Title: Phageome transfer from gut to circulation and its regulation by human immunity

Aleksander Szymczak[1*], Katarzyna Gembara[1,3], Stanisław Ferenc[3], Joanna Majewska[1], Paulina Miernikiewicz[1], Marek Harhala[1], Izabela Rybicka[1], Dominik Strapagiel[2], Marcin Słomka[2], Jakub Lach[2], Jan Gnus[3,4], Paweł Stączek[5], Wojciech Witkiewicz[3], Krystyna Dąbrowska[1,3,6*]

1. Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland
2. Biobank Lab, Department of Oncobiology and Epigenetics, University of Lodz, Lodz, Poland
3. Research and Development Center, Regional Specialist Hospital, Wroclaw, Poland
4. Wroclaw Medical University, Wroclaw, Poland
5. Department of Molecular Microbiology, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland
6. Faculty of Medicine, Wroclaw University of Science and Technology, Wroclaw, Poland

* correspondence to aleksander.szymczak@hirszfeld.pl and krystyna.dabrowska@hirszfeld.pl

Abstract

Certain parts of the human body, like the gut, serve as ecological niches where bacteriophages thrive. In contrast, the blood is not typically expected to host phageomes. However, bacteriophages have been shown to translocate into the bloodstream. The factors determining why some phages within phageomes translocate efficiently while others do not remain unclear.

We investigated the translocation of the phageome from the gut mucosa to the bloodstream using bioptates of large intestine mucosa collected via endoscopy (VLPs fraction) and serum samples (total cfDNA) from 38 human patients. We also performed immunoprecipitation of a phageome-wide epitope library covering the structural proteome of phages, followed by massive sequencing to identify phage epitopes targeted by patients' IgGs.

We found that Tequatroviruses and Kayviruses were the predominant phage groups in both the gut mucosa and blood of the patients. However, the overall correlation between the gut phageome composition and the phages present in the blood was moderate. Additionally, environmental sources appeared to contribute to the phages found in the blood. Phage translocation from the gut to blood likely involved epithelial transcytosis followed by lymphatic filtration, the latter limiting the translocation dynamics. Also, high levels of phage-specific IgGs negatively

correlated with the presence of targeted phage antigens in blood, indicating that these antibodies were a limiting factor for translocating phageome.

These observations suggest that fundamental factor, such as phage concentration in the gut, influence the composition of the circulating blood phageome. However, the blood phageome also includes phages that are low represented in the gut or originate from environmental sources. Further, immunological factors play a significant role, including the limiting effect of lymph node filtration and the exclusion effects of specific antibodies targeting certain antigens within phage populations. We believe that these observations contribute to the general understanding of phageome-body interactions and to the practical use of phages, such as antibacterial therapies.

Introduction

Phages constitute the largest known part of the microbiomes, with an estimated number of approximately 10^{31} bacteriophages existing worldwide (Bakhshinejad & Ghiasvand, 2017)positioning them as the most abundant biological entities on Earth. Originally, this hypothesis was derived from data pertaining to microbiotas in ocean environments. However, a revised calculation in 2016 yielded a virus-to-microbe ratio (VMR) ranging from 2.6 up to 160. These revised ratios far exceed earlier estimates that suggested a tenfold higher abundance of viruses compared to their bacterial hosts. Widespread bacteriophage host-virus relationship and its transformation to prophage forms play a pivotal role in regulating bacterial biomass, sustaining biodiversity, and facilitating biogeochemical cycles within Earth's biosphere.

Likewise, phages serve as a critical factor in the human microbiome, specifically in the intestinal tract. The density of phages in the large intestine ranges between 10^8 and 10^{10} phage virions per gram of feces (Guerin & Hill, 2020; Sutton & Hill, 2019; Zuppi et al., 2021). The gut phageome refers to the community of phages present in this region. Extensive research has demonstrated the abundant presence of phages in the gut, indicating their importance. These phages engage in complex interactions with the gut bacterial community, influencing microbial diversity, population dynamics, and functional capabilities. By selectively infecting and control-

ling specific bacterial species, phages contribute to the maintenance of microbial balance and stability. Additionally, phages facilitate horizontal gene transfer among gut bacteria, potentially influencing the evolution and adaptation of these microbial populations. Understanding the intricate role of phages in the gut is crucial for comprehending the dynamics of the gut microbiome and its implications for human health (Breitbart et al., 2008). Bacteriophages can persist in the environment, exhibiting two main lifestyles: lytic and lysogenic. The predominant lifestyle observed in gut phages is the lysogenic one (Benler et al., 2021; Manrique et al., 2017). Even though most gut phages adopt a lysogenic lifestyle, persisting as prophages within bacterial cells and becoming active under specific environmental conditions, their abundance in the gut remains remarkable.

Studies aiming to identify the most abundant phage groups within the gut microbiome in the majority related to previous phage taxonomy based on phage morphology; most of the bacteriophages identified in the gut belonged to Caudovirales and represented Siphoviridae, Podoviride, and Myoviridae families (Lin & Lin, 2019; Townsend et al., 2021). Most often isolated VLPs (Virus-like particles) targeted bacterial hosts like *Escherichia/Shigella*, *Enterococcus* sp, and *Clostridioides*. Studies that employed high throughput analysis of phageome with Next-Generation Sequencing revealed 80%-95% of unknown (not annotated previously nor unassigned to any taxonomic group or bacterial host) phage sequences (Barr et al., 2013a, 2013b, 2015; Benler et al., 2021; Wu et al., 2012).

In addition to the well-known body niches inhabited by phages, such as the gut, which harbors a multitude of bacteria, phages have also been observed in body areas where a normal bacterial microbiome is absent, such as the bloodstream. The concept of translocation suggests that phages originating from the gut or mucosal surfaces could breach host barriers, enter the bloodstream, and disseminate to various organs, and indeed, phages have been observed in blood and other tissues many times (Dąbrowska, 2019; Majewska et al., 2019). Barr and his group investigated mechanisms underlying this phenomenon and they demonstrated that bacteriophages could adhere to and be transported across epithelial cells (transcytosis). This suggests that phages can exploit cellular machinery to traverse host barriers(Barr et al., 2013a; Bichet et al., 2021). They further showed that phages could be encapsulated within intracellular

vesicles, thus being protected during transit (Van Belleghem et al., 2019) . Phages in the blood stream seem to some extent depend on systemically dispersed bacteria. When in the blood of septic patients, phages showed specific patterns associated with different pathogens (Bichet et al., 2021; Haddock et al., 2023). The translocation is linked to immune responses as expected effect of systemic dissemination of phages.

Immune responses have been identified as a crucial factor governing phage activity within the human body (Dąbrowska & Abedon, 2019). Conversely, the gut showcases a prominent phenomenon known as immunological tolerance. Immunological tolerance in the gut has a profound impact on the microbiota. By maintaining a state of tolerance, the immune system allows the microbiota to thrive, promoting microbial diversity and ecological balance. Disruption of immunological tolerance, such as in inflammatory bowel disease (IBD), can lead to dysbiosis and an altered microbial composition, exacerbating disease progression (Barr et al., 2013a; Belkaid & Hand, 2014; Round & Mazmanian, 2009). The gut employs several mechanisms to establish and maintain immunological tolerance. These mechanisms include the induction of regulatory T cells (Tregs) (Belkaid & Hand, 2014), the presence of a mucosal barrier that physically separates the microbiota from the underlying immune cells, and the production of secretory IgA antibodies that prevent the attachment of microbiota to the gut epithelium (MacPherson et al., 2018). However, it remains unclear whether translocating bacteriophages induce specific immune responses or if they benefit from the phenomenon of tolerance. Studies have shown that phages can translocate from the gut to systemic circulation, but this process is influenced by factors such as phage dose, morphology, and host physiological status (Dąbrowska, 2019). Particularly, the efficiency of translocation and immunoreactivity of phages in the context of the whole phageome has not been recognized so far.

Here we report a high throughput study of phageome translocation from mucosal surfaces in the human gut to circulation, along with identification of specific immune responses to translocating phages. The study involves human donors of large intestine mucosal bioptates and blood serum, and comparisons between phageome composition identified in the mucosa and blood serum of the same person. We identified major phage antigens that were recognized by specific IgGs in these patients. We sought to understand the phenomenon of translocated phages akin

to the age-old question of which came first, the chicken or the egg: does the presence of phage induce antibodies that effectively neutralize the phage, thereby preventing the detection of targeted phages in blood samples containing specific IgGs? Or conversely, must phages be present in the blood to sustain detectable levels of phage-specific IgGs?

