1   *Type of the Paper (Article)*

2 The antibiofilm activity of dual-function tail tubular
3 protein B from KP32 phage

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11

12   **Abstract:**

13   Background: Dual function tail tubular proteins (TTP) belonging to the lytic bacteriophages are the
14   interesting group of biologically active enzymes. Surprisingly, apart from their structural function,
15   they are also polysaccharide hydrolyzes destroying bacterial extracellular components. One of the
16   representatives of this group is TTPB from *Klebsiella pneumoniae* phage – KP32. TTPB hydrolyzes
17   exopolysaccharide (EPS) of *Klebsiella pneumoniae* and *Enterococcus faecalis* strain. This depolymerizing
18   feature was associated with the activity to prevent bacterial biofilm formation. TTPB can inhibit
19   biofilm formation by *K. pneumoniae*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Enterococcus faecalis*
20   and *Pseudomonas aeruginosa* strains. Moreover, synergistic activity with antibiotic action has been
21   observed, most likely due to depolymerases' facilitation of contact of antibiotic with bacterial cells.

22   Methods: TTPB was overexpressed in *E coli* system, purified and tested towards the bacterial strains
23   using agar overlay method. The hydrolytic activity of TTPB was performed using EPSs of *K.*
24   *pneumoniae* PCM2713 and *E. cloacae* ATCC 13047 as the substrates. Next, we determined the reducing
25   sugar (RS) levels in the TTPB/EPS mixtures, regarding the RS amount obtained after acidic
26   hydrolysis. The antibiofilm activity of TTPB has been set down on bacterial biofilm using a
27   biochemical method. Finally, we have demonstrated the synergistic activity of TTPB with
28   kanamycin.

29   Results: For the first time, the hydrolytic activity of TTPB towards bacterial EPSs has been shown.
30   TTPB releases about a half of the whole RS amount of EPSs belonging to *K. pneumoniae* PCM 2713
31   and *E. cloacae* ATCC 13047 strains. 1.12 μM of the phage protein reduces biofilm of both strains by
32   over 60%. Destroying the bacterial biofilm the phage protein improves the antibiotic action
33   increasing kanamycin effectiveness up to four times.

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

35   **Introduction**

36   *K. pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia*
37   *coli* belong to the most frequent biofilm-forming microorganisms [1]. The antibiotic- resistance is
38   related to the ability of biofilm production and bacteria survival in a hospital environment. Biofilm
39   is an organized structure, in which bacterial cells stick to each other on various solid surfaces
40   forming large aggregates. They are often enclosed by thick polysaccharide layer which makes
41   them resistant to antibiotics and thus very hard to eliminate [2]. The major biofilm adhesion factors
42   - a bacterial polysaccharide - is secreted outside the cell, and hence called exopolysaccharide (EPS)
43   [3]. EPS is a component of a bacterial capsule (capsular EPS) or is secreted by bacteria outside the

