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Of capsid structure and stability: The partnership between charged residues of E-loop and P-domain of the bacteriophage P22 coat protein

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ABSTRACT

Tailed dsDNA bacteriophages and herpesviruses form capsids using coat proteins that have the HK97 fold. In these viruses, the coat proteins first assemble into procapsids, which subsequently mature during DNA packaging. Generally interactions between the coat protein E-loop of one subunit and the P-domain of an adjacent subunit help stabilize both capsomers and capsids. Based on a recent 3.3 Å cryo-EM structure of the bacteriophage P22 virion, E-loop amino acids E52, E59 and E72 were suggested to stabilize the capsid through intracapsomer salt bridges with the P-domain residues R102, R109 and K118. The glutamic acid residues were each mutated to alanine to test this hypothesis. The substitutions resulted in a WT phenotype and did not destabilize capsids; rather, the alanine substituted coat proteins increased the stability of procapsids and virions. These results indicate that different types of interactions must be used between the E-loop and P-domain to stabilize phage P22 procapsids and virions.

1. Background

Viral capsids self-assemble from repeated use of one or just a few virally-encoded proteins, called coat or capsid proteins, leading to highly regular structures. Many viruses, in particular RNA viruses, undergo capsid assembly synchronously with nucleic acid packaging. On the other hand, tailed dsDNA bacteriophages, some archaeal viruses, and the herpesviruses actively package their dsDNA genomes into preassembled precursor structures known as procapsids or proheads, which are assembled from coat proteins having the HK97 fold. The genomes of these viruses can be packed into the matured capsids so tightly that it is liquid crystalline and causes high internal pressure (Booy et al., 1991; Evilevitch et al., 2003; Lander et al., 2013) Thus, these capsids require high stability to maintain genome integrity and to deliver the genome to the next host cell.

In the well-studied bacteriophage HK97, an isopeptide bond formed between K169 of the E-loop of one coat protein subunit and the N356 of the P-domain of an adjacent subunit crosslinks the capsid together (Wikoff et al., 2000). Beyond these crosslinks, ionic interactions between other amino acyl residues of HK97 coat proteins are important for assembly. For instance, reside E153 of the E-loop makes a stabilizing salt bridge with R210 of an adjacent subunit within a capsomer (Hasek et al., 2017). When either residue is mutated, aberrant assemblies form because the capsomers are unable to maintain the correct dome-shape required for assembly into proper procapsids and instead form sheets or tubes (Hasek et al., 2017). There are also electrostatic interactions that staple together subunits between and within capsomers (Tso et al., 2014). Amino acid substitutions made at some of these residues can prevent assembly of the HK97 coat protein beyond hexamers and pentamers, highlighting the importance of these residues for overall procapsid assembly (Gertsman et al., 2010).

Unlike HK97, bacteriophage P22's coat proteins do not crosslink. Rather, weak coat protein interactions are used to build the stable procapsid (Parent et al., 2006). The capsid is stabilized through an irreversible morphogenic maturation during DNA packaging (Teschke et al., 2003). Thermal denaturation of P22 capsids occurs in the range of 80–90 °C (Galisteo and King, 1993), while the melting temperature of the HK97 prohead I structure (prior to covalent crosslinks) is 82 °C (Duda et al., 2009). For phage P22, each coat protein subunit contributes ~6.5 kcal/mol to the thermodynamic stability of a procapsid, with additive energies yielding a robust particle (Parent et al., 2006; Zlotnick et al., 2012). Thus, even without crosslinking P22 is able to assemble a capsid that is very stable.

Phage P22 is a particularly tractable system for studying coat protein folding and capsid assembly (Asija and Teschke, 2018; Suhanovsky and Teschke, 2015; Teschke and Parent, 2010). Until recently there was no high-resolution P22 capsid structure; thus, experimental approaches were based on phenotypic analysis of mutants in different structural

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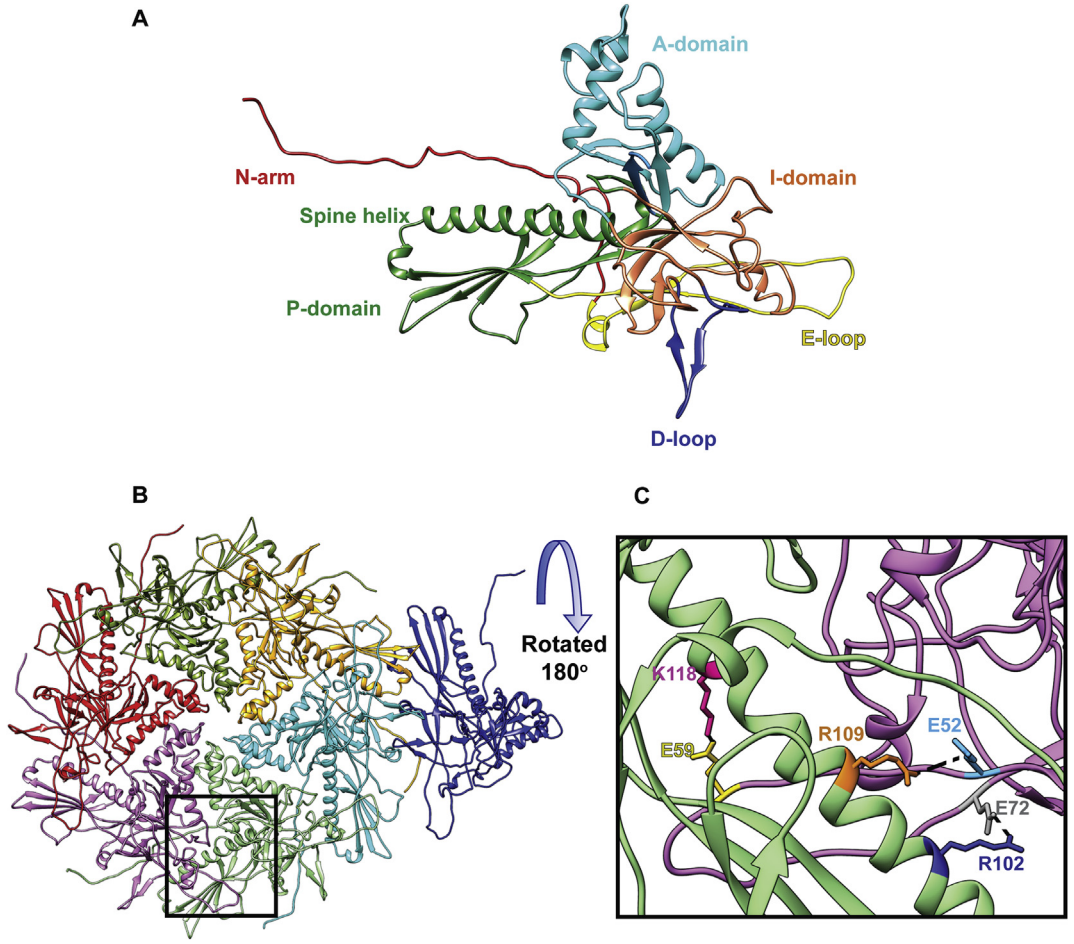

