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The Reduction of T7 Phage Adsorption in *Escherichia coli* B23 Cells Treated with Sub-Lethal Levels of Kanamycin

Joanne Bleackley, Jesse Cooper, Monica Kaminski and Stephanie Sandilands
Department of Microbiology & Immunology, UBC

The production of capsular material has been implicated as a bacterial defence mechanism against certain antibiotics and against phage adsorption. The capsule acts to block the phage from binding to its receptor on the bacterial cell which effectively inhibits phage adsorption. Previously, it has been shown that exposing *Escherichia coli* B23 cells to sub-inhibitory levels of the antibiotic kanamycin results in an increase in extracellular polysaccharide production, possibly in the form of a capsule. In the current study, this phenomenon was investigated using T7 phage adsorption as a model to assess the presence of a capsule. Phage adsorption assays were performed on both *E. coli* B23 cells treated with sub-lethal levels of kanamycin for 1 hour and untreated cells. T7 phage adsorption was shown to be diminished in *E. coli* treated with antibiotic, as compared to those which had not been treated with antibiotic. This decrease in phage adsorption to the antibiotic treated cells suggests that the induced extracellular polysaccharide produced by these cells is part of the capsule. Thus, this work provides further insight into the likely nature of the induced-extracellular polysaccharide produced by these cells following antibiotic treatment.

Bacteria have developed mechanisms to adapt to changes in their environment. The production of extracellular polysaccharides is a means to preserve bacteria in their natural habitats by excluding potentially harmful compounds (18). These extracellular polysaccharides are often assembled in a capsular formation, thus forming a physical barrier to the environment (13). Specifically, this protective layer is capable of shielding the cell from antibiotics, amoebic attack, desiccation, phagocytosis and bacteriophage infection (18). Capsules interfere with phage adsorption by either covering the receptor completely, or by interfering with receptor-ligand binding. In order for infection to occur, the phage must be able to inject its DNA into the cytoplasm of the bacteria. Therefore if the thickness of the capsule is greater than the length of the phage tail, the bacteria will be impermeable to phage infection. In this regard, capsular production acts as a non-specific phage inhibitor. Previous studies have shown that capsule thickness and phage resistance are proportional (18). The negative charge of the capsule is also an important factor in the resistance to phage adsorption (18).

The T7 phage adsorption system involves the interaction between the tail fibre protein of T7 phage and lipopolysaccharide (LPS) on the surface of *Escherichia coli* B23 (14). *E. coli* strains that are deficient in LPS, or have mutations in the heptose moiety of LPS, have been shown to be resistant to T7 phage adsorption (17). Binding of T7 phage to LPS can also be blocked by capsular molecules, including colonic acid. Colanic acid is one of the major components of the capsule produced by *E. coli* B23 in response to hostile environments; implicating colanic acid synthesis as a survival mechanism in damaged cells (13). It has been shown that sub-inhibitory concentrations of certain antibiotics, such as β-lactams, induce expression of the capsular polysaccharide operon (*cps)* which encodes genes required for colanic acid synthesis (13).

Previous groups investigating the effect of sub-lethal levels of aminoglycoside antibiotics on *E. coli*, have also reported an increase in extracellular polysaccharide production (3, 5, 9). Aminoglycosides, such as kanamycin, act within the bacterial cell to irreversibly bind the 30S subunit of the ribosome effectively inhibiting protein translation (9). Their effect on the transcription of *cps* operon has not yet been elucidated.

An experiment performed by Lu *et al*., involving the exposure of *E. coli* B23 to sub-inhibitory levels of kanamycin, found a significant increase in extracellular polysaccharide production (9). They theorized that this increase was due to the production of a capsule in the antibiotic-exposed bacteria. To test this hypothesis we used the T7 phage- adsorption system to determine if antibiotic treated cells would have reduced phage adsorption thus indicating increased capsule.

