Comparative genomics of four closely related *Clostridium perfringens* bacteriophages reveals variable evolution among core genes with therapeutic potential

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**Abstract**

**Background:** Because biotechnological uses of bacteriophage gene products as alternatives to conventional antibiotics will require a thorough understanding of their genomic context, we sequenced and analyzed the genomes of four closely related phages isolated from *Clostridium perfringens*, an important agricultural and human pathogen.

**Results:** Phage whole-genome tetra-nucleotide signatures and proteomic tree topologies correlated closely with host phylogeny. Comparisons of our phage genomes to 26 others revealed three shared COGs; of particular interest within this core genome was an endolysin (PF01520, an N-acetylmuramoyl-L-alanine amidase) and a holin (PF04531). Comparative analyses of the evolutionary history and genomic context of these common phage proteins revealed two important results: 1) strongly significant host-specific sequence variation within the endolysin, and 2) a protein domain architecture apparently unique to our phage genomes in which the endolysin is located upstream of its associated holin. Endolysin sequences from our phages were one of two very distinct genotypes distinguished by variability within the putative enzymatically-active domain. The shared or core genome was comprised of genes with multiple sequence types belonging to five pfam families, and genes belonging to 12 pfam families, including the holin genes, which were nearly identical.

**Conclusions:** Significant genomic diversity exists even among closely-related bacteriophages. Holins and endolysins represent conserved functions across divergent phage genomes and, as we demonstrate here, endolysins can have significant variability and host-specificity even among closely-related genomes. Endolysins in our phage genomes may be subject to different selective pressures than the rest of the genome. These findings may have important implications for potential biotechnological applications of phage gene products.

**Background**

Concerns over the spread of antibiotic resistances among bacteria have led to a ban on antimicrobial additives to animal feeds in the European Union (EU) [1,2]. Since its enactment in 2006, the EU-wide ban on the use of antibiotics in animal feed (Regulation 1831/2003/EC) has stimulated a renewed interest in bacteriophage biology and the use of phages and/or phage gene products as alternative antibacterial agents [3,4]. Prior to the discovery and widespread use of antibiotics, bacterial infections were commonly treated by administering bacteriophages which were marketed and sold commercially for human use up until the 1940’s. Bacteriophages continue to be sold in the Russian Federation and Eastern Europe as treatments for bacterial infections [5].

Recently our laboratory reported the genomic and molecular biological characteristics of two phages isolated from poultry intestinal material and poultry processing drainage water by screening for virulent
Clostridium perfringens bacteriophages [6,7] and demonstrated efficacy of the lytic proteins encoded by the bacteriophage endolysins as a C. perfringens antimicrobial [8]. These phages belonged to the Siphoviridae, a family within the tailed phages. The tailed bacteriophages belong to the order Caudovirales, have icosohedral heads, contain a linear, double-stranded DNA genome that can vary from 17 to 500 kb, and represent ca. 95% of all the bacteriophages examined by electron microscope [9]. Caudovirales are further divided into three families based on tail morphology: phages with contractile tails are placed in the Myoviridae, those with short tails are members of the Podoviridae, and phages with a long non-contractile tail belong to the Siphoviridae [10,11].

Clostridium perfringens is a Gram-positive, spore forming, anaerobic bacterium that is the 2nd leading bacterial cause of foodborne illness in the U.S., accounting for 10% of foodborne illnesses [12]. C. perfringens can cause food poisoning, gas gangrene (clostridial myonecrosis), enteritis necroticans, and non-foodborne gastrointestinal infections in humans and is a veterinary pathogen causing enteric diseases in both domestic and wild animals [13,14]. C. perfringens is considered the cause of necrotic enteritis among chickens, and although this does not generally present a threat to humans, it could potentially become a far greater problem for the poultry industry and consumers if antibiotics are withdrawn from animal feeds [13,14].

Bacteriophages have evolved a wide variety of antimicrobial compounds that can control C. perfringens and other pathogens and are of potential biotechnological importance. To realize this potential, it is essential to have a blueprint of the genomic machinery underlying phage-mediated bacterial lysis. Here we report the results of comparative analyses based on genome sequences of four newly isolated C. perfringens phages and focus on the genomic context and evolution of the phage endolysin genes.

