Diverse Virulent Pneumophages Infect *Streptococcus mitis*

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Abstract

*Streptococcus mitis* has emerged as one of the leading causes of bacterial endocarditis and is related to *Streptococcus pneumoniae*. Antibiotic resistance has also increased among strains of *S. mitis* and *S. pneumoniae*. Phages are being reinvestigated as alternatives to antibiotics for managing infections. In this study, the two virulent phages Cp-1 (*Podoviridae*) and Dp-1 (*Siphoviridae*), previously isolated from *S. pneumoniae*, were found to also infect *S. mitis*. Microbiological assays showed that both pneumophages could not only replicate in *S. mitis* but also produced more visible plaques on this host. However, the burst size and phage adsorption data were lower in *S. mitis* as compared to *S. pneumoniae*. A comparison of the genomes of each phage grown on both hosts produced identical nucleotide sequences, confirming that the same phages infect both bacterial species. We also discovered that the genomic sequence of podophage Cp-1 of the Félix d’Hérelle collection is different than the previously reported sequence and thus renamed SOCP.

Introduction

The *Mitis* group of streptococci is a member of the viridans group of streptococci [VGS], which includes several species that reside in the human oral cavity and the upper respiratory tract. In the *Mitis* group, *Streptococcus pneumoniae* and *Streptococcus mitis* are closely related species, making discrimination between them very difficult [1]. Although both bacteria are oral commensals they can cause a wide variety of human invasive diseases [2]. *S. pneumoniae* (pneumococcus) is the most common cause of community-acquired pneumonia worldwide and is also associated with a range of other diseases, including otitis media, meningitis, and septicemia [3]. *S. mitis* has traditionally been regarded as an innocuous commensal of the oropharynx, skin, and both the gastrointestinal and genitourinary tracts [4]. Some *S. mitis* strains have been shown to cause endocarditis and blood stream infections [5]. The transition from commensalism to pathogenesis is likely related to the acquisition of virulence genes.
S. pneumoniae and S. mitis have emerged as significant pathogens but differ in their expression of virulence factors. For example, phase variation is an important step in virulence and also an adaptive process that leads to the distribution of bacteria to other sites, causing disease [6]. The switching of phenotypes was noted in S. pneumoniae where phase variation changes the colony morphology from opaque to transparent without any change in the serotype. To our knowledge, this type of phase variation has not been observed for S. mitis [7], however, strains of S. mitis were recently shown to have a capsule, a well known virulence factor in S. pneumoniae [8]. In fact, genomic analysis of S. mitis revealed the presence of several virulence factors similar to those found in S. pneumoniae. However, it has not been confirmed whether these virulence factors are just implicated in adhesion and attachment of S. mitis or whether they are related to pathogenicity as in S. pneumoniae [5,9].

Strains nonsusceptible to the common antimicrobial drugs were detected among S. pneumoniae and S. mitis, especially to beta-lactams which have been widely used to treat such bacterial infections [10]. It was also reported for S. pneumoniae and other bacterial species that low concentrations of some antibiotics induces natural transformation and mutagenesis [11,12,13]. The ubiquitous nature of antibiotic resistance genes may also be due to horizontal gene transfer among strains of the naturally transformable Mitis group. Therefore, there is pressure to develop novel antibacterial alternatives.

Virulent phages may represent such an alternative strategy to combat antibiotic-resistant bacteria. Phages are recognized as the most abundant biological entities in the biosphere [14]. The study of phages of hemolytic streptococci began decades ago [15], and the vast majority of phages currently isolated from S. mitis and S. pneumoniae are temperate phages. In fact, both bacterial species contain many prophages in their genomes [16,17]. Prophage carriage was identified in clinical isolates of S. pneumoniae by hybridation and also via induction by mitomycin C [16,17]. At least seven phage-related gene clusters were detected in the genome of S. mitis B6 and two complete prophages (SM1 and phiB6) were isolated and sequenced [18,19]. Because temperate phages have the ability to transfer host DNA, which may encode virulence genes or toxins, into other bacterial strains [18,20], virulent phages are thought to be better suited for biocontrol purposes.

