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Novel evidence for the specific interaction between cholesterol and α-haemolysin of *Escherichia coli*

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Several toxins that act on animal cells present different, but specific, interactions with cholesterol or sphingomyelin. In the present study we demonstrate that HlyA (α-haemolysin) of *Escherichia coli* interacts directly with cholesterol. We have recently reported that HlyA became associated with detergent-resistant membranes enriched in cholesterol and sphingomyelin; moreover, toxin oligomerization, and hence haemolytic activity, diminishes in cholesterol-depleted erythrocytes. Considering these results, we studied the insertion process, an essential step in the lytic mechanism, by the monolayer technique, finding that HlyA insertion is favoured in cholesterol- and sphingomyelin-containing membranes. On the basis of this result, we studied the direct interaction with either of the lipids by lipid dot blotting, lysis inhibition and SPR (surface plasmon resonance) assays. The results of the present study demonstrated that an interaction between cholesterol and HlyA exists that seems to favour a conformational state of the protein that allows its correct insertion into the membrane and its further oligomerization to form pores.

Key words: cholesterol, cholesterol-recognition amino acid consensus, α-haemolysin (HlyA), monolayer, surface plasmon resonance (SPR).

INTRODUCTION

Microbial toxins whose targets are animal cells, such as HlyA (α-haemolysin), an exotoxin of wide lytic capability secreted by uropathogenic strains of *Escherichia coli*, have specific forms of interaction with membranes containing Cho (cholesterol) and SM (sphingomyelin). HlyA is a member of an extensive family of toxins, referred to as RTX (repeat in toxins), that are synthesized by Gram-negative organisms sharing several functional and genetic features [1]. Synthesis, maturation and secretion of *E. coli* HlyA are determined by the *hlyCABD* operon [2]. Gene product A is a 110 kDa polypeptide corresponding to a protoxin that is accordingly matured in the bacterial cytosol to the active form (HlyA) by HlyC-directed acylation at two internal lysine residues (Lys^{563} and Lys^{689}) [3]. HlyA is then secreted across both membranes by the type I secretion system employing an uncleaved C-terminal recognition signal [4]. Like other members of the RTX family, HlyA is extracellularly secreted as a soluble protein, although an alternative secretion mechanism by outer membrane vesicles was identified previously [5].

The lytic mechanism of HlyA is a complex process. Three stages seem to be involved that ultimately lead to cell lysis: binding, insertion and oligomerization of the toxin within the membrane. Studies that have explored the binding of the toxin to membranes and attempted to characterize a putative receptor for the toxin have produced contradictory results. One group demonstrated that HlyA binds non-specifically to rabbit erythrocyte membranes [6], whereas Cortajarena et al. [7] identified glycophorin as a receptor for HlyA in assays employing horse erythrocytes. With respect to the insertion of the toxin into the membrane, Hyland et al. [8] demonstrated that the principal region inserting into the membrane lies between residues 177 and 411. Furthermore, insertion is independent of membrane lysis, since totally non-lytic mutants are able to insert into lipid monolayers [9]. Once the toxin is inserted oligomerization occurs. We found that the fatty acids covalently bound to the toxin induce a molten globule state in the protein exposing intrinsically disordered regions that promote protein–protein interaction [10].

Upon characterization, the HlyA pore has proved to be a dynamic one, whose size depended on both time and toxin concentration [2]. The pore is proteolipidic in nature since its conductance and membrane lifetime in black-lipid-membrane experiments were found to be dependent on membrane composition [11].

Several previous results suggested the involvement of Cho and/or SM in the lytic mechanism of the toxin. Leakage experiments involving LUVs (large unilamellar vesicles) of different lipid compositions revealed that although HlyA can provoke the leakage of the contents of liposomes composed solely of PC (phosphatidylcholine), the percentage leakage increases when Cho and SM are present [12]. As to the oligomerization, we have reported previously that when erythrocytes were depleted of Cho by methyl-β-cyclodextrin treatment, toxin oligomerization, and hence haemolytic activity, diminished, suggesting that membrane microdomains enriched in SM and Cho might be implicated in the oligomerization [13]. These results have been largely attributed to a modification of membrane properties by Cho involving lateral segregation of lipids and the consequent

Abbreviations: AFM, atomic force microscopy; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; Cho, cholesterol; CRAC, cholesterol recognition/interaction amino acid consensus; DOPC, dioleoyl PC; DPH, diphenylhexatriene; DPX, *p*-xylenebispyridinium bromide; Hly, haemolysin; LUV, large unilamellar vesicle; PC, phosphatidylcholine; Pch, phosphorylcholine; PE, phosphatidylethanolamine; RTX, repeat in toxins, RU, resonance unit(s); SM, sphingomyelin; SMase, sphingomyelinase; SPR, surface plasmon resonance.

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formation of microdomains. Nevertheless, the nature and effects of specific lipid–protein interactions have not yet been directly investigated. In view of these considerations, the aim of the present study was to investigate the interaction between HlyA and Cho and/or SM and their roles in the mechanism of action of the toxin.

MATERIALS AND METHODS

Materials

Lipids were purchased from Avanti Polar Lipids. Pch (phosphorylcholine), glycophorin A (G5017), *Bacillus cereus* SMase (sphingomyelinase) and salts were from Sigma Chemicals. Polyclonal goat anti-rabbit antibodies were from Pierce and the remaining antibodies were from Abcam. Polyclonal rabbit anti-HlyA antibodies were raised in our laboratory following the protocol published by Cortajarena et al. [7].

Ethics statement

Blood from healthy sheep was obtained following a protocol approved by the Research Ethics Committee of the Facultad de Ciencias Veterinarias of the Universidad Nacional de La Plata (CICUAL 129/09) in accordance with international guidelines for care and use of laboratory animals.

All the studies performed with sheep erythrocytes were approved by the Institutional Review Board of the INIBIOLP and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Instituto de Investigaciones Bioquimicas de La Plata's Animal Welfare Assurance number A5647-01).

Erythrocyte isolation

Sheep blood obtained by jugular venipuncture was anticoagulated by manual defibrination. The total erythrocyte population was separated from the other blood components by centrifugation at 2000 ***g*** for 15 min at 20 °C. After washing the erythrocytes three times with 0.9 % NaCl and centrifugation at 2000 ***g*** for 5 min, packed erythrocytes were resuspended in Alsever's solution (Sigma Chemicals) for storage. The standardization of the sheep erythrocytes was performed just before the haemolytic assay. The erythrocytes were washed in 0.9 % NaCl and then diluted to 12.5 μl in 1 ml of distilled water to give a reading of 0.6 absorbance unit at 412 nm.

