The component parts of bacteriophage virions accurately defined by a machine-learning approach built on evolutionary features.

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1 ABSTRACT

2

3 Antimicrobial resistance (AMR) continues to evolve as a major threat to human health and new
4 strategies are required for the treatment of AMR infections. Bacteriophages (phages) that kill
5 bacterial pathogens are being identified for use in phage therapies, with the intention to apply
6 these bactericidal viruses directly into the infection sites in bespoke phage cocktails. Despite the
7 great unsampled phage diversity for this purpose, an issue hampering the roll out of phage
8 therapy is the poor quality annotation of many of the phage genomes, particularly for those from
9 infrequently sampled environmental sources. We developed a computational tool called STEP³
10 to use the “evolutionary features” that can be recognized in genome sequences of diverse phages.
11 These features, when integrated into an ensemble framework, achieved a stable and robust
12 prediction performance when benchmarked against other prediction tools using phages from
13 diverse sources. Validation of the prediction accuracy of STEP³ was conducted with high-
14 resolution mass spectrometry analysis of two novel phages, isolated from a watercourse in the
15 Southern Hemisphere. STEP³ provides a robust computational approach to distinguish specific
16 and universal features in phages to improve the quality of phage cocktails, and is available for
17 use at <http://step3.erc.monash.edu/>.

18

19 IMPORTANCE

20 In response to the global problem of antimicrobial resistance there are moves to use
21 bacteriophages (phages) as therapeutic agents. Selecting which phages will be effective
22 therapeutics relies on interpreting features contributing to shelf-life and applicability to
23 diagnosed infections. However, the protein components of the phage virions that dictate these
24 properties vary so much in sequence that best estimates suggest failure to recognize up to 90% of
25 them. We have utilised this diversity in evolutionary features as an advantage, to apply machine
26 learning for prediction accuracy for diverse components in phage virions. We benchmark this
27 new tool showing the accurate recognition and evaluation of phage components parts using
28 genome sequence data of phages from under-sampled environments, where the richest diversity
29 of phage still lies.

1 INTRODUCTION

2

3 Antimicrobial resistance (AMR) has risen to prominence as a major threat to human health (1,
4 2) and new strategies are required for the treatment of AMR infections (3-5). For example, the
5 Centers for Disease Control and Prevention have identified several species of microbes as
6 “Urgent” threats to human health by virtue of their AMR phenotypes, including *Escherichia*
7 *coli* and *Enterococcus faecalis*. As another prime example of one of these, the carbapenem-
8 resistant *Enterobacteriaceae* (CRE), *Klebsiella pneumoniae* infections represent a key target
9 for new therapeutics to treat AMR infections (3-5). Bacteriophages (phages) that kill bacterial
10 pathogens such as *Klebsiella* are being identified for use in phage therapies, with the intention
11 to apply these bactericidal viruses directly into the infection sites. Careful consideration is
12 needed in selecting the phages for use in therapeutic cocktails (4-6), considerations made
13 difficult because annotation of phage genomes is poor (7, 8), potentially obscuring phages with
14 therapeutic potential. For example, while structural motifs are now known (9) that will promote
15 phage virion stability (i.e. shelf-life), only with correct annotation of the major capsid, minor
16 capsid and other proteins involved can structural motifs be identified and evaluated.

17

18 Phage therapy has re-emerged because of its potential treatment for antimicrobial-resistant
19 infections, and a common protocol for treatments is to select two or more phages for
20 combination into a treatment cocktail (4-6). An ongoing issue is the establishment of criteria
21 used for selection of appropriate phages for a cocktail, to enhance production and maximize
22 efficacy, and to circumvent issues of phage-resistance and collateral induction of further drug-
23 resistance in the infection sites (4, 6). The phages used for phage therapy are *Caudovirales*
24 conforming to a blue-print of an icosahedral protein capsid housing the phage genome, and a
25 tail composed of 20-40 protein components (10). The tail of these phages can be considered as
26 a complex piece of molecular machinery, with component parts of the tail recognizing and
27 docking to a species-specific receptor on the host bacterium (11, 12). Penetration of the host
28 cell envelope depends on other components of the tail, which can have enzymatic functions to
29 locally hydrolyze each of the distinct layers of the bacterial envelope (12-14). An ultimate goal
30 for the development of personalized phage therapy is the recognition of all of these components
31 from genome sequence data, so that bespoke phage could be selected for specific therapeutic
32 purposes (5, 6). However, the annotation of phage genomes is poor, potentially obscuring
33 important features contributed by some component parts such as contributions to virion
34 stability and shelf-life, host-range and bacterial cell lysis (7, 8, 15).

1 RESULTS AND DISCUSSION

2

3 Currently phage genomes are assessed by tools such as multiPhATE (15) which provides a
4 bioinformatics pipeline for functional annotation using sequence-based queries. The annotation
5 accuracy of multiPhATE is limited by the extreme sequence diversity in phage genomes, likely
6 due to the rapid evolutionary rates of phages (16). This limitation has been addressed to some
7 extent with a neural network-based predictor iVIREONS (17) and further tools such as PVPred
8 (18), PVP-SVM (19), PhagePred (20), Pred-BVP-Unb (21) and PVPred-SCM (22). However,
9 recent evaluation of these tools in phage protein prediction showed less than satisfactory
10 performance (23). We developed an ensemble predictor, STEP³, to accurately call the protein
11 components of phage virions and visualize their predicted function-based relationships (Fig. 1).