44 cell as mucus (slime EPS) [4]**.** EPS forms a protective barrier against adverse environmental
45 conditions and biological factors. It also assists bacterial cells in settling in the biofilm structure.
46 EPS as the main component of the biofilm impedes the antibiotic penetration to the interior of the
47 biofilm structure [5]. On the other hand, the biofilm-associated antibiotic resistance may be
48 eliminated when polysaccharide depolymerase (PD) is used. A promising alternative in this field
49 seems to be bacteriophage derived PD application [6]. Based on the latest search, phage PDs are
50 very diverse in their molecular masses, structures, and mechanisms of action. Most of them are
51 encoded in the open reading frames of phage structural proteins and thus were considered as
52 structural proteins [7-9]. The diversity of phage PDs results from the co-evolution of phages and
53 their host bacteria [10]. Previously, we have reported the preliminary results of biological activity
54 of the tail tubular proteins A of the phages KP32 and KP34 [8,9]. These proteins were called the
55 dual-function proteins due to their structural and hydrolytic features related to binding and
56 hydrolysis of EPS obtained from *K. pneumoniae* PCM 2713. Here, we present a novel dual-function
57 phage protein named tail tubular protein B (TTPB) from KP32 phage. The protein possesses
58 antibiofilm activity towards *K. pneumoniae, Enterobacter cloacae, Staphylococcus aureus, Enterococcus*
59 *faecalis* and *Pseudomonas aeruginosa* strains. TTPB shows the highest inhibition activity of biofilm
60 formation by *E. cloacae* ATCC 13047 and *K. pneumoniae* PCM 2713 strains and hydrolyzes their
61 EPSs. Both bacterial strains are pathogenic. *K. pneumoniae* is Gram-negative causing different types
62 of healthcare-associated infections. While, *E. cloacae* is Gram-positive bacteria causing a range of
63 nosocomial, urinary tract, biliary tract infections, osteomyelitis, cholecystitis, and neonatal
64 meningitis in human [11, 5]. It is the most refractory pathogen causing biofilm-associated
65 infections. As we have shown TTPB facilitates antibiotic to break through the biofilm barrier and
66 cooperates with the antibiotic in the bacterial biofilm destruction. This finding suggests that the
67 protein could be applied as a therapeutic agent for chronic and nosocomial infections curing.

68 **Materials and Methods**

69   All of the bacterial strains used in the experiments were obtained from the Polish Collection of
70 Microorganisms (PCM) of the L. Hirszfeld Institute of Immunology and Experimental Therapy,
71 Polish Academy of Sciences (Wroclaw, Poland), namely: *Klebsiella pneumoniae* (PCM2713 and
72 PCM2715), *Enterobacter cloacae* (PCM533) ATCC 13047, *Pseudomonas aeruginosa* (PCM2710),
73 *Staphylococcus aureus* (PCM519), *Shigella flexneri* (PCM101), *E. coli O24* (PCM195), *Proteus mirabilis*
74 (PCM543), *Citrobacter freundii* O29 (PCM1562), *Hafnia alvei* (PCM1223) and *Enterococcus faecalis* VRE
75 (Vancomycin-resistance) which is a clinical isolate. All strains were stored at -80°C and cultivated in
76 Luria-Bertani (LB) broth medium (Difco). Bacteria were cultured at 37°C stationary or with shaking.

77 Cloning procedure

78 The genome of phage KP32 is deposited in the genomic database, GenBank: GQ413937. TTPB gene
79 of KP32 phage was obtained using polymerase chain reaction (PCR) with the following primers:
80 GP32FW – GGATCCCATATGGCTCTCGTATCACAATCA, GP32RV –
81 GAATTCAAAGCTTAATACCGTTAGCGCGTCT. The KP32 DNA template was obtained from the
82 Institute of Genetics and Microbiology, University of Wroclaw, Poland. PCR reactions were
83 conducted using a two-phase standard program. The first phase consisted of seven and the second
84 phase of 23 cycles. Taq polymerase (Fermentas) was used and the annealing time was max. 2 minutes.
85 Annealing temperature in the first phase was 48°C-52°C and in the second phase 55°C-65°C. PCR
86 product was cloned into the pGEM T-easy vector (T-vector, Promega) using T4 ligase. T-vector was
87 transformed into *E. coli* DH5α bacteria using the heat-shock method. The correctness of cloning was
88 analyzed using the white/blue colony selection system. Selected clones were inoculated and their
89 plasmid DNA was isolated using a mini-prep kit (Promega) and digested using EcoRI restriction
90 enzyme. The DNA fragments were electrophoretically analyzed and cut out from the agar gel and
91 then sequenced (Genomed) using T7 and SP6 primers. Correct sequences were cloned into pET28a
92 (Promega) expression vectors containing a gene for conferring kanamycin resistance. The vectors

93   express KP phage tail protein with an N-terminal six-histidine tag. Plasmid transformation into
94   competent *E. coli* BL21(DE3)plysS (Promega) cells was done using the heat shock method and then
95   inoculated on LB agar with the antibiotics kanamycin and chloramphenicol.

