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Properties of the Endolytic Transglycosylase Encoded by Gene 144 of *Pseudomonas aeruginosa* Bacteriophage phiKZ

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Abstract—Bacteriophage endolysins degrading bacterial cell walls are prospective enzymes for therapy of bacterial infections. The genome of the giant bacteriophage phiKZ of *Pseudomonas aeruginosa* encodes two endolysins, gene products (g.p.) 144 and 181, which are homologous to lytic transglycosylases. Gene 144 encoding a 260 amino acid residue protein was cloned into the plasmid expression vector. Recombinant g.p. 144 purified from *Escherichia coli* effectively degrades chloroform-treated *P. aeruginosa* cell walls. The protein has predominantly α-helical conformation and exists in solution in stoichiometric monomer : dimer : trimer equilibrium. Antibodies against the protein bind the phage particle. This demonstrates that g.p. 144 is a structural component of the phiKZ particle, presumably, a phage tail.

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Evolving antibiotic-resistant pathogenic bacteria require the design of new effective therapeutic methods. Lately, the use of bacterial viruses (bacteriophages) as alternative antibacterial agents [1, 2] is discussed. Also, lytic enzymes of bacteriophages, endolysins named enzybiotics, were recently reported to be used for therapy of bacterial infections [3].

Endolysins are lytic enzymes that specifically recognize and degrade peptidoglycans of bacterial cell walls. Generally, all enzymes of this type depending on the substrate degradation mechanism belong to one of the three following groups: lysozyme-like muramidases (EC 3.2.1.17), N-acetylmuramoyl-L-alanine amidases (EC 3.5.1.28), and peptidases (EC 3.4.17).

Bacteriophages employ endolysins at two stages of their development. In the infection process, the bacteriophage injects its nucleic acid inside the host bacterium using its own structural endolysin for local degradation of the cell wall. In the end of the lytic cycle, viruses leave the cell by destroying it. For that purpose many lytic viruses contain two late genes expressing endolysin and holin. Some phages encode a helper lysozyme mediating the bacterial cell wall lysis [4]. Phages that contain no endolysin at all are also known. They manage "lysis from inside" by invading the mechanism of the host cell autolysis [4-6].

Generally, all bacteria, including the pathogenic strains of *Streptococcus pneumoniae*, *Clostridium botulinum*, *Yersinia pestis*, *Bacillus anthracis*, *Mycobacterium tuberculosis*, *Salmonella typhi*, and *Borrelia burgdorferi* have their own specific bacteriophages. Binding specificity and lytic action of phage endolysins has been studied in detail for *in vitro* detection and elimination of *B. anthracis* [7] and *S. pneumoniae* [8].

Pseudomonas aeruginosa, a facultative pathogen, can cause prolonged and frequently lethal infections, especially with lower immunity patients [9] We investigate lytic bacteriophages infecting *P. aeruginosa* that differ in genome size and morphology. DNA sequencing of three genomes of bacteriophages belonging to different morphological groups—giant *Myoviridae* phage phiKZ [10], with several relatives known [11], T7-like *Podoviridae* bacteriophage phiKMV [12], and transposon phage D3112 [13]—has been completed and reported recently.

Bacteriophage phiKZ genome contains 280,334 bp (GeneBank accession number NC-004629) and encodes more than 300 genes. Two endolysins—gene products (g.p.) 144 and 181—were identified among them [10]. Gene 144 encodes a protein that consists of 260 amino

Abbreviations: PMSF) *o*-phenylmethylsulfonyl fluoride; IPTG) isopropyl β-D-thiogalactopyranoside; PCR) polymerase chain reaction; g.p.) gene product; bp) nucleotide base pairs.

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acid residues (a.a.), and gene 181 encodes a large polypeptide (2237 a.a.), where the endolysin domain (90 a.a.) is located near the C-terminus [14]. Here we present the results of the study of one of the phiKZ endolysins, g.p. 144, the recombinant form of which was obtained by *Escherichia coli* expression, purified, and characterized.