12

13 STEP³ extracted information from Position-Specific Scoring Matrix (PSSM) data (Fig. 1a), an
14 approach that tracks protein evolutionary histories (24, 25). In machine-learning evaluation of
15 protein sequences, “evolutionary features” refer to information within the amino acid sequences
16 that conceptually traces the evolutionary history of proteins, and their use often identifies highly
17 informative patterns (24, 25). Indirectly, these evolutionary features effectively capture structural
18 as well as physicochemical properties. STEP³ includes data visualization capabilities to
19 document relationships between virion components where the sequence similarity is sufficiently
20 strong to identify high confidence homologs from other phages (Fig. 1b, Supplementary Fig. 1).

21

22 There is power in integrating individual models within an ensemble framework for more robust
23 and stable predictions: trained with an individual model alone (AAC-PSSM), predictions
24 perform well with the 5-fold cross-validation test (Supplementary Table 1), but ranked only
25 fourth using the independent test (Supplementary Table 2). In contrast, combined with other
26 models into the ensemble model of STEP³, to draw on the best elements from all of the
27 individual models (Fig. 1a), the best prediction performance ranking was achieved
28 (Supplementary Tables 1, 2). In benchmarking against other available predictors, the ensemble
29 STEP³ achieved an improved performance, with the highest sensitivity (SN = 0.896), accuracy
30 (ACC = 0.891), F-value (0.891) and Matthews Correlation Coefficient (MCC = 0.781) using the
31 independent test (Supplementary Table 3). The superior performance of STEP³ can be attributed
32 to the integration of more informative evolutionary features, as well as the comprehensive and
33 up-to-date training dataset using experimentally verified inputs. It is worth noting that the
34 BLAST-based predictor, which represents the mode used for genome annotation had the lowest
35 accuracy (ACC) and F-value. This prediction bias is reflected by the extremely unbalanced
36 sensitivity (the lowest) and specificity (the highest) scores, so that the BLAST-based predictor

tended to predict positive samples as being negative. This quantifies and evidences past observations that pairwise sequence matching methods struggle to predict phage proteins (25).

As initial case studies we drew on three accounts published after STEP³ was trained, where phages had been discovered, the genome sequence data deposited for public access, and the protein composition virions had been determined by mass spectrometry. The mass spectrometry data is crucial as it enables a discrimination between false positive (FP; predicted but not present by mass spectrometry of the virion) and true positive (TP; predicted and found present by mass spectrometry of the virion). Phage vB_EfaS_271 infects *Enterococcus faecalis* (26), phage vB_PatM_CB7 infects *Pectobacterium atrosepticum* (27), and phage vB_Eco4M-7 infects enteropathogenic *Escherichia coli* (28). STEP³ was benchmarked against equivalent predictors: PVPred, PVP-SVM, PVPred-SCM and Pred-BVP-Unb (Fig. 2). STEP³ provided the greatest set of true positive predictions for each of the three phages, predicting 9 of the 12 virion components for phage vB_EfaS_271, 23 of the 26 protein components for phage vB_PatM_CB7 and 24 out of 33 components of the phage vB_Eco4M-7 virions. Making low FP predictions on each phage, STEP³ maintained a good balance between TP and FP and showcased robust prediction performance across the test cases. In the case of phage vB_PatM_CB7, where mass spectrometry data had shown the number of non-virion proteins is more than eight times as many as that of virion proteins, STEP³ generated an equal number of FP as that of TP. In this extreme case, STEP³ correctly predicts 23 out of 26 virion proteins with a false positive rate of 10.1% (23/227).

Oftentimes candidate phages that kill pathogens are isolated from hospital waste-water sources for their use in phage therapy (29, 30). This raises the issue of potential over-sampling of a common environmental source (*i.e.* wastewater) for phages, potentially limiting discovery of other, valuable phages and also potentially biasing the capability of predictors like STEP³. Therefore, as a further proof of principle test for STEP³ we sampled a natural watercourse with a strain of drug-resistant and hypervirulent *Klebsiella pneumoniae* as host. The Merri Creek, which forms a part of the larger Merri catchment, lies within Wurundjeri Woi Wurrung people's traditional homelands. Phages isolated from two separate sampling sites were characterized initially by genome sequencing and named in Woi wurrung language Merri-merri-uth nyilam marra-natj (MMNM) and Merri-merri baany-a bundha-natj (MMBB). These names translate as “Dangerous Merri lurker” and “Merri water biter”, respectively, in English.

Comparative genomic analysis revealed *Klebsiella* phages MMNM (Supplementary Fig. 2) and MMBB (Supplementary Fig. 3) to be distinct from previously sampled phages. In the case of

