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Author(s): Laanto, Elina; Mäntynen, Sari; De Colibus, Luigi; Marjakangas, Jenni; Gillum, Ashley; Stuart, David I.; Ravantti, Janne; Huiskonen, Juha T.; Sundberg, Lotta-Riina

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1 BIOLOGICAL SCIENCES

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3 **A new virus type found from a boreal lake links ssDNA and dsDNA viruses**

4 **Authors:** Elina Laanto^{1,4}, Sari Mäntynen^{1,4}, Luigi De Colibus^{2,4}, Jenni Marjakangas¹, Ashley
5 Gillum², David I. Stuart², Janne J. Ravantti^{1,3}, Juha T. Huiskonen^{2,3*}, and Lotta-Riina Sundberg^{1*}

6 **Affiliations:** ¹University of Jyväskylä, Centre of Excellence in Biological Interactions, Department of Biological and
7 Environmental Science and Nanoscience Center, P.O. Box 35, 40014 University of Jyväskylä, Finland

8 ²Division of Structural Biology, Wellcome Trust Centre for Human Genetics, Roosevelt Drive, OX3 7BN, University of
9 Oxford, Oxford, United Kingdom

10 ³Institute of Biotechnology and Department of Biosciences, P.O. Box 56, 00014 University of Helsinki, Helsinki,
11 Finland

12 ⁴These authors contributed equally to the study.

13

14 *Corresponding authors:

15 Juha Huiskonen: juha@strubi.ox.ac.uk, +441865287844

16 Lotta-Riina Sundberg: lotta-riina.sundberg@jyu.fi, +358408053931

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19 **KEY WORDS:** Cryo-electron microscopy, Flavobacterium, lipids, genome, virus structure

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26 **Summary**

27 Viruses have impacted the biosphere in numerous ways since the dawn of life. However, the
28 evolution, genetic, structural and taxonomic diversity of viruses remain poorly understood. This is
29 partially due to sparse sampling of the virosphere, which has mostly concentrated on exploring the
30 abundance and diversity of dsDNA viruses. Furthermore, viral genomes are highly diverse, and
31 using only the current sequence-based methods for classifying viruses and studying their phylogeny
32 is complicated. We describe the first virus, FLiP (*Flavobacterium* infecting, lipid-containing
33 phage), with a circular ssDNA genome and an internal lipid membrane enclosed in the icosahedral
34 capsid. The 9,174-nt long genome showed limited sequence similarity to other known viruses. The
35 genetic data implies that this virus might use similar replication mechanisms to those found in other
36 ssDNA replicons. However, the structure of the viral major capsid protein, elucidated at near-
37 atomic resolution using electron cryo-microscopy, is strikingly similar to that observed in dsDNA
38 viruses of the PRD1–adenovirus lineage, characterised by a major capsid protein bearing two beta-
39 barrels. The strong similarity between FLiP and another member of the structural lineage,
40 bacteriophage PM2, extends to the capsid organisation (pseudo $T=21$ *dextro*) in spite of the
41 difference in the genetic material packaged and the lack of significant sequence similarity.

42

43 **Significance**

44 We describe phage FLiP, the first ssDNA virus with an icosahedral capsid and an internal lipid
45 membrane. FLiP genome shows limited similarity to known sequences, although a ssDNA
46 replication mechanism was implied by genome analysis. However, as the capsid protein fold
47 indicates relatedness with the dsDNA viruses of the PRD1-adenovirus lineage, FLiP exhibits a
48 unique combination of structural and replication modules. It is suggested, that the capsid protein
49 structure could be used to complement the sequence data when classifying viruses, as well as
50 detecting their deep evolutionary relationships, especially in absence of sequence similarities.
51 Furthermore, these findings demonstrate the value of characterizing unknown viruses from diverse
52 environmental sources to understand the diversity of the microbial world.

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56 **Introduction**

57 Although conservative calculations estimate the number of viruses in the biome to exceed 10^{31}
58 virions (1) this enormous group of biological entities is largely unexplored, as only a fraction of
59 viruses have been studied in detail. In recent years, the enormous diversity of the viral world (2) has
60 been revealed by high-throughput sequencing of viral genomes. Whereas the traditionally used
61 virus detection methods (epifluorescence microscopy and pulse-field gel electrophoresis and early
62 metagenomic studies) have been selective for dsDNA viruses (3-5) recent metagenomic studies
63 have revealed that the number of ssDNA viruses in nature has been grossly underestimated. In fact,
64 ssDNA viruses are widespread and may predominate in certain habitats, including the oceans and
65 arctic freshwaters (6-10). However, only a few ssDNA viruses have been cultivated in laboratory
66 conditions and therefore detailed structural and biochemical analysis of distinct ssDNA viruses is
67 mostly lacking. Detailed characterization of novel virus isolates is essential to expand our
68 understanding of evolutionary relationships between viruses and their role in microbial
69 communities (11).