Fig. 1. Neighboring capsomer subunits involved in forming salt bridges. A. A coat protein subunit from the P22 capsid (PDB 5UU5) showing the different domains, important loops and features. **B.** An asymmetric unit of the P22 capsid comprised of seven subunits: six for a hexamer and one penton subunit. The box highlights two adjacent subunits, which are zoomed in and shown in greater detail in panel C. **C.** The zoomed in box from panel B rotated by 180° showing the salt bridge interactions between adjacent subunits. Spine helix residues R102 (blue), R109 (orange), and K118 (magenta) interact with E72 (gray), E52 (sky blue), and E59 (yellow), respectively.

proteins. Recent high-resolution structures of P22 virions have allowed specific structure-based inquiries (Hryc et al., 2017; Rizzo et al., 2014). For example, P22's coat protein has an extra domain (I-domain) inserted between stands β1 and β3 of the A-domain β-hinge that sits atop the surface of the capsid (Fig. 1A) (Parent et al., 2010; Rizzo et al., 2014). A loop in the I-domain, the D-loop, plays a similar role in capsid stabilization as the HK97 G-loop (Tso et al., 2014). The I-domain D-loops form crucial stabilizing ionic interactions across the two-fold axes of symmetry and absence of these interactions lead to assembly of coat protein into aberrant particles and tubes (D'Lima and Teschke, 2015).

Typically, in viruses formed from coat proteins having the HK97 fold, interactions between the E-loop of one subunit with the P-domain of an adjacent subunit are critical for assembly and capsid stability (Duda and Teschke, 2019). Residue W61 at the tip of the E-loop was recently identified as important for both intra- and inter-capsomer hydrophobic interactions (Asija and Teschke, 2019). In addition, a recent 3.3 Å structure of P22 virions showed several salt bridges between residues of the E-loop of one subunit and the P-domain of an adjacent subunit in a capsomer that were suggested to play an important stabilizing role for P22 capsids, in a fashion similar to those seen with phage HK97 (Hasek et al., 2017; Hryc et al., 2017). Here we have mutagenized these residues and analyzed the effects of the substitutions on procapsid and capsid stability, and assembly, both *in vivo* and *in vitro*. Mutating the residues did not result in destabilization of procapsids or virions, which suggests that P22 has evolved a different way to confer intra-capsomer stability between E-loops and the P-domains.

2. Methods

Bacterial and phage strains. *Salmonella enterica* serovar Typhimurium DB7136 (*leuA414*$^{-}$*am hisC525*$^{-}$*am sup*^{0}) was used as the host for bacteriophage P22 for procapsid and virion production (Winston et al., 1979). Strain *Salmonella enterica* serovar Typhimurium DB7155 (*supE20 leuA414*$^{-}$*am hisC525*$^{-}$*am*) is a su^{+} derivative of DB7136 (Winston et al., 1979). All the strains of P22 used in this study contained the c1-7 allele to prevent lysogeny. The phage strains also carried a gene 5 amber mutation so the coat protein is not expressed (5^{-}*am* N114), as well as an amber mutation in the gene responsible for cell lysis (13^{-}*am* H101). *Escherichia coli* BL21 (DE3) was used for the generation of procapsid-like particles from plasmids under the control of a T7 promoter (Studier and Moffatt, 1986).

Plasmids and site directed mutagenesis. Plasmid pMS11 has gene 5 cloned between the BamHI and the HindIII sites in the parent plasmid pSE380 (Invitrogen) and contains an isopropyl β-D-1- thiogalactopyranosidase (IPTG) inducible trc promoter (D'Lima and Teschke, 2015). Plasmid pPC was generated from pET3a parent plasmid with genes 8 (encoding scaffolding protein) and gene 5 cloned into it and was a kind gift from Dr. Peter E. Prevelige, Jr. Site-directed mutagenesis was used to introduce substitutions E52A, E59A, E72A and E52A-E59A-E72A (tripleA) in plasmids pMS11 and pPC, as described previously (D'Lima

and Teschke, 2015). Inverse polymerase chain reaction (PCR) was used to introduce point-mutations in gene 5 of plasmids by using non-overlapping primers (Silva et al., 2017). Phusion high-fidelity master mix with high-fidelity buffer (NEB) was used to prepare the PCR reaction. Since non-overlapping primers were used, following the completion of the PCR reaction, the plasmid was phosphorylated and re-circularized by using T4 polynucleotide kinase (NEB) and T4 ligase (NEB) respectively.

Efficiency of plating (EOP) assay. *Salmonella typhimurium* DB7136 cells containing plasmid pMS11 encoding WT or mutated gene 5 were grown to mid-log phase ($\sim 2 \times 10^8$ cells/ml) and harvested by centrifugation. The cells were then suspended in a small volume of ice-cold Luria-Bertani (LB) broth. Production of coat protein was induced with 1 mM IPTG when the cells were infected with 5$^-$*am* phages, added to soft agar and poured on LB plates containing ampicillin. Successful complementation of the 5$^-$*am* phages by the coat protein expressed from the plasmid results in plaque formation. Plaque formation was tested at 22 °C, 30 °C, 37 °C and 41 °C. The relative titer of the coat protein variants was calculated by comparing the titers produced at each temperature to the titer of 5$^-$*am* phages grown on DB7136 cells with the WT gene 5 expressed from plasmid pMS11 at 30 °C.