Results and Discussion

To first determine the whole-genome relatedness of phages ФCP9O, ФCP13O, ФCP26F, and ФCP34O to each other and to other Clostridial phages, we used two approaches: correlations of tetra-nucleotide frequencies and clustering of predicted proteins based on sequence similarities. The results of both methods were consistent with each other and demonstrated close genomic relationships among our phages, more distant relationships to other Clostridial phages, and consistent correlations between phage and host phylogenies. Our phages were generally quite closely related - both techniques showed that the genomes of ФCP34O and ФCP13O were most closely related to each other and formed a distinct group from ФCP26F and ФCP9O (Figure 1). All four genomes were similar to the genome of ФCP39O, previously published by our research group [6], and belonged to a larger clade (Figure 1B, 1C) containing ФCPV1, a C. perfringens phage isolated in Russia [7]. Genomic comparisons of our phages to two other C. perfringens phage genomes (ФSM101 and Ф3626), three C. difficile-infective phages (ФC2, ФCD27, and ФCD119), and one C. botulinum-infective phage (ФC-St) showed phage phylogeny closely associated with host phylogeny (Figure 1B, 1C). Our results of nearly identical topologies between tetra-nucleotide and proteomic trees is consistent with previous uses of tetra-nucleotide distributions as genomic signatures [15,16] and to infer co-evolution between virus and host [17].

Core and accessory genomes of Clostridial phages

To determine if our phages contain a common set of genes shared with other Clostridial phages, we compared predicted ORFs based on classifications of clusters of orthologous groups (COGs) among the three host groups shown in Figure 1. COGs represent individual proteins or groups of paralogs from at least three lineages corresponding to ancient conserved domains (http://www.ncbi.nlm.nih.gov/COG/) and thus provide an informative means to compare conserved functions across genomes [18].

Three COGs were shared among bacteriophages infecting C. perfringens, C. difficile, and C. botulinum (Figure 2). These shared COGs were COG5412, annotated as a phage-related protein of unknown function; COG0629, a single-stranded DNA-binding protein; and COG0860, a phage endolysin, N-acetylmuramoyl-L-alanine amidase (Figure 2). Endolysins, together with holins, are the key bacteriophage-encoded enzymes involved in cell wall degradation and lysis of the host and are typically transcribed from adjacent ORFs in the phage genome [8,19-21]. To better understand the evolution and natural variability of an endolysin in its genomic context, we investigated the phylogeny of the N-acetylmuramoyl-L-alanine amidase across multiple host genera and compared the phylogeny and host associations to the domain architecture of the endolysin-holin gene neighborhood.

Statistical associations between domain architecture and phylogeny

To compare our phage sequences and domain architecture to others, we retrieved amidase sequences belonging to the pfam protein family PF01520 from 26 publicly available bacteriophage genomes (Additional file 1, Table S1) and analysed these as fully described in the methods. Bacteriophage endolysins typically contain two domains: an enzymatically active domain and a cell wall
Figure 1 Whole-genome comparisons of Clostridial phages. A) Tetrancleotide-based comparisons of five genomes sequenced by our lab (ΦCP130, ΦCP26F, ΦCP34O, ΦCP39O, ΦCP9O) to three other publicly available *C. perfringens* phage genomes (ΦCPV1, ΦSM101, Φ3626). Lower panel shows scatter plots with linear models fitted to the 256 tetrancleotide z-scores for each pairwise genomic comparison. Upper panel represents Pearson correlation coefficients and significance (**p < 0.001**) of correlations. B) Cladogram representation of correlation matrix of tetrancleotide distributions from (a) with additional comparisons to *C. difficile* phages (ΦC2, ΦCD27, ΦCD119) and a *C. botulinum*-infective phage (ΦC-St). C) Proteome-based cladogram comparing the same phage genomes as in (b). Tree is based on all-versus-all sequence similarity comparisons of gene predictions using a custom analysis pipeline as fully described in the text. Note consistent and symmetrical topology of trees in (b) and (c) and consistent relationships to host as shown by shaded vertical bars.
binding domain, some of which have been elucidated with crystal structures [22]. We constructed an alignment of both putative domains after building a Hidden Markov Model from representative sequences in the Conserved Domain Database belonging to PF01520 and Markov Model from representative sequences in the
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Figure 2 Distribution of COGs across three host-infectivity groups. Each circle of the Venn diagram contains all COGs belonging to the host-infectivity group; intersections represent shared COGs. Groups shown are ‘Current’: C. perfringens phage genomes published here and previously by our group (ΦCP9O, ΦCP13O, ΦCP26F, ΦCP34O, and ΦCP39O), ‘Other C. perfr’: other C. perfringens-infective phages (Φ5626, ΦSM101, ΦCpV1), and ‘C. diff.’: C. difficile-infective phages (ΦC2, ΦCD27, and ΦCD19). COGs were classified according to the IMG pipeline as described in the text.