SM was removed from the erythrocytes by incubation with *B. cereus* SMase. A total of 1 ml of washed erythrocytes was incubated with 0.7 units/ml of *B. cereus* SMase for 60 min at 37 °C. Erythrocytes were washed extensively before use in the haemolytic assay. The removal of SM from erythrocyte membranes was confirmed by TLC. Total lipids were extracted using the procedure of Folch et al. [14] and subjected to analysis by TLC on high-performance TLC plates (Whatman), developed with a solvent system consisting of chloroform/methanol/water (25:10:1, by vol.). Lipid spots were detected by spraying with sulfuric acid/ethanol (5:100, v/v) and charring at 120 °C. A densitometric analysis was performed using ImageJ 1D Image Analysis software (http://rsb.info.nih.gov/ij/).

Protein purification

E. coli HlyA was purified from culture filtrates of overproducing strains of *E. coli* WAM 1824 (provided by Dr Rodney A. Welch, University of Wisconsin School of Medicine, Madison, WI, U.S.A.). Cultures of *E. coli* cells were grown to late log phase in LB medium to an attenuance at 600 nm (D_{600}) of 0.8–1.0. Cells were pelleted and the supernatant concentrated and partially purified by precipitation with 20 % (v/v) ice-cold ethanol at pH 4.5, the pI of the toxin. The precipitate containing the protein was collected by centrifugation (1 h at 14500 ***g*** in a Sorvall centrifuge with an SSA 34 rotor) and then resuspended in TC buffer [20 mM Tris/HCl and 150 mM NaCl (pH 7.4)] containing 6 M guanidinium chloride. Analysis by SDS/PAGE (10 % gel) of this preparation showed a main band at 110 kDa corresponding to more than 90 % of the total protein. Proteins of lower molecular mass were removed by dialysis (membrane cut-off, 30 kDa). The protein was stored at − 70 °C in guanidinium chloride. The proteins were dialysed in TC buffer (1:100, v/v) at 4 °C for 4 h before each experiment.

Surface pressure measurements

Surface pressure experiments were carried out with a NIMA Langmuir–Blodgett trough Model 102M (KSV-NIMA Biolin Scientific) with a Wilhelm platinum plate as the surface pressure sensor. The aqueous phase, or subphase, consisted of TC buffer containing 10 mM $CaCl_2$. The lipid, dissolved in chloroform/methanol (2:1, v/v), was gently spread over the surface of a Teflon microtrough containing 200 μl of subphase until the desired initial surface pressure was attained (25 mN/m). The protein was injected with a micropipette into the subphase bulk. The increment in surface pressure against time was recorded until a stable signal was obtained. All the experiments were repeated at least three times for each lipid monolayer to ensure consistent results.

The measurements of surface pressure as a function of time allows the membrane-insertion kinetics to be determined by applying eqn (1):

$$\pi - \pi_0 = \pi_\infty - \pi_0(1 - e^{-bt}) \quad (1)$$

where π is the surface pressure at time t, π_0 is the surface pressure at the moment of injection of the protein into the aqueous subphase, π_∞ is the surface pressure at the plateau and b is the rate constant of protein insertion into the lipid monolayer.

Lysis inhibition by Cho and Pch

HlyA was pre-incubated in a microtitre plate with increasing concentrations of Cho or Pch in a total volume of 100 μl of TC buffer with 10 mM $CaCl_2$ for 30 min at 22 °C. A 100 μl aliquot of standardized sheep erythrocyte suspension was then added to each well. After 30 min at 37 °C, the plates were centrifuged and haemolysis was quantified as the haemoglobin released by measuring the absorbance at 412 nm.

Cho stock solutions for this assay were prepared in 100 % ethanol. The final concentration of ethanol was always lower than 2 % (v/v). In controls carried out in order to check if ethanol affected the erythrocyte membrane integrity and HlyA activity, samples were incubated with identical volumes of ethanol or ethanol and Cho to those used in the inhibition tests. The inhibitory concentration (IC_{50}) was defined as the amount of Cho required to inhibit 50 % haemolysis and was calculated by fitting the data to a four-parameter logistic equation using SigmaPlot 11.0 software (Systat Software).

Lipid dot-blot assay

Lipids were spotted on to nitrocellulose membranes (GE Healthcare) from a 1 nmol/μl stock solution in chloroform. Membranes were left to dry at room temperature (25 °C) for 1 h in

order to evaporate all the solvent from the spotted lipids and were then blocked for 2 h in 3 % (w/v) non-fat dried powdered skimmed milk dissolved in TBS buffer. HlyA, at a final concentration of 30 nM, was then added in the presence of 10 mM $CaCl_2$ and incubated for 3 h at room temperature on a rotary shaker. Membranes were washed five times in TBS buffer, each time for 5 min, and then incubated with rabbit anti-HlyA antibodies in 3 % (w/v) non-fat dried powdered skimmed milk in TBS buffer (1:500 dilution) for 2 h. Next, the membranes were washed with the same buffer as described above and incubated with secondary goat anti-rabbit antibodies conjugated with horseradish peroxidase (1:1000 dilution) for 2 h. Finally, the membranes were washed with TBS buffer as above and the dot blots developed by chemiluminescence.

Liposome preparation

Lipids were dissolved in chloroform and dried on to a film under a stream of N_2. The lipids were then resuspended in TC buffer. LUVs were prepared by extrusion and sized through the use of 100 nm pore-size polycarbonate filters (Avestin) in a Lipofast Extruder (Avestin).

SPR (surface plasmon resonance) assays

SPR analysis was carried out using the Biacore T100 platform (GE Healthcare). HlyA was dialysed against sodium acetate (pH 4) and immobilized [450 RU (resonance units)] over a CM5 chip surface previously activated with an EDC [*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodi-imide]/NHS (*N*-hydroxysuccinimide) mixture as described previously [15]. The remaining coupling sites were blocked with 1 M ethanolamine. Analytes, liposomes of different composition and glycophorin, were prepared with running buffer (TC buffer with 10 mM $CaCl_2$).

Before the immobilization process of HlyA, the haemolytic activity of the toxin, kept at pH 4 for 1 day and then dialysed against TC buffer (pH 7.4), was assayed. Following this pH treatment the toxin maintained its activity. Analytes, in 2-fold dilutions, were applied at random concentrations over the immobilized HlyA. After each cycle, the surface was regenerated with 10 mM glycine/HCl buffer (pH 3) in order to return to the baseline. One flow cell was always left blank to serve as a reference surface and was subjected to the same treatment as described above.

The data analysis was performed using BIAevaluation™ software to fit to a kinetic one/one binding model or determining the dissociation constant (K_D) of the system under equilibrium conditions when the kinetic parameters fell outside of the limits of the equipment.

