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Research Article

Agtrevirus phage AV101 recognizes four different O-antigens infecting diverse *E. coli*

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Abstract

Bacteriophages in the *Agtrevirus* genus are known for expressing multiple tail spike proteins (TSPs), but little is known about their genetic diversity and host recognition apart from their ability to infect diverse *Enterobacteriaceae* species. Here, we aim to determine the genetic differences that may account for the diverse host ranges of *Agrevirus* phages. We performed comparative genomics of 14 *Agtrevirus* and identified only a few genetic differences including genes involved in nucleotide metabolism. Most notably was the diversity of the *tsp* gene cluster, specifically in the receptor-binding domains that were unique among most of the phages. We further characterized agtrevirus AV101 infecting nine diverse Extended Spectrum β-lactamase (ESBL) *Escherichia coli* and demonstrated that this phage encoded four unique TSPs among *Agtrevirus*. Purified TSPs formed translucent zones and inhibited AV101 infection of specific hosts, demonstrating that TSP1, TSP2, TSP3, and TSP4 recognize O8, O82, O153, and O159 O-antigens of *E. coli*, respectively. BLASTp analysis showed that the receptor-binding domain of TSP1, TSP2, TSP3, and TSP4 are similar to TSPs encoded by *E. coli* prophages and distant related virulent phages. Thus, *Agtrevirus* may have gained their receptor-binding domains by recombining with prophages or virulent phages. Overall, combining bioinformatic and biological data expands the understanding of TSP host recognition of *Agtrevirus* and give new insight into the origin and acquisition of receptor-binding domains of *Ackermannviridae* phages.

Keywords: Bacteriophages; *Ackermannviridae*; *Agtrevirus*; host range analysis; tail spike proteins; O-antigen receptors

Abbreviations

ESBL:	Extended Spectrum β-Lactamase
ETEC:	Enterotoxigenic *E. coli*
HMdU:	Hydroxymethyluracil
LB:	Luria–Bertani
LPS:	Lipopolysaccharide
NAMPT:	Nicotinamide phosphoribosyl transferase
NCBI:	National Center for Biotechnology Information
PFU:	Plaque formation unit
RPPK:	Ribose-phosphate pyrophosphokinase
TEM:	Transmission electron microscopy
TSP:	Tail spike protein
VriC:	Virulence-associated protein

Introduction

The recent establishment of genome-based taxonomy of phages has led to the classification of an increasing number of phage families and genera (Lefkowitz et al. 2018). Thus, based on genetic similarity, the *Ackermannviridae* family was established in 2017 and currently includes two subfamilies (*Cvivirinae* and *Aglimvirinae*) and 10 genera (*Kuttervirus*, *Agtrevirus, Limestonevirus Taipeivirus*, *Tedavirus*, *Nezavisimistyvirus*, *Miltonvirus*, *Campanilevirus*, *Vapseptimavirus*, and *Kujavirus*) (Kropinski et al. 2017, Adriaenssens et al. 2018). In the *Ackermannviridae* family, phages exhibit a conserved genome architecture including gene synteny in genomes of substantial size of ∼150 kb. In addition, these phages encode hydroxymethyluracil (HMdU) synthase leading to substitution of thymine with hydroxylmethyl uracil in their genomes (Adriaenssens et al. 2012b, Kutter et al. 2011, 2012a, Hsu et al. 2013). This nucleotide substitution has been suggested to prevent cleavage of the phage genomes by host-encoded restriction enzymes, which has been proposed to allow *Ackermannviridae* phages to infect a broad range of *Enterobacteriaceae* species (Adriaenssens et al. 2012b, Kutter et al. 2011, 2012a, Hsu et al. 2013).

The most well-studied feature of *Ackermannviridae* phages is their receptor binding properties arising from expressing up to four diverse tail spike proteins (TSPs). These four TSPs form a complex protruding from the baseplate in a star-like morphology that can be visually observed in transmission electron micrographs (TEMs) (Adriaenssens et al. 2012a, Plattner et al. 2019). The TSPs are hinged together in a complex, and to the baseplate, by protein interactions between the conserved N-terminal modules of each TSPs (Plattner et al. 2019, Chao et al. 2022). In contrast, the receptor-binding domain of these TSPs are highly diverse and binds to polysaccharide receptors like the O-antigen or K-antigen through conserved folds including a β-helix commonly observed for TSPs in the *Caudoviricetes* order (Barbirz et al. 2008, Andres et al. 2010, Lee et al. 2017a, Olszak et al. 2017, Prokhorov et al. 2017,

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Kunstmann et al. 2018). As the phages express multiple TSPs, each capable of binding to a specific O-antigen or K-antigen, their host ranges are broader compared to phages only expressing a single TSP targeting such polysaccharide receptor (Steinbacher et al. 1996, Plattner et al. 2019, Sørensen et al. 2021, Witte et al. 2021). For example, *Kuttervirus* CBA120 infects *Salmonella* O21, *Escherichia coli* O157, *E. coli* O77, and *E. coli* O78 strains through the specific binding of TSP1, TSP2, TSP3, and TSP4, respectively (Plattner et al. 2019). Both *E. coli* and *Salmonella* are common hosts for *Kuttervirus* and *Agtrevirus* phages, but these bacteria express more than 185 and 46 diverse O-antigens, respectively (Liu et al. 2014, 2020). Thus, *Ackermannviridae* phages match the diversity of O-antigens expressed the bacterial hosts by encoding diverse TSP. For example, we previously analyzed 374 TSPs encoded by 99 *Ackermannviridae* phages and found 96 diverse TSP subtypes each carrying unique receptor-binding domains (Sørensen et al. 2021). Furthermore, the TSP subtypes were strongly associated with phage genera. In addition, further analysis allowed us to predict the host recognition of TSP subtypes encoded by phages of the *Kuttervirus*, *Limestonevirus*, and *Taipei* genera, but due to lack of biological data, no information of *Agtrevirus* phages could be revealed (Sørensen et al. 2021).

The *Agtrevirus* genus is not very well studied in terms of host range and receptor recognition. The phages are known to infect Gram-negative bacteria like *Salmonella*, *E. coli*, *Enterobacter*, and *Shigella*. But, unlike other members of the *Ackermannviridae* family, such as phages in *Kuttervirus* genus that infect both *E. coli* and *Salmonella*, *Agtrevirus* phages are only known to infect one bacterial species each (Anany et al. 2011, Heyse et al. 2015, Soffer et al. 2016, Akter et al. 2019, Thanh et al. 2020, Kwon et al. 2021, Imklin et al. 2022). We recently isolated agtrevirus AV101 infecting Extended Spectrum β-Lactamase (ESBL) *E. coli* (Vitt et al. 2023). A large host range analysis of 198 ESBL *E. coli* strains showed that *Agtrevirus* phage AV101 had a narrow host range only infecting 9 of 198 strains tested (Vitt et al. 2023). In addition, we previously observed that five *Agtrevirus* phages express unique TSPs without similarity to other phages in the genus or *Ackermannviridae* family (Sørensen et al. 2021). Thus, the diverse hosts infected by *Agtrevirus* phages may be due to genetic differences between the phages including unique receptor-binding domains of the TSPs.

