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Ionic factors regulating the interaction of *Gardnerella vaginalis* hemolysin with red blood cells

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We have studied the hemolytic properties of an exotoxin released by *Gardnerella vaginalis* (Gvh). We found that hemolysis induced by Gvh is modulated by the composition of the isotonic buffer in which the red cells are suspended. In particular, low pH enhances its lytic activity, whereas low ionic strength and divalent cations diminish it. The inhibitory effects of reduced salt concentration and divalent cations occur despite normal binding of the toxin to the cells. This suggests that some post-binding step is impaired. The toxin is also able to damage cholesterol-containing lipid vesicles, and even on these model membranes it is more active at low pH. From this point of view, Gvh has some similarity to *Clostridium perfringens* θ-toxin, a membrane-damaging toxin belonging to the family of 'thiol-activated' cytolysins produced by Gram-positive bacteria.

Introduction

Gardnerella vaginalis is considered the main etiological agent of the so-called 'nonspecific' vaginitis or bacterial vaginosis. In fact, it is the prevalent organism present in the acute forms of this disease [1], although it is frequently associated with other anaerobic microorganisms [2]. *G. vaginalis* adheres to exfoliated epithelial cells forming the so-called 'clue cells' which constitute the main diagnostic marker for bacterial vaginosis [3]. Improved microbiological tests have led to more widespread detection of *G. vaginalis* also in other pathologies, like urological infections, especially post-surgical ones [4,5], gynecological disorders [6,7], and bacteremia in puerperal women and newborns [8]. A few cases of bacteremia in men have also been reported [9–11].

The Gram-characteristics of *G. vaginalis* have long been a subject of dispute. In fact, although initially it was often classified as a Gram-negative [12] or Gram-variable bacterium [13,14], it seems now unambiguously established that *G. vaginalis* has Gram-positive cell-wall organization [1,15,16].

Although in recent years interest in the potential invasiveness of this bacterium has increased, little is known about its virulence determinants. The hemolysin (Gvh), released in the culture broth [17,18] and responsible for the beta-hemolysis on human blood agar plates, is likely to represent an important factor in the pathology. A complete biochemical and functional characterization of Gvh is still lacking. In a previous paper we have demonstrated that Gvh is a pore-forming protein and that its damaging action is dependent on the amount of cholesterol and of negatively charged phospholipids in the target lipid bilayer [19]. Moreover, a specific immune response against the toxin was documented in the vaginal mucosal fluid of patients with recurrent bacterial vaginosis [19].

In this paper we have examined the influence of buffer composition on Gvh hemolytic activity. We asked the question whether Gvh conforms to what was proposed as a general rule for membrane-damaging toxins, i.e., to be inhibited by low pH, low ionic strength and divalent cations.

Materials and Methods

Gvh and other toxins. Gvh was prepared and stabilized as described in Ref. 19. Typically, the stock

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Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; calcein, fluorescein complexon; SUV, small unilamellar vesicles; RBC, red blood cells; HRBC, human red blood cells; Gvh, *Gardnerella vaginalis* hemolysin; PFO, *Clostridium perfringens* θ-toxin (perfringolysin O); HlyA, *Escherichia coli* hemolysin; octyl glucoside, n-octyl β-D-glucoside; HBS, Hepes-buffered saline; PEG1000, poly(ethylene glycol), average M_r 1000.

solution contained 1000 HU/ml in 0.7 M ammonium acetate, 6 mM octyl glucoside (pH 7.0). Purified *C. perfringens* θ-toxin (PFO) was kindly donated by Monica Thelestham.

Blood collection and preparation of red cells. Blood was collected by venipuncture either from animals or from healthy human volunteers. Red blood cells were washed thrice (470 $g \times 10$ min) with sterile buffered saline, then a 1% (v/v) RBC suspension was made in buffer HBS: 160 mM KCl, 1 mM EDTA, 10 mM Hepes (pH 7).