***In vivo* generation of procapsids and virions.** *Salmonella typhimurium* containing plasmid pMS11 was grown to mid-log phase and then infected with 5$^-$*am*13$^-$*am* P22 at a multiplicity of infection (MOI) of 5. IPTG (1 mM) was added simultaneously to induce expression of WT or mutated coat protein to complement the gene 5$^-$*am*. The infected cells were grown for an additional 4 h. The cells were harvested by centrifugation, the pellet was suspended in lysis buffer (50 mM ethylenediamine tetraacetic acid (EDTA), 0.1% Triton X-100, 200 µg/ml lysozyme) and stored at −20 °C. After 2–3 freeze-thaw cycles and the addition of 100 µg/ml each of RNase and DNase and 1 mM phenylmethyl sulfonyl fluoride (PMSF), the lysed cells were processed to purify procapsids and virions. The cell debris was removed by centrifugation and the procapsids and virions were pelleted from the supernatant by ultracentrifugation 60,000 rpm for 20 min (Sorvall, RP80-AT2). The pellet was suspended by gentle shaking in 20 mM sodium phosphate buffer (pH 7.6) with 20 mM magnesium chloride ($MgCl_2$) overnight at 4 °C.

Sucrose density gradients. Procapsids and phages obtained from the cell lysate experiment were separated using 5–20% linear sucrose density centrifugation. A gradient maker was used (Biocomp Instruments; model 106) to generate the gradients. About 100 µl of the procapsid and virion mixture was loaded on top of the gradient and centrifuged for 35 min in the Sorvall M120EX ultracentrifuge (rotor RP55S) at 104,812 × *g*. The gradients were fractionated from the top and the protein content of the fractions was analyzed by electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE).

Cesium chloride gradients to separate procapsids from phage particles. Separation of procapsids from phages was done using a cesium chloride step gradient. The procapsid and phage mixture from a cell lysate experiment was applied to the top of the gradient formed with a 25% sucrose solution layered above a step of 1.4 gm/cc cesium chloride and a step of 1.6 gm/cc cesium chloride (top to bottom). The sucrose and cesium chloride solutions were made in 20 mM phosphate buffer, pH 7.6. The sample was centrifuged in a Sorvall MX120 ultracentrifuge at 18 °C for an hour at 30,000 rpm. The phage band, which sediments at the interface between the 1.4 and 1.6 gm/cc steps in the gradient, was extracted and dialyzed against 2 l of 20 mM phosphate buffer, pH 7.6. This was repeated 3 times.

Burst size determination. *Salmonella enterica* DB7136 with plasmid pMS11 with either WT gene 5 or gene 5 mutated to produce coat proteins with the alanine substitutions was grown to mid-log phase ($\sim 2 \times 10^8$ cells/ml) at 30 °C. The plasmid was induced by the addition of 1 mM IPTG and infected with 5$^-$*am*13$^-$*am* phages at a MOI of 10. The infection was allowed to proceed for 2 h. Infected cells were lysed by the addition of $CHCl_3$. The number of infected cells was calculated as follows. The number of cells/ml before infection was determined by plating a dilution of the culture on an LB agar plate. The number of uninfected cells was determined by plating a dilution of the culture 10 min after phage infection. The number of infected cells is calculated as the initial cell count minus the number of remaining viable cells after infection. The number of phages produced per cell was calculated by determining the titer on the amber suppressing DB7155 strain.

Preparation of procapsid–like particles. Procapsid-like particles (PLPs) are assembled *in vivo* by the co-expression of genes 5 and 8 (encoding coat and scaffolding protein respectively) from the plasmid pPC in *E. coli* BL21 (DE3). The cells are grown to mid-log phase in LB containing 100 µg/ml of ampicillin. When the cells reach mid-log phase, protein expression is induced with 1 mM IPTG for 4 h at 30 °C. The cells were harvested by centrifugation, the pellets suspended in buffer B (25 mM Tris, 2 mM EDTA, 50 mM sodium chloride, pH 7.6) and frozen at −20 °C. The cells are lysed with 2–3 cycles of freezing and thawing. The PLPs were processed as described (D'Lima and Teschke, 2015), run over a 150 ml Sephacryl S1000 column (GE Healthcare) in buffer B for purification at 4 °C at a flow rate of 0.2 ml/min. The separated procapsids were pelleted by ultracentrifugation at 206,000 × *g* for 40 min. The PLPs were resuspended by gentle shaking in buffer B.

***In vitro* capsid maturation.** PLPs (1 mg/ml) were incubated at temperatures ranging from 22 °C to 72 °C for 15 min and then placed on ice. The samples were then run on a 1% SeaKem LE agarose gel in 1X TAE buffer (40 mM Tris base, 20 mM acetate, 1 mM EDTA).

Urea titration to test stability of procapsid-like particles. Freshly prepared 9 M urea in 20 mM sodium phosphate buffer (pH 7.6) was used to make 0–7 M urea solutions at 1 M intervals. The refractive index of the prepared urea was used to determine the concentration. PLPs were diluted in each concentration of urea to a final concentration of 0.5 mg/ml. The samples were left overnight in urea and about 5 µg of the sample was run on a 1% agarose gel using SeaKem LE Agarose in 1X TAE buffer.

Urea titration to test stability of viruses. WT and coat protein variant phages were prepared by complementation and purified on cesium chloride gradients, as described above. A stock solution of 9 M that was freshly prepared in 20 mM sodium phosphate buffer (pH 7.6) was used to make urea dilutions for titrations. The concentration of the prepared stock urea solution was confirmed by refractometry. Urea titrations were set up diluting the phages in 0–8 M urea in 20 mM sodium phosphate buffer (pH 7.6) at a final concentration of 10^4 phages/ml, and incubated overnight at 22 °C. For plating, 3 drops of DB7155 plating bacteria were mixed with 10 µl of the phage in urea solution in 2.5 ml of soft agar. The solution was mixed and poured over LB plates. The plates were incubated overnight at 30 °C and the plaques were counted.

Negative stain electron microscopy. 3 µl of the PLP samples from heat expansion as well as urea titration experiments were spotted onto carbon-coated copper grids (Electron Microscopy Sciences). They were washed with 2–3 drops of water, stained with 1% uranyl acetate for 30 s and the excess blotted. A Tecnai Biotwin transmission electron microscope was used to observe the grids at 68000X magnification.

