Xenorhabdus nematophila (Enterobacteriacea) Secretes a Cation-selective Calcium-independent Porin which Causes Vacuolation of the Rough Endoplasmic Reticulum and Cell lysis

Carlos RIBEIRO ε£, Michel VIGNES #$ and Michel BREHELINψ.

From the Departments of "Ecologie Microbienne des Insectes et Interactions Insecte-Pathogène" (EMIP) UMR 1133, INRA-UMII, Pl. E. Bataillon, 34095 Montpellier, France and "Plasticité et Synapse Glutamatergique", UMR 5102, CNRS-UMII, Pl. E. Bataillon, 34095 Montpellier, France

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ABSTRACT

Xenorhabdus nematophila and Photobacterium luminescens are two related enterobacteriaceae, studied for their use in biological control and for the synthesis of original virulence factors and of new kind of antibiotics. X. nematophila broth-growth exhibits different cytotoxic activities on insect (Spodoptera littoralis, lepidoptera) immunocytes (=hemocytes). Here we report the purification of the flhDC dependent cytotoxin, a 10,790 Da peptide we have called α-Xenorhabdolysin (αX). We show that plasma membrane of insect hemocytes and of mammal red blood cells, is the first target of this toxin. Electrophysiological and pharmacological approaches indicate that the initial effect of αX on macrophage plasma membrane is an increase of monovalent cation permeability, sensitive to potassium channel blockers. As a consequence several events can occur intracellularly such as selective vacuolation of the endoplasmic reticulum, cell swelling and cell death by colloid-osmotic lysis. These effects, inhibited by potassium channel blockers, are totally independent of Ca²⁺. However, the size of the pores created by αX on macrophage or red blood cells plasma membrane, increases with toxin concentration which leads to a rapid cell lysis.

INTRODUCTION

Enterobacteriaceae of the genus Xenorhabdus and Photobacterium are potent pathogens of various insect species (1), some strains being toxic for immunocompromised humans (2,3). The basis of this infectivity are still poorly understood whereas Photobacterium luminescens
was shown to have an oral insecticidal activity (4) due to entomotoxic proteins (5,6). Other insecticidal toxins, active after injection, are also produced by *P. luminescens* (7). Genes coding for similar entomotoxins were cloned in *Xenorhabdus nematophila* (8) and various cytotoxic factors were identified in bacterial broth-growth of this species (9, 10). Some of these factors are cytotoxic *in vitro* for insect immunocompetent cells and have also hemolytic activity on mammal red blood cells. Interestingly all these cytotoxic and hemolytic activities are absent in *P. luminescens* broth-growth (10), suggesting differences in the mode of virulence of these two related entomopathogenic bacteria species. We know that these toxins have very little homology with known sequences and represent new class of toxins (8). The first aim of this work was to study the mode of action of one of these new toxins on their cellular targets. In *X. nematophila* the existence of toxins active both on red blood cells and on hemocytes, allows us to study the mode of action of these molecules on mammal cells and on insect cells. Two different hemolytic activities were identified which appear sequentially in course of bacterial growth (10). The earliest hemolytic activity (activity C1) appears in broth-growth when bacteria culture reaches the stationary phase. It lyses sheep red blood cells (SRBC) but is inactive on rabbit red blood cells (RRBC) and is heat sensitive (30 min. at 60°C). It is under the control of *flhDC*, the flagellar master operon of *X. nematophila* (11). The second hemolytic activity (activity C2) appears late in the stationary phase, is heat resistant (one hour at 100°C), active on RRBC, inactive on SRBC and is not under the control of *flhDC*. Insertional inactivation of *flhDC* gene in *X. nematophila* leads both to loss of C1 activity (C2 being maintained) and to a very attenuated virulence phenotype.

As *X. nematophila* septicemia arises in the insect body, it is obvious that this bacteria is able to escape defence reactions and especially phagocytosis. The means by which entomopathogenic bacteria escape the defence reactions of insects is totally unknown. Hemocytes, the free cells in hemolymph, are the major immunocompetent cells in insects. Phagocytosis is mainly achieved by macrophage-like cells which belong to the morphotype "granular hemocyte 1" (GH1) (12). As GH1 are one of the targets of the cytotoxic activities evidenced in *X. nematophila* broth-growth (10), these activities appear as good candidates for supporting, at least in part, the immunosuppressive effect. In the present work we report the purification of the *flhDC* controlled lysin and the mode of action of this lysin was studied on SRBC and on insect hemocytes. We show that insect macrophages (=GH1) were the most sensitive hemocytes to the lysin and that this hemolysin was not recycled to react with multiple target cells but works as a porin. Finally, the swollen appearance of hemocytes incubated with *flhDC* controlled lysin prompted us to check for activity of this lysin on cell membrane permeability. We provide evidence that monovalent cation channels and larger pores are opened in the plasma membrane of the insect macrophages, leading to plasma
membrane depolarization and cell death through colloid-osmotic lysis, independently of Ca$^{2+}$ movements.

EXPERIMENTAL PROCEDURES

Bacterial strain, production and isolation of hemolysin- Xenorhabdus nematophila (strain F1, phase variant I, laboratory collection) were grown in Luria Bertani broth at 28°C. In these conditions the maximum production of flhD dependent cytotoxic activity (=C1) (10) was reached in 20 hours old cultures. A purification of the factor responsible for this activity was achieved from this C1 culture supernatant. Broth-growth was centrifuged (30 min at 12,10^3 g) and the supernatant was immediately precipitated in 50 % ammonium sulfate, centrifuged and the supernatant precipitated in 70 % ammonium sulfate. The 70 % pellet was dissolved and dialysed against water, concentrated by lyophilization and dissolved in low ionic strength buffer (phosphate buffer 10 mM, pH 7.8). This solution was submitted to chromatography on HiTrap Q column (Pharmacia) and elution was achieved with NaCl. Positive fractions, as assayed by measuring SRBC hemolysis and insect hemocyte lysis, eluted at 150 mM NaCl were pooled, lyophilised, dissolved in water and applied on a C18 Reverse Phase HPLC column. Active fractions, collected in a single and isolated peak, were immediately lyophilised. Before use, they were dissolved in phosphate buffer saline (PBS) or HEPES buffer. In this study PBS contained 1 mM of CaCl$_2$ and 2 mM of MgCl$_2$ unless otherwise stated. The titre of the lysin solution was evaluated by its hemolytic activity on SRBC (see below). Heat resistance was measured by incubating for 30 min at 60°C prior to test for cytotoxic and hemolytic activities. For trypsin resistance, fractions were incubated for 1h at 37°C with 30 units of trypsin (Sigma).