Determination of hemolytic activity at equilibrium. Hemolytic activity was measured as in Ref. 19. Briefly: RBC were incubated for 30 min at 37°C in HBS (adjusted to the indicated pH) plus or minus toxin. RBC titer gave a final reading of $A_{415} = 0.950$ after complete hemolysis (i.e., 0.05% for HRBC). Intact red cells were pelleted (1300 $g \times 5$ min) and the A_{415} of released hemoglobin was measured in a 1 cm optical path cell. Completely lysed erythrocytes were prepared by addition of pure water. The percentage of hemolysis was calculated as:

$$\text{hemolysis } (\%) = (A_{+\text{toxin}} - A_{-\text{toxin}})/(A_{\text{waterlysed}} - A_{-\text{toxin}}) \cdot 100 \quad (1)$$

One hemolytic unit (HU_{50}) was defined as the activity sufficient to cause 50% hemolysis under these conditions. Tests were performed in triplicate.

Kinetics of hemolysis in an automatic microplate reader. The time course of RBC lysis at room temperature was followed turbidimetrically at 650 nm using a kinetic 96-well microplate reader (Molecular Devices UVmax) as described [19]. Briefly: each well contained the indicated amount of Gvh, 0.1% (v/v) HRBC, 0.1 mg/ml of BSA and the specified amount of some effector. The entire plate was read, and stirred, every 8 s after starting of the hemolytic reaction and for 30 min. Both kinetic and end-point data were obtained. Percentage of hemolysis was calculated as:

$$\text{hemolysis } (\%) = (A_{\text{initial}} - A_{\text{final}})/(A_{\text{initial}} - A_{\text{lysed}}) \cdot 100 \quad (2)$$

where A_{initial} is the absorbance measured before toxin addition, A_{final} the absorbance at the end of the incubation time, and A_{lysed} that of completely lysed cells. The maximal rate of hemolysis, V_m, was the largest slope of the absorbance versus time curve (the absolute value was reported, in mA/min). Percentage and V_m had in general a similar trend; however, the latter was more sensitive at relatively high toxin doses, when the end-point hemolysis was always 100% but its rate was indeed different. During the experiment the pH of each microwell remained constant, within 0.2 units, as checked with a Lazar pH microelectrode.

Permeabilization of lipid vesicles. Gvh permeabilization of small unilamellar vesicles (SUV) was assayed as described earlier [20–22]. Briefly: lipids, at a starting concentration of 6 mg/ml, were sonicated in the presence of 70 mM calcein; the pH was adjusted to 7.0 by KOH. Sonication was thorough (usually about 40 min) to reach a final equilibrium in SUV size; titanium particles were then removed by centrifugation. SUV were washed through a Sephadex-G25 column using HBS buffer. Aliquots were put into a 1 cm semimicro quartz cuvette with 1 ml of a buffer containing 160 mM KCl, 1 mM EDTA, 10 mM Hepes, at the pH indicated, temperature was 37°C. The final lipid concentration was typically 2–4 μg/ml and the solution was continuously stirred. After mixing with toxin, the release of calcein from the vesicles produced an increase in the fluorescence F emitted at 520 nm (excitation at 494 nm), due to the dequenching of the internal dye into the external medium. Maximum release, yielding the fluorescence F_{max}, was determined by addition of 0.4 mM Triton X-100. The extent of permeabilization, P, was calculated as percentage of the maximum release obtained with Triton X100, as follows:

$$P(\%) = (F_{\text{fin}} - F_{\text{in}})/(F_{\text{max}} - F_{\text{in}}) \cdot 100 \quad (3)$$

where F_{in} and F_{fin} represent the initial and final value of fluorescence before and after toxin addition, respectively. Spontaneous release of calcein from SUV was negligible under these conditions.

Different lipid compositions were used as specified in the text. Lipids employed were: egg phosphatidylcholine, (PC, Calbiochem); phosphatidylserine, (PS, Lipid Products) and cholesterol (Serva). All lipids were more than 99% pure by TLC. Calcein was from Sigma.

Results and Discussion

Hemolysis by Gvh is modulated by the composition of the isotonic buffer in which the red cells are maintained during the assay. The effects of pH, ionic strength and divalent cations were studied.