TTPB expression and purification

97   TTPB was expressed in *E. coli* BL21 (DE3)plysS strain (Promega). Bacterial clones were propagated in
98   LB broth (37°C with shaking) with kanamycin and chloramphenicol to reach OD600 = 0.8. Induction
99   of gene expression was performed using 0.05 mM IPTG (Roche) and following overnight incubation
100   at 9°C cells were pelleted and suspended in 50 mM, Tris/HCl pH = 8.0 lysis buffer containing 1 M
101   NaCl and 10% glycerol supplemented with protease inhibitor cocktail tablets (Roche). Cells were
102   sonicated 8 times for 30 seconds with breaks lasting 1 minute. After debris removal via centrifugation
103   (14000g for 50 minutes) supernatant was mixed with Ni^{2+} chromatography gel and incubated for 1
104   hour at 37°C on a rotary shaker. After batch chromatography, the unbound fraction was removed on
105   a Buchner funnel. Proteins bound to Ni ions were eluted using lysis buffer containing 250 mM
106   imidazole. Imidazole was removed via dialysis on centrifugal filters containing membrane
107   (Millipore) with a cut-off of 30 kDa. The concentration of proteins was determined using the BCA
108   method by Smith et al. [12] and analyzed in SDS-PAGE electrophoresis using 7% gels according to
109   the method of Laemmli et al.[13].

Circular dichroism spectroscopy (CD)

111   CD spectra were recorded on a Jasco J-600 spectropolarimeter, at room temperature. Spectra were
112   measured in 50 mM Tris/HCl buffer pH = 8.0 containing 0.2 M NaCl and 5% glycerol. The path length
113   of 1 mm was used. Concentrations of the protein solutions were in the range of 0.07–0.1 mg/ml.

Agar overlay method

115   Bacteria were cultured overnight in 5 ml of LB broth at 37°C with shaking. The overnight cultures of
116   bacteria were diluted to OD = 0.2 and pipetted onto agar plates. 10 μl (1.12 μM) of TTPB and 10 μl of
117   the control sample (50 mM Tris/HCl buffer pH = 8.0 containing 0.2 M NaCl and 5% glycerol) were
118   dotted on a plate and incubated at 37°C overnight. All trials were performed in triplicate. The nutrient
119   agar (pH=7.2) composition was: beef extract (10 g) peptone (10 g) NaCl (5 g) and agar (20 g)
120   suspended in 1000 ml and sterilized at 121°C for 20 minutes. Samples of 1 ml of bacteria were
121   inoculated on agar plates and dried.

Hydrolytic activity

123   Slime exopolysaccharide of *K. pneumoniae* strain PCM2713 and *Enterobacter cloacae* (PCM533) ATCC
124   13047 and starch (POCH) were subjected to enzymatic degradation. Bacterial EPS was extracted using
125   3 volumes of 96% ethanol (4°C, overnight) from the supernatant obtained after centrifugation of one-
126   day bacterial culture. The pelleted slime was obtained after centrifugation (14000g for 30 minutes at
127   4°C) then dissolved in water, dialyzed against water, frozen and lyophilized. The reaction mixtures
128   contained 100 μg of EPS or starch and 100 μl of 0.56 μM of TTPB were incubated for 2 h at 37°C on a
129   rotary shaker. Enzyme activity was determined using reducing sugars (RS) determination with
130   glucose as a standard according to Nelson-Somogoi's method [14]. The RS amount was compared to
131   the total amount of RS released after EPS/starch (100 μg of dry mass) acid hydrolysis (10 mol l-1 HCl,
132   85°C, 25'). The negative control contained EPS/starch (100 μg) and buffer lacking the phage proteins.
133   The acid hydrolysis was performed according to the method of Kubler-Kielb et al. (2004) [15].

