To the Graduate Council:

I am submitting herewith a thesis written by Mathew Scott Greenberg entitled "The Identification and Partial Purification of the Tailspike Protein of the *Salmonella newington* Bacteriophage, ε^{34}." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Robert Villafane

Dr. Robert Villafane, Major Professor

We have read this thesis
and recommend its acceptance:

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Wayne L. Davis

Accepted for the Council:

Lew Minkel

Associate Vice Chancellor
and Dean of The Graduate School

THE IDENTIFICATION AND PARTIAL PURIFICATION OF THE TAILSPIKE PROTEIN OF THE *SALMONELLA newington* BACTERIOPHAGE, ε^{34}

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Mathew Scott Greenberg

May 1995

DEDICATION

This thesis is dedicated to my parents David and Marian, and my two brothers Daniel and Mitchell. They have been extremely supportive and understanding throughout this project. Their encouragement was my light at the end of the tunnel.

Out of the dark confinement! out from behind the screen! It is useless to protest, I know all and expose it.

Walt Whitman

"Song of the Open Road"

ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Villafane, for his support and help. I also would like to express my gratitude to Dr. Peterson and Dr. Savage for serving on my committee. To all of these professors I offer my gratitude.

I would like to express my thanks to Stephanie Botsford, Tim Rose, Tereza Marks, and Leann Lovin. All of your support and understanding helped me complete this project.

To everyone thank you for being helpful and patient.

Peace.

Mathew

ABSTRACT

The *Salmonella newington* bacteriophage, ε^{34}, must interact with the lipopolysaccharide (LPS) molecule of the host in order to start the infection process and was used in the investigations presented. An LPS-binding protein was identified and partially purified in these studies through standard biochemical techniques including salt precipitation and column chromatography. The protein identified was the tailspike protein of the ε^{34} virus. It was characterized by determining its stability in SDS and its ability to interfere with the *in vitro* assembly of a related virus. This study strongly suggests a close structural relationship between the ε^{34} tailspike protein and the tailspike of a related phage and that the protein identified will be suitable for future investigations into LPS-protein interactions. The purpose of this work was to identify and purify the tailspike protein (TSP) of ε^{34} so it could be utilized in comparative studies with the P22 TSP.

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ABBREVIATIONS

2-ME	2 mercaptoethanol
°C	degrees centigrade
APS	ammonium persulfate
AS	ammonium sulfate
bis	N,N'-methylen-bis-acrylamide
BSA	bovine serum albumin
ddH_2O	deionised, distilled water
DEAE	diethylaminoethyl
EDTA	ethylenediaminetetracetic acid
EtOH	ethanol
FPLC	fast protein liquid chromatography

HAc	acetic acid
kBp	kilo base pairs
kDa	kilo daltons
LB	Luria-Bertani
LPS	lipopolysaccharide
MeOH	methanol
MOI	multiplicity of infection
MWCO	molecular weight cut off
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PFU	plaque forming unit
ρ	density

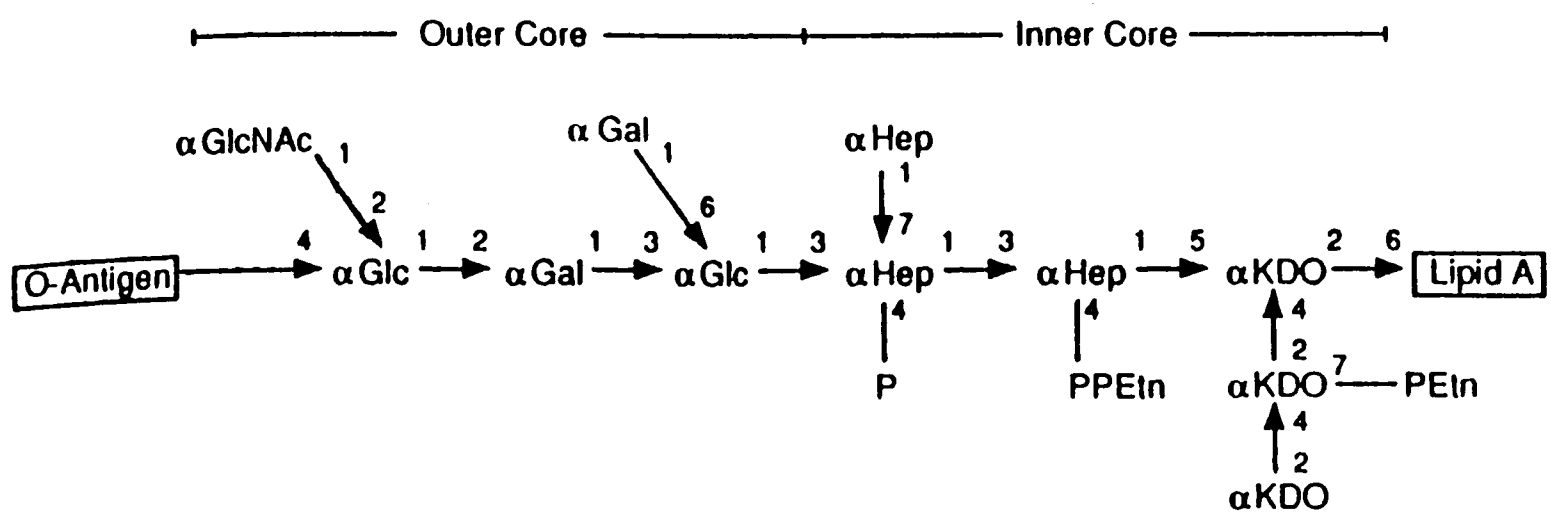
SDS	sodium dodecyl sulfate
TIA	P22 tailing interference assay
TIR	tailing interference ratio
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
TSP	tailspike protein

1. INTRODUCTION

The temperate bacteriophages ε^{34}, ε^{15}, and P22 can modify host cell LPS. This process has been termed phage antigenic conversion (Robbins and Uchida, 1962). Phage antigenic conversion involves the lysogenization of the host cell by a temperate virus capable of directing structural alterations in the LPS (Rick, 1987). Lysogenization is a process in which the viral DNA inserts itself into the host cell's chromosome (Birge, 1981; Susskind and Botstein, 1978). Alterations in the O-antigen portion of the LPS molecule by the phage proteins allow these temperate bacteriophages to exert control over the cell's own normal LPS production. Figure 1 depicts the structure of LPS of *Salmonella* species.

Components of the viral particle must interact with the host receptor sites in order for a productive infection cycle to begin (Kanegasaki and Wright, 1973). To gain insight into the interaction between viral particles and their host receptors, the adsorption protein of the *Salmonella* phage ε^{34} which interacts with the LPS of *S. newington* has been partially purified.
LPS has been shown to be a complex molecule that can be divided into two regions: the lipid A region and the

Figure 1. **Structure of the LPS Molecule from *Salmonella*.** The major regions of the LPS molecule are shown: O-Antigen, Outer and Inner Core, and Lipid A. Abbreviations: Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; Hep, heptose; KDO, 2-keto-3-deoxyoctanate; P, phosphate; PEtn, phosphoethanolamine; PPEtn, pyrophosphoethanolamine. (Adapted from: Raetz, C.R.H. 1990. Biochemistry of Endotoxins. Annu. Rev. Biochem. **59**:133.)


Outer Core
Inner Core
αGlcNAc
αGal
αHep
O-Antigen
αGlc
αGal
αGlc
αHep
αHep
αKDO
Lipid A
P
PPEtn
αKDO
PEtn
αKDO

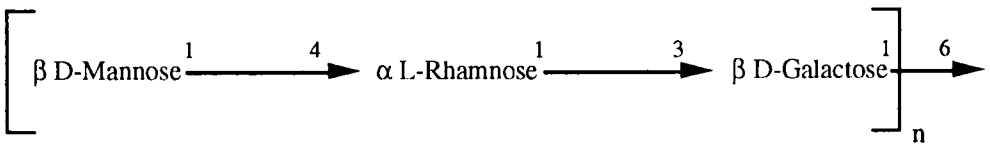
polysaccharide region (See Figures 1 and 2). The lipid A serves as an anchor into the outer membrane for the entire LPS molecule. The polysaccharide portion has been further divided into two regions: a core polysaccharide region, that serves to link the lipid A to the O-antigen, and the peripheral O-antigen region.

The O-antigen constitutes the immunodominant portion of the molecule, and the structural determinants of the O-antigens provide the basis for the serological classification of the *Enterobacteriaceae* (Rick, 1987). Groups A, B, D, E1, and E2 of *Salmonella* contain a common mannose-rhamnose-galactose repeating unit with varied linkage positions, configurations, and side chains in their O-antigens (Iwashita and Kanegasaki, 1975). *S. newington* belongs to group E2. *Salmonella anatum* is the host for ε^{15} and belongs to group E1. *Salmonella typhimurium* is the host for P22 and belongs to group B (Kanegasaki and Wright, 1973; Rick, 1987; Wright, 1971). Figure 2 shows a single trisaccharide repeating unit of the O-antigen for each of these bacteria.

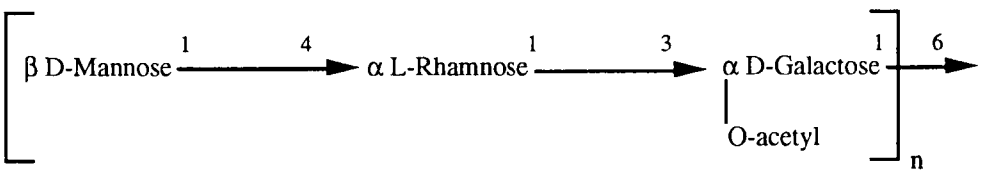
The tailspikes or tail fibers of bacterial viruses interact with bacterial cell surface molecules (receptors) in a process defined as adsorption (Casjens et al, 1992; Haggard-Ljundgguist et al, 1992; Montag et al, 1989). The entire adsorption process for the ε^{34} virus can be defined in three discrete and separate processes. First, there is

Figure 2. **Trisaccharide Repeats of Several *Salmonella* Species.** ε^{34} TSP is an endogalactosidase and it cleaves the bond between the galactose and mannose of *S. newington*. ε^{15} and P22 are endorhamnosidases and they cleave the bond between the rhamnose and galactose of *S. anatum* and *S. typhimurium*, respectively. (Adapted from: Rick, P.D. 1987. In Lipopolysaccharide Biosynthesis in *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, vol.I, ed. F.C. Neidhart, *et al.*: American Society for Microbiology pp. 648-662.)

