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Recognition of an α-helical Hairpin in P22 Large Terminase by a Synthetic Antibody Fragment

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Synopsis  Recombinant Fabs specific to P22 TerL.

Abstract  The genome packaging-motor of tailed bacteriophages and herpesviruses is a multisubunit protein complex formed by several copies of a large (TerL) and a small (TerS) terminase subunit. The motor assembles transiently at the portal protein vertex of an empty precursor capsid to power energy-dependent packaging of viral DNA. Both the ATPase and nuclease activities associated with genome-packaging reside in TerL. Structural studies on TerL from bacteriophage P22 have been hindered by the conformational flexibility of this enzyme and susceptibility to proteolysis. Here, we screened an unbiased, synthetic phage display Fab library and identified a panel of high-affinity Fabs against P22 TerL. This led us to the discovery of a recombinant antibody fragment, Fab4, that binds a 33 amino acid α-helical hairpin at the N-terminus of TerL with an equilibrium dissociation constant, Kd = 71.5 nM. A 1.51 Å crystal structure of Fab4 bound to TerL epitope (TLE) together with a 1.15 Å crystal structure of the unliganded Fab4, the highest resolution ever achieved for a Fab, elucidate the principles
governing recognition of this novel helical epitope. TLE adopts two different conformations in the asymmetric unit and buries as much as 1,250 Å$^2$ of solvent-accessible surface in Fab4. TLE recognition is primarily mediated by conformational changes in the third complementarity-determining region of Fab4 heavy chain (CDR H3) that take place upon epitope-binding. We demonstrate TLE can be introduced genetically at the N-terminus of a target protein where it retains high affinity binding to Fab4.

**Keywords:** viral genome-packaging motor; Large terminase; bacteriophage P22; antibody engineering; Fab–protein complex.

1. **Introduction**

Enterobacteria phage P22 is a member of the *Podoviridae* family of short-tailed bacterial viruses that infects *Salmonella* typhimurium (Teschke & Parent, 2010). P22 packages its ~43 kbs genome into an empty precursor capsid (or procapsid) using a ‘headful packing’ mechanism, a packaging strategy whereby the amount of DNA packaged inside the virion is determined by the interior volume of the mature particle (Casjens & Weigele, 2005, Catalano, 2005). Like many bacterial viruses and herpesviruses, P22 encodes two terminase subunits, known as large (TerL) and small (TerS)
terminase, whereas herpesviruses also have a third subunit (Heming et al., 2014). The packaging reaction requires the assembly of a genome-packaging motor (Bhardwaj et al., 2014, Sun et al., 2010, Casjens, 2011) that transiently forms onto the unique vertex of the icosahedral procapsid occupied by the portal protein (Chen et al., 2011). This ring-shaped dodecameric protein provides a conduit for DNA entry inside the capsid (Dedeo et al., 2019) while changing conformation during genome-packaging (Olia et al., 2011, Lokareddy et al., 2017). Both TerL and TerS are essential for genome-packaging, though the exact molecular mechanisms by which these two subunits assemble into and function as a molecular complex are poorly understood. In P22, TerL and TerS form a complex that can be purified from infected cells (Poteete & Botstein, 1979), or assembled in vitro from recombinant factors (McNulty et al., 2015).

We previously characterized TerL (499 amino acids, 57.6 kDa) (Roy & Cingolani, 2012) and TerS (162 amino acids, 18.6 kDa) (Roy et al., 2011, Roy et al., 2012) from phage P22. TerL, the packaging ATPase, binds directly to the procapsid conformation of P22 portal protein (Lokareddy et al., 2017). It contains an N-terminal ATPase domain (Sun et al., 2008, Zhao et al., 2013) with ATP-binding Walker A and B motifs and a C-terminal RNAse H-fold nuclease (Smits et al., 2009, Roy & Cingolani, 2012). The nuclease domain of TerL cleaves the concatemeric P22 genome at different stages of the packaging reaction (Wu et al., 2002). At the beginning of packaging, TerL makes sequence-specific cuts in the pac region known as “series initiation cleavage” to generate a DNA end that is inserted into the procapsid unidirectionally. At the end of packaging, TerL cleaves the DNA off the nascent virion, which is then sealed to prevent DNA leakage by three tail accessory factors, gp4 (Olia et al., 2006), gp10 (Olia et al., 2007) and gp26 (Bhardwaj et al., 2007, Bhardwaj et al., 2016). TerS also plays different roles in the genome-packaging reaction. As a specific DNA-recognition subunit, it binds to packaging initiation sites (pac) (Jackson et al., 1978, Wu et al., 2002) in the P22 genome promoting their recognition by TerL; it also stimulates the ATPase activity associated to genome-packaging (Roy et al., 2012). These two activities are likely coupled as the stimulation of the ATPase activity is enhanced by viral DNA (Roy et al., 2012). A C-terminal basic moiety in P22 TerS (residues 140-162) named LBD (Large terminase Binding-Domain) mediates association with TerL and viral DNA (Roy et al., 2012, McNulty et al., 2015). Although the LBD binds TerL in a 1:1 stoichiometry in vitro, the TerL:TerS complex purified from bacteria contains a sub-stoichiometric number of TerL subunits compared to the nonameric TerS (McNulty et al., 2015).

Synthetic antibodies developed using phage-displayed antibody technology are a powerful tool in biology that complement and expand the repertoire of natural antibodies. Synthetic Fabs can specifically target functional states of a protein, often trapping discrete protein conformations (Fellouse et al., 2007, Paduch et al., 2013), thereby enabling structural studies. This paper describes the identification and biophysical characterization of a novel synthetic Fab that binds an α-helical hairpin at the N-terminus of bacteriophage P22 TerL.
2. Materials and methods

2.1. Phage display

The synthetic antibody library built using the 4D5 Fab scaffold, selection criteria, and hit characterization have been described previously (Fellouse et al., 2007, Paduch et al., 2013). A biotinylated AviTag-TerL of phage P22 (see below) was subjected to phage display. After three rounds of library sorting, phages specific to TerL were identified by competitive phage ELISA that provided an estimate of the affinity and the conformational specificity of each clone. The Fabs, with variable regions in CDR-H3 listed in Table 1 from ten phagemids were PCR amplified and cloned into Sall/PaeI linearized pSFV4 vector using Gibson Assembly Cloning Kit (NEB).

