

# Two residues in *Staphylococcus aureus* $\alpha$ -hemolysin related to hemolysis and self-assembly

Yufeng Du<sup>1</sup>Li Liu<sup>2</sup>Chunping Zhang<sup>3</sup>Yani Zhang<sup>1</sup><sup>1</sup>College of Life Sciences, Northwest University, Xi'an 710069, China;<sup>2</sup>Ultrasonic Diagnosis Department, Shaanxi Provincial People's Hospital, Xi'an 710068, China; <sup>3</sup>College of Chemistry and Material Science, Northwest University, Xi'an 710069, China

**Abstract:** *Staphylococcus aureus* is becoming increasingly intractable because of its ability to acquire antimicrobial resistance and secrete numerous virulence factors that can exacerbate inflammation. Alpha-hemolysin (Hla) is a pore-forming virulence factor produced by *S. aureus* that can self-assemble into heptameric mushroom-structured pores in target cell membranes, leading to cell lysis and death. In the present study, we sought to better understand the mechanism underlying hemolysis and the oligomerization of Hla by creating nine mutants with single amino acid changes in different positions of the Hla protein: N17C, T18C, P103C, N105C, M113C, T117C, N121C, D128C, and T129C. The results showed that the P103C and N105C mutations, which are located in the triangle region, significantly diminished hemolysis and heptamer formation when compared with the wild-type Hla protein. This suggests that the P103 and N105 residues play key roles in the assembly of the Hla pore. These results improve our understanding of the mechanism underlying the pore-forming ability of Hla.

**Keywords:**  $\alpha$ -hemolysin, hemolysis, heptamer oligomers, cysteine mutants, assembly

## Introduction

*Staphylococcus aureus* is a major bacterial pathogen that invades and damages host tissue by expressing harmful toxins.  $\alpha$ -hemolysin (Hla), a pore-forming toxin secreted by *S. aureus*, is very important for pathogenesis. Hla can form a mushroom-shaped pore in eukaryotic cell membranes, causing cell damage and subsequent cell death.<sup>1,2</sup> Previous studies have reported that strains lacking Hla exhibited obviously attenuated pathogenicity in a mouse model of *S. aureus* infection.<sup>3</sup> Hla is a water-soluble monomer of 33.2 kDa, and seven copies of H1a self-assemble to form a heptameric pore of 232.4 kDa that is composed of three structural regions, the cap, rim, and stem, as observed based on X-ray diffraction.<sup>4</sup> However, the mechanism involved in Hla-induced hemolysis and oligomer pore assembly are not well understood. In this study, we generated nine mutants, each with a single amino acid change in different regions of the Hla protein, to investigate their role in Hla pore formation and cell hemolysis.

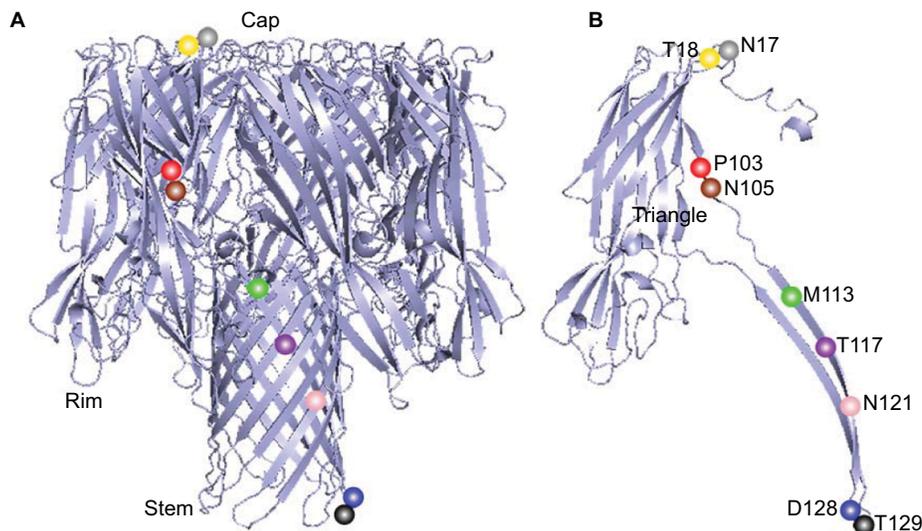
## Characterization of the assembly and hemolysis activities of nine different mutant Hla proteins

In the present study, nine different amino acids in wild-type (WT) H1a were individually changed to a cysteine residue to determine their role in pore formation. These nine sites, N17, T18, P103, N105, M113, T117, N121, D128, and T129, are located on the cap, rim, and stem regions of the heptameric mushroom-shaped pore structure (Figure 1).

