Towards determination of a high-resolution structure of the bacteriophage PR772

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Abbreviations List

µL = microliter
bp = base pair
Cu = Copper
Da = Dalton
dsDNA = double stranded DNA
E. Coli = Escherichia Coli
kDa = kilo Dalton
L = liter
mL = milliliter
mM = millimolar
NaCl = Natrium Chloride
nm = nanometer
PEG = poly ethylene glycerol
TAE = TRIS Acetate EDTA
V = voltage
XFEL = X-Ray Free-Electron Laser

Abstract

The Virus PR772 infects E. Coli and has an icosahedral shape with an inner lipid membrane. The diameter of the virus is 66 nm and a purification of the virus gave 0.8 mg/mL pure viral particles. The structures of the coat proteins of the virus PR772 are still unknown and it would be interesting to study them. Coat proteins will be expressed in a larger amount for crystallization. X-ray crystallography can, in principle, be used to determine high-resolution structures of the coat proteins, P6 and P30.
1 Introduction

1.1 Tectiviridae family
The bacteriophage PR772 is a member of the Tectiviridae virus family. Tectiviridae infect gram-positive and gram-negative bacteria. When their genomes were sequenced, it was found that they were highly identical, and therefore grouped as a family. They are divided into two subgroups that infect either gram-negative bacteria or gram-positive bacteria. The ones that infect gram-negative bacteria are PRD1, PR5, PR3, PR4, PR772 and L17. The others are Bam35, GIL01 and GIL16. The viruses have mosaic genomes, which means that they have several genotypes within one virus. They have an icosahedral structure and the particle contains a dsDNA. PRD1 is used as a model and it is the most well researched bacteriophage in this family. It has been studied in detail through X-ray crystallography and electron microscopy (Saren et al. 2005). The length of the genome sequences in this family does not vary much. It is 14 942 bp for PR722 and 14 927 bp for PRD1 (Saren et al. 2005). Bacteriophage were found in different parts of the world; PR772 was found in South Africa (Coetzee et al. 1979), PRD1 in Michigan, USA (Olsen et al. 1974), PR3 and PR4 in Melbourne, Australia (Stanisich 1974), PR5 in Edmonton, Canada (Wong & Bryan 1978) and L17 in the river Avalon, England (Bamford et al. 1981).

1.2 Bacteriophage PR772
The virus that we have studied is the coliphage PR772, the structure of which is still unknown. The first step for understanding the infection mechanism of this virus is to determine the structure of the virus particle. By knowing the structure it is possible to draw further conclusions on how the virus interacts with the host cell, how insertion of DNA works and viral reproduction. PR772 is a dsDNA icosahedral bacteriophage that infects gram-negative E. Coli F⁻-strain. The molecular weight of PR772 is 27 x 10⁶ Da (Coetzee et al. 1979). The capsid has a diameter of around 63-82 nm depending on how the virus is oriented, since it has an icosahedral shape. The genome of the virus has the GC-content of 48.3 % (Lute et al. 2004).

1.3 Coat proteins building up the virus capsid
To determine the structure of the bacteriophage PR772, it is crucial to determine the structure of the coat proteins that make up the major part of the viral structure. The capsid of the virus consists of major capsid protein P3, minor capsid proteins P6 and P30 and spike proteins, P2, P5, and P31 (Rydman et al. 2001). P30 works as a cementing molecule (Abrescia et al. 2004) while P6 is involved with DNA packaging in the virus (Karhu et al. 2007). The molecular weight of the coat proteins is: P3: 43 448 Da, P6: 17 573 Da and P30 has a considerably lower molecular weight of 9186 Da. The proteins therefore differ in both weight and the amount of amino acids (Lute et al. 2004). P3 is the main building block of PR772. 80 % of the virus' total gene expression consists of this single protein, so it has a huge importance. It is conceivable that the P3 protein supports the structure of the virus capsid but it is important for capsid assembly and folding of the membrane as well (Benson et al. 1999).
1.4 Comparison with PRD1
The virus PRD1 is the model organism for the Tectiviridae family. PRD1 and PR772 are both closely related with a sequence identity as high as 97.2 % and both infect gram-negative bacteria (Lute et al. 2004). Since PRD1 has a high similarity to PR772 (they are almost built up of the same proteins) it is an optimal starting point for studying PR772. There has been a lot of research about PRD1, but not so much of PR772. They have almost the same length with 14 927 bp for PRD1 and 14 942 bp for PR772 (Saren et al. 2005). PRD1 has a circular dsDNA (Dowd et al. 1998), and PR772 has a linear dsDNA (Lute et al. 2004). PRD1 consists of 16 % of lipid mass (Dowd et al. 1998). They both infect the same host and they share an icosahedral shape without tails (Lute et al. 2004).

