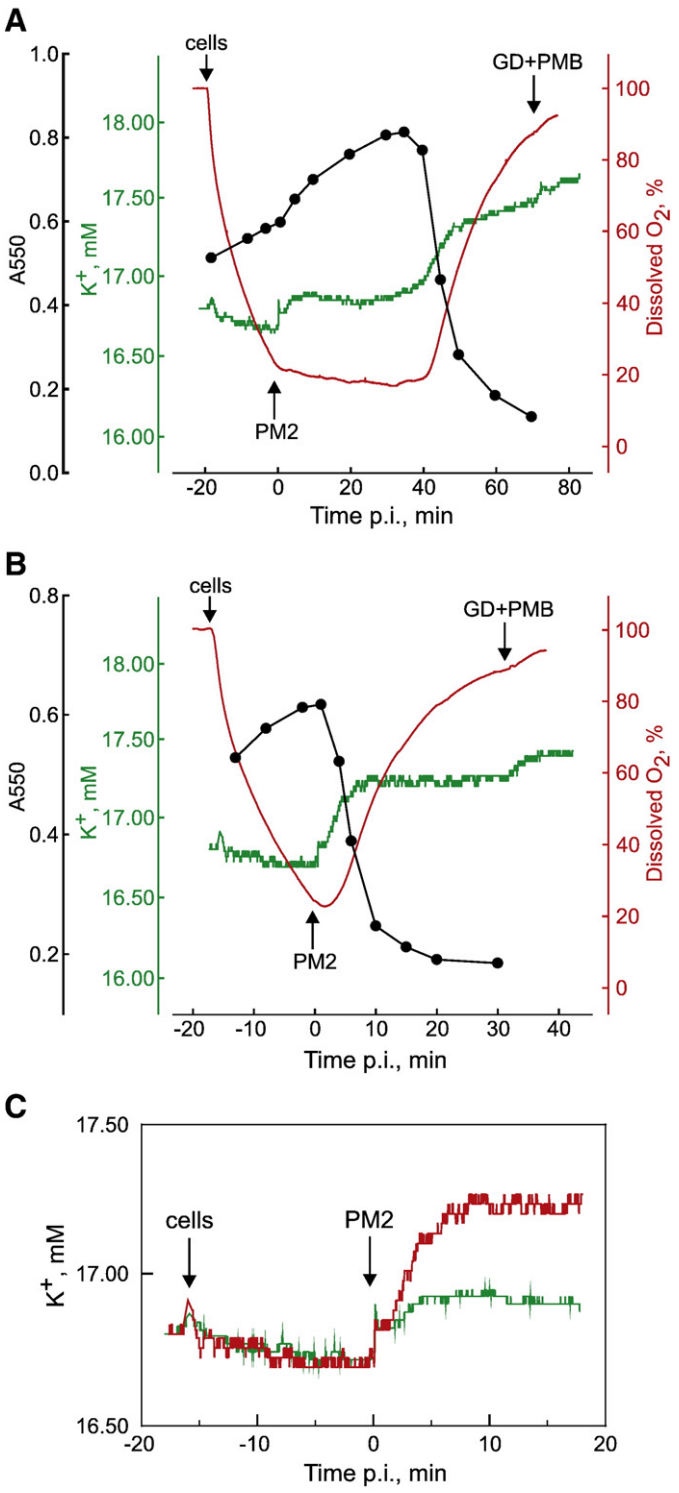

Fig. 4. Electrochemical measurements of PM2-induced changes in the physiology of ER72M2 cells in SB media containing 10 mM Ca^{2+} and 0 mM Mg^{2+} (A), and 2.5 mM Ca^{2+} and 0 mM Mg^{2+} (B). Fluxes of K^+ (green), and O_2 (red) were measured as described in Materials and Methods. A_{550} (black) was measured by taking samples from the electrochemical measurement vessels. PMB and GD were added at the time point indicated by the arrow to a final concentration of 100 μg/ml and 4 μg/ml respectively. (C) Close-up view of K^+ curves from panels A (green) and B (red). To evaluate the remaining intracellular K^+ concentrations and extent of respiration at the end of experiment the cells were perforated by adding polymyxin B (PMB) and gramicidin D (GD).

release-associated K^+ leakage started approximately 30 min p.i. and was followed (35–40 min p.i.) by an abrupt decrease in culture turbidity and complete cease in respiration (i.e., increase in dissolved O_2 concentration; Fig. 4A).