is immediately downstream of the amidase protein in the presumed direction of transcription, a reversed arrangement of the typical domain architecture (Figure 3). Interestingly, though Φ3626, ΦC2, and ΦCD27 belonged to a sister clade, this domain architecture was unique even among these other Clostridial phages (Figure 3). To confirm this domain architecture for our phages, we re-sequenced the appropriate regions of Φ9O, Φ13O, Φ26F, Φ34O, and several other phage isolates, all of which shared the amidase-holin arrangement. Holin genes were identified using multiple sequence-similarity approaches as described in detail in the methods, and included identifications of transmembrane domains. The association between gene phylogeny and domain architecture was strongly significant as determined by UniFrac (p < 0.001) and P tests (p < 0.001).

Because lysis of bacterial cells generally requires both an endolysin and a holin - membrane disruption (the function of the holin) is considered to be requisite for the endolysin to attack the peptidoglycan [19] - understanding the phylogenetics and genomic context of these genes are important milestones to develop biotechnological applications. The unusual domain architecture we observed suggests that either the typical gene order or the reverse is a successful evolutionary strategy. The transcriptional regulation of these genes in our phages remains unknown, but searches for transcriptional promoters and terminators using BPROM (Softberry, Inc., Mount Kisco, NY, USA; http://linux1.softberry.com/berry.phtml) and TransTerm (http://nbc3.biologie.uni-kl.de) did not find either within the regions of our endolysin and holin genes; these genes may be co-transcribed. Efficacy of the endolysin as recently demonstrated for phages ΦCP26F and ΦCP39O [8] could potentially be improved by successful holin purification.

Genomic arrangement and context of orthologs
Twenty-one pfam families were identified among the four phage genomes (Figure 4). Of these, only one, PF04233, annotated as a homolog of phage Mu protein gp30, was found in only one genome (ΦCP13O). Three other pfams were found in 2-3 genomes and were absent from the other(s). A prophage antirepressor (PF02498) was present and 100% identical in the genomes of ΦCP9O, ΦCP13O, and ΦCP26F, but, interestingly, a syntenous protein of ΦCP34O (gene product 22, Figure 4), had no significant sequence similarity to these sequences based on pairwise blastp and no significant matches to any pfams. Similarly, 3'-phosphoadenosine 5'-phosphosulfate sulfotransferase (PAPS reductase)/FAD synthetase (pfas01507) genes were present in the genomes of ΦCP13O and ΦCP34O with 100%
pairwise sequence similarity, but approximately syntenous ORFs in the genomes of FCp9O and FCp26F had no significant blastp similarity to COG0175 and did not match any pfam domains. The majority of pfams (17/21) were present in all four bacteriophage genomes (Figure 4). Detailed statistics for each genome are shown in Additional file 2, Table S3.