1.5 Research aim
The aim with the project is to express and determine the structure of the coat proteins, P6 and P30 of the coliphage PR772.
2 Materials and Methods

2.1 Virus culturing

*E. Coli ATCC 15597* was used as the host organism for culturing the virus. 30 g Tryptic soy broth was added to 1 L of deionized water and then autoclaved at 121°C. 900 µL *E. Coli ATCC 15597* was added to 25 mL media. It was then incubated at 37°C and the OD$_{600}$ were measured with a mass spectrophotometer every 20 minutes. At OD$_{600}$ ~ 0.5, 7 mL soft agar per plate was warmed up in a microwave oven and then put in a 45°C water bath. At OD$_{600}$ = 0.8 - 0.9 the bacterial culture was removed from incubation. 3 mL bacterial culture per plate was mixed with the 7 ml of soft agar. To culture the bacteriophage PR772, the *E. Coli* needs to be infected. 300µL bacteriophage PR772 stock per 10 mL of the soft agar with the bacterial culture was added and then mixed. 7 mL of this mixture was added to each plate and the plates were then sealed with plastic foil and incubated overnight in 37°C.

2.1.2 Viral Enumeration

A Plaque assay was done to measure the concentration of the viral particles. 10 mL of the viral culture was added to a falcon tube. It was serially diluted. The dilutions were added as droplets on to a pre-grown bacterial mat. The plate was then sealed with plastic foil and incubated overnight in 37°C. If the virus was present, clear plaques could be observed.

2.1.3 Purification of virus

The agar plates were taken out of incubation. The soft over layer of the agar plates was scraped into a sterile bottle and filled with TRIS buffer (50 mM TRIS HCl, 100 mM NaCl, 1 mM MgCl$_2$ at pH 8) up to 200 mL. It was then incubated in 4°C for 8 hours on a shaker in the cold room or overnight. The viral particle diffuses from the soft agar in to the TRIS buffer. The mixture was then centrifuged for 30 minutes at 8000 g at 4°C to remove agar and cell debris. The supernatant from the separation was collected and filtered through a 0.8-micron filter.

2.1.4 PEG-precipitation

The filtered supernatant was poured into a volume-measuring cylinder. 5.8 % of NaCl and 9 % PEG was added. The mixture was incubated at 4°C overnight on a magnetic stirrer. The solution was then centrifuged for 1 hour and 30 minutes at 8000 g at 4°C. The viral pellet was dissolved by adding 15-50 mL TRIS buffer. It was incubated in 4°C, for at least 12 hours or overnight on a shaker to dissolve the pellet.
2.1.5 Anion exchange chromatography
The HiTrap Capto Q column (GE healthcare), 5 mL was equilibrated with the TRIS buffer the viral mixture loaded onto the column and the sample eluted with the elution buffer (50 mM TRIS HCl, 1.5 M NaCl, 1 mM MgCl2 at pH 8). The single peak was collected.

2.1.6 Dialysis
To remover excess of salt from the virus, dialysis was performed. The dialysis membrane (MWCO: 12-14 000) was filled with the sample collected from the chromatography. It was sealed on both ends and dialyzed in 2 L TRIS buffer for two days on a magnetic stirrer.