The physiological response of ER72M2 cells to PM2 infection during the first 3 min after phage addition in SB containing 2.5 mM Ca^{2+} (Fig. 4B) was very similar to that in SB with 10 mM Ca^{2+} (Fig. 4A). However, as the infection proceeded further, drastic differences were observed (Fig. 4A, B). Instead of transient and reversible genome-delivery-associated K^+ efflux (Fig. 4A), abrupt and complete leakage of intracellular K^+ was recorded in the presence of 2.5 mM Ca^{2+} (Fig. 4C). Nevertheless, the temporary (10 mM Ca^{2+}) and the complete (2.5 mM Ca^{2+}) leakage of K^+ started approximately at the same time after phage addition (Fig. 4C), suggesting that the permeabilization of the CM in both cases was evoked by the same entry-related process. The leakage of K^+ in the presence of 2.5 mM Ca^{2+} was accompanied by a rapid decrease in culture turbidity and cessation of respiration (Fig. 4B), signifying the disruption of the infected cells. It is notable that early PM2 entry-related lysis events observed in the presence of low Ca^{2+} conditions (Fig. 4B) are reminiscent to those caused by the cationic antibiotic PMB, which forms large openings in the CM of ER72M2 cells (Krupovič et al., 2007b).

As mentioned above, PM2-infected ER72M2 cells in SB lacking divalent cations are very unstable and sensitive to stress. Electrochemical measurements of PM2 infection in the medium devoid of divalent cations always resulted in a gradual (but not abrupt) lysis of the infected cells (data not shown). Therefore, it was not possible to employ this technique to evaluate ER72M2 cell respiration and monitor the K^+ fluxes across the CM.

The arrest of PM2 infection can be relieved by addition of calcium ions

PM2 infection in the absence of divalent cations is arrested at an early stage of entry occurring after the fusion of the viral membrane with the OM of the host, but preceding the genome penetration through the CM (Kivelä et al., 2004). It was therefore interesting to investigate if the entry arrest can be removed by externally providing Ca^{2+} ions to the infected cell culture. For that, ER72M2 cells were infected in the medium devoid of divalent cations and 10 mM Ca^{2+} was added 5, 10, 20 and 40 min p.i. (Fig. 5). Surprisingly, this Ca^{2+} supplement was sufficient to remove the entry arrest leading to the productive infection and release of progeny virions (Fig. 5, Table 1). Notably, the time from Ca^{2+} addition to the onset of cell lysis was roughly the same as the duration of normal infection cycle (~50 min). In each case the replication of PM2 was not affected and the number of viruses released from infected cells upon lysis was of the same order of magnitude as during infection in normal SB (data not shown).

