CHAPTER V

ISOLATION AND CHARACTERIZATION OF PHAGE PIK RECEPTORS ON *P. AERUGINOSA* PAO1.
Studies on bacteriophage adsorption, which were once very popular (1-4) have been somewhat neglected for the past 25 years. There is, however, a certain renewal of interest in the subject (5), mainly because of the present emphasis on studies regarding the structure and function of biological membranes. Furthermore, the information now available on the structure of phage particles and the nature of their receptors allow one to approach the problem of phage adsorption in a more quantitative way than was possible 25 years ago.

The attachment of the phage to a specific receptor site on the bacterial surface is an essential step in the phage infection. Adsorption of a bacteriophage to its host requires the presence of specific receptor molecules on the bacterial surface. A successful adsorption may be followed by the ejection of nucleic acid from the phage and its penetration into the cells. These early steps of phage infection are yet, not well understood, although later events concerning the replication of nucleic acid and synthesis of phage particles have been well documented.

Almost every structure on the surface of a bacterial cell, or extending from it, can act as phage receptors (6). Consequently, phage receptors vary greatly in chemical nature and depend on the type of bacteria. Gram positive bacteria such as *Staphylococcus aureus* (7), *Streptococci* species (8)
and *Bacillus subtilis* (9) have been investigated and it was shown that the cell wall teichoic acid (10,11) or some moiety of the teichoic acid (12,13) was essential for phage adsorption. In *Lactobacillus casei* which lacks the teichoic acid in its cell wall, the phage J-1 receptor involves both D-galactosamine in the cytoplasmic membrane and L-rhamnose in the cell wall (14). The Gram negative cell wall has a mosaic of phage specific receptors present in lipopolysaccharide (LPS), protein and capsular polysaccharide portion of the outer surface (6). The receptors for coliphages T3, T4, and T7 of *E. coli* have been shown to be LPS and the one for coli phages T2, T6 and lambda were lipoprotein (15-19). The outer membrane of *E. coli* contains number of proteins primarily involved in some of the vital cell functions but which also serve as receptors for bacteriophage and colicins. The protein identified as a receptor for phage lambda allows maltose and maltotriose to diffuse through the outer membrane (20).

Receptors for phage T5, colicin M and colicin E3 are active even in the isolated state (21,22) and those for phages Tull and K3 and F-pilus are active only in the presence of other surface components (23,24,25).

The identification of the receptor has generally relied on the existence of a specific interaction between the
receptor and a given bacteriophage, and on the demonstration
that this receptor is absent or modified in extracts of
bacterial strains unable to adsorb the phage (6).

The first demonstration of the inactivation of bacterio-
phages by bacterial extracts was made by Levine and Frisch
(26). The most intensive study of the phage inhibiting
activity of purified bacterial antigens was made by Goebal
and collaborators using Shigella sonnei and the T-series of
coli dysentery phages (27,28,29).

Bacteriophage receptor materials for several bacterio-
phages of the T-series attacking E. coli and Shigella sonnei
have been isolated, purified and chemically characterized
(28,30-33). Phage inactivating materials have been prepared
from the Gram positive bacteria, Bacillus anthracis (34),
Staphylococcus species (7,35) and a group C Streptococcus
species (36).

The emergence of Pseudomonas aeruginosa as an important
human pathogen has resulted in a variety of serological, phage
and aeruginocin typing schemes. As yet, comparatively little
is known about the antigenic mosaic on the cell surface, and
in particular the nature of phage receptors. Although the
study of the cell wall and membrane structure of E. aeruginosa
has made great progress during the last few years (37-40),
there are still many unanswered questions particularly those
involving the relationship between the composition of bacterial envelope and the phenotypic properties of the organism, and the receptors of phages and aeruginocins. The surface properties have been shown to be involved in the resistance towards antibiotic and sensitivity towards detergents, as well as in the phage adsorption (41-47).

In the present investigation attempts have been made to isolate and characterize phage PIK receptor from P. aeruginosa PA01 in vitro. Phage PIK is a virulent phage specific for P. aeruginosa PA01 (48). In this chapter data is presented to show that phage PIK infecting P. aeruginosa strain PA01 interacts with the LPS of the cell wall which acts as the receptor. Components responsible for receptor activity in the LPS and some of the constituents of the receptor complex have been examined.