Table 1  List of anti-TerL Fabs generated in this study

<table>
<thead>
<tr>
<th>Fab</th>
<th>Variable region in CDR-H3</th>
<th>ELISA</th>
<th>SEC</th>
<th>Native GEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>YSKGWVYVIHSWVYVYAF</td>
<td>1.338</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>VKEYWNYVVYMYWSWYG</td>
<td>1.655</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>WEYYYSDRSYWSHSGM</td>
<td>1.004</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>YSWPWSYKPYGHLFSAM</td>
<td>0.962</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>SGWDSWLYSSFHSIG</td>
<td>1.036</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>SYWQYLFSYTPGL</td>
<td>0.988</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>7</td>
<td>GSHPGQWMGWVWYMAF</td>
<td>1.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>SPWLYNWSAL</td>
<td>1.394</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>GGYESIIMMYWSYKAAI</td>
<td>1.276</td>
<td>s.c.</td>
<td>n.t.</td>
</tr>
<tr>
<td>10</td>
<td>SESYSSWWVSWYYGWAL</td>
<td>1.165</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

+ = association detected by Size Exclusion Chromatography (SEC)
- = no association
s.c. = Fab stuck to the column;
n.t. = not tested

2.2. Biochemical techniques

Expression and purification of P22 TerL (plasmid pET30b-TerL) were previously described (Roy & Cingolani, 2012, Lokareddy et al., 2017, McNulty et al., 2015). An AviTag (MGLNDIFEAQKIEWHEGSS) was introduced at the N-terminus of 6His-TerL using site-directed mutagenesis (plasmid pET30b-AviTag-6His-TerL). AviTag-6His-TerL was expressed and purified like 6His-TerL. Purified AviTag-6His-TerL was biotinylated using the BirA-500 kit (Avidity, LLC). The efficiency of biotinylation was verified by binding to Streptavidin Sepharose resin (GE Healthcare). A peptide spanning P22 TerL residues 1-34 (referred to as TerL.pep) was synthesized by Peptide2go and purified to 90% homogeneity for crystallization. Recombinant Fabs were expressed as previously described (Bartesaghi et al., 2013). Briefly, Fab variants were sub-cloned into the
expression vector (plasmid pSFV4-Fab4). The protein was expressed using *Escherichia coli* BL21 cell line, where cells were grown in 2xYT, and expression was induced at OD$_{600}$ ~0.6. Induction proceeded for 5 h, at which point cell pellets were harvested. Fabs were purified as previously described using ProteinG-A1 resin for single-step purification (Bailey *et al.*, 2014). To form Fab:TerL complexes, a 2-fold molar excess of Fab was added to TerL, and the mixture was purified on a Superdex 200 16/60 gel filtration column (GE Healthcare) in ITC-buffer (20 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM MgCl$_2$, 5% glycerol). The gel filtration column was calibrated with MW markers, as previously described (Lokareddy *et al.*, 2013). To form Fab:TerL complexes, a 2-fold molar excess of Fab was added to TerL, and the mixture was purified on a Superdex 200 16/60 gel filtration column (GE Healthcare) in ITC-buffer (20 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM MgCl$_2$, 5% glycerol). The gel filtration column was calibrated with MW markers, as previously described (Lokareddy *et al.*, 2013). Fab4 and the Fab4:TerL complex were concentrated to ~15 mg ml$^{-1}$ using a 30 MWCO ultrafiltration spin column (Vivaspin 20, Sartorius). TerS-LBD was expressed and purified as a fusion protein with MBP, as previously described.$^{13}$ P22 TerL minimal epitope (residues 1-23) was introduced by PCR using asymmetric megaprimers between the N-terminal 6His-MBP affinity tag and the *Mycobacterium tuberculosis* necrotizing toxin (TNT) gene, in the plasmid pML1995 described earlier (Sun *et al.*, 2015). This modified plasmid (pML3977), encoding both 6His-MBP-TLE-TNT (69 kDa) and the anti-toxin IFT (19.8 kDa) was expressed in *E. coli* BL21 (DE3) LOBSTR and grown in LB medium to an OD$_{600}$ ~0.6 and induced with 0.5 mM IPTG for 3 h. The cells were pelleted by centrifugation (16,000 x g, 30 min, 4 °C), resuspended in 20 mM Tris-Cl pH 8.0, 200 mM NaCl, 1 mM PMSF, sonicated and centrifuged to recover soluble proteins (30,000 x g, 30 min, 4 °C). The 6His-MBP-TLE-TNT:IFT complex was purified from the supernatant using amylose resin. The 6His-MBP tag was cleaved with TEV protease and cleared by binding to Ni agarose resin. IFT was removed from the TLE-TNT:IFT complex by boiling at 70 °C for 10 min. The TLE-TNT (25.3 kDa) was further polished via SEC using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 20 mM Tris pH 8.0, 150 mM NaCl. TLE-TNT was incubated with a 2-fold molar excess of Fab4 and the complex purified on a Superdex 200 10/300 GL column. Native gel electrophoresis was performed on a 1.5% agarose gel, as previously described (Nardozzi *et al.*, 2010, Mitrousis *et al.*, 2008). In this assay, 20 µg of P22 TerL were mixed with 10 to 20 µg of Fab fragments or MBP-tagged LBD, and the mixture was separated on a 1.5% agarose gel at room temperature for 1 hour. After electrophoresis, the gel was fixed in Gel Fixing solution (25% (v/v) isopropanol and 10% (v/v) acetic acid) for 20 minutes and then equilibrated with 95% (v/v) ethanol for 2 h. Gels were then dried, stained for 10 minutes in 0.4% (w/v) Coomassie brilliant blue R250 in Gel Fixing solvent, and destained in Gel Fixing solvent until the background was clear.