Here, we investigated the genetic diversity of the growing number of phages belonging to the *Agtrevirus* genus and further characterized the host binding capabilities of agtrevirus phage AV101. Whole-genome comparison of 14 phages in the *Agtrevirus* genus showed that the *tsp* gene cluster represented the most diverse region. *In silico* analysis of the TSPs showed that the receptor-binding domains of phage AV101 were not observed in any *Agtrevirus* phages, but the receptor-binding domains share amino acid sequence similarity towards other phages and prophages. We further determined the host recognition of the four TSPs which correlates with the O-antigens of the bacterial hosts. Our work expands the understanding of TSP host recognition in the *Agtrevirus* genus and give new insight into exchange of receptor-binding domain between phages in different families and genera.

Material and methods

Bacterial strains and phages

All phage genomes used for genomic analysis are presented in Table S1 (Supporting Information). A list of the bacterial strains used in the study is presented in Table S4 (Supporting Information).

Bioinformatic analysis

All genomes of phages from the *Agtrevirus* genus and unclassified *Aglimvirinae* subfamily were extracted from NCBI. Phylogenetic analysis of all *Agtevirus* phages was performed using whole genomes sequence in CLC genomics version 22 (Qiagen) with default settings (date 04/01–23). To align and visualize all the genomes as well as the *tsp* gene cluster Easyfig version 2.2.5 or CLC genomics were used (Sullivan et al. 2011). 0.4 minimum identity was chosen as the BLAST setting. To identify homolog TSPs of the four TSPs of AV101 (TSP1: WJJ54142.1, TSP2: WJJ54143.1, TSP3: WJJ54145.1, and TSP4: WJJ54146.1), BLASTp was used with standard settings. Afterwards, the genomes identified was used to analysis of prophage in the *E. coli* genomes and was carried out with PHAge Search Tool Enhanced Release (PHASTER) with standard settings (Arndt et al. 2016).

Phage propagation

Agtrevirus AV101 was propagated on *E. coli* strain ESBL058 as described earlier (Sørensen et al. 2021). A single colony of *E. coli* ESBL058 was inoculated into LB media (Lysogeny Broth, Merck, Darmstadt, Germany) and incubated until exponential phase at 37°C at 180 rpm. A previous phage stock of AV101 (1.6×10^{10} PFU ml^{-1}) was 10-fold diluted followed mixing 100 µl of the dilutions with 100 µl of the ESBL058. On a LA plate (LB with 1.2% agar), 4 ml of molten top agar (LBov; LB broth with 0,6% Agar bacteriological no.1, Oxoid) was applied. 5 ml of SM buffer (0.1 M NaCl, 8 mM MgSO47H2O, 50 mM Tris-HCl, pH 7.5) was added to the plates after an overnight incubation at 37°C, and the plates were then incubated at 4°C at 50 rpm. The overnight samples were collected, centrifuged for 15 min at 11 000 rpm, then passed through a 0.2-M filter. By using a phage plaque assay (described below), the new phage stock was used to estimate the phage titre.

Phage DNA isolation

(Gencay et al. 2019) 1 ml of phage lysate was filtered three times with 0.22 µM syringe filters. RNase (10 µg ml^{-1}) and DNase (20 µg ml^{-1}) were added to the filtered lysate and incubated for 1 h at 37°C. The degradation was stopped by adding sterile EDTA (pH 8) at a final concentration of 20 mM. Afterwards Proteinase K was added (50 µg ml^{-1}) and incubate for 2 h at 56°C followed by cooling the sample to room temperature. To isolate the phage DNA, we used Genomic DNA clean and concentrator™ (Zymo research) following the manufactures instructions. The DNA concentration was measured using Qubit 2.0 Fluorometer (Invitrogen).

Transmission electron microscopy

To visualize phage AV101 with transmission electron microscopy we used a previously described method (Ackermann 2009). Briefly, bacteriophages from high-titer stock were sedimented at 12 000 *g* for 60 min at 4°C and washed three times with Ammonium Acetate (0.1 M, pH 7). Final sediment was used for imaging. 200 mesh copper coated carbon grids (Ted Pella, Inc.) were made hydrophilic by glow discharging the grids using a Leica Coater ACE 200 for 30 s at 10 mA. A volume of 6 µl of phages at a PFU ml^{-1} of 10^{12} were pipetted on the grids and incubated for 30 s. All liquid was removed with a Whatman filter paper. The phages were stained by incubating the grid with 6 µl of 2% uranyl acetate for 30 s. In a washing step, 6 µl of ddH2O was pipetted on the grid, incubated for 30 s, and removed with a Whatman paper. The phages where imaged using a CM100 microscope with a Bio TWIN objective lens and a LaB6 emitter. Pictures were taken with an Olympus Veleta

camera and analyzed using ImageJ to determine phage particle measurements.

Phage host range analysis

The newly prepared phage stock (3.4×10^{12} PFU ml^{-1}) was used to determine the host range of AV101 (Gencay et al. 2019). Bacterial strains were inoculated into 5 ml LB medium (Lysogeny Broth, Merck) and grown for 5 h. Afterwards, 100 µl of the strain were suspended into 4 ml of top-agar and poured onto LB plates left to solidify (~30 min). 10-fold serial dilutions of the phage stock were prepared, and three times of 10 µl of each dilution were spotted on the bacterial lawn. Subsequently the spots were dried the plates were incubated overnight at 37°C. The next day, dilutions with single plaques were counted to calculate the PFU ml^{-1}.

TSP cloning

Purified AV101 genomic DNA was used as a template for *tsp* cloning using *in vivo* assembly cloning (García-Nafría et al. 2016, Sørensen et al. 2021). The tsp genes [*tsp1*- (2202 bp.), *tsp2*- (2289 bp.), *tsp3*- (1947 bp.), and *tsp4*- (3279 bp.)] were individually amplified with Phusion™ High-Fidelity DNA polymerase (Thermo Scientific™) following the manufacture instructions with primers carrying homologous overhangs to the expression vector pET-28a (+). Furthermore, the expression vector with primers to linearize the vector were also added to each PCR reaction. The primers amplifying the *tsp* genes would allow for homologous recombination between the HinCII and Eco52KI restriction sites in the multiple cloning site of the vector. Primers (Table 1) were ordered from TAG Copenhagen A/S. Furthermore, the cloning site in the pET-28a (+) also express a his-tag upstream so the expressed TSPs could be purified with affinity chromatography. In order to degrade the parental pET28a-(+) plasmid, 1 µl of FastDigest DpnI enzyme (Thermo ScientificTM) was added to the PCR reactions after the amplification of the *tsp* genes and the expression vector. Each of the PCR samples were transformed into competent *E. coli* StellarTM cells (Takara Bio). The next day, pET_AV101_TSP1-4 extracted with GeneJET Plasmid Miniprep Kit (Thermo Scientific™). Vectors carrying the correct *tsp* insert (pET_AV101_TSP1-4) were confirmed by Sanger sequencing (Eurofins Genomics).