Very few lytic phages have been isolated for either of these two bacterial species. Despite the isolation of pneumophages, also called Omega phages [21], only two virulent pneumococcus phages are readily available today. Phage Dp-1 was the first virulent pneumophage isolated in 1975 [21,22] and belongs to the Siphoviridae family. Phage Cp-1 was isolated in 1981 [23] and is a member of the Picovirinae subfamily of the Podoviridae family, with a linear double-stranded DNA genome [24]. One virulent phage of S. mitis, vB_SmM_GEC-SmitisM_2, was isolated in 2012 from sewage water and belongs to the Myoviridae family [25].

Of interest, enzymes from virulent pneumococcal phages have also shown promise as antimicrobials [26,27,28,29,30,31,32]. At the end of their lytic cycle, virulent phages produce an enzyme (endolysin or lysine) that degrades the bacterial peptidoglycan to release new virions. A number of studies have demonstrated the potential of the lysin [Cpl-1] from phage Cp-1 to eradicate nasopharyngeal colonization and bacteremia [28], and to prevent otitis media [30]. The administration of Cpl-1 by inhalation [27] or by repetitive intraperitoneal injections even rescued mice from severe pneumococcal pneumonia [32].

In this study, we show that the virulent pneumophages Dp-1 and Cp-1 can infect S. mitis. Microbiological assays and genomic analyses were performed to compare phage behavior in both bacterial species.
Materials and Methods

Bacterial strains and culture conditions

The unencapsulated strain *S. pneumoniae* R6 (host of phage Cp-1), a derivative of *S. pneumoniae* strain R36A [host strain of phage Dp-1], was grown on TSA (Trypticase Soy Agar) containing 5% sheep’s blood at 37°C in the presence of 5% CO₂. Single colonies were suspended in filtered BHI (Brain heart infusion) broth containing 0.5% yeast extract and incubated at 37°C in the presence of 5% CO₂. *S. mitis* CCRI-15019 was grown at 30°C in BHI supplemented with 0.2 mM magnesium sulfate and 0.25 mM calcium chloride to prevent cell aggregation. The bacterial strains are stored at the Félix d’Hérelle Reference Center for Bacterial Viruses (www.phage.ulaval.ca).

Bacterial species identification

The bacterial housekeeping genes *recA*, *recP*, *HexB*, and *xpt* were amplified by PCR using the primers listed in S1 Table [22]. The PCR products were sequenced by the Plateforme de Séquençage et de Génotypage des Génomes service at the CHUL/CHUQ Research Center using the ABI data 3730XL DNA analyzer. Both DNA strands of the amplicons were sequenced using the same primer pairs used for PCR amplification. Sequences were aligned and analysed using the bioinformatics tools (Clustal W2 and BioEdit).

Phages

Pneumophages Cp-1 [23] and Dp-1 [22] were obtained from the Félix d’Hérelle Reference Center for Bacterial Viruses. For phage amplification, and to facilitate enumeration, we used liquid BHI+ medium, which consisted of BHI supplemented with 8 μM MnCl₂, 0.25 mM CaCl₂, 0.2 mM MgSO₄, 50 Mm Tris-HCl pH 7.5, 50 ng/μl choline chloride, 0.4% glycine, and 100 μl/ml catalase. Amplifications of phage Cp-1 on *S. pneumoniae* and on *S. mitis* were performed with agitation, as follows: a single colony, isolated from overnight culture on BHI blood agar, was used for cultivation in BHI. After overnight growth of the host strains an aliquot of the culture was transferred into fresh BHI and incubated until it reached an optical density at 600 nm (OD₆₀₀nm) of 0.06–0.08. The culture was diluted with an equal volume of BHI+, phages were added and incubated overnight at 30°C. Phage titers were obtained using a standard, double-layer agar assay method. To maximize plaque visualization, the bottom layer of BHI+ contained 1.5% agarose while the top agar contained 0.4% agarose.