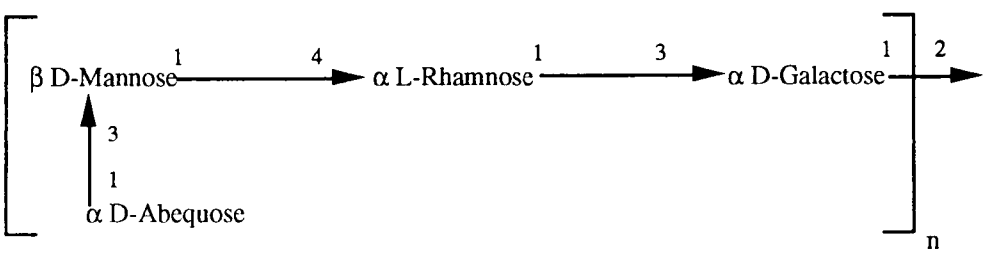
Salmonella newington



Salmonella anatum



Salmonella typhimurium



the initial interaction of the phage with a host surface component; for the *Salmonella* system, this has been shown to be the O-antigen of the LPS molecule. Second, there is enzymatic hydrolysis of the O-antigen by a phage component. It has been shown that the tailspike protein of ε^{34}, ε^{15} , and P22 have the ability to hydrolyze the O-antigen, which serve as their receptors (Eriksson et al, 1976; Iwashita and Kanegasaki, 1973, 1975; McConnell et al, 1979). Third, the injection of DNA into the host cell leads to a productive infection.

The bacteriophages ε^{34}, ε^{15}, and P22 are known to be similar in size and appearance. They are composed of a polyhedral head containing DNA, a short neck, a small base plate, and several identical spikes or tailspikes arranged symmetrically around the base plate (Chaby and Girard, 1980; Iwashita and Kanegasaki, 1973).

The P22 phage TSP has been intensively studied (Casjens et al, 1992; Danner et al, 1993; Goldenberg and King, 1982; King et al, 1990; Poteete, 1988; Prassad et al, 1993; Susskind and Botstein, 1978). It serves as a model system for the study of protein folding (Chen and King, 1991; Danner et al, 1993; Goldenberg and King, 1982; King et al, 1990; Villafane and King, 1988; Villafane et al, 1994a). The P22 TSP consists of a single protein species. It has been shown that each tailspike is a

homotrimer (Goldenberg and King, 1982; Sauer et al, 1982; Seckler et al, 1989). Each monomer of the trimer is a 72 kDa (666 amino acid residue) polypeptide chain. The P22 TSP has been purified, cloned, and sequenced (Berget and Poteete, 1980; Sauer et al, 1982; Schwarz and Berget, 1989). Some unusual characteristics of the TSP include resistance to denaturation by SDS, heat, and proteases (King, 1986). The TSP can bind to the head structure of P22 in vitro (Israel et al, 1966). The TSP of bacteriophage ε^{15} has been purified, and it exists as a polypeptide chain of 125 kDa (McConnell et al, 1979).

The ε^{34} virus and its TSP are closely related to the P22 virus and its TSP. The phage particles are similar in size, appearance, as well as other physical properties (Villafane et al, 1994b). Previous studies have shown that protein extracts containing the TSP of ε^{34} could form morphological hybrids with radioactive labeled P22 head structures as measured by the accumulation of P22 counts on the surface of A1ε^{15} cells (Iwashita and Kanegasaki, 1975). The hybrid phage could recognize neither the LPS of *S. typhimurium* host for the P22 phage) nor the LPS of *S. anatum* (host for the ε^{15} phage).

Recently, the three-dimensional structure of the P22 TSP has been determined by x-ray crystallography (Steinmacher et al, 1994). The P22 TSP is only the third member of a new structural motif class, the β-helix (Yoder

et al, 1993). Data reported in this thesis indicate a relationship between the ε^{34} and P22 TSPs. The three-dimensional structure of the P22 TSP may shed light on the structure of the ε^{34} TSP.

The purpose of this work is to identify and purify the TSP of ε^{34} so it can be utilized in comparative studies with P22.

2. MATERIALS and METHODS

Bacterial Strains and Phages.

S. newington, strain A1ε^{15}, and the phage ε^{34} are from our laboratory stock collection. *S. typhimurium* strain TP275 (leuAam414, supE) and the P22 virus, $5^{-}13^{-}$ (amber mutations in the coat and delayed lysis genes, respectively) were obtained from Dr. Tony Poteete.

Biochemical Solutions and Techniques.

Media and solutions.

Chemicals were reagent grade and purchased from Sigma unless otherwise noted.

LB broth contains 10 gm of Bacto Tryptone (Baxter), 5 gm of Bacto Yeast Extract (Baxter), 5 gm of NaCl, and 1 ml of 1.0 N NaOH per liter of ddH_2O and sterilized by autoclaving (25 min at 15 psi and 121°C).

LB agar plates were made from the components of LB broth plus 15 gm of Bacto Agar (Baxter) per liter of ddH_2O and sterilized by autoclaving.

LB top agar consists of 6 gm of Bacto Agar per liter of ddH_2O plus the components of LB broth, and sterilized

by autoclaving.

Phage Buffer consists of 50 mM Tris (pH 7.4), 100 mM $MgCl_2$, 10 mM NaCl and sterilized by autoclaving.

Protein Buffer contains 5 mM Tris (pH 7.5), 0.1 mM $MgSO_4$, 0.1% azide and sterilized by autoclaving (McConnell et al, 1979).

30% acrylamide/0.8% bis contains 30 gm of acrylamide (Bio-Rad Laboratories) and 0.8 gm of bis (Bio-Rad) in 100 ml of ddH_2O.

4XTris/SDS (pH 8.8) contains 91 gm Tris, 2 gm SDS and volume brought to 500 ml with ddH_2O.

4XTris/SDS (pH 6.8) contains 6.05 gm Tris, 0.4 gm SDS and volume brought to 100 ml with ddH_2O.

5XSDS/Running buffer contains 15.1 gm Tris, 72 gm glycine, 5 gm SDS and volume brought to 1 liter with ddH_2O.

2XSDS/Sample buffer contains 25 ml 4XTris/SDS (pH 6.8), 20 ml glycerol, 4 gm SDS, 2 ml 2-ME, 1 mg Bromphenol Blue and volume brought to 100 ml with ddH_2O.

Coomassie Blue Stain contains 50% MeOH, 10% HAc, 40% ddH_2O, and 0.05% Coomassie Brilliant Blue.

Destain solution contains 30% MeOH, 10% HAc, and 60% ddH_2O.

B buffer contains 50 mM Tris (pH 7.4), 2 mM EDTA, 3 mM 2-ME, 10% v/v glycerol, a subscript number indicates the numerical value for the salt concentration, in mM, and

was filter sterilized (Berget and Poteete, 1980).

FPLC Solution contains 150 mM NaCl, 20 mM Tris (pH 7.4), and 0.1 mM EDTA. The solution was degassed and filter sterilized.

Bacterial Growth Conditions.

S. newington was allowed to grow in LB broth in a NBS shaking water bath at 30°C to a concentration of 2 x 10^8 cells per ml (growth was monitored by using a Klett colorimeter). Bacterial cultures were maintained on LB agar plates and stored at 4°C.

Preparation of a Phage Stock.

Phage stock was made by diluting an overnight culture of *S. newington* cells, 1:100 in 30 ml of LB in a 125 ml sterile flask. A phage plug was added to the flask and the infected cells were incubated with shaking at 30°C until lysis (about 4 hours). To complete cell lysis, 0.5 ml of chloroform was added to the flask containing the infected culture and the culture was allowed to shake for 5 additional minutes. To pellet the bacterial debris, the lysed cells were centrifuged in a SS34 rotor for 10 min at 5000 revolutions per minute (RPM) and 4°C.

The resulting supernatant solution was brought to a final concentration of 0.5 M NaCl and 6% PEG and allowed to stir slowly overnight and maintained at 4°C. The

PEG/NaCl-phage complex was obtained after centrifugation in a GSA rotor for 20 min at 6000 RPM and 4°C as the pellet. The pellet was resuspended in 10 ml of Phage Buffer and purified by using a CsCl gradient. The CsCl gradient was formed by placing 2 ml of 1.7ρ CsCl at the bottom of a 13 ml polyallomer centrifuge tube (Beckman, for SW41Ti rotor), then carefully layering, (one on top of the other), 3 ml of 1.5ρ CsCl, 3 ml of 1.4ρ CsCl, 1 ml of 1.3ρ CsCl, and 3 ml of the phage sample was placed on top. The gradient was then centrifuged in a SW41Ti rotor for 1.5 hours at 28,000 RPM and 20°C. The resulting viral band, which appears blue, was carefully removed and placed in dialysis tubing of 12,000-14,000 molecular weight cut off (MWCO) value (Baxter) and dialyzed overnight against Phage Buffer.

Determination of the Concentration of a Phage Stock (Titering).

Titering must be performed in order to determine the concentration of the phage stock. The phage were titered on host cells (plating bacteria). These were prepared by taking an overnight culture of *S. newington* and diluting 1:100 in fresh LB. The new culture was allowed to grow for three hours. The cells were then centrifuged in a SS34 rotor for 20 min at 6,000 RPM and 4°C. The cell pellet was resuspended with LB in 1:10 of the original

volume. One drop of plating bacteria from a 1 ml pipette and 0.1 ml from a 0.2 ml pipette of a phage dilution (standard serial dilutions performed in Phage Buffer) were added to a tube of top agar. This was quickly mixed, poured on the surface of a LB agar plate and allowed to solidify. The plate was inverted and incubated at 30°C for 16-18 hours.

