CHAPTER VI

STUDIES ON EXOPOLYSACCHARIDE DEPOLYMERASE ENZYME ASSOCIATED WITH PHAGE PIK INFECTION.
Bacterial capsules are involved in a great variety of functions (1,2,3). The capsule of most Gram-negative bacteria are composed of hydrophilic polysaccharide (4) carrying negative charges (2,5). Those bacteriophages which require specific proteins and/or lipopolysaccharides for adsorption (6-9) are unable to infect capsulated cells. However, contrary to such a protective function of the capsule a small number of bacteriophages specifically require the presence of the capsule for adsorption and subsequent infection (10,11,12). The ability of bacteriophages to infect encapsulated bacteria and to remove capsules has been known for some time.

A common feature of phage infection of encapsulated bacteria is the induction of enzyme that degrade the capsule (or slime). Polysaccharide depolymerase has been examined from a number of phage host cell systems (13-18). Those phages which enzymatically hydrolyze the capsular polysaccharide are in analogy with the class of phages that are capable of depolymerizing o-antigen receptors (LPS) (19-21,12). Furthermore, the enzymes degrading LPS receptors, as well as the capsule degrading enzymes are structural components of the virus adsorption organelles (19-22) and may be found apart from the virion (23-28).

Uptill now most of the work has been done with phage
binding to \textit{E. coli} and \textit{Klebsiella aerogenses} capsules. \textit{E. coli} produces a number of different exopolysaccharides whose capsular antigens are subdivided into at least 3 varieties, A, B and L, based on differences in physical behaviour. In addition several strains produce a slime, "Colanic acid". At least 92 different K-antigens have been recognised (29). More than 10 different phages specific for capsules of \textit{E. coli} were isolated by Stirm and his co-workers (11,30). A detailed study of phage receptor interaction has so far been undertaken only for the system, phage 29 and capsule K29 (\textit{E. coli} K29), involving characterization of both phage and capsule. The phage is specific in action, only attacking capsulated bacteria with the K29 exopolysaccharide on the surface. The interaction between phage and isolated capsular polysaccharide results in hydrolysis of the \textit{Glucose-1 \rightarrow 3-Glucosamine} linkage within the polysaccharide (Table 4; Chapter I). However, the interaction does not result in triggering of DNA ejection from the phage (31). The primary structure of the serotype \textit{E. coli} K29 polysaccharide has been established (32). The phage 29 depolymerase enzyme is synthesized in free form in about 10 fold excess as compared to phage bound.

Strains of the \textit{Klebsiella-Enterobacter} (formerly \textit{Aerobacter}) genera are almost invariably mucoid i.e. they produce either a capsule or a slime or both. More than 80
different K-antigen have been identified in *Klebsiella* (29,33) whereas no established scheme exists for *Enterobacter*. Among the bacteriophages attacking *Klebsiella* strains, phage 11 is best characterized (34-37). The enzyme depolymerase is produced both in soluble (in excess) and phage bound form, and is associated with the spikes of the base plate of the phage. The depolymerase is an endoglycosidase hydrolysing the Glucose-1 $\rightarrow$ 3-Glucose linkage. Stirm and co-workers (26) studied the depolymerase of two other phages, 20 and 24, active on *Klebsiella* strains with capsules of types K-20 and K-24 respectively. The first detailed study of the interaction between an exopolysaccharide specific phage and its substrate, where the depolymerase was purified to homogeneity and the exopolysaccharide structure determined, was that of phage K-2 active on an unidentified *Enterobacter aerogenes* type (28,38). The morphology of the phage was not investigated.

Morphological examination of exopolysaccharide specific phages has revealed that most of them belong to group C. Bacteriophages representatives of groups A and B are however, also found (30). One common feature to all phages active on exopolysaccharides is that the base plates are provided with spikes and no tail fibres. Because of the relative simplicity of the group C phages in terms of number of phage proteins present, as compared to group A
and B, most of the work in characterization of phage associated depolymerases has been done with representative of group C phages.