70 Using solely sequence-based methods for virus classification and phylogeny is complicated due to
71 the enormous genetic diversity among viruses (11, 12). However, essential viral structures and
72 functions are often strongly conserved (13, 14). There seems to be only a limited number of folds a
73 functional protein may adopt and stringent structural constraints further decrease the number of
74 folds suitable for forming a capsid protein. This makes the serendipitous invention of new
75 architectures very unlikely, so that only a very limited number of lineages of virion architecture
76 have been observed (15, 16)(17). Therefore, it has been suggested, that the major capsid protein
77 (MCP) fold and overall virion structure could be used to classify viruses and track down their
78 deeper evolutionary relationships (18).

79 We describe here the first icosahedral virus with an ssDNA genome and an internal lipid membrane
80 designated as “FLiP” (*Flavobacterium* infecting, lipid-containing phage). The 9,174-nt long
81 genome of FLiP has limited sequence similarities with previously identified viruses. Based on
82 cryo-electron microscopy (at 4 Å resolution), the virion structure of FLiP displays pseudo T=21
83 *dextro* organisation, previously shown only for marine dsDNA phage PM2. The major capsid
84 protein (MCP) consists of two beta-barrels with jelly-roll topology and resembles closely dsDNA
85 viruses of the PRD1-adenovirus –lineage. Thus, these structural data indicate evolutionary
86 relatedness between some ssDNA and dsDNA viruses, and suggest that it would be beneficial to
87 complement traditional sequence-based systems by structure-based approaches.

88

89 **Results**

90 **FLiP is a novel phage infecting *Flavobacterium***

91 The virus, designated as “FLiP” (*Flavobacterium* infecting, lipid-containing phage), was isolated
92 together with the host bacterium from a boreal freshwater habitat in Central Finland in September
93 2010. The host bacterium was identified as a member of genus *Flavobacterium* (Bacteroidetes) by
94 16S rRNA sequencing. *Flavobacterium* species are important members of freshwater bacterial
95 communities in the boreal regions (19, 20). The greatest similarity to the obtained 1422 bp long 16S
96 rRNA-sequence was to several *Flavobacterium* sp. strains (99 %). Accordingly, the host bacterium
97 strain of FLiP was designated as *Flavobacterium* sp. B330.

98 FLiP virions were collected for purification from plate lysate. Phage particles were concentrated
99 from the filtered lysate with PEG-NaCl –precipitation and then purified by rate zonal and
100 equilibrium centrifugation to near homogeneity. The rate zonal centrifugation was optimized using
101 different gradient materials (iodixanol, glycerol and sucrose). In sucrose gradient the phage yield
102 was significantly higher, therefore this gradient material was routinely used for purification in both
103 rate zonal and equilibrium centrifugation. This method yielded highly purified particles with
104 specific infectivity of 1.28×10^{12} PFU/mg of protein. The typical recovery and specific infectivity
105 for each purification step is represented in Supplementary Table S1.

106 **FLiP genome is a circular ssDNA molecule with 16 putative ORFs**

107 Genomic nucleic acid of FLiP was extracted from purified virions. Nuclease treatments
108 (Supplementary Fig. S1) revealed that the genome is an ssDNA molecule. Genome sequencing
109 resulted in a 9,174 nt long molecule, with an overall GC content of 34 % (Fig. 1a). A total of 16
110 putative open reading frames (ORFs) were identified from the genome using programs Glimmer
111 (21) and Genemark (22). All the ORFs were oriented in the same direction. Most of the ORFs
112 showed limited similarity to other sequences in the public databases (Supplementary Table s2),
113 which is common for environmental phage isolates.

114 **Identification of FLiP structural proteins**

115 Purified virions were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis
116 (SDS-PAGE) analysis. The most abundant protein species detected were approximately 35 kDa in
117 size (Fig. 1b). Resolved structural proteins were identified by mass-spectrometry and N-terminal
118 sequencing was performed for the most abundant protein species (Supplementary Table S3).

119 According to these analyses five ORFs (7, 8, 9, 11 and 14) were designated as genes as their protein
120 products were identified (Fig. 1b). Due to the high expression level, gene 8 was predicted to encode
121 the major capsid protein. Translated gene 14 showed a high sequence similarity to several lytic
122 transglycosylases and contained the conserved transglycosylase domain (E-value 4.97×10^{-4}). This
123 indicates that a protein with lytic activity is present in the virion structure.