Correspondence: Yani Zhang  
College of Life Sciences, Northwest University, 229 North Taibai Road, Xi'an, Shaanxi 710069, China  
Tel/Fax +86 29 8830 3662  
Email [yani@nwu.edu.cn](mailto:yani@nwu.edu.cn)

Site-directed mutagenesis was used to change these nine different sites to cysteine codons by using the Muta-direct™ Kit (catalog number: SDM-15; SBS Genetech, Beijing, China) and a reconstructed WT Hla gene in a T7 plasmid (pT7-Hla) as the template. Then, the desired mutation was confirmed by sequencing. The primers used in this study are shown in Table 1. The pT7-Hla and mutant plasmids were individually

transformed into *Escherichia coli* BL21 (DE3) pLysS cells for protein expression. Ultrafiltration centrifuge tubes with three different pore sizes. 100-, 50-, and 30-kDa molecular weight cut off (MWCO) (catalog numbers: UFC910096, UFC905096, and UFC503096, respectively; Millipore, Burlington, MA, USA) were used for protein isolation and concentration, and all steps were conducted at 4°C. Initially,



**Figure 1** Ribbon representation of seven protomers from Hla heptamer (A) and a protomer from heptamer (B) in a PyMoL model.

**Notes:** The cap, rim, and stem domains are labeled. In (A) and (B), mutated positions in Hla nanopore structure were labeled with different colors. The figure was drawn using PyMoL software (<https://pymol.org>).

**Abbreviation:** Hla, alpha-hemolysin.

**Table 1** Primers used in the study

Position	Primer
N17C FWD	5'-ACCGGTACTACAGATATTGGAAGCTGTACTACAGTAAAAACAGG-3'
N17C REV	5'-CCTGTTTTACTGTAGTACAGCTTCCAATATCTGTAGTACCGGT-3'
T18C FWD	5'-ACCGGTACTACAGATATTGGAAGCAATTGTACAGTAAAAACAGG-3'
T18C REV	5'-CCTGTTTTACTGTACAATTGCTTCCAATATCTGTAGTACCGGT-3'
P103C FWD	5'-GTAGCTCAAATATCTGATTACTATTGCAGAAATTCGATTGATACA-3'
P103C REV	5'-TGTATCAATCGAATTTCTGCAATAGTAATCAGATATTGAGCTAC-3'
N105C FWD	5'-CAAATATCTGATTACTATCCAAGATGTTTCGATTGATACAAAAGAG-3'
N105C REV	5'-CTCTTTTGTATCAATCGACATCTTGGATAGTAATCAGATATTG-3'
M113C FWD	5'-CGATTGATACAAAAGAGTATTGCAGTACTTTAACTTATGGATTTC-3'
M113C REV	5'-GAATCCATAAGTTAAAGTACTGCAATACTCTTTTGTATCAATCG-3'
T117C FWD	5'-GATACAAAAGAGTATATGAGTACTTTATGTTTATGGATTCAACGG-3'
T117C REV	5'-CCGTTGAATCCATAACAATAAGTACTCATATACTCTTTTGTATC-3'
N121C FWD	5'-TTAACTTATGGATTCTGCGGTAATGTTACTGGTGATGATACAGG-3'
N121C REV	5'-CCTGTATCATCACCAGTAACATTACCGCAGAATCCATAAGTTAA-3'
D128C FWD	5'-ACGGTAATGTTACTGGTGATTGTACAGGAAAAATTGGCGGCCTT-3'
D128C REV	5'-AAGGCCGCCAATTTTTCTGTACAATCACCAGTAACATTACCGT-3'
T129C FWD	5'-GTTACTGGTGATGATTGTGGAAAAATTGGCGGCCTTATTGGTGC-3'
T129C REV	5'-GCACCAATAAGGCCGCCAATTTTTCCAACAATCATCACCAGTAAC-3'