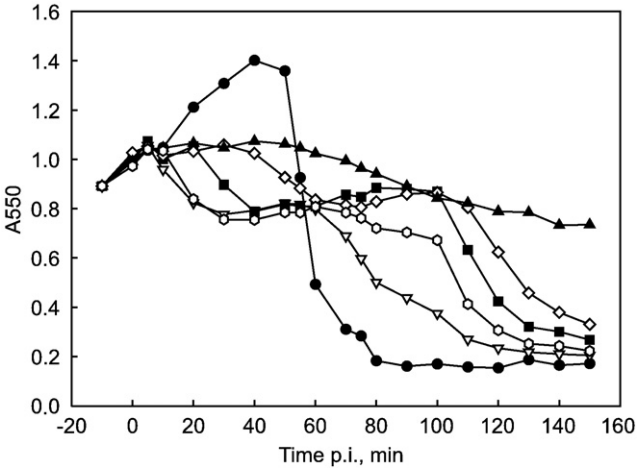

Fig. 5. Release of PM2 infection arrest by addition of calcium ions. ER72M2 cells were infected with PM2 using an MOI of 10 in SB medium without divalent cations. At different time points p.i., 10 mM calcium was added to infected cells. Filled circles- PM2 infection in SB; filled triangles—PM2 infection in SB without divalent cations; open triangles—Ca^{2+} added 5 min p.i.; open circles—Ca^{2+} added 10 min p.i.; filled squares—Ca^{2+} added 20 min p.i.; open diamonds—Ca^{2+} added 40 min p.i.

Discussion

The entry process of viruses starts with the recognition of a susceptible host through binding to a specific receptor. Using various metabolic poisons (Nicholls and Ferguson, 2002) we determined that PM2 adsorption is dependent on the intracellular ATP (Fig. 1A). It appears that in the course of evolution bacteriophage PM2 has developed a strategy to probe the wellbeing of its host, in order not to infect bacteria that would not support the progeny production. A similar strategy is employed by tectivirus PRD1 infecting a range of gram-negative bacteria. PRD1 binds to a transient transenvelope protein complex in an ATP-dependent manner (Daugelavičius et al., 1997). Other bacteriophages, such as cystoviruses ϕ6 and ϕ13 (Daugelavičius et al., 2005) or tailed viruses T1 and ϕ80 (Hancock and Braun, 1976) utilize receptors that are dependent on the energetic state of the CM rather than intracellular ATP pool. However, not all viruses seem to be equally fastidious. Another member of the *Tectiviridae*, bacteriophage Bam35, infecting *Bacillus thuringiensis*, not only adsorbs to the purified peptidoglycan of its host, but can be inactivated by *N-acetyl*-muramic acid, a building block of the peptidoglycan (Gaidelytė et al., 2006). Similarly, irreversible binding of tailed dsDNA bacteriophages T4 and T5 is also independent of the energetic state of their hosts (Filali Maltouf and Labedan, 1983; Labedan and Goldberg, 1979).

The PM2 receptor-binding protein P1 is structurally similar to sugar-binding proteins (Abrescia et al., 2008). Accordingly, we propose that the sugar moiety of the LPS might operate as a co-receptor and assist the irreversible binding of PM2 to its primary receptor. As cellular ATP is produced and maintained in the cytosol and not at the OM, where adsorption takes place, we suggest that the PM2 receptor is a protein or a protein complex spanning the entire cell envelope. The presence of the active receptor is dependent on the intracellular ATP concentration and when the ATP pool is depleted the PM2 receptor either disassembles or changes its conformation, rendering it incapable of binding the phage.

During the first 2 min of infection no phage-induced effects on the CM were observed (Fig. 4C), indicating that this period of time is required for irreversible adsorption to the receptor (Fig. 2A), dissociation of the capsid and fusion of the phage membrane with the OM as proposed previously (Kivelä et al., 2004). It was determined that shortly after PM2 addition, the OM of *Pseudoalteromonas* cells becomes permeable to GD (lasting for ~2 min.). However, when the cells were infected in the absence of Ca^{2+} sensitivity of the infected cells to GD was observed even several minutes after PM2 addition (Kivelä et al., 2004). Here we extended these experiments and found that in the absence of Ca^{2+} the so called GD "window" remains open even 20 min after infection (data not shown). It is possible that in the absence of calcium ions, after the fusion of PM2 membrane with the OM has taken place, the dsDNA genome holds the phage membrane "patch" in the OM through interactions with viral integral DNA-binding membrane proteins. This would correspond to the arrested state of PM2 infection, when infected cells neither grow nor lyse (Fig. 2B). Interestingly, tailed dsDNA bacteriophage 4Ic infecting *Bacillus subtilis* failed to complete the penetration if calcium ions were omitted from the growth medium. However, in contrast to PM2 (Fig. 5), addition of Ca^{2+} to the arrested infection did not rescue the phage 4Ic entry (Landry and Zsigray, 1980).