124 **FLiP obtains lipids selectively from the host**

125 Interestingly, FLiP is the first described ssDNA virus with an inner membrane. Low buoyant
126 density (1,21 g/cm³ and 1,18 g/cm³ in CsCl₂ and sucrose gradients, respectively) chloroform
127 sensitivity and positive Sudan Black staining (data not shown) indicated the presence of a lipid
128 membrane in the virion. The lipid class and molecular species composition of the virion and the
129 host bacterium inner membrane were analysed from the extracted lipids by mass spectrometry
130 (triple quadrupole ESI-MS/MS; Supplementary Fig. 2). FLiP contains significantly more ceramide
131 (60 % of the total lipid composition) than the host cytoplasmic membrane. Ornithine lipids are
132 enriched in the host membrane but present only in a negligible amount in the phage (approximately
133 7 %). The differing lipid compositions imply that the phage obtains the lipids from the host
134 selectively.

135 **Cryo-EM reconstruction of FLiP virion and MCP structure**

136 We solved the structure of the FLiP virion using cryo-EM (Fig. 2a). Micrographs of purified virions
137 revealed spherical particles that were ~60 nm in diameter (Supplementary Fig. S3a,b). Some of the
138 particles appeared hollow, suggesting they were empty, lacking the DNA genome and possibly also
139 the internal lipids (Supplementary Fig. S3c). Single particle analysis of 2,203 ‘full’ particles and
140 ‘gold-standard’ three-dimensional structure refinement (23) yielded an icosahedrally symmetric
141 reconstruction of the FLiP virion, solved from a final set of 934 particles (Fig. 2a; Supplementary
142 Table S3). The resolution of the viral protein capsid was on average 4.0 Å, as estimated by Fourier
143 shell correlation at a 0.143-threshold (Supplementary Fig. S3d). Some local areas of the viral
144 surface were less resolved while resolution in the capsid interior was approaching 3.9 Å resolution
145 (Supplementary Fig. S3e,f). Reaching such resolution from a relatively small number of particles
146 has been reported before, for example in the case of deformed wing virus (EMD-3570) (24). In the
147 FLiP reconstruction, pentameric spikes (12 nm tall) protrude from the twelve icosahedral vertices
148 (Fig. 2a,b). The major capsid proteins forming the outer protein shell follow a ‘pseudo $T=21$ *dextro*’
149 icosahedral capsid organisation (Fig. 2a), observed also in the dsDNA phage PM2 (25). The shape

150 of the shell is highly faceted, with facet-to-facet and edge-to-edge distance of 53 nm, and 55 nm,
151 respectively, compared to a vertex-to-vertex distance of 59 nm as measured from the base of the
152 spike. The outer protein shell covers a 5-nm thick lipid bilayer membrane (Fig. 2b). Minor
153 structural components, located between the outer protein shell and the membrane, bridge the two
154 together (Fig.2b).

155 The near-atomic resolution of the FLiP cryo-EM reconstruction facilitated building an atomic
156 model of the major capsid protein (MCP) *de novo* (Supplementary Fig. S4; Supplementary Table
157 S5). Minor structural proteins that were resolved in the reconstructions, such as the pentameric
158 spikes, were not built in the absence of sequence assignments. The MCP consists of two beta-
159 barrels/sandwiches, each of which is composed of two anti-parallel beta-sheets (Fig. 3a). Both of
160 the barrels have a jelly-roll topology (Fig. 3b). Strands C, H, E and F contribute to the sheets facing
161 the surface of the trimer whereas strands B, I, D and G contribute to the sheets facing towards the
162 centre of the trimer (Fig. 3a, b). Strand B is absent in the N-terminal beta-barrel. The two beta-
163 barrels are decorated by loops, in addition to alpha-helices embedded between strands F and G. A
164 long C-terminal alpha-helix resides horizontally at the base of the MCP. Trimers of the double-beta-
165 barrel MCP, are formed with the six beta-barrels of the MCP subunit arranged to form a pseudo-
166 hexameric molecule (Fig. 2).

167 **FLiP MCP fold suggests the virus belongs to PRD1-Adenovirus lineage**

168 The overall virion architecture and details of the MCP fold (including the topology and disposition
169 of the beta-barrels in addition to the position of the embedded alpha-helices) resemble closely those
170 observed in members of the PRD1–adenovirus lineage (14) (Fig. 3c, Fig. 4). This lineage of
171 icosahedral viruses has until now comprised solely dsDNA viruses, albeit infecting organisms
172 ranging from bacteria (e.g. PRD1 (26) and PM2 (27)) through archaea (*Sulfolobus* turreted
173 icosahedral virus (28)) to green algae (*Paramecium bursaria* *Chlorella* virus 1 (29)) and higher
174 eukaryotes (e.g. adenovirus (30)). We calculated a structure-based phylogenetic tree by aligning
175 MCPs of different members of this lineage against FLiP using Homologous Structure Finder
176 program (31) (Fig. 3c). FLiP branches from the bacterial/archaeal arm of the tree, being in no sense
177 an outlier, and is most closely structurally related to bacteriophage PM2. For these two viruses, the
178 structural homology extends beyond the conserved double-beta-barrel MCP fold. Notably, both
179 FLiP and PM2 share the same pseudo $T=21$ *dextro* capsid organization, although PM2 is slightly
180 larger in size than FLiP (vertex-to-vertex distance 63 nm vs. 59 nm; Fig. 4) (25). The two viruses
181 differ also in how the capsid is bridged to the internal lipid bilayer. In both viruses, minor

182 membrane proteins interact with the MCP inner surface via alpha-helices that are parallel to the
183 capsid (Fig. 4d). In FLiP, these parallel helices correspond to the C-terminus of the MCP (Fig. 2e;
184 Fig. 3a; α'' , Ser285–Lys360) whereas in PM2, the helices belong to another minor protein P3 (27).