Antibiofilm activity

135   One-day biofilm preparation: bacteria were cultured overnight in 5 ml of LB broth at 37°C with
136   shaking. Overnight cultures were diluted to OD600 = 0.2 using fresh LB. 100 μl of bacterial suspension

137   was inoculated into a 96-well plate (CytoOne) and incubated for 20 hours at 37°C. After that time
138   bacteria were removed and the plate was dried for 15 minutes up-side-down on a sterile paper towel.
139   TTPB was added (0.28, 0.56 and 1.12 μM) and incubated at 37°C for the next 24 hours. The next day,
140   the OD600 was measured and after that 50 μl of 0.1%, trimethyl tetrazolium chloride (TTC) was
141   added to each well. After 1 hour of incubation at 37°C, the OD540 was measured on a Biotec
142   microplate reader. Microtitre plate wells containing growth medium without any bacterial culture –
143   sterility control; wells containing cell cultures but without TTPB – control regarded as 100% cell mass.
144   All trials were performed in triplicate and the mean value was calculated with the standard deviation
145   range.

146   Synergistic action of TTPB with kanamycin on biofilm destruction

147   Bacteria were cultured overnight in 5 ml of LB broth at 37°C with shaking. Overnight cultures were
148   diluted to OD600nm = 0.2 using fresh LB. 100 μl of bacterial suspensions were inoculated into each
149   well of a 96-well plate and incubated at 37°C for 24 hours. The next day, we removed the bacterial
150   suspensions and inoculated 100 μl of fresh cultures (OD 600nm = 0.2). The above procedure was
151   repeating each day. On the fourth day, we removed the bacteria and inoculated 100 μl of TTPB
152   solutions (0.56 μM)/kanamycin (50 μg per well)/the mixture of TTPB and kanamycin and incubated
153   the plate at 37°C for 24 hours. The next day, we washed the plate 3 times with sterile Milli Q water.
154   Next, we added 125 μl of 0.1% crystal violet solution and stained for 10 minutes at room temperature.
155   After washing and drying the plate, we added 200 μl of 95% ethanol and incubated for 15 minutes
156   We measured the absorbance at 570 nm. The negative controls contained the biofilms untreated with
157   neither TTPB nor kanamycin.

158   **Results**

159   Cloning, gene expression, and protein analysis

160   Mostly TTPB was overexpressed as inclusion bodies. However, the use of elevated concentrations of
161   NaCl and glycerol in the lysis buffer allowed us to obtain almost 100% of soluble protein. TTPB was
162   purified using Ni^{2+} affinity chromatography and eluted by 250 mM imidazole in Tris/HCl buffer
163   containing 1 M NaCl and 10% glycerol. We have obtained 25 milligrams of the pure protein from 1 L
164   of the bacterial culture. After dialysis TTPB was analyzed in SDS-PAGE (Fig. 1) and by circular
165   dichroism spectroscopy. CD analysis revealed TTPB was folded after biosynthesis and purification.
166   It contained mostly β-strand secondary structure (data not shown). This CD analysis confirmed the
167   results obtained after YASPIN server analysis [16], which indicated that the secondary structure of
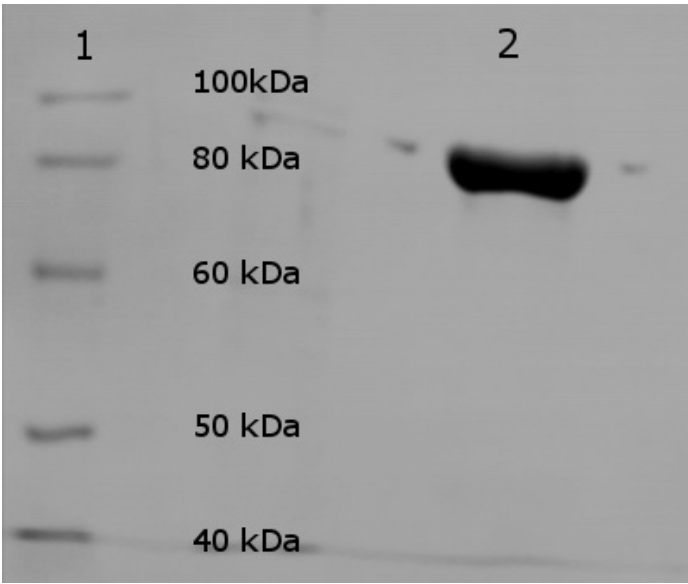168   gp32 consists of 53% β-strand.