Data on the composition and structure of exopolysaccharides produced by Gram-negative bacteria outside the Enterobacteriaceae family are fragmentary. Therefore, little information is available about phages active on exopolysaccharide producing *Pseudomonas*. Phages lysing only exopolysaccharide producing strains have been described for *P. putida* (18) and *P. aeruginosa* (17, 39, 40, 41). In the latter case six different bacteriophages were found, and classified them into two groups based on their substrate specificity (41). One of the phages, phage 2 (of *P. aeruginosa* BI) and its interaction with slime polysaccharide B have been investigated in more detail. Phage 2 belongs to group B of Bradley morphologically and has a long tail with a prominent knob like structure at the tip (39). This phage produces a depolymerase in excess when grown on the exopolysaccharide producing strains.

The present chapter describes in detail the results obtained on the characterization of a capsular depolymerase enzyme associated with the phage PIK. Phage PIK specific for *P. aeruginosa* PA01 classified into group 'A' of Bradley, produces an enzyme, the capsular or exopolysaccharide
Fig. 1: Zone of inhibition surrounding phage PIK spot on *P. aeruginosa* PA01 lawn after 48 hours of incubation at 37°C.
depolymerase during the course of its infection. Further attempts have been made to study the role played by this enzyme during phage adsorption and its release.

In many systems where bacteriophages have been isolated for capsulated species of bacteria, the typical plaque morphology consists of a large clear plaque surrounded by crater like depression or halo in the bacterial lawn has been found (14,42-45).

Plaques of phage PIK are clear and also shows a surrounding crater like depression or halo (Fig. 1; Chapter IV). These haloes are, at least partly, the result of diffusion of a phage induced enzyme which hydrolyzes the capsule without killing the bacteria. Within the halo, the bacterial lawn is decapsulated as evidenced by a diminished volume. This clearing of lawn can also be shown by spotting phage lysate on the mature lawn of *P. aeruginosa* PA01 (Fig. 1). The zone of clearance increases with time of incubation. The figure shows a large zone of clearance surrounding the phage spot after 48 hours of incubation at 37°C. Thus the production of halo, surrounding the plaque spot and its diameter increased with the time of incubation thereby indicating that phage PIK produces an enzyme which degrades or destroys the capsule. This ability of a phage enzyme to remove capsules from *Klebsiella pneumoniae* type 1
Fig. 2: Appearance of enzyme exopolysaccharide depolymerase during one step growth curve of phage PIK infection on *P. aeruginosa* PA01. Enzyme activity (o); Plaque forming units (●).
cells was first observed by Humphries (45). The important point to be noted is that this enzyme was produced only during infection by phage PIK on P. aeruginosa PA01. The supernatant fluids and homogenate obtained from uninfected culture of strain PA01 during log and stationary phases of growth have been repeatedly examined and found to be free from depolymerase.

In an attempt to show the time course of appearance of enzyme in extracellular fluids, strain PA01 was infected with phage PIK and one step growth curve was followed. It was found that appearance of this enzyme starts at the end of the latent period and then increases with time, reaches its maximum at 160 minutes of infection (Fig. 2).

The enzyme exopolysaccharide (EPS) depolymerase was purified to 881 fold from the lysate of phage PIK. Conventional methods of protein fractionation were employed namely, salting out with ammonium sulphate, sephadex gel filtration and high speed centrifugation (Table 1). At each step the activity of the enzyme was determined using purified EPS from P. aeruginosa PA01, which acts as a substrate for this enzyme.

A typical elution profile from Sephadex G-200 (second passage) is shown in Figure 3. Peak fractions (fractions 14-16) were pooled and reverse dialyzed against polyethylene glycol
Table 1. Purification of exopolysaccharide depolymerase from phage PIK infected *P. aeruginosa* PA01.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total proteins (mg)</th>
<th>Protein (mg/ml)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg protein)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude</td>
<td>600</td>
<td>2340.0</td>
<td>3.900</td>
<td>4100</td>
<td>1.75</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>2. Ammonium sulphate 40-45%</td>
<td>20</td>
<td>11.70</td>
<td>0.585</td>
<td>957</td>
<td>81.79</td>
<td>46.73</td>
<td>23</td>
</tr>
<tr>
<td>3. Sephadex G-200 (I)</td>
<td>60</td>
<td>0.67</td>
<td>0.011</td>
<td>671</td>
<td>1001.40</td>
<td>572</td>
<td>16</td>
</tr>
<tr>
<td>4. Sephadex G-200 (II)</td>
<td>30</td>
<td>0.24</td>
<td>0.008</td>
<td>370</td>
<td>1541.00</td>
<td>881</td>
<td>13</td>
</tr>
</tbody>
</table>

Units: µgs of aminosugars released per minute at 37°C.
Fig. 3: Purification profile of enzyme exopolysaccharide depolymerase from Sephadex G-200 column.