185

186 **Discussion**

187 Here, we describe a novel virus, FLiP, which infects a *Flavobacterium* host. The FLiP virion
188 consists of an icosahedrally symmetric protein capsid enclosing a circular ssDNA genome of 9,174
189 nucleotides in length (Fig. 1). The inner surface of the capsid is covered by a lipid membrane,
190 which makes FLiP unique among previously described ssDNA phages. Even outside of those
191 ssDNA genomes, few bacterial viruses with internal lipid membranes have been characterized (32).
192 The presence of the membrane evidently facilitates virus-host interactions (32), and similarly to
193 FLiP, some viruses have been shown to derive lipids selectively from the host (33, 34). FLiP
194 contained significantly more ceramide than the host cytoplasmic membrane. This may relate to the
195 cone-shaped structure of ceramide, which could favour the formation of the highly curved viral
196 membrane.

197 The FLiP genome is considerably larger than that of the previously isolated of non-tailed ssDNA
198 phages except for the *Cellulophaga* phage phi48:2 (35). FLiP shows limited sequence similarity to
199 any known sequences in the database (Supplementary Table S2), which is typical for environmental
200 phage isolates and probably reflects the limited number of cultured virus isolates. Similarities were
201 detected to *Flavobacterium* and *Zunongwangia profunda* as well as ssDNA phages infecting
202 *Cellulophaga* (35, 36). *Zunongwangia profunda* and *Cellulophaga* belong to the Bacteroidetes
203 phylum as the FLiP host *Flavobacterium*. Moreover, ORF4 contains conserved DNA-binding helix-
204 turn-helix domain of Xre family. Interestingly, ORF15 has resemblance to rolling circle replication
205 initiator proteins from *Staphylococcus aureus* and *Geobacillus stearothermophilus*, which suggests
206 that FLiP might use replication mechanisms similar to those found in other ssDNA replicons.

207 However, despite the unique combination of ssDNA genome and lipid membrane and the limited
208 sequence similarities, the further structural characterization of FLiP implies intriguing connections
209 to previously identified dsDNA viruses in the PRD1-adenovirus lineage. Although the number of
210 available high resolution structures of viral MCPs is increasing, most viruses seem to fit into just a
211 few structure-based lineages, to the extent that certain DNA viruses (e.g. ssDNA phage phiX174 as
212 well as papilloma and polyomaviruses with dsDNA genomes) have been assigned into the same

213 lineage together with some positive sense ssRNA viruses (15). The overall virion structure of FLiP
214 suggests a relationship with the members of the PRD1-adenovirus lineage and PM2 in particular. In
215 addition, the MCP of PM2 is the closest homolog of the FLiP MCP, strongly suggesting that FLiP
216 is a member of the PRD1-adenovirus lineage, despite the fact that the genomes of other members of
217 that lineage are dsDNA, as opposed to circular ssDNA of FLiP (Fig. 3c). This contrasts with the
218 traditional classification of viruses, accepted by the International Committee on the Taxonomy of
219 Viruses (ICTV), where genome type is used as the top level of classification, namely into ssDNA,
220 dsDNA, ssRNA and dsRNA virus groups. The data we present here, together with previous
221 observations (15, 37) raise the question, of whether it would be appropriate to place more emphasis
222 on the virion architecture and the structure of the capsid proteins, when classifying viruses and
223 assigning distant viruses to higher taxonomic levels. To gain a more comprehensive view on the
224 evolution of the virus world, it would seem beneficial to complement the genomic analyses with
225 structural data.

226 Pervasive shuffling of genes and gene modules is undoubtedly a key feature in the virus evolution.
227 Viral genomes commonly encompass structural and replication modules, which may have different
228 evolutionary provenances (38, 39). The recombination of these modules may provide adaptive
229 advantage for viruses and give rise to novel virus types (38). The genetic resemblance of FLiP
230 ORF15 to rolling circle replication initiation proteins implies, that this virus may display a
231 fascinating combination of a replication mechanism typical of ssDNA viruses and structural
232 characteristics of dsDNA viruses. However, the replication mechanism of FLiP is yet to be
233 confirmed. Interestingly, PM2-like prophages with differing replication machineries seem to be
234 common in aquatic bacteria, although their structural proteins have remain conserved (40).

