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Antibiofilm activity of dual-function Tail tubular protein B from KP32 phage on *Enterobacter cloacae*

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Abstract:

Background: Dual function tail tubular proteins (TTP) from lytic bacteriophage are novel interesting group of biologically active enzymes possessing antibiofilm activity. Surprisingly, apart from their structural function, they are also polysaccharide hydrolyzes destroying bacterial extracellular components. One of the representatives of this group is TTPB from KP32 phage. Here, we present its biological activity towards biofilm of *E. cloacae* ATCC 13047 strain and towards its exopolysaccharide (EPS).

Methods: TTPB was overexpressed in *E coli* system, purified and tested towards the pathogenic bacteria using agar overlay method. Hydrolytic activity of TTPB against bacterial EPS has been performed by reducing sugar (RS) determination in TTPB/EPS mixture regarding the RS amount obtained after acidic hydrolysis. Antibiofilm activity of TTPB has been set down on one-day *E. cloacae* biofilm using a biochemical method. Finally, the synergistic activity of TTPB with kanamycin has been demonstrated.

Results: For the first time the hydrolytic activity of TTPB towards bacterial EPS has been showed. TTPB releases about a half of the whole RS amount of *E. cloacae* ATCC 13047 EPS and can reduce its biofilm by over 60%. Destroying the bacterial biofilm the phage protein improves the antibiotic action increasing kanamycin effectiveness almost four times.

Keywords: tail tubular protein B, bacteriophage, biofilm, exopolysaccharide, hydrolytic activity

Introduction

Nowadays, drug-resistance of pathogenic bacteria is a serious problem. High degree of the antibiotic- resistance is related to biofilm production ability and bacteria survival in hospital environment. Biofilm is an organized structure, in which bacterial cells stick to each other on a various solid surfaces forming large aggregates. They are often enclosed by thick polysaccharide layer which makes them resistant to antibiotics and thus very hard to eliminate [1]. The major biofilm adhesion factors - bacterial polysaccharide - is secreted outside the cell, and hence called exopolysaccharide (EPS) [2]. EPS forms a shell (capsular EPS) or is secreted outside the cell as mucus (slime EPS) [3]. EPS assists in settling in the environment and forms a protective barrier against adverse environmental conditions and biological factors. EPS being a major component of the biofilm impedes antibiotic penetration to the interior of the biofilm [4]. On the other hand, the biofilm associated antibiotic resistance may be reduced by polysaccharide depolymerases (PD) use. A promising alternative in this field seem to be bacteriophage derived PD application [5]. Based on the latest search, phage PD are very diverse in their molecular mass, structure and

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mechanism of action and the huge majority of them are encoded in the same open reading frame of phage structural proteins, and were thus considered as structural proteins [6-8]. The huge diversity of phage PD is resulted from the co-evolution of phages and their host bacteria [9]. One of the most refractory pathogen causing biofilm associated infections is *Enterobacter cloacae. E. cloacae* is a Gram positive bacteria causing a range of nosocomial infections in human, urinary tract infections, biliary tract infections osteomyelitis, cholecystitis, and neonatal meningitis [10]. [4]. Here, we present a novel dual-function phage protein named tail tubular protein B (TTPB) from KP32 phage possessing antibiofilm activity on *E. cloacae* bacteria. TTPB inhibits biofilm formation by *E. cloacae* ATCC 13047 strain and hydrolyses its EPS. This phage protein facilitates antibiotic to break through the biofilm barrier and cooperates with the antibiotic in bacterial biofilm destruction.

Materials and Methods

Bacterial strain of *E. cloacae* ATCC 13047 was obtained from the Polish Collection of Microorganisms (PCM) of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw, Poland), the PCM number of 533. The strain was stored at -80°C and cultivated in Luria-Bertani (LB) broth medium (Difco). Bacteria were cultured at 37°C stationary or with shaking.