Protein (▲—▲), Plaque forming units (●—●) and Enzyme activity (■—■).
for protein concentration. The second step in Sephadex G-200 was carried out to remove the bacteriophage contaminants which were detected in the previous step in Sephadex G-200 because of overlapping of collected fractions. Thus all the bacteriophage activity was removed during the initial fractions (4 to 11) and the enzyme fractions obtained were free from any bacteriophage particles.

The enzyme depolymerase from PIK infected cultures of *P. aeruginosa* PA01 was purified to 881 fold. Yurewicz and his co-workers (28) obtained a 140 fold purification of capsular depolymerase from *Aerobacter aerogenes* infected cultures in DEAE cellulose and CM-cellulose columns.

Inspite of the extensive purification of the phage PIK, which was devoid of any free depolymerase it exhibited little depolymerase activity due to the bound enzyme. However, the amount of glucosamine released by purified phages (due to bound enzyme activity) was less as compared to those released by purified free depolymerase (Table 2).

Table 2: Free and bound exopolysaccharide depolymerase activity.

<table>
<thead>
<tr>
<th>Protein concentration (µg/ml)</th>
<th>Free depolymerase activity</th>
<th>Bound depolymerase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucosamine released (µg/minute)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.05</td>
<td>0.002</td>
</tr>
<tr>
<td>20</td>
<td>0.12</td>
<td>0.016</td>
</tr>
<tr>
<td>30</td>
<td>0.20</td>
<td>0.026</td>
</tr>
<tr>
<td>40</td>
<td>0.24</td>
<td>0.030</td>
</tr>
<tr>
<td>50</td>
<td>0.30</td>
<td>0.032</td>
</tr>
<tr>
<td>60</td>
<td>0.34</td>
<td>0.036</td>
</tr>
</tbody>
</table>
Fig. 4: Gel Electrophoresis of enzyme exopolysaccharide depolymerase at pH 8.0. The protein sample (approx. 180 μg/0.1 ml) in 5% sucrose was placed in a vertical gel electrophoresis cell (A); In a separate slot bovine serum albumin was run as a control (B).
Moreover, it was clear from the purification steps (Table 1) that free depolymerase gradually loses its total activity (units) with the fold purification, indicating that the depolymerase activity associated with phage PIK was lost with the gradual removal of phage PIK. Thus from these results it can be concluded that exopolysaccharide depolymerase in phage PIK exists in two forms: free and bound form. The free depolymerase was about 10 fold in excess than the bound enzyme.

Electrophoretic analysis of purified exopolysaccharide depolymerase on polyacrylamide gel exhibited a single protein component located at a distance of approximately 11 cms from the origin and approximately 3 cms away from the albumin front. The single band indicate the homogeneous purification of the enzyme (Fig. 4).

The molecular weight of the purified EPS depolymerase was estimated on Sephadex G-200 column using the molecular weights of the marker proteins as standards (Fig. 5). Based on these results, the EPS depolymerase of phage PIK was attributed as having molecular weight of approximately 200,000 daltons.

The EPS depolymerase produced by phage PIK during infection in P. aeruginosa PA01 was found to be highly specific. The induced lysates of other Pseudomonas strains
Fig. 5: Molecular weight determination of exopolysaccharide depolymerase on Sephadex G-200. A, Exopolysaccharide depolymerase (200,000); B, Lipoxidase (102,000); C, Bovine serum albumin (68,000); D, Lysozyme (14,000).
Fig. 6: Hydrolysis of exopolysaccharide (EPS) extracted from P. aeruginosa PA01 by exopolysaccharide depolymerase (substrate curve).
also tested and found to be free from depolymerase activity. The depolymerase activity could not be detected in uninfected cultures, nor was it present when infection was initiated by phages other than PIK on \textit{P. aeruginosa} PA01, e.g., phage E79. Phage E79 produces a clear plaque on bacterial lawns of \textit{P. aeruginosa} PA01 also reported by Meadow and Wells (47). This suggests that the genetic information required for biosynthesis of this particular enzyme is furnished by the phage PIK genome.

