The properties of the C-terminal domain of HlyIICTD suggest that *B. cereus* HlyII is a representative potential member of trimeric autotransporter adhesins among Gram-positive bacteria.

Siunov A.V. [a], *Nagel A. S.* [a], *Andreeva-Kovalevskaya Z. I.* [a], *Zamyatina A.V.* [b, c], *Rudenko N.V.*[b, c], *Karatovskaya A.P.*[c], *Borisova M. P.*[d], *Brovko F.A*[b, c], *Salyamov V. I.* [a], *A.S. Solonin* [a, b]

[a] G. K. Skryabin Institute of Biochemistry and physiology of microorganisms RAS 5 Prospekt Nauki, Pushchino, Moscow Region 142290, Russia [b] Pushchino State Institute of Natural sciences of the Ministy of Education and Science of the Russion Federation, 3 Prospekt Nauki, Pushchino, Moscow Region 142290, Russia [c] Pushchino Branch, Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, 6 Prospekt Nauki, Pushchino, Moscow Region 142290, Russia [d] Institute of Theoretical and Experimental Biophysics RAS, 2 Prospekt Nauki, Pushchino, Moscow region, 142290, Russia

INTRODUCTION

Hemolysin II of *B. cereus sensu lato* is synthesized in a bacterial cell in the form of a water-soluble secreted monomer and penetrates into eukaryotic membranes forms weakly anion-selective pores, the radius of which varies from 0.6 to 0.8 nm in both natural and model erythrocyte membranes [1]. The hemolysin II gene is found in all clades of *B. cereus* sensu lato; it is more common among various natural isolates of *Bacillus thuringiensis* [2]. The study of hemolysin II is becoming the most relevant since *B. thuringiensis* is widely used as a biological insecticide for plant protection [3] and currently makes up 85% of the global market for biological insecticides. Hemolysin II gene encodes a protein of 412 amino acid residues and, in the mature state, after separation of the signal peptide of 31 amino acids, it has a molecular weight of 42 kDa [4, 5]. In a mature state, this toxin is the closest homologue of *S. aureus* alpha toxin (38% amino acids identity). The HlyII protein has a C-terminal extension not previously described in this class of toxins, including 94 amino acid residues designated as HlyIICTD (C-terminal domain) [4]. Elimination of HlyIICTD from HlyII reduces the hemolytic activity of HlyII in the attack on rabbit erythrocytes eightfold, but the role of this region at pore formation is unknown. To date the function and role of HlyIICTD in secretion from bacterial cells and pore formation is not yet been clarified. To solve these tasks CTD domain (HlyIICTD) was cloned, purified, the portion was biotinylated and some properties were described.

RESULTS AND DISCUSSION

The interaction of HlyIICTD with erythrocytes was determined as part of HlyIICTD-MA-bio immunocomplexes [6].

Table 1. Identification by ELISA of biotinylated anti-HlyIICTD MA in erythrocyte lysates after the interaction of HlyIICTD-MA immune complexes with red blood cell membranes. The results are presented net of background values in the presence of biotinylated normal mouse Igs.

MA	A490nm	
	37 ^{0}C	Room Temperature
HlyIIC-16	0,512±0,02	0,348±0,01
HlyIIC-23	0,279±0,025	0,211±0,035
HlyIIC-15	0,0105	
HlyIIC-30	0,025	
HlyIIC-34	0,03	
HlyIIC-37	0,05	

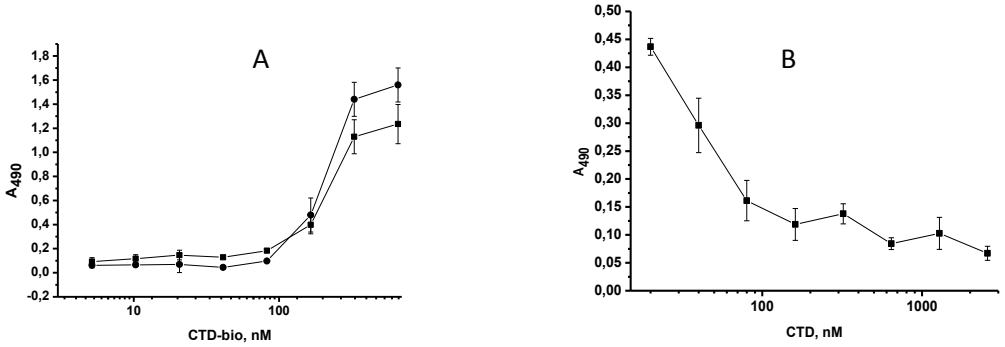


Fig. 1 (**A**). HlyIICTD-bio titration the affinity constant by the method of Beatty et al. [7] when loading 0.0125% and 0.025% Rrbc. (**B**) Inhibition of the interaction of HlyIICTD-bio with Rrbc by unlabeled HlyIICTD.