Leakage measurements

Leakage of the vesicle's aqueous content was measured using the ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid)/DPX (*p*-xylenebispyridinium bromide) assay. Vesicles of different lipid composition containing 12.5 mM ANTS, 45 mM DPX, 20 mM Tris/HCl and 70 mM NaCl (pH 7.4) were prepared by five cycles of freeze–thawing and subsequent polycarbonate filter extrusion of the lipid suspension as mentioned above. Untrapped material was removed by gel filtration on a Sephadex G-75 column in TC buffer. The lipid concentration of the LUVs after preparation was determined using the phosphorus colorimetric method [16]. HlyA was serially diluted in TC buffer containing 10 mM $CaCl_2$ in a 96-well microtitre plate. A 100 μl aliquot of the diluted suspensions were mixed with 100 μl of 200 μM LUVs in TC buffer. The plate was then incubated at 37 °C for 20 min and the fluorescence of the ANTS measured. The excitation and emission wavelengths were 355 nm and 525 nm respectively.

The fluorescence intensity corresponding to 100 % leakage was determined by the addition of 10 μl of 10 % (v/v) Triton X-100 to the vesicle suspension (0.25 % final concentration). The percentage leakage (%L) was calculated using eqn (2):

$$\%L = 100(F_p - F_0)/(F_{100} - F_0) \quad (2)$$

where F_p is the final fluorescence intensity after the addition of protein (20 min), and F_0 and F_{100} are the fluorescence intensities before protein and after the addition of Triton X-100 respectively.

Data presentation

Data are expressed as means ± S.D. for $n = 3$ independent experiments and analysed using Student's t test.

RESULTS AND DISCUSSION

Study of the insertion of HlyA into monolayers containing Cho and SM

We have reported recently that HlyA became associated with detergent-resistant membranes enriched in Cho and SM and that this association completely disappeared when erythrocytes were Cho-depleted by methyl-β-cyclodextrin treatment [13]. Moreover, after Cho depletion, toxin oligomerization, and hence haemolytic activity, diminished. Considering these results, we postulated that Cho- and SM-enriched domains had enabled the accumulation of a sufficient local concentration of HlyA to achieve the oligomerization that ultimately led to cell lysis [13]. The oligomerization of the toxin should occur if the toxin is simply inserted in the appropriate conformational state for allowing protein–protein interaction. Accordingly, in order to study the insertion process of HlyA, we used the monolayer technique. This technique, in particular, allows the direct observation of the insertion phenomenon separated from further changes in lipid architecture because, by design, the monolayer cannot undergo the 3D membrane restructuring that would be essential for altering the membrane-permeability barrier. Thus the lipid monolayer studies enabled a scrutinization of the insertion step itself apart from the membrane lysis that would subsequently occur [9].

Monolayers composed of DOPC (dioleoyl PC)/SM (16:0)/Cho (2:1:1 molar ratio) were chosen because of the ability that SM (16:0) has to form a two-phase system when mixed with a short or unsaturated PC and Cho, mimicking the outer layer of animal cell membranes (S.M. Maté, R.F. Vazquez, V.S. Herlax, M.A. Daza Millone, M.L. Fannani, B. Maggio, M.E. Vela and L.S. Bakas, unpublished work). The experiment shown in Figure 1 was performed with monolayers of pure DOPC or with the ternary lipid mixture composed of DOPC/SM (16:0)/Cho (2:1:1 molar ratio) being extended at the air/water interface of a Langmuir balance at an initial surface pressure (π_i) of 25 mN/m. HlyA (120 nM) was injected into the subphase in the presence of 10 mM Ca^{2+}. The results show that HlyA insertion into a pure DOPC monolayer and into a DOPC/SM (16:0)/Cho monolayer leads to a total increase in the surface pressure of 6 mN/m and 10 mN/m respectively, indicating that toxin insertion is favoured in the membrane containing SM and Cho.

The kinetics of insertion of the protein into both lipid/mixture monolayers exhibited an exponential dependency, such as the one

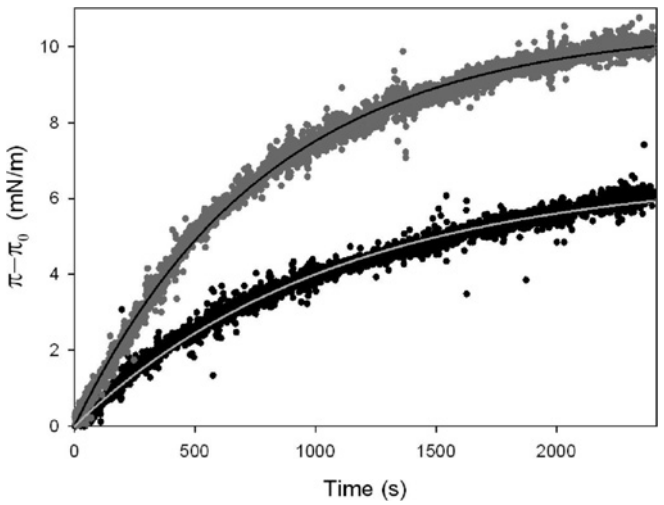

Figure 1 Time course insertion of HlyA into lipid monolayers

The experiment was performed at an initial lateral pressure of 25 mN/m in the presence of 10 mM $CaCl_2$. The lipid composition was DOPC (black line) or DOPC/SM/Cho (grey line), and the initial protein concentration in the subphase was 120 nM.

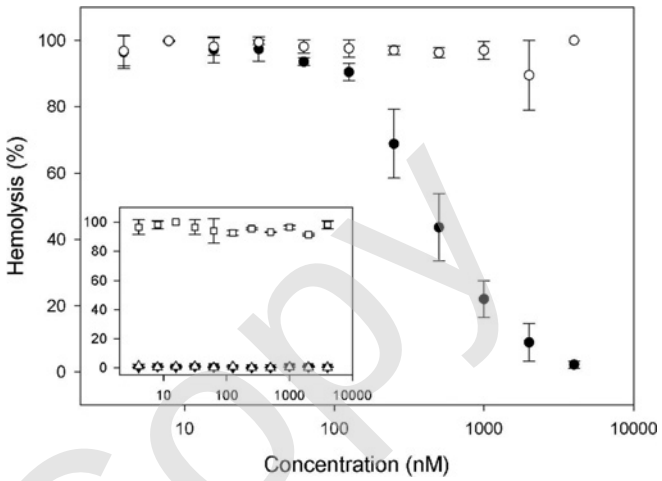

Figure 2 Lysis inhibition of sheep erythrocytes

Cells were incubated with 0.25 nM HlyA in the presence of Cho (●) or Pch (○) at the indicated concentrations and haemolysis read after 30 min at 37 °C. The inset shows control experiments. Erythrocytes were incubated with the same volumes of ethanol (△) or ethanol and Cho (▼) as those used in the inhibition assays. HlyA activity was tested in the presence of ethanol (□). Results are means ± S.D. for three independent experiments.

shown in eqn (1), characterized by rate constants of 9.14×10^{-4} s^{-1} and 1.25×10^{-3} s^{-1} for the DOPC and DOPC/SM/Cho monolayers respectively. This result indicated that HlyA inserted 37 % faster into a monolayer containing Cho and SM than in DOPC.