We propose that during PM2 infection in the medium supplemented with calcium ions a well-controlled pore in the CM opens. Most probably

the pore is comprised of host proteins, since all structural PM2 proteins either dissociate and remain in the medium upon phage adsorption (P1 and P2), or stay associated with the OM after the membrane fusion (P3-P10; Kivelä et al., 2004). The inner diameter of the pore is likely to be wide in order to allow the penetration of the supercoiled dsDNA genome. However, the possibility that the genome is relaxed by periplasmic nucleases cannot be excluded. Indeed, medium- and large-diameter pores have been observed in bacteria. Upon transformation of diverse gram-negative and gram-positive cells a pore of 6.5 nm in diameter is being opened to allow transport of dsDNA or DNA-protein complexes (Chen et al., 2005). Moreover, bacterial toxins are known to form transmembrane channels as wide as 26 nm in diameter (Tilley et al., 2005). We propose that PM2, in the presence of calcium ions, induces the opening of the pore large enough to release the cellular lytic factor (CLF), which locally degrades the peptidoglycan layer. Through the hole in the peptidoglycan layer the dsDNA genome gains access to the pore and penetrates into the cytosol. It appears that only the genome enters the cytoplasm, as electroporation of naked circular PM2 genome into the host led to normal virus reproduction without immediate requirement for virion associated components (Krupovič et al., 2006). Although the nature of the pore in the CM of *Pseudoalteromonas* cells remains enigmatic, it is tempting to speculate that the primary function of this channel is the uptake of DNA from the environment, i.e., natural transformation. In such case, viral DNA itself would be a signal for channel opening.

Divalent cations are known to play an important role during different stages of bacteriophage infection. In some cases divalent cations are required for phage adsorption (Landry and Zsigray, 1980), in others for viral genome penetration (Karnik and Gopinathan, 1980; Steensma and Blok, 1979; Watanabe and Takesue, 1972) or another intracellular step during virion development (Nagaraja and Gopinathan, 1980; Tucker, 1963). The ionic requirements might be non-specific, with different divalent ions acting interchangeably, or specific, with only certain ions facilitating the entry or reproduction of the phage. We observed here that the course of PM2 infection is dependent on the concentration of divalent cations in the medium (Fig. 2B). We show that if the OM is not stabilized by divalent cations, PM2 entry is lethal to the host cell (Figs. 2B and 4B). We believe that early lysis of PM2-infected cells is not caused by the genome entry itself, but rather is a consequence of the inability to re-establish the integrity of the cell envelope (Fig. 3B).

Here we suggest that phage PM2 uses a unique entry mechanism, different from those described for other dsDNA phages (for a review see Poranen et al., 2002). Experimental data described in this study in combination with results obtained previously allowed us to propose a sequential model describing the entry of bacteriophage PM2 into the *Pseudoalteromonas* host cells: (i) virus adsorbs to the receptor, which is in a competent conformation (or assembly state) that is dependent on the intracellular ATP concentration (Fig. 6, step 1). (ii) The interaction with the receptor triggers dissociation of the PM2 protein shell, composed of proteins P1 and P2 (Kivelä et al., 2004), and leads to the protein P10-mediated fusion of the viral membrane with the host OM (Kivelä et al., 2008; Fig. 6, step 2). (iii) The phage genome enters the periplasm and faces the peptidoglycan layer (Fig. 6, step 3). In the absence of Ca^{2+} the entry process is arrested with PM2-derived phospholipid membrane "patch" stably maintained in the OM. (iv) A virus-induced opening of a pore in the CM releases (in a controlled manner) the intracellular CLF into the periplasm, where it locally digests the peptidoglycan layer (Fig. 6, steps 4–5). This step is calcium ion-dependent and at least 2.5 mM Ca^{2+} is needed for this step to occur. (v) The same pore is used for the PM2 genome penetration into