1 MMNM, some similarities can be seen to phages belonging to the *Jedunavirus* genus according
2 to the most recent International Committee on Taxonomy of Viruses (ICTV) classification, but
3 the branch lengths on the tree designate diversity within this small group, comprising only eight
4 phages in the NCBI database (Fig 3a). Relatives of MMNM, isolated from hospital wastewater
5 in Russia, showed considerable diversity in gene content and arrangement (Fig 3b). Most
6 notably, MMNM encodes several genes that are absent in many of the other sequenced
7 *Jedunaviruses*, including previously uncharacterised proteins MMNM_5, MMNM_6
8 MMNM_45, MMNM_51, MMNM_56, MMNM_57 and the putative polynucleotide kinase
9 protein MMNM_50. Conversely, MMNM lacks the putative NHN endonuclease-like protein
10 encoded by both vB_KpnM_FZ14 and vB_KpnM_KpV52. Sequence annotations
11 (Supplementary Table 4) suggest that MMNM has a tail structure characteristic of *Myoviridae*,
12 including a baseplate protein (MMNM_21), a baseplate J-like protein (MMNM_23) and the
13 base-plate wedge protein (MMNM_26). In high resolution structural analyses of the *Myoviridae*
14 phage T4, each virion has 6 molecules of each of these proteins and 1-3 molecules per virion of
15 the hub proteins to which the baseplate is attached (31, 32).
16
17 MMBB belongs to the *Webevirus* genus, a group of phages that exclusively target *Klebsiella*
18 species (Supplementary Fig 3). MMBB is distinct from the other phages in this genus, with its
19 closest relationship being to a phage isolated in China called vB_KpnS_GH-K3 (also called
20 phage GH-K3) (33). Highlighting their differences, MMBB and GH-K3 show regions of
21 diversity in gene content and arrangement, this is observed for the gene encoding MMBB_16, a
22 putative AP2/HNH endonuclease previously found only in a small number of other *Siphoviridae*
23 phages including the *Escherichia* phage vB_EcoS_ESCO41 and *Escherichia* phage CJ19
24 (Supplementary Fig 2). Additional differences are seen in a contiguous cluster of four genes
25 encoding hypothetical proteins (MMBB_45-MMBB_48) that are absent in GH_K3.
26
27 Phenotypic characterization of the phages on lawns of *K. pneumoniae* (Methods) showed that the
28 plaque size for MMNM was smaller than MMBB (Fig 4a), and with liquid cultures of *K.*
29 *pneumoniae* (Methods) that MMNM had a shorter latent period (L) before host cell death as
30 determined by one-step growth curves (Fig 4b). Electron microscopy revealed that MMNM has
31 an icosahedral head and a tail tube of ~54 nm capped with a ~30 nm baseplate to generate thick
32 and straight tails (Fig 4c). The baseplate structure evident in MMNM (Fig 4c) is similar to that
33 seen for the T4 phage (31), which serves as a paradigm for the *Myoviridae* (34) (Fig 4d). By
34 contrast, MMBB has ~200nm long, slender and flexible tails (Fig 4c). The flexible, non-
35 contractile tail tube designate MMBB as a phage of *Siphoviridae*-like viruses (Fig 4d), consistent
36 with genome annotation data (Supplementary Table 5).

1
2 To directly test STEP³ prediction capability on the novel phages MMNM and MMBB, the
3 protein components contributing structurally to the virions were determined by high-
4 performance mass spectrometry (35, 36). To this end, samples of each virion were purified using
5 caesium chloride gradients. The MMNM virion is composed of 25 protein components
6 (Supplementary Table 6). Assuming a similar stoichiometry between MMNM virions and the
7 paradigm for *Myoviridae*, phage T4 virions, the identification of the lytic transglycosylase
8 MMNM_19 suggests that the proteomic analysis is sensitive enough to detect 3 or fewer
9 molecules per virion (31). From evaluation of the predicted proteins within the phage genomes,
10 together with this mass spectrometry data, the MMNM genome encodes 25 structural proteins
11 that serve as components of the virion and 42 proteins that would be expressed after infection of
12 the host, to drive phage replication (Fig 5a).
13
14 STEP³ successfully predicted 22 out of the 25 MMNM virion proteins (Fig 5b, Supplementary
15 Table 7). The other predictors gave poorer outcomes with these diverse protein sequences. For
16 example, second to STEP³ was iVIREONS which identified 19 virion proteins, but iVIREONS
17 also generated the largest number of false positives, 14, consistent with its high false positive
18 prediction rate in the independent tests (Supplementary Table 3). In one case, the initial STEP³
19 analysis made a false-negative prediction that was highly informative. The phage polynucleotide
20 kinase (PNK) is an enzyme that has been previously assumed to be a non-virion protein, and the
21 sequence was therefore included in that (non-virion) dataset from which STEP³ was trained.
22 However, mass spectrometry identified the putative PNK protein MMNM_50 as a component of
23 the virion (Supplementary Table 6). Note, an equivalent result was achieved with the prediction
24 for MMBB: protein MMBB_64 was detected by mass spectrometry (Supplementary Table 9)
25 and selected by STEP³ (Supplementary Table 8). We suggest that for some phages the PNK
26 remains associated with the packaged genome and is thereby incorporated within the capsid.
27 This suggestion explains the proteomics data herein, reconciles the false-negative prediction by
28 STEP³, and is consistent with the recent observation that the “gp44 ejection protein” is a virion-
29 protein in a *Staphylococcus* phage 80 α bound to genome ends and functioning as a putative PNK
30 would to protect the DNA from degradation upon phage entry into its host (37).
31
32 High-resolution mass spectrometry of the MMBB virions showed them to be composed of 29
33 protein components (Supplementary Table 9). Thus, the MMBB genome encodes 29 proteins
34 contributing structurally to the virions, and 50 non-virion proteins expressed only after infection
35 in the host bacterium (Fig 4c). For MMBB, STEP³ and iVIREONS retrieved 20 and 18 virion

proteins, respectively (Fig 4d, Supplementary Table 8). The other predictors achieved unsatisfactory prediction results, retrieving less than half of the 29 virion proteins.

The evolutionary features drawn on by STEP³ and iVIREONS are structure-informed, in that the patterns that they recognize are reflections of secondary and tertiary structure, and these patterns can also be used to suggest protein function. For example, a characteristic of the *Webervirus* has been suggested to be the presence of tail-spike proteins with polysaccharide degrading activity (38), and the sequence of MMBB_78 is suggestive of such a protein, as summarized in Supplementary Fig. 3. Conversely, pairwise sequence assessment is a poor means for recognition and characterization of virion proteins. For both MMNM and MMBB, sequence conservation alone proved the least satisfactory method for predicting phage virion proteins: the BLAST-based predictor recognized only 3 and 6 virion proteins, respectively (Fig 5b, 5d, Supplementary Tables 7, 8). This confirmed the independent test results that the BLAST-based methods commonly used for annotations are a poor means of recognizing and classifying sequence-diverse phage proteins.