There have been two principle approaches to the problem of the chemical nature of the bacterial receptor sites for phage adsorption. One approach involves study of the kinetics of phage adsorption to bacterial cells treated with various agents which might alter the cell surface. The second approach involves a study of the chemical properties of the receptor substances after their isolation from the bacterial cells.

The cell surface of P. aeruginosa strain PA01 was altered chemically to study the effect on phage PIK adsorption.
Actively growing cells were treated for 30 minutes with reagents indicated in Table 1, which shows that attachment of phage PIK appears to demand intact carboxyl groups, but is independent of the integrity of amino groups on the cell wall. The ineffectiveness of sulfhydryl-blocking reagents like p-chloromercuribenzoate indicates that cellular sulfhydryl groups play no significant role in the primary union of these bacteriophages to their host.

Table 1. The binding of PIK phage to chemically modified *Pseudomonas aeruginosa* PA01.

<table>
<thead>
<tr>
<th>Reagents used to modify bacteria</th>
<th>Adsorption to modified cell (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Carboxyl reagent</td>
<td></td>
</tr>
<tr>
<td>CH$_3$OH (80%) + HCl (0.1 M)</td>
<td>25.83</td>
</tr>
<tr>
<td>CH$_3$OH (80%)</td>
<td>28.17</td>
</tr>
<tr>
<td>HCl (0.1 M)</td>
<td>20.89</td>
</tr>
<tr>
<td>B. Amino reagents</td>
<td></td>
</tr>
<tr>
<td>Formaldehyde (1.2 M)</td>
<td>63.59</td>
</tr>
<tr>
<td>Acetic anhydride (0.3 M)</td>
<td>64.92</td>
</tr>
<tr>
<td>C. Other Reagents</td>
<td></td>
</tr>
<tr>
<td>I$_2$ (5 X 10$^{-4}$ M) + KI (0.5 M)</td>
<td>71.54</td>
</tr>
<tr>
<td>Na-p-Chloromercuribenzoate</td>
<td>69.36</td>
</tr>
<tr>
<td>D. Control</td>
<td>70.82</td>
</tr>
</tbody>
</table>
Experiments by Weidel (49) on *E. coli* B with formaldehyde decreases the receptor activity for phage T1, T3, T5 and T7 but not for T2, T4, and T6. Periodic, nitrous and other weak acids destroy the receptors for T1, T3, T4, T5 and T7 but not for T2.

One of the approaches in the study of bacteriophage adsorption and its infective process involves the use of mutants. The primary requirement of any genetic analysis is the ready availability of the stable mutants. Fortunately, by the use of various mutagens the proportion of the mutants in a bacterial population can be increased. Studies on mutation have facilitated our understanding of cellular processes at the molecular level in many important ways. One of the approaches in the study of bacteriophage adsorption involves the use of bacterial mutants from both the host and the virus.

A detailed study of induction of mutations in bacteria (*Escherichia coli*) by X-rays and ultra-violet light was first undertaken by Demerec and Latarjet (50) and since then bacteria have been increasingly used for both irradiation and mutational research. Ultraviolet irradiation induces both base substitutions and deletions in bacteria (51, 52). The weak induction of frame shift mutants has also been reported (53).

Ultraviolet irradiation was capable of inducing a variety of color mutants including the conspicuous greenish
Fig. 1: Ultraviolet irradiation survival curve of

*P. aeruginosa* strain PA01 (▲—▲) and its
nonpigmented mutant PAO (○—○).
Fig. 2: Adsorption kinetics of phage PIK on *P. aeruginosa* PA01 (●—●) and its mutants PA0 (○) (▲—▲); PA0 (R) (■—■); PA0 (N) (○—○); and PA0 (L) (△—△).
yellow and white types. The effect of UV irradiation on the survival of \textit{P. aeruginosa} strain PA01 and the non pigmented mutant PAO (0) is shown in Figure 1. The survival curves show an exponential decline with the single hit inactivation. The data indicate that the parent and the mutant PAO (0) are almost equally sensitive to UV irradiation. In both the cases about 91\% inactivation is obtained within 25 seconds.