**Notes:** Red indicates the mutated base.

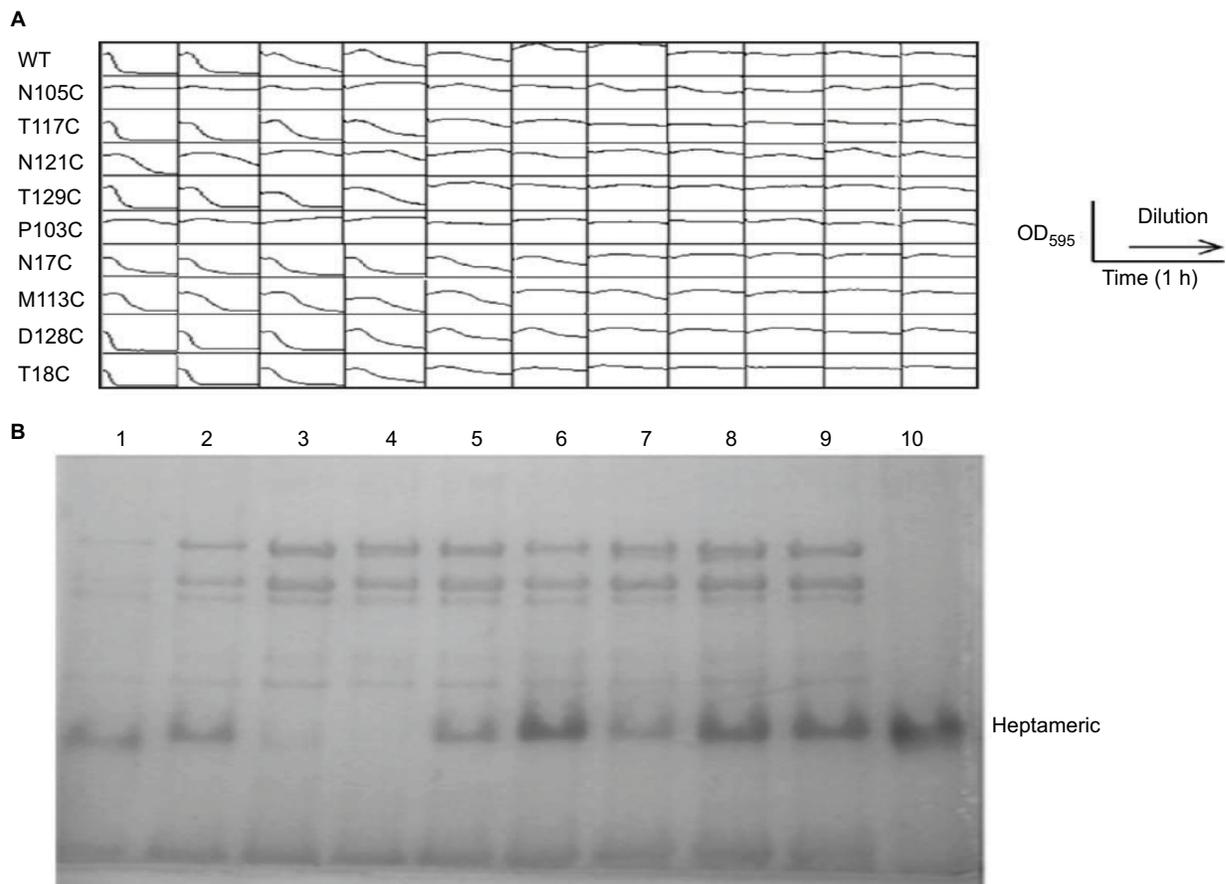
**Abbreviations:** FWD, forward; REV, reverse.

the 100-kDa MWCO centrifuge filter tube was used to eliminate macromolecular proteins from the cell lysate. Then, this filtrate was subsequently centrifuged in a 50-kDa MWCO tube, and then a 30-kDa MWCO tube. Finally, the retentate remaining on the top of the 30-kDa MWCO filter was used as the Hla protein solution. The protein solution was subsequently analyzed by SDS-PAGE.

To compare the hemolytic potencies of the WT and mutant Hla proteins, their pore-forming activities were measured by determining the rate of lysis of rabbit red blood cells (rRBCs) in a 96 well plate.<sup>5,6</sup> New Zealand white rabbit was used to prepare rRBCs as previously described.<sup>7</sup> All animal experiments were performed in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of China (November 14, 1988). All animal procedures were

approved by the Institutional Animal Care and Use Committee of the college of life sciences of Northwest University with a permit number: NW-02-2014. The protein solutions were incubated with the rRBCs at 37°C for 1 h. The results are shown in Figure 2A. Compared to WT Hla, the P103C and N105C mutants showed far less hemolytic activity toward the rRBCs, the N121C mutant showed decreased hemolytic activity, and the other six mutants showed activity similar to the WT protein. In all experiments, the concentration of the Hla protein was adjusted to 0.20 mg/mL.