2.1.7 Nano drop
2-5 µL of the sample was used to measure the concentration using OD

2.1.8 SDS-PAGE
1 mL of the sample was concentrated to 20 µL. 6.5 µl of distilled water, 2.5 µl NuPAGE LDS Sample Buffer (4X), 1 µl NuPAGE Reducing Agent (10X) and 5 µl of the concentrated PR722 were added into an Eppendorf tube. It was then warmed in 90°C for 3 minutes or 70°C for 10 minutes. It was run in a 1.5 % agarose gel at 120-150 V. The gel was washed with water and then stained with Lab Safe™ Gel Blue for 1-2 hours. Then the gel was washed with water for a couple of hours.

2.1.9 Electron microscopy
A droplet of the sample, PR772 was placed on a formvar/carbon 400 mesh Cu grid. The excess was soaked up with filter paper and the grid was left to dry. Then 1 % uranyl acetate (negative stain) was added to the grid, the excess soaked up and the grid was left to dry again. The grid was then transferred into the machine (Quanta FEG 650) and imaged.

2.1.10 Nano sight
PR772 was diluted to about 10^8 particles in TRIS buffer and injected into the sample chamber of the machine and analyzed.

2.2 Star Gate Cloning

2.2.1 Digestion and ligation
The genes of the coat proteins were constructed and ordered. Then they were diluted to 2 nM. 3.75 µL 5 ng Entry Vector pENTRY-IBA51, 6 µL 2 nM of the gene, 0.5 µL 12.5 mM ATP, 0.5 mM 12.5 DTT, 0.5 µL 1 U/µL T4-DNA-Ligase, 0.25 µL BamH1 restriction enzyme, 0.25 µL HindIII restriction enzyme and 1.75 µL Tango buffer were mixed. Then it was incubated at 30 °C for 1 hour.

2.2.2 Transformation
Sterile culture tubes (17 mm x 100 mm) were set on ice. E. Cloni® 10G chemically competent cells were taken out from the -80 °C freezer for thawing for 15 minutes. 40 µL E. Cloni® 10G and 5 µL of DNA sample were added to the sterile culture tubes. The mixture was then incubated on ice for 30 minutes. The bacteria were then heat shocked in a 42 °C water bath for 45 seconds. Then they were put on ice for 2 minutes. After that, the tubes were placed in a 37 °C shaking
incubator for 1 hour. The bacteria were then plated on kanamycin X-gal agar plates and incubated overnight at 37 °C.

2.2.3 Overnight culture
Overnight culture was done by taking single colonies into 10-20 mL LB medium with 50 μg/mL kanamycin. It was then incubated on a 37 °C shaker overnight.

2.2.4 Plasmid Mini-preparation
5 mL of the overnight culture were centrifuged at 6800 g for 2 minutes. The supernatant was poured out. 250 μL Resuspension solution was added and the tube was vortexed. Then 250 μL Lysis Solution was added and the tube inverted 4-6 times. After that, 350 μL of neutralization solution was added to the tube and inverted 4-6 times. It was then centrifuged at 12 000 g for 5 minutes. All the other steps with centrifuging is at 12 000 g. The supernatant was then transferred to a Gene JET spin column and centrifuged for 1 minute. 500 μL Wash solution was added and then the tube was centrifuged for 1 minute. This step was repeated and then centrifuged again for 1 minute. The Gene JET spin column was then transferred to an Eppendorf tube and 50 μL Elution Buffer was added and let be for 2 minutes. Then it was centrifuged for 2 minutes.

2.2.5 Restriction Digestion of Plasmid
11 μL sterile H₂O, 2 μL 10X FastDigest® Green buffer, 5 μL DNA from mini-preparation, 1 μL XBAI restriction enzyme and 1 μL HindIII restriction enzyme were mixed and incubated on a 37 °C heat block for 1 hour.

2.2.6 Gel electrophoresis
A 1.5 % agarose gel was used with SYBR® Safe DNA gel. The samples were added to the wells and the gel run at 70 V until the DNA is separated.