235 It is evident that bacterial viruses represent an enormous reservoir of genetic diversity. However,
236 only a small fraction of them have been structurally and biochemically characterized. The finding of
237 FLiP exemplifies the importance of detailed characterization of novel viruses from diverse
238 environments in obtaining deeper understanding of the microbial world. This is especially true for
239 ssDNA viruses whose diversity and ecological impact have remained poorly understood, despite
240 their abundance.

241

242 **Methods**

243

244 **Isolation of the bacteriophage and host bacterial strains**

245 Bacteriophage FLiP (*Flavobacterium* infecting, lipid-containing phage) and its host bacterium
246 *Flavobacterium* sp. B330 were isolated from a freshwater sample, taken from lake Jyväsjärvi in
247 Central Finland (N62°13.840' E 025°44.510) as described elsewhere (41). Afterwards B330 was
248 routinely grown in Shieh medium (42) at RT, using a constant agitation of 110 rpm. FLiP virions
249 were isolated from a filtered (pore size 0.45 µm, Micropore, MA, USA) water sample by enriching
250 host bacterium in Luria–Bertani (LB) liquid medium diluted to 1/5 with the filtrated water sample at
251 RT for 2 days (110 rpm). Enrichment culture was mixed with soft agar (0.7 % [w/v]) in 1/5 LB and
252 plated on solid agar. The plate was incubated at RT for 2 days, after which single plaques were
253 picked, suspended in 500 µl of 1/5 LB medium and stored at 4°C. Three rounds of plaque
254 purification were performed. Bacteriophage was propagated using the standard double agar layer
255 technique (43). In brief, 100 µl of the bacteriophage suspension in different dilutions was added to 3
256 ml of molten soft agar (0.7 % [w/v]) together with 200 µl of the host cell culture. The mixture was
257 poured onto the surface of Shieh agar plates (1 % [w/v]) and incubated overnight at RT.

258 **16S rRNA sequencing of the host bacterium**

259 Genomic DNA of B330 was extracted with GeneJET Genomic DNA Purification kit (Fermentas)
260 according to manufacturer's instructions. The gene encoding the 16S rRNA gene was amplified by
261 PCR using primers fd1 and rD1 (44). The PCR products were purified with QIAquick PCR
262 purification kit (Thermo Scientific) and sequenced using BigDye Terminator v3.1 kit and ABI
263 Prism Genetic Analyzer 3100 (Life Technologies). The sequence data was analysed in June 2015
264 using BlastN (45).

265 **Amplification, purification and stability of FLiP-virions**

266 For the preparation of high titer phage lysates, plates showing confluent lysis were used. 5 ml of
267 Shieh was added onto each plate and the suspension was incubated in cold (approximately 6°C) for
268 3 hours with agitation. Medium was collected from the plates and the resulting supernatant was
269 filtrated through a 0.45-µm-pore-size membrane filter (PALL Life Sciences). Viral titer of the
270 filtrated supernatant was determined by the plaque assay and the phage stock was stored at 4°C and
271 for longer periods at –80°C with 20 % glycerol.

272 For phage purification, the lysate was collected from several hundreds of confluent plates. The
273 lysate was filtered and virus particles were precipitated with 10 % (w/v) polyethylene glycol 6000

274 and 0.5 M NaCl. Phage precipitate was collected by centrifugation (Sorvall SLA3000, $11,000 \times g$,
275 30 min, 4°C) and suspended in 20 mM PPB (pH 7.2). Three different gradient materials (all in 20
276 mM PPB) were tested for optimal purification: glycerol (5–30 %), OptiPrep (5–20 %) and sucrose
277 (5–20 %), the latter of which was selected. The suspension was layered on top of a 5–20 % (w/v)
278 sucrose gradient (in 20 mM PPB pH 7.2) for subsequent rate zonal centrifugation (Beckman Optima
279 L-K90 ultracentrifuge, SW28 rotor, $104,000 \times g$, 1 h, 15°C). The light-scattering zone containing
280 the virus was collected and further purified by equilibrium centrifugation in 20–70 % (w/v) sucrose
281 gradient (in 20 mM PPB pH 7.2; Beckman Optima L-K90, SW41 rotor, $175,000 \times g$, 19 h, 5°C).
282 Light-scattering zones were collected, after which the phage particles were pelleted by
283 centrifugation (Beckman Optima L-K90, 70Ti rotor, $112,000 \times g$, 3 h, 5°C), suspended in 20 mM
284 PPB (pH 7.2) and stored at -20°C or -80°C .

285 **Analysis of phage proteins**

286 The protein concentrations of purified phage solutions were measured by the Bradford assay (46),
287 using bovine serum albumin as a standard. Structural proteins of the purified virions were resolved
288 by 16 % SDS-PAGE. The gel was stained with Coomassie brilliant blue to detect the proteins. For
289 structural protein identification, protein bands were cut from the gel.