MATERIALS AND METHODS

Vector construction and protein expression. Phage phiKZ gene 144 (780 bp) was amplified by polymerase chain reaction (PCR) using oligonucleotide primers 144 FWD 5′-TGTAGA**GGATCC**ATCATGAAAGTA and 144 REV 5′-TACATC**AAGCTT**TCTATGTGCT, that contain the *Bam*HI and *Hind*III sites, respectively. The PCR amplification product was purified using a Qiagen PCR kit, treated by *Bam*HI and *Hind*III enzymes, and then ligated to the pQE-30 vector (Qiagen, USA), pretreated by the same enzymes. The ligase mixture was transformed into *E. coli* Nova Blue cells (Novagen, USA). This vector employs a strong and tightly regulated phage T5 promoter. The presence of the insert in the plasmid was verified by *Bam*HI–*Hind*III restriction and DNA sequencing. The resulting plasmid vector denoted pKZ144 produces the g.p. 144 protein with six N-terminal histidine residues (affinity purification His-tag). The pKZ144 was transformed into *E. coli* AD494(DE3) (Novagen) for protein expression. The cells were pre-grown in 2xYT media at 37°C to OD_{600} ~ 0.6, then synthesis of the protein was induced by isopropyl β-D-thiogalactopyranoside (IPTG) addition to 0.5 mM concentration, and the cells were incubated for another 4 h at 37°C with moderate aeration.

Protein isolation and purification. *Escherichia coli* cells from 0.5 liter culture were centrifuged at 3500 rpm for 15 min, and the pellet was resuspended in 30 ml buffer A (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM *o*-phenylmethylsulfonyl fluoride (PMSF)). The cells were disrupted by sonication (Techpan MD20, Poland; five 15-sec bursts), and insoluble cell debris was removed by centrifugation at 17,000 rpm for 20 min. The supernatant was applied to a Ni-NTA-agarose (Qiagen) column. Affinity-bound protein was eluted in native conditions by 200 mM imidazole in buffer A. Purified protein was dialyzed against 20 mM Tris-HCl, pH 7.5, 50 mM NaCl buffer, and concentrated by Amicon Centriprep 10 (Millipore, USA). Protein concentration was determined by the Bradford method using a BioRad (USA) reagent kit.

Determination of the protein oligomeric state by gel filtration. Purified g.p. 144 protein was applied to a Superose 12 HR10/30 (Pharmacia, Sweden) column, equilibrated with buffer A, and eluted at 0.5 ml/min rate. The column was preliminarily calibrated with protein standards. The apparent molecular weight of g.p. 144 protein was determined according to the dependence of the molecular weight logarithm on the elution volume. Comparative peak areas were calculated using the FPLC Manager software.

Circular dichroism (CD) spectroscopy. The CD spectrum was recorded using a J-500 (JASCO, Japan) spectropolarimeter in 10 mM Na-phosphate buffer, pH 7.5, at room temperature. The secondary structure content was calculated by the Provencher [14] method using CONTIN [15] or Selcon [16] software.

Protein activity determination. To check the enzyme activity (at various purification stages or after storage) a 5-mm circle of sterile filter paper was moistened by protein solution (0.01-0.4 mg/ml) and placed onto a field of *P. aeruginosa* PAO 1 cells, pre-treated with chloroform vapor. After a 20-30-min incubation at 37°C, the active enzyme formed a transparent zone in the cell field.

Immunization. Female mice of BALB/cJ line were immunized intraperitoneally three times with two-week intervals. The animals were injected with suspension containing 50-100 μg recombinant g.p. 144 in 0.2 ml phosphate-saline buffer and 0.2 ml complete Freund's adjuvant for the first immunization or 0.2 ml incomplete adjuvant for the following immunizations. Blood was collected on the seventh day after the second or subsequent immunizations.

