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Increased Bacteriophage T4 Infectivity in Aging *Escherichia coli* Biofilms is Not Dependent on OmpC Expression

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Bacteriophage T4 infection within *Escherichia coli* biofilms demonstrates a significant increase in phage progeny production during the fourth day of biofilm growth. In this study Wild type and *ompC* (Outer Membrane Protein C) knockout strains of *E. coli* were analysed via phage assay and ELISA to assess bacteriophage productivity and levels of OmpC accumulation, respectively. Both phage production and levels of OmpC showed a corresponding increase on day four of biofilm growth in wild type cultures. However, phage production in both wild type and *ompC* knockout strains were comparable, indicating that the up regulation the phage co-receptor OmpC may not be the basis for increased phage production in four day old *E. coli* biofilms.

Biofilms are defined as multicellular microbial communities embedded within an extracellular matrix (1). Their formation can be induced by many factors including: cellular recognition of attachment sites, nutritional cues, and responses to quorum sensing molecules (2). Development is complex, highly regulated, and results in massive changes in gene expression making cells in biofilms phenotypically distinct from their planktonic counterparts (3**)**. One of these phenotypic changes includes alterations in the composition of the bacterial outer membrane (3). It has been previously documented that *Escherichia coli* cells in biofilms express up regulated levels of Outer Membrane Protein C (OmpC) as compared to cells in stationary phase (4). OmpC is a porin involved in osmoregulation and acts as co-receptor for Bacteriophage T4 attachment (4,7). It has been proposed that changes in membrane composition might enhance the infectivity of biofilms by T4 bacteriophage (5).

T4 is a member of the T even bacteriophages that infect *Escherichia coli* bacteria (6). Attachment occurs when the T4 tail fibers bind to the main host cell receptor lipopolysaccharide (LPS) as well as the OmpC co-receptor (7). *E. coli* cells harboring mutations in LPS have been shown have reduced susceptibility to infection by T4 phage (7). Furthermore, previous findings have demonstrated an increase in bacteriophage progeny production on day 4 of biofilm growth, which is not seen in aged planktonic cultures (5). Further studies have confirmed this finding; however the mechanism behind this link has not yet been determined (8). Here we investigated the mechanism behind this sudden increase in T4 phage production seen in day 4 biofilms. We proposed a causal link between OmpC levels, phage attachment and subsequent phage production in *Escherichia coli.* By monitoring the growth, phage infectivity and expression of OmpC over a seven day period we revealed the underlying cause behind this increase in phage production.

MATERIALS AND METHODS

Strains and Reagents. *E. coli* strains BW25113 (WT) and JW22031 ($OmpC^{-/-}$) were obtained from *E. coli* Genetic Stock Center and grown in Luria broth (LB) for 24 hours at 37°C in an New Brunswick Scientific Excella E24 Shaker. Eleven mg resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) tablets (Alied Chemical) were dissolved in 100 ml of distilled water to make 0.011% w/v working solution. TMB harnessed for ELISA (Sigma) was used at the supplied concentration. Glutaraldehyde used in ELISA (Sigma) was diluted in distilled water to a working concentration of 2.5% w/v. Rabbit-IgG-anti-OmpC (1mg/ml) antibody (Bioss) was diluted to a working concentration of 25 ug/ml in distilled water. Goat- anti rabbit IgG-HRP linked antibody (1mg/ml) (Kirkegaard & Perry Laboratories) was diluted to a working concentration of 25 ug/ml in distilled water. All strains and reagents were purchased by the UBC Department of Microbiology and Immunology.

Amplification of T4 phage stocks. An overnight culture of the WT *E. coli* strain was diluted into sterile LB until an OD of 0.15 was achieved. The diluted bacterial suspension was transferred to a sterile Erlenmeyer flask and $1x10^7$ pfu of T4 phage was added. The bacteria/phage mixture was incubated at 37°C for 6 hours. The mixture was centrifuged at 7,000 rpm. Supernatants containing the phage were removed and stored at 4˚C. Concentration of the stock was quantified by serial dilutions followed by plating and was found to be 8.64×10^9 pfu/ml.