Cloning procedure

Gene g32 of KP32 phage was obtained using polymerase chain reaction (PCR) with the following primers: GP32FW – GGATCCCATATGGCTCTCGTATCACAATCA, GP32RV – GAATTCAAAGCTTAATACCGTTAGCGCGTCT. The KP32 DNA template was obtained from the Institute of Genetics and Microbiology, University of Wroclaw, Poland. PCR reactions were conducted using a two-phase standard program. The first phase consisted of seven and the second phase of 23 cycles. Taq polymerase (Fermentas) was used and the annealing time was max. 2 minutes. Annealing temperature in the first phase was 48°C-52°C and in the second phase 55°C-65°C. PCR product was cloned into pGEM T-easy vector (T-vector, Promega) using T4 ligase. T-vector was transformed into *E. coli* DH5α bacteria using the heat-shock method. The correctness of cloning was analyzed using the white/blue colony selection system. Selected clones were inoculated and their plasmid DNA was isolated using a mini-prep kit (Promega) and digested using EcoRI restriction enzyme. The DNA fragments were electrophoretically analyzed and cut out from the agar gel and then sequenced (Genomed) using T7 and SP6 primers. Correct sequences were cloned into pET28a (Promega) expression vectors containing a gene for conferring kanamycin resistance. The vectors express KP phage tail protein with an N-terminal six-histidine tag. Plasmid transformation into competent *E. coli* BL21(DE3)plysS (Promega) cells was done using the heat shock method and then inoculated on LB agar with the antibiotics kanamycin and chloramphenicol.

TTPB expression and purification

TTPB was expressed in *E. coli* BL21 (DE3)plysS strain (Promega). Bacterial clones were propagated in LB broth (37°C with shaking) with kanamycin and chloramphenicol to reach OD600 = 0.8. Induction of gene expression was performed using 0.05 mM IPTG (Roche) and following overnight incubation at 9°C cells were pelleted and suspended in 50 mM Tris/HCl pH = 8.0 lysis buffer containing 1 M NaCl and 10% glycerol supplemented with protease inhibitor cocktail tablets (Roche). Cells were sonicated 8 times for 30 seconds with breaks lasting 1 minute. After debris removal via centrifugation (14000g for 50 minutes) supernatant was mixed with Ni^{2+} chromatography gel and incubated for 1 hour at 37°C on a rotary shaker. After batch chromatography the unbound fraction was removed on a Buchner funnel. Proteins bound to Ni ions were eluted using lysis buffer containing 250 mM imidazole. Imidazole was removed via dialysis on centrifugal filters containing membrane (Millipore) with a cut-off of 30 kDa.

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Concentration of proteins was determined using the BCA method by Smith et al. **(11)** and analyzed in SDS-PAGE electrophoresis using 12.5% gels according to the method of Laemmli et al.**[12]**.

Hydrolytic activity

EPS of *E. cloacae* ATCC 13047 was extracted using 3 volumes of 96% ethanol (4°C, overnight) from supernatant obtained after centrifugation of one-day bacterial culture. The pelleted slime was obtained after centrifugation (14000g for 30 minutes at 4°C) then dissolved in water, dialyzed against water, frozen and lyophilized. The reaction mixture contained 100 µg of EPS and 100 µl of 0.56 µM of TTPB and was incubated for 2 h at 37°C on a rotary shaker. Enzyme activity was determined using reducing sugars (RS) determination with glucose as a standard according to Nelson-Somogoi method **[13]**. The RS amount was compared to the total amount of RS released after EPS (100 µg of dry mass) acid hydrolysis (10 mol l-1 HCl, 85°C, 25'). The negative control contained EPS (100 µg) and buffer lacking the phage proteins. The acid hydrolysis was performed according to the method of Kubler-Kielb et al. (2004) **[14].**

Agar overlay method

Bacteria were cultured overnight in 5 ml of LB broth at 37°C with shaking. Samples of 1 ml of bacteria were inoculated on agar plates and dried. 10 µl (0.025 -0.1 nM) of TTPB and was dotted on a plate and incubated at 37°C overnight.