The adsorption kinetics of phage PIK on \textit{P. aeruginosa} PA01 and its UV mutants were shown in Figure 2. The data presented here indicate that the pigmentless mutant PAO (0) has almost equal adsorption efficiency with that of the parent while a green mutant PAO (L) shows lower rate of adsorption (10\%). The above results infer that phage adsorption was not related with pigment and that pigment pyocyanine of \textit{P. aeruginosa} PA01 might not be involved in the receptor complex of phage PIK. The pigment prodigio\text{cin} of \textit{Serratia marcescens} HY has been reported to be involved in phage kappa receptor complex (54).

These results were further confirmed from the one step growth curve of phage PIK with the mutant PAO (0) which showed that the latent period (70 minutes), rise period (90 minutes) and burst size (28) were similar to that of the parent PA01 strain (Fig. 3). Again clearly indicating that
Fig. 3: One step growth curve of phage PIK with non-pigmented strain PAO (o) of *P. aeruginosa* PA01.
pigment pyocyanine does not play any role either in phage PIK adsorption or its infective process.

2-Aminopurine (2 AP) and 5-bromouracil (5 BU) are analogues of normal purine and pyrimidine bases and are incorporated in DNA during replication. 5 BU, an analogue of thymine and 2 AP an analogue of adenine, both cause AT-GC transitions (55,56).

Variation in the individual pigment type mutations, as compared to the spontaneous mutation as well as in individual types, with respect to different doses of 5 BU and 2 AP is shown in Table 2. As the dose of 5 BU increased, there was a gradual fall in the number of green colonies with a concomitant increase in the white colonies. A similar effect was observed with 2 AP.

Table: 2. Mutagenesis by 5 BU and 2 AP in P. aeruginosa PA01 strain.

<table>
<thead>
<tr>
<th>Concentration of mutagen (µg/ml)</th>
<th>Total colonies (cfu/plate)</th>
<th>Type of Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous</td>
<td>138</td>
<td>119</td>
</tr>
<tr>
<td>5 BU (5)</td>
<td>121</td>
<td>89</td>
</tr>
<tr>
<td>5 BU (10)</td>
<td>127</td>
<td>80</td>
</tr>
<tr>
<td>2 AP (5)</td>
<td>119</td>
<td>110</td>
</tr>
<tr>
<td>2 AP (10)</td>
<td>103</td>
<td>83</td>
</tr>
</tbody>
</table>
Phage PIK adsorption with respect to various 5 BU and 2 AP mutants was studied (Table 3). It was clear from the efficiency of plating and adsorption percentage of mutants that the property of adsorption does not correlate with pigmentation because strain PA01 which is green in color gives maximum percentage adsorption (98%) while a mutant (also green in color) PA0 (P) gives less percentage of adsorption (42.7%). While a mutant PA0 (Q) white in color, shows nearly the same percentage of adsorption (80%) as compared to PA01 normal strain. Thus we conclude, pigmentation in *P. aeruginosa* PA01 does not play any role in phage adsorption. The variation observed in the phage adsorption may be due to mutation caused at the receptor site on the cell wall.

Table: 3. Efficiency of plating and percentage adsorption with selected mutants by 5 BU and 2 AP.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>EOP*</th>
<th>Percentage adsorption</th>
<th>Pigmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA0 (P)</td>
<td>0.37</td>
<td>42.7</td>
<td>Green</td>
</tr>
<tr>
<td>PA0 (Q)</td>
<td>0.83</td>
<td>80.0</td>
<td>White</td>
</tr>
<tr>
<td>PA0 (R)</td>
<td>0.68</td>
<td>63.03</td>
<td>Green</td>
</tr>
<tr>
<td>PA0 (S)</td>
<td>0.90</td>
<td>83.60</td>
<td>Yellowish green</td>
</tr>
<tr>
<td>PA0 (T)</td>
<td>0.92</td>
<td>87.92</td>
<td>Whitish green</td>
</tr>
<tr>
<td>PA0 (W)</td>
<td>0.51</td>
<td>57.68</td>
<td>Green</td>
</tr>
<tr>
<td>PA0 1 (normal)</td>
<td>1.0</td>
<td>98.00</td>
<td>Green</td>
</tr>
</tbody>
</table>

* EOP = Efficiency of plating
Fig. 4: N-Methyl N-Nitro-N'-Nitrosoguanidine (NTG) survival curve of P. aeruginosa PA01.