3. Results

The mechanism of capsid stabilization varies among viruses, even among those using the HK97 fold for their coat proteins (Duda and Teschke, 2019). In bacteriophage P22, the coat protein E-loop residues E52, E59 and E72 were suggested to play a role in stabilizing the capsid by making salt bridges with the P-domain residues R102, R109 and K118, respectively, of an adjacent subunit within a capsomer (Fig. 1) (Hryc et al., 2017). The distances between these amino acid pairs are given in Table 1. This hypothesis was directly tested by making amino acid substitutions at the E-loop sites.

Phage growth is not affected by amino acid substitutions forming inter-subunit salt bridges between the E-loop and P-

Table 1
Distances between proposed amino-acid pairs forming salt bridges.

Amino acid pair (Subunit 1- Subunit 2)	Distance (Å)[a]
E52-R109	4.33
E59-K118	3.33
E72-R102	2.89

[a] Distances were calculated using Chimera (Pettersen et al., 2004) based on the PDB structure 5UU5 (Hryc et al., 2017).

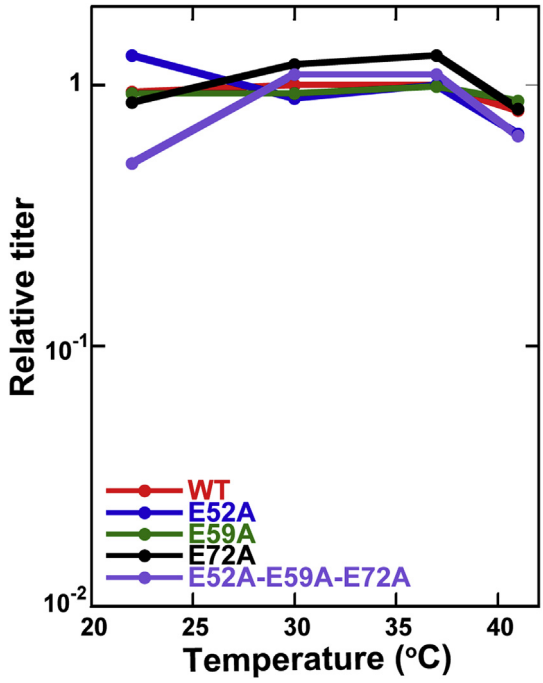

Fig. 2. Titer of phages assembled with alanine coat substitutions is the same as phages assembled with WT coat protein. The titers of phages with assembled with coat protein with the indicated alanine substitutions at temperatures 22 °C, 30 °C, 37 °C and 41 °C relative to those assembled with WT coat protein at 30 °C.

domain. Site-directed mutagenesis of plasmid-encoded gene 5 was used to make E52A, E59A, E72A and E52A-E59A-E72A (tripleA) coat protein variants, as described in the Methods. Each plasmid was transformed into host *Salmonella* cells and the cells were infected with a phage carrying a gene *5-am* mutation. In these complementation experiments, progeny virions are produced only if the plasmid-encoded gene 5 produces coat protein that is functional and can support phage growth. The plates were incubated at different temperatures (22 °C, 30 °C, 37 °C and 41 °C) and the relative titers were calculated (efficiency of plating; EOP) to observe the phenotype of phages assembled with the variant coat proteins. The EOPs of the coat variants at different temperatures were calculated relative to the titer when the 5-*am* phage was complemented with a plasmid encoded wild-type (WT) gene 5 at 30 °C (Fig. 2). All of the mutant proteins led to a WT phenotype in these assays. Thus, we show that the proposed salt bridge interactions were not crucial to the generation of P22 phages *in vivo* at the temperatures tested.

Since a plaque would be seen in the EOP assay provided a few phages are produced from an infected cell, a burst experiment was done to test the effect of disrupting the salt bridges on the ability of the E-loop mutant coat proteins to support phage production. A burst experiment determines the number of progeny phages produced per infected host cell after one round of infection (Table 2). As described above and in the Methods, 5-*am* phages were used to infect host *Salmonella* cells carrying a plasmid encoding WT or mutated gene 5 so that

Table 2
Burst size of phages with coat protein substitutions and with WT coat protein.

Coat protein variant	Burst size (phages produced per cell)[a]
WT	33 ± 2.5
E52A	50 ± 5.5
E59A	17 ± 6.6
E72A	39 ± 4.5
TripleA	34 ± 1.5

[a] Burst size is shown by the number of plaques formed by each sample as depicted by the number of progeny virions produced after one host cell is infected by one viral particle. The standard deviation is calculated from the experiment done in triplicate.

WT or variant coat proteins are produced. When the 5-*am* phages were complemented with WT gene 5, a burst of 33 progeny phages per cell was produced, which is a typical burst for P22 when using complementation from a plasmid (Gordon and King, 1994). All of the salt bridge mutant coat proteins supported production of essentially equivalent phage bursts, with E59A showing a modest decrease and E52A a modest increase in phages produced/cell. These effects could be due to the substitution affecting the burst, or from small changes in protein expression. The tripleA coat protein mutant had a burst that was the average of the three individual substitutions, indicating that the effects of the single substitutions are not additive. Thus, our data show that ability of phages to be assembled *in vivo* is not hindered by the interruption of the salt bridges formed between the E-loops and P-domains of adjacent capsomer subunits, at least in this laboratory condition. In nature where concentration of cells and phages is quite different, there could be an effect of these substitutions on the fitness of the phages.

Although phages were produced in the assays above, it was still possible that abnormal particles were being produced by the variant coat proteins. Thus, particles were purified from 5-*am*13-*am* phage infected *Salmonella* cells complemented with gene 5 to produce WT coat protein, or E52A, E59A, E72A and tripleA substituted coat proteins to determine if the particles had normal protein composition and sedimented as typical for procapsids and phages. The particles were separated on a 5–20% sucrose density gradient and the fractions analyzed on 10% SDS-PAGE. WT procapsids sediment around fraction 16, while abnormal petite procapsids sediment higher on the gradient around fraction 12 (Suhanovsky and Teschke, 2011). Mature phages and large aberrant particles sediment to the bottom of the gradient (fractions 22–23). Procapsids with the coat substitutions all had a normal amount of scaffolding protein and sedimented at approximately fraction 16, indicating that procapsid assembly of the salt bridge coat protein variants was normal (Fig. 3A). Micrographs of fraction 16 were taken to look for the presence of aberrant procapsids containing the alanine substitutions. These showed properly formed procapsids with little to no aberrant structures (Fig. 3B). Although not shown on the small gel slices shown in Fig. 3A, the procapsids and phages had normal levels of the ejection proteins and portal protein.