Antibiofilm activity

One-day biofilm preparation: Bacteria were cultured overnight in 5 ml of LB broth at 37°C with shaking. Overnight cultures were diluted to OD600 = 0.2 using fresh LB. 100 µl of bacteria suspension was inoculated into a 96-well plate (CytoOne) and incubated for 20 hours at 37°C. After that time bacteria were removed and the plate was dried for 15 minutes up-side-down on a sterile paper towel. TTPB was added (0.56 µM) and incubated at 37°C for the next 24 hours. The next day, the OD600 was measured and after that 50 µl of 0.1% trimethyl tetrazolium chloride (TTC) was added to each well. After 1 hour of incubation at 37°C the OD540 was measured on a Biotec microplate reader. Microtitre plate wells containing growth medium without any bacterial culture – sterility control; wells containing cell cultures but without TTPB – control regarded as 100% cell mass. All trials were performed in triplicate and the mean value was calculated with the standard deviation range.

Synergistic action of TTPB with kanamycin on biofilm destruction

Bacteria were cultured overnight in 5 ml of LB broth at 37°C with shaking. Overnight cultures were diluted to OD600 = 0.2 using fresh LB. 100 µl of bacteria suspension, 100 µl of protein solutions (0.56 µM) and 100 µl of kanamycin (50 µg per well) were inoculated into each well of a 96-well plate and incubated at 37°C for 24 hours. The next day, the bacteria/protein/antibiotic mix was removed and washed 3 times with sterile Milli Q water. Again, 100 µl of bacteria suspension and 100 µl of protein solutions with kanamycin were added into the plate and incubated at 37°C overnight. The above procedure was repeated for 3 days. On the fourth day after washing planktonic bacteria, 125 µl of 0.1% crystal violet solution was added and stained for 10 minutes at room temperature. The dye was decanted and the wells were washed with water and allowed to dry. Then 200 µl of 95% ethanol was added and after incubation for 15 minutes, the measurement was performed at 570 nm

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Results

Cloning, gene expression and protein analysis

TTPB was mostly expressed as inclusion bodies, however, the use of elevated concentrations of NaCl and glycerol in the lysis buffer allowed us to obtain almost 100% of soluble protein. TTPB was purified using Ni2+ affinity chromatography and eluted by 250 mM imidazole in Tris/HCl buffer containing 1 M NaCl and 10% glycerol. From one liter of bacterial culture 25 milligrams of the pure protein was obtained. After dialysis TTPB was analyzed in SDS-PAGE (Fig. 1) and the established molecular mass was 89 kDa.

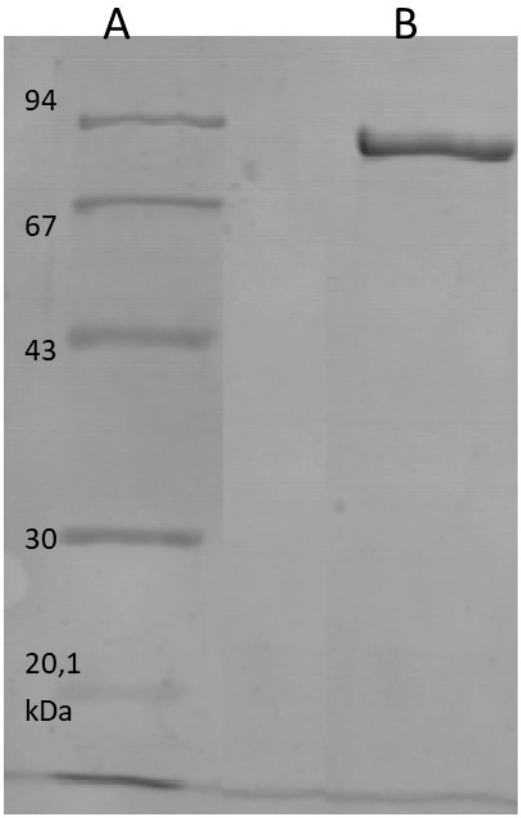

Figure 1. Electrophoretic analysis of TTPB after expression and purification. TTPB was analyzed in 12.5% SDS-PAGE; A) Low molecular mass standard, B) TTPB after purification on NI^{2+} affinity chromatography.