Insects, hemocyte monolayer preparation and test for cytotoxic activity - Larvae of the common cutworm Spodoptera littoralis (lepidoptera) were reared with a photoperiod of 12 hours on artificial diet at 24°C. Three days old sixth instar larvae were selected and surface sterilized with 70% (v/v) ethanol prior to collection of hemolymph in test tubes filled with anticoagulant buffer (62 mM NaCl, 100 mM glucose, 10 mM EDTA, 30 mM trisodium citrate, 26 mM citric acid see 13) at 4°C. After centrifugation, the hemocyte pellet was rinsed in PBS and resuspended in the same saline. Twenty µl of hemocyte suspension were layered on glass coverslips. Hemocytes were allowed to adhere on glass for 15 min in a moist chamber at 23°C then gently rinsed with PBS before being used as monolayers.

Cytotoxic activity was tested on monolayers. Excess PBS was pipetted off the coverslip, replaced by 20 µl of the solution of lysin in PBS and monolayers were incubated in a moist chamber at 23°C. Hemocyte mortality was checked by adding 2 µl of trypan blue dye (0.04 % final in PBS) and 5 min more incubation. In a preliminary experiment, results were expressed
as a percentage of mortality in total hemocyte population. In the other experiments, cytotoxic activity was expressed as percentage of dead cells among the GH1 population.

When potassium channel inhibitors (TEA and TBA) were used, they were added to a solution of the lysin in HEPES buffer (pH 7.2) at the concentrations of 50 mM to 300 mM. Results were expressed as a percentage of dead cells in the GH1 population. Osmolarity was measured in an automatic micro-osmometer (H. Roebling, Messtechnik, Berlin).

The role of Ca²⁺ ions was tested after extensive dialysis of lysin against PBS without Ca-Mg. Monolayers were prepared in the same buffer after rinsing hemocytes several times in anticoagulant buffer (deprived of Ca-Mg and with EDTA)

**Hemolytic activity on SRBC and titration of lysin**- Sheep red blood cells were provided by BioMérieux (France) at 50 % suspension. Before use, SRBC were extensively washed in PBS and adjusted to 5 % suspension in this buffer. Tests were achieved by using 50 µl of SRBC suspension to which 100 µl of the lysin solution were added. Incubation lasted 2 h at 37°C. Then the suspension was centrifuged at 3,000 g for 5 min. 130 µl of the supernatant were added to 770 µl of pure water and absorbance was determined at 540 nm. One unit of hemolytic activity (1 HU) was defined as the OD measured after total hemolysis of 50 µl of a 5 % SRBC suspension, in 900 µl of distilled water. Hemolytic titre of a solution was calculated using the formula deduced from numerous absorbance determinations with serial dilutions of lysin: 

$$\text{Titre (HU)} = 2^{10(\text{OD} - 0.72)}$$

In some experiments, TBA (final concentrations 100 to 300 mM) was added to red blood cell suspensions in HEPES buffer before incubations with hemolysin.

**Production of red blood cell ghosts**- They were obtained after 5 min incubation of one volume of a 5 % red blood cell suspension in PBS and 5 volumes of distilled water. Lysed red blood cells were centrifuged (10 000 g, 5 min) rinsed two times in distilled water then four times in PBS. For inhibition of hemolytic activity, lysin solutions were incubated with large amounts of ghost suspensions for 2 h at 37°C, then centrifuged and tested for hemolytic activity.

**Effect of incubation time and increasing target cell concentration on hemolytic activity**- In a first series of experiments, 100 µl of different concentrations of lysin in PBS (see results) were incubated with 50 µl of a 5 % suspension of SRBC at 37°C and absorbance of supernatant was determined from 0.5 h to 24 h. In a second series of experiments, a constant amount of lysin was incubated for 2 hours with increasing SRBC concentrations (5 %, 10 % and 20 %) and the percentage of hemolysis was determined for each red blood cell concentration.

**Neutral red uptake (NRU)**- We have used the procedure described by Szabo et al. (14). Hemocyte monolayers were prepared in 24 wells tissue culture plates (10⁶ cells per well) and were incubated in PBS for 30 min at 24°C with or without lysin at a titre which allowed
vacuolation of the cells (max. 0.02 HU) but gave a low percentage of lysis in time of the experiment (30 min). Data were expressed as the percentage of NRU values obtained in controls (no lysis treatment).

**Measurement of cytosolic free Ca^{2+} concentration**- Intracellular calcium concentration ([Ca^{2+}]_i) was measured with fluorescent indicator fura-2 (15). For this purpose, insect hemocyte monolayer was prepared on either rectangular (20 x 7 mm) or square (10 x 10 mm) glass coverslips. After plating, cells were loaded with fura-2 after incubation for 30 minutes at room temperature with the extracellular solution: 124 mM NaCl, 3 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 1.5 mM CaCl₂, 1 mM MgSO₄, 10 mM D-Glucose (bubbled with O₂/CO₂ : 95/5) containing 5 µM fura-2AM and 0.02 % Pluronic. [Ca^{2+}]_i was monitored either by spectrofluorimetry or videomicroscopy. After rinsing, a rectangular coverslip was inserted in the quartz cuvette of a Aminco-Bowman 2 spectrofluorimeter (SLM Instruments, USA) with an angle of 45° respective to the excitation beam. The toxin was applied directly in the quartz cuvette containing the extracellular solution magnetically stirred and thermostatted at 25°C. Fura-2 fluorescence was obtained by exciting the preparation alternatively at 340 and 380 nm and by monitoring emissions (F_{340} and F_{380}) at 510 nm. The ratio of emissions at 510 nm (F_{340} / F_{380}) was recorded every 0.5 seconds. Alternatively, a square coverslip was transferred to the recording chamber mounted on an inverted microscope (Leica, DMIRB). Fura-2 emission was obtained by exciting alternatively at 340 and 380 nm with a rotating filter wheel (Sutter Instruments). Fluorescent signals were collected with a CCD camera (Hamamatsu, Japan), digitized and analysed with an image analysis software ("Acquacosmos" Hamamatsu, Japan).