(White (△—△) and miscellaneous ( ■—■) mutants obtained from the percentage survival).
N-Methyl-N-Nitro-N' Nitrosoguanidine (NTG) is one of the most powerful mutagen known today. It is highly mutagenic in variety of systems. It has been found to have carcinogenic, antileukemic, antitumor (57) and antimalarial effects. It also produces chromosomal as well as nonchromosomal breaks and mutations (58,59).

Apart from the usual difficulties encountered with NTG in introducing "silent mutations", this compound also acts as an inducing agent for prophage and colicin production in bacteria (60).

Results from the NTG (100 μg/ml) survival curve of *P. aeruginosa* PA01 and the mutants obtained from this wild type are presented in figure 4. The results indicate the decline in viability of PA01 cells and the concomitant rise in the percentage of mutants. The white or completely non-pigmented mutants are easily counted, but the other mutants of lesser pigmentation than the parent, are difficult to enumerate because of subjective errors.

Apart from the mutants related to pigmentation, mutants with respect to antibiotic resistance or antibiotic super-sensitivity were also isolated by using NTG. A mutant designated as *P. aeruginosa* PBR1 (of *P. aeruginosa* PA01) shows resistance towards polymyxin B (MIC= 500 μg/ml). Another carbenicillin supersensitive mutant of *P. aeruginosa*
Fig. 5: PIK virions adsorbed at the cell wall of a strain PA01 cell x 63000. Arrow 'a' indicates empty virions at the cell surface. Arrow 'b' indicates contracted sheath and the appearance of rod after contraction, also show fine striations on the contracted sheath. Scale 300 nm.

Fig. 6: A magnified view of PIK virions adsorbed at the cell surface of PA01 cell x 105,000. Arrow indicates empty virions at the cell surface. Scale 200 nm.
PA01 designated as \textit{P. aeruginosa} CSS1 (MIC = 5 \textmu g/ml) was isolated. The minimum inhibitory concentration of Polymyxin B and carbenicillin for \textit{P. aeruginosa} PA01 were found to be 5 \textmu g/ml and 60 \textmu g/ml respectively. These two mutants were used in the further characterization of phage PIK receptors, described in the latter part of this chapter.

\textit{P. aeruginosa} phages utilizes a diverse of structures for adsorption on their hosts. A tail-less small RNA phage and filamentous phages utilizes pili for adsorption and infection (61). A few cell wall specific phages of \textit{P. aeruginosa} also have been reported. This includes phage 2 (62), \phi PLS-1 (63), phage 16, 44, 109 and F8 (64), 68 and PEI (65), E79, B3, D3 and G101 (66,67).

Electron micrographs of cells of \textit{P. aeruginosa} PA01 with phage PIK adsorbed at MOI 10, showed that the virions adsorb on the cell wall of host organism (Fig. 5 and 6). Some of the phage particles at the cell surface were empty (Fig. 5 and 6, arrow 'a') indicating the absence of DNA in the phage heads. A phage particle showing the contracted tail and the rod appeared after contraction (Fig. 5, arrow 'b'), indicates that phage PIK had a contractile tail. A complete bipyrimidal hexagonal shape could also be visualized from the same phage particle.

Lipopolysaccharide (LPS) together with lipoprotein and
Fig. 7: Phage PIK inactivation by *P. aeruginosa* PA01 LPS.

Mean ± S.D. from four experiments for each point was shown. Values of PhI\(_{50}\) (as derived from the figure), Br\(_{50}\) and adsorption rate constant \(k_{ARC}\) as calculated by the formula of Schlesinger (4) were also shown.
phospholipids are the main structural components of the outer membrane of Gram negative bacteria. These components possess phage receptor activity (68,6,69). The adsorption of phage to these receptors is known to be highly specific and this specificity is due to the molecular structure of both the receptor and the phage adsorption apparatus. Several Pseudomonas phages have been classified as being cell wall specific, however, the LPS components responsible for inactivation of these phages have not been well characterized.

