Phage-Produced Shigatoxigenic *Escherichia coli*-Specific Depolymerase Enzyme: Detection, Extraction, and Evaluation

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ABSTRACT

Shigatoxigenic *Escherichia coli* (STEC) belong to one of the major groups of foodborne pathogens in the United States, contaminating foods such as meats and vegetables, and food processing equipment. STEC can form strong biofilms, comprised of exopolysaccharides (EPS) which are difficult to remove by conventional sanitizers by preventing them from accessing the bacterial surface. Biofilms also provide a microenvironment for bacteria to survive despite harsh outside environments. Degrading these EPS substances to allow phages access to the bacterial surface, phage-produced depolymerase enzymes provide one potential means to control these bacterial biofilms. The objective of this study was to detect this depolymerase in the *E.coli* O45 specific phage P9, to extract this enzyme, and to test its biofilm degrading effects on *E.coli* O45. Polymerase chain reaction (PCR) was performed using primers from similar phages to detect the presence of the depolymerase enzyme in the P9 genome, and results were viewed by agarose gel electrophoresis. Following this, a crude enzyme extraction was performed, and enzyme activity was detected via spot-on-lawn assay. Next, the crude enzyme extract was tested for *E.coli* O45 biofilm degrading qualities using a biofilm disruption assay. *E.coli* O45 biofilm degradation was also visualized using Scanning Electron Microscopy (SEM). Presence of the depolymerase gene in the P9 genome was suggested by the presence of bands around the expected band size. Additionally, the presence of the enzyme in the crude enzyme extract was shown by haloes of inhibition on the spot-on-lawn assay. *E.coli* O45 biofilms grown for 24 h were reduced when treated with crude enzyme extract. When visualized using SEM, lower densities of bacteria without biofilms were observed on HDPE surfaces treated with depolymerase enzyme. In contrast, large aggregates of bacteria in biofilms were observed in the PBS control. This data suggests the potential use of the depolymerase enzyme produced by the phage P9 as a biocontrol agent for reducing *E.coli* O45 biofilms on food or food contact surfaces.

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1. Introduction

The Centers for Disease Control and Prevention (CDC) estimates that roughly 48 million Americans become sick each year as a result of foodborne pathogens, and nearly 3,000 of these succumb to the disease (Scallan et al. 2011, CDC, 2016). One such group of foodborne pathogens is Shiga-toxigenic *Escherichia coli*. Shigatoxigenic *E. coli* can produce strong biofilms on foods and food contact surfaces. These biofilms can be difficult to remove using conventional sanitizers; bacteria within the biofilm can generate extracellular polymeric substances (EPS) as a response to environmental stressors such as commercial sanitizers (Sutherland et al. 2001). One means of disrupting such EPS is through the use of STEC specific phages. These phages produce depolymerase enzymes which degrade bacterial EPS, allowing the phage to properly access and attach to the surface of the bacterium (Hughes et al, 1998 and Tait et al, 2002). Associated with the viral tail spikes of depolymerase-producing phages, these depolymerase enzymes allow phages to combat the protective bacterial biofilms. Use of phage-produced depolymerases as a potential means by which to reduce STEC biofilms posits a potential way of controlling such STEC. Previous work isolated 52 bacteriophages effective against various non-O157 STEC, and screened these phage for their biofilm degrading capability (Litt et al. 2016). Based on this data, phage P9 was selected as one of several phages with potential depolymerase activity. The objective of this study was to detect the presence of the depolymerase gene in the phage P9, to extract the potential enzyme from the phage, and to test its activity against *E.coli* O45 biofilms.

2. Experimental Details

Detection of the depolymerase gene:

To detect the presence of the depolymerase gene in the phage P9, a sequenced fragment of P9 was analyzed using the basic local alignment search tool (BLAST). Phages with 79% homology or higher were reviewed for the presence of tail spike proteins which are associated with depolymerase enzymes. These tail spike sequences from similar phage were used to design primers to detect the depolymerase gene in the phage P9. PCR was performed at different annealing temperatures to determine the presence of the depolymerase gene. The final PCR program was as follows:

Cycles	Temp (°C)	Time (min:sec)
1	95	130
35	95	0:45
	46	1:00
	72	0:45
1	72	5:00

Phage preparation:

E.coli O45 was cultured from a stock plate, and incubated in tryptic soy broth (TSB) for 16 hours at 37 °C. The phage was then propogated with the *E.coli* O45 culture on NZ-amine casamino acid yeast extract sodium chloride magnesium sulfate (NZCYM) agar using the double layer agar method. After incubation overnight at 37 °C, the soft agar was disrupted and the phage was eluted in phosphate buffered saline (PBS). Eluent was then centrifuged for 10 minutes at 13,000 rpm, and filtered through 0.2 µm filter. Chloroform (0.1%) was added to the filtrate, which was stored at 4 °C.

