Published in final edited form as:

Nat Microbiol. 2018 January ; 3(1): 38–46. doi:10.1038/s41564-017-0053-y.

Discovery of an expansive bacteriophage family that includes the most abundant viruses from the human gut

Natalya Yutin¹, Kira S. Makarova¹, Ayal B. Gussow¹, Mart Krupovic², Anca Segall^{1,3}, Robert A. Edwards³, and Eugene V. Koonin^{1,*}

¹National Center for Biotechnology Information, National Library of Medicine, Bethesda, Maryland 20894, USA

²Institut Pasteur, Unité Biologie Moléculaire du Gène chez les Extrêmophiles, 25 rue du Docteur Roux, 75015 Paris, France

³Viral Information Institute, Department of Biology, San Diego State University, San Diego, California 92182, USA

Abstract

Metagenomic sequence analysis is rapidly becoming the primary source of virus discovery^{1–3}. A substantial majority of the currently available virus genomes comes from metagenomics, and some of these represent extremely abundant viruses even if never grown in the laboratory. A particularly striking case of a virus discovered via metagenomics is crAssphage, which is by far the most abundant human-associated virus known, comprising up to 90% of the sequences in the gut virome⁴. Over 80% of the predicted proteins encoded in the approximately 100 kilobase crAssphage genome showed no significant similarity to available protein sequences, precluding classification of this virus and hampering further study. Here we combine comprehensive search of genomic and metagenomic databases with sensitive methods for protein sequence analysis to identify an expansive, diverse group of bacteriophages related to crAssphage and predict the functions of the majority of phage proteins, in particular, those that comprise the structural, replication and expression modules. Most if not all of the crAss-like phages appear to be associated with diverse bacteria from the phylum Bacteroidetes, which includes some of the most abundant bacteria in the human gut microbiome and are also common in various other habitats. These findings provide for experimental characterization of the most abundant but poorly understood members of the human-associated virome.

Viruses are the most abundant biological entities on earth: in most environments, from ocean water to the content of animal guts, the number of detected virus particles exceeds that of

Users may view, print, copy, and download text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms

*Correspondence and requests: All correspondence and requests should be addressed to Eugene V. Koonin at koonin@ncbi.nlm.nih.gov.

Authors' contributions

EVK conceived of the study; NY, KSM and MK performed research; NY, KSM, ABG, MK, AS, RE and EVK analyzed the data; EVK wrote the manuscript that was read, edited and approved by all authors.

Competing interests

The authors declare no competing financial interests.

cells by one to two orders of magnitude². Among these viruses, more than 90% are tailed bacteriophages¹. More than 99% of the prokaryotic diversity in the biosphere is represented by bacteria and archaea that fail to grow in laboratory cultures, and accordingly, the great majority of the viruses are thought to infect these uncultivated microbes¹. Moreover, analysis of the human gut virome shows that most of the sequences, in contrast to the bacterial and archaeal sequences, have no matches in the current sequence databases, suggesting a vast virome consisting primarily of ‘dark matter’^{5–7}.

The crAssphage is the utmost manifestation of this trend. The complete crAssphage (after Cross Assembly) genome was assembled by joining contigs obtained from several human fecal viral metagenomes as a circular double-stranded (ds) DNA molecule of approximately 97 kilobase (kb)⁴; the circular genome map apparently results from the terminal redundancy and/or circular permutation. The crAssphage is extremely abundant, accounting for up to 90% of the reads in the virus-like particle-enriched fraction of the gut metagenome and about 22% of the reads in the total metagenome. Numerous reads matching the crAssphage genome have been identified in numerous gut metagenomes collected in diverse geographic locations, indicating that crAssphage is not only the most abundant virus in the human gut microbiome but also a (nearly) ubiquitous one^{4,8,9}. Read co-occurrence analysis points to bacteria of the phylum Bacteroidetes as the host(s) of the crAssphage^{4,10}. This assignment is compatible with the presence, in the crAssphage genome, of a protein containing carbohydrate-binding domains (BACON domains) that is highly similar to a homologous protein from *Bacteroides* and with partial matches between two crAssphage sequences and CRISPR spacers from two species of *Bacteroides*⁴. Members of the Bacteroidetes dominate the gut microbiome but most of these bacteria so far have not been grown in culture^{11,12}. Thus, it is hardly surprising that the most abundant – but never isolated – phage from this environment appears to be a parasite of Bacteroidetes. Analysis of the protein sequences encoded in the crAssphage genome failed to identify specific relationships with other bacteriophages⁴. Several proteins implicated in phage genome replication have been identified including a family B DNA polymerase (DNAP), a primase and a flavin-dependent thymidylate synthase, but neither the major capsid protein nor other structural and morphogenetic proteins were detected. In an attempt to clarify the provenance of this most abundant but enigmatic human-associated virus, we re-analyzed the crAssphage genome using the most sensitive available methods for protein sequence analysis and taking advantage of the database growth since the time of crAssphage discovery. The result is the identification of a previously unknown, expansive bacteriophage family that appears to be associated with diverse members of Bacteroidetes and for which we now recognize the structural, replication and expression gene modules.