Separation of biofilm from planktonic cells. Biofilm growth was achieved by inoculating LB media with OmpC-/- and WT *E. coli* strains. Inoculated plates were incubated at 37 °C for seven days. Prior to running assays, all liquid culture was removed leaving behind only adherent biofilm. For ELISA and resazurin assays planktonic cells were discarded and each well gently washed 4 times with 0.9% NaCl solution. After washing. NaCl was added back to each well. For plaque assays planktonic cells were spun out of media in micro centrifuge tubes at 10,000 rpm for 2 minutes. After washing with 0.9% NaCl 4 times. Media supernatant was added back to each well.

ELISA of JW22031 and BW25113 *E. coli* biofilms. Sample and controls were set up in triplicate in 96 well plates. Following separation of biofilm from planktonic cells, wells were fixed with glutaraldehyde. Glutaraldehyde was removed and each well rinsed with 0.9% NaCl. Wells were blocked with 1% BSA. Following this, BSA was wells washed with 0.9% NaCl. One ug/ml of Rabbit-IgG-anti-OmpC antibody (catalogue #ABIN749318) was subsequently added to each well and incubated for 1 hr at 37°C. After removal of primary antibody, wells were washed 4 times with 0.9% NaCl. One ug/ml of goat-anti rabbit IgG-HRP linked secondary antibody (KPL catalogue # 075-1506) was added to each well and the plate was incubated for 40 minutes. Following incubation, each well was washed 4 times with 0.9% NaCl. Lastly, 3,3',5,5'-tetramethylbenzidine (TMB)

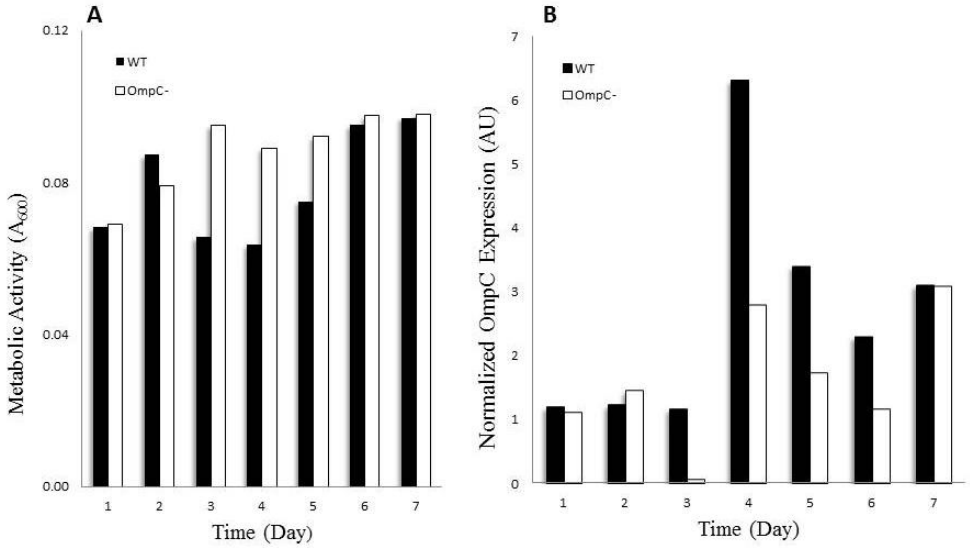

FIG 1 (A) Metabolic Activity of *E. coli* biofilms BW25113 and JW22031 (WT and OmpC$^{-/-}$ respectively) as measured by resazurin assay. (B) Expression of bound antibody WT and OmpC$^{-/-}$ *E. coli* biofilms as measured by ELISA assay. Expression of OmpC is normalized for differences in cell density. AU: Arbitrary Units.

and 0.9% NaCl was added to each well. Absorbance at 650 nm was read using a Biotek Epoch microplate spectrophotometer. Addition of TMB and 0.9% NaCl was added to empty wells was used as the blank.

Resazurin bioassay of JW22031 and BW25113 *E. coli* biofilms. Biofilms set up in triplicate in 96 well plates were inoculated as stated above. Following separation of biofilm from planktonic cells, 0.011% resazurin was added to each well. Plates were incubated at 37°C for 1 hr. Absorbance was read at 600 nm using a Biotek Epoch microplate spectrophotometer. 0.9% NaCl and 0.011% resazurin was used as the blank measurement.