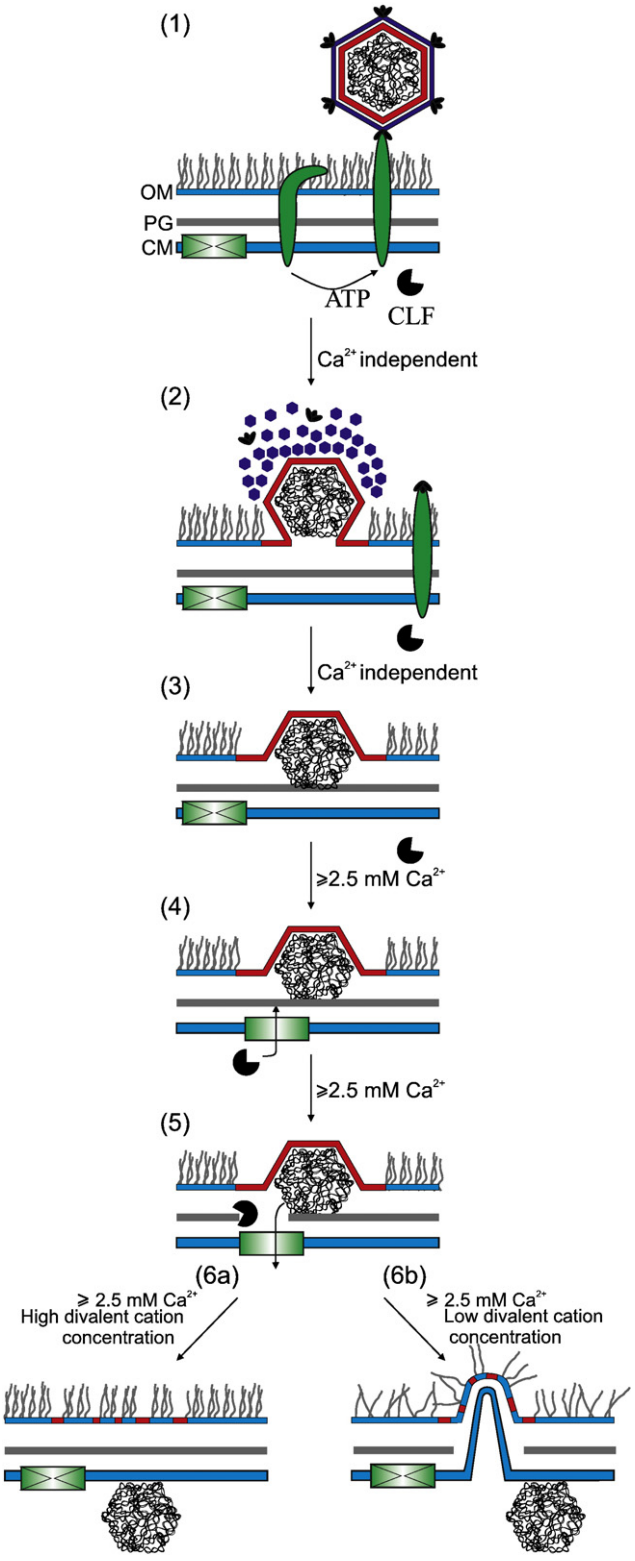

Fig. 6. Model for the PM2 entry into the host cell. (1) Adsorption to the receptor. OM-outer membrane; PG- peptidoglycan layer; CM-cytoplasmic membrane; CLF-cellular lytic factor. Green box depicts the pore in the CM. (2) Receptor binding-induced dissociation of the protein capsid and subsequent protein P10 mediated fusion of the viral membrane with the host OM. (3) In the absence of Ca^{2+} the infection is arrested at this stage with stable maintenance of the phospholipid "patch" derived from the viral membrane (4) opening of the pore in the CM associated with the efflux of intracellular K^{+}, release of CLF. (5) Peptidoglycan digestion by CLF and PM2 genome penetration through the CM. (a) Closing of the pore in the CM, peptidoglycan layer reparation and genome replication in the presence of Ca^{2+} and other divalent cations at concentrations sufficient to stabilize the OM. (b) Osmotic lysis of infected cell at conditions where OM is not stabilized by divalent cations. Requirement for divalent cations for each PM2 entry step is indicated next to the arrow.

the cytoplasm (Fig. 6, step 5). (vi) After the genome penetration the pore closes, the peptidoglycan is repaired and virus replication takes place (step 6a). However, this takes place only if the OM is stabilized by divalent cations. In the absence of divalent cations the genome enters the cytosol, the pore closes, but the lesion in the peptidoglycan layer and unstructured LPS lead to osmotic lysis of the infected cell (step 6b).

Materials and methods

Bacteria and phages

Wild type (*wt*) bacteriophage PM2 (Espejo and Canelo, 1968b) and PM2 mutant P85, that has LacZ′-Mu(NotI) transposon insertion in gene *XVIII* (Krupovič et al., 2006), were propagated on *Pseudoalteromonas* sp. ER72M2 cells in SB broth at 28 °C as described previously (Kivelä et al., 1999). PM2 nonsense mutant *sus2* with an amber mutation in the gene *X* was grown on a non-suppressor host and purified exactly as described by Kivelä et al. (2008).