The sequences of the crAssphage proteins were compared, using PSI-BLAST, to the non-redundant protein sequence database (nr) and the Whole Genome Shotgun (WGS) databases (NCBI, NIH, Bethesda) containing microbial genomic and metagenomic sequences. Sequences with significant similarity to crAssphage proteins were detected in four genomes of previously identified bacteriophages and numerous contigs assigned to bacterial genomes (possibly, prophages) and metagenomic contigs. These sequences were highly diverse, and most were not closely related (despite the statistical significance of the detected similarity) to the crAssphage proteins (Supplementary Table 1). Thus, crAssphage relatives identified

here might comprise a previously unidentified, large, diverse family of bacteriophages (henceforth crAss-like family), or potentially, even two or more families. Altogether, we identified several hundred putative representatives of the crAss-like phage family (Supplementary Figure 1); 37 diverse representatives, for which (nearly) complete genomes were available, were selected for in-depth analysis (Supplementary Table 1). We then constructed multiple alignments of the crAssphage proteins and their homologs, and used these alignments as queries for profile-profile searches against a comprehensive collection of protein families using the HHPred software, one of the most sensitive current methods for protein sequence analysis (see Methods for details). We identified a block of 5 genes that appear to comprise the structural module of the emerging family of bacteriophages. These genes encode a predicted major capsid protein (MCP) of the HK97 fold, portal protein, large terminase subunit, and two uncharacterized proteins that are conserved throughout the crAss-like family and, given the consistent adjacency to the MCP, could be components of the virion or the morphogenetic machinery (Table 1; Figure 1; Supplementary Table 2 and Supplementary Note 1).

Despite the low sequence conservation, even within the family, and remote similarity to proteins from other phages, the gene order in the capsid module of the crAss-like family is nearly invariant (Figure 1), suggesting congruent evolution of these genes. A concatenated alignment of all 5 genes was used to construct a phylogenetic tree of the crAss-like family which includes a strongly supported clade of crAssphage relatives and several other distinct groups of (predicted) bacteriophages (Figure 1). Three of these groups included previously identified phages, namely *Azobacteroides* phage ProJpT-Bp¹³, *Flavobacterium psychrophilum* phage Fpv3 and *Cellulophaga* phage phi14:2, widespread, although apparently not highly abundant phages in the oceans¹⁴. The same group that included the *Cellulophaga* phage also contained the genome of the IAS (immunodeficiency-associated stool) virus that is highly abundant in gut viromes of HIV-infected individuals¹⁵. Most of the other members of the crAss-like family are unassigned metagenomic sequences but several come from bacterial genome assemblies and might represent prophages. All experimentally characterized crAss-like phages are associated with Bacteroidetes. Among the other sequences included in the family, several are assigned to other bacteria, in particular, *Chlamydia trachomatis*, as well as several members of the recently identified candidate phyla radiation (CPR)¹⁶. However, in the phylogenetic trees of the predicted MCP, these sequences are embedded within groups consisting of sequences associated with Bacteroidetes (Figure 1 and Supplementary Figure 1), and none of these contigs contained genes that could be linked to the host bacteria. Thus, the available data appear compatible with exclusive association between the crAss-like phages and Bacteroidetes.

In the linear genome maps shown in Figure 2 (see Supplementary Table 1 and Supplementary Note 1 for the crAssphage and IAS phage gene annotations), the capsid structural module occupies about 10 kb near one end of the crAss-like phage genomes. Downstream of this module, are the genes encoding predicted tail proteins and two proteins homologous to bacterial Integration Host Factor (IHF) that is essential for chromatin packaging in bacteria and some phages¹⁷ (Figure 2; Table 1; Supplementary Table 2). The two most conserved tail proteins encoded by the crAss-like group are homologous to tail components gp4 (tubular tail protein) and gp10 (tail stabilization protein) of bacteriophage