### 2.3. In solution biophysical analysis

AUC velocity sedimentation analysis of Fab4 was carried out in a Beckman XL-A Analytical Ultracentrifuge available at the Sidney Kimmel Cancer Center X-ray Crystallography and Molecular Interaction Facility. Gel filtration purified Fab4:TerL complex dissolved at 0.5 mg ml$^{-1}$ in 20 mM Tris pH 8.0, 150 mM NaCl, 2.5% glycerol, 1 mM MgCl$_2$, 1 mM β-ME, 0.1 mM PMSF were spun at
Absorbance values at 280 nm were fit to a continuous sedimentation coefficient (c(s)) distribution model in SEDFIT (Schuck, 2000). ITC experiments were carried out at 25 °C using a nano-ITC calorimeter (TA Instruments). For ITC analysis, both Fab4 and P22 TerL were dialyzed overnight against ITC buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 5% glycerol, 1 mM MgCl₂, 1 mM β-mercaptoethanol, 0.1 mM PMSF) at 4 °C. Fab4 (160 µM) was injected in 2.0 µl increments into a calorimetric cell containing 195 µl of TerL (22 µM). The spacing between injections was 180 seconds. Titrations were performed in triplicate, and data were analyzed using the NanoAnalyze data analysis software (TA Instruments). Heats of dilution were determined from control experiments carried out by injecting Fab4 against ITC buffer and subtracted from enthalpies obtained by titrating Fab4 against TerL. Curve fitting was done in NanoAnalyze data analysis software using a single set of binding sites model. The concentration of Fab4 and TerL used for ITC was accurately determined using the Lowry protein assay and spectrophotometric determination with the theoretical extinction coefficient.

2.4. Crystallographic methods

All crystallization droplets were set up using the vapor diffusion hanging drop method by mixing 2 µl of purified protein at ~15 mg ml⁻¹ with an equal volume of crystallization solution. Crystals of Fab4:TerL₁⁻²³ were obtained from a gel filtration purified complex of Fab4 bound to the full-length TerL using as precipitant 100 mM KCl, 25 mM MgCl₂, 50 mM Na-Cacodylate trihydrate pH 6.0, 15% (v/v) 2-propanol. Crystals of Fab4:TerLₚₑₚ were obtained in the presence of 0.2 M Succinic acid pH 7.0, 20% (w/v) PEG3350. The unliganded Fab4 was crystallized in the presence of 0.1 M Tris pH 8.5, 25% (w/v) Polyethylene glycol 3350. Crystals were harvested in nylon cryo-loops, cryo-protected with 27% ethylene glycol, and flash-frozen in liquid nitrogen. Complete diffraction data were collected at beamline 9-2, at Stanford Synchrotron Radiation Lightsource (SSRL) and beamline 23-ID-D at the Advanced Photon Source (APS), using a Dectris Pilatus 6M detector (Table 2). The structure of Fab4:TerL was solved by molecular replacement (MR) using the heavy and light chains of a recombinant Fab against the HIV-1 Integrase catalytic core (PDB id 5EU7) as a search model, using PHASER (McCoy et al., 2007). The initial MR solution was refined using phenix.refine (Adams et al., 2002), and the variable loop and TerL residues (1-23) were built manually using Coot (Emsley & Cowtan, 2004). The model was then subjected to additional cycles of positional, real-space, and TLS B-factor refinement using phenix.refine (Adams et al., 2002). Final re-refinement using PDB_redo (Joosten et al., 2014) usually yielded the best R_cryst/R_free and stereochemistry. The final model includes Fab4 and TerL₁⁻²³ was refined to an R_cryst/R_free of 18.8/23.9% at 2.40 Å. The structures of Fab4:TerLₚₑₚ and unliganded Fab4 were solved by MR using the model of Fab4 and refined as described above to 16.7/20.3% at 1.51 Å, and 15.7/17.0% at 1.15 Å, respectively (Table 3). There are five cis-prolines in Fab4: three in the Heavy chain (P321, P383 and P385) and two in the
Light chain (P8 and P143). These cis-prolines have outstanding density in the 1.51 Å and 1.15 Å structures but are difficult to refine in the 2.4 Å structure. All models have excellent geometry, with >99.5% residues in the most favored regions of the Ramachandran plot, and root means square deviation (RMSD) of bond lengths/angles of 0.010Å/1.34° (Fab4:TerL_{(1-23)}), 0.012Å/1.09° (Fab4:TerL_{pep}), and 0.009Å/1.27° (unliganded Fab4).