Immunoblotting. Proteins were separated by SDS-PAGE [17] and electrotransferred to nitrocellulose membrane (Novex, USA) using 0.1 M Tris-borate buffer, 0.05 M EDTA, pH 8.3 [18]. The membrane was incubated for 1 h in 1% bovine serum albumin (BSA) solution in phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) at 20°C to eliminate nonspecific sorption, then incubated for 1 h at 20°C with the serum, and then with anti-mouse antibodies conjugated with horseradish peroxidase under the same conditions. At every stage, the membrane was washed not less than five times with PBS-T. Protein–antibody–enzyme complexes were identified by staining with 0.02% solution of 3,3′-diaminobenzidine (Sigma, USA).

RESULTS

Sequence analysis of g.p. 144. The amino acid sequence of g.p. 144 translated from the gene sequence [10] is presented in Fig. 1a. Homology search using BLAST [19] and CDD [20] systems demonstrated high relation of the C-terminal part of g.p. 144 (residues 92-181) to lytic transglycosylases. These enzymes belong to the same N-acetylmuramoyl hydrolase group (EC 3.2.1.17) as lysozymes. They degrade the cell wall peptidoglycans at the β-1,4-glycoside bond between N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) employing the same substrate site. However, the mechanism of transglycosylases differs from that of lysozymes by the formation of the 1,6-anhydro-N-MurNAc intermediate [21]. This difference is explained

a

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1                                                          60
MKVLRKGDRGDEVCQLQTLLNLCGYDVGKPDGIFGNNTFNQVVKFQKDNCLDSDGIVGKN
61
TWAELFSKYSPPIPYKTIPMPTANKSRAAATPVMNAVENATGVRSQLLLTFASIESAFDY
121
EIKAKTSSATGWFQFLTGTWKTMIENYGMKYGVLTDPTGALRKDPRISALMGAELIKENM
181
NILRPVLKREPTDTDLYLAHFFGPGAARRFLTTGQNELAATHFPKEAQANPSIFYNKDGS
241                        260
PKTIQEVYNLMDGKVAAHRK
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b

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75   YKTIPMPTANKSRAAATPVMNAVENATGVRSQLLLTFASIESAFDYEIKAKTSSATGWFQFLT 137
1866 WENIPMPQSNKSRDAAQATFKAVSEMTGVPVELLNIFCGIESSFNYNAKAPTSSAAGWFQFIK 1928

138  GTWKTMIENYGMKYGV-LTDPTGALRKDPRISALMGAELIKENMNILRPVLKREPTDTDLYLA 199
1929 STWKGMLAKYGAKFGIPADDENGSLRFDPRINALMGAMFLRDNYEYLENALGRAPTDVDLYLA 1991

200  HFFGPGAARRFLTTGQNELAATHFPKEAQANPSIFYNKDGSPKTIQEVYNLMDGKVAAHR 259
1992 HFMGPAGARKFLTRDQNSIGAEIFPDQARANRSIFFKTDGSARTLGEIYQVMENKVAKFR 2051
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Fig. 1. a) Structural and functional elements of g.p. 144 sequence. Peptidoglycan-binding domain residues are marked by single underlining and catalytic transglycosylase domain by double underlining. Active site residues are indicated in bold. Shading indicates proposed α-helical residues. b) Alignment of active site sequences of bacteriophage phiKZ g.p. 144 (top) and g.p. 181 (bottom).

by the absence of one of the negatively charged amino acid residues in the enzyme active site. In the case of egg lysozyme the protonated Glu35 residue serves as the catalytic acid (proton donor), and the deprotonated Asp52 residue is a nucleophile forming a covalent intermediate [22]. Transglycosylases have only one carboxyl group in the active site, i.e., Glu162 in the membrane-bound *E. coli* protein MltB [23], and the hydrolysis proceeds via oxazoline intermediate formation [24]. As a result of sequence alignment of ~40 transglycosylases from different organisms [20], we have revealed that the residue corresponding to g.p. 144 Glu115 is the most conservative and, probably, plays the role of the main functional group of the enzyme active site.