Plaque assay of T4 phage using *E. coli* BW25113. Biofilms were set up in triplicate in 96 well plates were inoculated as stated above. Following separation of the biofilm from planktonic cells, approximately 1.8 x 10^5 pfu were added to each well. The plate was incubated at 37°C for 2 hrs. After incubation, media was removed from each well and centrifuged at 8,000 rpm. Phage were quantified and measured via the soft-agar overlay method using WT *E. coli*. *E. coli* was grown in Luria Broth in a New Brunswick Scientific Excella E24 Shaker at 37°C for 24 hours. Luria Broth media for top and bottom agar contained 1% agar. Solidified bottom agar plates were warmed at 37°C while 3 ml molten bottom agar was maintained at 50°C until plating. *E. coli* WT was added to 1 % molton top agar followed by addition of T4 phage at 10^{-3} 10^{-4} and 10^{-5} dilutions of the centrifuged sample. Inoculated molten agar overlay was poured onto pre-warmed bottom agar and plates were incubated at 37°C overnight. T4 plaques were counted the following day.

RESULTS

Wild Type and *ompC*$^{-/-}$ mutant *E. coli* showed increased metabolic activity during biofilm development. A resazurin assay was used to measure the metabolic activity of wild type (BW25113) and *ompC*$^{-/-}$ (JW22031) cells during biofilm formation (Fig. 1 A). Both strains demonstrated a trend in which overall metabolic activity increased approximately 20% over the course of seven days. This trend suggested that these cells achieve metabolic equilibrium quickly after inoculation and the observed increase can be attributed to the activity of the cells near the surface of the biofilm as it expands over the time period.

Day 4 biofilms expressed highest amounts of OmpC. An ELISA was performed with a anti-OmpC antibody to measure the levels of OmpC expression during biofilm growth (Fig. 1 B). We analyzed an *ompC*$^{-/-}$ mutant and wild type strain to account for non-specific binding during the assay. It was necessary to determine this background signal over the entire time course because biofilms are dynamic and we expected it to vary. Using this approach, we were able to subtract background noise and produce a normalized measurement of OmpC expression during biofilm development (Fig. 2). It is important to acknowledge the inherent difficulties associated with the analysis of biofilms. Unlike planktonic cultures biofilms are highly heterogeneous and as such there is significant

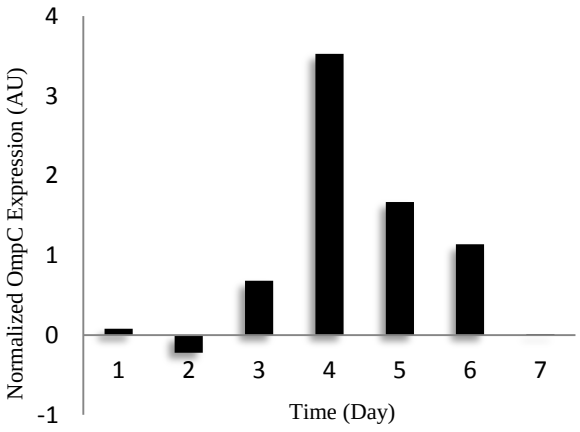

FIG 2 Effect of age on OmpC expression in *E. coli*. Expression normalized by subtracting *ompC*$^{-/-}$ ELISA values from WT values (Fig. 1b). AU: Arbitrary Units.

variation between duplicate wells. We used duplicate wells and normalization of data in an attempt to account for this natural variation, but the effectiveness of this technique is uncertain. The background levels throughout the ELISA were high and although trends could still be drawn from the data, this may limit the validity of our results. The study of biofilms is not trivial and given their broad implications a more representative model is needed. The theory behind the ELISA assay harnessed in this study was that biofilms can be directly stained (16) and therefore specific protein expression can be evaluated *in situ*. This approach, although appealing, is fraught with problems. Firstly the variability of biofilm growth even between duplicate wells is great, making comparison difficult. The reduction of background to an acceptable level was not feasible using direct staining. This may be due to a combination of factors. The thoroughness of the washing steps are limited by the fact that the biofilm must remain adherent. These "gentle" washing steps may not be sufficient to elute loosely bound antibody. Lastly the fact that biofilms are dense matrices increases the likelihood that antibody will be retained within the matrix without specific binding. The possibility of insufficient diffusion of antibodies into the biofilm may undermine its usefulness as a quantifiable measure. The development of a technique to accurately quantify protein or gene expression in a living biofilm is an important future step. With these considerations in mind, we observed that OmpC expression increased rapidly after day 2 reaching a peak of a nearly 4-fold increase in expression by day 4, before decreasing steadily to previous levels by day 7.