Some estimates put the number of phage virions in the world at 10^{31} , suggesting that there is a huge pool of phages that we know little about (39). This encourages a move towards informed bioprospecting for potentially useful phages from under-sampled environments. The effective use of these for therapy and other applications depends on a number of factors, not least of which is the sequence-based choices that must be made to identify novel phages warranting further characterization and potential development into phage therapy. We suggest that application of STEP³ will assist in distinguishing the specific and universal features in phages isolated from under-represented (under-sampled) geographical locations, with impact on the quality of future phage cocktails. Particularly in phage that might be highly divergent in their sequence characteristics, such as the MMNM and MMBB case studies here, STEP³ can predict the component parts of the virions with a confidence level well above other computational tools. The STEP³ toolbox is available at <http://step3.erc.monash.edu/>.

METHODS

Construction of the Klebsiella host strain

B5055 is a multidrug-resistant *K. pneumoniae* (40, 41) strain with a K2-type capsule considered indicative of hypervirulent *K. pneumoniae* (hvKp) (42). To avoid isolating phages that use the major porin for entry into *K. pneumoniae* (33) and, thus circumvent the prospect of phage-resistance acquired by decreased expression of porins (43) and collateral increases in drug-resistant phenotype in the infection (44), we constructed as bait a strain that has no OmpK36. This Δ ompK36 mutant strain of *K. pneumoniae* B5055 was constructed by “gene gorging” as previously described (45, 46) utilizing the donor and helper plasmids described in Supplementary Table 10.

Phage isolation and infection of Klebsiella

Water samples were collected from catchment locations along the Merri Creek in Melbourne, Australia (Reservoir, postcode 3073, yielded MMNM, and Pascoe Vale, postcode 3044, yielded MMBB). Samples were centrifuged at 10,000× *g* for 10 minutes and filtered through a 0.45 µm cut-off filter. Water samples (45 mL) were subsequently mixed with 5 mL of 10× concentrated Luria-Bertani (LB) media and 1 mL of a *K. pneumoniae* B5055 Δ ompK36 overnight culture and grown for a further 16 hours at 37°C. Cellular debris were pelleted by centrifugation at 10,000 × *g* for 10 minutes and the resulting supernatant was passed through a 0.45 µm filter. To monitor phage activity, 20 µL of the supernatant was then spotted onto LB agar plates containing a top layer of soft agar (4 mL LB and 0.35% (w/v) agar) and 200 µL of bacterial culture and incubated overnight at 37°C.

For liquid infections, the filtered supernatant was serially diluted with SM buffer (100 mM NaCl, 8 mM MgSO₄, 10 mM Tris pH 7.5) and added to 200 µL of *K. pneumoniae* B5055 Δ ompK36. Cultures were incubated for 20 minutes at 37°C to allow phage adsorption and were then added to soft agar and poured using the double overlay method. Plaques with distinct morphologies were isolated from the top agar, serially diluted in SM buffer and incubated with the bacterial host as described above. This was repeated 5 times to obtain pure phage stocks.

Phage amplification and purification

For large -amplification of the phages MMNM and MMBB, infections were performed using 14 cm petri dishes with 60 µL of phage preparation (10⁻⁴ dilution) added to 500 µL of an overnight culture and incubated for 20 minutes at 37°C. Ten millilitres of soft agar was then added to the culture and poured using the double agar layer method and incubated overnight at 37°C. Ten

1 millilitres of SM buffer were added to each plate and incubated at room temperature for 10
2 minutes. The soft agar layer was scraped off using a disposable spreader and chloroform was
3 subsequently added (1 mL/100 mL) to lyse bacterial cells to release the phages. The sample was
4 then subject to vigorous shaking, before the agar and bacterial cell debris were removed by
5 centrifugation at $11,000 \times g$ for 40 minutes (4°C). The supernatant containing the phages was
6 collected and DNase (1 $\mu\text{g}/\text{mL}$) and RNase (1 $\mu\text{g}/\text{mL}$) were subsequently added to the
7 supernatant and incubated for 30 minutes at 4°C . NaCl (1 M final concentration) was added and
8 incubated at 4°C for 1 hour with gentle mixing. Phages were precipitated from the media by
9 adding PEG 8000 (10% final concentration) and incubated at 4°C overnight. Precipitated phage
10 particles were collected by centrifugation at $11,000 \times g$ for 20 minutes at 4°C and resuspended in
11 SM buffer (1.6 mL/100 mL of precipitated supernatant). An equal volume of chloroform was
12 added to the resuspended phage suspension to remove residual PEG and cell debris and vortexed
13 for 30 seconds. The organic and aqueous phases were separated by centrifugation at $3,000 \times g$
14 for 15 minutes at 4°C .

15
16 For purification on caesium chloride (CsCl) gradients, the aqueous phase containing the phages
17 was removed and added to CsCl (0.5 g/mL of bacteriophage suspension) and mixed gently to
18 dissolve the CsCl. The suspension was layered onto a discontinuous CsCl gradient (2 mL of 1.70
19 g/mL, 1.5 mL of 1.50 g/mL and 1.5 mL of 1.45 g/mL in SM buffer) in a Beckman SW41
20 centrifuge tube. Gradients were centrifuged at 22,000 rpm for 2 hours (4°C). Phage particles
21 were collected from the gradient by piercing the side of the centrifuge tube with a syringe and
22 removing the visible band in the gradient. Residual nucleic acid was removed from the phage
23 preparation using floatation gradient centrifugation. Equal volumes of phage suspension (500
24 μL) and 7.2 M CsCl SM buffer were mixed and added to the bottom of a Beckman SW41
25 centrifuge tube. CsCl solutions (3 mL of 5 M and 7.5 mL of 3 M) were overlaid on top of the
26 phage sample and centrifuged at 22,000 rpm for 2 hours (4°C). Phage particles were collected
27 ($\sim 500 \mu\text{L}$) using a syringe as described above. CsCl was dialysed out of the phage stock twice
28 with 2 L of SM buffer overnight at 4°C .