TSP purification

pET_AV101_TSP1-4 were transformed into electrocompetent *E. coli* BL21 cells for the expression of the TSPs (Sørensen et al. 2021). A single colony of each of transformants carry one of the four pET_AV101_TSP1-4 was inoculated into LB medium with 50 µg ml^{-1} kanamycin and incubated overnight at 37°C and 170 rpm. Next day, 10 ml of the starter culture was added to 1 l of LB medium with 50 µg ml^{-1} kanamycin at 37°C and incubated until an OD_{600} value of 0.6. Then, a final concentration of 0.5 mM of isopropyl-b-D-thiogalactopyranoside was added to induce protein expression. The culture was incubated for an entire night at a reduced temperature of 16°C. The culture was centrifuged the following morning for 10 min at 13 000 *g*, and the pellet was then resuspended in 9 ml of lysis buffer (0.5 M NaCl, 20 mM Na2HPO4, 50 mM Imidazole, pH 7.4). Sonication was used to disrupt the cells, with a program of nine cycles lasting 30 s each at 80% power. Centrifugation at 9500 *g* for 30 min at 4°C separated cell debris. Using 0.22 mm filters, the supernatant containing the expressed proteins was filtered. HisGraviTrapTM (GE Healthcare) was then used to purify the proteins using an elution buffer (0.5 M NaCl, 20 mM Na_2HPO_4, 0.5 M Imidazole, pH 7.4). To transfer the TSPs into a new buffer [20 mM HEPES (pH 7.4)], Amicon Ultra-15 Centrifugal Filter Units with a 50 kDa cutoff (Merck Milipore) were utilized. Using a Qubit 2.0 Fluorometer and the QubitTM Protein Assay Kit, protein concentration was determined (Invitrogen).

TSP spot assay and inhibition assay

The TSP spot and inhibition assay was performed as previously described (Sørensen et al. 2021). Shortly, bacterial strains were grown to an OD_{600} value of 0.6. A volume of 100 µl of the bacterial culture was then added to 4 ml top-agar and poured onto an LB agar plate. After the bacterial lawns were solidified, 1.5 µg of the four TSPs were spotted onto the bacterial lawn and left to dry for 30 min. Phage AV101 and the protein buffer [20 mM HEPES (pH 7.4)] were used as a positive and negative control, respectively. The plates were incubated overnight at 37°C and the next day the presence of a translucent zone was evaluated. To further validate the spot assay, the inhibitory effect of the TSPs on the infectivity of the bacterial strains were evaluated. The four strains ESBL-038, -040, -058, and -144 were used as they represented strains that TSP1-4 recognize, respectively. A single colony of the bacterial strains was incubated in LB medium at 37°C at 170 rpm until OD_{600} reached 0.3. The cells were then cooled on ice before 100 µl of the cells were added to 5 mg of the TSPs in individually tubes. The cell-TSP suspension was then preincubated at 37°C for 20 min. Afterwards, the suspension was added to 4 ml top agar and poured onto an LB agar plate and left to solidify. Three times 10 µl phage AV101 dilutions (10^{-1}–10^{-8}) was spotted on top of the plate and incubated overnight at 37°C. Next day, the inhibitory effect of the TSPs were evaluated by comparing the PFU ml^{-1} of the phage sensitive strains with the PFU ml^{-1} with strains incubated with the respective TSPs. The inhibition assay was carried out in triplicates and the results were visualized in Graphpad Prism9 with the mean standard deviations shown in the figure.

Alphafold2 prediction

Models for the TPSs were predicted in a Nvidia Quadro RTX 8000 using Alphafold2-multimer (version 3.2.1) (Jumper et al. 2021, Evans et al. 2022). We utilized global search for the multiple sequence alignment, five recycling rounds, and the amber relaxation was skipped. The best model was ranked based on the iptm+ptm score and was the one selected for the analysis.

Results

Comparative genomics of *Agtrevirus* phages

Agtrevirus phages are known to infect species within the *Enterobacteriaceae* family like *Shigella*, *Salmonella*, *Enterobacter*, and *E. coli* including ESBL, however, each phage are only known to infect one of the species (Akter et al. 2019, Thanh et al. 2020, Kwon et al. 2021, Imklin et al. 2022, Vitt et al. 2023). To investigate the genetic differences that may account for such diverse host ranges, we extracted all *Agtrevirus* genomes as well as unclassified *Aglimvirinae* genomes, not to miss any potential *Agtrevirus* phages (Table S1, Supporting Information). We did not include the unclassified *Aglimvirinae Dickeya* phages: phiDP10.3 and phiDP23.1 as they have previously been suggested to belong to the *Limestonevirus* genus in the *Aglimvirinae* subfamily (Czajkowski et al. 2015). We confirmed that all phages in the genus as well as the unclassified phages belonged to *Agtrevirus* by aligning all genomes and demonstrate an overall average nucleotide identity (ANI) between 86% and 97% (Figure S1, Supporting Information). As phages PH4 and PC3 are 99.99% identical, the same phage may have been isolated twice. Phylogenetic analysis using whole

Table 1. Overview of primers for cloning of the four *tsp* genes.

Primers	Sequences (5'–3')
AV101_TSP1_F	GCAAGCTTGTCGACGGAGTTACTGGATAACAATACTACACTCCATATCTGAAACAAC
AV101_TSP1_R	CGCGGATCCGAATTCGAGATGAACGAAATGTTCTCACAAGGTGGTAAAG
AV101_TSP2_F	GCAAGCTTGTCGACGGAGTTAATTACTAATCTTACCACGTATGGATAGCTCAAGAGC
AV101_TSP2_R	CGCGGATCCGAATTCGAGATGACCAGAAACGTCGAGAGCATC
AV101_TSP3_F	GCAAGCTTGTCGACGGAGTTATAGCTGGCAAGTAAAGCTCAAGTCTATTGATGC
AV101_TSP3_R	CGCGGATCCGAATTCGAGATGATTTCTCAATTCAATCAACCACGCGG
AV101_TSP4_F	GCAAGCTTGTCGACGGAGTTATATTACTGACGTCACAGTTCCAGATATAGACGC
AV101_TSP4_R	CGCGGATCCGAATTCGAGATGGCTAATAAACCAACACAGCCTGTTTTC
pET-28a(+)_F	CTCGAATTCGGATCCGCG
pET-28a(+)_R	CTCCGTCGACAAGCTTGC

genome sequences demonstrated grouping of phages infecting *Salmonella* and *Shigella*, whereas *E. coli* phages formed a separate group with *Enterobacter* phage fGh-Ecl02. In contrast, *Enterobacter* phage EspM4VN and *Salmonella* phage SNUABM-02 did not group with any other phages (Fig. 1A).