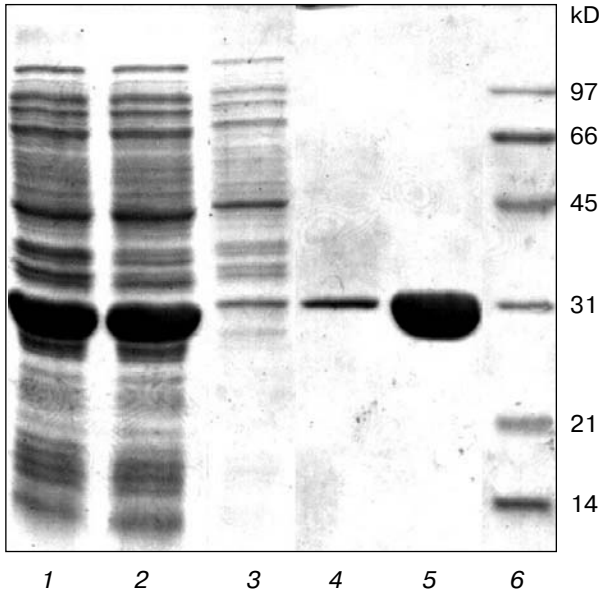

Fig. 2. Purification of recombinant g.p.144. 12% SDS-PAGE. *1*) AD494(DE3) cells after IPTG induction; *2*) soluble fraction after cell degradation; *3*) Ni-affinity column unbound material; *4*) purified g.p. 144; *5*) concentrated purified g.p. 144; *6*) BioRad molecular weight markers (in kD).

The N-terminal part of the g.p. 144 polypeptide chain (residues 9-69) contains an additional domain for binding the cell wall peptidoglycans (Fig. 1a). The search in the database of known and putative proteins has revealed no homology among viral proteins. However, a similar domain formed by three α-helices was found in bacterial autolysins responsible for cell wall degradation during cell growth and division [25]. The presence of such domain may explain the selectivity of full-length g.p. 144 when lysing *P. aeruginosa* cells "from outside".

The homology of active site domains of g.p. 144 and the other phage phiKZ endolysin, g.p. 181, is as high as ~52% identical amino acid residues (Fig. 1b). Glu1906 is possibly the catalytic carboxyl group of g.p. 181.

Expression, isolation, and properties of g.p. 144. Measurement of the g.p. 144 expression level in *E. coli* and enzyme activity in cell extracts has shown that addition of six histidine residues to the N-terminus has no effect on protein solubility and activity (data not shown). Therefore, we purified g.p. 144 by a convenient one-stage affinity chromatography method on Ni-NTA-agarose. The standard purification protocol yields ~15 mg protein of >90% purity (by SDS-PAGE analysis) from 1 liter of cell culture (Fig. 2).

The g.p. 144 protein is soluble and stable in solution at even relatively high concentrations (up to 7 mg/ml) (Fig. 2). Chromatogram analysis after gel filtration on a calibrated Superose 12 column (Fig. 3) showed that g.p. 144 in solution is a mixture of monomers, dimers, and trimers (peaks corresponding to apparent molecular weights of 128.3, 81.6, and 40.3 kD) in approximately 1 : 3 : 2 ratio, respectively. This is an equilibrium state because after separating material from individual peaks and re-chromatographing it on the same column we yielded the same three peaks in the same ratio.

Sequence analysis of g.p. 144 by the 3D-PSSM algorithm [26] shows predominantly α-helical protein conformation with the formation of seven prolonged α-helices (Fig. 1a). This result corresponds well to the CD data. Spectrum analysis by Provencher's method indicates that 72% of the residues of g.p. 144 are in α-helical conformation (Fig. 4).

Enzyme activity and specificity. G.p. 144 effectively degrades *P. aeruginosa* cell walls treated with chloroform. However, application of the enzyme solution to the growing cell field causes just temporary inhibition of bacterial growth. Similarly, the cell field lysis is observed in case of chloroform-treated *E. coli* cells, and live cells are not affected below enzyme concentration 2 mg/ml in the sample.