169

170 **Figure 1**. Electrophoretic analysis of TTPB after expression and purification. TTPB was analyzed in
171 7% SDS-PAGE; A) molecular mass standard (Thermo Scientific), B) TTPB after purification on Ni^{2+}
172 affinity chromatography.

173 Agar overlay and antibiofilm test

174 Initial screening tests that assess the enzymatic activity of TTPB were agar spot assays on plates with
175 mature bacterial lawns. We selected eleven strains belonging to Gram-negative and Gram-positive
176 groups. The agar overlay test showed that the phage protein causes plaques on the bacterial lawn of
177 *K. pneumoniae* PCM 2713, PCM 2715, *E. cloacae* PCM 533 ATCC 13047, *S. aureus* PCM 519, *P. aeruginosa*
178 PCM 2710 and E. *faecalis* VRE ATCC 13047. The activity zones were translucent, not clear plaques
179 suggesting that the enzymatic activity of TTPB was directed towards bacterial EPSs without the
180 bacterial cells damage. TTPB was no active towards *S. flexneri* Y PCM 101, *E. coli* O24 PCM 195, *P.*
181 *mirabilis* PCM 543, *C. freundii* O29 PCM 1562 and *H. alvei* PCM 1223. Further analysis showed that
182 1.12 μM of TTPB reduces bacterial biomasses of the sensitive strains more than 50%. The highest
183 activity of TTPB was observed towards *E. cloacae* ATCC 13047 strain. It inhibited the biofilm
184 formation over 80%. The biofilm inhibition activity of TTPB is shown in Figure 2.
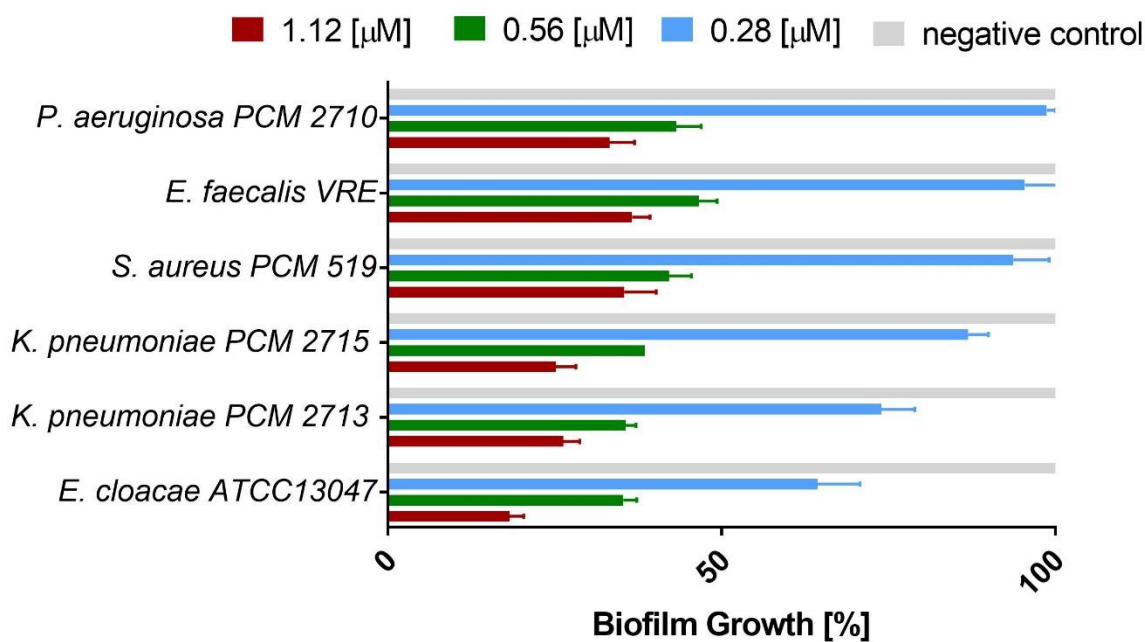
185