To further investigate the genetic differences of the *Agtrevirus* phages, we aligned all genomes using EasyFig (Sullivan et al. 2011). Overall, the phage genomes showed similar genetic organization, and all encode HMdU transferase genes used for synthesis of hydroxylmethyl uracil replacing thymine as previously described (Fig. 1B; Table S2, Supporting Information) (Adriaenssens et al. 2012a,b, Hsu et al. 2013). Interestingly, other nucleotide metabolism genes like nicotinamide phosphoribosyl transferase (NAMPT) and ribose-phosphate pyrophosphokinase (RPPK) were detected in eight of the 14 phage genomes analyzed (Fig. 1B; Table S2, Supporting Information). These genes were associated with the group of phages classified as *Salmonella* and *Shigella* phages, but also in *E. coli* phage AV101 and *Enterobacter* phage EspM4VN. Beside genes involved nucleotide metabolism, we observed diversity within genes encoding homing nucleases, yet with no corelation to the phylogenetic groups (Fig. 1B; Table S2, Supporting Information). Several other genes varied between phages, but all were annotated as hypothetical proteins. Finally, the gene cluster encoding TSPs expected to be responsible for host recognition exhibited the highest diversity (Fig. 1B). Our overall genomic analysis showed that the *Agtrevirus* phages grouped into two phylogeny groups with two outliners and that the phages are highly conserved, except for the *tsp* gene cluster.

Agtrevirus phages encode highly diverse TSPs

To further analyse the diversity of TSPs of *Agtrevirus* phages, we compared the *tsp* gene cluster of the 14 phages (Fig. 2A). While most *Agtrevirus* phages, like AV101, encode four *tsp* genes, phage ZQ1 and pSal-SNUABM-2 only encode two *tsp* genes. In phage AV101, we further confirmed the presence of multiple TSPs by performing transmission electron microscopy showing the expected star-like complex protruding the baseplate (Fig. 2B).

Analyzing the *tsp* sequences in more detail demonstrated that most *tsp* genes were similar in the N-termini, which are known to be conserved within phage genera due to their importance for the TSP complex formation and hinging to the baseplate (Plattner et al. 2019, Chao et al. 2022) (Fig. 2A). Furthermore, we observed a small region of nucleotide identity in the N-termini immediately upstream the β-helix responsible for receptor binding in *tsp1*, *tsp3*, and *tsp4* genes of some phages (Fig. 2A). This small region of nucleotide identity coincides with the tandem repeat domain previously suggested as a location for recombination between receptor-binding domains of *tsp* genes of *Kuttervirus* phages (Sørensen et al. 2021).

Even though comparative genomics revealed two groups of *Agtrevirus* (Fig. 1A), no apparent correlation between *tsp* genes and phylogenetic groups were observed. For instance, the group of *E. coli* phages encode diverse *tsp* genes expect from phage PH4 and PC3 (99.9% identical) and phage RPN242 that encode identical *tsp* clusters (Figs 1A and 2A; Figure S1, Supporting Information). In the group of *Shigella* and *Salmonella* phages, *Shigella* phage Ag3 encode *tsp4* with similarity to *Salmonella* phage P46FS4 and *tsp1* with similarity to *Shigella* phage ChubbyThor (Fig. 2B). Interestingly, the region coding for the receptor-binding domain of ChubbyThor *tsp2* was similar to the receptor-binding domain of *Shigella* phage Gloob *tsp1* gene (Fig. 2A). Thus, within this group, some of the *tsp* genes are similar but overall, most of the receptor-binding domains of the *tsps* are diverse. Furthermore, aligning all *tsp1*, *tsp2*, *tsp3*, and *tsp4* genes individually confirmed that *Agtrevirus* phages mainly express receptor-binding domains unique to each phage, thus suggesting a total of 35 different receptor recognitions (Fig. 2C). In summary, our analysis showed that most *Agtrevirus* phages encode *tsp* genes that only show N-terminus sequence similarity.

The TSPs of AV101 recognize specific O-antigens of *E. coli* hosts

The unique receptor-binding domains of *Agtrevirus* TSPs suggest that they recognize different bacterial receptors. To further investigate host recognition of *Agtrevirus*, we used AV101 as an example. AV101 was previously shown to infect 9 out of 198 ESBL *E. coli* strains tested (Vitt et al. 2023). To further investigate the host range, we spotted a serial dilution of phage AV101 on the *E. coli* ECOR strain collection ($n = 72$) as well as representative strains of *Salmonella enterica* subspecies Derby, Typhimurium, Enteritidis, Seftenberg, Anatum, Odersepoort, and Minnesota. None of the tested strains could be infected by phage AV101, as no single plaques could be observed by performing a standard plaque assay (data now shown). The host range of AV101 was thus limited to the previous identified nine *E. coli* hosts (Fig. 3A and B).

To identify the receptors of the individual TSPs encoded by *Agtrevirus* phage AV101, we cloned, expressed, and purified the four TSPs and spotted each of them on the nine known *E. coli* hosts. After incubation we noted if the TSPs were able to degrade the O-antigen by forming translucent zones on the bacterial lawns (Fig. 3B; Figure S2, Supporting Information). By this analysis, we observed a correlation between the TSP type and the O-antigen encoded by the individual bacterial hosts as TSP1, TSP2, TSP3, and TSP4 formed translucent zones only on O8, O82, O153, and O159 *E. coli* strains, respectively (Fig. 3B). Yet, TSP1 were not able to make translucent zones on the *E. coli* strains