Plaques, regions of cleared zones in bacterial lawns, were counted. The titer was determined by multiplying the number of plaques obtained by the dilution factor plated then dividing by volume plated. Typically the following dilutions were used for titering: 10^{-8} through 10^{-13}, with a resulting phage titer of 10^{12} plaque forming units (PFU) from 600 ml of infected cells.

Protein Extraction.

An overnight culture of *S. newington* cells was diluted 1:50 in 250 ml of LB at 30°C in a 1 liter side arm Erlenmeyer flask. The cells were allowed to grow with shaking to a concentration of 2 x 108 cells per ml in a NBS water bath at 30°C. An additional 250 ml of LB were added and the culture was allowed to shake for 5 min, after which $5x10^{11}$ PFU of ε^{34} phage were added (multiplicity of infection of 10). Approximately 80 to 90 min after infection, the cells were cooled at 4°C for 10 min and

then harvested by centrifugation in a SS34 rotor for 10 min at 7,000 RPM and 4°C. The cells were resuspended in 10 ml of cold Protein Buffer. Cells were lysed by sonication for 6 periods of 30 seconds each with a 1 min rest between each period. The sonicated cells were centrifuged in a SS34 rotor for 15 min at 7,000 RPM and 4°C to remove any cellular debris. To remove any fully assembled viral particles, the supernatant was further centrifuged in a SW41Ti rotor for 2 hours at 22,000 RPM and 4°C. The supernatant solution which contained the soluble cellular and viral proteins (protein extract) was stored at -80°C until used and was the protein source for all further studies (McConnell et al, 1979).

Preparation of LPS.

An overnight culture of *S. newington* cells was diluted 1:50 in a 2 liter Erlenmeyer flask containing 1 liter of LB. The culture was incubated with shaking at 30°C until a concentration of 2 x 10^8 cells per ml was reached. The cells were harvested by centrifugation in a GSA rotor for 10 min at 7,000 RPM and 4°C. The cells were resuspended in 30 ml of acetone and placed in a 60 ml lyophilization flask. The flask was placed in a ethanol/dry ice bath until the acetone cell mixture was frozen. The frozen mixture was lyophilized overnight.

The resulting bacterial mass, typically one gram per liter of culture, was ground up, using a spatula, to a fine powder and resuspended to a concentration of 5% w/v in $_{dd}H_2O$ and heated to 67°C in a water bath. An equal volume of 90% w/w phenol was heated to 67°C and added to the bacterial suspension. This was stirred by hand for 15 min while at 67°C. The mixture was subsequently distributed into 30 ml glass tubes (Corex) and allowed to sit at 4°C for 1 hour to induce phase separation. Complete phase separation was obtained by centrifugation in a SS34 rotor for 15 min at 8,000 RPM and 4°C.

The aqueous (upper) phase was carefully removed since it contained the majority of LPS. This was transferred to 3,500 MWCO dialysis tubing (Baxter) and dialyzed against running distilled water for at least 18 hours. The solution was centrifuged in a SS34 rotor for 15 min at 11,000 RPM and 4°C to remove any insoluble material. The solution was concentrated by rotary evaporation to 1/5 of its original volume and then centrifuged in a SW41Ti rotor for 3 hours at 24,000 RPM and 4°C. The resulting clear, gelatinous pellet was resuspended in 2 ml of $_{dd}H_2O$ and lyophilized. The resulting LPS was ready for use after resuspension with water (Hancock and Poxton, 1988).

Silver Staining for LPS.

After electrophoresis with a 14% PAGE gel, the gel

was placed in of 30% EtOH, 10% HAc and allowed to shake overnight. All future steps include shaking. The solution was replaced by 30% EtOH and allowed to rinse for an additional 30 minutes. This step was repeated twice. The gel was washed three times with $_{dd}H_2O$ for 10 min each wash, then oxidized with 60 mM periodic acid for 10 minutes. The gel was washed three times with $_{dd}H_2O$ for 10 min each wash. 0.1% silver nitrate (diluted from a 20% stock) was added to the gel and shaken for 30 minutes. The silver nitrate solution was removed by washing the gel for 5 min with $_{dd}H_2O$. A 100 ml solution containing 2.5% sodium carbonate and 0.02% formaldehyde was added in order to develop the gel. The reaction was stopped by washing the gel with 1% HAc. The gel was further washed with $_{dd}H_2O$ for 10 min (Harlow and Lane, 1988). The gel was transferred to 3mm Whatman paper (Whatman International Ltd.) and dried in a gel dryer (Hoefer Scientific Instruments) for 45 min at 80°C.

LPS Binding Experiments.

In order to determine whether any viral proteins in the protein extract had specific affinity for purified *S. newington* LPS, affinity binding experiments were performed. This was accomplished by taking 200 µl of protein extract and adding them to a 1 ml polyallomer

centrifuge tube (Beckman). Purified LPS was added to the protein extract to a final concentration of 1 mg/ml or 0.1 mg/ml (25 µl of a 8 mg/ml or 10 µl of a 2 mg/ml respectively). The mixture was incubated for 15 min at 4°C and centrifuged in a TLA100.2 rotor for 2 hours at 43,000 RPM and 4°C. The supernatant solution was collected and brought up to a total volume of 300 µl with ddH_2O. The pellet was resuspended in 200 µl of ddH_2O. The tube was washed with 100 µl of ddH_2O and this wash was added to the sample containing the resuspended pellet. Small volumes (35 µl) of the supernatant solution and pellet were then analyzed by SDS/PAGE and Coomassie Blue staining.

Protein Determination.

The Bradford method was used to quantify the protein content of extracts and to monitor protein levels during the purification steps of the ε^{34} TSP (Bradford, 1976). One hundred microliters of protein sample were mixed with 1 ml of the Bradford dye concentrate (Bio-Rad) in a microfuge tube and then vortexed. The solution was allowed to stand at room temperature for 5 minutes. The absorbance was measured at 595 nm in a 1 cm path length 1 ml cuvette. A standard curve was generated by using BSA as the protein standard.

Protein Analysis.

Vertical discontinuous gel electrophoresis was performed by the Laemmli method to analyze protein samples (Laemmli, 1970). Routinely polyacrylamide gels with dimensions of 12 cm x 14.5 cm x 1 mm were utilized. In order to make a 10% separating gel, 5 ml of 30% acrylamide/0.8% bis, 3.75 ml of 4xTris/SDS pH 8.8, and 6.25 ml $_{dd}H_2O$ were mixed and degassed in a 25 ml vacuum flask for 15 minutes. In order to make a 14% separating gel, 7 ml of 30% acrylamide/0.8% bis, 4.25 ml 4xTris/SDS pH 8.8, and 3.75 ml $_{dd}H_2O$ were mixed and degassed in a 25 ml vacuum flask for 15 minutes. Then 50 µl of 10% APS and 10 µl of TEMED (Bio-Rad) were added. The appropriate solution was applied between glass plates with a Pasteur pipette. A layer of $_{dd}H_2O$-saturated butanol was applied on top of the separating gel to promote a smooth boundary between the stacking and separating gel. The separating gel matrix was allowed to polymerize for 1 hour at room temperature.

To make a stacking gel, a mixture containing 0.65 ml of 30% acrylamide/0.8% bis, 1.3 ml 4xTris/SDS pH 6.8, 3.05 ml $_{dd}H_2O$ were mixed in a 25 ml vacuum flask and degassed for 15 minutes. Twenty-five microliters of 10% APS and 5 µl of TEMED were added. The butanol solution was washed

off with 1M Tris pH 6.8, and the stacking gel was applied by Pasteur pipette. The 15 well comb was placed between the two glass plates, and the gel was allowed to polymerize for 1 hour at room temperature. Afterwards, the wells were washed with 1XSDS/Running buffer. Protein samples were mixed with an equal volume of 2XSDS/Sample buffer, heated at 100°C for 10 min, and loaded onto the gel with a 100 µl Hamilton syringe (Hamilton Company). The samples were then electrophoresed at a constant voltage for a total of 600 VH (45 volts for 13 hours).

Visualization of the proteins was accomplished by staining the gel in Coomassie Blue Stain for 1 hour and destaining in Destain Solution for 2.5 hours with a change of Destain Solution at 1.25 hours. The gel was dried as described above.

Purification of the *S. newington* phage ε^{34} TSP.

A protein extract was made as described above. The protein extract was brought to a final concentration of 50% ammonium sulfate (AS) by slow stirring overnight at 4°C. The mixture was centrifuged in a SS34 rotor for 40 min at 12,000 RPM and 4°C. The resulting pellet was resuspended in 50 mM Tris (pH 7.4) and dialyzed overnight against the same buffer. Any fully assembled phage particles remaining in the protein extract were removed by

ultracentrifugation in a SW41Ti rotor for 1 hour at 22,000 RPM and 4°C. This step was repeated. The pH of the resulting supernatant solution was lowered to 4.5 (by the addition of 2N HCl by drops) and stirred for 1 hour at 4°C. The solution was brought to 35% AS by stirring slowly overnight at 4°C. The solution was centrifuged in a SS34 rotor for 40 min at 12,000 RPM and 4°C. The resulting pellet was resuspended in 50 mM Tris (pH 7.4) and dialyzed against the same buffer overnight. The above 3 steps were repeated. Then the mixture was centrifuged in a SS34 rotor for 40 min at 12,000 RPM and 4°C.

The resulting supernatant solution was loaded on a DEAE cellulose column. DEAE cellulose was hydrated per manufacturers instructions, 80 ml of the hydrated cellulose were utilized to make a column (30 x 1.76 cm, bed volume equals 50 ml). The supernatant solution was placed on the column and 5 ml fractions were collected (pump and fraction collector from Pharmacia Biotech Inc.). Proteins were fractionated by elution with a salt gradient (200 ml gradient from B25 to B200). Fractions were assayed for ε^{34} TSP by SDS/PAGE analysis and the use of a P22 tailing interference assay.