Figure 2. The antibiofilm activity of TTPB in the one-day biofilm test. Biofilm formation is expressed as a percentage relative to that produced by the untreated control (negative control), which is set at 100%. Error bars represent standard deviation (SD) of three biological samples performed in triplicate. Statistical significance ($p < 0.05$) was assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, (**www.graphpad.com**).

Hydrolytic activity

The hydrolytic activity of TTPB was performed using slime EPSs isolated from *K. pneumoniae* PCM 2713 and *E. cloacae* ATCC 13047 and starch as the substrates. The polysaccharides (100 µg of dry mass) were incubated with TTPB (100 µl of 0.56 µM) in 1 ml of reaction mixture. To determine their degradation level the amount of RS released during the hydrolysis was measured. The RS concentration after TTPB hydrolysis for both EPS was calculated as about 0.3 µM ml^{-1}, while the total amount of RS of *K. pneumoniae* EPS was 0.57 and of *E. cloacae* EPS it was 0.72 µM ml^{-1}. The calculation of the RS release has been performed considering the RS present in the control sample lacking TTPB (Fig. 3). TTPB does not hydrolyze the starch.
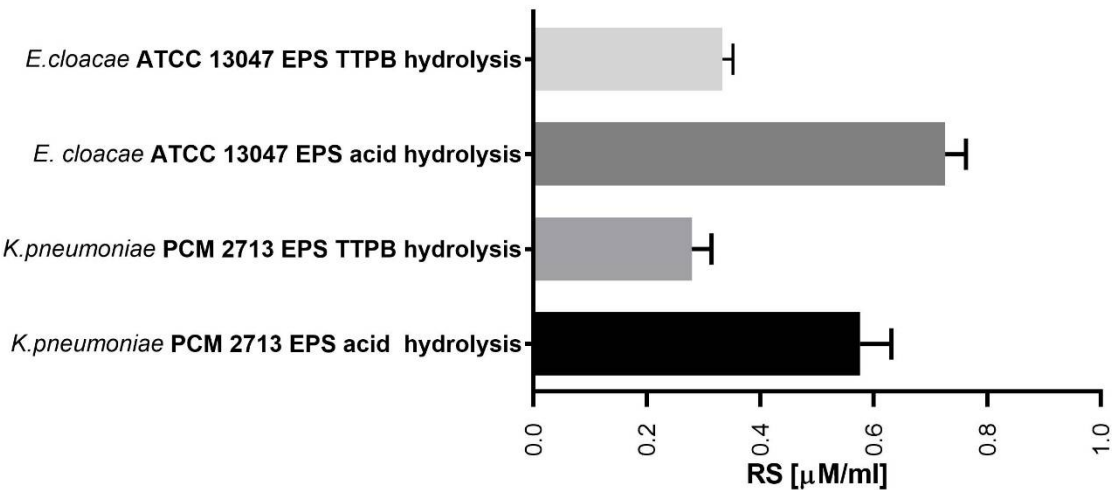
203

Figure 3. The hydrolytic activity of TTPB towards bacterial EPS. All results are presented as averages of results from three independent replicates in three parallel trials. Error bars represent the means, standard deviations (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, (**www.graphpad.com)**).