On the basis of studies on a series of proteins with different tertiary structures, Graham and Phillips [18] postulated that the increase in the surface pressure of a lipid monolayer can be caused either by the insertion of proteins or by a conformational rearrangement of the proteins in the adsorbed layer. These results could therefore reflect not only a favoured insertion of the toxin in the ternary lipid mixture, but also that the toxin can adopt competent tertiary structures to allow proper oligomerization in this lipid mixture compared with the pure DOPC monolayer.

Study of the interaction of HlyA with Cho

Since Cho and/or SM facilitate the insertion of the toxin into lipid monolayers, we studied the interaction between these two lipids and HlyA using the lysis-inhibition assay. For this purpose HlyA was treated with Cho and Pch (the headgroup of SM and PC) before the interaction of the toxin with erythrocytes.

Figure 2 shows that pre-treatment of the toxin with Cho, but not with Pch, inhibits erythrocyte lysis. Haemolysis was completely inhibited at a concentration of 4×10^3 nM Cho. The IC_{50} value for the inhibition of HlyA haemolysis by Cho was calculated from the data and estimated to be 440 ± 40 nM. In contrast, Pch was not able to inhibit HlyA-induced haemolysis at the concentrations assayed for Cho and inhibited only at the considerably higher concentration of 25 mM (results not shown). For control experiments, erythrocytes were incubated with identical volumes of ethanol or ethanol and Cho used in the inhibition tests. As shown in the inset of Figure 2, neither ethanol nor ethanol and Cho lysed erythrocytes and the haemolytic activity of HlyA was furthermore unaffected by ethanol.

The correlation between the SM content of the membrane and the lytic activity of HlyA was studied by measuring the haemolytic activity of HlyA on control and SMase-treated erythrocytes. The latter were obtained after the treatment of the erythrocytes with 0.7 units/ml *B. cereus* SMase at 37 °C for 1 h, which reduced the SM content to 32.5 ± 0.33 % (Figure 3B). To calculate the decrease in SM content in SMase-treated erythrocytes the attenuance (D) value corresponding to the SM spot was compared with the D value corresponding to the PE (phosphatidylethanolamine) spot, the major glycerophospholipid class in the sheep erythrocyte membrane (Table 1). Figure 3(A) shows that although erythrocytes treated with SMase are a bit more sensitive towards HlyA, the difference is not statistically significant compared with the control erythrocytes. This slight difference might be due to instability of these erythrocytes caused by SMase treatment. This result indicates that the SM content of the membrane does not modify HlyA activity. Altogether these results suggest that HlyA does not need to interact with SM to exert its activity.

Cho has a maximum solubility in aqueous solutions of 4.7 μM and undergoes a thermodynamically reversible self-association at a critical micellar concentration of 25–40 nM at 25 °C. This exceptionally low critical micellar concentration indicates that Cho micelles are stabilized by strong intermolecular attractive forces in addition to hydrophobic bonding. Since the binding of any amphiphilic ligand to a protein necessarily involves a competition with the self-association of the amphiphile, and because the free-energy gain on self-association is very large, a direct binding of Cho to a protein can occur only if that protein has a binding site for the sterol of a high affinity [19].

In order to corroborate a direct interaction between HlyA and Cho, a lipid dot-blot assay was performed. Figure 4 shows that HlyA interacted with only Cho in a concentration-dependent fashion. Only a very slight interaction was present for PC at 1 nmol of lipid, but not at lower lipid concentrations. No binding at all took place with SM. The specific interaction between Cho and HlyA was also demonstrated using SPR, a technique that has become one of the most effective for studying macromolecular interactions. Liposomes composed of DOPC/Cho (4:1 molar ratio) underwent a specific interaction with immobilized HlyA characterized by a K_D value of $1.6 \pm 0.7 \times 10^{-5}$ M as determined under equilibrium conditions (Figures 5A and 5B) after unspecific interaction with the carboxymethyl dextran matrix was subtracted.

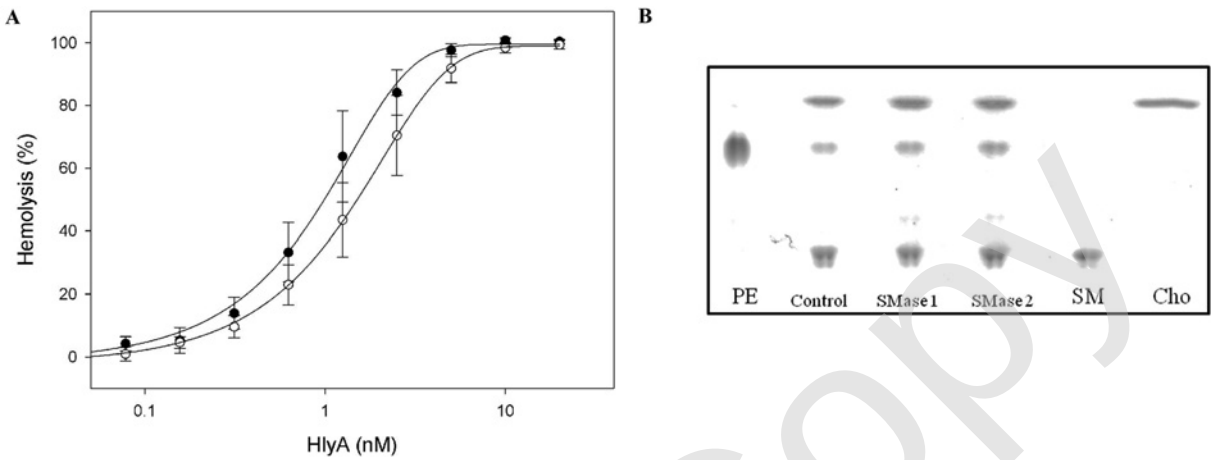

Figure 3 Haemolytic activity of HlyA on contral and SMase-treated erythrocytes

Haemolytic activity of HlyA on control and SMase-treated erythrocytes (**A**). Before the haemolytic assay erythrocytes were treated with 0.7 units/ml *B. cereus* SMase (1 h at 37 °C). Results are the mean ± S.D. haemolytic percentage of HlyA on control (○) and SMase-treated (●) erythrocytes ($n = 4$). (**B**) HPTLC of total lipids from control and SMase-treated erythrocytes (SMase 1 and SMase 2). Commercial lipid standards of PE, SM and Cho were used.