Wild type and *OmpC*$^{-/-}$ *E. coli* are most susceptible to T4 infection on day 4 of biofilm growth. Using a plaque assay we assessed the susceptibility of WT and *OmpC*$^{-/-}$ *E. coli* to bacteriophage T4 infection over seven days. We observed the viral production on days 1 through 3 was approximately $1x10^5$ pfu/ml, before increasing on day 4 to over $1x10^7$ pfu/ml. During the remaining days the viral production decreased steadily to final values of $3x10^6$ pfu/ml and $2.5x10^5$ pfu/ml for wild type and *ompC*$^{-/-}$, respectively. We did not observe substantial differences in progeny phage production between our Wild type and *OmpC*$^{-/-}$ strains.

DISCUSSION

We correlated the expression of OmpC, a known co-factor for T4 phage attachment, with an observed increase in phage production in day four of biofilm growth. ELISA readings indicate higher levels of OmpC expression on day 4-5 of growth in WT *E. coli* (Fig. 1b). Schembri *et al.* confirmed that OmpC is up regulated during biofilm growth (9). OmpC up regulation accounts for the increase in phage susceptibility during day four of biofilm growth (5). However, our plaque assay results showed a reliable increase in production at day 4 for both WT and OmpC$^{-/-}$ strains (Fig. 3). The fact that this increase persists in o*mpC*$^{-/-}$ mutants indicates that the absence of this co-receptor does not significantly impede phage production.

The observed increase in OmpC expression on day four can be explained by an increase buildup of microbial metabolites in the biofilm. This metabolite build up increases the osmolarity of the environment which induces the up regulation of OmpC to maintain osmotic balance(3). A subsequent decrease in OmpC expression occurred following day 4 of biofilm growth. This trend can be explained by a number of factors. First, it may be that as the biofilm matures its internal structure becomes more organized and better able to clear microbial wastes (10). The presence of aquaducts and internal channels may increase the drainage efficiency which would decrease the osmolarity of the immediate biofilm environment and decrease OmpC expression. A second possibility would be that as the biofilm grows in density and the extracellular matrix accumulates the antibodies ability to penetrate the biofilm and access its target may decrease. This would lead to reduced OmpC detection in our model.

We found *ompC*$^{-/-}$ cultures to have positive readings indicating that there may have been high levels of background (Fig. 1b). Both WT and OmpC$^{-/-}$ cultures follow similar trends (Fig. 1b). This is in contrast to the expected result in which OmpC$^{-/-}$ bacteria would show a flat trend with minimal signal. Assuming a true knockout, this can be explained by an extraneous variable contributing to background for both WT and *ompC*$^{-/-}$ cultures. For example, this curve may actually be representing two factors: biofilm density and OmpC levels. The nature of the structure of biofilms may contribute to trapping of excess antibody in extracellular matrix, which may inevitably contribute to the resulting increased signal. In this way, the difference between the two curves corresponds to actual levels of OmpC (Fig. 2). However, assuming a false knockout model these results can be explained by *ompC*$^{-/-}$ cells still expressing the OmpC porin, although not to the same degree as the WT counterparts. Transcription and subsequent translation of OmpC with a kanamycin insert could lead to both shorter mRNA half-life and more rapid degradation via the proteasome (11).

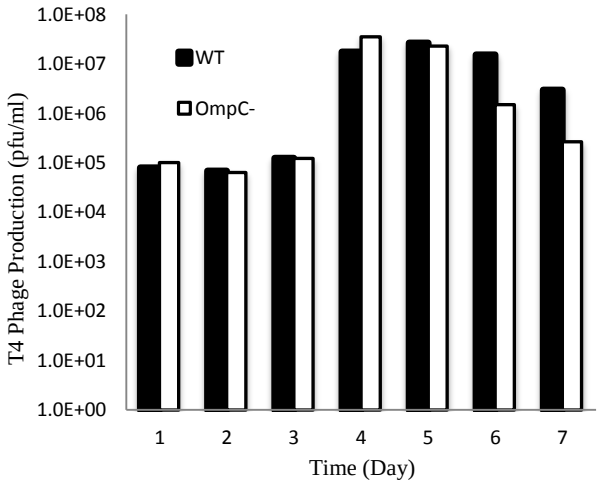

FIG 3 Effect of OmpC on the production of *E. coli* biofilms.