Microbiological assays

Phage lytic development was assessed using a one-step growth curve assay, in triplicate, as described elsewhere [33]. After overnight growth of the host strain in BHI, an aliquot of the culture was transferred into fresh BHI and incubated until it reached an OD₆₀₀nm of 0.06–0.08. The bacterial culture was diluted 1:3 in BHI+ and then infected at a multiplicity of infection of 0.05 at 30°C. Phages were allowed to adsorb to the host cells for 10 min. Unadsorbed phages were removed by centrifugation, the bacterial pellet was washed twice with BHI media and then we proceeded as described elsewhere [33]. The plates were incubated at 30°C overnight with 5% CO₂, and the plaque forming units (PFU) were counted. The burst size was determined by calculating the ratio of the average phage titer after the exponential phase to the average titer before the infected cells began to release virions [33]. Phage adsorption tests were also carried out on *S. pneumoniae* R6 and *S. mitis* CCRI-15019, in triplicate, essentially as previously reported [34]. Modifications: BHI+ broth was used, CaCl₂ was not added again at phage infection and the incubation was carried out at 30°C for 10 min.
Electron microscopy

Phage preparations produced on *S. pneumoniae* and *S. mitis* were purified using a CsCl gradient, as previously described [35]. Phages were then centrifuged and 100 μl of the pellet were kept and washed with 1.5 ml of ammonium acetate (0.1 M, pH 7.5). This process was repeated twice and 100 μl from the final wash was retained for observation by transmission electron microscopy. Grid preparation and observation were performed as previously described [36]. Phages were observed at 80 kV using a JEOL 1230 transmission electron microscope.

Phage DNA preparation and sequencing

Genomic DNAs of phages Cp-1 and Dp-1 amplified on *S. mitis* CCRI-15019 were isolated using a Lambda Maxi Kit (Qiagen). Phage Cp-1 DNA was also isolated after propagation on *S. pneumoniae* R6. Genome sequencing was performed on a 454 FLX instrument at the Plateforme d’Analyses Génomiques of the Université Laval (IBIS). The genomic sequences were completed by primer walking (primer sequences are listed in S2 Table) and by sequencing of PCR products. All mutations were also confirmed by primer walking.

Phage genome analyses

Genomic sequences were analyzed using BioEdit 7.2.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and the Staden package [37,38] (http://staden.sourceforge.net/). Sequence alignments were performed using BioEdit and Clustal W2 software (http://www.ebi.ac.uk/Tools/mga/clustalw2/). Open reading frames [ORFs] were identified using GenMark (http://exon.gatech.edu/) and ORFinder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Sequences were considered to be ORFs if they showed a putative ribosome binding site (RBS) at a reasonable distance from the starting codon (AUG, UUG, or GUG) and they consisted of at least 29 amino acids (aa). Function was attributed to an ORF by comparing the translated product to proteins available at the National Center for Biotechnology Information (BLASTp, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The annotations were reinforced by searching for protein functional domains using the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and EMBL InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). The theoretical molecular masses [MM] and isoelectric points [pI] of each deduced phage protein were determined using the ProtParam software tool available on the bioinformatics resource portal ExPASy Web site (http://ca.expasy.org/tools/protparam.html). The genomes of phages Cp-1 and Dp-1 were searched for tRNAs using tRNAscan-SE [39] and BLASTn from NCBI. Bacterial codon usage for the host strains was obtained from the Kazusa DNA Research Institute database (http://www.kazusa.or.jp/codon/) while codon usage for the phages was determined using the DNA 2.0 web server (Menlo Park, CA); the frequency per thousand codons was then calculated.

The complete genome sequence of phage SOCP has been deposited in GenBank under accession number KJ617393.

Results

Speciation of bacterial hosts

The species identification of *S. mitis* from the closely related *S. pneumoniae* represents a challenge. For example, significant sequence conservation of the 16S rRNA gene sequence within the Mitis group limits the use of these sequences for species differentiation [40]. To confirm the bacterial strains used in this study, degenerate primers were used and the PCR products of four housekeeping genes, *recA*, *recP*, *hex B* and *xpt* (S1 Table) [40] were amplified and
sequenced. Our results confirmed that the isolate CCRI-15019 is highly related to *S. mitis* B6 [18]. Our *S. pneumoniae* R6 and R36A strains were similarly confirmed.