Effects of pH on Gvh-induced RBC lysis

With HRBC the hemolytic activity of Gvh is a continuously decreasing function of the pH (Fig. 1), at least within the accessible range (which is restricted by the viability of the RBC itself at non-physiological pH). The pH effect is not limited to human erythrocytes, which are the most sensitive towards Gvh, but appears to be true also for other mammalian RBC (Table 1), despite their reduced sensitivity to the toxin. This would favor an effect of protons on Gvh itself rather than on membrane receptors which could be present on human RBC. This hypothesis is also consistent with the finding that low pH increases the permeabilizing

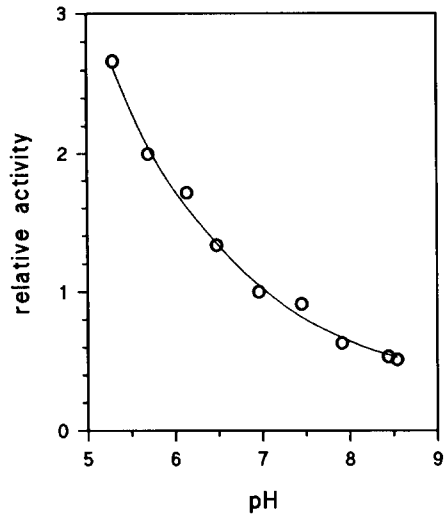

Fig. 1. Effect of pH on Gvh-induced HRBC lysis. The hemolytic activity of Gvh on 0.05% HRBC was measured after 30 min incubation at 37°C in HBS (adjusted to the indicated pH). Results are normalized by dividing by the activity at pH 7, which resulted in 417 HU/ml.

activity of Gvh against purely lipidic vesicles (as will be shown later). It has been demonstrated that in many cases low pH inhibits the activity of membrane-damaging agents, toxins and even detergents [23]. However, there are also a number of hemolytic toxins, particularly of bacterial origin, which, like Gvh, become more efficient around pH 5. Examples can be found both among bacteria of Gram-positive origin (e.g., *S. aureus* α-toxin [20,24], and *C. perfringens* PFO [25]) and of Gram-negative origin (e.g., *E. coli* HlyA [21,25])

Effects of low ionic strength on Gvh-induced HRBC lysis

An inhibition of toxin activity was observed at low ionic strength. Progressive replacement of NaCl with mannitol in an isotonic assay buffer led initially to a slight increase in activity, but then to a marked decrease (full protection from leakage was observed at mannitol concentrations above 250 mM – data not shown). The same results were obtained using sucrose or glucose, so this effect seems to be correlated only to the lowering of the ionic strength. Fig. 2 exemplifies

TABLE I

Relative hemolytic activity of Gvh on RBC of different origin

Activity on human RBC at pH 7.0 was arbitrarly set equal to 100%.

Species	Relative activity						
	human	cat	cow	dog	horse	pig	rabbit
pH 7.0	100	8.8	1.5	1.4	0.6	3.2	0.7
pH 6.0	165	14.5	3.1	1.6	0.6	5.4	3.7

Note: Sheep RBC were also tried and found to be completely refractory to the toxin at both pH values. They were not reported for this reason.

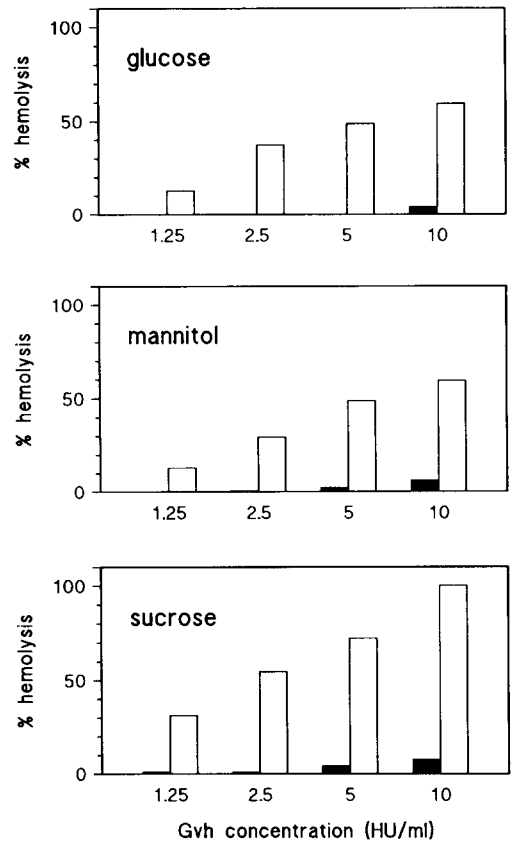

Fig. 2. Effect of low ionic strength on Gvh hemolytic activity. The extent of HRBC hemolysis elicited by different amounts of Gvh in low ionic strength solutions (300 mM sugar, 5 mM Hepes (pH 7.4)) is given by the filled bars. Solutions were kept iso-osmolar by either glucose or mannitol or sucrose, as shown. In normal saline, hemolysis is virtually 100% for Gvh activity $\leq$ 2.5 HU/ml (not shown). Empty bars represent the hemolysis obtained washing the cells (after incubation with the toxin in low salt) and resuspending them in normal saline.