Some of the basic properties of the capsular depolymerase of phage PIK has been studied in detail.

The enzyme depolymerase could hydrolyze the EPS extracted from \textit{P. aeruginosa} PA01, generating aminosugars (glucosamine equivalents). The typical substrate curve shows that when the EPS concentration reaches 1.2 mg/ml maximum amount of aminosugars (units) were liberated (Fig. 6) and thereafter it remains constant irrespective of the further increase in the substrate concentration. Paper chromatography analysis revealed only glucosamine spot (compared with standard D-glucosamine spot) from the enzymatic hydrolysate product.

The rate of depolymerase activity increased proportionally with increased concentration of the enzyme (Fig. 7) and exhibited a remarkable heat stability retaining about
Fig. 7: Rate of exopolysaccharide depolymerase activity with different concentrations of the enzyme.
Fig. 8: Heat Stability of enzyme exopolysaccharide depolymerase.
50% of the activity when heated for 20 minutes at 70°C, a temperature which destroyed the bacteriophage and the bacteria (Fig. 8). Similar kind of heat stability was reported for different capsular depolymerase enzymes in few systems (45,46).

Influence of incubation temperature on depolymerase activity was studied (Fig. 9). It was found that maximum activity of the enzyme was observed when the system was incubated at 37°C. However, even at 40°C enzyme depolymerase possess a remarkable activity, higher than what was observed at 30°C.

Rate of enzyme activity was determined with respect to optimum pH. It was found that the PIK depolymerase possess maximum activity at 7.2 pH in sodium phosphate buffer (5 mM) (Fig. 10). The enzyme activity decreases either with increase or decrease in pH. The enzyme also shows maximum stability at pH 7.2. Bartell et al. (46) have reported a pH optima of 7.5 for phage 2 enzyme specific for P. aeruginosa BI. Enzyme depolymerase from phage PIK however, shows less activity at pH 7.5 than at pH 7.2.

It was further studied that depolymerase activity was not inhibited by a variety of metal ions tested, including EDTA. However, the activity of depolymerase was slightly enhanced by the presence of manganese chloride (5 mM). The
Fig. 9: Effect of temperature of incubation on exopolysaccharide depolymerase activity.
Fig. 10: pH specificity for exopolysaccharide depolymerase enzyme.

Aminosugar Released (µg/min/mg protein)
enhanced activity of depolymerase in the presence of manganese ions could be correlated with the requirement of manganese chloride by phage PIK for its maximum adsorption (Table 2; Chapter IV).

For determination of Km value for the enzyme depolymerase Michaelis-Menton curve has been plotted (Fig. 11). From the plot Km value and Vmax value was found out to be 1.16 mg/ml and 12.5 µg/min/mg of protein respectively.

Many workers earlier noted that the bacteriophages active on EPS producing bacterial strains are generally EPS specific; non capsulate or non slime producing mutants are resistant to the phages (11,13,42,43). In view of this, attempts were made to find out other substrate for enzyme depolymerase from phage PIK. It was observed that isolated lipopolysaccharide (LPS) could also act as a substrate for enzyme depolymerase (Fig. 12). When equal amounts of EPS and LPS were incubated with equal amounts of enzyme depolymerase and for the same period of incubation, the amount of glucosamine released from EPS was more than that with LPS indicating a higher substrate specificity for EPS. However, it was interesting to note that enzyme depolymerase from phage PIK acts on both EPS and LPS unlike other depolymerases which act only on EPS. So far it was reported in literature that depolymerase from phage 2 (Bradley's group B)
Fig. 11: Michaelis - Menton plot of enzyme exopolysaccharide depolymerase showing the Km and Vmax values.
Fig. 12: Exopolysaccharide depolymerase activity with isolated lipopolysaccharide and Exopolysaccharide. 
A, Exopolysaccharide + enzyme (●); B, Exopolysaccharide control (▲); C, Lipopolysaccharide + enzyme (■); D, Lipopolysaccharide control (○).
acts on both LPS and EPS. extracted from *P. aeruginosa*
strain BI (39).

It is a general concept that interaction between phage
and EPS does not trigger the ejection of the phage nucleic
acid and the presence of the specific EPS does not always
correlate with the phage sensitivity (16,17,48). Thus the
phage has to interact with a second receptor for triggering
of nucleic acid ejection during the course of its infection.