2.2.7 Destination vector
The procedure was almost the same as for the Entry vector with digestion, ligation transformation and finally overnight cultured as earlier described. The vectors that were used are pASG-IBA3 and pASG-IBA5. pASG-IBA3 has a strep-tag on the 3’-end and pASG-IBA5 has a strep-tag on the 5’-end.

2.2.8 Digestion and ligation
3.75 μL IBA-3 or IBA-5 Acceptor Vector, 6.25 μL 2 ng/μL Donor Vector from the Entry Vector procedure, 0.5 μL 12.5 mM ATP, 0.5 mM 12.5 DTT, 0.5 μL 1 U/μL T4-DNA-Ligase, 0.5 μL 10U/μL Esp31 restriction enzyme and 1.25 μL Esp31 Buffer were mixed.

2.2.9 Overnight culture
A single colony was taken from a growing plate into 10-20 mL LB medium with 100 μg/mL Ampicillin and left over night.

2.2.10 Transformation
Agar plates with 100 μg/mL Ampicillin were used.
2.2.11 Destination Vector insertion into E. Coli
Vials of BL21-A1™ One Shot® Competent Cells were thawed on ice. 5 µL of 5-10 ng DNA from destination vector were added to the tube and the incubated on ice for 30 minutes. Then it was heat shocked in a 42 °C water bath for 30 seconds and then placed on ice. 250 µL Recovery medium was added and then incubated in a 37 °C shaker for 1 hour. Then the mixture was plated on the agar plate with 100 µg/mL Ampicillin and incubated at 37 °C, over night.

3 Result

3.1 Culture E. coli ATCC 15597
900 µL E. coli was cultured in 25 mL LB-medium. The OD$_{600}$ was measured to 0.745 after 2 hours and 30 minutes.

3.2 Viral Enumeration
A Plaque assay with droplet method was done to determine the concentration of viral particles. Single droplet with different dilutions was added on the plate. The viral concentration was determined to be $10^7$ viral particles per mL (Figure 1).

![Figure 1 A Plaque Assay with the droplet.](image)

3.3 Anion Exchange Chromatography
The method was done to purify the virus from all the excess proteins. The result shows one peak and the purification was done successfully. It was collected in sample 43 and measured to 1203 smAU (Figure 2).
Figure 2 Anion Exchange Chromatography showed the peak at sample 43 with 1203 mAU.

3.4 Nano sight
The diameter of a purified PR772 diluted to $10^8$ particles per mL was measured to be 66 nm (Figure 3).

![Figure 3 The mean diameter of the purified virus particle PR772.](image)

3.5 Nano drop
The concentration in each sample was measured in the Nano drop machine.

3.6 Anion Exchange Chromatography
The purification of the virus was measured after Anion Exchange Chromatography. The concentration in the sample with the most viral particles was measured to 0.83 mg/mL.

3.7 SDS_PAGE
PR772 contains 23 different proteins. To make sure that the coat proteins of interest were involved a SDS-PAGE gel was performed. The ladder used in this experiment was Spectra™
Multicolor Broad Range Protein Ladder that shows proteins from 10-260 kDa. The protein P3 has the size 43,448 kDa, P6 17,573 kDa and P30 9,186 kDa (Figure 4).

Figure 4 SDS-PAGE of the whole virus particle PR772. The proteins of interest, P3 with the mass of 42 448 Da, P6 with 17 573 Da and P30 9168 Da

### 3.8 Star Gate Cloning

After cloning, mini-preparation was done to purify the plasmids. The concentrations were measured using the Nano drop.