Materials and Methods

Biological samples

Biopsies from the colon mucosa were collected in the Endoscopy Laboratory of the Regional Specialist Hospital in Wrocław (Poland) by an expert physician from adult patients. These patients were also blood donors, with blood collected into clotting tubes, and blood serum separated and stored. The biopsies were performed following hospital procedures and guidelines to ensure accurate diagnosis and treatment of gastrointestinal conditions. Patients were recruited as qualified for colonoscopy examination due to adverse symptoms from the GI tract; Also, patients who in the interview reported antibiotic treatment in last six months or diabetes treated with metformin were excluded from the study. The group of patients included 22 women and 16 men (average age as 61, N=38) (Figure S1).

Bioethics statements

The study was conducted in accordance with the principles of the Declaration of Helsinki. The research was approved by the local Bioethical Commission of the Regional Specialist Hospital in Wroclaw (approval number: KB/nr8/rok2017). During the individual interview, all information about the study was provided and written consent was obtained from each participant. The written consent form was accepted by the local Bioethical Commission of the Regional Specialist Hospital in Wroclaw (approval number: KB/nr8/rok2017).

Next Generation Sequencing of blood and mucosal VLPs

Bioptates obtained during colonoscopy examination were floated in PBS for 2h and then forwarded to Cesium Chloride (CsCl) ultracentrifugation, preceded by filtration through 0.22 µm pore membrane filters. CsCl density gradient ranged from the highest density 1.7g/ml, followed by 1.5 g/ml, 1.35 g/ml and 1.15 g/ml. Centrifugation was performed overnight at 62,000 x g at 4^{0}C in a swinging bucket rotor. Half a milliliter of content between 1.35 and 1.5 of CsCL solution

was collected with a syringe for further phage DNA isolation. DNA isolation was performed with Sherlock AX (A&A Biotechnology) kit. Samples were proceeded to Genomiphi V2 DNA Amplification (Cytiva).

Whole blood collected from patients was centrifuged at 2000 RCF for 10 minutes at 4°C. The top layer, which contained the serum, was collected for total DNA isolation using the Sherlock AX kit (A&A Biotechnology). The samples were then subjected to GenomiPhi V2 DNA Amplification (Cytiva) before preparing the sequencing library.

Thirty-eight samples were analyzed by shotgun sequencing. Sequencing was carried out in the sequencing facility of the Biobank laboratory of the University of Lodz or in the sequencing facility unit of the Institute of Immunology and Experimental Therapy, Wrocław Poland. Both facilities make use of Illumina NextSeq 500/550 v2.5 Kit (Medium) for sequencing and DNA library preparation kit by Vazyme. A computer cluster available at the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences was used to analyze FASTQ sequences. The designed data analysis process at the very beginning rejected low-quality sequences using Trimmomatic 0.32 software. Then, obtained reads were mapped to the human GRCh38.1 genome from the UCSC (University of California Santa Cruz) repository. The program Bowtie2 2.4.5 was used for this purpose. Only sequences that did not map with the *"–very-sensitive"* parameter were used for further analysis. Alignment of bacteriophage sequences to the obtained reads was carried out using the BLAST (Basic Local Alignment Search Tool) algorithm. The BLAST algorithm was used for alignment along with an indication for e-value of 1e-50 and sequence identity of 99.

The database of bacteriophage sequences for the alignment step was created from resources available in the NCBI (National Library of Medicine) repository. Viruses containing genetic material in the form of RNA were not included into the analysis. Analysis of the data from the reports generated by the BLAST algorithm was carried out using python language libraries: "pandas 1.4.3", "numpy 1.23" and "seaborn 0.11.2". DNA viruses that are specific to human eukaryotic cells were excluded from the final transcytosis analysis. Only viruses specific to bacteria - bacteriophages - were subjected to statistical analysis. Normalization against the number of reads

obtained from the sequencer was used to correctly visualize the amount of penetration of each type of virus.

Library of phage-derived immune reactive epitopes

Identification of bacteriophage oligopeptides interacting with specific IgGs is presented in Figure 1. It was performed in accordance with the modified protocol published by Xu et al., 2015and adapted for our research using coding sequences of the investigated bacteriophages as the source for library design. The GenBank database (National Center for Biotechnology Information) was searched for all available phage genomes (accessed on: June 1, 2018). For further research, annotated open reading frames (ORFs) were selected, which were described with the keywords: 'structural', 'tail', 'decoration', 'capsid', 'scaffold', 'baseplate', 'coat', 'virion', 'structure', 'lysin', 'muramidase' or 'holin' but did not contain the keywords: 'initiator', 'chaperone', 'maturation protease', 'morphogenesis'. Phage enzyme-coding sequences were used as a reference for possible comparisons between structural proteins and propagation cycle-related proteins of phages. The amino acid sequences of selected ORF were virtually cut into 56 aa long fragments tailing through protein sequences, starting every 28 aa from the first amino acid (50% overlap was applied to avoid splitting potential epitopes). Sequences were deduplicated and then reverse-translated into DNA sequences using codons optimized for expression in E. coli; as a result, 277 051 oligopeptide sequences were included in the library. The oligopeptide library was synthesized using the SurePrint technology for nucleotide printing (Agilent). These oligonucleotides were used to create a phage display library using the T7Select 415-1 Cloning Kit (Merck Millipore) using Liga5™ (A&A Biotechnology) instead of T4 ligase and default restriction sites (EcoRI and HindIII).

Immunoprecipitation

Specific reactions between phage-derived oligopeptides and antibodies present in the investigated sera were identified by immunoprecipitation. Immunoprecipitation of the library was performed in accordance with a previously published protocol (Xu et al., 2015) . Briefly, the phage library was amplified in a standard culture as described in the manufacturer's manual, and then purified using a hollow fiber filtration membrane. In the final step, the purified and concentrated library was suspended in a Phage Extraction Buffer (20 mM Tris-HCl, 100 mM

NaCl, 60 mM MgSO4, pH 8.0). All plastic containers (96-well plates) used for immunoprecipitation were prepared by blocking with 3% Bovine Serum Albumin (BSA) in TBST buffer overnight on a rotator (40 rpm, 4°C). A sample representing an average of $2x10^5$ copies of each clone in 250 µL was mixed with 1 µL of human serum (two technical replicates were applied) and incubated overnight at 4°C with rotation (50 rpm). A 20µL aliquot of a 1:1 mixture of Protein A and Protein G Dynabeads (Invitrogen) was added and incubated for 4h at 4°C with rotation (50 rpm). The liquid in all wells was separated from Dynabeads on a magnetic stand and removed. Beads were washed 5 times with 280µL of a wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) and beads were resuspended in 60µL of water to elute the immunoprecipitated bacteriophages from the beads.

The immunoprecipitated part of the library was then used for amplification of the insert region according to the manufacturer's instructions with a Phusion Blood Direct PCR Kit (Thermo Fisher Scientific). The primers T7_Endo_Lib_LONG_FOR GCCCTCTGTGTGAATTCT and T7_Endo_Lib_LONG_REV GTCACCGACACAAGCTTA were used, and a second round of PCR was carried out with the IDT for Illumina UD indexes (Illumina Corp.) to add adapter tags. Sequencing of the amplicons in accordance with Illumina next generation sequencing (NGS) technology was outsourced (Genomed, Warszawa). A FASTA file containing the selected oligonucleotide sequences was utilized to construct a BLAST database. This was achieved by executing the "makeblastdb" command with the "nucl" dbtype option. Reads from sequencing were annotated using BLAST 2.12 algorithm.

Results

Phage genera detected in human gut mucosa and in blood serum

A total of thirty-eight patients were included in the study of phageome translocation from the gut mucosa to blood serum. These patients served as both colon mucosa bioptate donors and blood serum donors. Mucosal bioptates were specifically chosen instead of the gut content to exclude transient phages from food or water, as these phages may have limited access to transcytosing endothelium. To ensure the analysis focused on intact phage particles, VLPs were investigated instead of full metagenomic sequencing. This approach allowed the exclusion of partially digested or degraded phage particle remains that cannot represent phages capable of

translocation. Each mucosal sample underwent caesium chloride separation to isolate VLPs, followed by NGS shotgun sequencing. The obtained NGS results were subsequently analyzed to identify bacteriophage sequences by referring to the NCBI database, and comparisons were made between the mucosal (VLPs) and serum (total cfDNA) samples.

Initially, a potential correlation between the number of reads generated in the sequencing and the number of identified translocated phage genera was assessed, to validate the methodology for the identification of phage translocation. The analysis revealed the r-Pearson correlation coefficient of 0.236, with the a significance level of 0.154. Similarly, the rho-Spearman correlation coefficient was calculated as 0.283, with a significance level of 0.085. These values demonstrate that correlation is unlikely, indicating that the number of reads per sample cannot be considered as a determining factor in the detection of translocating phages.