Table 1 Attenuance of SM and PE spots on HPTLC

Densitometric analysis was performed using 1D Image Analysis software (ImageJ). To calculate the decrease in SM content in SMase-treated erythrocytes the attenuance (*D*) value corresponding to the SM spot was compared with the *D* value for the PE spot.

Lipid	Control erythrocytes	SMase 1-treated erythrocytes	SMase 2-treated erythrocytes
PE	8502.08	12181.81	13406.29
SM	24162.14	23228.63	25256.87
SM/PE	2.8	1.9	1.88

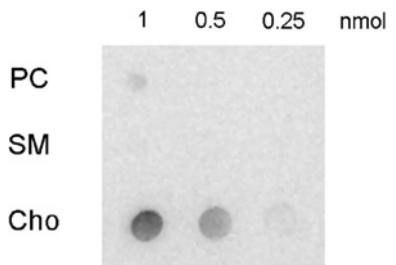

Figure 4 Direct binding of HlyA to Cho as demonstrated by a lipid dot-blot assay

PC, SM and Cho were spotted on to nitrocellulose membranes. The membranes were then blocked as described in the Materials and methods section and then treated with 30 nM HlyA in the presence of 10 mM $CaCl_2$. Bound toxin was analysed by immunoblotting with polyclonal anti-HlyA antibodies. Dot blots were developed by chemiluminescence. The first spot contains 1 nmol of lipid, followed by two successive 2-fold serial dilutions of the same lipid. An equal volume was spotted at each lipid concentration assayed.

The interaction resulted from the presence of Cho, since liposomes composed of either DOPC or SM alone did not interact with HlyA. This is illustrated clearly by the shape of the sensorgrams shown in Figure 5(D). This result is in agreement with the one obtained by the lipid dot-blot assay. In addition, to test the functional integrity of the immobilized HlyA, the interaction with its putative receptor glycophorin was tested. This interaction was characterized by a higher affinity of HlyA for glycophorin ($K_D = 6.1 \times 10^{-7}$ M) than for Cho, and one that, in turn, allowed an analysis of the binding kinetics (Figure 5C). All the K_D values were calculated from three independent sets of data.

In order to evaluate whether there is an effect of Cho concentration on the lytic activity we analysed the leakage of liposomes containing different amounts of Cho. Figure 6(A) shows that, as expected, the percentage leakage increased with the amount of toxin. A maximum leakage of 75 % was reached at 20 nM toxin and remained constant at higher toxin concentrations, independently of the vesicle lipid composition (results not shown). In contrast, at toxin concentrations below 10 nM, the leakage increased with the Cho content of the membrane. Figure 6(B) is a graphical illustration of these differences: the *y*-axis represents the increment in the percentage leakage resulting from the presence of different amounts of Cho relative to the percentage obtained for the pure DOPC liposomes at each toxin concentration assayed. This Figure indicates that at high concentrations, HlyA is able to disrupt both liposomes of pure DOPC and those of DOPC/Cho at different molar ratios with almost the same efficiency, probably through a non-specific destabilizing effect on the membrane; however, as the toxin concentration became lower, the presence of Cho markedly improved the permeabilizing activity of HlyA. This result suggests that the interaction between Cho and HlyA plays a critical role that is evidenced especially at low toxin concentrations.

Flanagan et al. [20] measured the steady-state anisotropy of DPH (diphenylhexatriene) of liposomes composed of DOPC/Cho at different molar ratios (from 7:3 to 4.5:5.5, equivalent to 30–55 % of Cho). These mixtures presented low anisotropy, suggesting a liquid-disordered phase, although the increase in Cho induced a slight increase in DPH anisotropy. Despite this increase, DOPC/Cho liposomes did not segregate into phases at those molar ratios assayed [20]. The Cho content in the liposome used in the present study ranges from 10 to 33 %, so we can state that the increased leakage activity of HlyA observed in Figure 6 is not a consequence of the insertion favoured by lipid phase segregation.

Furthermore, recently we have observed, using the monolayer technique, that the insertion of HlyA into ternary lipid mixtures containing DOPC/SM/Cho (2:1:1, molar ratio) (equivalent to 25 % Cho content) induces a similar increment in the surface pressure of monolayers independently of the presence of phase segregation (S.M. Maté, R.F. Vazquez, V.S. Herlax, M.A. Daza Millone, M.L. Fannani, B. Maggio, M.E. Vela and L.S. Bakas,