We have reported that LPS from *P. aeruginosa* PA01
possess phage PIK receptors (49) (Chapter V). It was also
noted that for phage PIK the value of PhI<sub>50</sub> by LPS
(4.8 μg/ml) was more as compared to Br<sub>50</sub> value (1.24 μg/ml).
In general, if at all phage has only one receptor then the
value of PhI<sub>50</sub> of phage by the receptor should not be higher
than Br<sub>50</sub> value. Jarrell and Kropinski (50) have also
suggests that a higher LPS- PhI<sub>50</sub> value as compared to
Br<sub>50</sub> value might indicate the interaction of phage with
another receptor on the cell wall surface.

Thus, attempts have been made to find out another
receptor for phage PIK on the PA01 cell surface. It was
found that the isolated EPS from PA01 cell surface also
causes PIK inactivation exhibiting a PhI<sub>50</sub> of 325 μg/ml
(Fig. 13). Thus, it was concluded that both EPS and LPS from
strain PA01 possess phage PIK receptors. This finding is
Fig. 13: Phage PIK inactivation by isolated exopolysaccharide.
further substantiated by the presence of glucosamine both in EPS and LPS which is a constituent of phage PIK receptors (49).

On the basis of our results we propose that phage PIK has two receptors on the cell surface. Castillo and Bartell (51) have reported earlier that phages can have more than one receptors. Phage 2 specific for P. aeruginosa BI can be inactivated by both slime and LPS. However, phage PIK is the only phage belonging to Bradley's group 'A' (possessing a contractile tail), possessing this property.

Early investigations on the location of the phage coded depolymerases show that they are invariably associated with the base plate and precisely on the tail spikes of the phages (34, 52, 35, 53, 38). Since the phages invariably adsorb through base plates and tail spikes the possibility of depolymerase playing a role in phage adsorption could not be ruled out. To show this possibility in phage PIK infective process we have carried out the following experiments.

The first of all these experiments was to prepare antidepolymerase and antiphage serum in rabbits. EPS and LPS from PA01 known to contain phage PIK receptors, were included in this experiment as positive controls since they readily inactivate phage PIK. When phage PIK was mixed with purified EPS and LPS from strain PA01, 87 and 96%
inactivation occurred respectively in comparison to control (Tris buffer 5 mM, pH 7.2, supplemented with 5 mM MnCl<sub>2</sub>). Similar buffer control was used in rest of the experiments. Nearly 100% inactivation occurred when phage PIK was reacted with antiserum prepared against it. Antiserum prepared against purified depolymerase also causes similar kind of inactivation in vitro (Table 3). The above data indicates that depolymerase enzyme is indeed associated with phage PIK and thus may play a role in its infectivity. This experiment also confirms that phage PIK depolymerase exists in two states: free, and bound to the phage particle as one of the structural proteins of the base plate. The relatedness of the free and bound depolymerase was determined by their serological cross-reactivity, since antiserum prepared against the purified free enzyme reacted with the phage associated enzyme.

Table 3. Inactivation of phage PIK by antiphage PIK and antidepolymerase sera.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Phage input (pfu/ml)</th>
<th>Phage recovered (pfu/ml)</th>
<th>Phage inactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>2.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.00 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>TSB</td>
<td>2.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.00 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>EPS (50 µg/ml)</td>
<td>2.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.26 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>87</td>
</tr>
<tr>
<td>LPS (25 µg/ml)</td>
<td>2.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.057 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>96.65</td>
</tr>
<tr>
<td>Antiphage PIK</td>
<td>2.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.003 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>99.85</td>
</tr>
<tr>
<td>Antidepolymerase</td>
<td>2.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.005 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>99.75</td>
</tr>
</tbody>
</table>

Antiphage PIK serum: 1:100 diluted serum incubated for 20 minutes.
Since the possible involvement of phage PIK depolymerase in the adsorption process has been anticipated, the effect of enzyme treatment on the ability of whole cells of PA01 to adsorb phage PIK was examined. It was observed that whereas 60% of adsorption of phage PIK to PA01 cells occurred (after 20 minutes of incubation), only 48% of adsorption took place when enzyme depolymerase was added simultaneously with the phage. Further, when the cells were pretreated with enzyme for 20 minutes before phage addition, no significant adsorption of phage PIK occurred (Table 4).