P22¹⁸. In the same putative operon, crAssphage also encodes a homolog of the tail needle protein gp26 of phage P22. These three proteins are sufficient for the formation of a short tail similar to that of bacteriophage P22¹⁹. The gp10 homologs of crAss-like phages are large (>1400 aa), apparently multidomain proteins in which the gp10-homologous region accounts only for about 150 aa. The additional domains of this protein could be involved in host recognition similarly to the tail spike protein of P22-like phages²⁰. Some crAss-like phages encode additional, auxiliary proteins in the tail module, e.g. an IAS protein homologous to the tail-associated lysozyme gp13 of short-tailed phage phi29²¹. Thus, the crAss-like phages can be predicted to possess short, stubby tails, a hallmark of the family *Podoviridae*. One of the isolated crAss-like phages, *Cellulophaga* phage phi14:2, is indeed a typical podophage¹⁴. Unlike phages with long tails (families *Myoviridae* and *Syphoviridae*), P22-like phages do not encode maturation proteases²², consistent with the apparent absence of such a protease among gene products of the crAss-like phages. However, in the midst of the genes for predicted tail components, some of the crAss-like phages, including the crAssphage group, encode a predicted Zn-dependent protease (Figure 2) that might be involved in processing of the tail and/or capsid proteins. In crAssphage group genomes, the protease gene is embedded within a block of genes encoding putative additional tail components which are highly similar to homologs from uncharacterized prophages integrated in *Bacteroides* genomes that are otherwise unrelated to crAss-like phages (Figure 2; also see Supplementary Table 2). Thus, evolution of the crAssphage group apparently involved relatively recent recombination with an unrelated (pro)phage from the same host(s).

The lytic replication module genes occupy about 30 kb on the opposite end of the genome from the capsid module and are transcribed towards the middle of genome (Figure 2). This module encodes a versatile suite of proteins implicated in DNA replication and repair, and shows a patchy gene distribution, without a single universally conserved gene, and a much greater variability within the crAss-like family than the structural modules (Table 1; Figure 3). The most conserved replicative gene is a predicted DnaG family primase; many crAss-like family members also encode a putative superfamily 2 (SNF2 family) helicase implicated in DNA replication (this helicase is inactivated in the crAssphage subfamily as indicated by multiple amino acid replacements in the catalytic sites), an ATP-dependent DNA ligase, a uracyl-DNA glycosylase (UDG), a flavin-dependent thymidylate synthase (ThyX), and one or two diverged single-stranded DNA-binding proteins (SSB) (Table 1; Figure 3). The crAss-like family viruses encode one of the two distinct DNA polymerases (DNAPs): the crAssphage clade has a family B DNAP, whereas the other family members have either a family B or a family A DNAP, or no DNAP at all (Table 1; Figure 3). Evolutionary reconstruction suggests that the family A DNAP is ancestral in crAss-like phages and was lost or replaced with the family B DNAP on several occasions (Supplementary Figure 2). The only near universal replicative protein, DNA primase, appears to be monophyletic in the crAss-like family and forms a strongly supported clade with the primases of *Bacteroidetes* (Supplementary Figure 2). This is the only conserved crAss-like family gene that shows a deep connection to *Bacteroidetes*, indicating that a founder crAss-like phage acquired this gene from a *Bacteroidetes* host and implying that the virus-host link is evolutionarily ancient. An unusual evolutionary connection was detected for the phage ligase: in the phylogenetic tree, the crAss-like family ligases clustered with those of eukaryotic giant

dsDNA viruses suggesting that these viruses acquired the ligase from crAss-like phages (Supplementary Figure 2). Portions of the replicative gene block of the IAS virus and its closest relatives show high similarity to homologs from putative prophages of Bacteroidetes, suggesting another recombination event (Figure 2 and see Supplementary Table 2).

The structural and replicative gene blocks of the crAss-like phages are separated by an array of uncharacterized genes that are transcribed in the same direction as the structural genes and are universally conserved across the crAss-like family (Figure 2 and Table 1). Several of these genes encode giant proteins, up to 6000 amino acids in size. An HHpred search initiated with the multiple alignment of the homologs of one of these large proteins (crAssphage gene 46 product) identified a small region of similarity with the β' -subunit of the bacterial RNA polymerase (RNAP), which contained the signature catalytic loop with the metal-binding DxDxD motif²³ (Supplementary Figure 3). Detailed sequence analysis resulted in the identification of two additional conserved motifs typical of the RNAP β subunit²³, suggesting that the two subunits are fused in this protein (Figure 4 and Supplementary Figure 3). Although the similarity between these crAss-like family protein sequences and the large RNAP subunits was limited, the strict conservation of several predicted key motifs that comprise the RNAP catalytic site across the crAss-like family, the fusion of the putative homologs of two RNAP subunits in a single large protein compatible with the combined lengths of the β and β' RNAP subunits, and the compatibility of the predicted secondary structure elements with the RNAP core structure (Supplementary Figure 3) strongly suggest that the crAss-like family phages encode an active RNAP.