Table 2 Data collection and processing
Values for the outer shell are given in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Fab4:TerL_{(1-23)}</th>
<th>Fab4:TerL_{pep}</th>
<th>Fab4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffraction source</td>
<td>SSRL 9-2</td>
<td>SSRL 9-2</td>
<td>SSRL 9-2</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.980</td>
<td>0.978</td>
<td>0.978</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Detector</td>
<td>Pilatus 6M PAD</td>
<td>Pilatus 6M PAD</td>
<td>Pilatus 6M PAD</td>
</tr>
<tr>
<td>Crystal-detector distance (mm)</td>
<td>350</td>
<td>250</td>
<td>200</td>
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<tr>
<td>Rotation range per image (°)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Total rotation range (°)</td>
<td>250</td>
<td>225</td>
<td>250</td>
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<tr>
<td>Exposure time per image (s)</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>77.7, 139.3, 163.7</td>
<td>74.1, 86.3, 86.1</td>
<td>64.7, 65.8, 107.4</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90.0, 98.2, 90.0</td>
<td>90.0, 97.7, 90.0</td>
<td>90.0, 99.8, 90.0</td>
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<tr>
<td>Mosaicity (°)</td>
<td>0.25</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>50-2.49 (2.49-2.40)</td>
<td>15-1.51 (1.56-1.51)</td>
<td>15-1.15 (1.19-1.15)</td>
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<tr>
<td>Total No. of reflections</td>
<td>3,100,030</td>
<td>3,417,037</td>
<td>5,789,033</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>127,623</td>
<td>161,937</td>
<td>293,620</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>94.0 (94.2)</td>
<td>96.0 (94.3)</td>
<td>93.8 (59.8)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>2.5 (2.3)</td>
<td>2.9 (2.7)</td>
<td>4.7 (3.0)</td>
</tr>
<tr>
<td>&lt;I/σ(I)&gt;</td>
<td>11.3 (1.6)</td>
<td>32.7 (1.9)</td>
<td>50.4 (1.9)</td>
</tr>
<tr>
<td>R_{symm}</td>
<td>11.8 (63.9)</td>
<td>9.2 (71.3)</td>
<td>5.4 (77.9)</td>
</tr>
<tr>
<td>R_{r.i.m.}</td>
<td>8.5 (55.5)</td>
<td>4.2 (57.3)</td>
<td>2.7 (55.9)</td>
</tr>
<tr>
<td>CC1/2 outer shell</td>
<td>0.508</td>
<td>0.456</td>
<td>0.511</td>
</tr>
<tr>
<td>Overall B factor from Wilson plot (Å²)</td>
<td>32.5</td>
<td>20.4</td>
<td>15.9</td>
</tr>
</tbody>
</table>

Table 3 Structure solution and refinement
Values for the outer shell are given in parentheses.
2.5. Structure analysis and modeling

All ribbon diagrams and surface representations were prepared using the program Pymol (The PyMOL Molecular Graphics System, Version 2.0 Schrodinger, LLC, https://pymol.org/2/). Nonlinear Poisson–Boltzmann electrostatic calculations were performed using APBS Tools (Dolinsky et al., 2004). Secondary structure superimpositions were carried out in Coot (Emsley & Cowtan, 2004). The free energy of assembly dissociation (ΔGdiss) was calculated by PISA (Krissinel & Henrick, 2007), and intramolecular contacts were measured using PDBsum (Laskowski et al., 1993). A 3D-model of P22 TerL was calculated using Phyre2 (Kelley et al., 2015) and docking with ZDOCK (Pierce et al., 2011).

3. Results and discussion

3.1. Isolation of recombinant Fabs that bind TerL from P22
P22 TerL is a two-domain enzyme of 499 amino acids that contains an N-terminal ATPase (residues 1-285) connected to a C-terminal nuclease domain (residues 294-482) by a protease-sensitive loop (‘GIPTMGSG’). The purified enzyme is poorly stable in solution and prone to degradation (Roy & Cingolani, 2012). Our previous attempt to crystallize the full-length protein yielded crystals of the C-terminal nuclease domain alone, which we solved to 2.02 Å resolution. Similarly, in vitro assembled TerS:TerL complex is heterogeneous (Roy et al., 2012) and unsuitable for high-resolution structural analysis. In the attempt to identify a crystallization chaperone for TerL, we screened an unbiased, chemically diverse synthetic phage Fab library against TerL. Phagemid hits were characterized using an in vitro binding assay, which led to the identification of ten putative binders for P22 TerL (Table 1). We cloned, expressed, and purified all ten recombinant Fabs and screened them for binding to the purified TerL using size exclusion chromatography (SEC). Four Fabs, Fab1, Fab4, Fab6, and Fab10 markedly shifted TerL migration on a Superose 6 column (Table 1), though we were not able to make large quantities of Fab10 for biochemical studies. We further validated the association of Fabs with TerL by native gel electrophoresis. Though all Fab1, Fab4, and Fab6 shifted TerL mobility on an agarose gel (Fig. 1), Fab4 gave the most quantitative mobility shift. Thus, we focused on Fab4 that is well-expressed in bacteria, easy to purify in milligram-quantities and highly soluble, as expected for a crystallization chaperone.

3.2. Biophysical characterization of Fab4 binding to TerL

To investigate the binding-stoichiometry of Fab4 for TerL, we added a 3-fold molar excess of Fab4 to TerL and subjected the mixture to SEC. The two proteins eluted as a major peak of ~100 kDa (peak 1), preceded by a smaller peak of ~300 kDa that eluted more rapidly (peak 2) (Fig. 2a). Next, we subjected three fractions of the Fab4:TerL complex (#68 and #72 from peak 1, and fraction #34 from peak 2) to analytical ultracentrifugation (AUC) sedimentation velocity analysis. Fig. 2b shows a typical sedimentation profile of Fab4:TerL fractions obtained in 150 mM sodium chloride and 2.5% glycerol, at 10 °C (Table 4). In a range of concentration between 1-10 µM, samples from peak 1 (fractions #68 and #72) migrated as homogeneous species with an apparent sedimentation coefficient (s*) of ~3.4S (absolute sedimentation coefficient, S20, w = ~3.8S) corresponding to a mass of 109.9 kDa, unambiguously consistent with one copy of TerL bound to Fab4 (expected M.W. ~108.1 kDa). The frictional ratio estimated based on sedimentation data was f/fo = 2.2, suggestive of an elongated molecular assembly. In contrast, fraction #34 corresponding to peak 2 (Fig. 2a, b), which also had stoichiometric bands for Fab4 and TerL on gel, appeared polydisperse, possibly indicative of a soluble aggregate. We used nano Isothermal Titration Calorimetry (nano-ITC) to quantify the binding affinity of Fab4 for TerL. We measured the heat released upon titration of increasing concentrations of 160 µM Fab4 inside a cell containing purified 22 µM TerL at 25 °C (Fig. 3a). We observed an exothermic reaction with ΔG = -9.87 kcal/mol, which saturated within 17-18 injections. Binding data were fit
using a one independent binding site model yielding an equilibrium dissociation constant, \( K_d \approx 71.5 \) nM, and a \( n \)=value of \(-0.992\), also consistent with a 1:1 interaction. Interestingly, Fab4 association to TerL (Fig. 3b) involves a negative enthalpy value (\( \Delta H = -10.97 \) kcal/mol), indicating the formation of favorable ionic and hydrogen bonds as well as van der Waals interactions. The negative entropy (\( \Delta S = -3.50 \) kcal/mol) suggests Fab4-binding to TerL leads to a reduction in the overall conformational entropy of the complex, possibly explained by a reduction of mobility and/or flexibility of either protein upon complex formation. Thus, ITC analysis at 25 °C revealed Fab4 binds P22 TerL in a 1:1 stoichiometry with \( K_d \approx 71.5 \) nM. Attempts to repeat the same titration at a higher temperature (e.g., 30-37 °C) were unsuccessful, due to TerL tendency to aggregate and come out of solution. This problem was alleviated, but not eliminated at 25 °C, suggesting the \( K_d \) determined at this temperature may be slightly underestimating Fab4 equilibrium binding affinity for TerL.