Nevertheless, binding controls for secondary antibody and staining of an empty well indicated high levels of background. Binding controls for antibody resulted in absorbance readings greater than 50% of those seen in WT samples. This is most likely due to antibodies becoming trapped in the biofilm extracellular matrix, as mentioned previously. Staining of empty wells to control for background signal resulted in absorbance readings greater than 30% of those seen in WT samples. This can be explained by non-specific binding of both primary and secondary antibodies to the walls of the well. These observations give support to the initial claim that the *ompC*$^{-/-}$ *E. coli* strain was a true knock out model of OmpC as opposed to a false knockout model.

We expected the WT strain to show increased phage production on day 4 coinciding with OmpC up regulation. Unexpectedly we also observed an increase in phage production on day 4 for the *ompC*$^{-/-}$ mutant. Indeed, our results showed low phage production during days 1-3 followed by a significant increase on days 4-5 tapering off from days 5 through 7 for both strains (Fig. 3). This predicted increased phage production seen in the WT strain is in agreement with Chan et al (5). However, our *ompC*$^{-/-}$ results are in stark contrast with the findings of Wang *et. al*., in which *ompC*$^{-/-}$ mutants demonstrated decreased susceptibility to phage infection (12). However in their model they were using a chemical mutagen with many off target mutations limiting the validity of these results (12). These off target mutations may have altered the growth and/or permissibility of the *E. coli* cells to T4 infection. Further supporting this, more recently Yu *et al*. indicate that knocking out OmpC alone was not sufficient to impede T4 production (13). These authors proposed that OmpC merely acts to expose a critical glucose residue within lipopolysaccharide (LPS) and is not centrally involved in viral attachment (13). Yu *et al.* demonstrated that only double knockouts of both OmpC and the glucose residue of LPS displayed reduced susceptibility to T4 infection. In accordance with this, our results show near identical trends for both WT and *ompC*$^{-/-}$ strains which fully supports the fact that OmpC may not be required in the infection process. Alternatively, this result could indicate a problem with the *ompC*$^{-/-}$ strain used in this experiment. It is possible that the environmental pressure to retain a functional OmpC protein is so great that reversion of the mutation after many generations of growth becomes more likely (14).

Previous research has shown that the production of T4 heavily depends on the metabolic activity of the host cell (15). To rule this out as a possible explanation for the observed increased phage production on day 4, we conducted a resazurin assay to measure *E. coli* metabolic activity over the time course of the experiment. Our results show that metabolic activity remained fairly constant with no observed increase on day 4 (Fig. 1a). This indicates that an increase in metabolic activity is most likely not the cause of increased phage production seen in day 4 biofilms.

Our data reconfirmed the findings by Chan *et al.* in which wild type *E. coli* biofilms experienced an increase in T4 infectivity on day 4 of growth (5). Furthermore, our results also demonstrated that OmpC is up regulated during biofilm growth; however an OmpC deletion does not appear to have a significant functional consequence for T4 bacteriophage infectivity. Therefore the increase in productivity is not due to OmpC up regulation.

FUTURE DIRECTIONS

We demonstrated that increased levels of OmpC are not the sole cause of increased biofilm susceptibility to T4 infection. It would be interesting to see if the structure or expression of LPS changes during the biofilm life cycle thereby affecting the infectivity at particular time points. This could be accomplished by growing biofilms over various time points followed by extraction of LPS and subsequent disaggregation. Biochemical analysis could shed light on the differing levels of different forms of LPS over the biofilm life cycle. Alternatively, the increased phage production seen in day four biofilms may not be an issue of attachment but rather of varying productivity at different time points. Although results from the resazurin assay did not show a corresponding spike at day 4 as compared to ELISA or phage assay results, a more sensitive assay measuring the average burst size over time could be more revealing. This could be done by performing a fluorescein diacetate hydrolysis (FDA) assay in addition to the resazurin assay (18). A final approach that could be harnessed to better study the interaction of OmpC with T4 phage binding could involve use of other *E. coli* knockout models of OmpC. These strains could be used in a phage assay in order to study binding of different mutants with T4 phage. This should help to solidify the results depicted in this study, in which OmpC was observed to not be necessary for T4 phage binding.

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