HlyIICTD-bio interaction inhibition curve (165 nM) with unlabeled HlyIICTD is presented (titration with double dilution from 18-fold excess). The use of biotinylated CTD allowed us to demonstrate direct binding of CTD to Rrbc. Calculated K_{aff} was equal $2{,}6\times10^6$ M^{-1}. Using monoclonal antibodies against recombinant HlyIICTD as well showed the possibility to bind effectively to red blood cells [6]**.** Using cells different from red blood cells revealed

that the effectiveness of the interaction of HlyIICTD with human cells of the J774 and Jurkat lines is significantly different (Table 2). Thus, the effectiveness of the interaction depends on the specific components of the cell wall of eukaryotes.

Table 2. CTD-bio titration the affinity constant by the method of Beatty et al. [7]

Used cells	Affinity constants
Rrbc	$2{,}6\times10^{6}\ M^{-1}$
Mrbc	$2{,}6\times10^{6}\ M^{-1}$
Hrbc	$2{,}6\times10^{6}\ M^{-1}$
J774	$7{,}8\times10^{6}\ M^{-1}$
Jurkat	$15{,}8\times10^{6}\ M^{-1}$

As seen in Fig. 2, after incubation of CTD with Rrbc, a number of oligomers are detected, while monomeric forms are almost completely absent. Formation of CTD molecular forms under the action of Rrbc led to a change in the efficiency of interaction with MA. The use of MA HlyIIC-15 and HlyIIC-40 in experiments on oligomerization at the presence of liposomes showed similarity to oligomerization on red blood cells. Thus, the efficiency of oligomerization does not depend on the presence of additional components in the cell membranes.

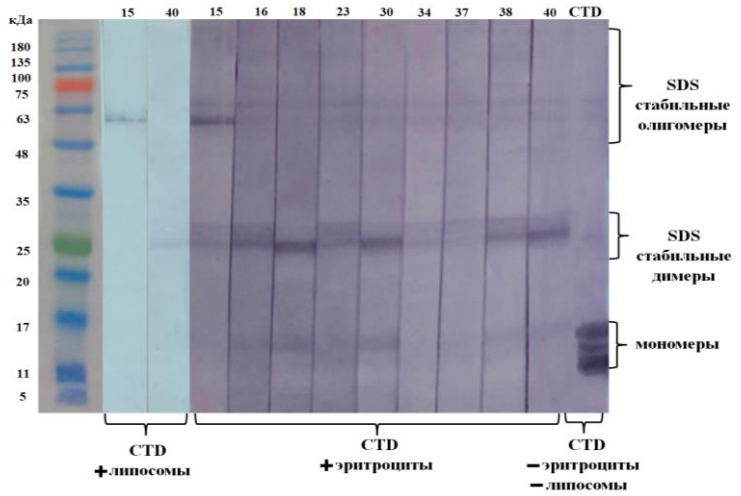


Fig. 2. Immunoblotting of HlyIICTD after and before interaction with Rrbc and liposomes. The numbers of MA are above the tracks

Additionally, purified HlyIICTD preparations are capable to trimerization in the presence of 4M urea (Fig. 3A, B) and, forming a possibly beta-barrel-like structure, integrate into the artificial bilayer membrane with the formation of pores (Fig. 4 A, B).

A **B**

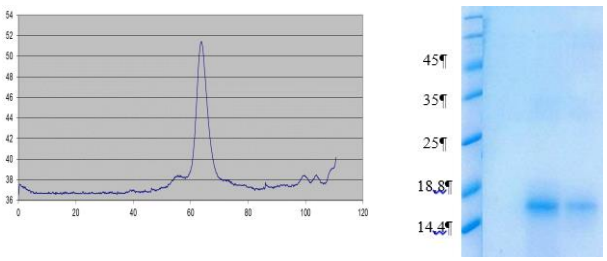


Fig. 3 (**A**) HlyIICTD gel filtration, column HiLoad 16/60 Superdex 75 prep grade. (**B**) SDS PAGE of HlyIICTD gel filtration peak

Testing the C-terminal domain of HlyII protein on artificial membranes showed its channel-forming activity. When HlyIICTD was added on one side of the membrane at a concentration of tens to hundreds of μg per ml of 1M KCl solution, channels of different conductivity from 0.5–4 pA are formed.

Fig. 4 (**A**) shows an example of the operation of one channel with a conductivity of 0.5 pA that is anion selective at neutral pH. The current-voltage characteristic shown in Fig. 4 (**B**), taking into account the potential that has appeared, shows the same current through the membrane at different signs and the same voltage. In contrast to the characteristics of the ionic pores formed by the full-sized HlyII (the radius of which varies from 0.6 to 0.8 nm), its HlyIICTD forms ion channels of a smaller size and has lower conductivity at comparable effective concentrations.