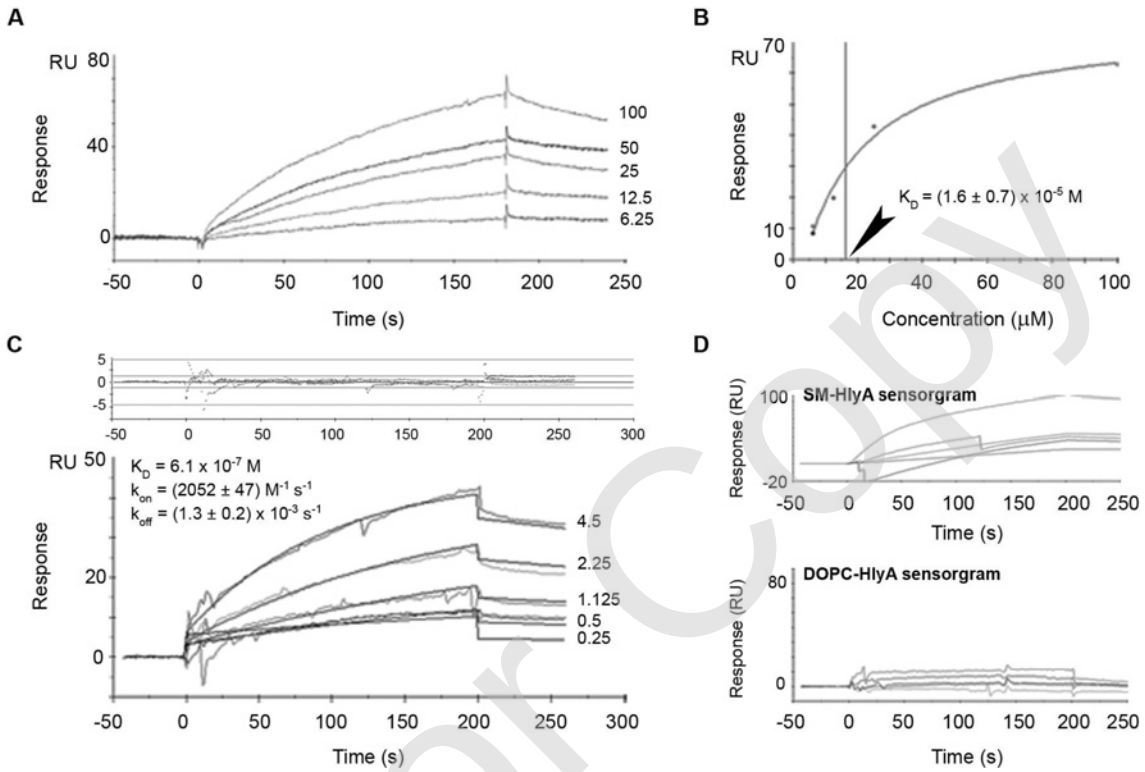

Figure 5 HlyA interaction with lipids determined by SPR

DOPC/Cho (4:1) liposomes (**A** and **B**), glycophorin (**C**) or DOPC and SM (**D**) were injected at the indicated concentrations (μM) over immobilized HlyA (450 RU). The curves show specific (**A** and **C**) or non-specific (**D**) binding after subtracting the values obtained over a control surface with no protein immobilized. (**B**) The K_D for the HlyA–DOPC/Cho liposome interaction was determined by non-linear analysis. (**C**) Sensorgrams were fitted to a 1:1 binding model and the kinetic parameters k_{on} and k_{off} were determined. Residuals are shown in the upper panel.

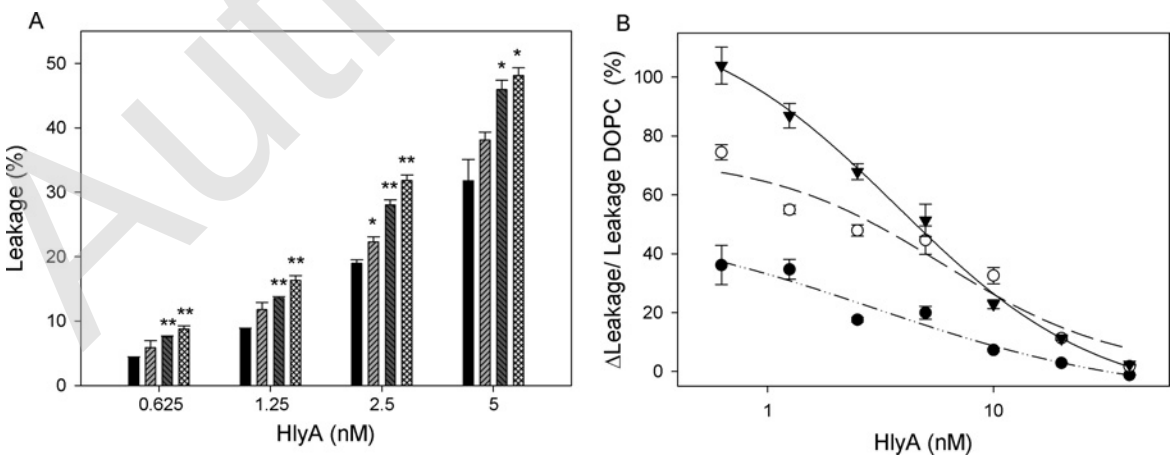

Figure 6 Leakage of liposomes of different lipid composition

HlyA was serially diluted in TC buffer containing 10 mM $CaCl_2$ in a 96-well microtitre plate. Of the diluted suspensions, 100 μl were mixed with 100 μl of 200 μM LUVs. After 20 min at 37 °C the fluorescence of ANTS was measured. The excitation and emission wavelengths were 355 and 525 nm respectively. (**A**) The percentage leakage at different toxin concentrations of liposomes containing DOPC (black bars), DOPC/Cho (9:1) (dashed light grey bars), DOPC/Cho (4:1) (dashed dark grey bars) and DOPC/Cho (2:1) (squared light grey bars) liposomes. (**B**) The percentage increase in leakage resulting from the presence of different amounts of Cho in the Cho-containing liposomes relative to pure DOPC liposomes. The [%*L* DOPC/Cho(X:X) − %*L* DOPC liposome]/%*L* DOPC liposome plotted against the HlyA concentration assayed is shown. DOPC/Cho (9:1) (●), DOPC/Cho (4:1) (○) and DOPC/Cho (2:1) (▼). Results are means ± S.D. analysed by Student's *t* test (**P* < 0.05 and ***P* < 0.01 compared with pure DOPC liposomes).

unpublished work). In that study we investigated the phase lateral behaviour of DOPC/SM/Cho lipid mixtures where different SM molecular species were used: SM (16:0) and SM (24:1). The lipid mixture containing SM (24:1) did not present lipid segregation, whereas the one containing SM (16:0) did, as confirmed by Brewster angle microscopy and AFM (atomic force microscopy) (S.M. Maté, R.F. Vazquez, V.S. Herlax, M.A. Daza Millone, M.L. Fannani, B. Maggio, M.E. Vela and L.S. Bakas, unpublished work). These results confirmed that it is indeed the presence of Cho that facilitates HlyA insertion. Moreover, it was demonstrated by real-time AFM studies that HlyA interacts preferentially with liquid-disordered phases (S.M. Maté, R.F. Vazquez, V.S. Herlax,

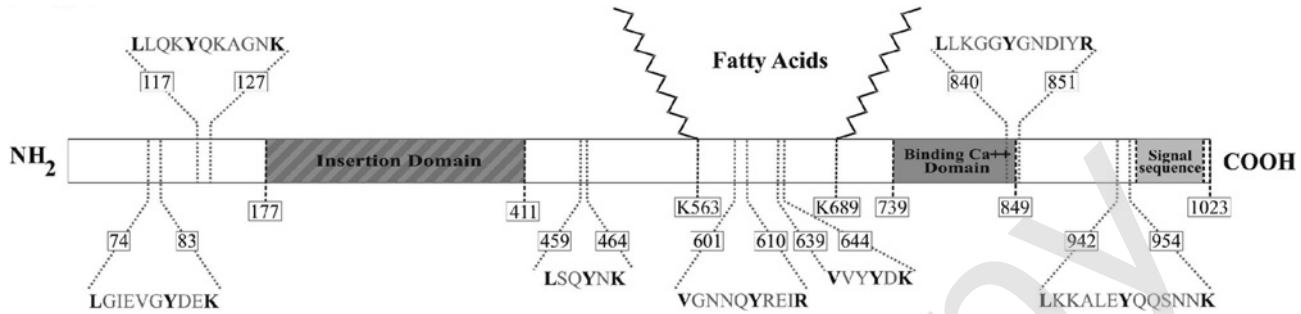

Figure 7 Location of seven putative Cho-binding motifs present in the HlyA sequence (UniProt accession number P09983)

The consensus sequence for the Cho-binding motif searched for in the N- to C-terminus direction: L/V($X_{1–5}$)Y($X_{1–5}$)R/K. The conserved tyrosine residue along with leucine/valine towards its N-terminus and lysine/arginine towards the C-terminus are highlighted. Motifs were identified using the ScanProsite tool (http://prosite.expasy.org/scanprosite). Important functional domains, such as the insertion and Ca^{2+} -binding domain, both sites of acylation and the signal sequence, are indicated.