290 Structural proteins were identified by nano liquid chromatography-electrospray ionization-tandem
291 mass spectrometry (nanoHPLC-ESI-MS/MS) in Proteome Factory (Proteome Factory AG, Berlin,
292 Germany). The LCMS system included an Agilent 1100 nanoHPLC system (Agilent, Waldbronn,
293 Germany), PicoTip electrospray emitter (New Objective, Woburn, MA) and a LTQ-FT or an
294 Orbitrap XL Ultra mass spectrometer (ThermoFisher, Bremen, Germany). For LC-MS/MS analysis
295 SDS-PAGE-separated proteins were excised from the gels and in-gel digested by trypsin. Peptides
296 were first trapped and then desalted on the enrichment column (Zorbax SB C18, 0.3×5 mm,
297 Agilent) for 5 minutes (solvent: 2.5 % acetonitrile/0.5 % formic acid), after which they were
298 separated on a Zorbax 300SB-C18, $75 \mu\text{m} \times 150$ mm column (Agilent) using a linear gradient from
299 10 % to 32 % B (solvent A: 5 % acetonitrile in water, solvent B: acetonitrile, both with 0.1% formic
300 acid). Ions of interest were data-dependently subjected to MS/MS according to the expected charge
301 state distribution of peptide ions. Proteins were identified by database search against a database
302 containing the RefSeq entries of *Flavobacterium johnsoniae* UW101 (National Center for
303 Biotechnology Information, Bethesda, USA) and FLiP sequences using MS/MS ion search of the
304 Mascot search engine (Matrix Science, London, England). Only peptides matches with a score of 20
305 or above were accepted.

306 **Gel electrophoresis and Edman degradation**

307 Gel electrophoresis and Edman degradation were conducted in Proteome Factory (Proteome
308 Factory AG, Berlin, Germany). 5–23 μg of sample were loaded onto 15 % SDS–PAGE under
309 reducing conditions. After electrophoresis, proteins were transferred onto an Immobilon-P
310 membrane (PVDF, pore size 0.45 μm ; Millipore, Bedford, MA) using a Trans-Blot SD Semi-Dry
311 Transfer Cell (Biorad, München, Germany; 5 V; 16 h, 4 °C; blot-buffer: 40 mM borat [pH 9], 20 %
312 methanol) and visualized by Coomassie blue staining. Afterwards an ABI Procise 491 Protein
313 Sequencer (Applied Biosystems) was used for automated N-terminal sequencing according to
314 manufacturer's protocol.

315 **Isolation, sequencing and bioinformatics of phage genome**

316 To isolate phage DNA, purified phage particles were disrupted by treatment with 2 % SDS and 1.2
317 $\mu\text{g}/\text{ml}$ proteinase K (37 °C, 45 min), followed by phenol-ether extraction and precipitation with
318 sodium acetate and ethanol. The purified phage genome was digested with different nucleases
319 [DNase I (Fermentas), RNase A (Sigma-Aldrich), RNase I (Thermo Scientific), S1 nuclease
320 (Thermo Scientific), Mung bean nuclease (Promega) and *EcoRI* (Fermentas)], according to the
321 manufacturer's instructions. The same treatments were performed for the single-stranded DNA
322 genome of ϕX174 (Thermo Scientific) as control. Digestion products were analysed by agarose gel
323 electrophoresis (1 % agarose in $1\times$ Tris-acetate-ethylene diamine tetra-acetic acid buffer).

324 Complementary strands were synthesized for the single-stranded DNA genome of FLiP using
325 random hexamer primers and Klenow Fragment. Subsequently, the double-stranded complementary
326 DNA was amplified by Illustra GenomiPhi V2 DNA Amplification Kit, and sequenced using Roche
327 454 GS FLX+ -sequencer at the Institute of Biotechnology, University of Helsinki, Finland. This
328 initial sequence data was utilized to design specific oligonucleotides for primer walking to fill in the
329 sequence gaps between six contigs. Klenow Fragment was again utilized in these complementary
330 PCR reactions. The resulting PCR products were first amplified by the Illustra GenomiPhi V2 DNA
331 Amplification Kit and then sequenced using conventional Sanger sequencing with BigDye
332 Terminator v3.1 Cycle Sequencing kit and ABI Prism Genetic Analyzer 3100 (Life Technologies)
333 to obtain the complete genome sequence of FLiP. Finally, the whole genome was covered by primer
334 walking as described above.