**Morphological analysis of phages Dp-1 and SOCP**

As documented previously [40], phage Dp-1 belongs to the *Siphoviridae* family. It has an icosahedral capsid with an estimated diameter of 69.8 ± 1.2 nm (Fig. 1A) and a long non-contractile tail of 169.9 ± 3.6 nm in length and 19.6 ± 2 nm in width. Phage Cp-1, used in this study and stored at the d’Hérelle Center, was re-named SOCP due to genome variations (see below). Phage SOCP belongs to the *Podoviridae* family and has a hexagonal capsid of 65.8 ± 1.1 nm (top to bottom) and 42.1 ± 1.7 nm in width (Fig. 1D). This phage has a short non-contractile tail, 19.3 ± 1 nm in length and 7.5 ± 1.2 nm in width. Observations of phages Dp-1 and SOCP, amplified on both bacterial species, confirmed that the general morphology of these virions was not affected by growth in either hosts.

**Microbiological assays**

One of the great difficulties of working with virulent pneumophages is the ability to visualize plaques consistently. We were able to observe plaques for both phages using BHI+ media (Figs. 1B & E). We also noticed that liquid cultures of *S. pneumoniae* grew better in BHI+ when started from colonies grown on blood agar. Finally, replacing agar by agarose in both top and bottom media led to an improvement in the plaque size and visibility.
The availability of an improved plaque assay allowed us to investigate the host range of phages Dp-1 and SOCP. Interestingly, we observed that they could both replicate on a *S. mitis* strain (CCRI-15019). Moreover, even larger phage plaques were obtained on *S. mitis*, compared to *S. pneumoniae* host cells grown under the same conditions (Figs. 1BC & EF). To further investigate the behavior of the two phages on both bacterial species, one-step growth curve assays were performed using *S. pneumoniae* R6 and *S. mitis* CCRI-15019. The latent period of phage Dp-1 was calculated to be 71 ± 4 min when amplified on *S. pneumoniae* and 59 ± 4 min on *S. mitis*. The burst sizes were 73 ± 6 plaque-forming units (PFU) per infected cell and 63 ± 6 PFU for *S. pneumoniae* and *S. mitis*, respectively. Similarly, the latent period of phage SOCP was estimated to be 78 ± 2 min on *S. pneumoniae* and 66 ± 5 min on *S. mitis*, while the burst size was 94 ± 4 PFU on its pneumococcal host and 53 ± 4 PFU on *S. mitis*. These data indicated a lower burst size for both phages on *S. mitis* cells but a shorter latent period.

Adsorption to the cell surface was also evaluated for the two phages on both strains. Under the conditions tested, the percentage of adsorption of phage Dp-1 on *S. pneumoniae* R6 was 95% ± 1.8% and was 79% ±1% on *S. mitis*. The adsorption of phage SOCP was 94% ± 0.6% on *S. pneumoniae* R6 and 86 ± 1.3% on *S. mitis* B6. Thus, both pneumophages could also readily adsorb to the *S. mitis* cell surface.

The efficacy of plaquing (EOP) of both phages on the two propagating hosts was determined. When phages Dp-1 and SOCP were amplified on their *S. pneumoniae* hosts they had EOPs of $10^{-1}$ and $10^{-4}$ on *S. mitis*, respectively. Similarly, when Dp-1 and SOPC were amplified on *S. mitis*, their EOPs on *S. pneumoniae* were $10^{-1}$ and $10^{-3}$, respectively. These data suggest the presence of host factors that modify the phage behavior.

**Genome analysis**

To determine if replicating these phages on various hosts had an impact at the nucleotide level, we determined the complete genome sequence of both phages after amplification on *S. pneumoniae* or *S. mitis*. The double-stranded DNA (dsDNA) genome of phage Dp-1 is made of 56,506 bp. The analysis of the sequence found no nucleotide variation compared to the genomic sequence recently reported for Dp-1 [41]. Moreover, the sequence was identical when Dp-1 was amplified on both *S. pneumoniae* and *S. mitis* hosts, indicating that propagating this siphophage on two distinct streptococcal species did not lead to genomic changes.