#### Table 1

The concentration of the plasmid containing the gene, P30 throughout the Star Gate cloning. P30 is inserted to the Entry Vector (pENTRY-IBA51), then Destination vector (pASG-IBA3 and pASG-IBA5).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration [ng/µL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>P30_pENTRY-IBA51.1</td>
<td>136,5</td>
</tr>
<tr>
<td>P30_pASG-IBA3.1</td>
<td>105,5</td>
</tr>
<tr>
<td>P30_pASG-IBA3.2</td>
<td>86,6</td>
</tr>
<tr>
<td>P30_pASG-IBA3.3</td>
<td>99,1</td>
</tr>
<tr>
<td>P30_pASG-IBA5.1</td>
<td>89,6</td>
</tr>
<tr>
<td>P30_pASG-IBA5.2</td>
<td>88,9</td>
</tr>
<tr>
<td>P30_pASG-IBA5.3</td>
<td>123,0</td>
</tr>
<tr>
<td>P30_BL21-A1.3.1.1</td>
<td>143,6</td>
</tr>
<tr>
<td>P30_BL21-A1.3.1.2</td>
<td>235,7</td>
</tr>
<tr>
<td>P30_BL21-A1.3.1.3</td>
<td>240,9</td>
</tr>
<tr>
<td>P30_BL21-A1.5.3.1</td>
<td>231,8</td>
</tr>
<tr>
<td>P30_BL21-A1.5.3.2</td>
<td>265,4</td>
</tr>
<tr>
<td>P30_BL21-A1.5.3.3</td>
<td>233,9</td>
</tr>
</tbody>
</table>

#### Table 2

The concentration of the protein, P6 throughout the Star Gate cloning. P6 is inserted to the Entry Vector (pENTRY-IBA51) and then Destination vector (pASG-IBA3 and pASG-IBA5).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration [ng/µL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>P6_pENTRY-IBA51.1</td>
<td>109,9</td>
</tr>
</tbody>
</table>
### 3.9 Gel-electrophoresis of the proteins

The cloning took part in many steps that will be described down below. To control all the steps, gel-electrophoresis was done to see that the right vector and gene was in the samples.

The gene for the protein P30 was inserted into the Entry Vector, pENTRY-IBA51. The gel-electrophoresis clarifies the sizes. The sizes were measured by comparison with the ladder, GeneRuler™ 1 kb DNA Ladder. The sizes are: P30 with 309 bp and pENTRY-IBA51 with 1860 bp (Figure 5).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P6_pENTRY-IBA51.2</td>
<td>128,2</td>
</tr>
<tr>
<td>P6_pENTRY-IBA51.3</td>
<td>133,2</td>
</tr>
<tr>
<td>P6_pASG-IBA3.1</td>
<td>154,4</td>
</tr>
<tr>
<td>P6_pASG-IBA3.2</td>
<td>146,6</td>
</tr>
<tr>
<td>P6_pASG-IBA3.3</td>
<td>162,1</td>
</tr>
<tr>
<td>P6_pASG-IBA5.1</td>
<td>187,9</td>
</tr>
<tr>
<td>P6_pASG-IBA5.2</td>
<td>191,9</td>
</tr>
<tr>
<td>P6_pASG-IBA5.3</td>
<td>150,4</td>
</tr>
</tbody>
</table>

![Figure 5 Gel-electrophoresis for protein P30 inserted to the Entry Vector, pENTRY-IBA51. It indicates the bands at 309 bp for P3 and 1860 bp for pENTRY-IBA51.](image)

The gene for the protein P30 was inserted into the Destination Vector, pASG-IBA3. The sizes were measured by comparison with the ladder, GeneRuler™ 1 kb DNA Ladder. The sizes are: P30 with 309 bp and pASG-IBA3 with 3877 bp (Figure 6).
Figure 6 Gel-electrophoresis for protein P30 inserted to the Destination Vector, pASG-IBA5. It indicates the bands at 309 bp for P3 and 3892 bp for pASG-IBA5.

The gene for the protein P30 was inserted into the Destination Vector, pASG-IBA5. The sizes were measured by comparison with the ladder, GeneRuler™ 1 kb DNA Ladder. The sizes are: P30 with 309 bp and pASG-IBA5 with 3892 bp (Figure 7).

Figure 7 Gel-electrophoresis for protein P30 inserted to the Destination Vector, pASG-IBA5. It indicates the bands at 309 bp for P3 and 3892 bp for pASG-IBA5.