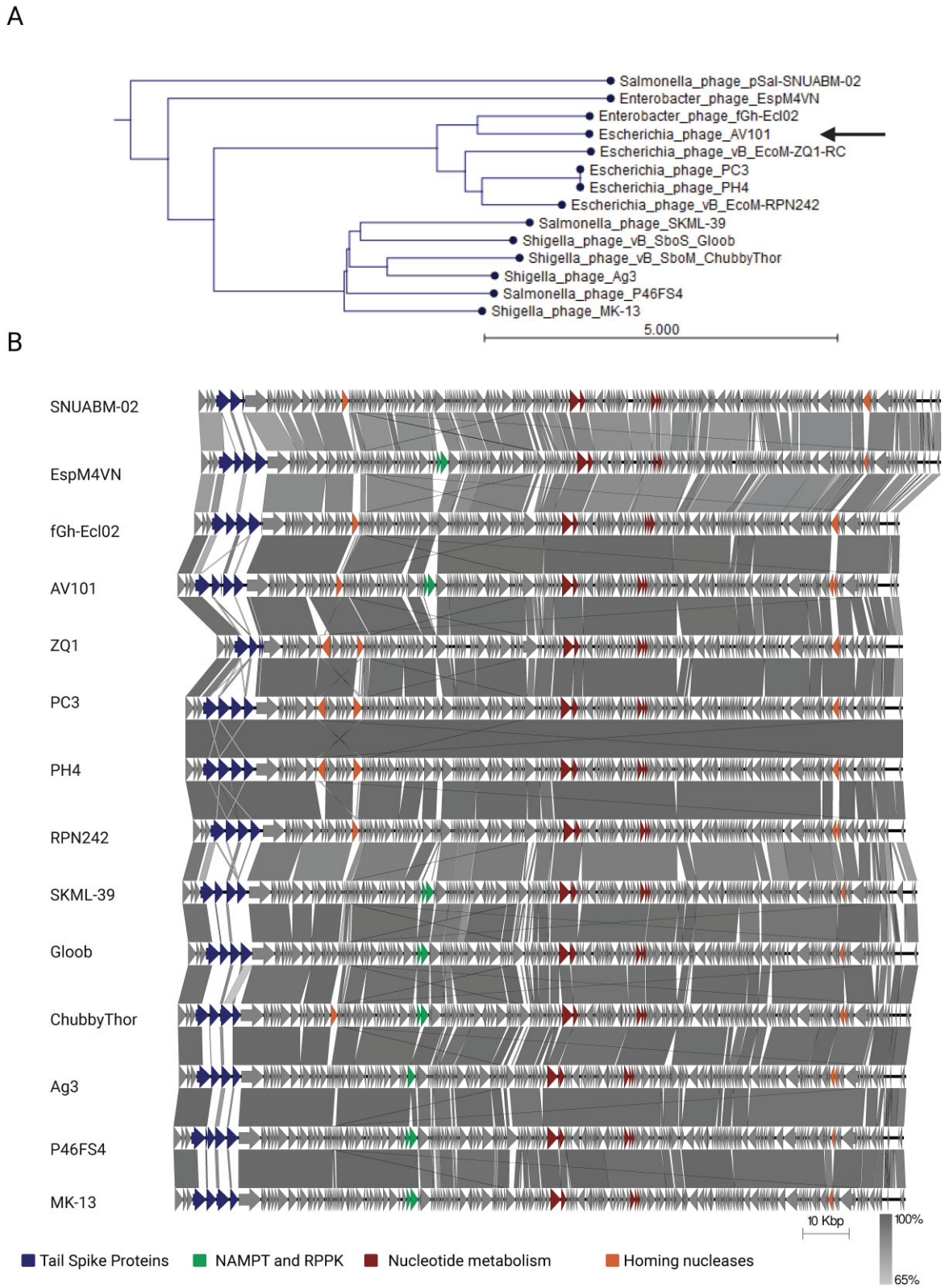


Figure 1. *Agtrevirus* phages are genetic similar with a few expectations. (A) ANI phylogenetic analysis of phage AV101 and the other 13 extracted *Agtrevirus* phages using CLC workbench 22. The analysis showed that AV101 closes relative is Enterobacter phage fGh-Ecl02. (B) Based on the phylogenetic analysis the *Agtrevirus* genomes were aligned using EasyFig. Genetic differences are highlighted with colours; Genes encoding nicotinamide phosphoribosyl transferase (NAMPT) and ribose-phosphate pyrophosphokinase (RPPK) (green), other nucleotide metabolism genes (red), homing nucleases (orange), and the *tsp* gene cluster (blue). Phage genomes and the variable genes are listed in Table S2 (Supporting Information).

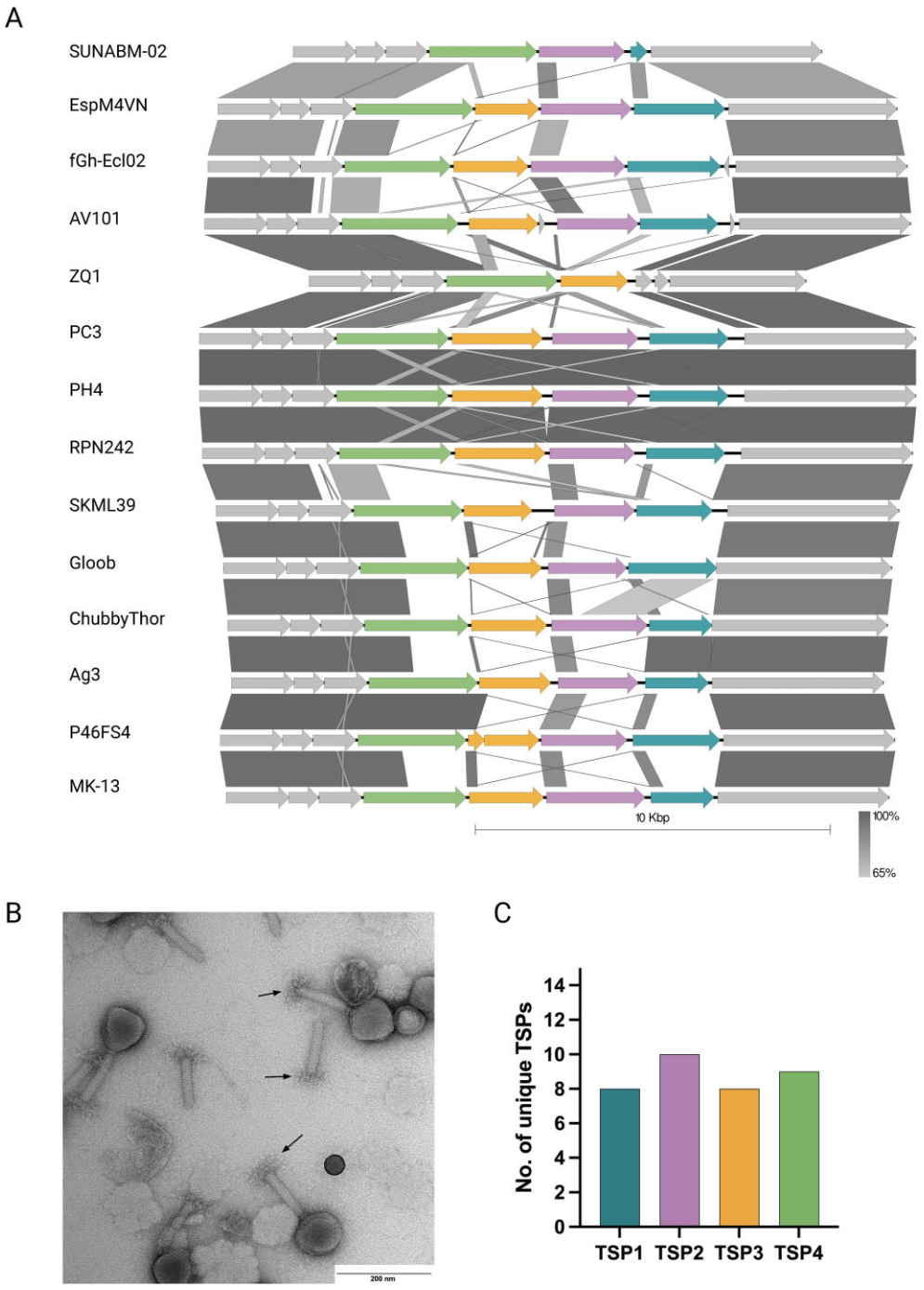
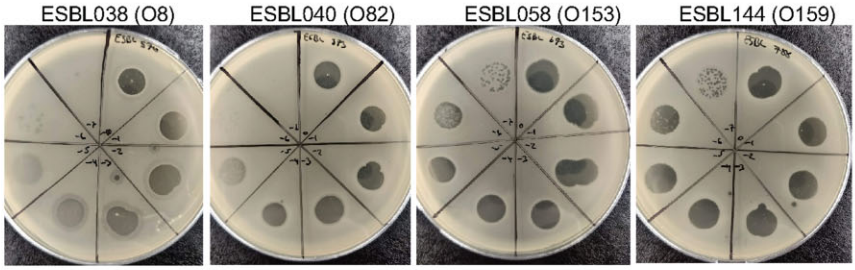


Figure 2. The majority of *tsp* genes in *Agtrevirus* phages encode unique receptor-binding domains. (A) Alignment of the *tsp* gene cluster located between the Virulence associated gene (*VriC*) and baseplate wedge genes (grey) of all available *Agtrevirus* phages showed high diversity of the *tsp* genes among the phages. Colours; Turkish: TSP1, purple: TSP2, yellow: TSP3, green: TSP4, and grey: hypothetical proteins, baseplate wedge, and *VriC*. B) TEM photo of AV101 showing virion morphology similar to other *Agtrevirus* phages. The arrows points to the distinctive star-like TSP complex. (C) All *tsp1*, *tsp2*, *tsp3*, and *tsp4* genes were individually aligned and the number of dissimilar genes of *Agtrevirus tsp* genes were visualized plotted.