Receptor for phage PIK on P. aeruginosa PA01 was studied in detail. Incubation of phage PIK with different preparations of lipopolysaccharide (LPS) at various concentrations resulted in marked inactivation of the phage (92-98%). The PhI value was found to be 4.8 μg/ml of LPS (Fig. 7). The adsorption rate constant (ARC) and Br values as calculated by the method of Schlesinger (4) were also incorporated in the same figure. Phage PIK inactivation rate with the isolated LPS was also studied. Purified phage particles were incubated with LPS (25 μg/ml) at 37°C and at different time intervals from the unadsorbed phage particles phage inactivation (%) was calculated. 50% of phage inactivation (PhI) was observed within 23 minutes at 37°C (Fig. 8).

On the basis of these results we propose that a positive correlation may exists between the kinetics of
Fig. 8: Phage PIK inactivation rate with the isolated *P. aeruginosa* PA01 LPS.
phage interaction with isolated receptor substances (PhI

and their interaction with cell bound receptors (Br

P. aeruginosa PA01 contain 26.4 X 10^{-9} \mu g LPS/cell (based
on KDO and glucose determination). It is in good agreement
with the value (38.4 X 10^{-9} \mu g LPS/cell) calculated by
Kropinski and his coworkers (64).

The data for PhI

(4.8 \mu g/ml) and Br

(1.24 \mu g/ml)
values obtained in phage PIK were also in close agreement
with those obtained by Castillo and Bartell (68) for phage 2
and by Jarrell and Kropinski (63) for phage \Phi PLS-1.

Several mono- and disaccharides were tested to
determine the effect on phage PIK neutralization by PA01-LPS,
with the idea that any sugar which initiates the receptors
determinant group should inhibit inactivation by interacting
with phage adsorption apparatus. The degree of inhibition
should increase as the similarity between the sugar and
the determinant group in the receptor increases. D-Galactose,
D-fructose, D-glucose, D-xylose, sucrose and cellobiose
reduced inactivation from a value of 97% in control (Fig. 7)
to 50% or less even at the concentration of 1.6 M (Fig. 9).

D-Mannose, D-maltose and L-rhamnose reduced inactivation
to 50% when added at 0.25-0.35 M concentration (Fig. 10A),
while D-glucosamine at the concentration of 0.045 M was
found to be more active inhibitor (in term of concentration)
Fig. 9: Inhibition of phage PIK inactivation by sugars. The mixture contained phage (10^3 pfu/ml) and PA01-LPS (15 μg/ml) plus the indicated concentration of the following sugars:

- D-glucose (●●●)
- D-fructose (■■■)
- D-galactose (▲▲▲)
- D-xylose (□□□)
- Sucrose (○○○)
- Cellobiose (△△△)
Fig. 10 A, B: Inhibition of phage PIK inhibition by sugars. The mixture contained phage PIK (10^3 pfu/ml) and PA01-LPS (15 μg/ml) plus the indicated concentration of the following sugars: A D-mannose (■—■); Maltose (●—●); L-rhamnose (▲—▲) and B D-glucosamine (●—●).
than all other sugars tested (Fig. 10B).

Considering the structure of various carbohydrate molecules and from the extent of their involvement in phage PIK receptor the following conclusions can be made:

(a) Phage PIK recognises a pyranose ring structure rather than a furanose, because fructose and sucrose which have furanose rings shows less than 50% inhibition of PIK inactivation by PA01-LPS even at the concentration of 1.6 M.

(b) The –NH₂ group at the C-2 position in glucose resulted in a very strong recognition as was observed in the case of D-glucosamine.

(c) A –CH₂ group at C-6 position as in rhamnose causes more than 50% inhibition and is more than the inhibition caused by glucose and galactose with CH₂OH group in the same C-6 position. Further the absence of a C-6 e.g. in xylose caused less inhibition than rhamnose.