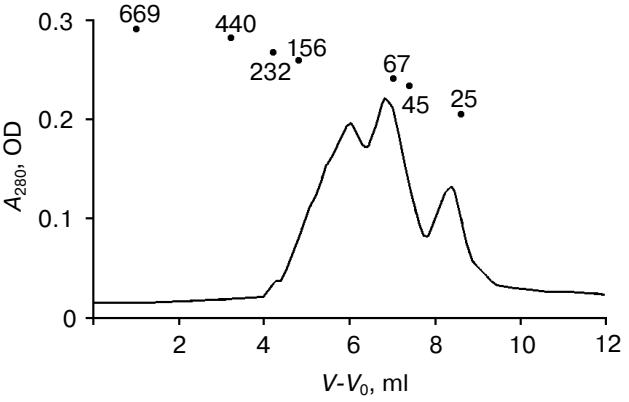

Fig. 3. Resolution of g.p. 144 oligomeric forms by Superose 12 HR 10/30 gel filtration. Elution volumes of protein standards with known molecular weight (kD) are indicated by dots.

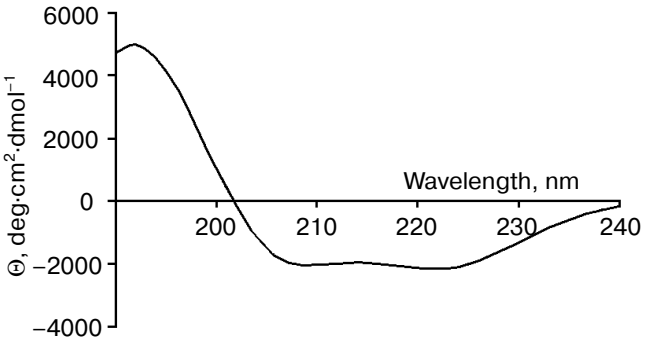

Fig. 4. CD spectrum of g.p. 144 recorded in 10 mM Na-phosphate buffer.

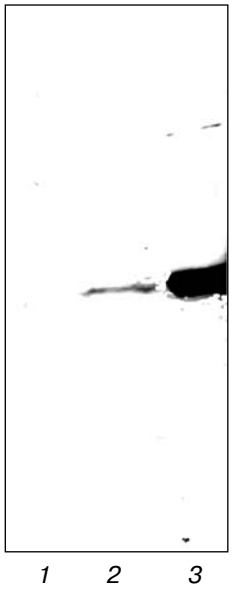

Fig. 5. Protein immunoblotting after 12% SDS-PAGE resolution. Staining with anti-g.p. 144 antibody serum. *1*) Phage phiKZ capsids produced by ts13 mutant; *2*) bacteriophage phiKZ; *3*) recombinant g.p. 144.

Immunodetection. The localization of g.p. 144 in the bacteriophage phiKZ particle was studied by immunoblotting. For this purpose whole phage particles or its capsids (we have obtained a thermosensitive phiKZ ts13 mutant which forms only capsids while infecting *P. aeruginosa* PAO1 cells at 42°C (unpublished data)) were purified by CsCl gradient centrifugation and degraded by several freeze–thaw cycles. Structural proteins of phage particles were then resolved by SDS-PAGE with subsequent transfer to nitrocellulose membrane and detected with antibodies against recombinant g.p. 144. Immuno-blotting results presented in Fig. 5 show that g.p. 144 is a structural protein of the whole phiKZ particle and is not found among capsid proteins. Preliminary results of mass-spectrometric characterization of isolated phage head proteins also indicate that g.p. 144 is absent in capsids (data not shown; results are prepared for publication). Thus it is possible to propose that g.p. 144 is a structural protein of the bacteriophage tail, possibly the virion base plate.