The putative β - β' RNAP fusion protein contains another large region of similarity with other phages, which in some of them resides in a separate protein (e.g. gene₅₃ product of Azobacteroides phage ProJPT-Bp1; Figure 4). Another protein conserved in most of the crAss-like phages (crAssphage gene 47) is typically encoded next to or is fused to the β - β' RNAP (Figure 4). Most likely, all three proteins are functionally linked and form a multisubunit RNAP; although no homologs of the gene 47 product were detected, the size and association with the RNAP subunits suggest that this could be a highly diverged α subunit. In most crAss-like phages that encode fused subunits of the predicted RNAP, a putative zincin-like protease domain is encoded in the vicinity, e.g. within the gene 45–46 fusion product of the crAssphage, whereas most of the phages that encode the RNAP subunits in separate genes lack the predicted protease (Figure 4 and Supplementary Figure 3). Thus, the fused RNAP subunits might be cleaved by the Zn-dependent protease to produce the mature proteins. Fusions of zincin family proteases are typical of different multidomain phage proteins²⁴, which is compatible with the RNAP cleavage hypothesis. Nonetheless, it cannot be ruled out that the fusion protein is the active form of the phage RNAP. Only a few groups of phages encode their own multisubunit RNAPs, including *Lactococcus* phage 1706, *Rhodococcus* phage ReqiPepy6, *Bacillus* phage SPbeta, *Thermus* phages P74 and P23, and giant phages of *Pseudomonas*^{25–28}. In most of these phages, β and β' subunits are fused^{25,29}, whereas in *Pseudomonas* group phages, each subunit is split into two proteins²⁸. The phage RNAPs belong to diverged families that can be considered signatures of each respective phage group^{26,28}. The crAss-like family RNAPs are even more extremely divergent than those of other phages. To our knowledge, processing of RNAP polyproteins by a dedicated protease so far has not been identified in viruses or cellular

organisms. Phage RNAPs transcribe either early (replicative)²⁸ or late (structural)²⁷ genes; in the former case, the RNAP presumably is packaged into the virion. We attempted to identify promoters of early and late genes of crAss-like phages by searching the sequences upstream of the genes for potential conserved nucleotide motif, but no such motifs were detected.

The discovery of crAssphage, the most abundant virus in the human gut virome, appeared particularly striking because the genome was *terra incognita*, with few homologs detected in other viruses or bacteria, and the virion proteins not identified⁴. The present analysis changes this by showing that crAssphage belongs to an expansive phage family that is only distantly related to other known phages and has unusual predicted features, in particular, a previously unknown putative mechanism of RNAP maturation via polyprotein processing. The MCP of these phages, a distinct form of the HK97 class of icosahedral capsid proteins³⁰, is now confidently predicted and amenable for experiments aimed at direct identification and characterization of the phage. Altogether, homologs with characterized functions have been detected for 53% (48 out of 91) of MetaGeneMark-predicted crAssphage proteins (compared to 26% in the original analysis and 14% in the current RefSeq annotation; Supplementary Table 2).

The crAss-like family includes at least one additional phage, IAS, that can reach high abundance in the human gut¹⁵. Generally, the crAss-like family appears to be abundant and widespread in diverse habitats, both animal-associated and environmental. Various bacteria of the phylum *Bacteroidetes* appear to be the primary hosts of crAss-like phages as indicated by the presence of several genes apparently derived from these bacteria including the DNA primase that is ancestral in the family and the BACON domain protein implicated in phage adhesion to mucus that could increase the frequency of the encounters with the host bacteria³¹. This virus-host association is supported by CRISPR spacer analysis; in addition to the previously reported imperfect matches to *Bacteroides* genomes⁴, we detected perfect matches of crAssphage sequences to two spacers from *Porphyromonas* sp. (Supplementary Note 2). It seems likely that crAssphage has a broad host range among the *Bacteroidetes*, which could contribute to the (near) ubiquity of this phage in humans. For the IAS virus, spacers with partial matches were detected in CRISPR arrays of *Prevotella* (Supplementary Note 2), again indicating a *Bacteroidetes* host. Although some of the crAss-like family sequences identified here are assigned to genomes of other bacteria, these assignments could be erroneous (see above), suggesting that the crAss-like virus family is *Bacteroidetes*-specific. Our results indicate that some of the crAss-like phages are temperate and lysogenize their hosts by integrating into their genomes with the aid of phage-encoded tyrosine integrases. The temperate life style increases the opportunities for recombination with other phages and could account for the presence of regions with high similarity to otherwise unrelated *Bacteroidetes* prophages.

Our analysis of the predicted tail proteins indicates that the crAss-like phages possess short, podovirus-like tails. Thus, under the classical morphology-guided classification scheme, these phages would be classified into the family *Podoviridae*. However, given that phage taxonomy is moving towards sequenced-based approaches³², crAss-like phages are likely to become a family within the order *Caudovirales*. The general lesson from this study is that,

with the current proliferation of genomic and especially metagenomic sequence databases and advances in database search approaches, any discovered abundant virus or microbe is likely to become a prototype of a previously undetected, often highly diverse group of organisms. Such advanced analyses can guide experimental study of viruses and microbes that are currently known only through genomic sequences but could be major players in the microbiota.