Table 4. Effect of depolymerase enzyme on adsorption of phage PIK to *P. aeruginosa* PA01.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Phage input (pfu/ml)</th>
<th>Phage recovered (pfu/ml)</th>
<th>Phage adsorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSB + buffer</td>
<td>$1.5 \times 10^5$</td>
<td>$1.52 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>TSB + depolymerase</td>
<td>$1.5 \times 10^5$</td>
<td>$1.50 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>Cells$^a$ + buffer</td>
<td>$1.5 \times 10^5$</td>
<td>$0.60 \times 10^5$</td>
<td>60</td>
</tr>
<tr>
<td>Cells + depolymerase (at zero time)</td>
<td>$1.5 \times 10^5$</td>
<td>$0.78 \times 10^5$</td>
<td>48</td>
</tr>
<tr>
<td>Cells + depolymerase (pretreated for 20 minutes)</td>
<td>$1.5 \times 10^5$</td>
<td>$1.48 \times 10^5$</td>
<td>1.34</td>
</tr>
</tbody>
</table>

$^a$ *P. aeruginosa* PA01 cells ($2 \times 10^8$ cells/ml) diluted and added to get MOI1 approximately.
From the earlier results it is clear that EPS and LPS components constitute the receptors for phage PIK. Thus it was of interest to study the effect of the phage PIK depolymerase on EPS and LPS. When EPS and LPS were treated with the enzyme before addition of phage, they completely lost phage PIK inactivating ability (Table 5). These experiments suggest that phage PIK receptors were either modified and/or destroyed by the action of the enzyme. Alternatively, this may indicate the necessity of enzyme depolymerase for the recognition of phage receptors, thus, indicating its role in phage PIK adsorption.

Table 5. Effect of depolymerase on phage PIK inactivation by purified EPS and LPS from *P. aeruginosa* PA01.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Phage input (pfu/ml)</th>
<th>Phage recovered (pfu/ml)</th>
<th>Phage inactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>$2.75 \times 10^5$</td>
<td>$2.75 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>Buffer + depolymerase</td>
<td>$2.75 \times 10^5$</td>
<td>$2.77 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>EPS (50 µg/ml) + buffer</td>
<td>$2.75 \times 10^5$</td>
<td>$0.29 \times 10^5$</td>
<td>89.46</td>
</tr>
<tr>
<td>EPS (50 µg/ml) + depolymerase</td>
<td>$2.75 \times 10^5$</td>
<td>$2.74 \times 10^5$</td>
<td>0.36</td>
</tr>
<tr>
<td>LPS (25 µg/ml) + buffer</td>
<td>$2.75 \times 10^5$</td>
<td>$0.21 \times 10^5$</td>
<td>92.36</td>
</tr>
<tr>
<td>LPS (25 µg/ml) + depolymerase</td>
<td>$2.75 \times 10^5$</td>
<td>$2.80 \times 10^5$</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig. 14: Clear plaques of phage PIK mutant (Pdm1).
The above findings could be explained in more detail by isolating a phage PIK mutant by NTG mutagenesis, which produces hololess plaques, designated as Pdm1 (Fig. 14). The mutant was able to infect and replicate on \textit{P. aeruginosa} PA01, the normal host for wild type PIK. The growth curve of Pdm1 was found to be similar to that of phage PIK, having a latent period of 70 minutes, a rise period of 90 minutes and an average burst size of 30 (Fig. 15).

Phage Pdm1, in spite of possessing similar infective properties like PIK, depolymerase activity has not been observed to be associated with this mutant, since the lysate of Pdm1 was unable to release any glucosamine residues either from EPS or LPS. Thus the absence of halo around the plaque of Pdm1 may be interpreted in three ways: (I) Absence of depolymerase or (II) the structural presence of depolymerase which lacks the hydrolytic activity or (III) the presence of depolymerase enzyme in a different conformational form which probably lacks the hydrolytic activity.