29

30 ***Phage growth***

31 One-step growth curve experiments were performed on *K. pneumoniae* as previously described
32 (29). Mid-log-phase cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.5,
33 pelleted, and suspended in 0.1 volume of SM buffer. Phage lysate was subsequently added at a
34 multiplicity of infection (MOI) of 0.01 and was allowed to adsorb for 10 minutes at 37°C .
35 Following centrifugation at $12,000 \times g$ for 4 minutes, the pellet was washed twice with SM
36 buffer, resuspended with 30 mL of fresh LB broth, and incubated at 37°C . Samples were

collected at 10-minute intervals for 120 minutes and titrated to determine PFU/mL. Growth experiments were performed in biological triplicates.

Electron microscopy

From the CsCl-purifications, phage preparations (4 µL) were added to freshly glow-discharged CF200-Cu Carbon Support Film 200 Mesh Copper grids (ProScieTech) for 30 seconds. The sample was blotted from the grid using Whatman filter paper and samples were subsequently stained with 4 µL of Nano W Methylamine Tungstate (Nanoprobes) for 30 seconds and blotted again. Grids were imaged using a 120keV Tecnai Spirit G2 transmission electron microscope (Tecnai).

Genomic DNA extraction, sequencing and annotation

Phage genomic DNA was isolated and samples were sequenced as 2× 250bp paired-end reads using Illumina MiSeq (36). The obtained reads were trimmed using Trimmomatic (47) and *de novo* assemblies of each genome were made using Burrows-Wheeler aligner (48) and Spades (49). The genomes were annotated using Prokka (50). The consensus sequences were then screened against the GenBank database using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), date 29 April 2020. The genome data is available at Genbank with Accession ID: Klebsiella_phage_MMNM (MT894004) and Klebsiella_phage_MMBB (MT894005).

Comparative genome analyses and BLAST

Proteomic trees were constructed using nucleotide genome sequences using the double stranded (ds) DNA nucleic acid type and Prokaryote host category database from ViPTree v1.9 (51) which also included a list of curated phage genomes (Supplementary Table 11). Refined trees were regenerated to analyse the phylogeny of either *Myoviridae* or *Siphoviridae* that infect *Gammaproteobacteria*. Each predicted open reading frame was analysed using BLASTP (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), Pfam HMMER (<https://www.ebi.ac.uk/Tools/hmmer/>) and HHpred (<https://toolkit.tuebingen.mpg.de/>) using the default settings.

A BLAST-based predictor was implemented during the evaluation of STEP³. It ran using blast-2.2.26+ For a query protein, the BLAST-based predictor will predict it to be positive if there is a BLAST hit against the training positive samples with a specified E-value. The E-value was set to 0.01 in this study, optimized on the independent dataset with a range of: 0.001, 0.01, 0.1, 1, and 10.

Mass spectrometry

Each CsCl purified phage sample was solubilized in sodium dodecyl sulfate (SDS) lysis buffer (4% SDS, 100 mM HEPES pH8.5) and sonicated to assist protein extraction. The protein concentration was determined using a BCA kit (Thermo Scientific). SDS was removed according to previous work (52) and the proteins were proteolytically digested with trypsin (Promega) and purified using OMIX C18 Mini-Bed tips (Agilent Technologies) prior to LC-MS/MS analysis. Using a Dionex UltiMate 3000 RSLCnano system equipped with a Dionex UltiMate 3000 RS autosampler, an Acclaim PepMap RSLC analytical column (75 $\mu\text{m} \times 50 \text{ cm}$, nanoViper, C18, 2 μm , 100Å; Thermo Scientific) and an Acclaim PepMap 100 trap column (100 $\mu\text{m} \times 2 \text{ cm}$, nanoViper, C18, 5 μm , 100Å; Thermo Scientific), the tryptic peptides were separated by increasing concentrations of 80% acetonitrile / 0.1% formic acid at a flow of 250 nL/min for 120 minutes and analyzed with a QExactive Plus mass spectrometer (Thermo Scientific) using in-house optimized parameters to maximize the number of peptide identifications. To obtain peptide sequence information, the raw files were searched with Byonic v3.0.0 (ProteinMetrics) against the *K. pneumoniae* B5055 GenBank file FO834906 that was appended with the phage protein sequences. Only proteins falling within a false discovery rate (FDR) of 1% based on a decoy database were considered for further analysis.

Raw data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (53) partner repository with the dataset identifier PXD020607.

Username: reviewer30311@ebi.ac.uk, **Password:** ggYKM6wi

Homology Modelling

Structural homologues were selected by querying the MMBB_78 sequence via the BLASTp webserver against the Protein Databank (PDB). In addition, this same sequence was probed using the Phyre2 software suite to identify local homology (54). Residues 186-872 of MMBB_78 were modelled against the enzymatic domain of the bacteriophage CBA120 tail-spike protein (PDB ID: 5W6P (55)). MODELLER v9.19 (56) was used with custom in-house scripts to generate 1000 potential models. These models were validated and sorted by their Discrete Optimised Protein Energy (DOPE) score, followed by visual inspection. An additional atomic model was calculated by the predictive software GalaxyTBM using the full length MMBB_78 sequence, as part of the GalaxyWEB (57) software suite.