Agar overlay and antibiofilm test

Purified TTPB was tested towards *E. cloacae* ATCC 13047 strain to determine the range of lytic activity. The agar overlay test showed that the phage protein causes plaques on the bacterial lawn (Fig. 2A). The activity zones were translucent not clear plaques suggesting that the enzymatic activity was directed towards bacterial EPS leaving the bacterial cells intake. Further analysis showed that TTPB can reduce bacterial biomasses of above strain. 0.56 μM of TTPB can reduce about 65% of *E. cloacae* strains' biomass after one-day biofilm formation assay. The antibiofilm activity of TTPB is shown in Figure 2B.

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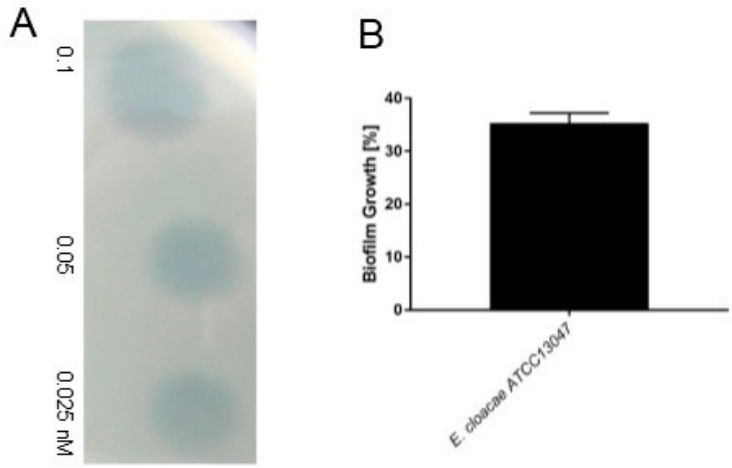

153

154 **Figure 2.** Antimicrobial activity of TTPB in overlay method towards *E. cloacae* ATCC 13047 (A) and
155 towards the bacterial biofilm (B).

156 Hydrolytic activity

157 100 μg of EPS of *E. cloacae* ATCC 13047 strain contained 0.71 μM ml^{-1} of total RS. This total amount
158 of RS was established after EPS acidic hydrolysis. The RS calculation was made using Nelson-
159 Somogoi assay. The EPS hydrolyzed by TTPB gave 0.32 μM ml^{-1} of RS and represents a significant
160 proportion (45%) of the total RS of the EPS. The calculation of the RS release has been performed
161 considering the RS present in the control sample lacking TTPB (negative control).

162 Synergistic activity

163 Further analysis showed that TTPB exhibits synergistic antimicrobial activity with kanamycin (Fig.
164 3). Kanamycin was first tested on *E. cloacae* ATCC 13047 lawn to make sure it kills the bacteria.
165 Next, the 4-day old biofilm was cultured on 96-well plates according to the method described in
166 Material and Methods section. It was shown that, the presence of TTPB increased antibiotic activity
167 by almost four times. Kanamycin (10 μg per well) alone inhibited biofilm formation by about 70%
168 and with phage proteins the inhibition percent increased to 84% with respect to control – bacterial
169 biomass obtained in culture without any additives including TTPB. It was interesting result
170 showing the additive effect of antibiotic and the phage protein.

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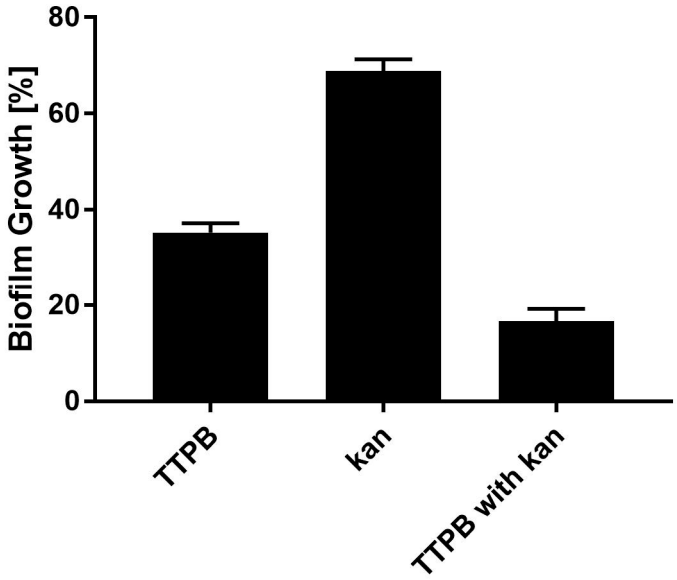