Crude enzyme extraction:

One large colony of *E.coli* O45 from a stock plate was inoculated into 10mL TSB, and incubated at 37 °C for 16 hours. The resultant culture was then added to 90mL of TSB, and incubated at 37 °C for 16 hours to mid-log phase. To this culture was added 10mL of high titer phage and culture was incubated for 6 hours. The culture was then centrifuged at 20,000g for 20 minutes to pellet bacteria. The supernatant was dialyzed at 4 °C for 48 hours in distilled water. The dialysate was then concentrated against PEG overnight. This concentrate was then filtered through Millipore Vivacell 70 membrane filters (<100 kDa cut-off size). Crude enzyme extract was then evaluated via a spot on lawn assay; lawns of *E.coli* O45 on NZCYM agar was prepared using the double layer agar method, and 10µL of crude enzyme extract was spotted on the lawn. Plates were incubated overnight at 37 °C and examined for the presence of haloes, indicated decapsulated bacteria.

Biofilm formation

Crude enzyme extract was evaluated for its biofilm degrading ability using a biofilm inhibition assay. To prepare an *E.coli* O45 biofilm, one to two colonies were selected from stock plates, inoculated in 9mL of Luria broth (LB), and incubated at 37 °C for 16 hours at 180 rpm. The resultant culture was then subcultured; 100µL of the resultant culture was added to 9.9mL of supplemented M9 media, which was incubated at 37 °C for 24 hours at 180rpm. This culture was then diluted 1:100 in supplemented M9 media, and 150µL of

culture was used to inoculate each well of a 96 well microtiter plate, which was incubated at 37 °C for 24 hours to form biofilms.

Biofilm degradation assay

Bacterial culture was carefully removed from each well of the 96-well microtiter plate, and 24h biofilms were washed three times with 150 µL of PBS to remove unattached cells. Biofilms were dried for 15 minutes at 37 °C, and treated at 37 °C with either crude enzyme extract for 30, 60, or 90 minutes, or PBS control. Crude enzyme extract/PBS control was then removed, and biofilms were again washed three times with PBS. Biofilms were dried for 15 minutes at 37 °C, and stained with crystal violet solution (0.1% w/v) for 2 minutes. Wells were washed three times with PBS to remove excess crystal violet, and wells were dried for 15 minutes at 37 °C. Stain was released with ethanol/acetone solution (80/20 v/v), and absorbance was measured spectrophotometrically at 595nm. Surviving bacterial populations were also enumerated via serial dilutions and spread plating on tryptic soy agar (TSA).

Scanning electron microscopy (SEM)

E.coli O45 biofilms were formed on HDPE coupons using the above mentioned method. Coupons were treated for one hour with either crude enzyme extract or PBS control. Samples were then fixed and visualized at magnifications of 8,000 and 13,000x.

3. Results

Detection of the depolymerase gene

Primers from three phage were used: *Enterobacteria* phage Bp4, a *Shigella* phage psb-1, and *Escherichia* phage EcoM. Potential positive bands were seen around 750-800bp with the primers designed from the *Enterobacteria* and *Shigella* phages (Figure 1).