Synergistic activity

Further analysis showed that TTPB exhibits synergistic antimicrobial activity with kanamycin (Fig. 4). TTPB increased the antibiotic activity by 30% towards *K. pneumoniae* PCM 2713 strain and over 50% towards *E. cloacae ATCC 13047* strain. We performed the tests on the 4-day-old biofilm according to the method described in Material and Methods section. The negative controls contained no TTPB and kanamycin.
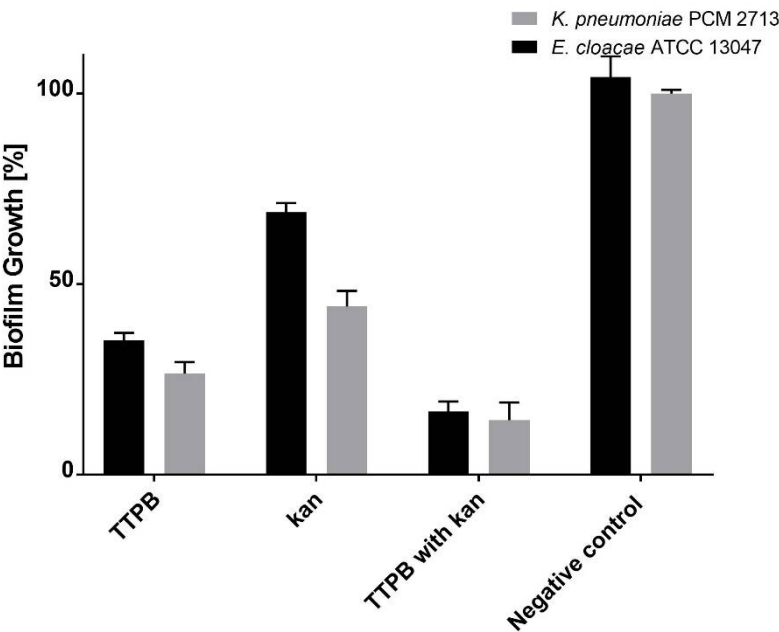

Figure 4. Synergistic activity of TTPB with kanamycin measured with reduction of biofilm formed by *K. pneumoniae* PCM 2713 and *E. cloacae* ATCC 13047. All results are presented as averages of results

227   from three independent replicates in three parallel trials. Error bars represent the means, standard
228   deviations (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California
229   USA, (www.graphpad.com)).

230   Primary structure analysis

231   The primary structure of TTPB is very similar among tail tubular proteins B of *Klebsiella pneumoniae*
232   phage family. For these proteins the amino acid identity is in the range of 84 – 98%. The Blast analysis
233   showed that amino acid sequence of TTPB is similar in 60 - 64% to tail proteins of the phages of
234   *Pectobacterium, Enterobacteria, Yersinia, Serratia, Citrobacter, Escherichia coli*, and *Erwinia*. The primary
235   structure identity of TTPB in the range of 51 - 59% was calculated for tail proteins for phages of
236   *Pasteurella, Morganella, Pseudomonas* and also some *Yersinia, Citrobacter* and *Escherichia coli*. So far,
237   most of phage tail proteins mentioned above were characterized very poor or at all. One of the best-
238   known tail tubular protein B is that coming from Enterobacteria phage T7 (EPT7), named gp12. TTPB
239   of KP32 phage is similar in 61% to gp12 of EPT7 in term of primary structure. Based on literature
240   reports gp12 is known as the part of the phage-extracted tail complex that forms the end of the tail,
241   shaped like conical tube, the nozzle [17]. There is no information about its enzymatic activity. Our
242   results showed any doubts that TTPB of KP32 phage can hydrolyze bacterial EPS. Our trials of TTPB
243   3D structure predictions or hydrolytic domain indications were not successful. But on the base of
244   our previous results regarding TTPA from KP32 phage [8, 9], we could suspect that the enzymatic
245   activity of TTPB could be due to the presence of the catalytic triad D-X-D. We found this sequence
246   in two places of polypeptide chain: D331-X-D333 and D633-X-D635.