Phage adsorption

For the phage adsorption tests ER72M2 cell were grown in SB medium to a density of 5.8×10^8 CFU/ml, then collected by low speed centrifugation (Sorvall SLA3000 rotor, 7000 rpm, 30 min, 4 °C). The pellets were re-suspended in an ice cold SB medium (for adsorption in the absence of divalent cations SB was not supplemented with Ca^{2+} and Mg^{2+}) and the cell suspension was kept on ice until used (maximum for 6 h). For the adsorption tests, concentrated cell suspension was added to 10 ml of SB broth (with or without Ca^{2+} and Mg^{2+}) to a cell density of 5.8×10^8 CFU/ml, incubated for 5 min at 28 °C with aeration, and then infected with fresh PM2 stock using a multiplicity of infection (MOI) of 0.2. After different incubation times at 28 °C with aeration, the adsorption was stopped by immediate centrifugation (Heraeus Biofuge; 13000 rpm, 5 min, at room temperature; RT), and the number of PFUs in the supernatant was determined by plating on a lawn of ER72M2 cells.

When energy poisons were used, cells were grown and prepared as described above. After nigericin (NG; final concentration 24 μg/ml), potassium cyanide (KCN; 20 mM), sodium arsenate (20 mM), sodium azide (NaN_3; 20 mM), dinitrophenol (DNP; 1 mM), or valinomycin (VAL; 12 μg/ml) were added, cells were incubated for another 5 min at 28 °C with aeration and then infected with fresh PM2 stock using an MOI of 0.2. After 15 min of incubation the number of unbound PM2 particles was determined as described above.

Phage stability was monitored by incubation of phage particles in different media for 30 min and titers were then determined by plating on the lawn of ER72M2 cells. Adsorption rate constants were calculated as described by Adams (1959).