**Measurement of intracellular K⁺ concentration**- In order to record K⁺ efflux from insect hemocyte, intracellular K⁺ concentration was measured with fluorescent K⁺-binding benzofuran isophtalate dye (or PBFI). For this purpose, cells were plated on a square (10 x 10 mm) glass coverslip and incubated for 30 minutes at room temperature with 5 µM PBFI-AM and 0.02 % Pluronic diluted in the extracellular solution. After rinsing, the coverslip was transferred to the stage of an inverted microscope (Leica DMIRB). PBFI fluorescence was obtained by exciting the preparation at 380 nm and collected at 510 nm. Analysis and digitization were performed as described in the upper section.

**Electrophysiology**- For electrophysiological recordings, insect hemocyte monolayers were prepared on square (10 x 10 mm) glass coverslips. After plating, a coverslip was transferred to the recording chamber of an inverted microscope (IMT2, Olympus, Japan), continuously superfused (flow rate : 5 ml/min.) with the extracellular solution described above and containing 10 mM HEPES (pH = 7.4), at room temperature. Patch-clamp experiments were performed in the cell-attached and the inside-out configurations with glass microelectrodes (4-5 MΩ resistance). According to the experiment, electrodes were filled with various
solutions: the extracellular solution (as described above), potassium rich solutions comprising 150 mM potassium gluconate and 50 mM HEPES (pH = 7.4) or 150 mM potassium chloride and 50 mM HEPES (pH = 7.4), a tetraethylammonium (TEA)-based solution containing 150 mM TEA chloride and 50 mM HEPES and a tetrabutylammonium (TBA)-based solution comprising 150 mM TBA chloride and 50 mM HEPES (pH = 7.4). For both cell-attached and inside-out experiments, the toxin \( \alpha \)X was used at a concentration of 0.062 HU either diluted in the intra-electrode solution or bath-applied, respectively. Recordings were performed in the voltage clamp mode and command voltage (Vcmd) refers to the voltage applied in the recording electrode. Transmembrane voltage (Vm) recorded in the cell attached configuration equals \( V_i - V_{cmd} \), where \( V_i \) is the voltage of the inner face of the patch (16). On graphs, only Vcmd is given since \( V_i \) is not known. The voltage dependency of the currents recorded in the presence of \( \alpha \)X in the cell-attached mode was studied by stepping Vcmd from \(-80\) to \(+80\) mV with an increment of 20 mV. In this protocol, each voltage step lasted 2 seconds. Single channel currents were recorded with a patch-clamp amplifier (Axopatch 200 B, Axon Instruments, USA) and digitized (Digidata 1200 Interface, Axon Instruments, USA). Signals were filtered at 1 kHz and sampled at 10 kHz. Continuous recording and analysis of the currents were performed with John Dempster’s softwares ‘WinCDR’ and ‘WinWCP’.

**Osmotic protection**- Possible osmotic protection of insect hemocytes and SRBC was tested with protectants of different sizes: polyethylene glycol 6,000 and 4,000, Dextran 1,000 all at 30 mM, Raffinose (MW 504) and Sucrose (MW 342) both at 50 mM. These protectants were added to the lysin solutions and hemocytes or red blood cells were incubated as described above. In a series of experiments, after incubation and measurement of the optical density of supernatant (hemolysis), the red blood cells pellet was resuspended and incubated five minutes more in PBS and measured again for hemolysis. For cytolysis, two series of monolayers were incubated with lysin and protectant. In one series, the percentage of macrophage lysis was determined at the end of incubation. In the other series, at the end of incubation, monolayers were washed and incubated five minutes more in PBS. Then the percentage of lysis was determined and compared to that obtained without rinsing the cells.

**Electron microscopy**- Hemocyte monolayers were incubated for 0.5 hour with lysin diluted in PBS (titre 0.02 HU) or in PBS for control, fixed in 5 % glutaraldehyde then in 1 % osmium tetroxide and embedded in Epon. Ultrathin sections were stained according to Reynolds (17).

**RESULTS**

**Purification of the lysin**- It was achieved through the method described in Material and Methods and is summarized in Table 1. MALDI-TOF analysis of the C18 active fraction gives
only one peak with a MW of 10,790 Da. The profile mass fingerprint (PMF) after trypsin digestion of this molecule, was determined (thanks to N. Galeotti, P. Marin and E. Demay from CCIPE, Montpellier). This PMF was used to search protein databases but this analysis did not yield to any protein identification. We called this lysin α-Xenorhabdolysin (αX). It was hemolytic for SRBC but not for RRBC (Tab. 2).

**Vacuolation and lysis of hemocytes**- The effects of different dilutions of αX on *Spodoptera littoralis* hemocytes were compared to the effects of culture supernatant with C1 activity (10) under the same experimental conditions (Tab. 2). With αX solutions of 0.02 hemolysis units (HU, see Mat and Meth.) or more, death of the hemocytes occurred by necrosis as tested by trypan blue uptake, in less than one hour. The main hemocyte types, which are Plasmatocytes (Pl) and Granular Hemocytes 1 (GH1 = insect macrophages), were unequally sensitive, GH1 showing higher percentage of lysis than Pl did for the same αX titre (not shown). So most of the numerations reported in this study were achieved on GH1 alone. Before lysis, Pl and GH1 exhibited extensive vacuolation (Figs. 1A, B) suggesting a modification of plasma membrane permeability by the toxin. Neutral red uptake quantification did not show any significant difference between vacuolated cells and untreated hemocytes (not shown). Transmission electron micrograph of αX treated cells showed the presence of numerous ribosomes on the cytoplasmic side of the vacuole membrane. These vacuoles were dilated endoplasmic reticulum cisternae and perinuclear cisterna (Fig. 2). Other cellular organelles, especially mitochondria, Golgi apparatus or lysosomes appeared only very slightly altered if any.

Cytolytic and hemolytic activities were lost after incubation of αX or C1 broth growth at 60°C for 30 min or incubation in presence of trypsin (Tab. 2). The lytic effects on hemocytes and on SRBC were still observed in a non-added calcium medium (Tab. 2). The absence of Ca²⁺ did not alter the difference in sensitivity between Pl and GH1 to αX (not shown).

**αX molecules are not recycled**- To test for the possible recycling of αX after a first exposure to cells, we conducted three kinds of experiments.

In the first series, a suspension of SRBC was incubated with different dilutions of αX (Fig. 3). Incubations lasted up to 24h with a measurement of OD at different incubation times. In these experiments we observed an increase of hemolysis up to 2 hours then the OD reached a plateau value. Such long term incubations were not performed with insect hemocytes.