MATERIALS AND METHODS

Phage preparation. A stock solution of T7 phage and *Escherichia coli* B23 culture were obtained from the MICB 421 culture collection in the Department of Microbiology and Immunology at the University of British Columbia. To enumerate phage, an overnight culture was prepared by inoculating 15 ml of H-media (10.00 g/L Bactopeptone, 3.00 g/L beef extract, 5.00 g/L NaCl, 1.00 g/L glucose) with a loop full of *E. coli* B23. The culture was incubated overnight at 37°C in an air shaker at 100 rpm. Phage bottom agar (PBA) plates (13.0 g/L Bactotryptone, 8.0 g/L sodium chloride, 2.0 g/L sodium citrate, 1.3 g/L glucose, 15.0 g/L agar, at pH 7.2) were warmed in a 37°C incubator 30 minutes prior to utilization. 2.5 ml aliquots of Phage Top Agar (PTA) (13.0 g/L Bactotryptone, 8.0 g/L sodium chloride, 2.0 g/L sodium citrate, 3.0 g/L glucose, 7.5 g/L agar, at pH 7.2) were boiled at 100°C to melt and then kept at 48°C in a water bath. T7 stock solution was serially diluted to 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}. 200 µl of overnight *E. coli* B23 culture and 200 µl of diluted phage were added to 2.5 ml of liquid PTA at 48°C. Tubes were mixed and then poured onto warmed PBA plate. PTA solidified for 15 minutes at room temperature and then the inverted plates were incubated at 37°C for 18-24 hours. Plaques were counted and phage stock concentration was determined.

Growth of *E. coli* B23. An overnight culture was prepared by inoculating 15 ml of H-media with a loop full of *E. coli* B23. The next morning two flasks containing 25 ml of H-media were inoculated with 200 µl of the overnight culture. The newly inoculated cultures were grown for three hours at 37°C in an air shaker at 100 rpm. Kanamycin monosulfate (Gibco-BRL#11815-024) was mixed with distilled water to give a final concentration of 20 mg/ml and then filter sterilized. 25 µl was then added to one of the 25 ml *E. coli* B23 to make a final antibiotic concentration of 20 µg/ml. The antibiotic treated culture was then incubated at 37°C in an air shaker at 100 rpm for 1 hour. After incubation a phage binding assay was performed on both the antibiotic treated sample and the control sample.

Phage binding assay. A culture of indicator cells was prepared by inoculating 5 ml of H-media with a loop full of *E. coli* B23 the afternoon before the phage assay was to be performed. The culture was incubated overnight at 37°C in an air shaker at 100 rpm. Phage binding assay was performed according to the protocol outlined by Adams *et al* with some modifications (1). Each *E. coli* culture for the phage binding assay was diluted with H-media to an OD_{660} of 0.4. For the phage binding assay, 120 µl of 2 x 10^5 PFU/ml phage solution were added to 6 ml of diluted bacterial culture and were mixed. 1 ml samples were removed at 0, 1, 4, and 7 minutes and centrifuged in an Eppendorf 5415D centrifuge for 1 minute at 5000 x g in order to isolate the unadsorbed phage in the supernatant. Between time points the bacterial-phage culture was kept in a 37°C 25 rpm shaking water bath. To enumerate the unadsorbed phage in each sample, either 20 µl or 100 µl of the supernatant was added to a test tube containing 2.5 ml of molten PTA (48°C) and 200 µl of *E. coli* indicator cells. Each tube was then mixed by phage style mixing and poured over a pre-warmed PBA plate. Plates were left at room temperature for 15 minutes in order for the PTA to solidify and then inverted plates were incubated at 37°C for 18-24 hours. Plaques were enumerated on each plate in order to determine the titre of unadsorbed phage in each sample.

Capsule stripping. Two flasks containing 90 ml of H-media were inoculated with 1 ml of overnight *E .coli* B23 culture. The inoculated 90 ml flasks were incubated at 37°C in an air shaker at 100 rpm for 3 hours. After incubation, 90 µl of 20 mg/ml kanamycin stock solution was added to each flask to a final concentration of 20 µg/ml. The antibiotic treated cultures were incubated at 37°C in an air shaker at 100 rpm for 1 hour. The capsule stripping was performed according to the protocol outlined by Egorenkova *et al* (4). Cells were incubated at 4ºC during this time. Following centrifugation the supernatants were discarded.