### Table 4  List of AUC parameters

<table>
<thead>
<tr>
<th></th>
<th>Fraction #34</th>
<th>Fraction #68</th>
<th>Fraction #72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein conc (mg ml(^{-1}))</td>
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<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Apparent Sedimentation Coef., s (S)</td>
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<td>3.409</td>
</tr>
<tr>
<td>Absolute Sedimentation Coef., s20,( w ) (S)</td>
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<td>3.870</td>
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<tr>
<td>Frictional Ratio, ( f/f_0 )</td>
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<td>2.17</td>
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<tr>
<td>Abundance, %</td>
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<td>77.42</td>
</tr>
<tr>
<td>M.W. (kDa)</td>
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<td>108.65</td>
</tr>
<tr>
<td>Oligomeric state (Fab4:TerL)</td>
<td>Polydisperse</td>
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<td>1:1</td>
</tr>
</tbody>
</table>

#### 3.3. Structure of Fab4 bound to TerL N-terminal \( \alpha \)-helical hairpin

To shed light on the structure of P22 TerL, we crystallized the TerL:Fab4 complex eluted from SEC in peak 1 (Fig. 2a) and obtained large plate-like crystals after a month that diffracted X-ray to 2.4 Å resolution (Table 2). Crystallographic analysis revealed a large unit cell containing six copies of Fab4 arranged in a monoclinic asymmetric unit that has about 50% solvent but not enough room for TerL. Interestingly, all six Fabs displayed strong and continuous electron density in the antigen-binding site (Fig. 4a). Despite the modest resolution (~2.4 Å), the electron density in the antigen-binding site was sufficiently clear to allow for unambiguous tracing of 23 amino acids, organized as an \( \alpha \)-helical hairpin (Fig. 4a). A BLAST search revealed the sequence of this epitope matches perfectly to residues 1-23 of P22 TerL (Fig. 4b). Thus, we fortuitously crystallized Fab4 bound to a cleavage product of TerL that consists of an N-terminal \( \alpha \)-helical hairpin. This epitope contains two \( \alpha \)-helices: \( \alpha_1 \), spanning residues 3-9, and \( \alpha_2 \) that encompasses residues 12-23. As for the structure of P22 TerL
nuclease domain (Roy & Cingolani, 2012), our attempts to crystallize the full-length TerL yielded a proteolytic fragment of TerL, further confirming the extreme conformational flexibility of this protein that has eluded crystallization efforts for a decade. The final structure of Fab4:TerL_{(1-23)} was refined to a R_{cryst}/R_{free} of 18.8/23.9% at 2.4 Å resolution (Table 3).

Inspecting the Fab4:TerL_{(1-23)} interface, it became clear the third complementarity-determining region of Fab4 heavy chain (CDR H3) extends past TerL residues 23 in helix \( \alpha_2 \) (Fig. 4a), suggesting Fab4 recognizes a longer epitope in TerL than just the first 23 residues visible in the crystal structure. In support of this idea, a secondary structure prediction of TerL N-terminal suggested the helix \( \alpha_2 \) (residues 11-23) seen in the crystal structure could extend for an additional ten amino acids C-terminal of residue 23 (Fig. 4b). To test this hypothesis, we synthesized a peptide spanning residues 1-34 of TerL (TerL_{pep}, Fig. 4b) that we co-crystallized with Fab4. Large crystals were obtained in only two days that yielded complete diffraction data to 1.51 Å resolution. We solved this structure by MR using the model of Fab4 previously determined and found two Fab4s in the asymmetric unit arranged in an antiparallel fashion (Table 3). The electron density for TerL epitopes was exceptionally well resolved (Fig. 5a), which allowed up to build an unambiguous atomic model. In one of the two asymmetric unit assemblies (complex A), TerL helix \( \alpha_2 \) extends up to residue 33, making intimate contacts with the Fab4 CDR-H3 (Fig. 5b). In the other assembly (complex B), helix \( \alpha_2 \) ends at residue 23, while residues 24-30 adopt a random coil conformation, stabilized by the constant domain (C_{H}) of Fab4 from complex A (Fig. 5c). In complex B, helix \( \alpha_2 \) melts at the beginning of a stretch of four glutamates (23-Glu-Glu-Glu-Glu-26), which are fully helical in complex A. The average B-factor of TerL residues 1-23 is 40.6 Å^2 and 35.9 Å^2 for complex A and B, respectively, which raises to 53.3 Å^2 and 60.1 Å^2 for the remainder C-terminal residues. The final crystallographic model of Fab4:TerL_{pep} was refined to a R_{cryst}/R_{free} of 16.7/20.3, at 1.51 Å resolution (Table 3).