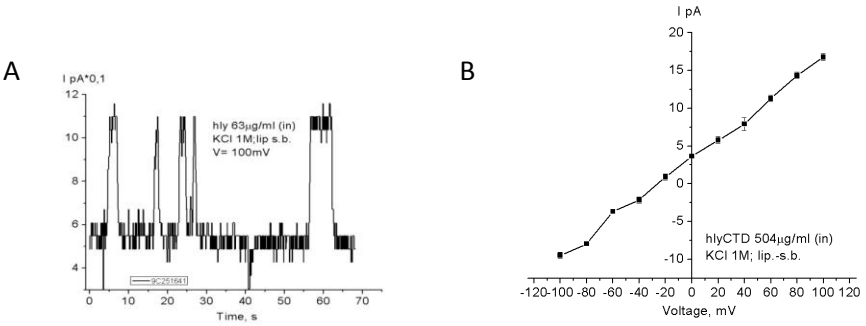
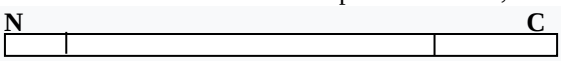


Fig. 4.**(A)**. An example of the operation of one channel with a conductivity of 0.5 pA, (**B**). Single channel current–voltage relationship.

CONCLUSION AND OPEN QUESTIONS

We assume that HlyIICTD during trimerization is able to form a β-barrel structure, since pore-forming proteins can spontaneously form oligomers that are resistant to SDS but break down when boiled, which is a feature of many pore-forming proteins with a β-barrel structure [8]. The possibility to form of trimeric oligomers a channel in bilayer membranes by the C-terminal domain of HlyII and and the manifestation of its adhesive properties suggest that HlyII can be presumably attributed to trimeric autotransporter proteins. In this case, HlyII itself stands out as a passenger of the trimeric autotransporter adhesin. The topographic scheme of HlyII (Fig. 5] is similar to that described for trimeric autotransporter adhesins, and the protein itself can be assigned to this family [9].



Signal peptide **passenger (HlyII)** **412 aa HlyII** **β-barrel domain (HlyIICTD)**

Fig.5. The topographic scheme of HlyII

Future detailed analysis of HlyIICTD donate the application this pore-forming protein in nanopore technology for sequencing of long DNA and RNA.

ACKNOWLEDGMENT

The study was supported by a grant from the Russian Science Foundation (no. 14-04-00592) and by the Ministry of Science and Higher Education of the Russian Federation (unique project number RFMEFI60419X0218).

REFERENCES:

1. Z.I Andreeva., V.F. Nesterenko, M.G. Fomkina, V.I. Ternovsky, N.E. Suzina, A.Y. Bakulina, A.S. Solonin, E.V. Sineva, "The properties of *Bacillus cereus* hemolysin II pores depend on environmental conditions," Biochim. Biophys. Acta. 2007. V.1768. P.253–263.
2. Z.I. Budarina, M.A. Sinev, S.G. Mayorov, A.Y. Tomashevski, I.V. Shmelev, N.P. Kuzmin. "Hemolysin II is more characteristic of Bacillus thuringiensis than *Bacillus cereus*,"Arch Microbiol. 1994;161(3):252-257.
3. A Chattopadhyay, N.B. Bhatnagar, R. Bhatnagar.. "Bacterial insecticidal toxins", Crit. Rev. Microbiol. 2004, 30: 33-54. https://doi.org/10.1080/10408410490270712.
4. G. Baida, Z. I. Budarina, N.P. Kuzmin, A.S Solonin. "Complete nucleotide sequence and molecular characterization of hemolysin II gene from *Bacillus cereus*," FEMS Microbiol. Lett. 1999. V.180. P.7–14.
5. Z.I. Andreeva, V.F. Nesterenko, I.S. Yurkov, Z.I. Budarina, E.V. Sineva, A.S. Solonin. "Purification and cytotoxic properties of *Bacillus cereus* hemolysin II". Protein Expr Purif. 2006 May; 47(1):186-93.
6. N. V Rudenko., A. P. Karatovskaya, A. V. Zamyatina, A. V. Siunov, Zh. I. Andreeva-Kovalevskaya, A. S. Nagel, F. A. Brovko, and A. S. Solonin " C-Terminal Domain of *Bacillus cereus* Hemolysin II Is Able to Interact with Erythrocytes", Russian Journal of Bioorganic Chemistry, 2020, Vol. 46, No. 3, pp. 321–326.
7. J.D. Beatty, B.G. Beatty, W.G. Vlahos, "Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay" J. Immunol. Methods. 1987, 100, 173–179. https://doi.org/10.1016/0022-1759 (87) 90187-6.
8. S.P Hardy., T Lund., P.E Granum. „CytK toxin of *Bacillus cereus* forms pores in planar lipid bilayers and is cytotoxic to intestinal epithelia". FEMS Microbiol. Lett. 2001;197:47–51.
9. A. R. Kiessling, A.Malik, A. Goldman, „Recent advances in the understanding of trimeric autotransporter adhesins" Medical Microbiology and Immunology https://doi.org/10.1007/s00430-019-00652-3 published on line 2019.