Coat protein variant procapsid-like particles undergo *in vitro* heat expansion similar to WT PLPs. Procapsids undergo a maturation event during DNA packaging, during which the subunits change conformation and the capsids expand in diameter and gain stability. As the structure from which the salt bridges were identified was that of the mature virion, it is conceivable that the salt bridges are only formed after capsid maturation. Therefore, it is possible that the substitutions might affect the maturation reaction, yet still result in mature virions. The ability of procapsids to undergo maturation was tested using an *in vitro* heat expansion assay on procapsid-like particles (PLPs). PLPs, composed of only coat and scaffolding proteins produced from the plasmid pPC as described in Methods, can be induced to expand by incubation at high temperatures. As the incubation temperature is increased, the PLPs release the internal scaffolding protein, the particles

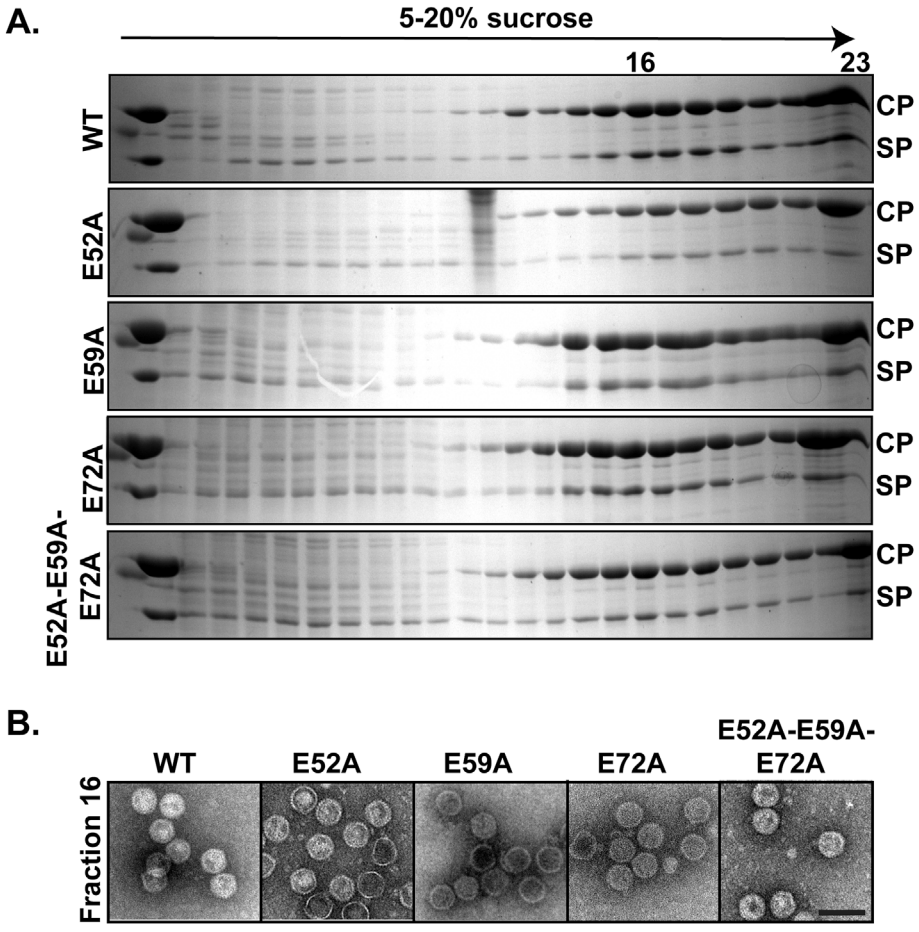

Fig. 3. Sucrose gradient sedimentation profiles of procapsids and phages with alanine coat variants are similar to procapsids and phages with WT coat protein. A 10% SDS-PAGE of 5–20% sucrose gradient fractions from a cell lysate experiment. Coat and scaffolding proteins in the samples have been shown in the gel slices. Procapsids sediment at about fraction 16 and mature phages sediment to fraction 23. CP, coat protein; SP, scaffolding protein. **B.** Micrographs of procapsids sedimenting at fraction 16 from the cell lysate experiment. Procapsids either contain the WT coat protein or coat protein with E52A, E59, E72A, E52A-E59A-E72A coat substitutions. Scale bar is 100 nm.

expand and ultimately lose their pentons yielding the 'whiffle ball' form of the capsid (Galisteo and King, 1993; Morris and Prevelige, 2014; Teschke et al., 2003). PLPs assembled with the WT and alanine variant coat proteins were incubated at 22 °C–72 °C for 15 min, followed by analysis with native agarose gels, where the expanded heads run slower than PLPs. WT PLPs undergo expansion around 67 °C (Fig. 4). Most of the coat protein variants underwent expansion between 67°C and 70 °C, similar to the WT PLPs. A fraction of the E72A and the tripleA mutant PLPs did not expand even at 72 °C, indicating an increased resistance to expansion (Fig. 4A). Furthermore, the PLPs were observed by negative stain transmission electron microscopy to compare the difference in the structures at the temperatures of expansion. All of the expanded PLPs assembled with the alanine mutant coat proteins undergo capsid expansion similar to that of PLPs made with WT coat protein, in that none of the capsids were disrupted at their expansion temperature (Fig. 4B). Thus, these substitutions neither affected the ability of the PLPs to expand nor the ability of the PLPs to withstand high temperature treatment.