3.4. Intimate recognition of TerL by Fab4

TerL_{pep} helices \( \alpha_1 \), and \( \alpha_2 \) expose an acidic bonding surface for Fab4 (e.g., the calculated isoelectric point of TerL_{pep} is 3.7). In the extended conformation of helix \( \alpha_2 \) seen in complex A (Fig. 5b) the hairpin projects 21 residues toward the epitope-binding site of Fab4, including 9 Glu and 3 Asp, that make 11 hydrogen bonds, one salt bridge, and 101 van der Waals contacts within a cut-off distance of 4 Å (Fig. 6a). Residues 1-23 in TerL make up the majority of bonds with Fab4. Only two residues C-terminal of Glu23, namely, Glu26 and Arg29, make contacts with Fab4, which may explain why this region of the helix \( \alpha_2 \) adopts a random coil conformation in complex B (Fig. 5c). TerL \( \alpha \)-helical hairpin is stabilized intramolecularly by seven hydrophobic residues (e.g., Met1, Ile6, Leu7, Leu10, Leu18, Leu19, and Leu22) that form a hydrophobic core. PISA (Krissinel & Henrick, 2007) estimate
a solvation free energy gain upon the formation of the Fab4:TerL<sub>pep</sub> interface of $\Delta G = -7.30$ kcal/mol, which is slightly lower than the value experimentally observed by ITC: $\Delta G = -9.87$ kcal/mol. We attribute this difference to entropic effects such as conformational changes or solvent hydration that depend on the geometry and dynamics of the Fab4:TerL<sub>pep</sub> interface (Krissinel, 2011) and are not captured by in silico analysis of interface properties. The Fab4:TerL<sub>pep</sub> binding interface buries a total solvent-accessible surface upon assembly formation of 1,250 Å$^2$, mostly involving the heavy chain. For comparison, Fab-protein complexes bury, on average, 777 ± 135 Å$^2$ of surface area (Ye et al., 2008). Thus the large surface complementarity between Fab4 and TerL<sub>pep</sub> observed in the structure is more similar to those found in protein:protein binding interfaces than Fab:peptide complexes.

To decipher how Fab4 remodels in response to TerL-binding, we also solved a high-resolution structure of the unliganded Fab4, that we refined to a $R_{cryst}/R_{free}$ of 15.7/17.0% at 1.15 Å resolution (Table 3). To our knowledge, this is the highest resolution ever achieved for a Fab, which sheds light on the atomic details of this synthetic Fab. The 1.15 Å structure contains two Fabs arranged in the asymmetric unit that had excellent electron density for the CDR loops. Superposition of the Fab4 structure in the antigen-bound and unliganded state results in a root mean square deviation of only 0.503 Å (Fig. 6b). Deviations are located mainly in the CDR-H3, between heavy chain residues H<sub>323</sub>–H<sub>333</sub>. In the unliganded-state, the CDR-H3 loop swings away from the light chain, whereas in the TerL-bound conformation of Complex A (Fig. 5b), it collapses onto the helix $\alpha_2$. Overall, there is a 5 Å displacement in the main chain position of the CDR-H3 in two states and, notably, Tyr328 and Tyr329 swing 180° toward the epitope in the bound state with a total displacement of ~13 Å compared to the unliganded Fab. Since the CDR-H3 loop is well-defined in both electron density maps, the conformational change described here are directly induced by the binding of TerL<sub>pep</sub> to Fab4 (Fig. 6b). In support of this idea, Tyr328 and Tyr329 have no discernible side-chain electron density in complex B of Fab4:TerL<sub>pep</sub> (Fig. 5c), where helix $\alpha_2$ is too short of making direct contacts with these residues in the CDR-H3 loop. The closing of CDR-H3 loop toward the antigen is consistent with the negative variation in entropy upon complex formation calculated from ITC data (Fig. 2b), pointing to a reduction in the conformational entropy of Fab4 upon TerL recognition.

### 3.5. Protein engineering with TerL epitope (TLE)

We took a protein engineering approach to determine if the TerL epitope could be introduced at the N-terminus of a target protein and retain high affinity binding to Fab4. We fused the minimal P22 TLE spanning residues 1-23 to the *Mycobacterium tuberculosis* necrotizing toxin (TNT), an exotoxin we previously determined crystallographically in complex with the antitoxin IFT (Sun et al., 2015).

First, we cloned TLE at the N-terminus of TNT, which was expressed as 6His-MBP-tagged fusion (69 kDa) in the presence of the anti-toxin IFT (19.8 kDa) to avoid the cytotoxic effect of TNT, which is a potent NAD$^+$/NADP$^+$ glycohydrolase (Tak et al., 2019) (Fig. 7a, lane 1). We then cleaved off 6His-
MBP with TEV Protease (Fig. 7a, lane 2), incubated the mixture with Ni-agarose resin to capture the 6His-MBP tag (Fig. 7a, lane 3), and finally, boiled off IFT to isolate TLE-TNT (25.3 kDa, Fig. 7a, lane 4). The toxin was then incubated with a 2-fold molar excess of Fab4 and analyzed by SEC to assess if Fab4 retains activity toward TLE fused to an exogenous protein. Remarkably, Fab4 associated stoichiometrically with TLE-TNT, shifting the migration of this protein by 3 ml (Fig. 7b). The TLE-TNT:Fab4 complex (fractions a-g in Fig. 7b) was visualized by SDS-PAGE under non-reducing and reducing (Fig. 7c) conditions, confirming the presence of Fab4 in the peak fractions. Thus, a minimal TLE encompassing just 23 residues retains high affinity binding to Fab4 when fused to the N-terminus of TNT.