(d) Similarly the C-2 position was important. The –NH₂ group in C-2 position in glucose was very important as was observed in D-glucosamine. The presence of –OH at C-2 position in mannose causes more than 50% inhibition which was more than that observed for glucose. The acetylation of the C-2 amino group as in N-acetyl glucosamine shows the loss of recognition of receptor activity.
The data clearly indicates that phage PIK has the capacity to recognise specific carbohydrate residues. The C-2 and C-6 positions are seems to be important for the recognition by the phage PIK. From these results it appears that D-glucosamine or a related molecule could be an important constituent of the phage PIK receptor. Although maltose had the same intermediate activity as D-mannose or L-rhamnose, it can not be detected in PA01-LPS (69), therefore, must not be a part of the receptor. D-mannose and L-rhamnose in addition to D-glucosamine also represent the constituents of the receptor. It must be stated here that the specificity must be determined not only by a given sugar or combination of sugars but also by the type of linkage(s) joining them and/to the other component in the LPS. For example in Salmonella anatum phage $\epsilon_{15}$ O-antigen of LPS representing the receptor consists of trisaccharide unit (O-acetyl $\alpha$-D-galactosyl- rhamnosyl-D-mannose) joined by $\alpha$-linkages and when the trisaccharides are $\beta$-linked, the phage $\epsilon_{15}$ no longer binds to the receptor (70).

On the basis of these results we suggest that D-glucosamine, D-mannose and L-rhamnose present in PA01-LPS represents the receptor complex for phage PIK. Either one of the latter two could be at the terminal position alpha linked to the adjacent residue or located internally in the lipopolysaccharide chain linked through its C-4 position. However,
studies on the linkage configuration existing between these residues were not carried out.

Further attempts were made to study phage PIK receptors by using phage resistant mutant. A carbenicillin supersensitive mutant designated as CSS1 (resistant to phage PIK infection) was isolated using NTG (100 μg/ml). The minimum inhibitory concentration (MIC) of carbenicillin for *P. aeruginosa* PA01 (wild type) was 60 μg/ml whereas that for mutant CSS1 was only 5 μg/ml. In the following study a correlation between antibiotic supersensitivity and phage resistance was carried out.

The LPS was isolated from the mutant CSS1 using the same procedure as used for isolation of PA01-LPS (71) and phage PIK inactivation was carried out. It was found that CSS1-LPS does not show any significant PIK inactivation even at a very high concentration (1000 μg/ml) (Fig. 11). This indicates that the LPS from mutant CSS1 does not possess PIK receptors activity and that the LPS of mutant CSS1 might be defective in some of the essential components responsible for phage PIK receptor activity.

Further attempts were made to find out the components in the LPS responsible for phage PIK receptor activity by analysing LPS from wild type PA01 as well as from the mutant CSS1. Both the LPS were partially degraded by using 1% acetic
Fig. 11: Phage PIK inactivation by *P. aeruginosa* CSS1-LPS.
acid. This partially degraded polysaccharide, from which lipid part has been removed, were fractionated by elution from a column of sephadex G-25. The various fractions were analysed for total carbohydrate and aminosugars. Detection of few sugars like glucosamine, mannose and rhamnose were done by paper chromatography on Whatman No. 1 filter paper. The wild type strain PA01 gave a high molecular weight (H, side chain), a middle molecular weight (M) and a low molecular weight (L) fractions. Both the 'H' and 'M' fractions contained detectable amount of glucosamine (more predominant in 'H' fraction), glucose (predominant in 'M' fraction), rhamnose and mannose. Low molecular weight material did not contain any glucosamine (Fig. 12).

Chester and his coworkers (72) have reported in *P. aeruginosa* PAC1 that the 'M' fraction constitute mainly glucose, rhamnose, heptose, galactosamine, ketodeoxyoctonate, alanine and phosphate. The 'H' fractions constitute mainly aminosugars and glucose. The 'L' fractions do not contain any neutral sugars but contains ketodeoxyoctonate and phosphate. In the present investigation we have only carried out the detection of glucosamine, rhamnose and mannose since we were interested only in finding out the presence or absence of these moieties in wild type and carbenicillin supersensitive mutant (CSS1); because these carbohydrate moieties were found to involve in phage PIK receptor complex (71).
Fig. 12: Fractionation of polysaccharide obtained from P. aeruginosa PA01 LPS after partial degradation with 1% acetic acid. The material was eluted from a column of 'Sephadex' G-25 and analysed for total carbohydrate (•—•) and aminosugars (▲—▲). The three regions marked represent low molecular weight material (L), the core region (M) and the high molecular weight region (H).
The elution profile from the mutant CSS1-LPS shows very little high molecular weight material (Fig. 13). A small amount of material which reacted with phenol sulphuric acid was detected from the 'H' fractions. No detectable amount of glucosamine, rhamnose and mannose were found in the 'H' fractions. Like in the 'M' fractions of PA01-LPS both glucose and glucosamine were found to be present but rhamnose and mannose were not detected.