Stability of PLPs assembled with coat protein variants is comparable to WT PLPs. Although the E-loop to P-domain salt bridges did not appear to be critical for phage production *in vivo*, or capsid expansion *in vitro*, the overall stability of the particles could still be affected by the substitutions. PLPs assembled with WT or E52A, E59A, E72A or the tripleA substituted coat protein were incubated in increasing concentrations of urea (0–7 M) overnight at 22 °C to assess the effect of the coat protein variants on the stability of the particles to denaturant treatment. Urea titrations are a typical method used to assess stability of proteins (Matthews, 1987), which we have adapted to compare the stability of WT and variant PLPs and virions (below). Approximately 5 µg of each sample was loaded onto a native agarose gel to determine the urea concentration required to dissociate the PLPs and unfold the proteins (Fig. 5A). All the PLPs disassembled at ~6 M urea, indicating that the stability of the PLPs to denaturant is unaffected by the alanine substitutions in coat protein. At low urea concentrations the E72A and the tripleA substituted PLPs have a secondary band that migrates slower than the normal PLP band. WT PLPs and PLPs with the alanine substitutions were observed by electron microscopy at 0 M urea and at the urea concentration at which the samples denatured (Fig. 5B). The secondary band in the E72A and tripleA mutants can be attributed to the presence of aberrant structures highlighted by white arrows in Fig. 5B. Aberrant structures were also seen in PLP sample with the E52A substitution (white arrows, Fig. 5). Although there are aberrant particles in these samples, which were generated by cloned gene overexpression, they are not observed in particles purified from phage infected cells and the coat proteins are clearly able to support assembly of phages, indicating their presence is insignificant.

Stability of virions with coat protein alanine substitutions is higher than virions with WT coat protein. Our data indicate that the salt bridges are not critical to either the thermal or denaturant stability of PLPs. However, the salt bridges could stabilize virions to harsh conditions found in nature. Therefore, the sensitivity of phages assembled with the salt bridge variants to denaturant treatment was determined. Approximately 10^4 phages of the WT virions and virions assembled with E52A, E59A, E72A or tripleA coat variants were diluted in 0–8 M urea and incubated overnight at 22 °C, and plated on an appropriate host. The titer of the phages was determined at each urea concentration. The concentration of urea at which 50% of the titer remained (relative to the 0 M sample) is plotted in Fig. 6. The number of virions assembled with WT coat protein decreased to 50% at 2 M urea. Phages assembled with E52A, E59A, E72A and tripleA coat protein variants all showed increased stability (Fig. 6). Thus, these E-loop to P-domain salt bridges do not seem to be important for procapsid or capsid

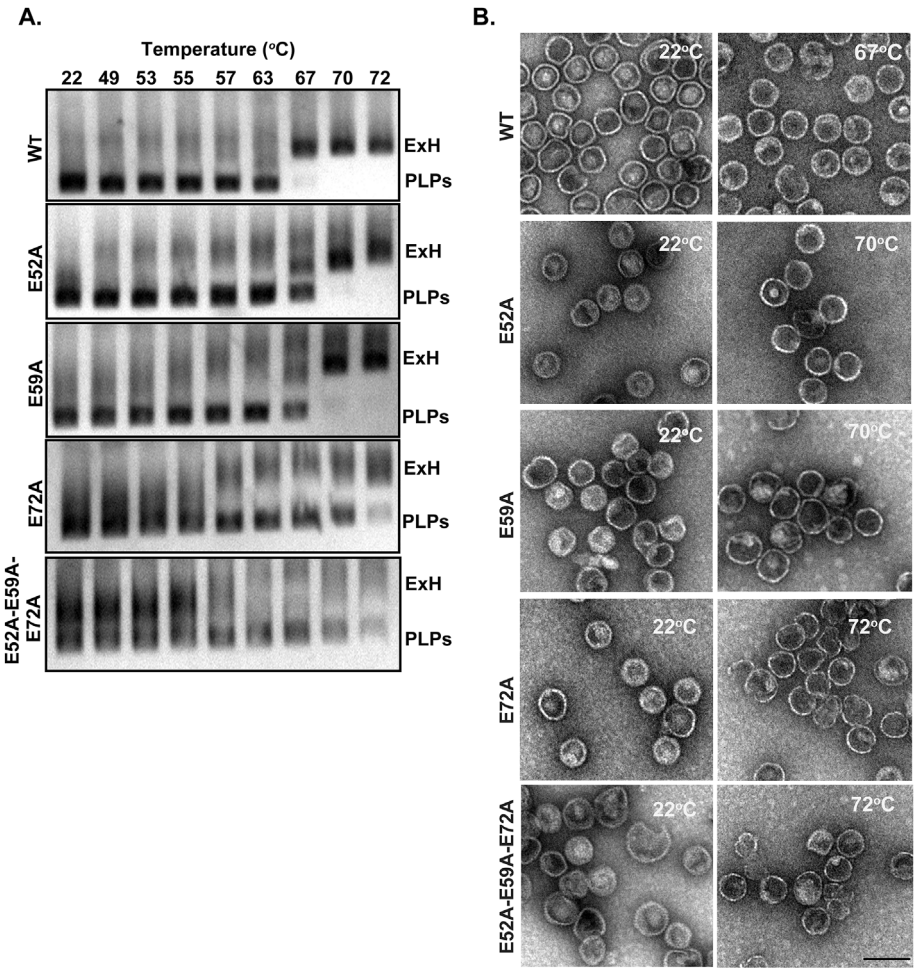

Fig. 4. Heat expansion temperatures of PLPs with coat substitutions are similar to WT PLPs. A. *In vitro* heat expansion of PLPs assembled with WT and alanine coat variants. The PLPs were subjected to temperatures ranging from 22 °C to 72 °C and run on a 1% native agarose gel. Slower migration of PLPs occurs owing to capsid expansion. PLPs, procapsid-like particles; ExH, expanded heads. **B.** Electron micrographs of WT PLPs and coat variant PLPs at room temperature and at the temperature at which they undergo expansion. Scale bar represents 100 nm.

stability, assembly or maturation.

4. Discussion

Interactions important for capsid stability— The stability of a viral capsid is governed by many factors including inter- and intra-subunit electrostatic and hydrophobic interactions, as well as by the addition of decoration proteins to the capsid surface. Electrostatic interactions are important in capsid stability for many viruses. For instance, the retroviral HIV capsid protein (CA) requires a salt bridge in the amino terminal β-hairpin to form dimers and assemble a proper capsid (Cortines et al., 2011a). When the β-hairpin is disrupted by mutagenesis, aberrant structures form. In P22, electrostatic interactions between the N-arm of coat protein and the C-terminal helix-turn-helix of scaffolding protein drive assembly of procapsids (Cortines et al., 2011b, 2014; Padilla-Meier et al., 2012). As expected for electrostatic interactions, this reaction is exquisitely sensitive to the solution salt concentration (Parent et al., 2005). Additionally, electrostatic interactions across icosahedral two-fold axes of symmetry between D-loop residues in the coat protein I-domain are surprisingly important for proper capsid assembly (D'Lima and Teschke, 2015). Investigation of stabilizing electrostatic interactions in the capsid of phage HK97 between the E-loop and the P-domain of an adjacent subunit provides additional evidence for the importance of electrostatic interactions (Hasek et al., 2017).