To further explore the properties of these polypeptides, the ability of the mutant monomers to form heptameric oligomers in rRBC membranes (rRBCMs) was evaluated. The mutant monomers were reacted with 0.5% rRBCMs for 3 h at 37°C to allow oligomer assembly.<sup>8</sup> Then, oligomer formation was evaluated by SDS-PAGE. The oligomers were



**Figure 2** The hemolytic and heptamer-forming activities of the mutant proteins.

**Notes:** (A) The hemolytic activity of WT Hla and mutant proteins against rRBC. Briefly, 5  $\mu$ L of the Hla was diluted with 95  $\mu$ L maleylated bovine serum albumin buffer containing 10 mM sodium 3-(N-morpholino) propanesulfonic acid, 150 mM sodium chloride, and 1 mg/mL BSA (pH 7.4). Then, this solution was further subjected to 10 serial twofold dilutions with the same buffer across the row of a 96 well plate, which left 50  $\mu$ L in each well, and then an equal volume of 1% (mass/volume) rRBC in MBSA was added to each well to obtain a final volume of 100  $\mu$ L. The hemolysis was monitored for 1 h at 37°C using a microplate reader (Tecan infinite M200; Tecan, Männedorf, Switzerland) at 595 nm. In all cases, the initial concentration of WT Hla and mutant proteins was adjusted to 0.20 mg/mL. (B) Heptamers formed from Hla mutant protein monomers shown using 7% SDS-PAGE. 1, N17C; 2, T18C; 3, P103C; 4, N105C; 5, M113C; 6, T117C; 7, N121C; 8, D128C; 9, T129; 10, WT. MoPSNa (Catalog number: 71119-22-7, Seebio Biotech, Shanghai, China); BSA (Catalog number: A116563, Aladdin, Shanghai, China).

**Abbreviations:** Hla, alpha-hemolysin; rRBC, rabbit red blood cell; WT, wild-type.

separated by electrophoresis using 7% SDS-PAGE gels with a prestained protein marker at 120 V for 6 h at 4°C. As expected, SDS-PAGE revealed that compared to WT Hla, the extent of oligomer formation by the P103C and N105C mutants was very low but detectable, which was consistent with the results showing low hemolytic activity. In contrast, the mutations at positions 17, 18, 113, 117, 121, 128, and 129 did not appear to affect binding and oligomer formation, and these seven mutants had phenotypes similar to that of WT Hla (Figure 2B).

## Conclusion

Hla plays a critical role in the virulence of *S. aureus*. Here, we used single amino acid Hla mutants to examine the mechanisms involved in hemolysin and oligomer assembly, which are important for understanding the actions of cytolytic toxins and immune proteins. In previous studies, using cysteine mutants and generating targeted chemical modifications at various positions, it was shown that some residues and regions of Hla are critical for heptamer assembly.<sup>9</sup> Specifically, conversion of three residues in the triangle region, R104, K110, and D152, to cysteine residues diminished membrane binding, cell lysis, and heptamer-forming activities.<sup>4</sup> Two other residues, D108 and K154, were shown to be critical for assembly of Hla but not for membrane binding.<sup>9</sup> Here, our experiments showed that the P103C and N105C mutations diminished the hemolytic and heptamer-forming activities of the Hla protein. These results suggest that P103 and N105 play important roles in hemolysis and heptamer formation. The triangle region of the Hla heptameric oligomer is the linker between the protomer core and the stem-forming strands, and it participates in crucial protomer-protomer interactions.<sup>4,10</sup> The cysteine mutants at positions 103 and 105 in the triangle region failed to form oligomers, which may explain their low hemolytic activity. These results

confirmed that the triangle region plays an essential role in the conversion of monomers to heptamers. The glycine-rich stem region is translocated across the membrane. The five cysteine mutants we generated in this region, at residues 113, 117, 121, 128, and 129, did not alter monomer assembly in cell membranes. These findings provide a foundation for new studies into the mechanism underlying Hla assembly and function.

## Disclosure

The authors report no conflicts of interest in this work.

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