Further experiments were performed to examine these possibilities and the results obtained are presented in Table 6.
Fig. 15: One step growth curve of phage Pdm1 mutant on *P. aeruginosa* PA01.
Table 6. Inactivation of phage mutant Pdm1 by antiphage PIK and antidepolymerase sera.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Phage input (pfu/ml)</th>
<th>Phage recovered (pfu/ml)</th>
<th>Phage inactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>$2.5 \times 10^5$</td>
<td>$2.5 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>EPS (50 µg/ml)</td>
<td>$2.5 \times 10^5$</td>
<td>$2.8 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>LPS (25 µg/ml)</td>
<td>$2.5 \times 10^5$</td>
<td>$2.9 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>Antiphage serum</td>
<td>$2.5 \times 10^5$</td>
<td>$0.009 \times 10^5$</td>
<td>99.64</td>
</tr>
<tr>
<td>Antidepolymerase serum</td>
<td>$2.5 \times 10^5$</td>
<td>$0.027 \times 10^5$</td>
<td>98.92</td>
</tr>
</tbody>
</table>

Antiphage PIK serum: 1:100 diluted serum incubated for 20 minutes.

Antiserum prepared against purified phage PIK was found to inactivate phage Pdm1 completely, indicating a strong serological relatedness between phage PIK and its mutant Pdm1. Antiserum prepared against the purified depolymerase similarly inactivated the mutant. This was taken as an indication that a cross-reacting protein/polypeptides (5), antigenically similar to the depolymerase, was/were present in Pdm1 particles in a different conformational form than present in the wild type PIK.

It was interesting to note that the cellular components EPS and LPS were able to inactivate wild type PIK (Table 3) but not the mutant Pdm1 (Table 6). These results may suggest that (A) the structural depolymerase carried by Pdm1 has
more stringent requirement for recognition of receptors in EPS and LPS or (B) the purification of EPS and LPS induces modification(s) in the tertiary structure of these moieties which limit or modify the availability of the receptors or (C) secondary receptors may be necessary for the inactivation of Pdm1 and were either missing or hidden in the purified EPS and LPS. Alternatively, the free and bound enzymes, although serologically similar might have a different mechanism of interaction with the phage receptors.

Effect of enzyme depolymerase was studied on the adsorption of phage Pdm1 to PA01 cells. In the adsorption mixture enzyme depolymerase was added simultaneously with phage Pdm1. It was found that adsorption of phage Pdm1 was reduced to 43% from the normal 57% of adsorption. Further, when the cells were pretreated with the enzyme for 20 minutes before Pdm1 addition no significant adsorption was achieved (only 7% adsorption) (Table 7). These experiments confirm that although Pdm1 could not be inactivated by either EPS or LPS, its adsorption was significantly reduced by purified depolymerase enzyme thereby affecting receptors for phage Pdm1 similar to wild type PIK phage.
Table 7. Effect of depolymerase on adsorption of phage mutant Pdm1 to *P. aeruginosa* PA01.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Phage added (pfu/ml)</th>
<th>Phage recovered (pfu/ml)</th>
<th>Phage adsorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSB + buffer</td>
<td>2.0 x 10^5</td>
<td>2.0 x 10^5</td>
<td>0</td>
</tr>
<tr>
<td>TSB + depolymerase</td>
<td>2.0 x 10^5</td>
<td>2.3 x 10^5</td>
<td>0</td>
</tr>
<tr>
<td>Cells + Buffer</td>
<td>2.0 x 10^5</td>
<td>0.86 x 10^5</td>
<td>57</td>
</tr>
<tr>
<td>Cells + depolymerase (at zero time)</td>
<td>2.0 x 10^5</td>
<td>1.14 x 10^5</td>
<td>43</td>
</tr>
<tr>
<td>Cells + depolymerase (pretreated for 20 minutes)</td>
<td>2.0 x 10^5</td>
<td>1.86 x 10^5</td>
<td>7</td>
</tr>
</tbody>
</table>

a *P. aeruginosa* PA01 cells (2 x 10^8 cells/ml) diluted and added to get MOI of 1 approximately.

On the basis of above results (with phage mutant Pdm1) the question arises, now, that if enzyme depolymerase plays any role in the attachment of the wild type phage PIK to PA01 cells, then Pdm1, devoid of any depolymerase activity, should be incapable of adsorption or show a slower rate of adsorption, as would have been reflected from longer latent and rise periods than those of wild type phage PIK. However, surprisingly the same values, 70 minutes for the latent period, and 90 minutes for the rise periods were observed with mutant Pdm1 (Fig. 15). Further, similar
effect of enzyme depolymerase on adsorption of phage Pdm1 to viable cells of *P. aeruginosa* PA01 was observed. These results may suggest that although the active enzyme was not essential in the adsorption process, the presence of structural enzyme in an inactive form might be still involved in the adsorption process.