M.A. Daza Millone, M.L. Fannani, B. Maggio, M.E. Vela and L.S. Bakas, unpublished work). In this context, the results obtained in the present study are in agreement with a preferential interaction of HlyA with Cho located at liquid-disordered phases.

Previous results also agreed with the notion that Cho played a significant role in the lytic mechanism of HlyA. Bakás et al. [11] found that the percentage of HlyA binding remained constant when small amounts of Cho were added to PC liposomes (up to 10%), without a temperature dependence (same at 10°C and 37°C). In contrast, when the irreversible HlyA insertion into LUVs was tested by the accessibility of HlyA previously bound to DMPC/Cho multilamellar vesicles to erythrocytes, the tendency is an increase in irreversible binding with the increment in Cho liposome content. This finding indicated a stabilization of the membrane-bound form of the toxin that facilitated the irreversible insertion of HlyA [21]. As the irreversible property of the binding is given by the formation of oligomers of the toxin in the membrane [13,22], and considering that the toxin decreases its oligomerization in Cho-depleted erythrocytes by β-cyclodextrin treatment [13], we are tempted to state that the interaction with Cho facilitates the insertion of the toxin in a competent conformation to induce oligomerization.

Putative Cho-binding motifs in the HlyA sequence

The Cho polar section is restricted to a single hydroxy (OH) group which can form two distinct types of hydrogen bond (acceptor and donor) with a polar group belonging to either a membrane lipid or protein. The apolar section of Cho has an asymmetric structure with two distinct faces, referred to as α and β. The α face displays a planar surface, in contrast with the β face which has a significantly rougher surface due to the presence of several aliphatic groups (two methyl groups and a terminal isooctyl chain that are linked to the sterane backbone) [23]. Several proteins that interact with Cho have a highly conserved sequence, referred to as CRAC (cholesterol recognition/interaction amino acid consensus) [24]. The consensus sequence for the Cho-binding motif is in the N- to C-terminus direction, L/V($X_{1–5}$)Y($X_{1–5}$)R/K, and consists of a branched apolar leucine or valine residue followed by a segment containing 1–5 of any residue, then an aromatic residue that is mandatory tyrosine, then again 1–5 of any residue, and finally a basic lysine or arginine. Leucine and valine can interpenetrate their apolar side chain with the β face of Cho through van der Waals interactions. Lysine and arginine are commonly found flanking transmembrane domains because they have long apolar side chains buried in the apolar section of the membrane, whereas the positively charged basic group can 'snorkel' at the surface of the membrane [25].

Molecular modelling studies have shown that a CRAC motif belonging to transmembrane domains can have a good fit for Cho. This was demonstrated for the leukotoxin of *Aggregatibacter actinomycetemcomitans* (LtxA) an RTX toxin. Brown et al. [26] demonstrated that a CRAC motif situated between residues 333 and 339 is conserved among the RTX toxins (in that paper HlyA of uropathogenic *E. coli* was not considered). They demonstrated that a peptide corresponding to this CRAC motif inhibited the ability of LtxA to kill Jurkat (Jn.9) cells. Although peptides corresponding to both CRAC(333–339) and CRAC(501–505) bind Cho, only CRAC(333–339) competitively inhibited LtxA binding to this sterol. This last motif is present in the hydrophobic domain (residues 1–420), which is strongly predicted to interact with membranes [26].

Considering that the results of the present study indicate that HlyA interacts with Cho to favour its insertion into the membrane, we investigated the existence of such a motif in the toxin sequence. Figure 7 shows the location of seven putative Cho-binding motifs present in the HlyA sequence. It is strongly expected that if Cho favours HlyA insertion, the CRAC motif should be situated in the insertion region of the toxin (residues 177–411) [8]. However, none of the predicted CRAC regions are present in this domain, although the motif located at positions 117–127 is close to the insertion region. Interaction of the toxin with Cho through this motif would be a stabilizing feature for monomers to facilitate their insertion.

A new Cho-binding domain similar to CRAC, but with the opposite orientation along the polypeptide chain, has been identified: the CARC motif K/R($X_{1–5}$)Y/F($X_{1–5}$)L/V from the N- to C-terminus [27]. The presence of the basic residues ensures that the CARC motif is correctly positioned at the polar/apolar interface of a transmembrane domain, exactly where Cho is supposed to be. This is due to the 'snorkelling' effect attributed to the fact that the long and flexible side chain of lysine (or arginine) is buried in the hydrophobic part of the membrane, whereas the cationic group is present at the membrane surface. Interestingly, arginine and lysine residues are more frequently found in the N-terminal rather than the C-terminal section of a transmembrane domain, indicating that snorkelling is an asymmetric phenomenon [25]. The specific topology of the CARC sequence implies that the key residues following K/L in the motif (i.e. Y/F and L/V) actually belong to a transmembrane domain, and this is consistent

with the fact that both aromatic and aliphatic residues have an apolar side chain [28].

Thirteen CARC motifs were found in the HlyA sequence (Supplementary Table S1 at http://www.biochemj.org/bj/458/bj4580481add.htm); however, in the context of this work, the most relevant one is **RFKK**LG**Y**DGDS**LL** (residues 341–353; conserved motif residues in bold), which is present in the insertion region of the toxin (residues 177–411). Using a secondary structure prediction server (Jpred) [29], in the region where the CARC motif is located two α-helixes are predicted and this region was shown to be highly conserved in the RTX toxins (Supplementary Figure S1A at http://www.biochemj.org/bj/458/bj4580481add.htm). Surprisingly, the sequence RFKK (residues 341–344) is located at the N-terminal end of a predicted α-helix that may be transmembrane (Supplementary Figure S1A). Even more surprisingly, a similar (residues 341–353) HlyA CARC motif is also present in *A. actinomycetemcomitans* LtxA, besides the CRAC motif (residues 333–339), which was found to interact with Cho (Supplementary Figure S1B) [26]. Moreover, this CRAC motif is also found in HlyA with the difference that the N-terminal end of the CRAC motif (residue 336) was isoleucine rather than leucine. Taking all of these results together, it is tempting to speculate that the CRAC and CARC motifs present between residues 336 and 353 can bind Cho in order to stabilize the insertion of the predicted α-helixes located at both sides of these motifs.