In the second series of experiments, increasing SRBC concentrations (5 to 20 %) were incubated for 2 hours in 0.2 or in 0.05 HU of αX. Fig. 4 shows that the percentage of hemolysis elaborated, decreased with increasing target cell concentration.
In the last series of experiments, different dilutions of αX were first incubated with SRBC ghosts and the supernatant was further incubated with a 5% suspension of SRBC or with S. littoralis hemocyte monolayers. Neither hemolytic nor cytolytic activity was detected (Tab. 2).

Effects of αX on hemocyte Ca²⁺ and K⁺ ions concentrations- We have investigated whether potential toxin-mediated modifications of membrane permeability could result in a change in hemocyte Ca²⁺ and K⁺ cytosolic concentrations. In order to record [Ca²⁺]i changes, fura2-loaded cells were exposed to increasing concentration of αX (from 0.032 to 0.25 HU) and the fluorescence ratio 340/380 of hemocyte monolayer was recorded at 510 nm in a spectrofluorimeter. Toxin at all these concentrations led to cell death. At a concentration of 0.25 HU, a transient rise in [Ca²⁺]i could be recorded which was apparently regulated quickly. Lower concentrations of toxin, ranging from 0.062 to 0.032 HU led to a dose- and time-dependent increase in [Ca²⁺]i (Fig. 5A). In a Ca²⁺ free external medium (no added Ca²⁺), the toxin at concentration of 0.25 HU had almost no detectable effects. Therefore, this suggests that toxin-induced [Ca²⁺]i rise results from a Ca²⁺ influx from the external medium (Fig. 5A). The analysis of the fura2 emissions at 510 nm, obtained by exciting at 340 nm (F340) and 380 nm (F380), indicates that although the initial increase in fluorescence ratio in the presence of αX was effectively due to an increase in [Ca²⁺]i as indicated by the variations of fura2 emissions in opposite directions (Fig. 5B), the decrease of fluorescence ratio reflects more likely leakage and dilution of the probe in the medium, as both F340 and F380 decrease in parallel. The toxin at 0.062 HU elicits rapid and unregulated increases in [Ca²⁺]i in visually identified GH1 and plasmatocytes, as observed using videomicroscopy (Fig. 5C). Observation in phase contrast microscopy of these cells during the experiment, confirms that the cells were lysed at the time of [Ca²⁺]i rise (Fig. 5D). Since the toxin is able to induce cell death in the absence of external calcium, it can be postulated that the increase in [Ca²⁺]i detected upon exposure of cells to the toxin is not responsible for its toxicity but more likely reflects ionic disturbance across cell membrane and cell lysis.

Cytoplasmic concentration of K⁺ was monitored in PBFI-loaded hemocytes. At each αX (0.02 HU) application to the medium, an immediate loss of K⁺ could be recorded which was in part regulated up to the cell lysis. This lysis was evidenced by a large decrease in PBFI fluorescence revealing leakage of the probe in the medium, as illustrated in 3 visually identified GH1 (Fig. 5E).

Channels opened on insect macrophage membrane by αX are monovalent cation selective- From the videomicroscopy data, one can hypothesize that αX mediates to an ionic imbalance leading to cell death. This imbalance could be initially due to a K⁺ efflux. Therefore, we have examined whether αX could alter K⁺ ions permeability through native GH1 membranes by using patch-clamp recordings. In a first attempt to study membrane
modifications elicited by the toxin, conventional patch-clamp whole cell recordings (WCRs) were undertaken in visually identified GH1. It has to be mentioned that experiments were conducted only within 90 minutes following hemocytes plating. Indeed, the hemocytes would naturally deteriorate after 90 minutes and give unreliable data. Stable WCRs were almost impossible to get from GH1 since these cells sealed almost immediately after obtaining a gigaohm seal and rupturing the patch by applying a negative pressure (n = 20). By contrast, plasmatocytes gave easily access to WCRs (n = 5). This discrepancy between these two cell populations could be attributed to specific membrane properties. Indeed, GH1 have a rough plasma membrane with invaginations and pseudopods while plasmatocytes have a smoother plasma membrane allowing easier access to microelectrode (not shown). This is the main reason why cell-attached recordings were preferred to WCRs. In addition, in order to examine the pore-forming activity of the αX and to avoid a lethal exposure of the cells to this toxin, αX was applied directly within the recording electrode. For this purpose, a fraction of the toxin was diluted in the internal medium of the electrode and, just like in the perforated patch technique using pore-forming antibiotics (16, 18), toxin-induced modifications could be recorded underneath the electrode. It must be emphasized that a concentration of 0.062 HU was chosen since it gave a good activity without damaging the seal. Indeed, in the presence of higher concentrations of the toxin, tight seals could not be established. In a first set of experiments, we have therefore examined the selectivity of these αX pores for K⁺ ions. For this purpose, recordings were performed under symmetrical concentrations of K⁺, obtained by filling electrodes with K⁺-based solutions (K gluconate and KCl). In this respect, we have tested first K gluconate containing solutions. In the absence of the toxin, no current could be recorded when stepping Vcmd from −80 to +80 mV (Fig. 6A). In the presence of the toxin, channel formation could be observed after 3 to 20 minutes of contact (n = 5) (Fig. 6B). These currents reversed when Vcmd = 0 mV and displayed a linear voltage dependency. The conductance of αX-generated channels was evaluated at 21 ± 2 pS (n = 4) from the current-voltage relationship (Fig. 6C). The effect of the toxin was time-dependent since after 30 to 45 minutes of contact (n = 4), longer openings could be observed prior to the rupture of the seal (Fig. 6D). Similar data were obtained when recording electrodes were filled with KCl-based intracellular solution (Fig. 6E). This suggests that αX generates pores permeant to K⁺ ions. In order to confirm this possibility, conventional K⁺ conductance blockers i.e. tetrasubstituted ammonium derivatives, tetraethylammonium chloride (TEACl) and tetrabutylammonium chloride (TBACl), were further tested for their ability to block currents recorded in the presence of αX. When K⁺ was substituted for TBA⁺ ions, no currents could be recorded from the patches when αX was applied (n = 4) (Fig. 6F). Similar result was obtained with TEA⁺ ions (data not shown). Therefore, ammonium derivatives are blocking αX-generated
conductances. In addition, since, firstly, the counter anion of these compounds was Cl\(^-\) ion in both cases and since, secondly, similar data were obtained with both KCl and K gluconate filled electrodes, one can suggest that \(\alpha\)X-formed pores are rather not permeant for Cl\(^-\) ions.