Appropriate fractions were pooled and concentrated by a Centriprep-30 Concentrator (Amicon Inc.). The retentate was dialyzed overnight against FPLC Solution. The retentate was further concentrated by ultrafiltration in a

Centricon-30 Concentrator (Amicon) to a final volume of 500 µl. This sample was injected into a FPLC device (Pharmacia) and passed through a size exclusion column, Superose 12 (Pharmacia) and 1 ml fractions were collected. Fractions were analyzed by SDS/PAGE for size determination.

P22 Tailing Interference Assay (TIA).

ε^{34} TSP was monitored by assaying protein extracts or column fractions in a TIA. TIA took advantage of the fact that both the *in vitro* assembly of P22 heads and P22 TSP, and binding of P22 heads and ε^{34} TSP had been previously shown (Israel, et al, 1967; Iwashita and Kanegasaki, 1975). Since the binding of ε^{34} TSP to purified P22 heads would be expected to occur at the same site as the P22 TSP binds; thus the prior binding of ε^{34} TSP to P22 heads should interfere with the binding of P22 TSP to P22 heads. It was possible to develop an assay in which the presence of an interfering agent (ε^{34} TSP) could be detected.

The TIA assay was initiated by incubating 180 µl of protein sample or a DEAE column fraction with 10 µl of P22 heads (10^{-2} dilution) for 30 min at 30°C. Ten microliters of a 10^{-3} dilution of P22 TSP ($5^{-}13^{-}$, amber mutations in the coat and delayed lysis genes, respectively) were added to the TIA reaction and incubation continued for another

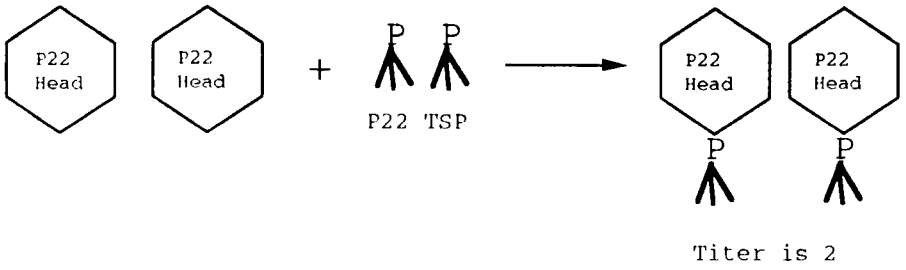
30 min at 30°C. The total volume was 200 µl.

Serial dilutions of the reaction mixture were made, and then 50 µl of that mixture and the dilutions were plated on TP275 cells (*S. typhimurium* host for the P22 5^{-} 13^{-} virus). After the top agar had solidified the plates were inverted and incubated overnight at 30°C.

Plaques were then counted and the tailing interference ratio (TIR) was calculated for each sample. This was accomplished by taking the titer for the sample and dividing it by the titer of the control reaction (P22 heads plus P22 TSP). The TIR was used to determine whether the sample had the ability to block the *in vitro* assembly of P22 heads with P22 TSP. Figure 3 shows a schematic of the TIA. Panel A shows the P22 heads plus P22 TSP (control reaction). Panel B shows the interference of the P22 tailing reaction by the ε^{34} TSP.

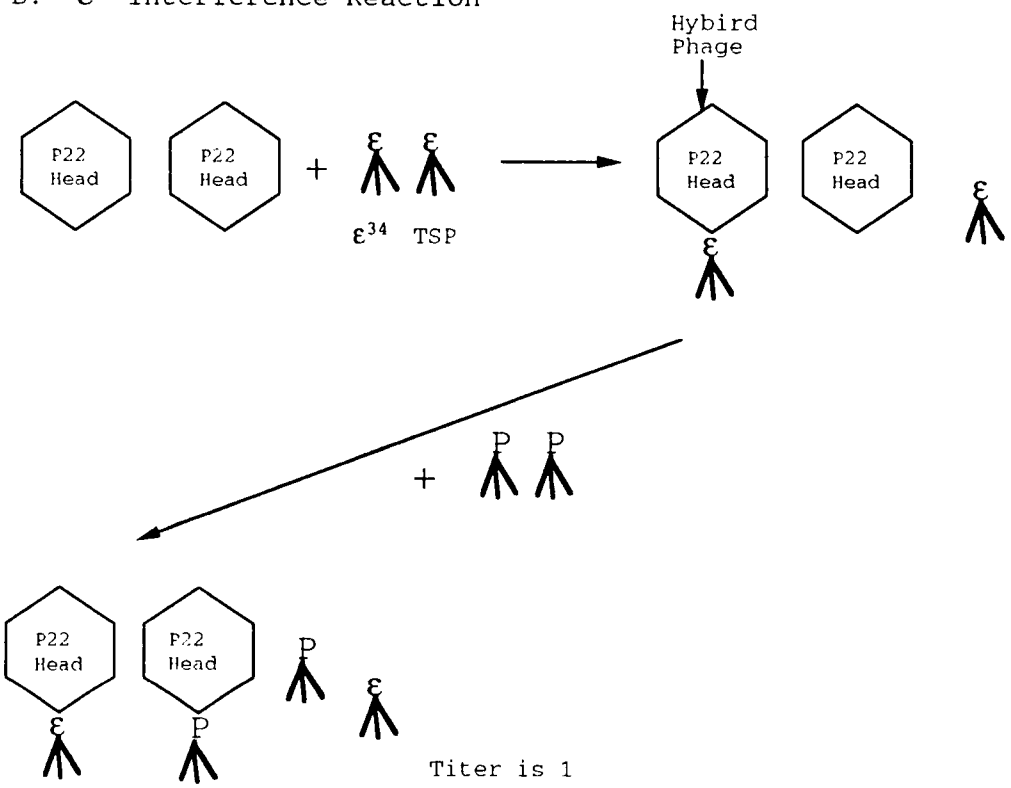
Figure 3. **P22 Tailing Interference Assay.** Panel A: P22 heads bind with P22 TSPs. The resulting titer is 2. Panel B: Interference of the P22 tailing reaction by the ε^{34} TSP. The resulting titer is 1. The resulting tailing interference ratio is 0.5.

A. P22 Tailing Reaction



Titer is 2

B. ε^{34} Interference Reaction



Titer is 1

Tailing Interference Ratio is 1/2 = 0.5

3. RESULTS

Physical Characterization of the ε^{34} Phage Particle.

The ε^{34} virus has been studied by electron microscopy to reveal its size and composition for comparison to the P22 virus (Figure 4A, Villafane, et al, 1994b). The phage particles located in the center of the figure are completely assembled virus particles. The dimensions (width x length) of the head (62 x 61 nm), and TSP (5 x 10 nm) of ε^{34} have been previously determined and they have compared favorably with the P22 phage particle dimensions (Villafane et al, 1994b).

The protein composition of the ε^{34} virus was determined by SDS and heat denaturation of a CsCl concentrated phage stock followed by PAGE separation of the phage proteins. As shown in Figure 4B, the ε^{34} viral particle contains at least six proteins which are termed P1 through P6 (Villafane et al, 1994b). The largest protein, located near the top of the gel, having the slowest electrophoretic mobility was named P1. The smallest protein, located near the bottom of the gel, having the fastest electrophoretic mobility was named P6.

The molecular weights of the ε^{34} viral particle

Figure 4. **Structural Proteins of Bacteriophage ε^{34}.** Panel A: Electron micrograph, magnification is x280,000 (J. Dunlap, UTK Electron Microscope Facility). TSP can be seen on the phage particle on the right. Panel B: SDS/PAGE of ε^{34} structural proteins. Lane 1 contains High Molecular Weight Standards (Sigma) from the top to the bottom of the gel; 205 kDa, Myosin (rabbit muscle); 116 kDa, β-galactosidase (*Escherichia coli*); 97.4 kDa, Phosphorylase b (rabbit muscle); 66 kDa Albumen (bovine); 45 kDa, Albumen (egg); 29 kDa, Carbonic anhydrase (bovine erythrocyte); Lane 2 contains 40 µl of denatured ε^{34} phage proteins. They have been termed P1 through P6 from the top to bottom of the gel. (Source: Villafane, R., D. Tremblay, J. Dunlap, and M. Greenberg. 1994. Characterization of the *Salmonella newington* ε^{34} Phage System. Submitted J. Struct. Biol.).