the results obtained in 300 mM sugar, 5 mM Hepes (pH 7.4). Viscosity effects, which could decrease the mobility of the toxin in solution, seem not to be involved. In fact: (a) the relative increase in viscosity is expected to be very small, e.g., 1.16 with 300 mM glucose and 1.33 with 300 mM sucrose (data from Handbook); (b) the viscosity increase is larger with sucrose but the protection from hemolysis is larger with glucose; (c) larger sugars as PEG1000 at 30 mM elicited a larger increase of solution viscosity but no protection [19].

We found that cells washed after incubation with the toxin in low salt, and resuspended in normal saline, were lysed, indicating that toxin binding was not prevented at low ionic strength (Fig. 2). These findings agree with what appeared to be a quite general rule for membrane-damaging agents [26], i.e., that they are inhibited by low ionic strength. However, it should be noted that even in this case some exceptions have been documented (e.g., *Cerebratulus lacteus* cytolysin A-III [27]).

Effects of divalent cations on Gvh-induced HRBC lysis

A protective effect against lysis was also elicited by divalent cations (Fig. 3), which decreased, in a dose dependent way, both the extent of hemolysis (panel A) and its rate (panel B). Comparing panels A and B it can be noted that the cation concentrations which decrease hemolysis to $\approx 50\%$ attain a decrease of V_m to a value of ≈ 2 mA/min. We found that this is true in general, i.e., under different experimental conditions, and thus we considered these two concentrations as equivalent estimates of the inhibition efficacy. Such concentrations (expressed in mM) were found to be: $Mg^{2+} = 37.5 \pm 2.8$ (4), $Ca^{2+} = 34.8 \pm 2.3$ (4), $Sr^{2+} = 26.0 \pm 1.0$ (3), $Ba^{2+} = 29.3 \pm 0.8$ (3), $Co^{2+} = 23.7 \pm 1.3$ (2), $Cd^{2+} = 6.0 \pm 1.9$ (2), $Zn^{2+} = 0.68 \pm 0.15$ (3) where values are mean $\pm$ S.E. and figures in parentheses are the number of experiments. Thus, no major difference was observed between magnesium, calcium, strontium, barium and cobalt, which all require a very high concentration to completely abolish hemolysis (≈ 0.1 M), suggesting that some unspecific screening of negative charges is probably involved. Protection by Ca^{2+} was abrogated when the red cells were washed, after exposure to toxin in the presence of the divalent ion, and resuspended in normal saline buffer (not shown). Therefore, it appears that, at least in the case of Ca^{2+}, the primary action of the divalent cation is not to prevent toxin binding to the erythrocytes. A similar behavior was documented for other pore-forming agents and it was postulated that divalent cations could block leakage at a stage of lesion formation subsequent to toxin binding, probably by somehow closing already formed pores [25,26].