Next, the most abundant genera of bacteriophages present in the large intestine mucosa and in blood were identified. Full list of phage genera detected in the investigated patients is presented in Table S1. The ten most abundant genera are presented in Table 1, with the first 3 in gut including *Tequatrovirus*, *Kayvirus*, and *Skunavirus*, representing coliphages, staphphages, and lactococcal phages, respectively (Table 1 column 3.). In turn, phage genera found to be the most abundant in human blood were *Tequatrovirus*, *Pahexavirus* (bacterial host: *Cutibacterium*, previously *Propionibacterium*), and *Kayvirus*, as outlined in Table 1 (column 4.). Interestingly, while *Tequatroviruses* and *Kayviruses* have the most probably been translocated from gut where they represent dominating fraction of bacterial viruses, the origin of *Pahexaviruses* in blood does not correlate to their abundance in gut. Since these phages predate on bacteria often present on the skin, phageome transfer between skin and blood circulation can play a role in shaping blood phageome.
A comprehensive list of phage genera detected in the studied patients is presented in Table S1.

Phage transcytosis from human gut mucosa to blood serum

Bacteriophage transcytosis was considered positive if the same bacteriophages were present in both mucosal and serum samples; this was detected by comparing mucosal and serum samples from the same patient. Transcytosis was confirmed in 33 out of 38 patients (genus level) (86%) (Table 2). The most commonly transcytosed group of bacteriophages turned out to be *Kayviruses* (myoviruses predating on *Staphylococcus*). They were detected in both gut mucosa and blood serum in 23 different individuals. The second group was *Tequatrovirus* (myoviruses, specific to *Enterobacteriaceae*, in the majority *Escherichia coli*) detected in 22 individuals. These were followed by the group *Gamaleyavirus* (in the majority podoviruses predating on *Escherichia coli*), detected in 12 samples.

The frequency of transcytosis in the investigated group (Table 2) overall correlated to phage abundance in the investigated individuals (Table 1), for instance two most often transcytosed genera of bacteriophages (*Kayviruses* and *Tequatoviruses*) were the groups most abundant in human gut. However, Pearson test (0.58, $p<0.05$) shows, that correlation between phage abundance in the gut and phage transcytosis frequency was moderate. If so, other factors like the physicochemical properties of phage particles or their interactions with the human immune system may have a significant effect on phage's ability to translocate from gut to circulation. Also, some phage groups were detected only in serum while not present in the gut mucosa, including *Foxborovirus* (bacterial host: *Gordonia*), *Corndogvirus* (bacterial host: *Mycobacterium*), *Paopuvirus* (bacterial host: *Microbacterium*), *Omarvirus* (bacterial host: *Streptomyces*), and *Salasvirus* (bacterial host: *Bacillus*) (Table S1), again suggesting that gut phageome shapes the phageome found in circulation only in part. Since the majority of these phages are linked to environmental bacteria, they have probably been able to enter circulation from environmental sources, such as food, water, dust, etc.

Antibodies specific to bacteriophage proteins

Due to significant homologies between some phage proteins, antibodies targeting phages (that is: targeting phage proteins) often demonstrate cross-reactions between different phage strains (Gembara & Dąbrowska, 2021). To avoid ambiguous results, we investigated specific immune responses to the translocating phages at phage protein level. We used adapted VirScan method

(Xu et al., 2015), with phage-derived epitope library containing 277 051 oligopeptides covering the structural proteome of phages available in GenBank (for details see Materials and Methods section and Figure 1). Briefly, we applied the library immunoprecipitation with each patient serum sample, followed by massive sequencing of the immunoprecipitated part of the library to identify phage proteins recognized by the patients' sera. Immunoreactive phage proteins were found to represent differentiated taxonomic groups of bacteriophages (Figure 2).
Similarly to the identification of phage genera transcytosis, phage proteins were also identified as transcytosed based on sequencing results from gut and serum phageomes. In total, 201 specific proteins were identified as crossing gut-blood barrier. Comparison of abundance of identified protein groups by their function is presented in Figure 3 A.

We further investigated how phage-specific immune responses regulate phageome that has been translocated to blood. We sought to understand which mechanism predominated: (i) the efficient neutralization and removal of targeted phages mediated by phage-specific antibodies, resulting in the undetectability of these phages in blood samples containing significant levels of specific IgGs; or conversely, (ii) the correlation between the presence of phages in blood and the detection of phage-specific IgGs, suggesting that the presence of relevant phages was essential for sustaining significant levels of phage-specific IgGs. We used GenBank metadata on phage protein functions or annotated structural homologies. We compared the functional profile of the proteins targeted by specific IgGs (as identified by immunoprecipitation with the phage epitope library) to the profile of phage proteins identified as present in the blood of investigated patients (Figure 3B). Only some major capsid proteins maintained noticeable representation within the blood phageome despite pronounced pressure from specific IgGs (Figure 3B), while most proteins targeted by specific antibodies were not detectable in blood phageome. These results were consistent with those observed when protein annotated structural homologies were analyzed instead of functions (Figure S2). Further, no significant Pearson correlation was observed between these two groups (p-value > 0.05). These observations strongly suggest that the first proposed mechanism of interaction between translocating phageome and human immunity plays a major role: phages that induce specific antibodies are effectively neutralized and removed, thus not detectable in circulation.

Discussion

Transcytosis (translocation) of bacteriophages in animals and humans is a relatively new field of phage biology. Nguyen et al. (2017) demonstrated transcytosis of a few different types of tailed phages *in vitro* in epithelial and endothelial cell lines including mucosal-related ones. Those phages efficiently travelled between two chambers of a culture device via a cellular monolayer. Moreover, cellular uptake of phages was later evidenced by visualization within cells (Bichet et al. 2021). This indicates that phages detected in a mucosal layer in the gut should also be detected in the blood sera of animals and humans. Many animal models and human trials confirmed that for individual phages applied orally (Majewska et al., 2015; Monsur et al., 1970; Oliveira et al., 2009; Wolochow et al., 1966), but so far it remains unclear how transcytosis is regulated in the context of phageomes as the whole, as well as how it interplays with immune responses to phages. Here we have compared phageome composition in the human gut mucosa and blood of the same patients. In the gut, we observed a significant contribution of bacteriophages targeting typical gut bacteria, like *Lambdavirus*, *Teseptimavirus*, *Tequatrovirus,* and *Mosigvirus*, that parasitize mainly *Escherichia coli*, *Salmonella,* and *Shigella,* or *Skunavirus* that parasitize *Lactobacilli* (Table 1). Another highly represented group of gut phages were *Kayviruses* which are staphylococcal phages; this is in line with the common identification of *Staphylococcus* as an asymptomatically carried member of human microbiomes including gut (Raineri et al., 2022) .

We further compared gut phageome composition to blood phageome. In the majority, the expectation that phages found abundant in gut microbial communities can also be found circulating in blood was confirmed. *Tequatrovirus* and *Kayvirus,* identified within the three most abundant phage groups in gut phageomes, were also found within the three most abundant phage groups in the patients' sera. Further, individual analyses of each patient's data confirmed the translocation of these groups in 22 and 23 patients (out of 38), respectively. To some extent, other phage groups were also consistently represented both in the gut mucosa and in blood sera (Table 1), however, Pearson test (0.58, $p<0.05$) demonstrated, that the overall correlation was moderate. Also, some phage groups found abundant in gut, like lactococcal phages from *Skunaviruses* were less abundant in blood, or conversely, those less represented in the gut were

however numerous in blood, like *Pahexaviruses*. If so, other factors, like physicochemical properties of phage particles or their interactions with human immune system, may have a significant effect on phage ability to translocate from the gut to circulation. Also, despite the high density of phages in the gut, contribution of other (than gut) sources of phages to the composition of blood phageomes cannot be excluded. That includes phages that parasitize environmental bacteria, meaning they are more likely to be found abundantly in the environment rather than in the human body. Examples include *Foxborovirus* (bacterial host: *Gordonia*), *Corndogvirus* (bacterial host: *Mycobacterium*), *Paopuvirus* (bacterial host: *Microbacterium*), *Omarvirus* (bacterial host: *Streptomyces*), and *Salasvirus* (bacterial host: *Bacillus*) (Table S1).

Phage transcytosis has been postulated as a dynamic and intense phenomenon(Nguyen et al., 2017). In our study, number of phages detected in blood serum was rather low, and efficient detection of phageome composition required viral DNA amplification. Also, only 21 phage groups (out of 51 detected in the mucus layer) were detected simultaneously in the blood (Table 2). What is more, a high abundance of bacteriophages in the human gut was found only moderately positively correlated with abundance of phage in blood. The overall frequency of translocation confirmed in an individual patient (Table 2) was moderate. This may suggest that translocation is not as intensive as it could be suggested by *in vitro* models. Possibly, transcytosis which seemed to be relatively dynamic, (Nguyen et al., 2017), plays only a role in the first stage of translocation to the systemic circulation. Before reaching circulation, infiltrates from the gut are first filtered by gut lymph nodes. Lymph nodes, in turn, act as filters with a 70-kilodalton cutoff for molecules entering the parenchyma and high endothelial venules whereas phages are typically measured in megadaltons (Hons & Sixt, 2015). Concordantly, previously reported i*n vivo* experiments revealed phage arrest in lymph nodes (Dąbrowska, 2019).We also assessed the ability of lymph nodes to filtrate phages translocating from gut to blood circulation in a mouse model using a model phage T4 (representing *Tequatrovirus* genus found within the most frequently translocated phage groups in this human study). We observed that despite high T4 concentration in murine intestinal content, its translocation to blood demonstrated limited efficacy and phage harvest in lymphatic tissue was marked (Figure S3).