Under symmetrical K\(^+\) conditions, equilibrium potential (\(E_{K^+}\)) equals 0mV as calculated with the Nernst equation. Under these conditions, currents mediated by K\(^+\) fluxes are expected to reverse when \(V_m = E_{K^+} = 0\text{mV}\). Here, reversal of the currents obtained in the presence of \(\alpha\)X was observed when \(V_{cmd} = 0\text{mV}\). Therefore, this suggests that \(V_m = V_{cmd}\) in the presence of the toxin in the recording electrode.

In a second set of experiments, cell attached recordings were performed with electrodes filled with the extracellular solution. In the absence of toxin, voltage-dependent ionic channels could not be evidenced in GH1 since no microscopic currents could be recorded by stepping \(V_{cmd}\) from \(-80\) to \(+80\text{mV}\) (Fig. 6G). In the presence of the toxin, channel-like openings could be recorded at extreme membrane potentials, i.e. \(-80\) and \(+80\text{mV}\). No current could be detected when \(V_{cmd} = 0\text{mV}\) (Fig. 6H). This tends to indicate that cations flow not selectively through \(\alpha\)X-generated pores. Indeed, currents flowing through non selective cationic channels are expected to reverse when \(V_m = 0\text{mV}\).

We have next examined whether the effect of the toxin was selective for the outside domain of GH1 membrane. For this purpose, patch-clamp recordings were performed in the inside-out configuration. Patches of GH1 were held at -60mV under symmetrical conditions (extracellular medium in the electrode and in the bath) and allowed to equilibrate 5 min after excision. Exposing the inner face of GH1 membrane to \(\alpha\)X (0.062 HU, \(n=4\)) resulted then in the occurrence of large inward currents followed by a rapid loss of the seal. This indicates that \(\alpha\)X may have an effect on both sides of the plasma membrane of GH1 (Fig. 6I).

**Osmotic protection**- Osmotic protection of insect hemocytes and of SRBC was tested with potential protectants of different sizes. Polyethylene glycols (PEG) 4,000 and 6,000 and dextran 1,000 were used at a final concentration of 30 mM and raffinose and sucrose at a final concentration of 50 mM. Protection of hemocyte cytolyis (Fig. 7) and of SRBC hemolysis (not shown) were obtained with all these protectants, depending on the concentration of \(\alpha\)X solution. For the same \(\alpha\)X concentration, protectant were more efficient against cytolyis of insect macrophages than against hemolysis of SRBC. Whereas a total inhibition of cytolyis was obtained with PEG 4,000 at the highest toxin concentration (1 HU), hemolysis was only reduced of one half by PEG 4,000 at this concentration. Total hemolysis inhibition was observed with PEG 6,000. No change OD was recorded when the different protectants were added to the supernatant obtained after incubation of red blood cells with the toxin. This shows that there was no direct effect of protectants on hemoglobin absorbance.
We have next examined whether protectants were either inhibiting the insertion of the toxin in the plasma membrane or blocking pores formed by the toxin. For this purpose the following protocols with insect hemocyte monolayers were designed. In a first series of experiments, the percentage of GH1 lysis was determined in monolayers incubated 45 min in PBS (control 1), in PBS containing lysin (0.2 HU)(control 2), and in PBS containing lysin (0.2 HU) and PEG 4,000 as protectant. In a second series of experiments, monolayers were incubated in same conditions and then hemocytes were rinsed in PBS and incubated 5 min more in PBS. Percentage of GH1 lysis was determined. The percentage of cell lysis obtained after washing monolayers incubated with lysin and protectant, was the same as the one determined after incubation with lysin in absence of protectant (Tab. 3).

In the last series of experiments, after incubation of SRBC with lysin solution (0.5 HU) and protectant, the optical density of supernatant (hemolysis) was measured and the red blood cells pellet was resuspended in PBS. After 5 min more incubation, red blood cells were pelleted again and OD of the supernatant measured. The sum of optical densities of the two supernatants was close to the OD of the supernatant of SRBC incubated with αX in absence of protectant (Fig. 8).

As cytolysis and hemolysis were restored after washing off the protectant, we conclude that the observed inhibitions were not due to direct inactivation of αX by PEG.

Effect of potassium channel blockers on hemocyte cytolysis and red blood cell lysis- TEA and TBA were tested on cytotoxic and TBA on hemolytic activities triggered by αX. In a first series of experiments, they were added to solutions of αX giving almost 50% (TEA) or 80% (TBA) GH1 mortality (from 0.04 to 0.2 HU). The cytolytic activity on hemocytes was almost totally inhibited with 100 mM TEA or 50 mM TBA in the time of experiments (not shown). In experiments conducted with lower toxin concentration (0.01 to 0.02 HU) and a shorter incubation time, vacuolation of hemocytes was also extremely reduced when TEA (100 mM) or TBA (50mM) were added to incubation medium (Fig. 1C for TBA). The best protection achieved with TBA over TEA was attributed to the larger size of the TBA molecule, which allows a better blockade of the K+ channels (del Camino et al., 2000).

In a second series of experiments, SRBC were incubated with different dilutions of αX with 100 to 300 mM of TBA. TBA solutions were able to inhibit the hemolytic activity, depending on the αX and TBA ratio, the lower lysin concentration the better protection (not shown).

DISCUSSION

Two different cytotoxic activities (C1 and C2) on insect hemocytes were evidenced in the culture medium of the entomopathogenic bacteria X. nematophila (10, and this work Table
2), one of them, C1, being under the control of \textit{flhDC}, the flagellar master operon (11). This \textit{flhDC} dependent activity was also hemolytic for SRBC but did not lyse RRBC. SRBC hemolysis and insect hemocyte cytolysis were equally sensitive to heat or trypsin treatments of the culture medium and independent of Ca\textsuperscript{2+} ions. In the present work the \(\alpha\)-Xenorhabdolysin (\(\alpha\X\)), a 10.8 kDa toxin responsible for this \textit{flhDC} dependent activity, was purified from \textit{X. nematophila} broth-growth with C1 activity. Culture medium and the present purified toxin had the same heat and trypsin sensitivity, the same range of specificity for insect hemocytes and hemolytic activity on SRBC, RRBC being insensitive. After incubation of broth-growth or purified lysin solution with SRBC ghosts, hemolysis as well as hemocyte cytotoxicity were lost. We conclude that the purified lysin is responsible for both SRBC hemolysis and insect hemocyte cytolysis. It is also responsible for the C1 activity previously evidenced in \textit{X. nematophila} culture medium. Titration curves show that there was no linear relationship between lysin concentration and hemoglobin release (optical density). This suggests that \(\alpha\X\) has a concentration-dependent binding affinity to its target, as shown for \(\alpha\)-toxin of \textit{Staphylococcus aureus} (20). Alternatively, possible interactions between lysin molecules could occur as degrees of polymerisation depending on toxin concentration.