RESULTS

Phage Adsorption Assay. The percentage of adsorbed phage after 4 and 7 minutes of incubation with the untreated cells during the phage adsorption assay was 14.5 and 26.9 % respectively. In contrast, for cells treated kanamycin the percentage of adsorbed phage after 4 and 7 minutes was 2.4 and 5.2, respectively. The slope of the line for the kanamycin treated cells was -0.007 and the correlation between variables was 0.83 (figure 1). In contrast, the slope of the line for the untreated cells was more than fivefold steeper, with a value of -0.04 and a correlation of 0.99. The steeper slope for the untreated cells indicates greater phage adsorption for this sample than for the kanamycin treated cells. The increase of unbound phage to 102 % of the initial titre at 1 minute for the kanamycin treated cells does not likely represent a true increase in the titre of phage, since progeny phage would not have been produced within one minute. In order to represent the fraction of unbound phage as positive values, the P/Po ratio was multiplied by 10 before taking the logarithm.

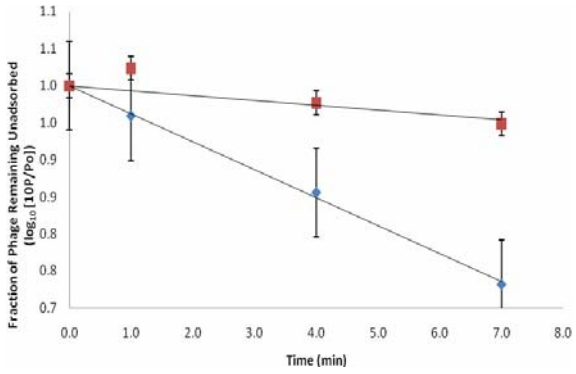

FIG. 1 Effect of kanamycin on the adsorption of T7 phage to *E. coli* B23. Error bars indicate calculated standard error at each time point. Data represents the average of three replicates. 10P: Tenfold unbound phage at indicated time point; Po: Unbound phage at time 0. Red squares: kanamycin treated cells; blue diamonds: untreated cells

Capsule Stripping. To directly assess the effect of the suspected induced capsule on phage adsorption, cells were treated with antibiotic, subsequently stripped of their capsule with PBS, then enumerated and assessed for phage adsorption. However, results from the phage adsorption assay indicated numerous (>10^3) PFU per plate, indicating that very few phage had adsorbed to cells. Consistent with this finding, enumeration of the capsule-stripped cells determined that the cells were non-viable after the stripping treatment. Almost all of the cells died in both the stirred and non-stirred samples (data not shown).

DISCUSSION

It was found that adsorption of T7 bacteriophage to *E.coli* B23 treated with sub-lethal levels of kanamycin was decreased in comparison to T7 adsorption to untreated *E.coli* B23.

Binding of T7 bacteriophage to *E. coli* B23 is expected to exhibit first order kinetics wherein the amount of free phage and time are negatively correlated (7). The amount of unbound phage should decrease over time as more phage come into contact with the appropriate receptors on the bacterial surface. The slopes of the lines indicate the adsorption rate constant for each of the treatments (Fig. 1). The adsorption rate constant is independent of the concentration of bacterial or phage treatment, and therefore the data from different trials could be combined (7).

It can be observed that the non-antibiotic treated control sample showed an increase in phage adsorption over time as shown by the decreasing levels of free phage. The antibiotic treated cells exhibited a considerably smaller increase in phage adsorption during the time course of the experiment. Adsorption of phage is affected by the growth phase of cells, temperature and the presence of salts and organic compounds in the media (7). The two treatments differed only in the addition of antibiotic to the growth media thereby indicating that any differences observed in phage adsorption can be attributed to the addition of antibiotic.