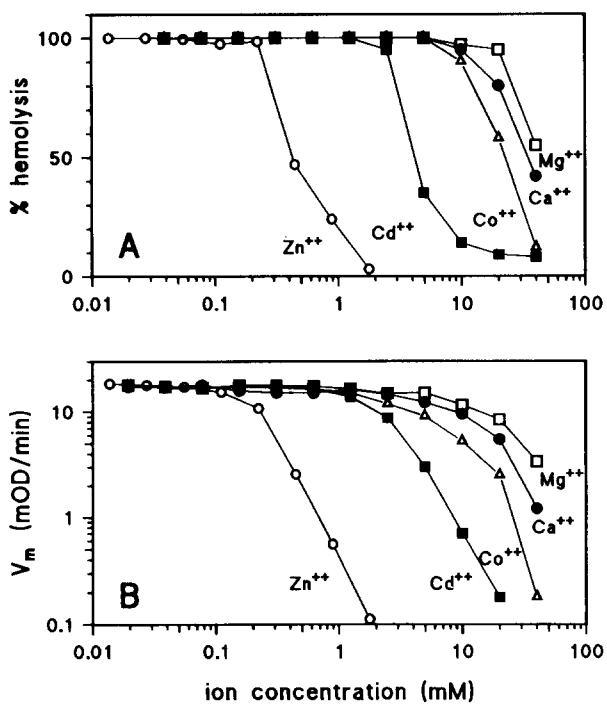

Fig. 3. Effect of divalent cations on Gvh-induced HRBC lysis. Hemolysis was measured in a microplate reader (as described in the Methods section) in the presence of varying divalent-cation concentrations. The extent of hemolysis (% lysis) and its maximal rate (V_m) are reported in panels A and B, respectively. Each well contained, in a total volume of 200 μl HBS: 4 HU/ml of Gvh, 0.1% HRBC, 0.1 mg/ml of BSA and various concentrations of divalent cations as indicated. The initial A_{650} was about 0.13 A. The protocol used for preparing the microplate was: first buffer, then the appropriate amount of divalent cation, then HRBC, finally toxin.

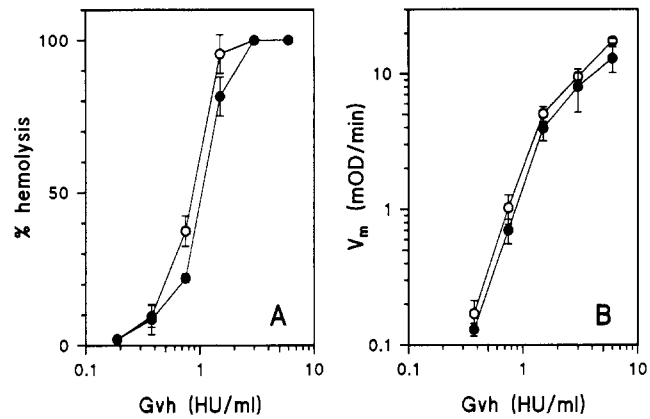

Fig. 4. Effects of preincubation with Zn^{2+} on Gvh hemolytic activity. Gvh was preincubated with 1 mM Zn^{2+} for 30 min at room tenperature, then its hemolytic activity was measured in a microplate reader (as described in Fig. 3) in the presence of EDTA in excess of the residual Zn^{2+}. The extent and the rate of hemolysis at different toxin concentrations are reported in panels A and B, respectively. Each well contained a final volume of 200 μl HBS with different amounts of Gvh (either preincubated with Zn^{2+}, filled circles, or with buffer, empty circles), 0.1 mg/ml of BSA, and 0.1% HRBC. Protocol for preparing the microplate: first buffer, then the appropriate amount of Gvh, finally HRBC.

We investigated whether Zn^{2+} (the most effective divalent ion) protects HRBC by binding with high affinity to the toxin. We found that preincubation of Gvh with 1 mM Zn^{2+} for 30 min at room temperature, does not appreciably reduce its hemolytic activity tested in the presence of excess EDTA in the assay buffer (Fig. 4). This excludes the possibility that Zn^{2+} could promote an irreversible aggregation of the toxin, as was observed in the case of perforin and complement [28–30], and indicates that, if Zn^{2+} binding sites are present on the protein, they have an affinity lower than EDTA. In all these experiments (Fig. 3 and 4) hemolysis was measured turbidimetrically (with a microplate reader) independently of hemoglobin concentration. Hence, we can rule out that the observed Zn^{2+} effects are due to a nonspecific Zn^{2+}-induced precipitation of hemoglobin as was suggested in some cases [31,32]. In any case, we did not observe substantial precipitation of hemoglobin.