To our knowledge, an hemolysin secreted by an entomopathogenic bacteria, which is also active on insect hemocytes, is purified here for the first time.

The total disappearance of cytotoxic activity after incubation with SRBC ghosts and the results of osmotic protection and patch-clamp experiments show that the plasma membrane is a target of \(\alpha\X\). Furthermore, these results are consistent with data obtained with long term incubations (up to 24 hours) of \(\alpha\X\) with SRBC and with data obtained with increasing target cell concentration. In these last experiments, the amount of hemolysis did not increase later than 2 hours incubation and the percentage of hemolysis decreased when target cell concentration was increased. We conclude that once fixed on a plasma membrane site, \(\alpha\X\) is not recycled to react with multiple target cells at least with red blood cells. According to Rowe and Welch (21) these data show that \(\alpha\X\) looks like a pore-forming toxin rather than to a lysin with an enzymatic activity. Results of microspectrofluorimetry, of patch-clamp studies and of osmotic protection experiments, are consistent with a such pore-forming activity.

Microspectrofluorimetry data suggest that the toxin-induced calcium rise after a latency-period, resulted from Ca\textsuperscript{2+} influx from the external medium. But, when the maximal increase of the ratio 340/380 nm has been obtained, fura2 emissions tend to display parallel decreases. The more likely explanation is that the toxin induced membrane disruption leading to leakage of fura2 in the medium. Since the toxin was able to induce cell death in the absence of external calcium, it can be postulated that the increase in cytosolic Ca\textsuperscript{2+} detected upon exposure of the cells to the toxin was correlated neither with specific Ca\textsuperscript{2+}
entry nor with mobilization of Ca$^{2+}$ from internal stores. More likely it reflects cell lysis, as shown by light microscopic observation (Fig.5D). On the other hand, αX induced an immediate loss of K$^+$, which could be temporarily regulated by the cell until lysis. These last observations are in accordance with the results of patch-clamp experiments.

Cell attached patch-clamp experiments were designed to evaluate the pore-forming activity of αX in native GH1 membranes. It must be emphasized that no voltage dependent channels could be detected in the absence of the toxin. However, this does not imply that GH1 are 'electrically' silent. Indeed, the activity of Ca$^{2+}$ activated K$^+$ channels or second messenger-operated channels, for instance, remains to be established in these cells. Electrophysiological recordings to study αX actions had to be adapted to the specific membrane properties of GH1 and to the very high sensitivity of these cells to αX. For this purpose, αX was directly applied in the recording electrode. This procedure enabled us to evaluate the pore-forming activity of this toxin. Interestingly, the bacterial toxin, α-toxin from *Staphylococcus aureus*, has previously been used for its pore-forming activity in the perforated patch method (16). In order to observe microscopic currents due to channels formation, low concentrations of αX had to be applied. Indeed, as evidenced also for *Helicobacter pylori* (14) for instance, high concentrations of αX could prevent tight seal formation between cell membrane and recording electrodes. In the presence of αX, the activity of cation selective channels could be evidenced. These currents could be totally blocked by K$^+$ channel antagonists (TEA$^+$ and TBA$^+$). This tends to indicate that αX alters primarily membrane permeability by forming K$^+$ permeable pores. Under physiological ionic concentrations, non-selective cationic currents could be recorded in the presence of the toxin. Under symmetrical K$^+$ conditions, the conductance of αX-generated pores was rather small (21 ± 2 pS). This is probably due to the fact that the toxin was applied at a low concentration and had a small area of contact with the membrane of GH1. However, the effect of the toxin on channel forming was time dependent. Indeed, an increase in currents was observed with time. This could be due to an increase in the number of pores and/or in the size of these pores, as demonstrated for other porins (22).

Taken together, these results show that the first effect of αX on the insect hemocyte membrane was an increase in ionic permeability, mainly for monovalent cations. Modification of ions permeability by bacterial toxins in eukaryotic cells is well documented. Anion selective channels are formed in SF-9 insect cells by the δ-endotoxin from *Bacillus thuringiensis* (23) and in HeLa cells by VacA from *Helicobacter pylori* (14). Channels with weak discrimination among different cations are formed in human macrophages by HlyA from *E. coli* (24). Alpha-X from *X. nematophila* was more specific as it created channels rather selective for monovalent cations, as did the major cytolysin of *Staphylococcus aureus* (25). In time of our
experiments, inhibition of the current recorded in the presence of αX by the specific blockers of potassium channels, TEA and TBA, shows that the cation channels opened or created by αX, could be rather specific for potassium. This specificity has been evidenced for aerolysin from Aeromonas hydrophila on BHK (mammalian) cells (26). TEA and TBA also inhibited both ER vacuolation and cell lysis, showing that disturbance of potassium permeability induced by αX could be sufficient to lyse the target cells. Furthermore, as in experiments conducted with VacA toxin on HeLa cells (14), inhibition of insect macrophage lysis by blockers was more effective at the lowest αX doses. We cannot dismiss the possibility that αX activates unknown endogenous channels rather than forming new ones. However different evidences are in favour of a pore-forming activity. Firstly, the results of osmotic protection experiments are consistent with the formation of pores. Secondly, the pore size increase with toxin concentration is well documented for pore-forming molecules such as complement (pore sizes ranging from 0.7 to 15nm) (27) or E. coli toxin (0.6 to 1.3nm) (28) and other RTX toxins (29). A total protection of insect macrophage cytolyis was obtained with PEG 4,000 (pore radius: 1.9 nm) (30) whatever the αX concentration but protection of SRBC, at the highest αX concentrations, was only obtained with PEG 6,000 (pore radius: 2.9 nm). This suggests that the maximum size of the pores formed in red blood cells plasma membrane would be larger than that of pores made by the same lysin in insect macrophages plasma membrane. A larger size of pore created in red blood cells than pore in nucleated cells, was already reported for ShlA from S. marcescens (31). But the reason for this variability is unknown. Finally our results on osmotic protection led us to conclude that αX processes through colloid-osmotic lysis (32). In colloid-osmotic lysis, transmembrane channels allow only ions and small molecules to pass freely across the cell membrane. So the osmotic pressure generated by the high concentration of macromolecules inside the cell causes a water influx that leads to cell swelling and sometimes to cell burst. This colloid-osmotic process is consistent with our results on Ca\textsuperscript{2+} and K\textsuperscript{+} movements through hemocyte plasma membrane. Low doses of αX mediate a selective leakage of K\textsuperscript{+} (and possibly other monovalent cations) from hemocytes. However low doses of αX do not enhance membrane permeability for Ca\textsuperscript{2+} or larger molecules. In this respect, intracellular Ca\textsuperscript{2+} entry was observed only upon cell lysis. Therefore, Ca\textsuperscript{2+} entry was not a cause but a consequence of cell lysis.