Previous studies have shown that *E.coli* B23 grown in the presence of sub-lethal levels of kanamycin tend to produce a capsule made up of additional polysaccharides on the surface of the cell (5). By showing that less phage was able to adsorb to the antibiotic treated bacteria we are able to provide further evidence that the increased extracellular carbohydrate production was due to capsule formation at the surface of *E. coli* B23.

In order to determine whether the decrease in phage binding due to antibiotic treatment was solely the result of capsule formation or if the difference was also influenced by other physiological effects of the antibiotic on the bacteria, we attempted to perform a capsule stripping procedure on a sample of our bacteria. Unfortunately after the treatment, the cells were non-viable, and because T7 phage does not tend to adsorb to bacterial debris (10) we were unable to perform kinetic analysis on this data. We were also unable to attribute capsule formation as the sole causation of decreased phage adsorption. It has been proposed that increased capsular production in antibiotic treated cells may involve the action of caseinolytic proteases (Clp) and Rcs proteins (8). It has been shown that the activities of Clp proteases in E.coli are altered by stress and by the presence of antibiotics (12). One of the substrates of the Clp proteases is the RcsA protein which is involved in the regulation of the expression of capsular polysaccharide (*cps)* operon genes (8). RcsA, in conjunction with other Rcs proteins, works to induce the transcription of the *cps* operon which encodes colanic acid –the major component of the *E.coli* B23 capsule (11). This knowledge could be used to produce mutants in which the production of capsule is prevented by knocking out a component of the pathway. These mutants could be used as controls to determine if capsule formation is the only factor that prevents the adsorption of phage.

The polysaccharide capsule acts a physical barrier to T7 bacteriophage; the mechanism may involve capsular molecules covering the LPS receptor so the phage is unable to bind (14). Extracellular polysaccharide molecules are bulky compounds that may sterically hinder the interaction between phage and LPS. Capsules are hydrophilic and give the surface a characteristic negative charge which may interfere with phage binding (18). From the results it can be noted that capsular formation decreases phage adsorption but does not entirely inhibit the process (Fig. 1) indicating that capsular production alone is not completely effective in eliminating phage entry.

Although production of extracellular polysaccharides appears to account for the differences in adsorption observed between treatments, it must also be noted that the decreased growth rate of *E. coli* in antibiotics may have an affect on the adsorption of phage (6). Previous groups have found that *E. coli* exhibits a decreased growth rate when exposed to sub-lethal levels of antibiotic and other groups have demonstrated a slower rate of adsorption at these lowered growth rates (2, 6). Therefore, the decrease in growth rate may be a partial cause of the decreased adsorption rate in the antibiotic treated samples (Fig. 1).

Although the results from the phage binding assay indicate there is a difference in the phage adsorption between antibiotic treated and control cells, we cannot conclusively state that this difference is due to capsular formation. In order to conclusively determine the relationship between phage adsorption and capsule it is essential that the capsule stripping procedure maintains viable cells or that a mutant unable to form capsule in the presence of antibiotic is generated as a control. If the production of capsule is the major reason for the difference in phage adsorption, then levels of phage adsorption in cells grown in antibiotic but lacking capsule should be similar to those of untreated control samples.

FUTURE EXPERIMENTS

In future studies it would be necessary to repeat the phage binding assay using a method for capsule

stripping that did not kill the bacterial cells. This experiment is essential to provide concrete evidence that capsular formation does occur in the presence of sub-lethal levels of antibiotic. One such method is the use of sodium salicylate, which can reduce the production of capsular polysaccharide in heavily encapsulated strains of *Klebsiella pneumoniae* by more than 50% (19). By treating one sample of *E.coli* B23 cells with both sodium salicylate and kanamycin, it would be possible to see if the effects of kanamycin treatment on T7 phage binding were due to increased capsule production or other changes within the cell. Alternatively, one could compare the effects of kanamycin treatment on a strain of *E.coli* capable of producing a capsule to a strain of *E.coli* B23 with a mutation in the Rcs protein, which confers the cell capsule-deficient, on phage adsorption, which may provide a definitive answer as to whether kanamycin treatment increases capsule production.

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