Construction of STEP³

Dataset construction. 481 phage virion proteins were collected from the UniProt database with the “reviewed” tag and from the NCBI database following extensive literature searches.

1 Redundant sequences were removed using the CD-HIT program (58) at a cut-off threshold of 0.4.
 2 As a result, 339 virion proteins with less than 40% sequence similarity were obtained. These
 3 proteins were further divided into two parts as positive samples: 243 in the training dataset and
 4 96 in the independent dataset. For negative samples, 694 and 96 phage non-virion proteins were
 5 collected from UniProt to make up the training and independent datasets, respectively. Finally, a
 6 training dataset (243 positive samples and 694 negative samples) and an independent dataset (96
 7 positive samples and 96 negative samples) were obtained, where each had less than 40%
 8 sequence similarity against each other. The two newly sequenced phage genomes MMNM and
 9 MMBB in this study were used to validate the prediction capability of STEP³ in practical
 10 scenarios.

11 *PSSM generation.* PSSM is a L*20 matrix, where L is the length of its original protein sequence
 12 and 20 is the number of amino acids. The (i, j)-th element ($1 \leq i \leq L$, $1 \leq j \leq 20$) in a PSSM
 13 corresponds to the probability of j-th amino acid to appear in the i-th position of its protein
 14 sequence. To generate a PSSM, blast-2.2.26 resource
 15 (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables>) was used to search the protein sequence against the
 16 UniRef50 dataset (<https://www.uniprot.org/help/uniref>) with an E-value of 0.001 and the
 17 iteration of 3.

18 *Feature encoding.* Instead of extracting features directly from the protein sequences,
 19 evolutionary features mine patterns from a more informative profile in the format of PSSM. Five
 20 types of evolutionary features were generated using the POSSUM toolkit (59), including AAC-
 21 PSSM (60), PSSM-composition (61), DPC-PSSM (60), AADP-PSSM (60), and MEDP (62). For
 22 a given PSSM, their calculations are briefly described as follows: 1) AAC-PSSM generates a 20-
 23 dimensional vector through summing up and averaging all rows of the PSSM (60). 2) PSSM-
 24 composition further divides PSSM rows into 20 groups according to their corresponding amino
 25 acids in the original protein sequence (61). The rows in each group are summed up and
 26 normalized, and as a result the PSSM are transformed into a 20*20 matrix. Converting this
 27 matrix into a vector by row, PSSM-composition finally generates a 400-dimensional vector. 3)
 28 DPC-PSSM generates a 400-dimensional vector $(y_{1,1}, \dots, y_{1,20}, y_{2,1}, \dots, y_{2,20}, \dots, y_{20,1}, \dots, y_{20,20})^T$
 29 through taking into account the local sequence-order effect (60). Among the vector, $y_{i,j}$ can be
 30 calculated by $\frac{1}{L-1} \sum_{k=1}^{L-1} p_{k,i} \times p_{k+1,j}$ where i and j are between 1 and 20, and $p_{k,i}$ denotes the
 31 (k,i)-th element in PSSM. 4) AADP-PSSM combines AAC-PSSM and DPC-PSSM (60) as a
 32 420-dimensional vector. 5) Likewise, MEDP generates a 420-dimensional vector through
 33 combining another two features EEDP and EDP (62). Among them, EEDP generates a 400-
 34 dimensional vector similarly to DPC-PSSM but using different transformation methodologies.

1 EDP further sums up and averages all rows of the EEDP matrix to generate a 20-dimensional
2 vector.
3

4 Additionally, four commonly used features were additionally implemented for comparison
5 purpose, including the amino acid composition (AAC), dipeptide composition (DPC), QSOrder
6 (63) and PAAC (64). AAC and DPC count the frequencies of residues and dipeptides in a protein
7 sequence, respectively. QSOrder and PAAC extract features from a protein sequence as well,
8 incorporating the physicochemical properties of its individual amino acids. Among them,
9 QSOrder adopts Schneider-Wrede physicochemical distance matrix (65) and Grantham's
10 distance matrix (66), while PAAC takes hydrophobicity value from Tanford (67) and from Hopp
11 and Woods (68), as well as amino acid side chain.

12 *Model training on imbalanced data.* Our imbalanced training dataset is to reflect the fact that the
13 number of virion proteins is usually smaller than that of the non-virion proteins in a phage isolate.
14 We combined all of the virion proteins with the same number of randomly selected non-virion
15 proteins to generate a new balanced subset. This procedure was repeated five times, to generate
16 five balanced subsets. For each feature, five individual models were trained based on five
17 balanced subsets, and their prediction scores were averaged to obtain an ensemble model as the
18 baseline model. Support vector machine (SVM) with a radial basis function kernel was used to
19 train each model, implemented by the e1071 package ([https://CRAN.R-](https://CRAN.R-project.org/package=e1071)
20 [project.org/package=e1071](https://CRAN.R-project.org/package=e1071)) in the R language (<https://www.r-project.org/>). The two parameters
21 of SVM, including the Cost and Gamma, were optimized by a grid search between 2^{-10} and 2^{10}
22 with a step of 2^1 using the same R package.

23 *Model integration.* Training a model with each of features and then integrating them as an
24 ensemble model usually have better and more robust performance, when compared with simply
25 training a model with all features (69). Accordingly, the five baseline models (corresponding to
26 five evolutionary features) were further integrated as the final ensemble model of STEP³ through
27 averaging their prediction scores (Fig 1a).