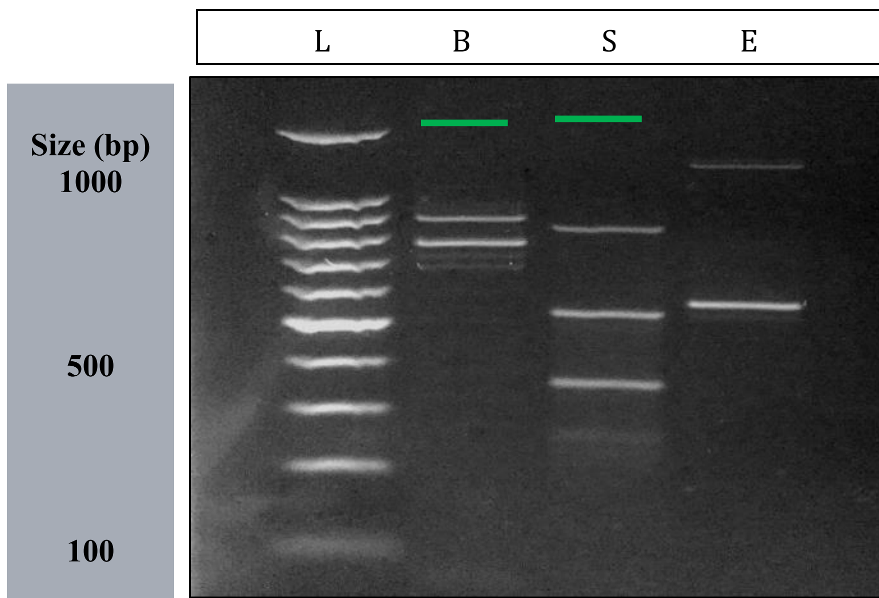
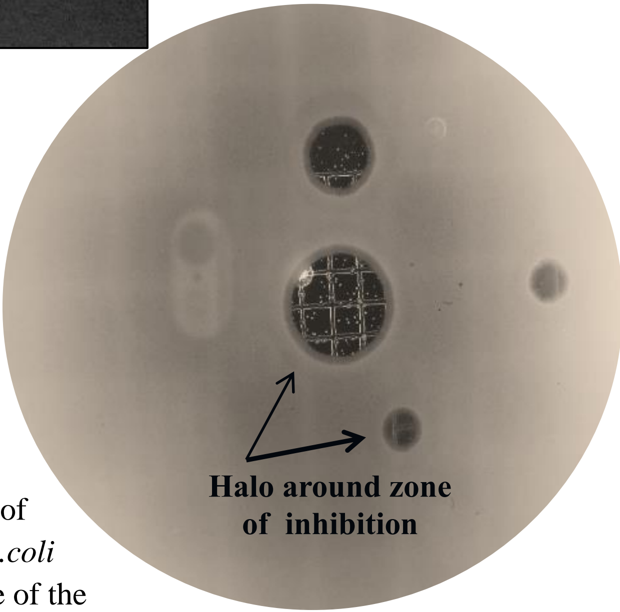


Figure 1:

(left) PCR products visualized with agarose gel electrophoresis show positive bands around 750-800 base pairs in lanes B (*Enterobacteria* phage) and S (*Shigella* phage), but not E (*Escherichia phage*). Ladder shown for size

Figure 2:

(right) Lawns of *E.coli* O45 were inoculated using the double layer agar method. Crude enzyme extract was spotted onto these lawns, and incubated overnight. Haloes around the zones of inhibition indicate the presence of the depolymerase enzyme.



Crude Enzyme Extraction:

Haloes of inhibition were visible around the zone of inhibition when crude enzyme extract was spotted onto *E.coli* O45 lawns (Figure 2). These haloes indicate the presence of the depolymerase enzyme in the crude enzyme extract.

Table 1: Biofilm Disruption (A595) by Crystal Violet Assay				
	Treatment Time (h)			
Treatment	**0**	**0.5**	**1.0**	**1.5**
PBS Control	3.107	2.376	2.679	2.613
Enzyme Treatment	3.054	1.610	2.581	1.475

Table 1: Biofilm disruption quantified spectrophotometrically at A_{595}. Enzyme treatment showed disruption of the biofilm, increasing with treatment time, compared to PBS control.

Biofilm disruption by crude enzyme extract:

E.coli O45 24 h biofilms were treated with crude enzyme extract for 30, 60, or 90 minutes. Biofilm reduction was quantified by crystal violet assay (table 1). Surviving bacteria were quantified via spread plating (table 2).

Table 2: Surviving Bacterial Population Log_{10} CFU/mL				
	Treatment Time (h)			
Treatment	**0**	**0.5**	**1.0**	**1.5**
PBS Control	8.64	8.60	8.61	9.07
Enzyme Treatment	8.51	8.51	8.23	8.42

Table 2: After treatment with enzyme or PBS control, surviving bacteria in wells were enumerated via spread plating. Resultant populations have been reported in Log_{10} CFU/mL

Scanning electron microscopy

HDPE coupons with 24h *E.coli* O45 biofilms were treated with enzyme for 1h, or PBS control, then washed and fixed for SEM. Images were viewed at 8,000 and 13,000x. Images under SEM show higher densities of bacteria residing in intact biofilms on PBS control coupons (figure 3).

