Identification of TSPphg as a Bacteriophage Endolysin with Peptidoglycan-Degrading and Bacteriolytic Activity

Feng Wang*, Xinyu Ji and Lianbing Lin

Faculty of Life Science and Technology, Kunming University of Science and Technology, 727 South Jingming Road, Kunming, Yunnan Province 650500, China.

*Corresponding author. E-mail: wangfeng076@126.com (F.W.)

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Abstract: With the serious emergence and dissemination of bacterial drug resistance worldwide, there is a critical need to find alternatives to common antibiotics for the treatment of bacterial infections. Previously, by decoding the complete genome of *Thermus* phage TSP4 belonging to the family of Siphoviridae, we have identified a bacteriophage lytic protein named TSPphg, which is heat-stable below the temperature of 70 °C and has considerable bacteriolytic activity. Here, we further performed the molecular modelling of TSPphg and found that His24 in motif HXXXD and His109 in motif HXH are functional residues that are critical for its antimicrobial activity. LC-MS analysis further confirmed that TSPphg has specific peptidoglycan-degrading activity, causing lysis of bacterial cell walls and thus bacteria destruction. These results suggest the potential application of TSPphg in combating antibiotic-resistant pathogenic bacteria and provide insights into the bacteriophage-based strategies to tackle the menacing bacterial infections.

1. Introduction

At present, multidrug-resistant (MDR) bacterial pathogens are the serious threats to public health both in hospitals and community settings [1, 2]. The most difficult-to-treat MDR strains are the ESKAPE representatives consisting of *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species [3, 4]. One of the promising ways to cure bacterial infections caused by these antibiotic-resistant ESKAPE pathogens is bacteriophage endolysins that are capable of degrading bacterial peptidoglycan, resulting in a rapid lysis of cell walls (CW) and thus bacteria destruction [5-7]. Previously, we have reported TSPphg, an endolysin originated from thermophilic bacteriophage TSP4 [8], can be recovered by rapid heat treatment method and be used in bacterial cell disruption [9]. Here, we further identified the critical residues within the M23 peptidase domain of TSPphg that are responsible for its bacteriolytic activity and confirmed the CW peptidoglycan-degrading activity of TSPphg by liquid chromatography-mass spectrometry (LC-MS) analysis.

2. Materials and methods

Bacterial strains and culture conditions. All bacteria used in this study were cultivated at 37 °C in LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl). *Escherichia coli* strains BL21 (for protein overproduction) and DH5a (for plasmid construction) were stored in our laboratory, and when necessary, the media were supplemented with ampicillin at 100 μg/mL or kanamycin at 50 μg/mL.

Production and purification of TSPphg. E. coli BL21 cells containing our previously constructed pET-28a-*TSPphg* vector was employed as the host for recombinant TSPphg protein expression [9]. The purification procedure was performed using HisTrap™ HP according to the manufacturer's instructions (GE Healthcare, Pittsburgh, PA), and the purity of TSPphg was analyzed by 12% SDS-PAGE.

Computational *analysis and molecular modelling.* SWISS-MODEL server was used to construct the 3D structure of TSPphg and find templates for protein structure prediction by comparative

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modelling (http://www.swissmodel.expasy.org/) [10]. The target sequence of TSPphg was searched with BLAST and HHBlits [11] against the SWISS-MODEL template library (SMTL). Based on the combined criteria of high sequence identity and high resolution of the experimentally confirmed structure, we chose the crystal structure of lysostaphin-type metalloprotease (LytM, PDB id: 1qwy) from *Staphylococcus simulans* [12] as the potential best template for TSPphg molecular modelling, and model building was carried out based on the target-template alignment using ProMod3. After evaluation of the global and per-residue model quality using the QMEAN scoring function, the results were revealed through Swiss-PDBViewer [13].

Site-directed *mutagenesis.* Two residues (H24 in motif HXXXD and H109 in motif HXH) of TSPphg were substituted with Cys and Thr, and the resulting protein variants were designated as H24C and H109T, respectively. Site-directed mutagenesis was performed using a Site-directed Gene Mutagenesis Kit (Beyotime Institute of Biotechnology, Shanghai).

Liquid chromatography-mass spectrometry (LC-MS) analysis. For LC-MS analysis of the cleavage sites of TSPphg on peptidoglycan, soluble products from TSPphg digestion of 5 mg of peptidoglycan at 37 °C overnight were boiled for 10 min to terminate the reaction, and further purified on a ACQUITY UPLC system (Waters, Sparta, NJ, USA) using a Venusil MP C18 column and a linear water/acetonitrile gradient that contained 0.1% trifluoracetic acid. Molecular masses of the released products were then determined by Xevo G2-S QTof MS (Waters), and the results were analyzed with MassLynx software (Waters) and MZmine2 research tools [14].

Nucleotide *sequence accession number.* The complete genome sequence of *Thermus siphoviridae* phage TSP4 was deposited at GenBank under accession number MH992131.1.