The above hypothesis was again supported by the fact that the mutant Pdm1, although devoid of any depolymerase activity, reacted with anti-depolymerase serum getting completely inactivated. These results clearly indicated the presence of wild type depolymerase cross-reacting polypeptide(s) in the base plate, which although devoid of enzymatic activity might retain the ability to recognise complementary receptors and allow phage to attach to the cell surface.

In this connection, it is worthwhile to discuss the results obtained by Kozloff et al. (54) who mentioned that coliphage T4D possesses an enzyme, dihydrofolate reductase, as one of the structural components of the base plate and plays a role in the adsorption process. Two mutants of T4D, neither of which induces the production of active enzyme, are fully infective. They isolated a protein which resembles the enzyme dihydrofolate reductase in binding to the substrate but does not hydrolyze the substrate. They
proposed that the protein that resembles the enzyme may serves a structural rather than an actual enzymatic role in the phage base plate. Another hypothesis was also proposed by Dowes and Goldberg (55), who stated that the dihydrofolate reductase plays no enzymatic role in the virion, but is structurally important during the early stages of phage adsorption. A similar explanation could be attributed to the role of phage PIK depolymerase in the adsorption process, thereby clarifying the fact that Pdm1 is fully infective. Alternatively, phage PIK depolymerase may possess a bifunctional role involving the ability to hydrolyze the EPS and LPS and to recognize the attachment of the phage PIK receptors present in EPS and LPS.

The presence of receptors for phage PIK in EPS and LPS and the destruction of these receptors by enzyme depolymerase in vitro and in situ is of great significance because when new phage progeny is produced after replication, an excess amount of free depolymerase is produced, which protects these newly formed phage progeny from inactivation by EPS and LPS that are present on the surface of the cell.

Apart from the involvement of enzyme depolymerase in phage PIK adsorption the present investigation was aimed to study the role of this enzyme in phage PIK release after its reproduction. For this purpose, we have carried out the
Fig. 16: Optical density of *P. aeruginosa* PA01 suspension during various treatments with the following substances:
- Sodium phosphate buffer (pH 7.2, 5 mM) (●);
- Exopolysaccharide depolymerase (250 µg/ml) (●);
- Lysozyme (300 µg/ml) (▲); Lysozyme + exopolysaccharide depolymerase (300 + 250 µg/ml respectively) (□);
- EDTA (300 µg/ml) (■); EDTA + Lysozyme (300 µg/ml each) (▼).
following experiment to show cellular lysis of PA01 cells by enzyme dipolymerase when incubated together. Similar lytic activity has also been carried out in presence of EDTA, lysozyme and also in combination. Enzyme depolymerase and lysozyme in combination slightly increased the cell lysis (after 50 minutes) in comparison to lysozyme alone (Fig. 16). From these results, it appears that depolymerase supports the lysozyme in lysing the infected cells.

The above hypothesis can be explained using few known facts. In Gram-negative bacteria, generally the surface layer has different composition and structure represents a mechanical barrier that might impede the release of mature phage particles from the infected cell. Phage release may be accompanied by the production of specific enzymes that disrupt the structural integrity of these layers (e.g. lytic enzyme like lysozyme). The peptidoglycon layer is usually considered to be responsible for maintaining the rigidity of the cell in the majority of Gram-negative bacteria. However, in P. aeruginosa the situation seems to be somewhat different. Although, some lesions were observed after treatment of the cells with lysozyme, the cell wall structure remained intact (56). This indicates that peptidoglycan is not solely responsible for the rigidity of the cell in P. aeruginosa. Moreover, if the outer membrane is disrupted by treatment with Tris buffer, EDTA
or a combination of both, cell lysis ensues (56-60). As reported here, treatment of PA01 cells with phage PIK depolymerase in hypotonic media induced cell lysis. Thus under natural conditions, depolymerase activity may facilitate to some extent the release of phage particles by eliminating a portion of the structural layers of the cell envelope. In addition, progeny phage PIK may be protected from the receptors present in LPS and EPS which are hydrolyzed or possibly blocked by the depolymerase.

Thus, we conclude that phage. PIK depolymerase appears to play a role in two important steps of the phage PIK life cycle: phage adsorption and to some extent in phage release.
References