Interaction with model membranes

Since Gvh-induced HRBC lysis appears to result from the formation of toxin channels into the cell

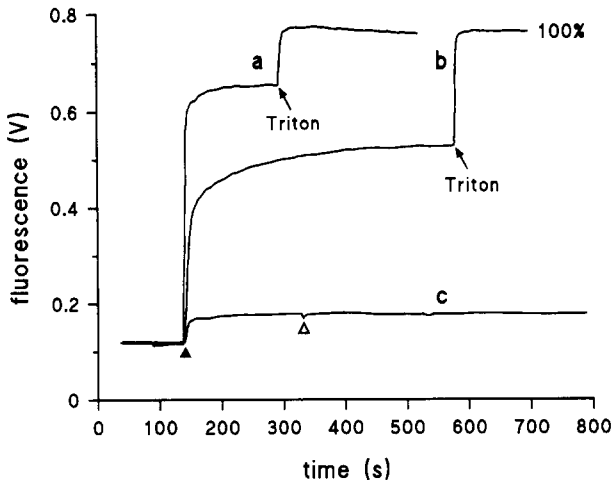

Fig. 5. Gvh-induced permeabilization of lipid vesicles loaded with calcein. The full arrow head indicates the addition of 60 μl of Gvh, dissolved in a buffer containing 6 mM octylglucoside, to PS/cholesterol vesicles (1/1 molar ratio) in 1 ml of solution containing 160 mM KCl, 1 mM EDTA, 10 mM Hepes, buffered either at pH 4 (trace a) or at pH 7 (trace b) and thermostatted at 37°C. The fluorescence increase after toxin addition indicates that the dye is released from the vesicles. Fluorescence is measured in volts using a unitary gain of the fluorometer. 100% release was obtained by the addition of 1 mM Triton X-100 (indicated by arrows). Final lipid concentration was 20 μg/ml (about 33 μM), final toxin concentration was 600 HU/ml. Trace c is a control showing the effects of 60 μl octyl glucoside buffer alone at pH 4, a second addition of further 60 μl octylglucoside is indicated by the empty arrow head. At pH 7 the detergent was even less effective (not shown).

membrane [19] we were prompted to investigate whether the toxin can form pores also in model lipid bilayers (like it was shown for many other cytolytic toxins [33,34]). We observed that addition of Gvh to a solution containing SUV loaded with calcein (at a self-quenching concentration) could indeed promote the release of the dye, indicated by a fluorescence increase (Fig. 5). Gvh-induced SUV permeabilization improves when they contain cholesterol (a major component of the plasma membrane of mammalian cells and particularly of RBC) and also by lowering the pH of the bathing solution. The percent of calcein release at pH 7 and 5 was 40 and 66, respectively, for SUV comprised of PS/cholesterol (1:1 molar ratio), 5 and 30 for SUV comprised of PC/cholesterol (1:1 molar ratio) and almost nil for SUV comprised of either pure PC or pure PS. It can be observed that release is more relevant with vesicles containing negatively charged lipids, in agreement with the toxin's capacity to bind to lipid bilayers. A similar pH and cholesterol dependence was observed also with PFO [25].

Conclusions

We demonstrated that low pH enhances Gvh damaging activity. This finding is not general among hemolysins [23], and suggests that the toxin may be an important factor in the invasiveness of *G. vaginalis* at an early stage. In fact, the normal pH of the vagina is around 4.5, a value which is unfavourable for bacterial proliferation but which potentiates Gvh action. Colonization of the vagina leads then to an increase in pH toward a value of 7, which is more favourable for the bacterium, but somehow inhibitory for the toxin. Hence, Gvh appears to be an important factor for survival of *G. vaginalis* under the unfavourable conditions encountered during the early stages of colonization.

Divalent cations are neither required for, nor strongly inhibitory toward, Gvh action, suggesting that the toxin is perfectly functional under normal serum conditions. This finding is confirmed by the very limited capacity of blood serum itself to prevent the function of the toxin [19].

The extremely high specificity of Gvh for human erythrocytes may suggest that a receptor is present on these human cells. However, the ability of the toxin to attack SUV in a similar pH-dependent way indicates that not necessarily a protein receptor has to be present on the target bilayer for the damage to occur. In the case of SUV, cholesterol seems to be involved (see also Ref. 19). It is possible that the lipid components of the cell membrane have an anchoring effect on the toxin and that small variations in lipid composition can attain big changes in cell response.

It was suggested that most membrane-damaging toxins conform to the following behaviour: they are inhibited by low pH, low ionic strength and divalent cations. Gvh does not conform completely to this rule, becoming more active at low pH. In this sense it is similar to PFO and HlyA, although genetically unrelated [19]. Inhibition by low ionic strength and divalent cations is not due to a decreased binding, suggesting that a subsequent step, necessary for pore opening, is impaired.

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