This suggests that even an intensive transcytosis at gut epithelium monolayer may be insufficient for the abundant presence of phages in blood serum due to immunological barriers preventing phage from entering circulation.

Phages that manage to enter systemic circulation act as foreign antigens. Foreign antigens disseminated systemically in the human body typically induce specific immune response manifested by high serum levels of antigen-specific antibodies, mainly IgGs. Likewise, phage-specific IgGs have been demonstrated as easily inducible for virtually all phages and potentially efficient in systemic phage neutralization. According to previous observations, phage-specific IgGs production is dose dependent, so higher phage concentration in blood typically results in higher levels of phage-specific IgGs (Gembara & Dąbrowska, 2021; Ledeboer et al., 2002). In this study we sought to understand if phages present in the blood sustain detectable levels of phage-specific IgGs or conversely, once antibodies have been induced, they efficiently mediate phage neutralization and removal. To find the predominant mechanism, we compared the composition of patients' blood phageomes to the specificity of their antibodies, identified by IgGs interaction with a library of phage epitopes (technology modified from (Xu et al., 2015), for details see Materials and methods section). This comparison (Figure 3B) revealed that phage antigens targeted by specific IgGs in the vast majority were very different to those detected in blood phageome. Also, no significant Pearson correlation was observed, which strongly suggests that once induced, phage-specific antibodies predominate phage translocation and make phage delivery to circulation inefficient. Of note, in this study we used total metagenomic sequencing of serum samples to detect the potential presence of phages; while genetic methods based on phage DNA analysis (in contrary to microbiological cultures) are capable of detecting phages even when captured by specific antibodies (Kłopot et al., 2017). Here, in the patients' blood we have not found phage DNA coding for epitopes targeted by IgGs. We conclude that phages targeted by specific IgGs were indeed prevented from entering circulation (or immediately removed) not only "hidden" by phage-specific antibodies.

Interestingly, particularly phage tail proteins appeared to be targeted by specific IgGs at the same time being undetectable in the sera. Despite the presence of specific IgGs, major capsid proteins still maintained a significant presence within the blood phageome (Figure 3B). It is un-

clear how the function of phage protein targeted by specific IgG may determine the biological effects of IgGs on phage in the environment where phage propagation on host bacteria has limited (if any) availability. Nevertheless, phage tail proteins are typically engaged in phage propagation and antibodies targeting these proteins are typically efficient neutralizers of phage activity. Major capsid proteins, in turn, are rather "containers" for phage nucleic acid and potential IgGs binding these proteins are rather inefficient in direct neutralization (Gembara & Dąbrowska, 2021). This observation calls for further investigation, since it suggests that phage-specific IgGs may differ in their effect on phageome translocation depending on the functions of phage proteins that are specifically targeted by antibodies.

Bacteriophages are a crucial part of the body's microbiome and they are considered potential antibacterial agents that could enhance the medical arsenal against bacterial infections (Kutter et al., 2015; Schooley & Strathdee, 2020; "The Promise of Phages," 2023). However, it becomes evident that each phage should be studied within the context of the entire body phageome. The key features of phage as a drug, such as pharmacokinetics and bioavailability, may not solely depend on the body's response to a single therapeutic phage, but rather on complex interactions within the patient's phageome and immune system. Here we demonstrate phage groups that either follow or avoid simple extrapolation from gut phageome to systemically disseminated phages. We also propose the limiting effects of lymph node filtration and the exclusion effects of specific antibodies targeting certain antigens within phage populations. We however believe that our observations call for further investigation of phage interaction with the human body, particularly in the context of body phageome and human immunity responses.

Conclusions

- *Tequatroviruses* and *Kayviruses* have been found among most represented phage groups in gut mucosa of patients investigated herein, and at the same time they have been found most frequently translocated to blood circulation

- Overall correlation between phageome composition in gut and translocated phage groups is moderate, thus other factors (than phage concentration in gut) may affect efficacy of phage translocation
- The gut phageome contributes to the composition of the phageome circulating in the blood, but other sources of phages also appear to contribute, including environmental ones
- We propose to consider epithelial transcytosis of phage as the first step of multi-step phenomenon of phage translocation from gut to blood circulation; since transcytosis is followed by filtration in lymphatic tissue, resulting dynamics of phage translocation *in vivo* is limited
- Significant levels of phage-specific IgGs were negatively correlated to the presence of targeted phage antigens in blood circulation, thus phage-specific antibodies are the limiting factor for translocating phages
- Negative correlation between specific antibodies in human blood and circulation of phage-derived antigens was common for tail antigens, while less pronounced for head antigens (like major capsid proteins); this indicates differences in neutralizing efficiency of IgG fractions targeting different phage proteins, with moderate effect of those targeting phage head and stronger effect of those targeting proteins necessary for phage propagation, however, this problem calls for further investigation

Acknowledgments

This work was supported by National Science Center (Poland) grants: Preludium16 UMO-2018/31/N/NZ6/02584, Opus18 UMO-2019/35/B/NZ7/01824, and by the subsidy for scientific activity provided to the Department of Molecular Microbiology by the University of Lodz (B2111000000038.01).

Conflicts of interest

Authors declare no conflicts of interest.

Data Access

https://www.ncbi.nlm.nih.gov/sra/PRJNA874475

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Figures and Tables

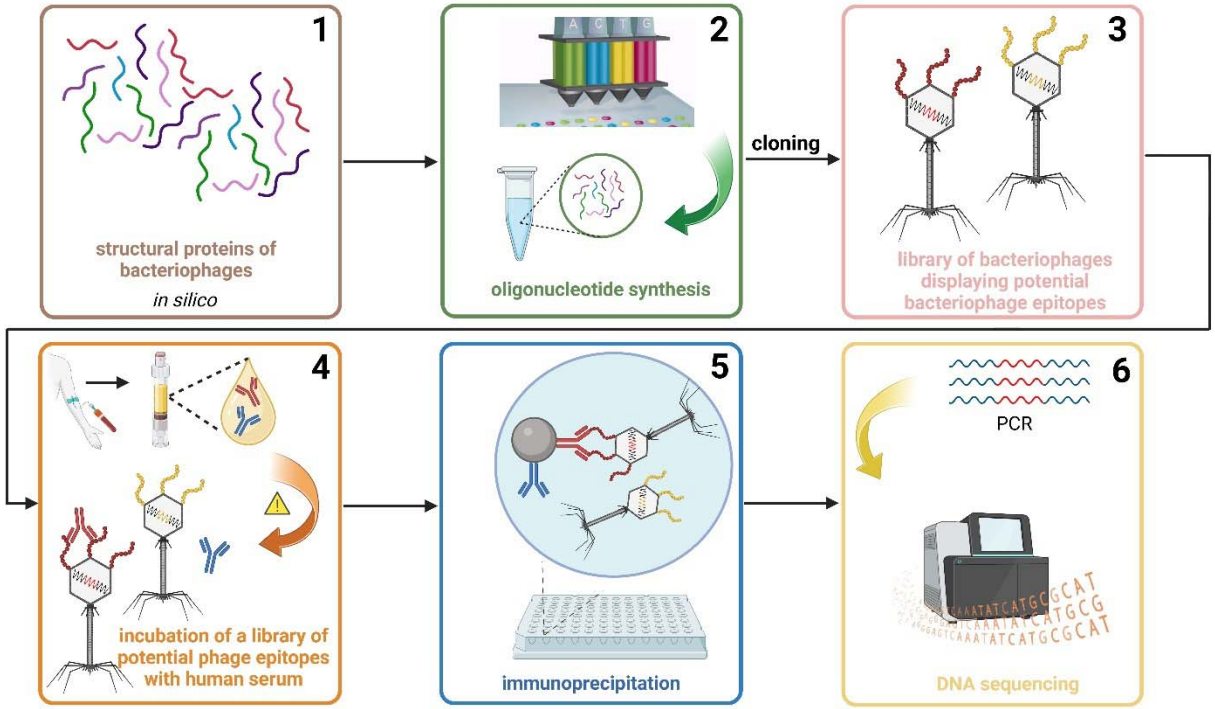


Figure 1. PhageScan technology (adapted from VirScan, Xu et al. (2015) (BioRender)