Figure 3. Synergistic activity of TTPB with kanamycin towards *E. cloacae* ATCC 13047 biofilm

Primary structure analysis

Our analysis of amino acid sequence showed that in term of primary structure TTPB from bacteriophage KP32 displays a very high identity (61%) with TTPB from T7 bacteriophage. It suggests these two proteins should display a very similar functional and structural properties. TTPB from T7 bacteriophage was not considered as a hydrolytic enzyme. We can only suggest that function. So we can not compare and point any enzymatic active sites of these two tail B proteins.

4. Discussion

Most infections caused by bacterial strains which are capable of biofilm formation are difficult to treat because of their increased resistance to antibiotics. This resistance of biofilmed bacteria is attributed to a barrier function of the biofilm which binds the antimicrobial agents within a matrix and also the metabolic change in the biofilmed bacteria cells [15]. It is suggested that microbial cells forming a biofilm are even 1000-fold more resistant to antimicrobial agents than their planktonic counterparts [16]. Bacteriophages seem to be attractive and very effective antibiofilm agents, because of their capacity for exopolysaccharide matrix degradation, bacterial cell infection and biofilm structure disruption [17]. Phages can recognize bacterial receptors by specific structural proteins that can also have enzymatic activity able to degrade sugar polymers on the outer side of the cell wall of bacteria [18]. It has been shown that *Klebsiella pneumoniae* phages can produce polysaccharide depolymerases which are associated with the tail spike and even after phage inactivation the enzymes are still active and can form characteristic halo zones in spot agar plate assay [19]. In most cases these hydrolase enzymes are located on the phage tail and spikes, but they can also be produced as soluble proteins which are secreted to the environment [9].

In our previous work we have reported dual-function tail tubular proteins A (TTPA) containing hydrolytic activity towards bacterial polysaccharides [8,7]. Here, we show next Tail tubular protein from KP32 bacteriophage classified as form B (TTPB). It has been consider as structural protein, however, according to our results it is polysaccharide hydrolyzing enzyme. The screening analysis revealed that the protein is the most active towards *E. cloacae* strain. Its activity towards *K. pneumoniae* EPS has been also observed (data not shown). EPS produced by *K. pneumoniae* and by *E. cloacae* are closely resembling, hence both are TTPB's substrates [20].

The analysis of similarities in amino acid sequences using the BLAST program showed TTPB is over 60% homologous to tail B protein (gp12) of T7 bacteriophage. Gp12 is classified as structural protein, located in the bacteriophage tail core and it is important for phage adsorption to the bacterial cell wall and host infection [21]. It has not been considered as an enzyme and its three-dimensional structure has not been known so far. Thus it is not possible to predict the active site of our TTPB comparing with TTPB from T7 bacteriophage.

The most important observation was the additive effect with kanamycin against bacterial biofilm formation. The antibiotic could not reach the biofilmed bacterial cells and the situation was completely different when the phage protein was present. To accurately characterize TTPB's hydrolyzing activity it is necessary to conduct further biochemical studies using polysaccharides of known structures. The study would provide extremely valuable information

In summary, in the presented study an attempt was made to demonstrate the utility of bacteriophage tail tubular protein B to bacterial biofilm destruction. For the first time the hydrolytic activity of the phage protein has been shoved rendering it as dual-function protein. However, the mechanism of action of this protein is still unclear and needs further investigation. Moreover, the biochemical, physico-chemical and tertiary structure analyses of the protein should provide us more information about the substrate binding site and catalytic domain occurrence.

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Author Contributions: EB, KP, AP conceived and designed the experiments. EB, AP performed the experiments. EB, AP, KP analyzed the data. EB,AP,KP,AG contributed to the writing of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest

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