ECOR7 and ECOR72 expressing O8 O-antigen (data not shown). Moreover, phage AV101 is not able to infect these strains either, which may be due to modification of the O-antigen (Knirel et al. 2015, Liu et al. 2020). Surprisingly, TSP3 also formed a translucent zone on O8 hosts although smaller compared to TSP1 (Figure S2, Supporting Information), suggesting that TSP3 may be able to degrade both O153 and O8 antigens, even though the two O-antigens does not share any similarity in their sugar composition (Liu et al. 2020).

To show that binding of the individual TSPs to specific *E. coli* O-antigens are important for AV101 infection, we carried out an inhibition assay. For this experiment, we chose ESBL038 (O8), ESBL040 (O82), ESBL058 (O153), and ESBL144 (O159) to represent hosts recognized by TSP1, TSP2, TSP3, and TSP4, respectively. The strains were grown to exponential phase and mixed with the individually TSPs, allowing the TSPs to bind to and degrade their receptor before plating to form a lawn. Afterwards, AV101 were spotted on the lawn to evaluate the ability of the phage to access the receptor

A



B

Host	I.D.	O-antigen	AV101	TSP1	TSP2	TSP3	TSP4
E. coli	ESBL038	O8	+	+	-	(+)	-
E. coli	ESBL048	O8	+	+	-	(+)	-
E. coli	ESBL040	O82	+	-	+	-	-
E. coli	ESBL079	O82	+	-	+	-	-
E. coli	ESBL082	O82	+	-	+	-	-
E. coli	ESBL058	O153	+	-	-	+	-
E. coli	ESBL078	O153	+	-	-	+	-
E. coli	ESBL089	O153	+	-	-	+	-
E. coli	ESBL144	O159	+	-	-	-	+
E. coli	MG1655	-	-	-	-	-	-

C

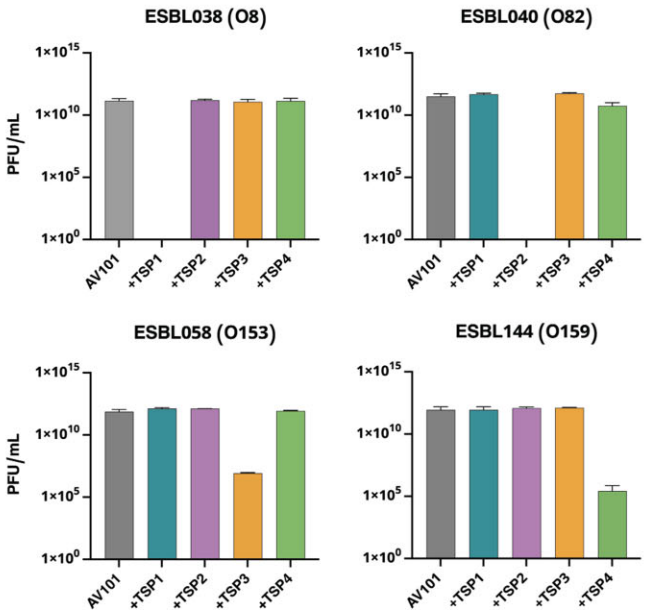


Figure 3. The four TSPs of AV101 recognize different O-antigens of ESBL *E. coli*. (A) Phage AV101 infection on different bacterial hosts showing diverse plaque morphologies. (B) Successful phage infection or detection of a clear translucent zone on bacterial lawns are indicated with plus sign. TSP3 makes small translucent zones on two *E. coli* strains expressing the O8 O-antigen (+). Big clear translucent zone is indicated with a + sign. Small translucent zone is indicated with (+) sign. (C) Inhibition of AV101 infectivity on ESBL *E. coli* host after individual TSPs was preincubated with the ESBL hosts. TSP1 and TPS2 were able to completely block the infection of AV101 on their respective hosts, whereas only a partial inhibition with TSP3 and TSP4 could be observed.

and subsequently form plaques. TSP1 and TSP2 completely abolished the infection of AV101 on ESBL038 and ESBL040, respectively (Fig. 3C). In contrast, TSP3 and TPS4 had an inhibitory effect on infection of ESBL058 and ESBL144, respectively, leading to ∼5-log reduction of the phage titre (Fig. 3C). The differences in the ability of the TSPs to inhibit infection may suggest different kinetics of the enzymatic activity of the receptor-binding domains. Finally, while we observed small translucent zones on ESBL038 (O8 O-antigen) when spotting TSP3, the protein did not inhibit infection of phage AV101 of ESBL038. This suggest that TSP3 does not degrade the O8 O-antigen but may degrade another surface polysaccharides of this strain, thus forming a translucent zone unrelated to O-antigen degradation. Overall, our results demonstrate phage AV101 express four TSPs that are unique for the *Agtrevirus* genus and each of the TSPs recognize distinct O-antigens required for infection of *E. coli*.

The receptor binding module of phage AV101 TSPs show similarity to prophages and virulent phages

While we only observed little *tsp* sequence similarity between AV101 and other *Agtrevirus* phages, it is known that the receptor-binding domain of TSPs may be subjected to horizontal gene transfer between phages from distant related families (Pires et al. 2016, Latka et al. 2019, Sørensen et al. 2021). Thus, to further investigate if the receptor-binding domain could be found in other phages than *Agtrevirus*, we extracted the amino acid sequence and conducted a BLASTp analysis of the four TSPs of phage AV101. TSP1, TSP2, and TSP3 did not show overall similarity to any virulent phage genomes except for the conserved N-termini of TPSs of *Ackermannviridae* phages (data not shown). Instead, similarities in the C-terminal were found to *E. coli* genome sequences, suggesting that these TSPs share sequences of the receptor-binding domain to diverse prophages (Fig. 4A; Table S3, Supporting Information). To identify and further classify the corresponding prophages, we extracted and analyzed these *E. coli* genomes using PHASTER (Arndt et al. 2016). Indeed, the analysis identified prophages in all the *E. coli* genomes that were either intact or questionable (Table S4, Supporting Information), suggesting that these prophages express TSP with similar receptor-binding domains. Furthermore, we investigated if the TSPs shared receptor-binding domains in prophages encoded by our own ESBL *E. coli* strain collection ($n = 198$). However, none of the four TSPs shared similarity to prophages in our collection (data not shown). In contrast, TSP4 showed similarity to virulent phages infecting *E. coli* as well as *Salmonella* including kuttervirus LPST94, kayfunavirus ST31, and phapecoctavirus Ro121c4YLVW (Fig. 4B). The phages ST31 and Ro121c4YLVW infects *E. coli* and are only distantly related to *Agtrevirus*, whereas LPST94 belongs to *Ackermannviridae* and infects *Salmonella enterica* subspecies (Liu et al. 2018, Yan et al. 2020, Khalifeh et al. 2021). Still, the receptors recognized by these TSPs have not been identified (Yan et al. 2020). To get a better understanding of the similarity in relation to the structural domains, we used Alphafold2-multimer to predict the structure of the four TSP of AV101 (Fig. 4B). All the TSPs shared a modular fold, with an anchor domain in the N-terminus, and a receptor-binding domain close to the C-terminal. Beside the β-helix, the N-terminal head-binding domains of the TSPs had a low prediction score (Figures S3 and S4, Supporting Information). When we compared the alignment of TSPs and the structures, we observed that the amino acid similarity coincides with the β-helix carrying the receptor-binding domain (Fig. 4A and B). Thus, we expect that the prophages and virulent phages bind to the same O-antigen receptors as the TSPs of phage AV101.