Conservation and variability of core genome
To investigate shared genes in more detail and to classify the majority of predicted ORFs which were not assigned to COGs or pfams, we next compared the distributions of pfams and sequence-similarity groups derived by clustering of all predicted ORFs across all four genomes to determine a core and accessory genome (Figure 5). Most gene clusters (41/61) were shared by all four genomes on the basis of sequence similarity (Figure 5a). Of the 17 pfam families that were common to all four genomes, we considered 12 to represent a ‘conserved core genome’, and five to represent a ‘variable core genome’ based on pairwise sequence similarities (Figure 5b). The five pfam families in the core genome containing highly variable genes were: PF01520, the N-acetylmuramoyl-L-alanine amidase (COG0860); PF11753 of unknown function; PF10145, a tail tape measure protein (COG5412); PF02511, a thymidylate synthase (COG1351) involved in...
nucleotide transport and metabolism; and PF11651, a P22 coat protein (Figure 5b).

In the conserved core genome, genes within each of the 12 pfam families were very similar to each other, with a maximum pairwise sequence difference of 8% based on amino acid alignments with bl2seq (Figure 5b). Genes belonging to these 12 pfam families were involved in the following functions: tail protein, phage anti-repressor, ssDNA binding, portal protein, minor structural protein GP20, hydrolase, CHC2 zinc finger, terminase large subunit, virulence-associated protein E, and the holin (Figure 5b).

The holin genes were among the most conserved, with 100% identity among all sequences, and the amidase genes were the most variable (Figure 5b), suggesting these two genes are subject to very different rates of evolution despite their colocation in the genome and paired function in the lytic cycle. Holins target the relatively invariant cytoplasmic membrane, while phage endolysins recognize and degrade the cell wall, which is highly variable. It has been suggested that holins may function as a type of lysis clock, governing the timing of lysis of the host [26]. As the primary determinant of the length of the infective cycle, holins can be considered to experience stabilizing selection as there are opposing fitness advantages to extending the vegetative cycle and allowing phage replication versus lysing the host to release progeny phage to infect new host cells [19].

**Figure 4** Genome maps for phage \( \Phi \text{CP}90 \), \( \Phi \text{CP}130 \), \( \Phi \text{CP}26F \), and \( \Phi \text{CP}340 \). Arrows are shown in presumed direction of transcription. Grey arrows indicate genes of unknown homology or function, colored arrows indicate genes belonging to pfam families or clusters of orthologous groups (COGs) as classified by IMG. Functional categorizations of pfam designations are shown in the legend. For predicted ORFs with unknown function, sequence similarity clustering at a 40% similarity cutoff is indicated by shading.
contrast, the phage endolysins generally contain an enzymatically active domain and a cell-wall binding domain which recognizes highly-specific ligands on the host cell surface [27], and thus each domain is under strong directional selective pressures. Our data clearly show strong sequence conservation of the holin protein, and very distinct sequence types within the associated amidase for a group of closely related phages.

**Detailed sequence comparisons of variable core genome and association with host genotype**

For the four pfam families with known functions in the variable core genome, multiple-sequence alignments of the four genomes presented here and \( \Phi CP390 \), \( \Phi CP130 \), \( \Phi CP26F \), and \( \Phi CP34O \) showed strong sequence conservation of the holin protein, and 473AA residues in length with a 225 AA distance of the protein, corresponding to the cell wall binding domain (CBD) of \( \Phi CP26F \) and \( \Phi CP34O \). Genes belonging to the five pfam families shown in grey were defined as a conserved core genome for the structure and function of the protein, but clearly the ancestral state remains unknown, as do the consequences for the structure and function of the protein, but clearly these questions warrant further study.

For the thymidylate synthase (PF02511/COG1351), the protein encoded by a single reading frame, the N-terminal portion of the protein encoded by another ORF immediately upstream in the genome. For the por-
Figure 6 Multiple-sequence alignments for each of the four Pfam families of the variable core genome with known functions. Dots represent conserved positions relative to the first sequence, topologies of sequence-similarity groupings are shown to the left of each alignment. Alignments illustrate distinct sequence types defined by sequence variation and major deletions/insertions within each gene for A) PF01520/COG0860, N-acetylmuramoyl-L-alanine amidase, B) PF10145/COG5412, tape measure protein, C) PF02511/COG1351, thymidylate synthase, and D) PF11651, P22 coat protein. Whole genome relationships of host C. perfringens as determined by rep-PCR are shown in (E). Scale represents % dissimilarity. For each gene, topologies of neighbor-joining trees are outlined to the left of the alignment. For A and C, alignments of entire protein are shown; for B, the entire protein was 780 AA, not shown is 220 AA N-terminal deletion for FCP13O and FCP34O which is encoded by another ORF immediately upstream. Alignments were done using MUSCLE with default parameters as described in the text. Inferred EAD (N-terminal) and CBD (C-terminal) domains of the amidase joined by a linker region are designated by boxes in (A).
those shared by ΦCP9O, ΦCP26F, and ΦCP39O (Figure 6d).