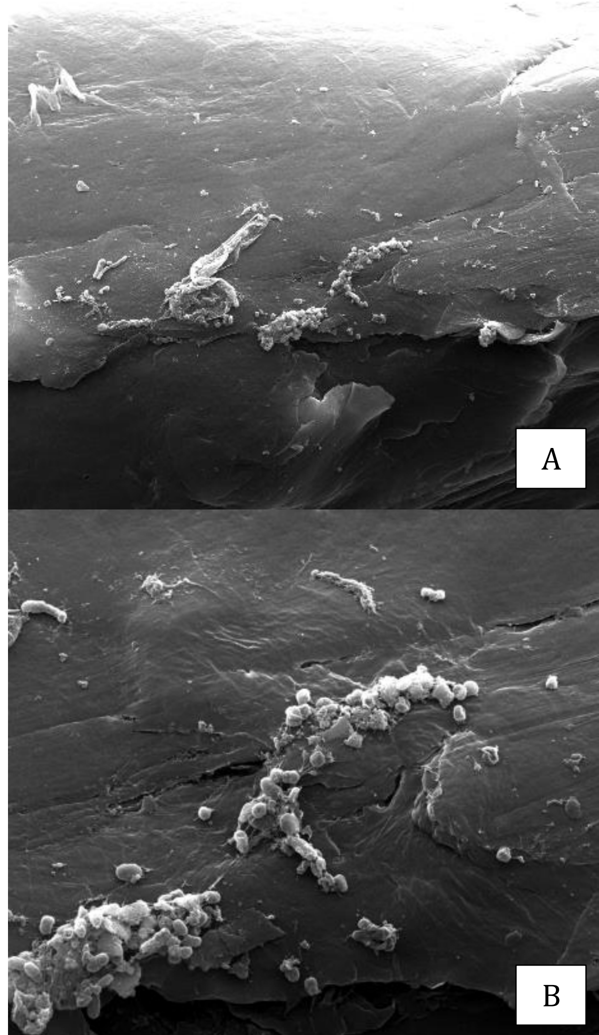


Figure 3: (Right) (Image A: 8,000x, Image B: 13,000x) PBS control 24 h *E.coli* O45 biofilms showed aggregates of intact bacteria encapsulated in exopolysaccharide. Bacteria appear in clusters on the HDPE surface.

Coupons treated with depolymerase enzyme showed lower numbers of scattered bacteria across the surface of the HDPE coupon. Remaining bacteria showed no protective exopolysaccharides (figure 4)

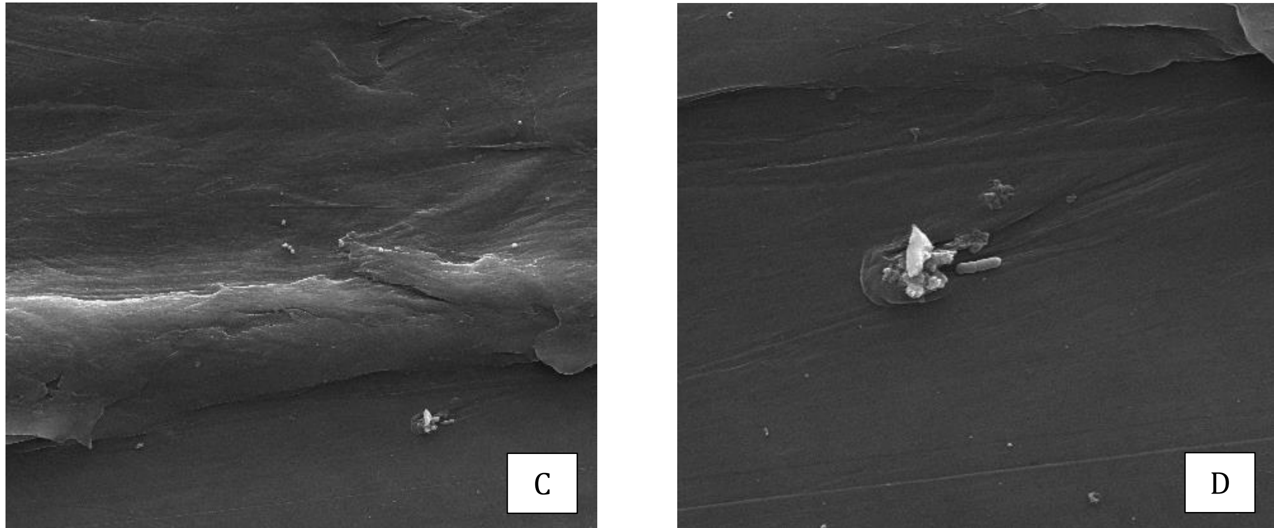


Figure 4: (Image C: 8,000x, Image D: 13,000x) *E.coli* O45 24h biofilms on HDPE surfaces treated with depolymerase enzyme for 1h. Images show few remaining bacteria on the surface. Remaining bacteria show no EPS surrounding them.