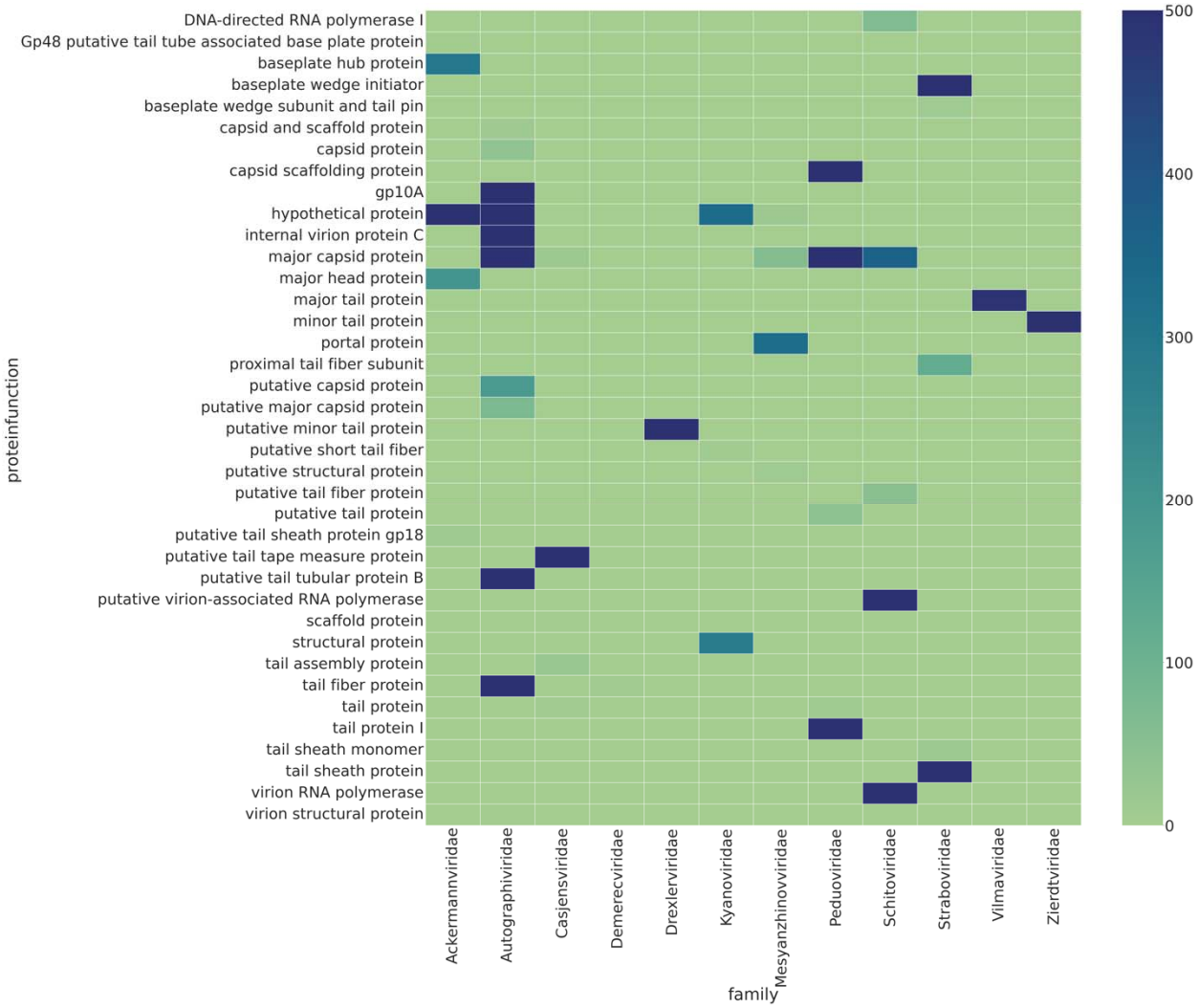
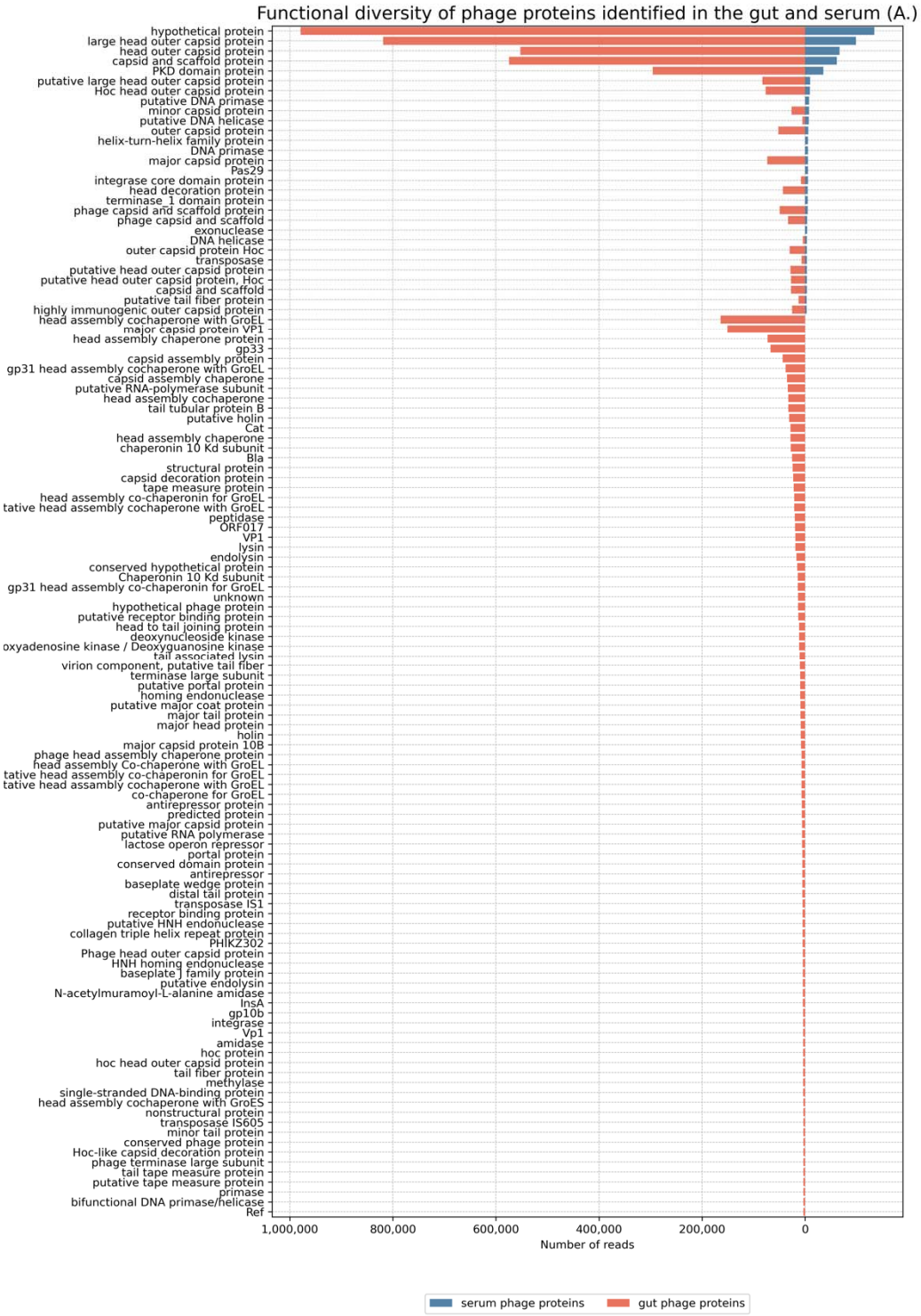
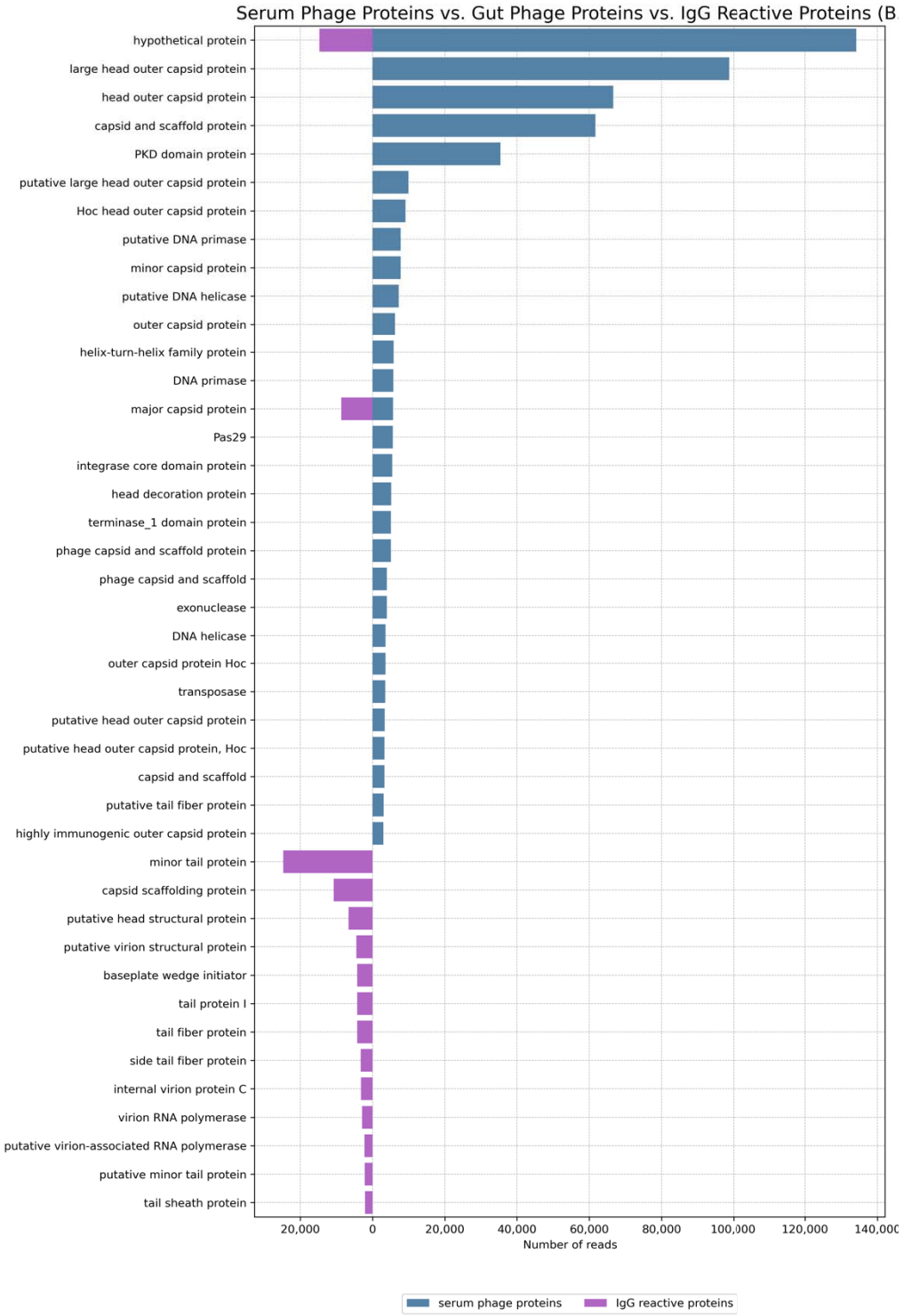