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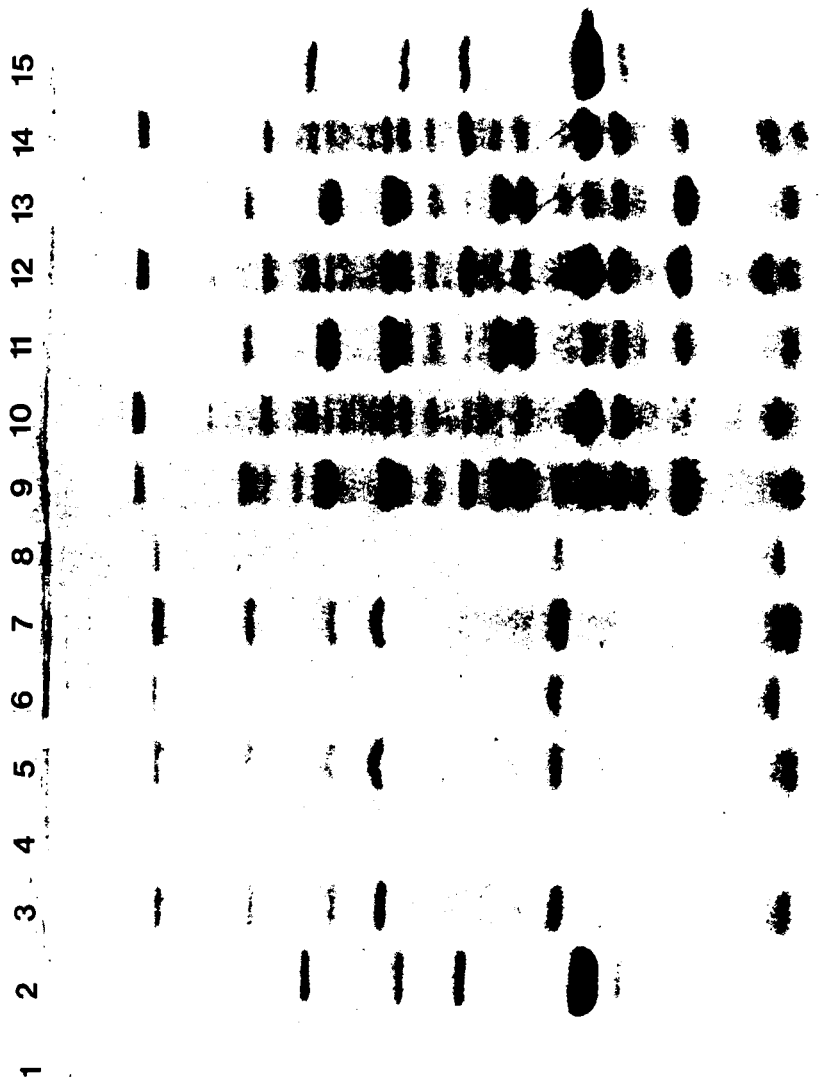
proteins have been estimated by comparison to a standard curve generated with proteins of known molecular weight. The resulting molecular weights were as follows: P1, 87 kDa; P2, 72 kDa; P3, 60 kDa; P4, 52 kDa; P5, 50 kDa; and P6, 47 kDa. Proteins P4 and P6 were present in very small amounts and were located above and below the major protein band (P5) in Lane 2 of Figure 4B.

P3 of ε^{34} Binds LPS.

Since the TSPs of P22 and ε^{15} bind to LPS of their hosts, it was expected that the tailspike of ε^{34} would also bind to the LPS of its host. If a viral protein was shown to bind LPS, it could be tentatively identified as the TSP of ε^{34}. LPS binding experiments were performed in order to determine whether any structural protein of ε^{34} from an ε^{34} infected protein extract could preferentially bind to purified LPS of *S. newington* (McConnell et al, 1979). LPS was purified from *S. newington* by the aqueous phenol method (Hancock and Poxton, 1988). This LPS was utilized in LPS affinity experiments.

The results of an affinity binding experiment are shown in Figure 5. Denatured ε^{34} phage particles (Lanes 2, 15) were used as a guide in detecting viral bands in the other lanes. The gel revealed that there are protein

Figure 5. **LPS Affinity Experiment.** Supernatant solutions and pellets were from the LPS affinity reactions. Lane 1 contains 50 µl of purified LPS (1 mg/ml). Lanes 2 and 15 contain 20 µl of denatured ε^{34} phage particles. Lanes 3-14: odd numbered lanes contain supernatant solutions and even numbered lanes contain the pellets. Lanes 3-8 contain uninfected A1ε^{15} protein extracts. Lanes 9-14 contain ε^{34} infected A1ε^{15} protein extracts. LPS was added to a final concentration of 1 mg/ml (Lanes 5, 6, 11, 12) or to a final concentration of 0.1 mg/ml (Lanes 7, 8, 13, 14). 50 µl of supernatant solution or pellets were loaded onto the gel (Lanes 3-14).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bands migrating with the same mobility as the viral protein standard bands in the lanes containing extracts made from ε^{34} infected cells (compare Lane 2 with 3, 4, 9, 10).

When LPS was added to extracts made from ε^{34} infected cells (Lanes 11-14), the P3 of ε^{34} bound to the LPS and was removed from the protein extract after sedimentation (compare Lanes 9 and 10 with 11, 12 and 13, 14). In addition LPS added to the protein extracts nonspecifically bound or trapped cellular and viral proteins (compare Lane 6 with 12, 14). The data from Figure 5 suggests that the ε^{34} structural protein, P3, is an LPS binding protein. The TSP of P22 is the only LPS binding protein produced by the phage (Iwashita and Kanegasaki, 1973; Poteete, 1988; Susskind and Botstein, 1978). Therefore; the ε^{34} P3 protein might be the ε^{34} LPS binding protein and TSP. The presence of P3 was used as a purification criteria.

Purification of the TSP of ε^{34}.

A classical purification approach (Figure 6) was undertaken (Takeda, 1994). The concentration of protein at each step of the procedure is shown in Table 1.

An aliquot of protein from the purification scheme was electrophoresed on a 10% SDS/PAGE gel in order to

Figure 6. **Steps in the Purification Protocol of ε^{34} TSP.**

1. A1ε^{15} cells are infected with ε^{34}

2a. Harvest cells

2b. Resuspend cells in 5 mM Tris (pH7.4), 0.1 mM $MgSO_4$, 0.1% azide

3. Sonicate the cells

4. Spin 7K, 15 min, 4°C

4a. Supernatant go to step 5

4b. Pellet repeat steps 2b through 4a

5. Spin 22K, 2 hours, 4°C

5a. Supernatant go to step 6

5b. Pellet not used

6. Make 50% AS stir slowly overnight at 4°C

6a. Spin 12K, 40 min, 4°C

6b. Supernatant not used

6c. Pellet resuspended in and dialyzed overnight against 50 mM Tris (pH 7.4)

7. Spin 22K, 1 hour, 4°C (do two times)

7a. Supernatant go to step 8

7b. Pellet not used

8. Make pH 4.5 by drop addition of 2N HCl

8a. Spin 12K, 20 min, 4°C

8b. Supernatant go to step 9

8c. Pellet not used

9. Make 35% AS stir slowly overnight at 4°C

9a. Spin 12K, 40 min, 4°C

9b. Supernatant not used

9c. Pellet resuspended in and dialyzed overnight against 50 mM Tris (pH 7.4)

10. Repeat step 9, 9a and supernatant goes to step 11

11. Load onto a DEAE cellulose column and elute with B_{25} to B_{200}

12. Collect fractions

13. Concentrate by ultrafiltration

13a. Filtrate not used

13b. Retentate go to step 14

14. Dialyze overnight against 20 mM Tris, 150 mM NaCl, 0.1 mM EDTA (pH 7.4)

15. Repeat step 13 and retentate goes to step 16

16. Load onto a FPLC / Superose 12 H/R column and collect fractions

Figure 6 (continued)

Table 1: **Protein Concentration of the Various Steps Involved in the Purification of the TSP of ε^{34}.**

Protein Sample	Volume (ml)	Concentration (mg/ml)	Total Protein (mg)
Lysed Cells	79	0.427	33.7
22K Spin	78	0.182	14.2
50% AS Supernatant	116	0.900	104
50% AS Pellet	35	0.706	24.7
22K Spin x 2	34	0.473	16.1
Post pH 4.5	33	0.480	15.8
35% AS Supernatant	45	n.d.	n.d.
35% AS Pellet	21	0.210	4.41
Filtrate	40	n.d.	n.d.
Retentate*	2.7	0.099	0.267

* determined by UV absorbance and extinction coefficient of 1 was used; all others determined by Bradford method.
n.d. not able to determine

determine if the P3 protein was present in each of the purification steps. The results of this gel indicated that most of purification steps were not effective in separating cellular proteins from viral proteins as seen in Figure 7 (compare Lanes 2 and 3 with Lanes 4 through 11). The purification step that was most effective in removing cellular proteins (compare Lanes 2 and 4 with Lanes 12 and 13) was the DEAE column. Lanes illustrative of the DEAE column are 12, 13; these have been combined and concentrated by ultrafiltration (Lane 12 the filtrate and Lane 13 the retentate). The use of the DEAE column resulted in a protein sample containing 4 different protein species (Figure 7, Lane 13) compared to over 20 species in the original extract (Figure 7, Lane 4).

The ammonium sulfate treated extract was eluted from a DEAE column in two steps: a gradient from 25-200 mM NaCl and a second gradient from 200-500 mM NaCl. Aliquots from every fourth fraction from the DEAE column over the NaCl concentration of 25 mM to 500 mM, were electrophoresed and are shown in Figure 8. The elution profile of 25 mM to 200 mM and of 200 mM to 500 mM are located in Figure 8A and 8B respectively. The ε^{34} P3 protein started to elute from the column in fraction number 24 (Figure 8A Lane 7). Lane 8 of Figure 8A contained the first cellular protein.

The conductivity was then measured for each of the

Figure 7. **Protein Purification Steps.** Lanes 1, 15 are molecular weight markers (See Figure 4). Lanes 2, 14 contain 20 µl of denatured ε^{34} phage proteins. Lane 3 contains uninfected A1ε^{15} extract. Lane 4 contains ε^{34} infected A1ε^{15} extract after sonication and a 7K spin. Lane 5 contains the infected extract after a 2 hour 22K spin. Lane 6 contains the supernatant solution and lane 7 contains the pellet from the 50% AS treatment. Lane 8 contains the infected extract after it has undergone two 1 hour 22K spins. Lane 9 is the resulting extract after low pH treatment. Lane 10, 11 contain the supernatant solution and pellet, respectively from 35% AS treatment. Lanes 12 and 13 contain pooled fractions from the DEAE column and concentrated by ultrafiltration with Lane 12 containing the filtrate, and Lane 13 containing the retentate.