Similarly, the genome of the podophage was also identical when propagated on either *S. pneumoniae* or *S. mitis*. However, analysis of the genomic sequence of the phage Cp-1 used in this study showed variations compared to the GenBank sequence no. NC_001825.1. As indicated above, we thus renamed our Cp-1-like phage SOCP due to these differences. The dsDNA genome of SOCP is 19,347 bp long, while the reported genome of phage Cp-1 is 19,343 bp. Comparative analyses revealed 31 variations in the genome of SOCP as compared to Cp-1 (S3 Table). These differences were confirmed through primer walking directly on the phage genome.

The genome of phage SOCP has a GC content of 38.8%. It has 27 open reading frames, each preceded by a putative ribosome-binding site (RBS) (Table 1). Putative functions could be attributed to only 12 ORFs (44%). Interestingly, we found two additional ORFs in SOCP as compared to Cp-1, namely ORF23 and ORF25 (Fig. 2). Of interest were also the products of genes *orf5* and *orf6*, which likely code for DNA polymerase subunits [42,43], whereas only one (*orf5*) was found in Cp-1. Conversely in the case of *orf18*, one gene was found in SOCP whereas it was split into two genes (*orf17* and *orf18*) in Cp-1. The major capsid protein ORF10 was also affected by mutations, although its size remained the same (S3 Table). Other notable ORFs containing mutations included the tail protein ORF19, the collar protein ORF12, as well as ORF4, ORF9, ORF12, ORF16, ORF23, ORF25, and ORF27.
| ORF | Start | End  | Strand | Length [aa] | ORF in Cp⁻¹ | # of identical aa/ size of the alignment [% aa identity] | Length [aa] | E-value | Accession number [GenBank]* |
|-----|-------|------|--------|-------------|-------------|----------------------------------------------------------|-------------|--------|--------------------------------|
| 1   | 376   | 642  | +      | 88          | Cp⁻¹, p01    | 1                                                        | 88          | 3E-57  | NP_044813.1                   |
| 2   | 657   | 947  | +      | 96          | Cp⁻¹, p02    | 2                                                        | 96          | 5E-61  | NP_044814.1                   |
| 3   | 1074  | 1346 | +      | 90          | Cp⁻¹, p03    | 3                                                        | 63          | 3E-36  | NP_044815.1                   |
| 4   | 1351  | 2043 | +      | 230         | Cp⁻¹, p04    | 4                                                        | 229         | 7E-165 | NP_044816.1                   |
| 5   | 2040  | 2609 | +      | 189         | Cp⁻¹, p05    | 5                                                        | 178         | 3E-123 | NP_044817.1                   |
| 6   | 2681  | 3745 | +      | 354         | Cp⁻¹, p05    | 5                                                        | 341         | 0      | NP_044817.1                   |
| 7   | 3702  | 4148 | +      | 148         | Cp⁻¹, p06    | 6                                                        | 148         | 1E-100 | NP_044818.1                   |
| 8   | 4141  | 4653 | +      | 170         | Cp⁻¹, p07    | 7                                                        | 170         | 5E-118 | NP_044819.1                   |
| 9   | 4875  | 5165 | +      | 96          | Cp⁻¹, p08    | 8                                                        | 95          | 4E-59  | NP_044820.1                   |
| 10  | 5409  | 6506 | +      | 365         | Cp⁻¹, p09    | 9                                                        | 352         | 0      | NP_044821.1                   |
| 11  | 6563  | 7576 | +      | 337         | Cp⁻¹, p11    | 10                                                       | 337         | 0      | NP_044823.1                   |
| 12  | 7563  | 8210 | +      | 215         | Cp⁻¹, p12    | 11                                                       | 192         | 2E-137 | NP_044824.1                   |
| 13  | 8223  | 8807 | +      | 194         | Cp⁻¹, p13    | 12                                                       | 194         | 5E-140 | NP_044825.1                   |