Methods

The search for crAssphage structural proteins was performed as follows. The sequences of the crAssphage proteins were first used as queries in a PSI-BLAST search³³ of the NCBI non-redundant (nr) database. Proteins that produced no statistically significant hits to nr proteins with predicted functions were considered as candidates for crAssphage structural proteins. For each of these proteins, homologs from both nr and environmental (env_nr) protein sequence databases were collected (using PSI-BLAST with default parameters until convergence or for 5–6 iterations). The homologs detected in this search were aligned with the query crAssphage protein, and the alignments were used as queries for HHPred³⁴ searches. Three of these queries produced statistically significant hits to phage structural proteins (terminase large subunit, portal, and HK97 family MCP).

The sequences of the predicted crAssphage MCP and its homologs (“initial MCP set”) were used as queries for translating blast (TBLASTN) searches against the wgs and nr nucleotide databases. Nucleotide sequences of the hits were retrieved and translated in 6 frames, and the sequences of the putative MCP homologs were validated by comparison to the initial MCP set. The extracted protein sequences of the putative MCP homologs (either complete proteins or fragments longer than 150 aa) were clustered with blastclust at 90% identity, aligned with MUSCLE³⁵, and used for the phylogenetic reconstruction shown in Supplementary Figure 1.

In addition, other conserved crAssphage proteins were searched against GenBank databases by protein blast (BLASTP) and TBLASTN³³. These searches led to the identification of several complete and partial crAss-like viral genomes in the nr database as well as several hundred crAss-like contigs in the wgs databases (Supplementary Figure 1; see ftp://ftp.ncbi.nih.gov/pub/yutinn/crassphage_2017/ for additional data).

For in-depth analysis, 37 representative genomes (contigs) were selected among the crAss-like family members identified in the nr and wgs databases. In some cases (namely, CVNZ01000019ext, CVNZ0100007ext, contig0001, contig0002, and contig0005), representative contigs were obtained by additional assembly using the Geneious software. The original contigs used for these assemblies are listed in Supplementary Table 1.

All representative genomes were translated using MetaGeneMark³⁶. The set of open reading frames produced by this translation for crAssphage was virtually identical to the original set that was produced using the Glimmer software³⁷; all genes detected by MetaGeneMark, for which a function was predicted, were represented also in the original Glimmer translation⁴ (Supplementary Table 2). Homologous protein sequences were

aligned using MUSCLE³⁵. In order to identify putative homologs outside the crAss-like family, the alignments of all conserved proteins were used as queries for PSI-BLAST³³ and HHPred³⁴ searches.

The analysis reported here differed from the original analysis of the crAssphage genome in the following respects: i) expanded databases of genomic and metagenomics sequences were searched, ii) the updated versions of the PSI-BLAST and HHPred software were used, iii) multiple alignments of crAssphage proteins and their homologs, rather than individual crAssphage protein sequences, were used to initiate the searches. Furthermore, no pre-set cut-offs were used in database searches, and all search results and alignments were examined individually for conservation of diagnostic sequence motifs. Together, these amendments to the sequence analysis protocol yielded substantially enhanced search results.

For phylogenetic reconstruction of crAss-like family MCP, PolA, PolB, primase, and ligase (Supplementary Figures 1 and 2), gapped columns (more than 30% of gaps) and columns with low information content were removed from the alignments³⁸; filtered alignments were used for tree reconstructions using the FastTree program³⁹. For the phylogenetic tree shown on Figure 1, the alignments of five conserved proteins of the capsid module were concatenated and used for phylogenetic analysis with PhyML program (<http://www.atgc-montpellier.fr/phyml-sm/>)⁴⁰. The best model identified by PhyML was LG +G+I+F (LG substitution model, gamma distributed site rates with gamma shape parameter estimated from the alignment; fraction of invariable sites estimated from the alignment; and empirical equilibrium frequencies).

Search for nucleotide sequence motifs was performed using the MEME⁴¹ and Gibbs Centroid Sampler⁴² programs.

Data availability

All the data used for the analysis reported in this work are publicly available through GenBank. Genbank Accession Numbers for the representative set of contigs that have been analyzed in detail are given in the Supplementary Dataset 1, and the complete set of Accession Numbers for all crAss-like contigs is available at ftp://ftp.ncbi.nih.gov/pub/yutinn/crassphage_2017/MCP_containing_contig_sources.xlsx. The annotation of the crAssphage and IAS virus genes is given in the Supplementary Dataset 1. Further supporting information is available in Supplementary Note 1 and at ftp://ftp.ncbi.nih.gov/pub/yutinn/crassphage_2017/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Yuri I. Wolf and Sergey Shmakov for invaluable technical help, and Koonin group members for helpful discussion. NY, KSM, ABG, and EVK are supported by intramural funds of the US Department of Health and Human Services (to the National Library of Medicine).