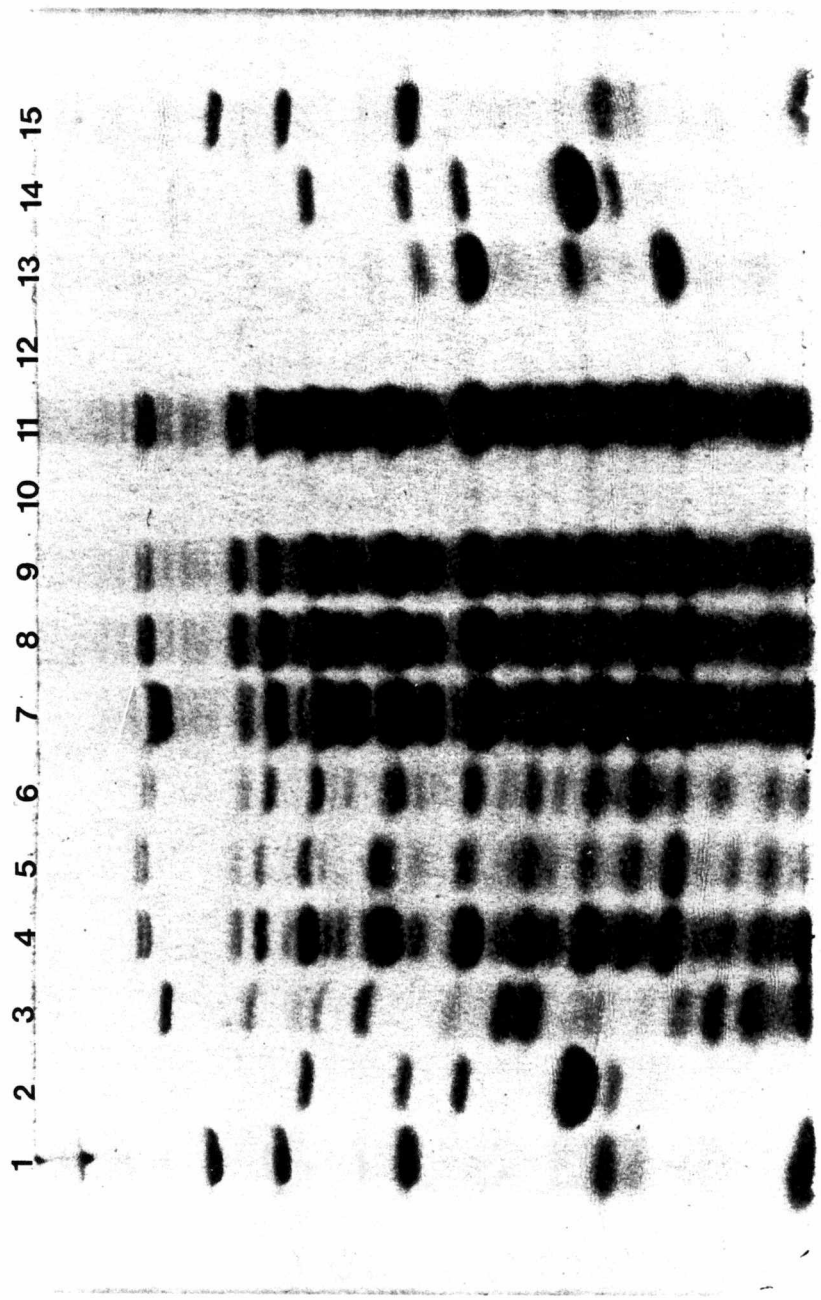
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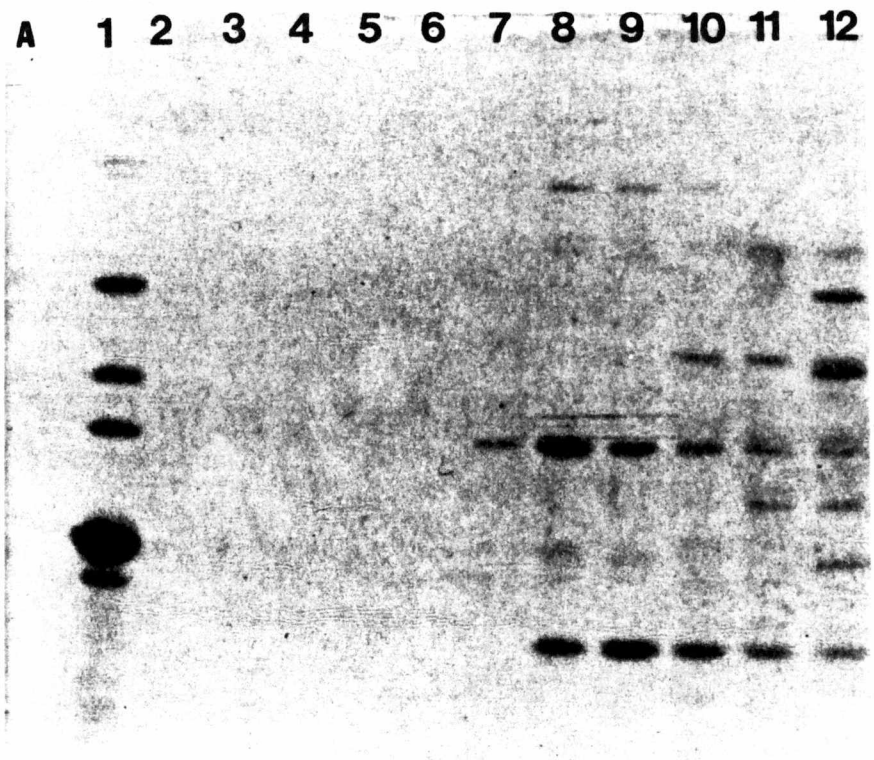
Figure 8. **DEAE Column Fractions.** Panel A: Lane 1 contains 20 µl of denatured ε^{34} phage proteins. Lanes 2-12 contain 50 µl of the fractions from the DEAE column; starting with fraction #4 in lane 2 and ending with fraction #44 in lane 12 (every fourth fraction). Panel B: Lanes 1-8 contain 50 µl of the fractions from the DEAE column; starting with fraction #48 in lane 2 and ending with fraction #76 in lane #8. Lane 9 contains 20 µl of denatured ε^{34} phage proteins.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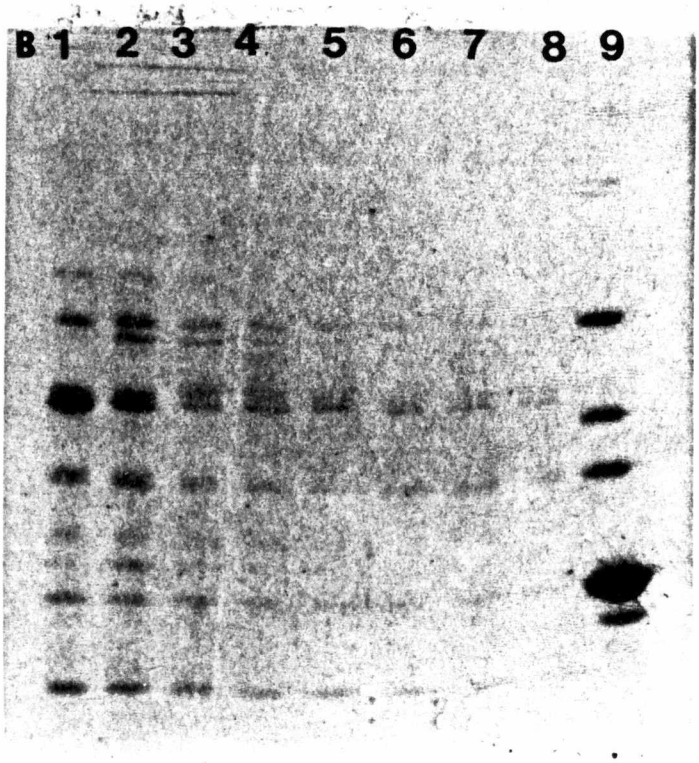
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fractions represented on the PAGE gel (Figure 8) and the NaCl concentration computed from a standard curve. The resulting NaCl concentration from lanes 7 and 8 (Figure 8A) was 76 mM and 88 mM respectively. Figure 8 shows where other viral protein begin to elute from the column. The P1 and P5 proteins of ε^{34} virion (Figure 8A, Lane 12) eluted at 131 mM NaCl, while the P2 protein of ε^{34} (Figure 8B, Lane 1) eluted at 141 mM NaCl.

At this point, the total amount of protein present was 250 µg (absorbance data; Stoscheck, 1990). Because the 250 µg of protein was not enough material to use in FPLC gel filtration, it was necessary to process frozen infected extracts from other experiments in order to purify more of the TSP. Since the DEAE column gave the best separation, it was utilized in conjunction with a 35% AS precipitation on this second protein extract.

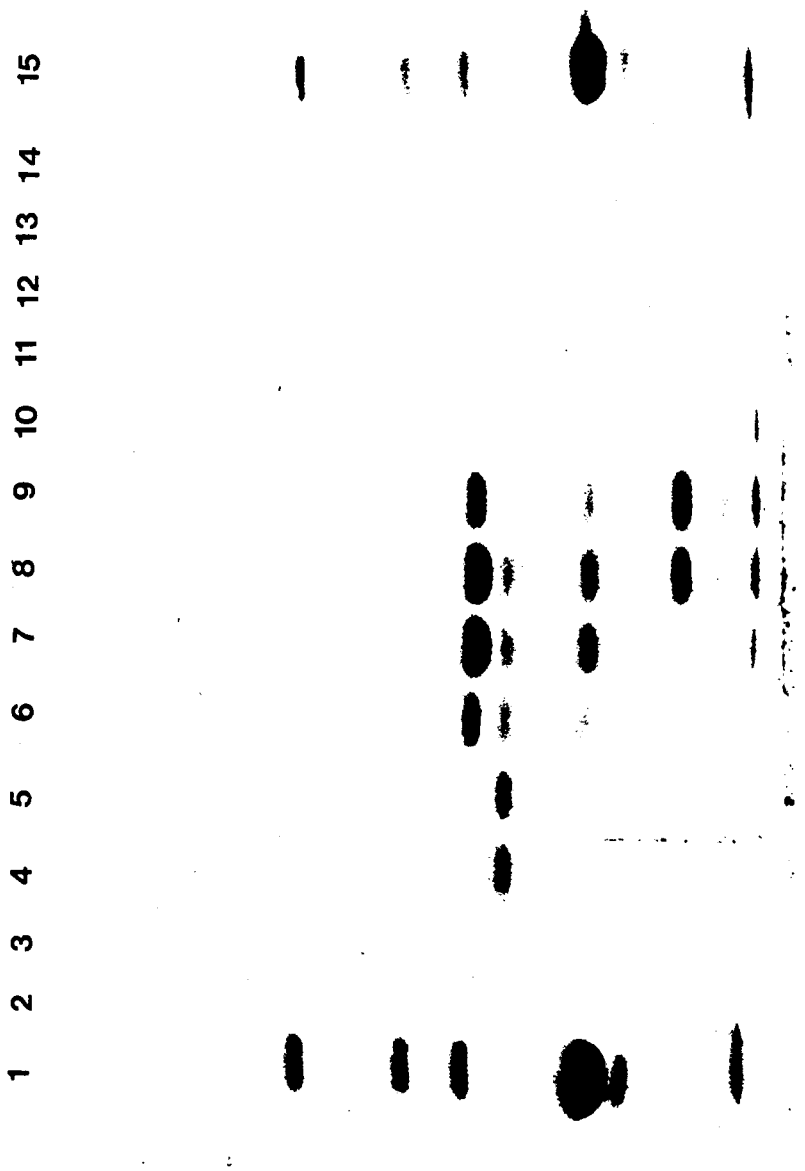
The resulting pellet was collected and dialyzed against 50 mM Tris (pH 7.4). This was passed through the DEAE column, eluted with the same salt gradient. The results of these steps on the purification of the TSP were similar to those shown in Figure 7 and 8. Data from this second DEAE column are not shown. DEAE fractions were pooled, combined with the first protein sample, and dialyzed against the FPLC buffer for concentration by ultrafiltration. The final protein concentration at this