In contrast to these groupings, genomic fingerprints of the *C. perfringens* host based on rep-PCR defined three main host groups: 1) Cp34O and Cp9O, 2) Cp13O and Cp39O, and 3) Cp26F as a more distantly related group (Figure 6e). Interestingly, the single gene phage similarities based on the tape measure protein, the thymidylate synthase, and the coat protein reflected the whole-genome groupings shown in Figure 1 with ΦCP13O and ΦCP34O most similar to each other and ΦCP9O, ΦCP26F, and ΦCP39O forming a separate group. In contrast, sequence similarities based on the amidase protein were not concordant with the other genes in the core genome or the whole-genome clustering. Based on these data, we concluded that the selective pressures on the amidase genes for these phages are somehow unique from the rest of the genome. This result may have important implications for potential biotechnological applications in which amidase proteins are used separately or together with other gene products such as holins for bacterial control.

**Endolysin protein structure**

To investigate the association between the sequence variability of our phages and the structure of the EAD and the CBD of the amidase, we constructed a structural model using as a template a related structure from a *Listeria* phage (PDB: 1XOV) previously solved with crystallography [22]. Comparative modelling of bacteriophage lytic enzymes is becoming a common tool to inform the development of phage lysis-based biocontrol agents [28]. N-acetylmuramoyl-L-alanine amidases are one of at least six types of phage endolysins and attack the amide bonds between the amino sugar MurNAc and L-Ala of the cross-linking peptide stem in the peptidoglycan layer of the host cell wall [21]. The specificity of the enzyme is thought to be due to recognition of specific ligands on the host cell surface by the CBD [21]. Our modeling revealed that the enzymatic core is formed by a twisted, six-stranded β-sheet flanked by six helices (α1–α6) linked through a loop region to the cell wall binding domain which consists of two anti-parallel β-sheets (Figure 7). The areas of highest sequence conservation were concentrated in the CBD and the central portion of the enzymatic domain (Figure 7). Several point mutations within the CBD may contribute to its specificity, but interestingly, for our phages, the N-terminal EAD was much more variable than the CBD, suggesting much higher diversifying selective pressures on this portion of the protein.

**Conclusions**

Comparisons of genome sequences from four newly isolated *C. perfringens* phages and related sequences previously published has provided new insights into genomic conservation and variability. Sequence and structural variability of the endolysin EAD may have important implications for the potential to target specific strains of pathogenic bacteria. Sequence and structural conservation of the CBD suggests the potential to tailor specificity for detection and differentiation of target cell populations, extending previous work [29]. Holins and endolysins represent conserved functions across divergent phage genomes and, as we demonstrate here, endolysins can have significant variability and host-specificity even among closely-related genomes. Endolysins in our phage genomes may be subject to different selective pressures than the rest of the genome, with important implications for potential biotechnological applications of these phages and their gene products.

**Methods**

**Bacteriophage Genome Sequencing**

Purification and propagation of bacteriophages and subsequent genomic DNA purification was carried out as previously described in detail [6]. Sequencing of the bacteriophage genomes was completed by MWG Biotech, Inc High Point, NC by Sanger and pyrosequencing to 14-fold redundancy that included primer-walking to fill gaps.