3.6. Modelling the full-length structure of P22 TerL

The fold of TerL ATPase and nuclease domains are conserved among tailed bacteriophages and herpesviruses, despite low sequence similarity. The relative orientation of these two domains varies dramatically in different crystal structures, due to the flexibility of the linker that connects the two domains (Zhao et al., 2013), which is protease-sensitive in P22 TerL (Roy & Cingolani, 2012). To generate an accurate model of the full-length TerL from P22, we took advantage of two lines of evidence. First, the atomic structures of both the C-terminal nuclease domain (residues 289-482) (PDB 4DKW), and N-terminal α-helical hairpin (residues 1-33) of P22 TerL have been determined. Residues 32-288 in the ATPase domain represent the only structurally uncharacterized part of TerL, which is less than half of TerL’s 499 residues. Second, the ATPase domain is conserved in other viral TerLs of known structure. A database search reveals P22 TerL is 13% identical to the TerL subunits (Fig. 8a-d) from the thermophilic bacteriophages D6E (a Myoviridae) (Xu et al., 2017) and P74-26 (a Siphoviruses) (Hilbert et al., 2015), 9% identical to the TerL from the Podoviridae Sf6 (Zhao et al., 2013), and 8% identical to gp17 from the Myoviridae T4 (Sun et al., 2008). Lower sequence identity is also detectable with the N-terminal domain of the DNA packaging ATPase from bacteriophage Phi29 (Mao et al., 2016). As a starting point to model the unknown residues of P22 TerL, we focused on the central β-sheet of the ATPase domain, which consists of eight β-strands sandwiched by α-helices (Fig. 8a-d). In all TerLs, the β-sheet starts with a α1-β2-α2 motif (in yellow in Fig. 8a-d) whereby helix α1 connects to the second strand (β2) of the β-sheet that continues into helix α2 and from there to the fifth strand (β5) of the β-sheet. The nucleotide is held between helix α1 and helix α2, which harbours a classical phosphate-binding loop (P-loop). Interestingly, the first helix of the α1-β2-α2 motif is amino-terminal in the TerLs of D6E (Fig. 8a), P74-26 (Fig. 8b), and Sf6 (Fig. 8c) while it contains a 138 amino acids extension in T4 gp17 (in red in Fig. 8d), that was previously hypothesized to function as the transmission of a car (Sun et al., 2008). With this in mind, we generated an atomic model of P22 TerL residues 40-499 using Phyre2 (Kelley et al., 2015), which is shown in Fig. 9a. Next, we docked TerL_{pep} from complex A against the predicted ATPase core either by letting the
docking software to probe the entire predicted structure of TerL (40-499) or by restricting the docking area to a region within ~30 Å from residue 40 of TerL. This distance mimics the length of a seven amino acid linker between residues 34-40, assuming ~4 Å per amino acid (Ainavarapu et al., 2007). In either case, automated docking positioned the acidic cradle of TerL_{pep} recognized by Fab4 against a patch of basic residues at the interface between TerL ATPase and nuclease domains, which includes the C-terminal β-hairpin involved in portal protein-binding (residues 480-497) (McNulty et al., 2015, Lokareddy et al., 2017) (Fig. 9a). This model predicts an intramolecular association between TerL_{pep} and the C-terminal β-hairpin that ‘locks’ the protein in a closed, possibly less active conformation, as previously suggested for the large terminase from bacteriophage D6E (Xu et al., 2017). This model explains the slow turnover of P22 TerL measured in vitro and the need for TerS to stimulate the weak ATPase activity associated with genome-packaging 25. Because both TerS and Fab4 bind the N-terminus of TerL between residues 1-58 (McNulty et al., 2015), we asked whether the two proteins make simultaneous or mutually exclusive interactions with TerL. A native gel electrophoresis assay confirmed TerS-LBD binds TerL (McNulty et al., 2015) (Fig. 9b, lane 4), and this complex is super-shifted by the addition of an equimolar quantity of Fab4 (Fig. 9b, lane 5), indicative of a trimeric complex. Thus, Fab4 and TerS harbour distinct binding sites on TerL, as explained by at least two models of association. TerS could bind C-terminal of Fab4, between residues 34-58, although this region (coloured in yellow in Fig. 8 and 9a) is partially buried inside the ATPase core. Alternatively, TerS-LBD could bind the helical surface of TerL helixes α1 and α2 (Fig. 5c), which is solvent-exposed when Fab4 is bound. Future structural studies will clarify the interaction between TerL and TerS and the interplay with Fab4.