References

1. Rohwer F. Global phage diversity. *Cell*. 2003; 113:141. [PubMed: 12705861]
2. Suttle CA. Marine viruses--major players in the global ecosystem. *Nat Rev Microbiol*. 2007; 5:801–812. nrmicro1750 [pii]. DOI: 10.1038/nrmicro1750 [PubMed: 17853907]
3. Simmonds P, et al. Consensus statement: Virus taxonomy in the age of metagenomics. *Nat Rev Microbiol*. 2017; 15:161–168. nrmicro.2016.177 [pii]. DOI: 10.1038/nrmicro.2016.177 [PubMed: 28134265]
4. Dutilh BE, et al. A highly abundant bacteriophage discovered in the unknown sequences of human faecal metagenomes. *Nat Commun*. 2014; 5:4498. ncomms5498 [pii]. [PubMed: 25058116]
5. Dutilh BE. Metagenomic ventures into outer sequence space. *Bacteriophage*. 2014; 4:e979664. 979664 [pii]. [PubMed: 26458273]
6. Ogilvie LA, Jones BV. The human gut virome: a multifaceted majority. *Front Microbiol*. 2015; 6:918. [PubMed: 26441861]
7. Hurwitz BL, U'Ren JM, Youens-Clark K. Computational prospecting the great viral unknown. *FEMS Microbiol Lett*. 2016:363. fnw077 [pii].
8. Yarygin K, et al. Abundance profiling of specific gene groups using precomputed gut metagenomes yields novel biological hypotheses. *PLoS One*. 2017; 12:e0176154. PONE-D-16-15668 [pii]. [PubMed: 28448616]
9. Manrique P, et al. Healthy human gut phageome. *Proc Natl Acad Sci U S A*. 2016; 113:10400–10405. 1601060113 [pii]. DOI: 10.1073/pnas.1601060113 [PubMed: 27573828]
10. Ahlgren NA, Ren J, Lu YY, Fuhrman JA, Sun F. Alignment-free $\hat{\epsilon}$ oligonucleotide frequency dissimilarity measure improves prediction of hosts from metagenomically-derived viral sequences. *Nucleic Acids Res*. 2017; 45:39–53. gkw1002 [pii]. DOI: 10.1093/nar/gkw1002 [PubMed: 27899557]
11. Wexler AG, Goodman AL. An insider's perspective: Bacteroides as a window into the microbiome. *Nat Microbiol*. 2017; 2:17026. nmicrobiol201726 [pii]. [PubMed: 28440278]
12. Whitaker WR, Shepherd ES, Sonnenburg JL. Tunable Expression Tools Enable Single-Cell Strain Distinction in the Gut Microbiome. *Cell*. 2017; 169:538–546 e512. S0092-8674(17)30370-7 [pii]. DOI: 10.1016/j.cell.2017.03.041 [PubMed: 28431251]
13. Pramono AK, et al. Discovery and Complete Genome Sequence of a Bacteriophage from an Obligate Intracellular Symbiont of a Cellulolytic Protist in the Termite Gut. *Microbes Environ*. 2017
14. Holmfeldt K, et al. Twelve previously unknown phage genera are ubiquitous in global oceans. *Proc Natl Acad Sci U S A*. 2013; 110:12798–12803. 1305956110 [pii]. DOI: 10.1073/pnas.1305956110 [PubMed: 23858439]
15. Oude Munnink BB, et al. Unexplained diarrhoea in HIV-1 infected individuals. *BMC Infect Dis*. 2014; 14:22. 1471-2334-14-22 [pii]. [PubMed: 24410947]
16. Brown CT, et al. Unusual biology across a group comprising more than 15% of domain Bacteria. *Nature*. 2015; 523:208–211. nature14486 [pii]. DOI: 10.1038/nature14486 [PubMed: 26083755]
17. Burroughs AM, Kaur G, Zhang D, Aravind L. Novel clades of the HU/IHF superfamily point to unexpected roles in the eukaryotic centrosome, chromosome partitioning, and biologic conflicts. *Cell Cycle*. 2017:1–11. DOI: 10.1080/15384101.2017.1315494
18. Lander GC, et al. The P22 tail machine at subnanometer resolution reveals the architecture of an infection conduit. *Structure*. 2009; 17:789–799. S0969-2126(09)00186-5 [pii]. DOI: 10.1016/j.str.2009.04.006 [PubMed: 19523897]
19. Casjens SR, Molineux IJ. Short noncontractile tail machines: adsorption and DNA delivery by podoviruses. *Adv Exp Med Biol*. 2012; 726:143–179. DOI: 10.1007/978-1-4614-0980-9_7 [PubMed: 22297513]
20. Bhardwaj A, Molineux IJ, Casjens SR, Cingolani G. Atomic structure of bacteriophage Sf6 tail needle knob. *J Biol Chem*. 2011; 286:30867–30877. M111.260877 [pii]. DOI: 10.1074/jbc.M111.260877 [PubMed: 21705802]