Figure 2. Antibodies targeting specific protein epitopes across various bacteriophage families. The x-axis represents the bacteriophage families, the color gradient indicates the quantity of antibodies, with lighter green representing lower quantities and darker blue representing higher quantities. This variability suggests distinct immune recognition patterns associated with the epitopes of these bacteriophage proteins


Functional diversity of phage proteins identified in the gut and serum (A.)
hypothetical protein
large head outer capsid protein
head outer capsid protein
capsid and scaffold protein
PKD domain protein
putative large head outer capsid protein
Hoc head outer capsid protein
putative DNA primase
minor capsid protein
putative DNA helicase
outer capsid protein
helix-turn-helix family protein
DNA primase
major capsid protein
Pas29
integrase core domain protein
head decoration protein
terminase_1 domain protein
phage capsid and scaffold protein
phage capsid and scaffold
exonuclease
DNA helicase
outer capsid protein Hoc
transposase
putative head outer capsid protein
putative head outer capsid protein, Hoc
capsid and scaffold
putative tail fiber protein
highly immunogenic outer capsid protein
head assembly cochaperone with GroEL
major capsid protein VP1
head assembly chaperone protein
gp33
capsid assembly protein
gp31 head assembly cochaperone with GroEL
capsid assembly chaperone
putative RNA-polymerase subunit
head assembly cochaperone
tail tubular protein B
putative holin
Cat
head assembly chaperone
chaperonin 10 Kd subunit
Bla
structural protein
capsid decoration protein
tape measure protein
head assembly co-chaperonin for GroEL
tative head assembly cochaperone with GroEL
peptidase
ORF017
VP1
lysin
endolysin
conserved hypothetical protein
Chaperonin 10 Kd subunit
gp31 head assembly co-chaperonin for GroEL
unknown
hypothetical phage protein
putative receptor binding protein
head to tail joining protein
deoxynucleoside kinase
oxyadenosine kinase / Deoxyguanosine kinase
tail associated lysin
virion component, putative tail fiber
terminase large subunit
putative portal protein
homing endonuclease
putative major coat protein
major tail protein
major head protein
holin
major capsid protein 10B
phage head assembly chaperone protein
head assembly Co-chaperone with GroEL
tative head assembly co-chaperonin for GroEL
tative head assamblу cochaperone with GroEL
co-chaperone for GroEL
antirepressor protein
predicted protein
putative major capsid protein
putative RNA polymerase
lactose operon repressor
portal protein
conserved domain protein
antirepressor
baseplate wedge protein
distal tail protein
transposase IS1
receptor binding protein
putative HNH endonuclease
collagen triple helix repeat protein
PHIKZ302
Phage head outer capsid protein
HNH homing endonuclease
baseplate J family protein
putative endolysin
N-acetylmuramoyl-L-alanine amidase
InsA
gp10b
integrase
Vp1
amidase
hoc protein
hoc head outer capsid protein
tail fiber protein
methylase
single-stranded DNA-binding protein
head assembly cochaperone with GroES
nonstructural protein
transposase IS605
minor tail protein
conserved phage protein
Hoc-like capsid decoration protein
phage terminase large subunit
tail tape measure protein
putative tape measure protein
primase
bifunctional DNA primase/helicase
Ref
1,000,000
800,000
600,000
400,000
200,000
0
Number of reads
serum phage proteins
gut phage proteins


Serum Phage Proteins vs. Gut Phage Proteins vs. IgG Reactive Proteins (B
hypothetical protein
large head outer capsid protein
head outer capsid protein
capsid and scaffold protein
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putative large head outer capsid protein
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putative DNA primase
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DNA primase
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Pas29
integrase core domain protein
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terminase_1 domain protein
phage capsid and scaffold protein
phage capsid and scaffold
exonuclease
DNA helicase
outer capsid protein Hoc
transposase
putative head outer capsid protein
putative head outer capsid protein, Hoc
capsid and scaffold
putative tail fiber protein
highly immunogenic outer capsid protein
minor tail protein
capsid scaffolding protein
putative head structural protein
putative virion structural protein
baseplate wedge initiator
tail protein I
tail fiber protein
side tail fiber protein
internal virion protein C
virion RNA polymerase
putative virion-associated RNA polymerase
putative minor tail protein
tail sheath protein
20,000
0
20,000
40,000
60,000
80,000
100,000
120,000
140,000
Number of reads
serum phage proteins
IgG reactive proteins

Figure 3: Functional diversity of phage proteins identified in the human intestinal mucosa compared to those identified in sera from the same patients (A), and phage proteins targeted by phage specific IgGs compared to phage protein presence in the same human sera (B). Phage protein sequences were analyzed based on annotations found in the NCBI database. The y-axis lists various phage protein functions based on NCBI annotations, while the x-axis represents the number of reads for each protein function.
A) The left panel displays the functional diversity of phage proteins identified in both the gut and serum of patients. **(B)** The right panel compares the presence of specific phage proteins in the serum (green bars) to those targeted by IgG reactive proteins.

Table 1 Most abundant genera of bacteriophages in patients' large intestines' mucosa. Ratio was calculated based on readings assigned to a genus of virus relative to all reads in the samples.