Nonetheless, capsids are complex structures and capsid protein electrostatic interactions cannot be the sole driver of assembly or stability. For example, during Hepatitis B (HBV) self-assembly, hydrophobic interactions balance the repulsive electrostatic interactions (Ceres and Zlotnick, 2002; Kegel and van der Schoot, 2004). The balance between forces leading to weak protein interfaces allows for thermodynamic editing of kinetically trapped capsid intermediates (Lutomski et al., 2017; Parent et al., 2005, 2006). In general, there is a fine balance between stability of a capsid with its requisite functions, which could include capsid disassembly to deliver the genome to a eukaryotic host cell, or the conformational changes that occur during assembly and genome packaging. Given this, the suggestion that salt bridges between the E-loop and P-domain spine helix should stabilize P22's capsid was reasonable (Hryc et al., 2017).

The association energy between two adjacent coat protein subunits during the initial steps of the virus assembly process is around −2 to −7 kcal/mol in HBV, chlorotic mottle virus, phages HK97 and P22 (Bahadur et al., 2007; Ceres and Zlotnick, 2002; Katen and Zlotnick, 2009; Parent et al., 2007b; Ross et al., 2005). In P22, the amalgamation of these weak forces leads to stable procapsids (Parent et al., 2006; Zlotnick et al., 2012). Procapsids then undergo structural rearrangements during the process of maturation as a mechanism to guarantee capsid stability, including an irreversible increase in diameter of the capsid (Casjens et al., 1992; Galisteo and King, 1993; Parker and Prevelige, 1998). The maturation reaction is exothermic, and in P22 the

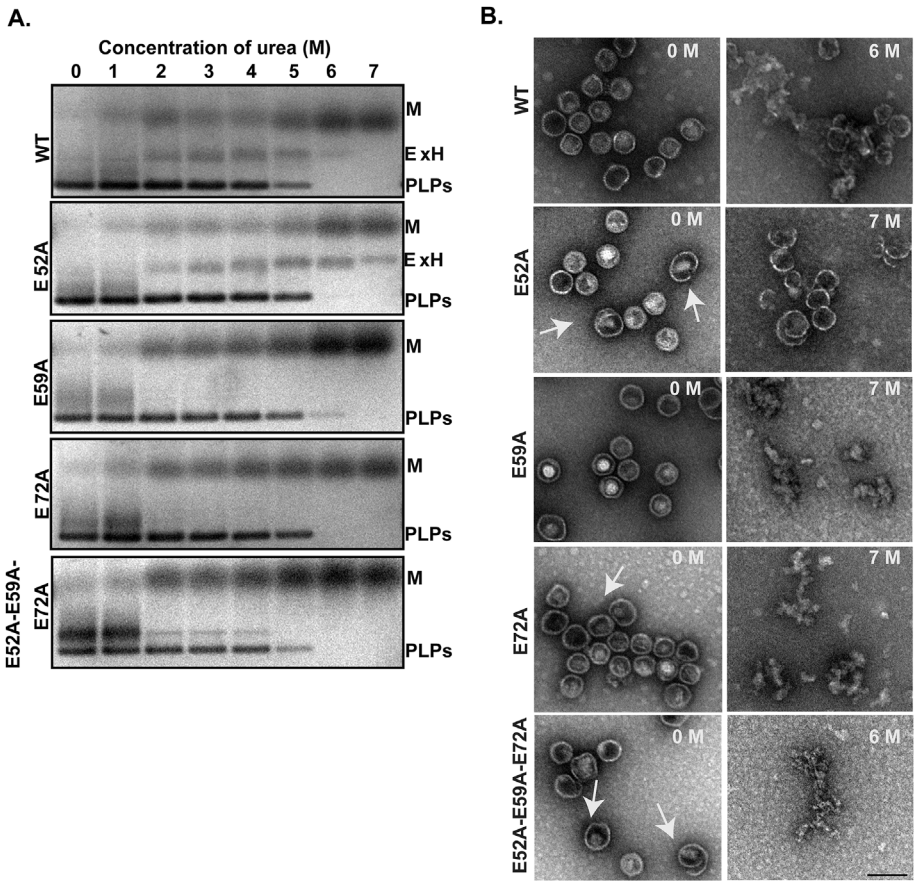

Fig. 5. Stability of PLPs with coat protein ala substitutions is comparable to WT PLPs. A. Urea denaturation of PLPs. 1% native agarose gel of PLPs with alanine coat variants treated with 0–7 M urea. Procapsid-like particles denature to form coat and scaffolding protein monomers. PLPs, procapsid-like particles, ExH, expanded heads; M, monomers. **B.** Electron micrographs of WT PLPs and PLPs with coat proteins substitutions at 0 M urea concentrations and the concentration of urea at which they denature. White arrow shows aberrant procapsids formed by the PLPs with E52A, E72A and tripleA coat substitution. Scale bar is 100 nm.

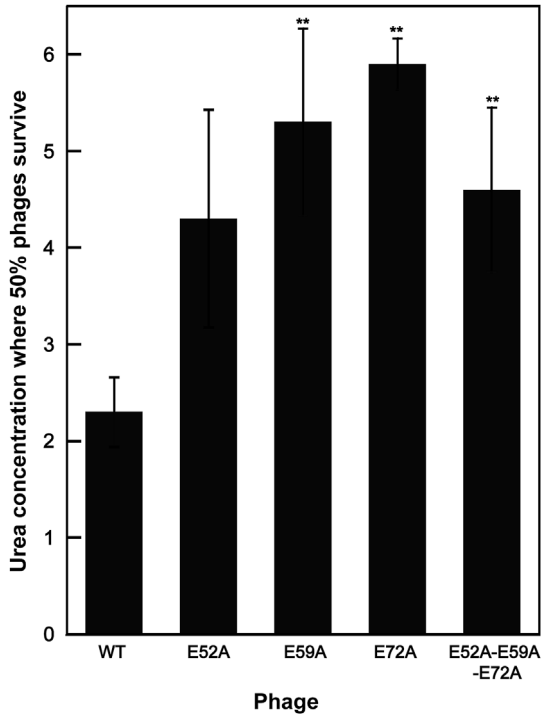

Fig. 6. Phages with coat substitutions are more stable to urea than phages WT coat protein. Concentration of urea at which 50% of the phage titer is remaining relative to the titer after incubation in 0 M urea. **p value < 0.05 was calculated using two-sample student t-test with unequal variance.