4. Discussion and Conclusions

The study showed the presence of EPS-degrading depolymerase enzyme in *E. coli* O45 phage P9. A PCR-positive band at ~800bp was observed indicating the existence of enzyme-encoding sequence in the phage P9 DNA (Figure 1). The PCR amplified DNA sequences of approximately 750-800bp size most likely indicate the presence of the tail spike protein gene in the genetic material of phage P9. Tail spike proteins are associated with depolymerases (Cornelissen et al. 2011), thus the presence of such a sequence indicates the presence of an EPS depolymerase in the phage P9 in the current study. The presence of depolymerase enzyme in the extract was confirmed by spotting it on the lawn of host bacteria. The spots of crude phage enzyme extract showed partially cleared areas of de-capsulated host bacteria around the zone of inhibition, indicating the presence of an EPS-degrading enzyme in the extract (Figure 2). The presence of haloes around an inhibition spot suggest that the bacterial EPS acted as a substrate for the depolymerase enzyme, thereby causing EPS degradation (Hughes et al. 1998, Tait et al. 2002). Our results are consistent with the study conducted by Hughes et al. (1998), where the EPS-degrading properties of the depolymerase enzyme, produced by *Enterobacter agglommerans*-specific phage, was confirmed by halo formation.

The crude enzyme extract, in the present study, was also tested for *E. coli* O45 biofilm disruption *in vitro* via a biofilm disruption assay. Results showed that the enzyme extract effectively reduced *E. coli* O45 biofilms after 0.5, 1.0, and 1.5 h treatment. Compared to the control, a reduction (1.497) in absorbance of biofilm was documented after 0.5 h treatment with enzyme extract (Table 1). In a similar study (Migl et al. 2012), the phage depolymerase reduced (0.060) absorbance of *Acinetobacter baumannii* biofilms after 9 h treatment compared to the control. Higher reduction in biofilm absorbance was observed in the current study which could be due to the differences in EPS composition of the bacterial strains used in the two studies.

Bacterial populations, surviving within the biofilms, were also determined by direct plating on TSA. No changes in bacterial populations were observed upon treatment with enzyme, compared to the control. Similar results were recorded by Tait et al. (2002), when *E. agglomerans* biofilms were treated with phage depolymerase. These results are consistent with the enzyme mechanism proposed by Tait et al (2002), suggesting that phage depolymerases degrade only the EPS, leaving bacteria unaffected by the enzyme.

In the present study, biofilm disruption was also observed visually under SEM. Surfaces treated with PBS-control showed aggregates of bacteria encapsulated in EPS in the biofilms (Fig 3 A,B). On the other hand, phage-enzyme treated surfaces showed an absence of *E. coli* O45 biofilms as well as bacteria in the biofilms, with segregated un-encapsulated bacteria (Fig 4 C,D).

Overall, the study confirmed the presence of the EPS-degrading depolymerase enzyme in the *E. coli* O45 phage P9. This enzyme was successfully extracted from the phage and shown to be effective in its biofilm disrupting capabilities. The phage depolymerase enzymes with biofilm-degrading ability, could be employed as bio-control agents against biofilm-forming STEC in the food industry.

5. Summary

Overall, the study confirmed the presence of the EPS-degrading depolymerase enzyme in the *E. coli* O45 phage P9. This enzyme was successfully extracted from the phage and shown to be effective in its biofilm disrupting capabilities. The phage depolymerase enzymes with biofilm-degrading ability, could be employed as bio-control agents against biofilm-forming STEC in the food industry.

6. Appendices

- **Papers Published**

Mackenroth, B., Litt, P.K., and Jaroni, D., 2017 *Detection, extraction, and evaluation of phage produced depolymerase enzyme against Shiga-toxigenic E.coli biofilms*. IAFP July 9-12, Tampa FL.

Mackenroth, B., Litt, P.K., and Jaroni D., 2017 *Detection and extraction of shiga-toxigenic Escherichia coli-specific bacteriophage depolymerase enzyme* OSU Research week poster symposium Feb 23, 2017

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- **Literature Cited**

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