4. Conclusions

In this paper, we present the identification of a synthetic Fab that recognizes a 33 amino acid α-helical hairpin at the N-terminus of P22 TerL. High-resolution crystal structures of the unliganded Fab4 and Fab4 bound to TerL_{pep} revealed the detailed molecular recognition of this helical epitope. Furthermore, we show a minimal epitope of TerL encompassing residues 1-23 can be genetically introduced at the N-terminus of a target protein, TNT, retaining high-affinity binding to Fab4. Although Fab4 did not help get crystals of the full-length TerL from P22, which is unstable and short-lived (Roy & Cingolani, 2012), future studies will have to determine if Fab4 can be used as a tool for protein engineering and structural studies.
Figure 1  Identification of recombinant Fabs specific to P22 TerL. Native gel electrophoresis on agarose showing binding of purified Fab1, Fab4, and Fab6 to 200 pmole of P22 TerL. 1x molar ratio is equal to 200 pmole of Fab and 2x = 400 pmole.
Figure 2 Stoichiometry of Fab4 binding to P22 TerL. (a) Highlighted in light blue is a typical elution profile of the Fab4:TerL complex analyzed on a Superdex 200 (16/60) gel filtration column (e.g., the green trace in the background is for TerL alone). Fractions from the main eluted peak were analyzed by SDS-PAGE (bottom gel). Fab4 excess is not visible as an individual peak as the antibody alone is ‘sticky’ and binds the Superdex matrix non-specifically, eluting as a smaller-than-expected species at around 108 ml (Hornsby et al., 2015). (b) Sedimentation velocity profiles of three fractions of Fab4:TerL eluted from SEC (fractions #34 from peak 2 and fraction #68 and #72 from peak 1). All
samples were measured in 20 mM Tris-Cl pH 8.0, 150 mM NaCl, 3 mM DTT, 5% glycerol, 1 mM MgCl₂ at 6 °C. Top panel: raw absorbance at 280 nm plotted as a function of the radial position. Data at intervals of 20 min are shown as dots for sedimentation at 40,000 rpm. Middle panel: the residuals between the fitted curve and raw data. Bottom panel: the fitted distribution of the apparent sedimentation coefficient (s*) calculated for Fab4:TerL in fractions #68 and 72 is about 3.4S (~80% sample) corresponding to an estimated molecular mass of ~109 kDa (Table 2).
Figure 3 Calorimetric analysis of the interaction of Fab4 with P22 TerL. (a) Titration of 160 μM Fab4 (in the syringe) in a cell containing 22 μM TerL. Top panel: raw injection heats. Bottom panel: integrated, buffer-subtracted binding enthalpy plotted as a function of the Fab4:TerL molar ratio. (b) Histogram showing the overall variation of enthalpy (ΔH), entropy (TΔS), and Gibbs (ΔG) energy upon titration of TerL against Fab4.
Figure 4  Structure of the Fab4 bound to TerL. (a) A 2.4 Å Fo-Fc electron density difference map visible in the antigen-binding site of Fab4 cocrystallized with TerL (Table 3). The difference map (in gray) is displayed at 2.25σ above background and is overlaid to residues 1-23 of the refined TerL_{(1-23)} model. The CDR-H3 is also shown in yellow. (b) Amino acid sequence of P22 TerL residues 1-40. Underlined is the region of TerL built in the electron density displayed in panel A. In red, is the amino acid sequence of TerL_{pep}. ‘H’ stands for residues in helical conformation in TerL_{pep} (black residues are always helical, while grey residues in helix α₂ are helical only in complex A, see Fig. 5b).
**Figure 5** 1.51 Å crystal structure of Fab4 bound to TerL_pep. The structure revealed two Fab4:TerL_pep complexes (referred to as ‘A’ and ‘B’) assembled in an antiparallel fashion in a monoclinic asymmetric unit. 

(a) Refined 2Fo-Fc electron density map for TerL_pep residues 1-8 displayed at 1.6σ above background. The refined model is overlaid to the density. 

(b) Ribbon diagram of complex A with Fab4 colored in cyan (light chain) and yellow (heavy chain) and TerL_pep, which is visible between residues 1-33, in red. 

(c) Ribbon diagram of complex B, with Fab4 colored in cyan (light chain) and yellow (heavy chain), and TerL_pep visible between residues 1-30 in green. Region 24-30 of TerL in complex B adopts a random coiled conformation. C_H/C_L and V_H/V_L are Constant and Variable domains for Heavy and Light chains, respectively.
Figure 6 Schematic of TerL N-terminal hairpin recognition by Fab4. (a) Schematic summary of interactions made by Fab4 with TerL_{ pep}. Eleven hydrogen bonds (shown as blue dash line) and one salt bridges (red dash line) are displayed as dash lines. Nineteen non bonded interactions are shown as circle schematics. Residues from Fab4 heavy and light chains are colored in yellow and cyan, respectively. TerL residues making intramolecular contacts between the helices $\alpha_1$ and $\alpha_2$ are colored in green. (b) Superimposition of Fab4 bound to TerL_{ pep} and unliganded showing conformational changes in the CDR-H3 loop that is colored in yellow in the bound-state and light orange in the unliganded conformation.
Figure 7 Fab4 binds TLE-tagged TNT. (a) Purification of TLE-TNT. A complex of 6His-MBP-TLE-TNT (69 kDa) and IFT (20 kDa) purified on amylose beads (lane 1), was subjected to TEV protease cleaving 6His-MBP (44 kDa) from TLE-TNT (25 kDa) (lane 2). The mixture after the recapture of 6His-MBP over Ni-agarose resin (lane 3) was boiled off at 70 °C to remove IFT and obtain TLE-TNT (lane 4). (b) SEC profile on a Superdex 200 10/300 of TLE-TNT alone and in complex with Fab4 showing a 3ml shift in the complex elution. (c) SDS-PAGE showing 1:1 stoichiometric complex of TLE-TNT and Fab4 purified on Superdex 200 10/30 shown in (a). The TLE-TNT:Fab4 complex fractions (a-g) were visualized under both non-reducing and reducing conditions.
Figure 8 The conserved topology of the TerL ATPase domain. Ribbon diagrams of the ATPase domain of TerL from (a) D6E (PDB 5OE8), (b) P74-26 (PDB 4ZNI), (c) Sf6 (PDB 4IDH), (d) T4 (PDB 3CPE). In all panels, the eight-stranded β-sheet is colored in gray with the α1-β2-α2 motif in yellow. The long N-terminal insertion domain found in T4 is colored in red. An N-terminal 16 amino acid insertion that contains a short α-helix (7-SDKFFELL-14) is also present at the N-terminus of P74-26 TerL, but not shown in panel (b).
**Figure 9** A complete 3D-model of the full-length TerL of phage P22. The model of TerL spanning residues 40-499 was generated using Phyre2 (Kelley et al., 2015). The ATPase and nuclease domains are colored in gray and cyan, respectively. The α₁-β₂-α₂ motif in the ATPase domain is colored in yellow, like in Figure 6. The N-terminal α-helical hairpin TerL_{hel} is colored in red and was docked onto the structure using ZDOCK (Pierce et al., 2011). (B) Native gel electrophoresis on agarose showing binding of purified Fab4 (lane 1), TerL (lane 2), and TerS-LBD (lane 3). LBD binds TerL stoichiometrically (lane 4), and the addition of Fab4 yields a super-shift (lane 5), while Fab4 and LBD do not bind to each other.
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References