4. Discussion

248   Most infections caused by bacteria which form a biofilm are difficult to treat. This resistance of
249   bacteria in biofilm is attributed to a barrier function of the biofilm which binds the antimicrobial
250   agents within a matrix [18]. Biofilm bacteria show much greater resistance to antibiotics than their
251   free-living counterparts [19]. Bacteriophages, thanks to their capability of an exopolysaccharide
252   matrix degradation and bacterial cells infection are attractive agents of biofilm destruction [20].
253   Phages can recognize bacterial receptors by the specific proteins that also have enzymatic activity.
254   These enzymes degrade extracellular sugar polymers produced by bacteria [21]. It has been shown
255   that *Klebsiella pneumoniae* phages produce polysaccharide depolymerases associated with the tail
256   spike. Even after the phage inactivation, the enzymes are still active forming characteristic "halo"
257   zones observed in the spot assay [22]. Mostly, these hydrolyzing enzymes are part of the phage tail
258   and spikes, or they are produced as soluble proteins secreted outside the cell [10].
259   In our previous work, we have reported dual-function tail tubular proteins A (TTPA) containing
260   hydrolytic activity towards bacterial polysaccharides [8 ,9]. Here, we show next tail tubular protein
261   from KP32 bacteriophage classified as form B (TTPB). It has been considered as the structural protein
262   so far. According to our results, it is the polysaccharide-hydrolyzing enzyme either.
263   We have shown that TTPB is active against strains of *Klebsiella pneumoniae, Enterobacter cloacae,*
264   *Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeruginosa*. A lack of activity was observed
265   in the case of bacterial strains producing less mucus or not at all such as *S. flexneri, E. coli, P. mirabilis,*
266   *C. freundii* and *H. alvei*. In the screening assay their enzymatic activity has been displayed as the
267   translucent halo zones creating on the bacteria lawns. The zones were not clear plaques meaning that
268   this protein did not lyse the bacterial cells. The results suggested that TPPB destroyed the components
269   localized outside the cells such as slime giving the translucent, but not clear zones. The antibacterial
270   activity has been confirmed in antibiofilm assay. We used trimethyl tetrazolium chloride (TTC) assay
271   that is versatile, high-throughput method of biofilm measure, applicable to a broad range of
272   microorganisms. Interestingly, the proteins were active towards different bacterial strains – both
273   Gram-positive and Gram-negative. TTPB was the most active towards *E. cloacae*, that is why we took
274   this bacteria strain and *K. pneumoniae* PCM 2713 being the KP32 phage host for further studies. The
275   hydrolytic activity of the protein was confirmed in a test using EPSs of the two strains as the
276   substrates. TTPB hydrolyzed both EPSs. It has been shown that EPSs of the members of the family

Enterobacteriaceae most frequently belonging to the genera *Enterobacter* and *Klebsiella* contain charged groups in the form of uronic acid or pyruvate revealing the polyanionic nature of these polysaccharides [23]. TTPB was not active towards starch unlike TTPA belonging to the same phage and reported by us earlier [8, 9]. To show the synergistic features of TTPB with antibiotic, we have chosen the aminoglycoside antibiotic kanamycin. The antibiotic is used to treat infections caused by as well Gram-negative as Gram-positive bacterial strains (according to DrugBank https://www.drugbank.ca/drugs/DB01172). For that reason we could use it towards *K. pneumoniae* and *E. cloacae* strains. However, the most important was the observation of the additive effect of TTPB and the antibiotic against the biofilms formation. The antibiotic could not reach the bacterial cells in biofilm and the situation was completely changed 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.

The amino acid sequence analysis by BLAST program showed that TTPB is homologous in over 60% to tail B protein (gp12) of T7 bacteriophage. Gp12 is classified as the structural protein, located in the bacteriophage tail core and it is important for phage adsorption to the bacterial cell wall and host infection [24]. 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.

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 shown rendering it as dual-function protein. However, the mechanism of action of this protein is still unclear and needs further investigation. Moreover, the biochemical, physicochemical and tertiary structure analyses of the protein should provide us more information about the substrate binding site and catalytic domain occurrence.

Author Contributions: EB, KP, AP conceived and designed the experiments. EB, AP, SG 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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