point was determined by absorbance to be 550 μg. The next purification step was a FPLC gel filtration column.

A gel filtration column (Superose 12, Pharmacia) was used to separate the protein sample based on size. The standards (Pharmacia), albumin (67 kDa), aldolase (158 kDa), and catalase (232 kDa), were utilized to develop a standard elution profile so that the molecular weight of the proteins in the protein sample could be determined. The protein sample was resolved into two dominant peaks by FPLC gel filtration. The peak of interest had a retention time between that of the standards aldolase and catalase and an estimated molecular weight of 177 kDa.

Fractions from the FPLC were then analyzed by SDS/PAGE. The results are shown in Figure 9. Lane 6 contained a protein that has a similar mobility as the P3 of denatured ε^{34} particles while a protein located in Lane 7 has a mobility similar to the P5 of denatured ε^{34} particles. A contaminating host protein located in lane 8 migrates faster than the P5 of ε^{34}. An additional protein species of unknown origin was present in the fractions. It first appeared in lane 4 of Figure 9 and has a mobility faster than that of the P3 of ε^{34}.

Figure 9. **FPLC/Gel Filtration Column Fractions.** Lanes 1, 15 contain 20 µl of denatured ε^{34} phage proteins. Lanes 2-14 contain 50 µl of the fractions from the column: 5, 7, 8, 10, 11, 12, 13, 14, 16, 18, 21, 25, and 35 respectively.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P22 Tailing Interference Assay.

This assay was developed in order to monitor ε^{34} TSP in different protein extracts or DEAE column fractions. If the sample being tested contains a protein that has the ability to interfere with the tailing reaction of P22 heads and P22 TSP, then a lowering in the resulting titer can be expected (see Materials and Methods, Figure 3). The results of the assay are shown in Table 2. The tailing interference ratio (TIR) was calculated by dividing the resulting titer of the sample by the titer of the P22 control (P22 heads plus P22 TSP). If the resulting value was greater than 1, then no interference would be postulated. If the value was less than 1 then interference would be postulated. From this table it can be inferred that a protein that interferes with the P22 tailing reaction can be detected in the steps of the protein purification scheme as well as those DEAE fractions tested. Although the data presented in Table 2 is suggestive of the ability of ε^{34} P3 protein to interfere with the P22 tailing reaction, this interference study will have to be repeated.

Table 2: **P22 Tailing Interference Data.**

Protein Sample	Titer ($\times 10^4$)	Tailing Interference Ratio
P22 TSP Control	3.1	1
A1ε^{15} Uninfected Lysed Cells	13	4.2†
A1ε^{15} Infected w/ ε^{34} Lysed Cells	2.2	0.7
22K Spin	0.4	0.1
50% AS Supernatant	*	*
50% AS Pellet	0.1	0.01
22K Spin x 2	1.7	0.6
Post pH 4.5	*	*
35% AS Supernatant	*	*
35% AS Pellet	0.2	0.1
Fractions from DEAE Column		
4	3.2	1.0
8	3	1.0
12	3.5	1.1
16	3.7	1.2
20	3.2	1.0
24	2.4	0.8
28	1.8	0.6

Table 2 (continued)

Protein Sample	Titer ($\times 10^4$)	Tailing Interference Ratio
Fractions from DEAE Column		
32	2.6	0.9
36	2.9	0.9
40	2.8	0.9
44	3	1.0

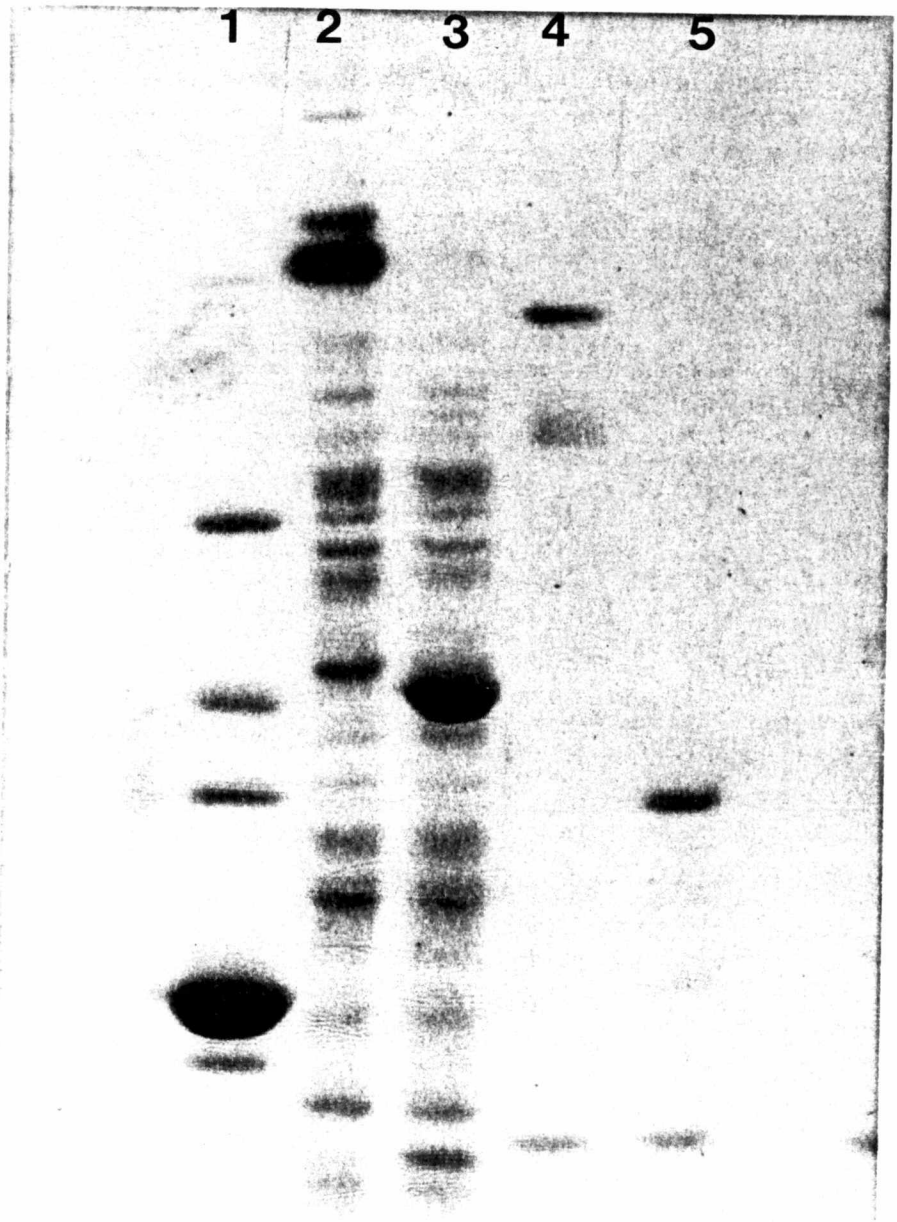
* Lack of production of phage plaques under these condition could be due to the effect of high salt or low pH.

† The value for this sample is expected to be 1, however the larger value may be due to the addition of a solution containing a large number of proteins which may cause macromolecular crowding to occur (Zimmerman and Pheiffer, 1983).

The ε^{34} Viral Band, P3, is Analogous to the P22 TSP.

The P22 TSP exists as a trimer (210 kDa) that is resistant to SDS denaturation in the absence of heat (Goldenberg and King, 1982; Seckler et al, 1989). To determine whether the ε^{34} structural protein, P3, shares this characteristic of the P22 TSP, its resistance to SDS in the presence or absence of heat was studied. The results of incubating the P22 TSP or the ε^{34} P3 with SDS in the presence or absence of heat (100°C, 10 min) are shown in Figure 10. Lane 2 shows that the P22 TSP exists as a stable trimer (most abundant protein band). Lane 3 shows that the P22 TSP migrated as a monomer (72 kDa), the same molecular weight as the P2 of the denatured ε^{34} particle. Lane 4 shows that the ε^{34} P3 protein retained the ability to form a multimer (top band) by comparing this to the trimer of P22. Lane 5 shows that when the ε^{34} P3 protein was heated in the presence of SDS it migrated with the P3 of the denatured ε^{34} particle. Thus, the P3 protein exhibited similar migration patterns as the P22 TSP when treated with SDS in the presence or absence of heat.