335 GC content of the genome was calculated using Infoseq-program from EMBOSS-package. Putative
336 protein-encoding open reading frames (ORFs) were predicted from the genome using the

337 algorithms of Genemark (22) and Glimmer (21). Proteins homologous to translated FLiP ORFs
338 were found using NCBI programs BlastP and PSI-Blast (47) against the nonredundant GenBank
339 protein database. NCBI Conserved Domain Search was used to search for protein motifs. Translated
340 ORFs were characterized by molecular mass and isoelectric point using programs included in the
341 ExPASy Proteomics tools. Transmembrane domains were predicted using TMHMM-program (48).

342 **Extraction of cytoplasmic membrane (CM) and lipid analysis**

343 Extraction of cytoplasmic membrane (CM) from the cells of *Flavobacterium* sp strain B330 was
344 done according to Laurinavičius et al (33) using a floatation gradient for separation of membranes.
345 Lipid extraction and analysis of CM and purified phage was done commercially by the Lipidomics
346 Unit at the University of Helsinki, using mass spectrometry (triple quadrupole ESI-MS/MS).

347 **Electron cryo-microscopy**

348 An aliquot (3 μ l) of purified particles was applied on a glow-discharged electron microscopy grid
349 (C-flat; Protochips, Raleigh, NC) and plunge frozen into liquid ethane using a vitrification
350 apparatus (Vitrobot mark IV, FEI, Hillsboro, OR), operated at room temperature and above 80%
351 relative humidity. Data were acquired using a 300-kV transmission electron microscope (Tecnai
352 F30 'Polaris'; FEI), operated at liquid nitrogen temperature and equipped with an energy filter (GIF
353 Quantum LS; Gatan, Pleasanton, CA; zero-loss mode with 20-eV slit width) and direct electron
354 detector (K2 Summit; Gatan). Movies (22 frames, total electron dose 22 $e^-/\text{\AA}^2$) were collected in
355 electron counting mode using a dose rate of 8 $e^-/\text{pix/s}$, frames were aligned in MotionCor2 to
356 compensate for specimen drift and electron beam induced damage, and averaged together (49).
357 Particles were picked from averaged images automatically using ETHAN (50). The picked particles
358 were manually divided into two datasets, consisting of full and empty particles. CTF parameters
359 were estimated locally using GCTF (51). The 3D structure full particles was determined in Relion
360 using established protocols for image classification and gold-standard structure refinement (23). As
361 a starting model, the previously published structure of PM2 (EMDB:1082) was used and it was
362 filtered to low resolution (40 \AA) to avoid bias. The resolution of the reconstruction was estimated by
363 Fourier shell correlation using threshold of 0.143. Map was sharpened by applying an inverse B-
364 factor of -100\AA^2 and local resolution was estimated in Relion.

365 **Model building and refinement**

366 PM2 major capsid protein (pdb:2VVF) was used to generate the FLiP asymmetric unit; 10 protein

367 chains were fitted in the FLiP cryo-EM density using Chimera (52). Subsequently the density
368 corresponding to the asymmetric unit was extracted with Phenix suite (53) phenix.map_box and
369 CCP4 suite (54). To facilitate the sequence assignment the map features were improved by B-factor
370 sharpening in REFMAC (55) and density modification in PHENIX (53). The atomic model of a
371 single MCP protomer was manually traced in the density map filtered to 3.9 Å using COOT (56).
372 Initial model was re-built and refined in Rosetta release version 2016.32.58837 using protocols
373 optimized for cryo-EM maps (57). The best-scoring model as estimated by density fit and geometry
374 was selected and used in COOT to guide further model building and optimization. Final model was
375 refined with Phenix suite phenix.real_space_refine using icosahedral constrains. Model was
376 validated by calculating model to map real-space cross-correlation and Fourier shell correlation
377 with and without masking the map around the model. Side-chains were validated by EMRinger (58)
378 and model geometry by Molprobity (59). Further experimental details on model building and
379 refinement will be published elsewhere. Structural alignment of FLiP MCP and other viral MCPs
380 with a double-beta-barrel fold and a phylogenetic tree were calculated using HSF (Homologous
381 Structure Finder) software (31). Figures were generated with Chimera (52).

382

383

384 **References**

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513

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528

529 **Author Contributions**

530 E.L., S.M., and J.M. isolated FLiP and its host and performed the biological and genomic
531 characterisations. A.G. and J.T.H. collected and analysed the cryo-EM images. L.D.C. performed
532 the model building, density modification and structure refinement. D.I.S. and J.T.H. supervised the
533 structural analysis. J.J.R. and L.R.S. designed and supervised the project. E.L., S.M., L.D.C. and
534 J.T.H. wrote the manuscript. All authors discussed the results and commented on the manuscript.

535 **Data accessibility**

536 The genome sequence of FLiP will be submitted to GenBank upon acceptance. The cryo-EM 3D
537 reconstruction map of FLiP will be deposited with the Electron Microscopy Data Bank (EMDB)
538 under accession code EMD-XXX. The modeled structure of the virus MCP shell will be deposited

539 with the Protein Data Bank (PDB) under the accession code XXXX. The authors declare no
540 competing financial interests.