As it was indicated earlier that glucosamine, rhamnose and mannose were the main acting sugars in the PIK receptors in LPS (71) we hereby conclude that these sugars, constituting phage PIK receptor complex lies in the higher molecular weight fractions of PA01-LPS i.e. in the side-chains of PA01-LPS. We also suggest that there might exists some kind of correlation between antibiotic supersensitivity (carbenicillin in our case) and bacteriophage resistance and this supersensitivity of CSS1 mutant towards carbenicillin might be due to absence of higher molecular weight fractions in CSS1-LPS. Koval and Meadow have reported similar kind of carbenicillin supersensitivity in mutants of P. aeruginosa PAC 1 (73).

From the above results it appears that the polysaccharide component of LPS contains receptors for phage PIK. Phage PIK inactivation was studied with the isolated polysaccharide component from the wild type PA01 cells (Table 4). The data clearly indicates insignificant inactivation. It can be
Fig. 13: Fractionation of polysaccharide obtained from the carbenicillin supersensitive phage PIK resistant mutant (P. aeruginosa CSS 1) after partial degradation with 1% acetic acid. The material was eluted from a column of 'Sephadex' G-25 and analysed for total carbohydrate (•--•) and aminosugars (▲—▲). The three regions marked represent low molecular weight material (L), the core region (M) and the high molecular weight region (H).
inferred from the above results that the isolated polysaccharide contains the receptor site, but without the lipid component it may lack the proper spatial requirements to render the receptor amenable to phage binding. Alternatively, the PIK receptor may in fact be contained in both the polysaccharide and the lipid component and hence isolated polysaccharide or isolated lipid would not be able to inactivate the phage PIK. Lipid A is known to form part of the receptor site for $\phi 5$ in *E. coli* K-12 (74). A third possibility is that phage PIK, which is known to possess a structure similar to the T-even phages (Chapter IV, Fig. 17), except that this phage does not contain tail fibres, may require specific binding sites for several components. The polysaccharide isolated here is known to be partially degraded and so may not contain, in fact, all of the sites required.

Table 4. Phage PIK inactivation by isolated polysaccharide component from *P. aeruginosa* PA01.

<table>
<thead>
<tr>
<th>Polysaccharide concentration (µg/ml)</th>
<th>Phage input (pfu/ml)</th>
<th>Phage remaining (pfu/ml)</th>
<th>Phage inactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>$5 \times 10^3$</td>
<td>$5.10 \times 10^3$</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>$5 \times 10^3$</td>
<td>$4.90 \times 10^3$</td>
<td>2.0</td>
</tr>
<tr>
<td>50</td>
<td>$5 \times 10^3$</td>
<td>$5.00 \times 10^3$</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>$5 \times 10^3$</td>
<td>$4.85 \times 10^3$</td>
<td>3.0</td>
</tr>
<tr>
<td>200</td>
<td>$5 \times 10^3$</td>
<td>$4.71 \times 10^3$</td>
<td>5.8</td>
</tr>
<tr>
<td>500</td>
<td>$5 \times 10^3$</td>
<td>$5.00 \times 10^3$</td>
<td>0</td>
</tr>
</tbody>
</table>
In order to decipher the above possibilities for the involvement of lipids in the phage PIK receptor complex a few experiments were carried out. Adsorption of phage PIK was studied with respect to polymyxin B and avidin. Polymyxin B acts to disorganise the LPS layer of the cell wall, probably, by intercalating with the negatively charged phospholipids in the membrane (75). Polymyxin B was reported to cause inactivation of receptors for phage T3, T4, and T7 of E. coli (15) and kappa phage of Serratia marcescens (76).