21. Xiang Y, et al. Crystal and cryoEM structural studies of a cell wall degrading enzyme in the bacteriophage phi29 tail. *Proc Natl Acad Sci U S A*. 2008; 105:9552–9557. 0803787105 [pii]. DOI: 10.1073/pnas.0803787105 [PubMed: 18606992]
22. Casjens SR, Thuman-Commike PA. Evolution of mosaically related tailed bacteriophage genomes seen through the lens of phage P22 virion assembly. *Virology*. 2011; 411:393–415. S0042-6822(10)00814-7 [pii]. DOI: 10.1016/j.virol.2010.12.046 [PubMed: 21310457]
23. Lane WJ, Darst SA. Molecular evolution of multisubunit RNA polymerases: sequence analysis. *J Mol Biol*. 2010; 395:671–685. S0022-2836(09)01321-7 [pii]. DOI: 10.1016/j.jmb.2009.10.062 [PubMed: 19895820]
24. Iyer LM, Burroughs AM, Anand S, de Souza RF, Aravind L. Polyvalent Proteins, a Pervasive Theme in the Intergenomic Biological Conflicts of Bacteriophages and Conjugative Elements. *J Bacteriol*. 2017;199. e00245-17 [pii]. JB.00245-17 [pii].
25. Berdyuglova Z, et al. Temporal regulation of gene expression of the *Thermus thermophilus* bacteriophage P23-45. *J Mol Biol*. 2011; 405:125–142. S0022-2836(10)01179-4 [pii]. DOI: 10.1016/j.jmb.2010.10.049 [PubMed: 21050864]
26. Iyer LM, Aravind L. Insights from the architecture of the bacterial transcription apparatus. *J Struct Biol*. 2012; 179:299–319. S1047-8477(11)00361-3 [pii]. DOI: 10.1016/j.jsb.2011.12.013 [PubMed: 22210308]
27. Yakunina M, et al. A non-canonical multisubunit RNA polymerase encoded by a giant bacteriophage. *Nucleic Acids Res*. 2015; 43:10411–10420. gkv1095 [pii]. DOI: 10.1093/nar/gkv1095 [PubMed: 26490960]
28. Lavysh D, et al. The genome of AR9, a giant transducing *Bacillus* phage encoding two multisubunit RNA polymerases. *Virology*. 2016; 495:185–196. S0042-6822(16)30097-6 [pii]. DOI: 10.1016/j.virol.2016.04.030 [PubMed: 27236306]
29. Ruprich-Robert G, Thuriaux P. Non-canonical DNA transcription enzymes and the conservation of two-barrel RNA polymerases. *Nucleic Acids Res*. 2010; 38:4559–4569. gkq201 [pii]. DOI: 10.1093/nar/gkq201 [PubMed: 20360047]
30. Krupovic M, Koonin EV. Multiple origins of viral capsid proteins from cellular ancestors. *Proc Natl Acad Sci U S A*. 2017; 114:E2401–E2410. 1621061114 [pii]. DOI: 10.1073/pnas.1621061114 [PubMed: 28265094]
31. Barr JJ, et al. Subdiffusive motion of bacteriophage in mucosal surfaces increases the frequency of bacterial encounters. *Proc Natl Acad Sci U S A*. 2015; 112:13675–13680. 1508355112 [pii]. DOI: 10.1073/pnas.1508355112 [PubMed: 26483471]
32. Krupovic M, et al. Taxonomy of prokaryotic viruses: update from the ICTV bacterial and archaeal viruses subcommittee. *Arch Virol*. 2016; 161:1095–1099. 10.1007/s00705-015-2728-0 [pii]. DOI: 10.1007/s00705-015-2728-0 [PubMed: 26733293]
33. Altschul SF, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 1997; 25:3389–3402. [PubMed: 9254694]
34. Soding J. Protein homology detection by HMM-HMM comparison. *Bioinformatics*. 2005; 21:951–960. [PubMed: 15531603]
35. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. 2004; 32:1792–1797. [PubMed: 15034147]
36. Besemer J, Borodovsky M. GeneMark: web software for gene finding in prokaryotes, eukaryotes and viruses. *Nucleic Acids Res*. 2005; 33:W451–454. 33/suppl_2/W451 [pii]. DOI: 10.1093/nar/gki487 [PubMed: 15980510]
37. Kelley DR, Liu B, Delcher AL, Pop M, Salzberg SL. Gene prediction with Glimmer for metagenomic sequences augmented by classification and clustering. *Nucleic Acids Res*. 2012; 40:e9. gkr1067 [pii]. [PubMed: 22102569]
38. Yutin N, Makarova KS, Mekhedov SL, Wolf YI, Koonin EV. The deep archaeal roots of eukaryotes. *Mol Biol Evol*. 2008; 25:1619–1630. msn108 [pii]. DOI: 10.1093/molbev/msn108 [PubMed: 18463089]
39. Price MN, Dehal PS, Arkin AP. FastTree 2--approximately maximum-likelihood trees for large alignments. *PLoS ONE*. 2010; 5:e9490. [PubMed: 20224823]