541

542 **Figure 1 | Circular ssDNA genome of FLiP. a**, A graphic representation of the genome (9,174 nt,
 543 numbering starting from a unique *EcoRII* restriction site) with predicted open reading frames
 544 labeled and shown in blue. Those identified by proteomics to encode for structural proteins are
 545 indicated in purple. **b**, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (16 %
 546 acrylamide) analysis of the structural proteins. Molecular weight standards (Fermentas #26619) are
 547 shown in lane 1. Major protein bands (lane 2), which were subjected to mass spectrometry, are
 548 indicated by an asterisk. N-terminal sequences determined by N-terminal protein sequencing are
 549 indicated.

550

551 **Figure 2 | Three dimensional cryo-electron microscopy reconstruction of the FLiP virion. a**,
 552 Surface rendering of the FLiP virion. The major capsid proteins are colored in yellow, green, blue,
 553 and red. Other structural components are colored in grey. Icosahedral 2-fold (ellipse), 3-fold
 554 (triangle) and 5-fold (pentagons) axes of symmetry are indicated. The triangulation number $T=21$ of
 555 the icosahedral lattice of the capsid is calculated as $T=h^2 + h \times k + k^2$ where lattice indices are $h=4$
 556 and $k=1$ as indicated. The lattice is handed, and is right-handed (*dextro*) as indicated by the right
 557 turn (arrow) **b**, A section of virion density is shown from the area indicated in *a*. Spike structure,
 558 minor proteins (asterisk), outer (OL) and inner (IL) leaflet of the lipid bilayer and density
 559 corresponding to the ssDNA are indicated. **c**, The capsid is composed of 20 faces, each consisting a
 560 group of 10 pseudo-hexameric MCP trimers (ribbons). Each face is divided in three asymmetric
 561 units (one outlined), each consisting of three MCP trimers (1,2, and 4) and one chain from MCP
 562 trimer 3 (in addition to the base domain of the spike). **d–e**, Group-of-ten seen from the side (*d*) and
 563 below (*e*). Approximate footprints of the minor capsid proteins indicated in *b* are outlined.

564

565 **Figure 3 | FLiP major capsid protein fold and structure-based phylogeny. a**, The structure of
 566 FLiP major capsid protein (MCP) is shown as a ribbon representation, colored from the N-terminus
 567 (blue) to the C-terminus (red). The beta-strands in the N-terminal beta-barrel are labeled C–I and
 568 the beta-strands in the C-terminal beta-barrel B'–I'. Alpha-helices are labeled α , α' and α'' . **b**, A
 569 schematic diagram to illustrate the topology of the double-beta barrel jelly-roll fold. The secondary
 570 structure elements are labeled and colored as in *a*. The N- and C-termini are labeled with the
 571 respective letters. **c**, Structure-based phylogenetic tree derived from alignment of MCPs (31) from
 572 FLiP, bacteriophage PM2 (pdb:2WOC), bacteriophage PRD1 (pdb:1HX6), Sulfolobus turreted
 573 icosahedral virus (STIV; pdb:2BBD), Paramecium bursaria chlorella virus 1 (PBCV-1; pdb:1M3Y),
 574 Vaccinia virus Western Reserve D13 (D13; pdb:2YGB), Sputnik virophage (pdb:3J26), and human
 575 adenovirus 5 (h-Ad5; 1P30) in addition to three other adenoviruses (2OBE, 1P2Z, 2INY; not
 576 shown). The region of each protein used in the structure-based alignment is colored in green. A
 577 generalized common core of the fold is shown as a C-alpha trace in the inset. Branches
 578 corresponding to viruses with dsDNA genomes are shaded in light blue. The branch corresponding
 579 to FLiP, the only characterized member the lineage with a ssDNA genome, is highlighted in red.

580

581 **Figure 4 | Comparison of FLiP and PM2 capsid architecture. a**, Surface rendered views of FLiP
 582 (this study) and PM2 (EMD-1082) virion structures are shown. Both maps have been low-pass
 583 filtered to 8-Å resolution. Major capsid protein (MCP) trimers are colored in yellow, blue, green
 584 and red, depending on their location in the icosahedral capsid. Other densities, including the spikes
 585 in the icosahedral vertices, are colored in gray. **b**, A central section of density is shown for the two
 586 virions. Coloring is as in *a*. Capsid-to-membrane contacts are indicated with arrowheads. In FLiP,

587 these densities have not been assigned. In PM2, they correspond to minor protein P3 (27). **c–d**, a
588 ribbon representation of four different types of MCPs seen from outside (*c*) and inside (*d*) of the
589 virion. In addition to the MCP, alpha-helices of the PM2 minor protein P3 interacting with the MCP
590 (27) are shown in dark blue.

591

592