Figure 10. **Resistance of ε^{34} TSP to SDS.** Lane 1 contains 20 µl of ε^{34} phage proteins. Lanes 2 and 3 contain 50 µl of P22 TSP. Lanes 4 and 5 contain 50 µl of fraction #28 from the DEAE column. The samples in lanes 2 and 4 were held at room temperature for 10 min; while the samples in lanes 3 and 5 were heated at 100°C for 10 min.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4. DISCUSSION

Structural Proteins of ε^{34}.

Comparative studies of phage repressor proteins have enabled investigators to determine which amino acids are critical for biological function. Therfore, comparative studies with the structural proteins of the related phage, P22, could suggest the identification of several of the structural proteins of ε^{34}. The largest structural protein of P22 is the portal protein. The function of this protein has been shown; it allows the passage of the viral DNA into the host bacterium during infection; it also functions intracellularly in the assembly of the virion (Susskind and Botstein, 1978). Since it is a multifunctional protein, it is not surprising that the portal protein could be so large. The largest protein of the ε^{34} phage particle, P1 may fill a similar role in that bacteriophage.

Phage capsids are normally composed of one or a few proteins. Therefore, these proteins must be produced in a large quantity compared to other viral proteins. The head structure encapsulates the DNA of the virus. The ε^{34} viral protein, P5, could therefore be a candidate for the capsid protein. In other viral systems, the most abundant

protein has been shown to be the coat protein. Amino acid sequencing of the N-terminal of the ε^{34} P5 protein has shown striking similarities to the coat protein of P22 (Murphy and Villafane, unpublished observations).

The results, reported in this thesis, have identified the ε^{34} viral structural protein, P3 as the ε^{34} TSP. This has been shown by 1) LPS binding study, 2) P22 tailing interference assay, and 3) shared characteristics of the purified P22 TSP with the partially purified ε^{34} TSP. P2, P4, and P6 have not been studied nor identified.

Protein Identification.

The P3 protein of ε^{34} was identified as a LPS binding protein. Therefore, in order to compare the P22 and ε^{34} TSPs, an ε^{34} purified TSP is necessary. The P22 TSP is the only LPS binding protein produced by the phage (Iwashita and Kanegasaki, 1973; Poteete, 1988; Susskind and Botstein, 1978). Thus, if a LPS binding protein is identified in the ε^{34} system it is possible that this protein could also serve as the TSP. A LPS binding experiment is utilized to determine whether a LPS binding protein is present in ε^{34} infected protein samples. This approach was successful in identifying the TSP of the related ε^{15} phage (McConnell et al, 1979).

A LPS binding protein is detected by adding purified LPS from *S. newington* to a protein extract from ε^{34} infected cells The results show that the P3 protein of ε^{34} does bind to the LPS (Figure 5, Lanes 12, 14). Aggregation and centrifugation leads to accumulation of proteins in the pellet (Figure 5, Lane 10) and has prevented this experiment from being further explored. Experimental conditions such as buffer, centrifugation time, and speeds can be modified in attempts to optimize this experiment.

Purification Procedure.

As seen in Figure 6 the protocol for the purification of the ε^{34} TSP involves many steps. However the overall goal of each step is twofold: to concentrate the protein extract and to remove unwanted cellular and viral proteins from the extract. The results of this purification scheme are shown in figures 7, 8, and 9.

Protein samples from the purification steps (Figure 6) are analyzed by SDS/PAGE (Figure 7). The purification steps leading up to the utilization of the DEAE column are only effective in concentrating the protein but not effective at removing any of the unwanted cellular or viral proteins from the extract (Figure 7 lanes 4-11). The

purification step that is effective in removing cellular or viral proteins is the utilization of the DEAE column. This purification step has removed all but four of the proteins from the extract and thus is the single most effective step in the purification process (Figure 7 Lanes 12,13).

Comparison of the lane 13 of Figure 7 suggests the origin of these protein bands. The largest and smallest proteins are probably from the host. Both of these proteins seem to have been induced by the infection of the host cell. Perhaps these host cell proteins are *Salmonella* phage shock proteins, Psp (Brissette et al, 1990). The second largest protein has the same migration as the P3 protein of ε^{34} which we have identified as the ε^{34} TSP. The third largest protein has the same migration as the P5 of ε^{34} and based on quantity would most likely be the coat protein of ε^{34}.

The protein sample from the DEAE column is concentrated by ultrafiltration and separated by FPLC gel filtration (Figure 9). This separation is not effective. Prior to FPLC analysis the protein sample consists of four protein species (Figure 9 lane 13). After FPLC again the sample consists of four protein species. Inspection of Figure 9 indicates that protein species before and after FPLC are not identical. The FPLC step eliminates a

protein that has a molecular weight greater than the TSP of ε^{34} (Figure 7 lane 13). However, a new protein appears whose molecular weight is slightly smaller than the TSP of ε^{34} (Figure 9 lanes 4-9). This additional new band could have resulted from: 1) proteolytic cleavage of the ε^{34} TSP, 2) contaminating protein in the sample prior to injection on the FPLC device, or 3) elution of a contaminating protein from the column.

There is precedence for a functional truncated P22 trimer (Chen and King, 1991). By removing the first 100 amino acids at the N-terminus of the P22 TSP with proteases, a truncated trimer is produced. This truncated trimer migrates slightly faster in SDS/PAGE then the full length trimer. The truncated P22 TSP folded properly and formed a SDS resistant trimer (Danner et al, 1993).

If a small amount of protease was present in the protein sample, ε^{34} TSP could have been truncated. However, if a protease is present then a smaller truncated multimer should be expected to elute later from a size exclusion column. Also a contaminating protease is unlikely since there is no other evidence to suggest degradation of the other proteins present.

If there is a contaminating protein prior to FPLC injection the source of this protein can not be determined at this time. It is also possible that the protein can be

located in the column apparatus or column material prior to loading of the protein sample. However, this is unlikely because the column is washed with the FPLC Buffer before use, and high monomeric molecular weight standards are injected into the column before the protein sample.

Also the fractions chosen to be applied to the FPLC gel filtration column need to be more carefully picked. Since choosing a fraction that contains a large amount of the targeted protein does not ensure that it is also the only protein present.

Characterization of Structural Protein P3 of ε^{34}.

To determine which of the ε^{34} viral structural protein was the TSP, two studies were initiated in this study. One takes advantage of the known property of the P22 TSP to bind to P22 head structures lacking tails (Berget and Poteete, 1980; Israel et al, 1967). The other study compares the characteristics of the TSP of P22 with ε^{34}.

The results of the P22 TIA show that when ε^{34} P3 protein is detected in a protein extract or a column fraction, the amount of P22 tails binding to P22 heads is reduced (Table 2, Figure 3, Figure 7, and Figure 8). Thus a correlation exists between the appearance of the P3 protein (Figure 8, Lane 7) and a reduced TIR (Table 2).

The P22 TSP has several unique properties: resistance to denaturation by heat, resistance to denaturation by SDS, and resistance to proteases. The ε^{34} phage particle was thermostable as indicated by the ability of the virion to form plaques after incubation at high temperature (Villafane et al, 1994b). Since the ability of the virus particle to form plaques was dependent on the TSP, it therefore must also be stable.

The native P22 trimeric TSP is stable in the presence of SDS if unheated. If the P22 TSP is heated in the presence of SDS prior to electrophoresis, the TSP will migrate as a monomer on a SDS/PAGE gel. To determine whether ε^{34} TSP shares with P22 TSP this unusual stability in SDS, ε^{34} P3 protein was incubated with SDS and then heated or held at room temperature (Figure 10). The data indicate that the two proteins share this property. Both proteins migrate in the high molecular weight range when incubated with SDS but not heated. Under these conditions the P22 TSP has been shown to be a trimer with a molecular weight of 210 kDa. Under similar conditions the ε^{34} P3 protein appears to migrate as a multimer with a predicated molecular weight of 180 kDa (FPLC gel filtration predicated 177 kDa). Thus its functional form may be a trimer.

The TSP of P22 and ε^{15} bind to LPS (Iwashita and

Kanegasaki, 1973; McConnell et al, 1979; Poteete, 1988; Susskind and Botstein, 1978). The P3 protein of ε^{34} has been shown to bind LPS (Figure 5). The above properties strongly suggest that the P3 protein of ε^{34} is the TSP of this virus.

Future Studies.

Purification.

Several avenues can be pursued in the purification of the TSP of ε^{34}. With the preparation currently available, separation of the four protein species may be achieved by isoelectric focusing or FPLC ion exchange.

Since the protein concentration of the ε^{34} TSP is small in the protein extract, larger samples of protein extracts from infected cells should be prepared. Once that is achieved, the material can be concentrated by ammonium sulfate precipitation. The resulting material can be passed through a DEAE column. As shown in Figure 7 lane 7, P3 starts eluting at a concentration of 76 mM NaCl. At this concentration of salt, the only protein present is P3 ε^{34}. Therefore, only this fraction would be further concentrated; however, the bulk of the TSP is located in fractions that elutes at higher salt

concentrations. Since the bulk of the protein is impure it must be processed further. For example, hydroxylapatite chromatography, different FPLC matrices, or isoelectric focusing could be utilized.

Genetics and Structure-Function Studies.

Once the ε^{34} TSP is completely purified then several other experiments could be performed: 1) reverse genetics can be used to obtain the TSP gene (Carter et al, 1992); 2) once the gene is obtained it can be cloned into a high expression vector (Tabor and Richardson, 1985); 3) the cloned gene can be mutagenized to determine critical amino acid residues needed for function (Humphreys et al, 1976); 4) high levels of protein can be obtained from the expression vector; 5) attempts will be made to obtain crystals (Steinbacher et al, 1994); 6) should crystals be obtained, the three dimensional structure will be determined by x-ray diffraction methods (Steinbacher et al, 1994).

The research described in this thesis has led to the identification of the ε^{34} TSP. Additional studies have insured that the purification of this TSP will be accomplished in the near future.

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