The cell burst was the most obvious effect of αX from X. nematophila, on hemocytes. But prior to lysis, insect hemocytes showed extensive vacuolation of the cytoplasm. Vacuolation is a non classical pathway of toxicity of bacterial toxins. However it is achieved by cereulide from Bacillus cereus (33), VacA toxin from Helicobacter pylori (34), aerolysin from Aeromonas hydrophila (26), ShlA hemolysin from Serratia marcescens (31) and HlyA
hemolysin from *Vibrio cholerae* (35) in different mammalian cell types *in vitro*. But among these toxins, only vacuolation triggered by ShlA and HlyA is followed by a lysis of cells in culture. As all these toxins (cereulide, VacA, aerolysin, ShlA and HlyA) triggered the vacuolation of different cellular organelles, we conducted two kinds of experiments to characterize which cell organelle was subjected to vacuolation by αX. Neutral red is a supravital dye of the endosome/lysosome system (36). Data obtained with NRU experiments suggest that the vacuoles observed in hemocytes after αX incubation do not belong to this endosome/lysosome system. This conclusion is not in agreement with studies performed on mammal cells with VacA, ShlA or HlyA. Examination of αX treated hemocytes under electron microscopy, lead us to conclude that the vacuoles are in fact dilated cisternae of the ER. Vacuolation of ER by a bacterial toxin is reported here for the first time in non-mammalian cells. An other case of ER vacuolation by bacterial toxin was reported by Abrami *et al.* (26) for aerolysin on BHK cells. For aerolysin (37, 38, 39) as for αX (this study), the first target of the toxins is the plasma membrane where they form channels selective for small cations before they trigger ER vacuolation. But a fundamental difference between aerolysin and αX is their respective effect on intracellular Ca$^{2+}$ concentration. Indeed, aerolysin induces Ca$^{2+}$ release from intracellular stores as well as a Ca$^{2+}$ influx (39), while αX does not. In this respect the activity of αX on its target cells appears different from the different actions already described and studied for all other bacterial toxins.

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FOOTNOTES

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ψ To whom all correspondence should be addressed: Tel.: (33) 4 67 14 46 72; Fax: (33) 4 67 14 46 79; E-mail: brehelin@crit.univ-montp2.fr
£ Present address: Secção de Biologia Celular e Molecular, Universidade dos Açores, 9501-801 Ponta Delgada, Açores, Portugal
$ C.R. and M.V. contributed equally to this study

¹ The abbreviations used are: αX, alpha-Xenorhabdolysin; GH1, granular hemocyte 1; HU, hemolysis unit; NRU, neutral red uptake; OD, optical density; PBFI, potassium binding benzo furen isophtalate; PBS, phosphate buffered saline; PEG, polyethylene glycol; PI, plasmatocyte; PMF, peptide mass fingerprint; RRBC, rabbit red blood cells; SRBC, sheep red blood cells; TBA, tetrabutyl ammonium; TEA, tetraethyl ammonium; WCR, whole cell recording.

FIGURE LEGENDS.

**Fig. 1.** Vacuolation of insect hemocytes by αX and inhibition by TBA. Hemocytes monolayers were incubated for 30 min in PBS without toxin (A), or in presence of 0.02 HU of αX (B) or with 0.02 HU of αX and 50 mM TBA (C) Arrowheads: vacuoles in macrophages (arrow) or in plasmatocytes (double-arrow). Bar = 10 µm.
**Fig. 2.** $\alpha X$ triggers endoplasmic reticulum vacuolation in insect hemocyte (0.02 HU for 30 min). Vacuoles are dilated vesicles of rough endoplasmic reticulum (er) or nuclear cisterna (arrow). m = mitochondria; n = nucleus; arrowheads = ribosomes. Bar = 1 $\mu$m.

**Fig. 3.** Time course of $\alpha X$-induced hemolysis of SRBC. 5 % SRBC were incubated with 0.03 to 1 HU of $\alpha X$ for 0.5 to 24 hours then centrifuged and optical density of supernatant was determined. This result is representative of three distinct experiments.

**Fig. 4.** Decrease in percent hemolysis with increase in SRBC concentration. 5, 10 and 20 % SRBC in PBS were incubated for 1 hour with 0.05 or 0.2 HU of $\alpha X$. Results were expressed as a percentage of total hemolysis. Data are means of three distinct experiments $\pm$ S.E.M.

**Figs. 5:** Effect of $\alpha X$ on cytosolic Ca$^{2+}$ and K$^+$ concentrations. **A, B.** After loading with fura-2 (30 minutes, room temperature), hemocyte preparation plated on rectangular coverslips was transferred to the recording chamber of a spectrofluorimeter. Fluorescence ratio was collected every 0.5 seconds. **A:** Concentration dependency (from 0.032 to 0.25 HU) of $\alpha X$ effect on [Ca$^{2+}$]i. **B:** Analysis of respective emissions recorded at 510 nm by excitation at 340 and 380 nm after the application of $\alpha X$ at 0.25 HU. **C, D** Effect of $\alpha X$ (0.062 HU) in visually identified GH1 and plasmatocyte. **C** Time course of [Ca$^{2+}$]i increase after the application of the toxin in a GH1 and a plasmatocyte. **D** Phase contrast microphotographs obtained (upper) prior to the application of $\alpha X$ and (lower) at the time of [Ca$^{2+}$]i peak in these two cells. Scale bar represents 20 $\mu$m. **E** Effect of $\alpha X$ on [K$^+$]i in visually identified GH1. Hemocyte monolayer was loaded with PBFI (30 minutes, room temperature) and then used for videomicroscopy. Potassium leakage was measured in identified GH1 after successive applications of $\alpha X$ (0.02 HU). On all graphs, vertical arrows indicate the time of application of $\alpha X$. Traces are representative of at least three distinct determinations.