OD measurements

ER72M2 cells were grown with aeration at 28 °C to a cell density of 5.6×10^8 CFU/ml collected as described above, re-suspended in SB lacking Ca^{2+} and Mg^{2+} and infected with *wt* or mutant PM2 using an MOI of 10. The number of *sus2* particles was estimated from the protein amount of the 1× purified virus preparation as compared to the *wt* PM2 (5×10^{12} PFU/mg of protein). The concentrations of Ca^{2+} and Mg^{2+} were adjusted by adding $CaCl_2$ and $MgSO_4$. When appropriate, $BaCl_2$ was added to the final concentration of 50 mM. Absorbance ($\lambda=550$ nm) was measured at different time points using Selecta Clormic digital colorimeter (J. P. Selecta). The experiments were done in triplicate with the *wt* PM2 infection as a control. To monitor virus production at the end of the experiment cell debris was removed by centrifugation (Heraeus Biofuge; 13,000 rpm for 5 min, RT) and the number of infectious particles in the supernatants was determined by plating on the ER72M2 cell lawn. The titers are presented in Table 1.

Measurements of ion fluxes

Ion flux measurements were performed as described previously (Krupovič et al., 2007a) with minor modifications. Briefly, *Pseudoalteromonas* sp. ER72M2 cells were grown to a density of 5.6×10^8 CFU/ml, collected by centrifugation (Sorvall SLA3000 rotor, 7000 r.p.m., 30 min, 4 °C), re-suspended in the ice-cold SB medium without Ca^{2+} and Mg^{2+} to obtain 1/100 of the original volume. The cells were kept on ice until used (maximum for 6 h). The concentrated cell suspension was added to 30 ml of SB broth (containing appropriate concentrations of divalent cations) in a thermostated (28 °C) vessel to obtain a density of 5.6×10^8 CFU/ml (when K^+ fluxes were monitored) or $\sim2.8\times10^8$ CFU/ml (when dissolved oxygen was measured), incubated for 10 min with intensive stirring, and then infected with freshly made PM2 virus stock using an MOI of ~10. The concentration of K^+ ions in the reaction medium was monitored by a K^+-selective electrode. The electrode was calibrated at the very beginning of each experiment. The K^+-selective electrode (Orion model 9319) and the dissolved oxygen electrode (Orion model 970800) were from Thermo Inc. When the dissolved oxygen concentration was measured, at the end of each experiment, solid $Na_2S_2O_5$ was added. Oxygen concentration in the medium after $Na_2S_2O_5$ addition was referred to as 0%, while concentration before the cell addition was set to 100%. For the turbidity ($\lambda=550$ nm) measurements (J. P. Selecta), 70 μl samples from the measuring vessel were taken at different time points throughout the experiment. To evaluate the remaining intracellular K^+ concentrations and extent of respiration at the end of experiment the cells were perforated with additions of polymyxin B (PMB) and gramicidin D (GD).

To investigate the PM2-induced increase in permeability of the OM in the absence of Ca^{2+}, the experiments were carried out in 5-ml thermostated (28 °C) vessel with aeration as described previously (Kivelä et al., 2004). The TPP^+ and K^+-selective electrodes and dissolved oxygen electrode were connected to the electrode potential-amplifying system, based on an ultralow input bias current operational amplifier AD549JH (Analog Devices, USA). The amplifying system was connected to a computer through the data acquisition board AD302 (Data Translation, Inc., Malboro, USA). The Ag/AgCl reference electrodes (Orion Research Inc.; model 9001) were indirectly connected to the measuring vessels through an agar salt bridge. PMB and GD were purchased from Sigma.

Transmission electron microscopy

For thin-section electron microscopy, ER72M2 cells were infected with fresh PM2 stock using an MOI of 10. The samples were taken at different time points after infection. The infection mixtures (4 ml) were fixed with 3% (v/v) glutaraldehyde (30 min, RT), collected by centrifugation (Sorvall SS34 rotor, 6000 rpm, 15 min, 4 °C), washed with 2 ml of 20 mM potassium phosphate buffer (pH 7.4), and pelleted (Heraeus Biofuge; 13,000 rpm for 5 min, RT). The samples for transmission electron microscopy were prepared as described previously (Bamford and Mindich, 1980). The micrographs were taken with a JEOL 1200EX II electron microscope operating at 60 kV at EM unit, Institute of Biotechnology, University of Helsinki.

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