The gene for the protein P6 was inserted into the Entry Vector, pENTRY-IBA51. The gel-electrophoresis clarifies the sizes. The sizes were measured by comparison with the ladder, GeneRuler™ 1 kb DNA Ladder. The sizes are; P6 with 555 bp and pENTRY-IBA51 with 1860 bp (Figure 8).
Figure 8 Gel-electrophoresis for protein P6 inserted to the Entry Vector, pENTRY-IBA51. It indicates the bands at 555 bp for P3 and 1860 bp for pENTRY-IBA51.

The gene for the protein P6 was inserted into the Destination Vector, pASG-IBA3. The sizes were measured by comparison with the ladder, GeneRuler\textsuperscript{TM} 1 kb DNA Ladder. The sizes are: P6 with 555 bp and pASG-IBA3 with 3877 bp (Figure 9).

Figure 9 Gel-electrophoresis for protein P6 inserted to the Destination Vector, pASG-IBA3. It indicates the bands at 555 bp for P6 and 3877 bp for pASG-IBA3.
The gene for the protein P6 was inserted into the Destination Vector, pASG-IBA5. The sizes were measured by comparison with the ladder, GeneRuler™ 1 kb DNA Ladder. The sizes are: P6 with 555 bp and pASG-IBA5 with 3892 bp (Figure 10).

Figure 10 Gel-electrophoresis for protein P6 inserted to the Destination Vector, pASG-IBA5. It indicates the bands at 555 bp for P6 and 3892 bp for pASG-IBA5.
4 Discussion

It is important to optimize the purification steps of the virus PR772 in order to get a higher concentration of PR772. To find the best way to determine the virus structure it is crucial to look at the parameters that can affect PR772 in all ways. The bacteriophage infects best when the E. coli is in their most active phase and that is when they are in the late log growth. This can be checked by a spectrophotometer, when the OD$_{600}$ should be at 0,8-0,9. It is possible to culture the E. coli in both liquid and on solid media. In the liquid solution it is easier for the stalk on the virus to break during infection. It is also difficult have to calculate the optimal ratio between the bacteria and the bacteriophage to get high concentration. Therefore it is easier to culture on a solid media. There, to enumerate how many viral particles are produced, plaque assays may be used to determine the concentration of infectious phage particles. It is also possible to produce higher concentration of the virus by increasing the number of plates and producing larger batches. With more than ten plates, there will be a higher risk of the soft agar hardening before it is poured on the plates. After PEG-precipitation, a small amount of TRIS buffer is used for dissolution and the concentration of the viral particles is kept high.

We chose the StarGate Cloning method instead of the classical Cloning method. The StarGate decreased the time required for cloning and it is more efficient. In the experiment we used two different destination vectors, pASG-IBA3 and pASG-IBA5. The vector pASG-IBA3 has a strep-tag on the 3’-end and pASG-IBA5 has a strep-tag on the 5’-end. Since the structures of these proteins are unknown, we were not sure if the 3’-end or 5’-end was freely available to bind with the affinity column, so we used strep-tags on both ends. The Destination Vectors has a strong T7-promotor, which can be induced with L-Arabinose. By controlling the concentration of L-Arabinose, we can control the expression level of the protein.

After the proteins have been expressed through cloning, the next step would be to perform crystallization. This step is hard and time consuming. There is a need to try different crystal screen combinations to get crystals, that later could be analyzed at a synchrotron. When the diffraction patterns are collected and the structure solve, it will be possible to compare it with earlier structure in a lower resolution to see if there is any difference.

Further, crystal structures for the minor coat protein that can be produced by X-ray crystallography can be used along with the data generated from Cryo Electron Microscopy to get an overall structure of the bacteriophage PR772. Having such a structure would help tremendously in understanding the mechanism of infection of the phage into the host.
Acknowledgements

I would like to sincerely thank my supervisor Martin Svenda for giving me the opportunity to work in this amazing project and working along in his research group. Especially thanks goes to my other supervisor Hemanth Kumar for all the guidance, teaching and patience throughout the project. Last but not least it is important to thank the whole department of Biophysics for the engagement and great team spirit that made this experience truly interesting.
References