**Figs. 6:** Patch clamp recordings obtained from GH1. **A to H:** Cell attached recordings performed in the presence of $\alpha X$ applied directly in the recording electrode at 0.062 HU. **I** Inside-out recording obtained from a patch of GH1 membrane and exposed to $\alpha X$ at 0.062 HU. **A** Recording obtained with Kgluconate-based (150 mM Kgluconate, 50 mM HEPES, pH = 7.4) filling solution without the toxin. **B and D** Recordings obtained with K gluconate-based (150 mM K gluconate, 50 mM HEPES, pH = 7.4) filling solution in the presence of the toxin. In **D**, the recording obtained 60 minutes after getting the seal is shown. **C** Averaged current-voltage relationships obtained from four distinct experiments performed with K gluconate-based filling solution. On graph, data are presented as means $\pm$ S.E.M. **E** Recording obtained with KCl-based (150 mM KCl, 50 mM HEPES, pH = 7.4) filling solution with the toxin. **F** Recording obtained with TBACI-based (150 mM TBACI, 50 mM HEPES, pH = 7.4) filling solution with the toxin. **G and H** Recordings obtained with extracellular filling solution without the toxin (**G**) and with the toxin (**H**). **I** Inside-out recording was performed under...
symmetrical conditions achieved with extracellular medium and at a holding voltage of –60 mV. The arrow indicates the time of application of αX (0.062 HU). Traces are representative of at least three distinct determinations.

**Fig. 7.** Effect of protectants on αX induced GH1 lysis. On graph, the columns represent the percentages of GH1 lysis (trypan blue staining) after 1 hour incubation of hemocyte monolayers in PBS (0) or in different dilutions of αX supplemented with various protectants (PEG and dextran: 30 mM; raffinose and sucrose: 50 mM). Data are means of three independent experiments ± S.E.M.

**Fig. 8.** Dissociation of toxin binding and hemolytic activity. 5 % SRBC were incubated for 45 min with αX (0.5 HU) supplemented or not (=control) with PEG, then centrifuged and OD of supernatant was determined (1st incubation). Then the red blood cell pellet was suspended and incubated 5 min more in PBS with neither toxin nor PEG added, centrifuged again and OD of supernatant determined (2nd incubation). There was an actual osmotic protection in first incubation. Removal of first supernatant washed off the protectant but not the lysin which was bound to red blood cell membrane. In these conditions hemolysis was recovered. Experiments were carried out in quadruplicate and similar results were obtained.

### TABLES.

**Table I**

*Purification of α-Xenorhabdolysin from bacteria culture medium.*

| Purification steps | Vol. (ml) | Act./100µl | Total act. | Yield |
|--------------------|-----------|------------|------------|--------|
| Crude extract      | 620       | 0.5        | 3100       | /      |
| Pption-dialysis     | 19        | 10.4       | 1984       | 64 %   |
| HiTrap Q            | 7         | 18.1       | 1271       | 41 %   |
| C18                 | 0.9       | 16         | 144        | 4.6 %  |

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Table II

Insect hemocyte cytolysis and red blood cell hemolysis.

Insect hemocyte cytolysis and red blood cells hemolysis induced by purified αX is compared to X. nematophila culture supernatant with C1 activity (Brillard et al., 2001). Lysin concentration was lower in tests for hemocytes (0.2 HU) than in tests for red blood cells (0.85 HU).

|                  | S. littoralis hemocytes | SRBC (OD) | RRBC (OD) |
|------------------|-------------------------|-----------|-----------|
|                  | (percent of lysis)(a)   | (b)       | (b)       |
| Control (Ca-Mg)  | 11.5±5.7                | 0.01±0.02 | 0.030±0.001 |
| PBS (with Ca-Mg) | C1 89.3±3.7             | 0.710±0.01| 0.050±3.3 |
|                  | αX 76.2±4.9             | 0.696±0.062| 0.021±0.001 |
| Temperature (60°C, 30 min) | C1 15.9±4.4 | 0.033±0.02 | NT |
|                  | αX 12.9±4.3             | 0.054±0.01 | NT |
| Trypsin (30 U, 1 h) | C1 13.2±3.7            | 0.041±0.01 | NT |
|                  | αX 14.7±7.2             | 0.034±0.009| NT |
| PBS (w/o Ca-Mg)  | C1 90.6±7.2             | 0.700±0.1 | NT |
|                  | αX 77.6±5.1             | 0.684±0.057| NT |
| SRBC ghosts      | C1 12.8±3.0             | 0.031±0.06 | NT |
|                  | αX 10.5±2.2             | 0.026±0.007| NT |

(a) Means ± S.E.M., on 12X2 monolayers from 6X2 larvae
(b) Means ± S.E.M., on 4 measurements from 6 different extracts. For SRBC total hemolysis gave an OD of 0.72.

NT: not tested

Table III

Dissociation of binding and cytolytic activity.

Monolayers were set up and incubated for 45 min in the absence of αX (control 1), in αX solution (0.2 HU) (control 2) or αX solution in PEG 4,000. In the first series of monolayers, percentage of GH1 lysis was determined at the end of 45 min incubation. In the second series, monolayers were first incubated for 45 min and then rinsed in PBS, incubated for 5 min more in this saline and GH1 lysis was determined. Data are presented as means ± S.E.M. of independent experiments where each experimental value is determined in duplicate.

|                  | Percentage of lysed GH1 |
|------------------|-------------------------|
|                  | No lysin (control 1)    | Lysin in PBS (control 2) | Lysin in PBS + PEG 4,000 |
| First series of monolayers | 12.0±1.5            | 98.1±1.2             | 23.2±11.7             |
| Second series of monolayers | 15.4±2.6             | 97.3±1.1             | 98.5±1.1             |
Fig. 5

A

B

C

D

cell 1: GH1 (granulocyte)
cell 2: plasmatocyte

E

PBE fluorescence (arbitrary units)

Time (seconds)

Time (seconds)

Time (seconds)
Fig. 7

Fig. 8
Xenorhabdus nematophila (Enterobacteriacea) secretes a cation-selective calcium-independent porin which causes vacuolation of the rough endoplasmic reticulum and cell lysis

Carlos Ribeiro, Michel Vignes and Michel Brehélin

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