3. Results

Previously, we have identified a novel phage endolysin named TSPphg derived from the genome of *Thermus* phage TSP4 (GenBank accession number MH992131.1) which was isolated from Tengchong hot spring in China [8, 9]. In this study, a molecular model of TSPphg was established with the SWISS-MODEL server based on the crystal structure of lysostaphin-type metalloprotease (LytM, PDB accession no. 1qwy, resolution 1.30 Å) from *Staphylococcus simulans* [12]. Figure 1 illustrates the three-dimensional structure of TSPphg and its two important catalytic sites, namely His24 in motif HXXXD and His109 in motif HXH, which were predicted to be highly conserved and functionally similar to the members of M23 peptidase family [15, 16]. Site-directed mutagenesis was then carried out to confirm whether these residues of TSPphg are involved in the functional process. We substituted His24 and His109 with Cys and Thr, and designated the resulting variants as H24C and H109T, respectively. It turned out that after the mutations occurred, both variants H24C and H109T severely lost their bactericidal activity; consequently, we speculate that the studied residues are critical for the antibacterial activity of TSPphg.

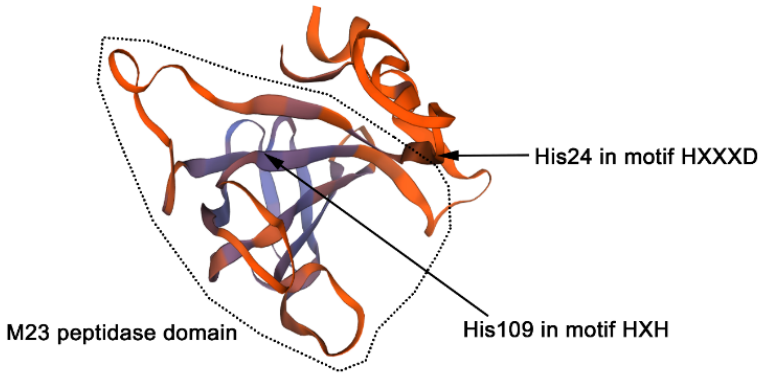

Figure 1 The homology model of TSPphg was created through the SWISS-MODEL server based on the crystal structure of lysostaphin-type metalloprotease (LytM, PDB accession no. 1qwy, resolution 1.30 Å) from *Staphylococcus simulans*. Two important and highly conserved active sites of TSPphg are shown as arrows.

Considering that TSPphg is a putative endolysin identified from *Thermus* phage TSP4 using a bioinformatics approach and it belongs to the M23 peptidase family comprising primarily hydrolytic activity on bacterial peptidoglycan (Figure 1), we further extracted peptidoglycan from *S. aureus* cell walls and examined the capability of TSPphg to degrade peptidoglycan. As shown in Figure 2, LC-MS analysis further confirmed that TSPphg has the specific hydrolytic activity on peptidoglycan by cleaving its glycosidic bonds; thus it could subsequently result in lysis of bacterial cell walls. These findings are consistent with the function of TSPphg as a phage lysin belonging to the M23 family of peptidoglycan hydrolase.

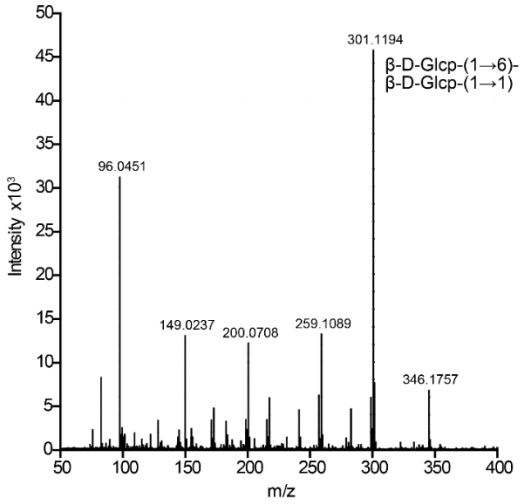

Figure 2 The MS spectrum of peptidoglycan hydrolysate released by TSPphg suggest that TSPphg has specific hydrolytic activity on bacterial peptidoglycan by cleaving its glycosidic bonds.

4. Discussion

It has previously been shown that many proteins with the M23 peptidase domain have specific peptidoglycan-degrading and bacteriolytic activities, causing hydrolysis of bacterial cell walls [17, 18]. Perhaps the most famous examples of these lytic peptidase-containing proteins are lysostaphin from Staphylococcus *simulans biovar staphylolyticus* [17], ALE-1 from *Staphylococcus capitis* [19] and zoocin A produced by *Streptococcus Zooepidemicus* [20]. These proteins all carry an M23 domain and are conserved for the M23 protease motifs of HXH (HLH or HVH) or HXXXD [16]. The findings in this study that His24 in motif HXXXD and His109 in motif HXH within M23 peptidase domain of TSPphg are critical for its bactericidal activity are fairly consistent with these reports. It is possible therefore that TSPphg might have a broad bacteriolytic activity against many other closely related bacteria, and thus has a promising application in biotechnology. Future structure-based functional studies should contribute to delineating the molecular mechanisms involved more precisely. Summarily, our results suggest the great potential of TSPphg as a valuable alternative to antibiotics for therapeutic use to combat multidrug-resistant bacterial pathogens.

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