28 *Performance evaluation.* The STEP³ predictor was extensively validated, with the baseline
29 models and existing state-of-the-art tools on the 5-fold cross-validation and independent tests.
30 Five performance metrics were used, including sensitivity (SN), specificity (SP), accuracy
31 (ACC), F-value and Matthews correlation coefficient (MCC) (70). For each model, 5-fold cross-
32 validation tests were conducted 5 times based on the 5 balanced training datasets, and then the
33 performance metrics were averaged as the final performance result. The other tools compared to
34 STEP³ were iVIREONS (<https://vdm.sdsu.edu/ivireons>), PVPred ([14](http://lin-</p>
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1 group.cn/server/PVPred), PVP-SVM (<http://www.thegleelab.org/PVP-SVM/PVP-SVM.html>),
2 PVPred-SCM (<http://camt.pythonanywhere.com/PVPred-SCM>) and Pred-BVP-Unb (21). With
3 no available tool for Pred-BVP-Unb, we developed one based on our training dataset by strictly
4 following its methods, including its synthetic minority oversampling technique (SMOTE) to
5 cope with the imbalance dataset, feature encodings, feature selection (a more generalized
6 method GainRatio used) and the same grid search for parameter optimization. The prediction
7 threshold for Pred-BVP-Unb is a standard cut-off of 0.5, which is the same as STEP³.
8
9 *Server construction and usage.* The STEP³ server contains a client web interface and a server
10 backend. The client web interface was implemented by the JAVA (<https://www.java.com/>)
11 server development suite, JSP, CSS, jQuery (<https://jquery.com/>), Bootstrap
12 (<https://bootstrapdocs.com/>) and their extension packages. The server backend was used by the
13 Perl CGI (<https://metacpan.org/pod/CGI>). For visualization purposes, the blast 2.8.1+
14 (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/2.8.1/>) was used to search each predicted
15 virion protein against known virion proteins to generate sequence similarities, which was
16 visualized by BlasterJS (71). The MAFFT v7.271 (<https://mafft.cbrc.jp/alignment/software/>) was
17 used to generate multiple alignment results between each predicted virion protein and known
18 virion proteins, which was visualized by jsPhyloSVG (72). The all-against-all BLAST (version
19 blast-2.2.26) was used to generate the sequence similarity network, visualized by ECharts
20 (<https://echarts.apache.org/>). A queuing system was implemented using the Gearman framework
21 (<http://gearman.org/>) to store the jobs the client deposits and dispatch them to idle threads
22 maintained in the server backend. In this way, it links the two parts of STEP³, but decouples the
23 prompt-response required in a client web interface and the time-consuming server backend for
24 better user experience. To use the STEP³ server, users submit their protein sequences in FASTA
25 format, and obtain a unique link to track the prediction progress or obtain the results once
26 finished. In default mode, i.e. ‘For normal use’, the known virion proteins were marked with
27 ‘exp.’ with an external link to the UniProt or NCBI database, while the predicted virion proteins
28 were marked with ‘pred.’ with detailed annotations and options for visualization. Through
29 interactive visualization, users could tentatively annotate the putative virion proteins with their
30 potential subtype or functions, based on the sequence similarity or phylogenetic analysis
31 considerations. For users who want to benchmark the STEP³ server, a ‘For benchmarking test’
32 option is available to obtain prediction scores for all their sequences.
33

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36

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6 Merri-merri-uth nyilam marra-natj (MMNM) and Merri-merri baany-a bundha-natj (MMBB)
7 translate as “Dangerous Merri lurker” and “Merri water biter”, respectively, in English. Our
8 future work in this field will be pursued according to a Memorandum of Understanding (MoU)
9 between the Monash Centre to Impact AMR and the Wurundjeri Woi wurrung Cultural Heritage
10 Aboriginal Corporation (<https://www.wurundjeri.com.au/>) the peak body representing the
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24

25 **AUTHOR CONTRIBUTIONS**

26

27 TTY, MEW and JJW performed biological experiments. WD and YZ performed computational
28 experiments. DW, RSB and SMcG performed structural calculations and modelling, and RSB
29 performed electron microscopy. EJ, JJB, AR, C.J.S., CH and RS analyzed data. JW, RAD and TL
30 supervised project, analyzed data and wrote the paper. All authors contributed critical evaluation
31 to the final version of the manuscript.

32

33 **COMPETING INTERESTS**

34

35 The authors declare no competing interests.

1 FIGURE LEGENDS

2

3 **Figure 1. Construction and workflow for STEP³.** (a) Graphic summarizing the construction
4 and prediction process of STEP³. A set of experimentally validated virion proteins and non-
5 virion proteins was compiled and sequence data fed into five PSSM models, including AAC-
6 PSSM(60), PSSM-composition(61), DPC-PSSM(60), AADP-PSSM(60), and a MEDP(62)
7 model. The five individual models were trained based on five balanced subsets, and their
8 prediction scores were averaged to obtain an ensemble model. Finally, five baseline models
9 (corresponding to five evolutionary features) were further integrated as the final ensemble model
10 of STEP³ through averaging their prediction scores. Support vector machine (SVM) with a radial
11 basis function kernel was used to train each model. This ultimately provides a prediction of a
12 “virion protein” which would be a structural component of the phage virion. (b) STEP3 data
13 visualization provides a means to document relationships between a protein of interest. The
14 example given is the protein component gpE from phage λ , which shows clear similarity to
15 major capsid proteins from other phages. Structural studies confirm that despite limited sequence
16 similarity, gpE is part of a family of major capsid proteins(9). Alternative visualization features
17 are available in STEP³ (Supplementary Fig. 1).