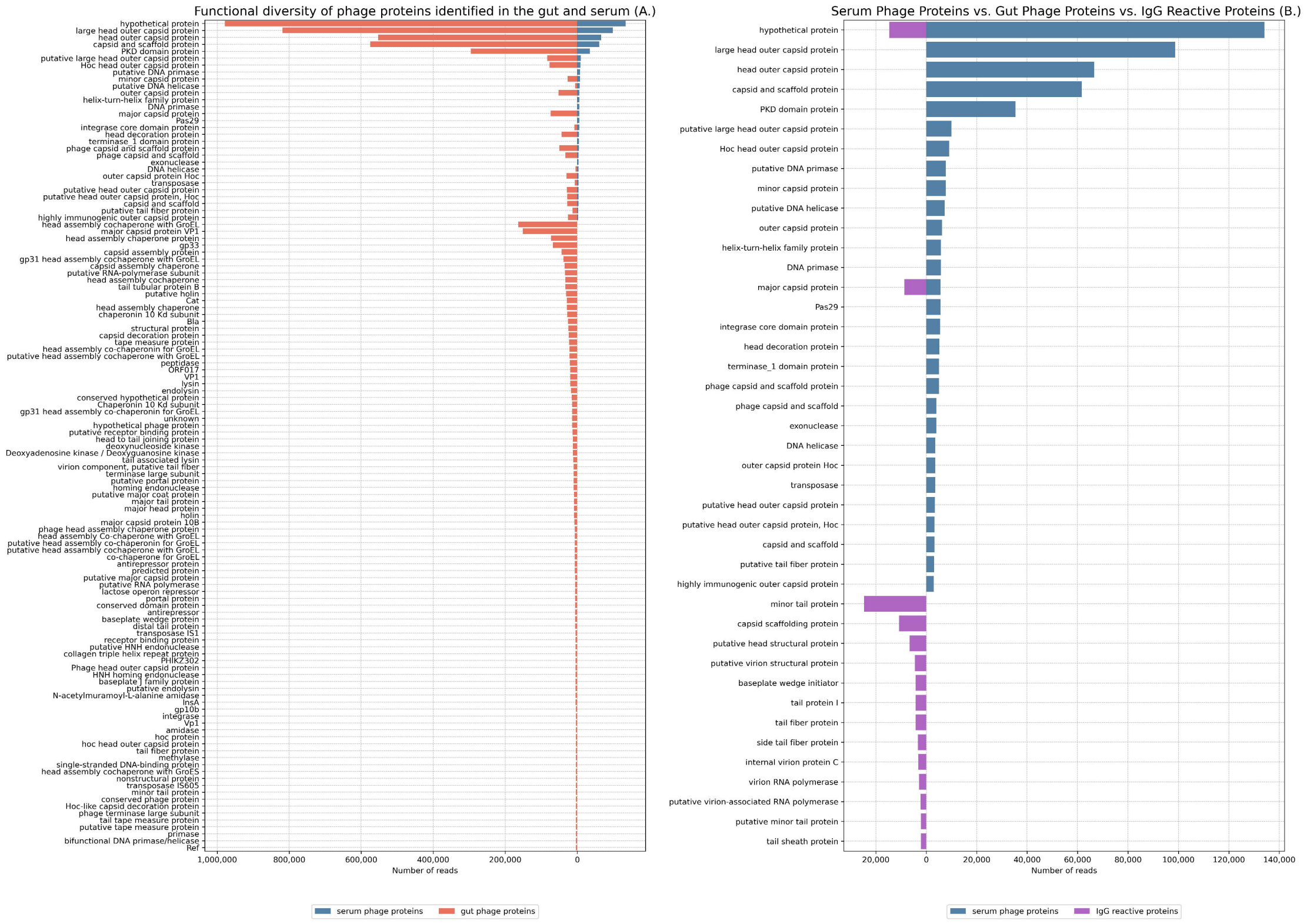
Genus	Major hosts	Presence ratio – large intestines	Presence ratio - serum	Morphology
Tequatrovirus	*Escherichia coli sp.*	0.436254	0.362355	myovirus
Kayvirus	*Staphylococcus sp.*	0.140325	0.085876	siphovirus
Skunavirus	*Lactobacillus sp.*	0.039254	0.00110751	siphovirus
Mosigvirus	*Escherichia coli sp.*	0.034812	0.026797	podovirus
Shenzhenvirus	*Aeromonas sp.*	0.023566	4.04E-05	myovirus
Lambdavirus	*Escherichia coli sp*	0.012607	0.00198961	siphovirus
Teseptimavirus	*Escherichia coli sp.*	0.011867	0.030024	podovirus
Rockefellervirus	*Staphylococcus sp.*	0.009914	0.00067102	siphovirus
Dhakavirus	*Escherichia coli sp.*	0.009354	0.00423459	myovirus
Kehishuvirus	*Uncultured crAssphage*	0.008801	9.12E-06	Non-enveloped, head-tail structure.
Chlamydiamicrovirus	*Chlamydia*	0.008516	1.30E-06	Non-enveloped, round, T=1 icosahedral symmetry
Pahexavirus	*Cutibacterium sp.*	0.00514331	0.094851	siphovirus

Traversvirus	*Escherichia spp.*	0.00585324	0.011539	head-tail
Diegovirus	*Shigella sp.*	0.0001381	0.007664	podovirus
Affertcholeramvirus	*Vibrio sp*	5.22E-06	0.007562	inovirus
Sepunavirus	*Staphylococcus sp.*	0.0003644	0.006788	myovirus
Berlinvirus	*Escherichia spp.*	0.00413165	0.006762	head-tail
Hemphillvirus	*Bacillus sp*	4.21E-05	0.004976	Non-enveloped, head-tail structure

Table 2 The genus of bacteriophages most frequently transcytosed in the investigated patients. In each patient gut phageome sequencing reads were aligned to blood phageome sequencing reads and positive alignment was considered as detected transcytosis.

genus	**No of samples where transcytosis was observed**	**Major host**	**Morphology**
Kayvirus	23	*Staphylococcus sp.*	myovirus
Tequatrovirus	22	*Escherichia sp.*	myovirus
Gamaleyavirus	12	*Escherichia sp.*	myovirus
Atlauavirus	11	*Synechococcus sp.*	myovirus
Punavirus	9	*Escherichia sp.*	myovirus
Lambdavirus	8	*Escherichia sp.*	siphovirus
Pahexavirus	7	*Propionibacterium sp.*	myovirus
Lowelvirus	7	*Synechococcus sp.*	myovirus
Teseptimavirus	7	*Escherichia sp.*	podovirus
Phietavirus	7	*Staphylococcus sp.*	myovirus
Traversvirus	7	*Escherichia sp.*	myovirus
Nereusvirus	7	*Synechococcus sp.*	myovirus