P. aeruginosa PA01 when treated with various concentrations of polymyxin B, loses its phage PIK adsorption efficiency. Polymyxin B (100 μg/ml) causes nearly 90% inhibition of phage PIK adsorption efficiency in sensitive culture (PA01) while only 30% inhibition was observed in case of resistant culture (PBR1) (Table 5).

To confirm these results effect of polymyxin B on the isolated LPS was studied. LPS layer which causes nearly 90% inactivation of phage PIK in vitro as indicated by the control, loses this inactivation to 50% when treated with polymyxin B (27.5 μg/ml) (Fig. 14). These results indicate that polymyxin B has a direct effect on phage PIK receptor sites. Thus inactivation might be due to a complex formation between polymyxin B and LPS layer thereby mimicking the phage receptors as suggested by Tsang et al. (77).
Fig. 14: Dose response of polymyxin B for the adsorption efficiency of phage PIK to *P. aeruginosa* LPS.
Table 5. Effect of polymyxin B on phage PIK adsorption on *P. aeruginosa* PA01 and its variant PBR1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Polymyxin B concentration (µg/ml)</th>
<th>Number of free phage after adsorption ($x10^7$ pfu/ml)</th>
<th>Absorption efficiency of phage* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> PA01</td>
<td>0</td>
<td>0.83</td>
<td>91.70</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.03</td>
<td>79.70</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.19</td>
<td>78.10</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3.92</td>
<td>60.80</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.08</td>
<td>39.20</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>6.62</td>
<td>33.80</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8.83</td>
<td>11.70</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PBR1</td>
<td>0</td>
<td>0.59</td>
<td>90.10</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.87</td>
<td>81.30</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.69</td>
<td>73.10</td>
</tr>
</tbody>
</table>

* Phage PIK was added at 2.5 multiplicity of infection.
Lipids also play a major role in the phage receptor sites (74-75; 78). An indirect approach was made in order to find out whether lipids play any role in the receptor complex for phage PIK. The host bacteria was grown in normal media together with the supplementation of avidin. Avidin is known to cause biotin deficiency in the cells leading to the inhibition of lipid synthesis (79) and also reduction in the cell wall lipids (80,81). Biotin deficiency in avidin grown culture was confirmed by microbiological assay. Nearly 50% reduction in total lipid synthesis was observed in avidin grown culture in comparison to control (Table 6). This effect was reversed by the addition of biotin, to the avidin supplemented culture resulting to the original lipid.

<table>
<thead>
<tr>
<th>Condition of growth*</th>
<th>Number of free phage after adsorption (x 10^6 pfu/ml)</th>
<th>Adsorption efficiency (%)</th>
<th>Total lipids (mg/g dry wt. of cells**)</th>
<th>Total lipids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>03.80</td>
<td>92.40</td>
<td>20</td>
<td>1.07</td>
</tr>
<tr>
<td>Avidin</td>
<td>19.60</td>
<td>60.80</td>
<td>08</td>
<td>0.75</td>
</tr>
<tr>
<td>Avidin + Biotin</td>
<td>06.14</td>
<td>87.72</td>
<td>18</td>
<td>1.62</td>
</tr>
<tr>
<td>Biotin</td>
<td>09.34</td>
<td>81.32</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Cells were grown under experimental conditions for 24 hours at 37°C and were used for adsorption experiments.

** Lipids extraction was carried out according to the method described by Goldfine and Bloch (82).

ND = Not determined.
synthesis. Similarly adsorption efficiency of phage PIK was also decreased in avidin grown culture from 92.40% to 60.80% (83). Phage PIK adsorption was almost restored to normal with the addition of biotin to the avidin supplemented culture. This suggests the possibility of involvement of lipids in phage PIK receptors on LPS layer.

In summary we conclude that polymyxin B causes inactivation of phage PIK receptors on the LPS. We also suggest the possible involvement of lipids in the phage PIK receptor sites. However, what type of lipids plays the major role for phage PIK adsorption in the LPS layer was not investigated.
References

   61, 371.