**Genome Annotations and comparisons**

Gene predictions and genome annotations were performed with the IMG pipeline [30], which uses a combination of Hidden Markov Models and sequence similarity searches. Briefly, gene predictions were performed with GeneMark [31] and then compared to COG PSSMs obtained from the CDD database [32], searched against the KEGG genes database [33] with BLASTp, and then searched against the Pfam [34] and TIGRfam [35] databases using BLAST prefiltering and subsequent comparison to HMMs using hmmssearch [36]. To compare the phylogeny and protein domain architecture of phage-encoded endolysin and holin genes, genomes of 26 bacteriophage were retrieved from IMG (Additional file 1, Table S1) based on top ortholog hits to COG0860. Genome accession numbers and basic summary statistics are shown in Additional file 1, Table S2. Gene predictions, annotations, and genome coordinates are listed for each genome in Additional file 2, Table S3.

Tetra-nucleotide distributions for Clostridial phage genomes and correlation coefficients between genomes were calculated with TETRA [37]. Correlation coefficients were transformed to a dissimilarity matrix for tree construction using the hierarchical clustering algorithm hclust in R [38], which was also used to generate dendrograms and visualize tetra-nucleotide distributions.
Proteomic comparisons of Clostridial phage genomes was performed with a custom analysis pipeline we constructed using CD-HIT [39] for clustering of predicted ORFs. Output was parsed with a series of perl scripts, and dendrograms constructed in mothur [40] using the Jaccard similarity index. COG and pfam designations from IMG for each genome were used to determine shared and accessory functions across the 12 Clostridial phage genomes. To construct genome maps, annotated genome files were transferred to Artemis [41] and genome maps constructed with DNA Plotter [42]. rep-PCR of host genomes was performed as previously described [43].

**Tree construction**

Bacteriophage endolysin sequences belonging to COG0860 and/or PF01520 were retrieved from IMG and Genbank genomes using BioPerl. A seed alignment of 100 representative sequences belonging to conserved domain cd0269 in the CDD (10) was used to build a
Hidden-Markov profile and the phage sequences shown in Figure 3 were aligned to this HMM model using Hmmer 3.0 (14). Aligned sequences were imported into ARB [44] where trees were constructed with neighbor-joining and maximum-likelihood methods restricted to columns sharing at least 10% sequence identity. When identical topologies were obtained with both methods, tree files were exported and visualized with ITOL [45]. The significance of associations between phylology and host, and phylology and protein domain architecture was assessed with UniFrac [23] and Parsimony tests [24], which use a Monte Carlo approach to compare observed phylogenies with a null model derived from random permutations.

Designation and comparisons of core versus accessory genomes
Shared and unique genes, COGs, and pfams were determined by two methods. First, the same analysis pipeline described above was used to group predicted ORFs on the basis of sequence similarity as determined by CD-HIT [39]. Second, classifications from IMG were used to determine shared and unique COGs and pfam families. The similarity of genes belonging to each pfam family in the core genome was determined by pairwise blastp implemented with the b12seq algorithm in a perl script.

Structural Modeling
The 3D structure of the endolysin from ΦCP26F (ORF22, pfam01520) was modeled using the HHpred server with default settings [46]. Briefly, the HHpred method is specialized in remote homology detection using hidden Markov models (HMMs) built from PSI-BLAST profiles and secondary structures. The crystal structure of *Listeria* PlyPSA (Protein Data Bank code 1XOV chain A, [22]) was used as a template since it had the highest sequence and secondary structure scores. Lastly, a 3D model was generated using MODELLER [47] and visualized using the UCSF Chimera molecular analysis program [48]. Sequence conservation among our five phages was calculated using the mavPercentConservation method based on the AL2CO algorithm [48] which performs calculations in two steps. First, amino acid frequencies at each position are estimated and then the conservation index is calculated from these frequencies. The results were then mapped to the predicted protein structure of ΦCP26F using the following color parameters: lowest (60%) and highest (100%) sequence conservation.

Additional file 2: Additional_File2_TableS3.xls Gene predictions, annotations, and genome coordinates.

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Authors’ contributions
BBO annotated genomes, analyzed data, and wrote the ms; ET performed the structural modeling and contributed to the ms; CAM provided technical support; NNV and KLH contributed to genome sequencing efforts; GRS initiated the study, isolated the phages, and performed rep-PCR; and BSS oversaw the genome sequencing and supervised the project. All authors read and approved the final manuscript.

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