energy released is of the order of −21 kcal/mol (Steven, 1993). During this maturation reaction, more inter-subunit interactions are formed that 'stitch' the capsid subunits together (D'Lima and Teschke, 2015; Hasek et al., 2017; Parent et al., 2007a; Wikoff et al., 2000). Undoubtedly, there are inter-subunit interactions important for procapsid assembly and for procapsid stability that are not important for in the mature virion, and vice versa (Duda and Teschke, 2019).

While electrostatic interactions are important for intra- and inter-capsomer contacts for phage HK97, P22 and others, here we show the P22 coat protein E-loop residues E52, E59, and E72, which make salt bridges with basic residues in an adjacent subunit within a capsomer, are not important for stability of procapsids, PLPs, matured particles or virions – at least in laboratory conditions. This result is surprising given that P22 capsids are not stabilized by inter-subunit crosslinks like HK97, so non-covalent interactions such as these salt bridges might be important for procapsid or capsid stability. However, in nature the salt bridges could contribute to fitness in a way that our laboratory assays cannot assess. We chose to make alanine substitutions to remove the charge from the glutamic acid but without increasing the electrostatic repulsion that would occur if lysine or arginine substitutions had been made. Repulsion between the E-loop residues and spine helix of the adjacent subunit could certainly destabilize the capsids.

Comparison within the P22-like phage cluster— The P22-like phage cluster has three branches: P22-like, Sf6-like and CUS-3-like (Casjens and Thuman-Commike, 2011). The residues of the salt bridge pairs are not conserved in the P22-like phage cluster when their amino acid sequences are aligned with Clustal Omega (Madeira et al., 2019), suggesting that members of the P22-like, Sf6-like and CUS-3-like branches may have evolved distinct capsid stabilization mechanisms. Coat proteins within the P22-like phage cluster are only about 15–20% identical (Casjens and Thuman-Commike, 2011), so an amino acid sequence alignment could miss conservation of important interactions

that might structurally align. Thus, we did a structural comparison of P22 (PDB 5UU5) and Sf6 (PDB 5L35) coat proteins. Unfortunately, there is no high-resolution structure of CUS-3 virions. The E-loop intersubunit salt bridges in an Sf6 asymmetric unit were identified using Chimera (Pettersen et al., 2004). The Sf6 structure has a salt bridge between E-loop residue R60 of one subunit and the P-domain spine helix residue E123 of an adjacent subunit within a capsomer (Zhao et al., 2017). This salt bridge structurally aligns with P22's E59-R118 salt bridge, though with the charges in reversed positions, suggesting that this interaction might be important and conserved. Substitution of P22's E59 with an alanine produced a small decrease in the phage burst, though had no effect in the other assays. There is also an intersubunit salt bridge tying the Sf6 E-loop residue K71 to E92 in a P-domain β-strand, rather than the spine helix. There is not an equivalent salt bridge at this site in the P22 structure. P22's E52-R109 and E72-R102 salt bridge interactions do not have equivalent interactions in Sf6 coat protein. The lack of conservation of two of P22's and one of Sf6's salt bridge interaction sites also suggests the evolution of distinct capsid stabilization mechanisms.

A recent comparison of inter-subunit interactions also revealed that P22 and Sf6 build their capsids rather differently (Stone et al., 2019). P22 and Sf6 have about the same average intersubunit interface area. However, between two adjacent subunits P22 has about 42 hydrogen bonds and 9 salt bridges compared with Sf6's 36 hydrogen bonds and 5 salt bridges. One possible reason for the overall lack of destabilization due to eliminating the P22 E-loop salt bridges is that small energetic changes caused by these single amino acid substitutions could be readily compensated for by the myriad of other stabilizing interactions, including hydrophobic interactions that could occur with the E-loop salt bridge residues altered to alanine. On the other hand, we readily see changes in capsid stability caused by single amino acid substitutions in other regions of coat protein (Capen and Teschke, 2000; D'Lima and Teschke, 2015; Doyle et al., 2004; Doyle et al., 2003; Morris and Prevelige, 2014). The insertion of additional loops and domains into the HK97 fold of the coat proteins of φ29, T4, T7 and P22 phages are known to confer capsid stability (Chen et al., 2017; D'Lima and Teschke, 2015; Guo et al., 2014; Hryc et al., 2017; Parent et al., 2012; Rizzo et al., 2014). Interactions from these insertions likely compensate for the lack of crosslinks such as seen in HK97 to increase capsid stability. These large contributions to stability may mask smaller effects due to changes at other locations.

While there are many interactions that stabilize the capsid of P22, the salt bridges pairs E52A-R109, E59A-K118 and E72A-R102 are not obviously contributing to the stability of the virion, at least in laboratory conditions. However, we have shown that tryptophan 61 at the tip of the E-loop makes stabilizing hydrophobic interactions within and between capsomers (Asija and Teschke, 2019). Mutating the W61 leads to a lethal phenotype for several substitutions. In the mature capsid, the N-arm also winds between subunits and links together capsomers (Hryc et al., 2017). These interactions may ultimately prove to be more important stabilizing interactions than the E-loop salt bridges.

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List of abbreviations

HBV	Hepatitis B Virus
EOP	Efficiency of plating
TripleA	E52A-E59A-E72A
LB	Luria-Bertani
IPTG	Isopropyl β-D-1- thiogalactopyranosidase
MOI	Multiplicity of infection
EDTA	Ethylenediaminetetracetic acid
$MgCl_2$	Magnesium chloride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
WT	wild type
PLPs	Procapsid-like particles
HSV	Herpes Simplex Virus
TAE	Tris-acetate-ethylenediaminetetraacetic acid

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Consent for publication

All authors approve the final manuscript.

Conflicts of interest

There are no competing interests.

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