18

19 **Figure 2. Prediction details from STEP³ and other tools.** (a) For phage vB_EfaS_271,
20 horizontal bars denote the number of virion and non-virion proteins. The bar chart counts the
21 virion proteins correctly retrieved as true positives (TP), i.e. confirmed by mass spectrometry
22 (26), and non-virion proteins mistakenly predicted as virion proteins (denoted by false positives,
23 FP). (b) For each protein in the phage vB_EfaS_271 virion defined by mass spectrometry, a
24 green circle represents a successful hit by a predictor. (c) For phage vB_PatM_CB7, the bar chart
25 counts the virion proteins correctly retrieved as TP and non-virion proteins mistakenly predicted
26 as FP. (d) Detailed predictions from STEP³ and other tools for vB_PatM_CB7 virion proteins
27 defined by mass spectrometry (27). (e) For phage vB_Eco4M-7, the bar chart counts the virion
28 proteins correctly retrieved as TP and non-virion proteins mistakenly predicted as FP. (f)
29 Detailed predictions from STEP³ and other tools for vB_PatM_CB7 virion proteins defined by
30 mass spectrometry (28).

31

32 **Figure 3. Comparative genome analysis of *Klebsiella* phage MMNM.** (a) Proteomic tree
33 analysis of *Myoviridae* that infect *Gammaproteobacteria*. The branch lengths represent genomic
34 similarity based on normalised pairwise sequence similarity scores plotted on a logarithmic
35 scale. The tree was constructed using sequences from the default ViPTree dataset and phage
36 genomes listed in Table S13. Viral subfamilies or genera are highlighted in the coloured bars.

1 Gray bars represent phages that are currently unclassified. All known members of the
2 *Jedunavirus*, including *Klebsiella* phage MMNM (*), are highlighted in red. (b) Whole genome
3 alignment of *Klebsiella* phage MMNM, vB_KpnM_FZ14 and vB_KpnM_KpV52. Each genome
4 has been oriented to start with the gene encoding the putative tape measure protein. The
5 sequences are linked by colored bars highlighting sequence identity values as shown in the key.

6

7 **Figure 4. Morphological characterization of phage MMNM and MMBB.** (a) Plaque
8 morphology analysis was performed using the double overlay method. Phages MMNM and
9 MMBB were serially diluted with SM buffer and spotted onto LB agar plates containing a top
10 layer of soft agar and *K. pneumoniae* B5055 $\Delta ompK36$. Plaque morphologies of MMNM and
11 MMBB were determined after overnight incubation at 37°C. Scale bars represent 10 mm. (b)
12 One-step growth curve of MMNM (left) and MMBB (right) was performed by co-incubation
13 with the host strain for 10 min at 37°C for phage adsorption, after which the mixture was
14 subjected to centrifugation to remove free phage particles. The resuspended cell-phage pellets
15 were incubated at 37°C and sampled at 10 min intervals for 120 min. L, latent period; B, burst
16 size. Data points are the mean of n=3 biologically independent samples and the error bars are the
17 standard deviation. (c) Transmission electron micrographs of MMNM (left) and MMBB (right).
18 The scale bars represent 100 nm. (d) Based on EM micrographs, illustrations of MMNM (left)
19 and MMBB (right) note the cognate features in *Myoviridae* and *Syphoviridae* with annotation.

20

21 **Figure 5. Prediction details from STEP³ and other tools applied to MMNM and MMBB.** (a)
22 The statistics of the prediction results on MMNM. Horizontal bars on top describe the number of
23 virion and non-virion proteins in the phage isolates. The bar chart counts the virion proteins
24 correctly retrieved (denoted by true positives [TP], i.e. confirmed by mass spectrometry) and
25 non-virion proteins mistakenly predicted as virion proteins (denoted by false positives [FP]). (b)
26 Detailed predictions from STEP³ and other tools for MMNM the virion proteins defined by mass
27 spectrometry. The green circles represent a successful hit by a predictor. The green stars denote
28 the proteins that have not previously been identified in phages. The red stars denote those with
29 activities that have been previously identified in phages, but not previously found as protein
30 components of purified virions. (c) Prediction statistics for MMBB. (d) Detailed predictions
31 from STEP³ and other tools for MMBB virion proteins defined by mass spectrometry.

SUPPLEMENT

Supplementary Figure 1. Sequence analysis and data visualization of the major capsid protein from λ phage.

Supplementary Figure 2. Comparative genome analysis of *Klebsiella* phage MMBB.

Supplementary Figure 3. Structure informed analysis of *Klebsiella* phage MMBB virion components.

Supplementary Table 1. Prediction performance of STEP³ and baseline models on the 5-fold cross-validation test.

Supplementary Table 2. Prediction performance of STEP³ and baseline models on the on the independent test.

Supplementary Table 3. Prediction performance of STEP³, other available predictors and the BLAST-based baseline predictor on the independent dataset.

Supplementary Table 4. Annotation of *Klebsiella* phage MMNM genome.

Supplementary Table 5. Annotation of *Klebsiella* phage MMBB genome.

Supplementary Table 6. Mass spectrometry and STEP³ analysis of *Klebsiella* phage MMNM virions.

Supplementary Table 7. Detailed prediction of STEP³, other available predictors and the BLAST-based baseline predictor on the phage *Klebsiella* phage MMNM.

Supplementary Table 8. Detailed prediction of STEP³, other available predictors and the BLAST-based baseline predictor on the phage *Klebsiella* phage MMBB.

Supplementary Table 9. Mass spectrometry and STEP³ analysis of *Klebsiella* phage MMBB virions.

Supplementary Table 10. Strains, plasmids and primers used in this study.

Supplementary Table 11. Genomes of phages used for tree analysis.

Phage: vB_EfaS_271
Host: *Enterococcus faecalis*

Phage: vB_PatM_CB7
Host: *Pectobacterium* spp.

Phage: vB_Eco4M-7
Host: *Escherichia coli* O157

a **b**

MMNM

MMBB

a

b

c

d

a

c

b

d