Discussion

With the rise of genome-based phage taxonomy, the investigation of biological functions of a single phage can provide a general understanding of other phages within the same family or genus (Turner et al. 2021). *Agtrevirus* phages infect different *Enterobacteriaceae* species, but not much is known about the genetic differences between the phages allowing them to infect such diverse hosts (Akter et al. 2019, Thanh et al. 2020, Kwon et al. 2021, Imklin et al. 2022). Receptor-binding proteins are responsible for the initial binding to the bacterial hosts and the characterization of such proteins can provide important biological information, crucial for understanding phage host ranges. Here we investigated phages belonging to the *Agtrevirus* genus and observed that while all phages show high nucleotide identity and a similar genome structure, the genes encoding TSPs were highly diverse. Furthermore, as an example, we investigated the specific host recognition of the four TSPs encoded by *Agtrevirus* phage AV101 previously isolated on ESBL *E. coli* (Vitt et al. 2023).

To investigate similarities and difference among *Agtrevirus* phages, we carried out a comparative *in silico* analysis of all available *Agtrevirus* genomes. Besides observing diversity in the *tsp* gene region, we also observed that eight of the phages encoded NAMPT and RPPK. These genes have also been observed in distant related phages including *Babavirus* Baba19a, *Schizotequatrovirus* KVP40, and polybotosvirus Atu_ph7 phages (Lee et al. 2017b, Attai et al. 2018, Nilsson et al. 2019). RPPK convert ribose 5-phosphate and ATP to phosphoribosyl pyrophosphate (PRPP) and AMP. PRPP is a precursor of purine and pyrimindine that is used by ribonucleotide reductases, also encoded by all *Agtrevirus* phages, for nucleotide production during the phage replication (Nilsson et al. 2019). Given that not all *Agtrevirus* phages encode these genes, they may not be required for phage genome replication but provide other advantages, yet to be discovered, during certain conditions or when infecting specific hosts.

Previously, we have characterized TSPs encoded by phages of the *Ackermannviridae* family and observed that receptor-binding domains were conserved between phages especially within the *Kuttervirus* genus (Sørensen et al. 2021). In contrast, we showed that the receptor-binding domain are not as well conserved within the *Agtrevirus* genus, as most phages encode unique domains, indicating specific host recognition. Instead, our protein alignment and AlphaFold2 predictions showed that the receptor-binding domain of phage AV101 TSPs share sequence similarity and are structural similar to distant related virulent phages as well as prophages. Likewise, the receptor-binding domain of TSP1 of kuttervirus CBA120 show nucleotide similarity to TSP encoded by a prophage found in a *Salmonella* Minnesota strain (Plattner et al. 2019) and nucleotide similar receptor-binding domain sequences were identified in kuttervirus Det7 and temperate lederbergvirus P22 (Walter et al. 2008). Thus, more receptor-binding domains of TSPs found in the *Ackermannviridae* family may share receptor binding abilities as prophages suggesting exchange of structural domains between phages and prophages. Furthermore, the receptor-binding domain of TSP4 of AV101 had amino acid sequence similarity to TSP1 encoded by phage *Kuttervirus* LPST94, demonstrating exchange of receptor-binding domains between phages of different genera within the *Ackermannviridae* family. So far, host range analysis demonstrated that phage LPST94 infects several *Salmonella enterica* subspecies expressing different O-