Namakavirus	7	*Synechococcus sp.*	myovirus
Karamvirus	6	*Enterobacter sp.*	myovirus
Dubowvirus	6	*Staphylococcus sp.*	myovirus
Skunavirus	6	*Lactococcus sp.*	myovirus
Mosigvirus	6	*Escherichia sp.*	myovirus
Bcepfunavirus	6	*Burkholderia sp.*	myovirus
Jiaodavirus	5	*Klebsiella sp.*	myovirus
Dhakavirus	5	*Escherichia sp.*	myovirus
Harbinvirus	5	*Lactobacillus sp.*	myovirus
Gaprivervirus	5	*Escherichia sp.*	myovirus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putative tail fiber protein
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head assembly cochaperone with GroEL
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head assembly cochaperone
tail tubular protein B
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Cat
head assembly chaperone
chaperonin 10 Kd subunit
Bla
structural protein
capsid decoration protein
tape measure protein
head assembly co-chaperonin for GroEL
putative head assembly cochaperone with GroEL
peptidase
ORF017
VP1
lysin
endolysin
conserved hypothetical protein
Chaperonin 10 Kd subunit
gp31 head assembly co-chaperonin for GroEL
unknown
hypothetical phage protein
putative receptor binding protein
head to tail joining protein
deoxynucleoside kinase
Deoxyadenosine kinase / Deoxyguanosine kinase
tail associated lysin
virion component, putative tail fiber
terminase large subunit
putative portal protein
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major tail protein
major head protein
holin
major capsid protein 10B
phage head assembly chaperone protein
head assembly Co-chaperone with GroEL
putative head assembly co-chaperonin for GroEL
putative head assembly cochaperone with GroEL
co-chaperone for GroEL
antirepressor protein
predicted protein
putative major capsid protein
putative RNA polymerase
lactose operon repressor
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baseplate wedge protein
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putative tape measure protein
primase
bifunctional DNA primase/helicase
Ref
1,000,000
800,000
600,000
400,000
200,000
0
Number of reads
serum phage proteins
gut phage proteins
Serum Phage Proteins vs. Gut Phage Proteins vs. IgG Reactive Proteins (B.)
hypothetical protein
large head outer capsid protein
head outer capsid protein
capsid and scaffold protein
PKD domain protein
putative large head outer capsid protein
Hoc head outer capsid protein
putative DNA primase
minor capsid protein
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DNA helicase
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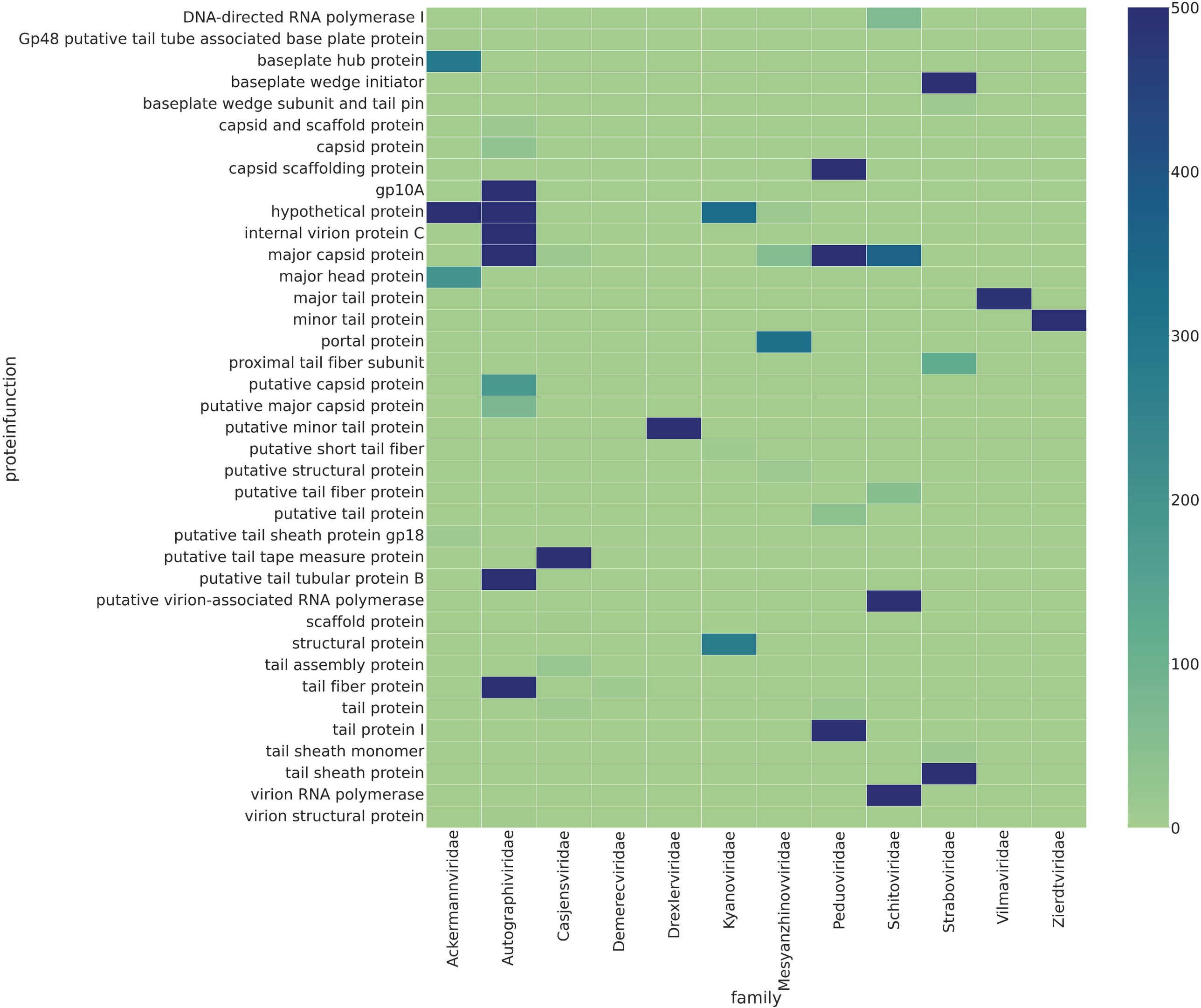
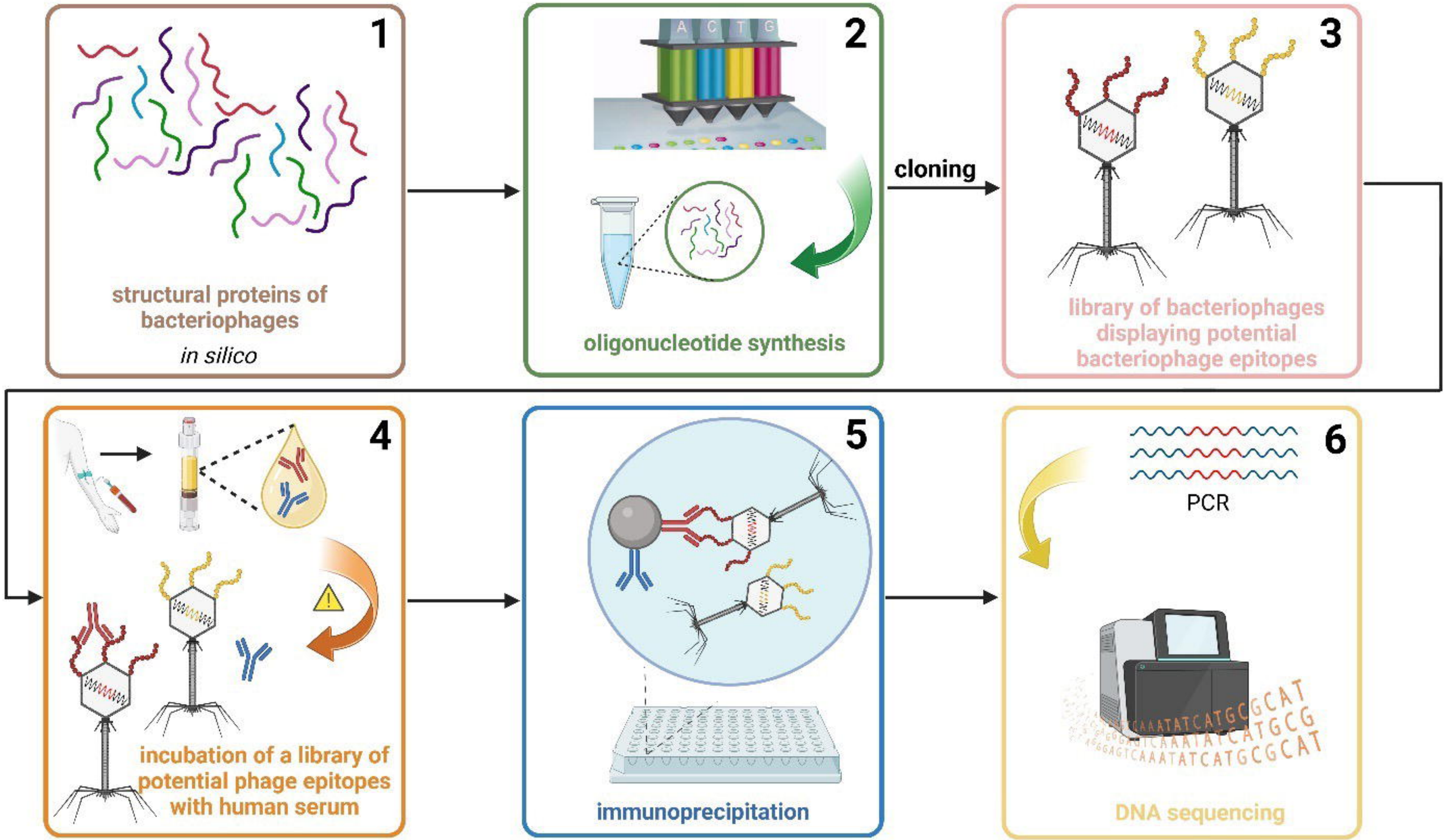
transposase
putative head outer capsid protein
putative head outer capsid protein, Hoc
capsid and scaffold
putative tail fiber protein
highly immunogenic outer capsid protein
minor tail protein
capsid scaffolding protein
putative head structural protein
putative virion structural protein
baseplate wedge initiator
tail protein I
tail fiber protein
side tail fiber protein
internal virion protein C
virion RNA polymerase
putative virion-associated RNA polymerase
putative minor tail protein
tail sheath protein
20,000
0
20,000
40,000
60,000
80,000
100,000
120,000
140,000
Number of reads
serum phage proteins
IgG reactive proteins


DNA-directed RNA polymerase I
Gp48 putative tail tube associated base plate protein
baseplate hub protein
baseplate wedge initiator
baseplate wedge subunit and tail pin
capsid and scaffold protein
capsid protein
capsid scaffolding protein
gp10A
hypothetical protein
internal virion protein C
major capsid protein
major head protein
major tail protein
minor tail protein
portal protein
proximal tail fiber subunit
putative capsid protein
putative major capsid protein
putative minor tail protein
putative short tail fiber
putative structural protein
putative tail fiber protein
putative tail protein
putative tail sheath protein gp18
putative tail tape measure protein
putative tail tubular protein B
putative virion-associated RNA polymerase
scaffold protein
structural protein
tail assembly protein
tail fiber protein
tail protein
tail protein I
tail sheath monomer
tail sheath protein
virion RNA polymerase
virion structural protein
proteinfunction
Ackermannviridae
Autographiviridae
Casjensviridae
Demerecviridae
Drexlerviridae
Kyanoviridae
Mesyanzhinovviridae
Peduoviridae
Schitoviridae
Strabovirdae
Vilmaviridae
Zierdtviridae
family
500
400
300
200
100
0


1
structural proteins of bacteriophages
in silico
2
A C T G
oligonucleotide synthesis
cloning
3
library of bacteriophages displaying potential bacteriophage epitopes
4
incubation of a library of potential phage epitopes with human serum
5
immunoprecipitation
6
PCR
DNA sequencing