Concluding remarks

In the present study we have demonstrated and report for the first time the interaction of HlyA with Cho. This novel finding is highly relevant to the mechanism of action of HlyA since up to now Cho has only been associated with the physical properties that the sterol imparts on membranes in order to facilitate an irreversible binding, insertion or oligomerization of the toxin [21]. The interaction of HlyA with Cho facilitates the insertion of the toxin into membranes in a competent conformation of the protein, i.e. one allowing not only that insertion, but also the toxin oligomerization necessary for pore formation. Putative Cho-binding sites have been identified in the HlyA sequence that may participate in the interaction with Cho. This Cho-binding motif has also been reported in an another RTX toxin [26] and it is a highly conserved motif in all the RTX toxins, suggesting that the interaction of toxins with Cho may be conserved in all the toxin family. Other non-RTX toxins that lyse a wide range of target cells, such as is true of CDCs (cholesterol-dependent cytolysins) and the actinoporin family of toxins, utilize Cho or SM in their mechanism of action. The role that these lipids play is to act not only as mere receptors, but also as stabilizers of monomers, promoters of oligomerization and inducers of the phase segregation that facilitates toxin concentration [30]. A phenomenon similar to the above interactions is observed with HlyA, a member of a totally unrelated group of toxins that also permeates a wide range of target cells. It is therefore tempting to suggest the hypothesis that, despite the origin of the various toxins, many have evolved to be able to use the Cho and/or SM of the eukaryotic cell outer plasma membrane in order to optimize their invasiveness.

AUTHOR CONTRIBUTION

Romina Vazquez performed all the haemolysis, leakage and lipid dot-blot assays. Sabina Maté performed the surface pressure experiments and HPTLC. Marisa Fernández and Emilio Malchiodi performed the SPR assays. Romina Vazquez, Sabina Maté, Laura Bakás and Vanesa Herlax planned the experiments, analysed the experimental data and wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

Novel evidence for the specific interaction between cholesterol and α-haemolysin of *Escherichia coli*

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Table S1 CARC motifs in the HlyA sequence (UniProt accession number P09983)

The conserved tyrosine (Y) or phenylalanine (F) residue, along with leucine (L)/valine (V) towards the C-terminus and lysine (K)/arginine (R) towards the N-terminus, are highlighted in bold. Motifs were identified using the ScanProsite tool (http://prosite.expasy.org/scanprosite).

Residues	Sequence
90–99	**K**QV . . . **F**GTAEK**L**
105–114	**R**GVTI.**F**APQ..**L**
341–353	**R**FKKLG**Y**DGDSL**L***
413–420	**K**QAM..**F**EH . . . **V**
457–468	**K**ILSQ.**Y**NKEYS**V**
464–472	**K**E . . . .**Y**SVERS**V**
500–512	**K**SYIDY**Y**EEGKR**L**
514–523	**K**KPDE.**F**QKQ..**V**
521–527	**K**QV . . . **F**DP . . . **L**
558–570	**R**RQSGK**Y**EYITE**L**
563–572	**K**YE . . . **Y**ITELL**V**
586–595	**K**GSVYD**Y**SN . . . **L**
954–964	**K**ASYV.**Y**GNDA.L

*Present in the insertion region of the toxin (residues 177–411).

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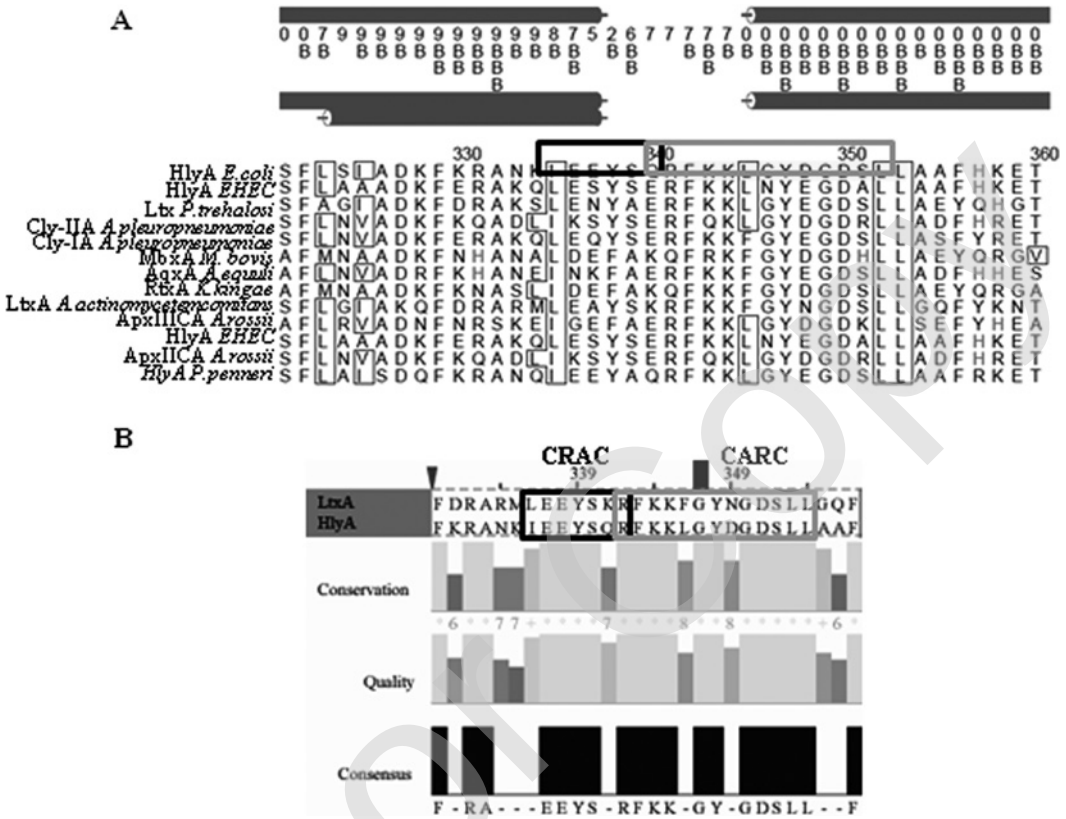

Figure S1 Putative α-helixes in the insertion region of HlyA

(**A**) Results were obtained using the Jpred 3 secondary structure prediction server [28] (http://www.compbio.dundee.ac.uk/www-jpred/). (**B**) Amino acid alignments between HlyA of *E. coli* (UniProt accession number P09983) and LtxA of *A. actinomycetemcomitans* (UniProt acession number Q43892) round the LtxA CRAC region 333–339. The sequence alignment was performed using CLUSTALW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Histogram summarizes the amount of amino acid conservation.

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