40. Guindon S, et al. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol.* 2010; 59:307–321. [syq010 \[pii\]](#). DOI: 10.1093/sysbio/syq010 [PubMed: 20525638]
41. Bailey TL, Williams N, Misleh C, Li WW. MEME: discovering and analyzing DNA and protein sequence motifs. *Nucleic Acids Res.* 2006; 34:W369–373. 34/suppl_2/W369 [pii]. DOI: 10.1093/nar/gkl198 [PubMed: 16845028]
42. Thompson WA, Newberg LA, Conlan S, McCue LA, Lawrence CE. The Gibbs Centroid Sampler. *Nucleic Acids Res.* 2007; 35:W232–237. [gkm265 \[pii\]](#). DOI: 10.1093/nar/gkm265 [PubMed: 17483517]

Figure 1. Architecture and evolution of the capsid gene module of the crAss-like phage family
 The phylogenetic tree was constructed from concatenated multiple alignments of the 5 proteins of the capsid module. The genomic maps of the capsid gene block are shown for each branch. The 5 genes of the capsid module are color-coded, and uncharacterized adjacent genes are shown by empty block arrows. The colors of labels and branches indicate host or metagenome source: Red, human gut or fecal metagenome; Green, termite gut metagenome; Purple, terrestrial/groundwater; Brown, Soda lake (hypersaline brine); Turquoise, Marine sediment; Orange, populus root microbiome; Black, *Flavobacterium psychrophilum* 950106-1/1 (fish pathogen). Tree branch colors indicate the DNA polymerase family represented in the respective genome (contig): Purple, family B DNAP; Red, family A DNAP; Green, no DNAP; Black, unknown (incomplete genomes). Support values were obtained using 100 bootstrap replications; values greater than 50% are shown. The scale bar represents the number of amino acid substitutions per site. No outgroup was included due to the low (or absent) similarity between the crAss-like family protein to homologs from other phages.

Figure 2. Whole genome maps of crAssphage and IAS virus, the two members of the crAss-like family that are abundant in the human gut virome

Conserved crAss-like family genes are color-coded. Dashed boxes highlight capsid, tail, replication, and transcription gene blocks. Genome regions encoding proteins with strong similarity to Bacteroidetes are shaded in pale green. Gene numbers are according to the crAssphage and IAS virus MetaGeneMark translations. Abbreviations: ssb, single-stranded DNA-binding protein; SF1, SF1 helicase; UDG, uracyl-DNA glycosylase; PolB, DNA polymerase family B; SF2, SNF2-family helicase; RecT, phage RecT recombinase; primase, DnaG family primase; ligase, ATP-dependent DNA ligase; dNK, deoxynucleoside monophosphate kinase; ThyX, flavin-dependent thymidylate synthase; Gp157, Siphovirus Gp157; dUTP, dUTPase; N4_gp49, phage protein of N4_gp49/Sf6_gp66 family; RepL, plasmid replication initiation protein RepL; IHF, integration host factor IHF subunit; PD-(D/E)XK, PD-(D/E)XK family nuclease; Rep_Org, putative replisome organizer protein; DnaB, DnaB replicative DNA helicase; AAA, AAA domain ATPase; rIIA, rIIA-like protector protein; rIIB, rIIB-like protector protein; NRDD, anaerobic ribonucleoside-triphosphate reductase; RNR, anaerobic ribonucleoside-triphosphate reductase activating protein; PolA, DNA polymerase family A; DprA, DNA processing protein DprA. For further details on the annotation, see Supplementary Table 2.

A

B

Figure 3. The replicative gene module of the crAss-like phage family

A. The crAssphage group

B. The rest of the crAss-like family

Homologous genes are marked by the same colors and labels. Genes with no predicted function are numbered according to crAssphage translation, OBV-13 virus translation (suffix 'b'), *Cellulophaga* phage phi14:2 translation (suffix 'c'), or IAS virus translation (suffix 'i'). Abbreviations: RNRm, class II ribonucleotide reductase; RNRa, ribonucleoside reductase alpha chain; RNRb, ribonucleoside reductase beta chain; GGCT, Gamma-glutamyl cyclotransferase; Gn_AT, glucosamine-fructose-6-phosphate aminotransferase; NTP-PPase,

nucleoside triphosphate pyrophosphohydrolase. The rest of the abbreviations are as in Figure 2.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. The genome expression gene module of the crass-like phage family
The predicted RNAP subunits as well as the RNAP and protease motifs are color-coded as shown at the bottom of the figure. The PD-DxK nucleases are most likely encoded in Group I introns.