DISCUSSION

The analysis of the bacteriophage phiKZ genome sequence has revealed two genes, 144 (780 bp) and 181

(6726 bp), products of which are supposed to have endolysin activity [10]. It is known that gene 181 is completely translated to a protein, which is included into the phage particle after a proteolytic processing, the deletion of 150 N-terminal residues. Judging from the band intensity after the electrophoresis of bacteriophage phiKZ proteins, the particle contains not less than six copies of g.p. 181 [10]. Analysis of genome reading frames shows a high number of conservative residues typical for transglycosylases in the C-terminal part of g.p. 144 and 181 (Fig. 1). This domain enriched with α-helices catalyses the degradation of cell wall peptidoglycans at the β-1,4-glycoside bond between N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) and is abundant among lytic transglycosylases of bacteria [23, 27, 28], enterobacterial phages P1 and PRD1 [29], and was found in eukaryotic "goose type" lysozymes [30].

Compared to other transglycosylases, Glu115 is the most conservative residue of g.p. 144. It possibly serves as a carboxyl group of the enzyme active site. Other amino acid residues of g.p. 144 (Ser116, Gln134, and Thr139) may be essential for proper orientation of the substrate in the active site cavity [23].

Most known endolytic enzymes of phages and bacteria have modular layout of the polypeptide chain [25]. Domains providing primary receptor binding and the fixation of the enzyme on the cell surface or the penetration through the cell membrane play an important role in the selectivity of endolytic proteins.

Database analysis [19, 20] allows classification of the N-terminal domain of g.p. 144 as a member of the domain group that includes about 60 amino acid residues clustered as a bundle of three α-helices. This structure was first identified as a part of Zn-dependent Ala-Ala-carboxypeptidase [31] and later was found in a number of bacterial cell wall-degrading enzymes [25]. The peptidoglycan-binding domain of g.p. 144 has substantially lower homology with other modules of this group unlike the g.p. 144 catalytic domain and other transglycosylases. This is possibly explained by conservation of just general domain architecture while amino acid residues responsible for binding to particular peptidoglycan sites may differ. The presence of an additional substrate-binding domain besides the active site of the enzyme may explain high selectivity of g.p. 144 and low cytotoxic effect to *E. coli*.

Morphologically, bacteriophage phiKZ belongs to the *Myoviridae* family possessing a contractile tail. So, we suggested the mechanism of phiKZ cascade of endolytic proteins to be the same as well studied representatives of T-even bacteriophages infecting *E. coli*. Similarly to g.p. 5 of bacteriophage T4 [32], the endolytic domain of g.p. 181 is found among structural proteins of the phage virion [10]. Hence, the second protein, g.p. 144, should have been the helper lysozyme assisting the phage release from the host cell, like g.p. *e* of phage T4. However, immunological analysis revealed that g.p. 144 is also a virion protein, being a part of the tail or base plate because the phiKZ mutant ts13 producing only phage capsids has no immune response to g.p. 144-specific antibodies.

Thus, we can propose the following scheme of phiKZ endolysin cascade: g.p. 144 lysozyme is a part of the protein complex of the phage tail and participates in the primary recognition and infection of the host cell. This local interaction is highly specific but not lethal for the cell. The mode of action of g.p. 144 on live *P. aeruginosa* cells *in vitro* supports this suggestion.

A role of the lysozyme domain of g.p. 181 in the bacteriophage phiKZ life cycle is still unclear. Probably like bacteriophage T4 g.p. 5 [32], it is a part of the "molecular needle" complex and provides the injection of the viral DNA inside the cell along with g.p. 144. So the later stages of the phage particle assembly may be accompanied with autocatalytic activation of the transglycosylase domain of g.p. 181 as happens with bacteriophage T4 g.p. 5 lysozyme. It is also possible that g.p. 181 may participate in the final lysis of the host cell to release newly synthesized phage particles.

Small phages of the *Tectiviridae* family—PRD1 *Salmonella entrica* [29] and GIL01 *Bacillus thuringiensis* [33]—contain two structural endolysins. However for more complicated tailed bacteriophages, such organization of enzymes like we found in phiKZ was not previously known.

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