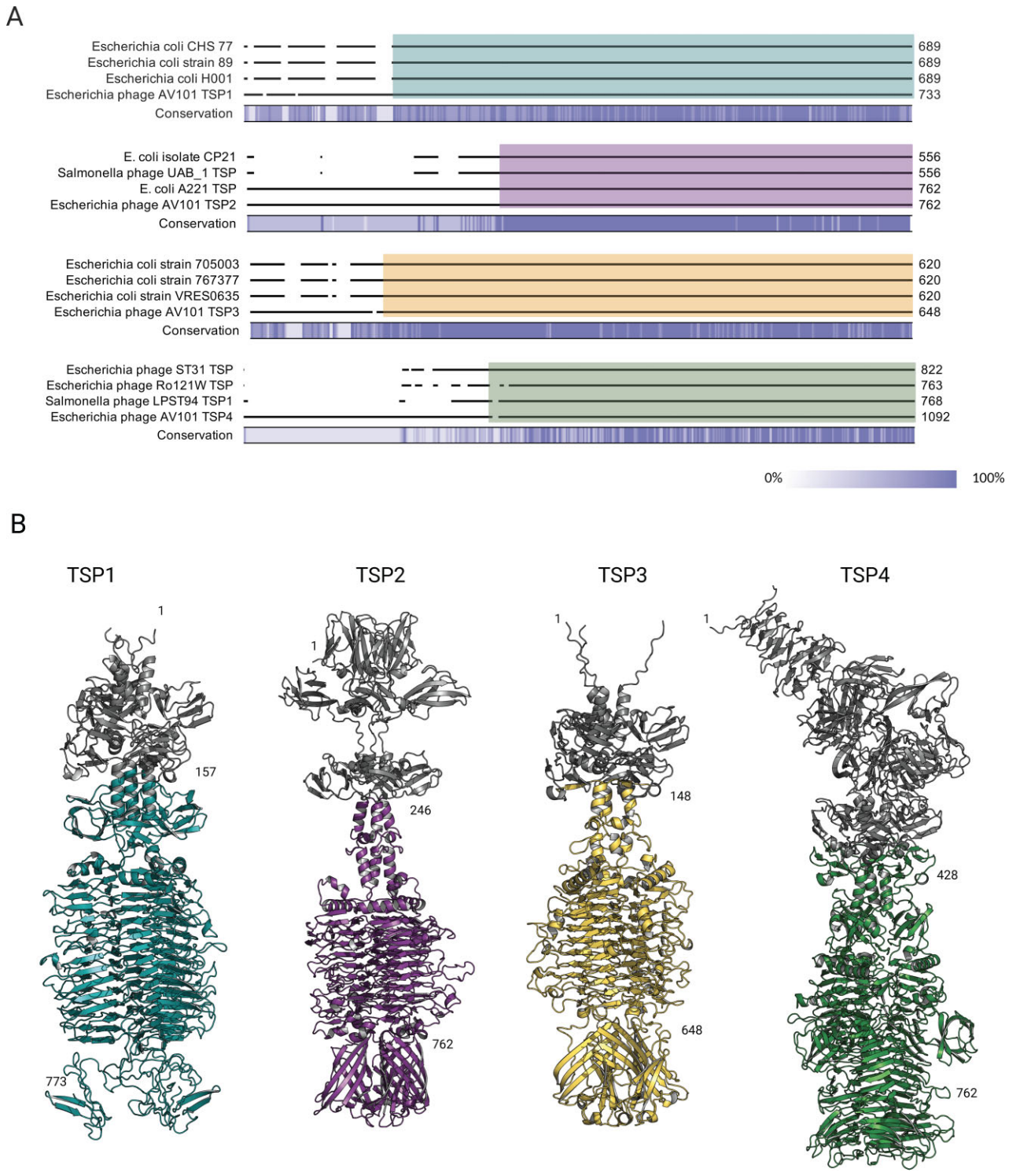


Figure 4. The receptor-binding domain of AV101 TSPs show similarity to prophages and distant-related lytic phages. (A) BLASTp analysis of the four TSPs showed that TSP1, TSP2, and TSP3 share similarity towards proteins found in *E. coli* strains. Furthermore, TSP2 and TSP4 had similarity towards distant-related lytic phages. A detailed overview of the sequence similarity and the accession numbers of the BLASTp hits can be found in Table S3 (Supporting Information). The coloured boxes represent the region of similarity visualized in the structures. (B) AlphaFold 2 was used to prediction and visualize the four TSPs of AV101. The four TSPs folds like other known TSPs with the conserved β-helix serving as the receptor-binding domain. Moreover, the analysis showed that the amino acid sequence similarity to other TSPs were found in the receptor-binding domains, thus are highlighted in the same colours as in (A). The grey colour represents the N-termini structural domains; TSP1 amino acids 1–157; TSP2 amino acids 1–246; TSP3 amino acids 1–148, and TSP4: amino acids 1–428.

antigens (Yan et al. 2020), yet phage AV101 could not infect any of these *Salmonella enterica* subspecies. In addition, phage LPST94 could infect six *E. coli* strains, but information about their O-antigens was not included in the study (Yan et al. 2020). Still, our study suggests that TSP1 of phage LPST94 may recognize O8 O-antigen, but it remains to be experimental verified. Overall, combining bioinformatic, structural predictions and biological data of phage AV101 TSPs gives an insight into the origin and acquisition of the receptor binding abilities of *Ackermannviridae* phages.

ESBL *E. coli* are of high concern as they encode ESBLs conferring resistance to antibiotics such as penicillin and cephalosporins commonly used in treatment human infections (Paterson and Bonomo 2005, Benz et al. 2021). To propose alternative solutions targeting ESBL *E. coli*, we recently established a collection of phages, including phage AV101, infecting ESBL *E. coli* (Vitt et al. 2023). Using this collection, we composed phage cocktails that prevented growth of ESBL *E. coli*, thus suggesting that phages may indeed be promising alternative antimicrobials (Vitt et al. 2023). Phage AV101 was not included in these cocktails, but may be used to target ESBL *E. coli* expressing O8, O82, O153, and O159 O-antigens due to the specificities of the four TSP. It should be noted though, that AV101 did not infect two other *E. coli* strains tested (ECOR7 and ECOR72) carrying the O8 O-antigen, suggesting that internal defence mechanism or modification of the O-antigen, like glycosylation or acetylation may influence phage infection (Knirel et al. 2015, Egido et al. 2022). While we have tested AV101 on ESBL *E. coli* strains, the four O-antigens are indeed expressed in diverse pathogenic and commensal *E. coli* strains, hence AV101 most likely also infect commensal *E. coli* strains expressing the same four O-antigens. (Tamaki et al. 2005a, Marin et al. 2022). For example, O159 O-antigen are often expressed by enterotoxigenic *E. coli* (ETEC) causing diarrhoea in humans (Tamaki et al. 2005b, Linnerborg et al. 1999), suggesting that AV101 may be used in phage therapy or biocontrol of such strains. Thus, identification of receptor recognition of AV101 may allow design of phage applications dedicated to pathogenic *E. coli* like O159 ETEC or O8, O82, and O153 *E. coli* including ESBL-producing *strains*.

Purified TSPs have also demonstrated promising potential as therapeutics or diagnostic tools (Wang et al. 2023). For instance, oral administration of purified TSP of *Lederbergvirus* phage P22 dramatically decreased *Salmonella* colonization in the chicken gut and reduced penetration into internal organs (Waseh et al. 2010). TSPs from bacteriophages 9NA and P22 have been used as tools to detect *S. typhimurium* (Schmidt et al. 2016) and TSP3 of kuttervirus Det7 was used as a biosensor using surface plasmon resonance to detect *S. typhimurium* (Hyeon et al. 2020). Thus, instead of using phage AV101 as an antibacterial agent, AV101 TSPs targeting these specific O-antigens on *E. coli* or O159 of ETEC strains could be of interest. Furthermore, other phage-based solutions for combating pathogenic bacteria based on exploring the knowledge of binding abilities TSPs proteins may be developed. For example, pyocins are phage tail-like particles encoded by *Pseudomonas aeruginosa* showing bactericidal activity against other *Pseudomonas* strains (Ge et al. 2020). Such pyocins have been engineered to successful kill other bacteria by exchanging the receptor binding protein of the native pyocin with phage receptor binding proteins (Williams et al. 2008, Scholl et al. 2009). Similarly, TSPs targeting specific O-antigens, like those encoded by AV101, may be used to retarget pyocins to other hosts. TSPs may also be utilized for developing Innolysins, which are novel antibacterials fusing a receptor binding protein or domain to an endolysin, allowing the engineered enzyme to target gram negative bacteria (Zampara et al. 2020). So far Innolysins have been created and shown to kill specifically *Campylobacter jejuni* or commensal *E. coli* (Zampara et al. 2020, 2021), and the use of TSP4 could allow development of novel Innolysins targeting *E. coli* O159 strains including ETEC. Thus, investigation of the receptor recognition of TSPs is not only crucial for understanding the host range of phages but can be further exploited for the development of therapeutics against pathogenic bacteria.

Author contribution

Anders Nørgaard Sørensen (Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing—original draft, Writing—review & editing), Dorottya Kalmár (Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing—review and editing), Veronika Theresa Lutz (Investigation, Formal analysis, Writing—review and editing), Victor Klein-Sousa (TSPs modelling, Writing—review & editing), Nicholas M. I. Taylor (Writing—review & editing), Martine C. Sørensen (Conceptualization, Visualization, Writing—review & editing, Funding acquisition), Lone Brøndsted (Conceptualization, Project administration, Supervision, Visualization, Writing—review & editing, Project administration, Funding acquisition).

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Supplementary data

Supplementary data is available at *FEMSML Journal* online.

Conflict of interest: The authors declare no competing interests.

Data availability

The genome sequence data of phage AV101 has been submitted to the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/nuccore/) under accession number OQ973471.

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