| 14  | 8804  | 9118 | +      | 104         | Cp⁻¹, p14    | 13                                                       | 104         | 7E-67  | NP_044826.1                   |
| 15  | 9102  | 9989 | +      | 295         | Cp⁻¹, p15    | 14                                                       | 295         | 0      | NP_044827.1                   |
| 16  | 10011 | 10787| +      | 258         | Cp⁻¹, p16    | 15                                                       | 192         | 4E-132 | NP_044828.1                   |
| 17  | 10787 | 11548| +      | 253         | Cp⁻¹, p18    | 16                                                       | 253         | 0      | NP_044830.1                   |
| 18  | 11515 | 13077| +      | 520         | Cp⁻¹, p19 / Cp⁻¹, p20 | 17 | 210/224 | 6e-149/ 2E-164 | NP_044831.1 / NP_044832.1 |
| 19  | 13081 | 14919| +      | 612         | Cp⁻¹, p21    | 19                                                       | 582         | 0      | NP_044833.1                   |
| 20  | 14993 | 16039| +      | 348         | Cp⁻¹, p23    | 20                                                       | 348         | 0      | NP_044835.1                   |
| 21  | 16029 | 16433| +      | 134         | Cp⁻¹, p24    | 21                                                       | 104         | 1E-89  | NP_044836.1                   |
| ORF | Start | End   | Strand | Length [aa] | IP[^b] | Mw [kDa] | Putative RBS[^d] and start codon | Putative function | Best hit with Blast ‘Locus tag’[^e] | ORF in Cp-1 | # of identical aa/ size of the alignment [% aa identity] | Length [aa] | E-value | Accession number [GenBank][^f] |
|-----|-------|-------|--------|-------------|--------|---------|--------------------------------|------------------|--------------------------------|------------|--------------------------------|-------------|---------|-----------------------------|
| 22  | 16433 | 17452 | +      | 339         | 4.6    | 39.2    | AAAGGAGAAAGAAATAATG           | Lysozyme         | Cp-1, p25                         | 22          | 339/339 [100%]                  | 339         | 0       | NP_044837.1                  |
| 23  | 17480 | 17896 | -      | 139        | 5.62   | 15.8    | AAAACGTAGGGGTTAATACTATG        | Hypothetical protein | SP058_00395                | 24.65/37% | 234                             | 6E+00       | 0       | YP_008239483.1                |
| 24  | 17901 | 18143 | -      | 80         | 9.9    | 9.4     | AAATTGAGTTAAGAAAATG            | Hypothetical protein | Cp-1, p26 [orf]                | 80/80 [100%] | 80                             | 5E-50       | 0       | NP_044838.1                  |
| 25  | 18148 | 18423 | -      | 91         | 5.1    | 10.9    | AAGGAGCGGTACTAGATG             | Hypothetical protein | AGR66263                       | 14/14 [32%] | 410                             | 8E-01       | gb|AGR66263.1                   |
| 26  | 18424 | 18693 | -      | 89         | 7.7    | 10.8    | ACAATAGGAGGGTAACATG            | Hypothetical protein | Cp-1, p27[orf]                | 89/89 [100%] | 89                             | 5E-58       | 0       | NP_044839.1                  |
| 27  | 18704 | 18973 | -      | 89         | 5.0    | 10.2    | AAAGAGGTATAACAAAATG            | Hypothetical protein | Cp-1, p28 [orf][a]            | 60/61 [98%] | 62                             | 7E-35       | 0       | NP_044840.1                  |

[^a]: Number of amino acids (aa),
[^b]: IP, isoelectric point and
[^c]: Mw, molecular mass.
[^d]: RBS, ribosomal binding site. Bases in bold correspond to nucleotides identical to the RBS consensus; lowercase indicates.
Three open reading frames were identified on the negative strand of the SOCP genome, specifically within three other ORFs (major capsid protein, tail protein and one hypothetical protein). These ORFs were not included in the annotation of the phage genome since no BLAST data support these proteins as valid protein products.

Codon usage

No tRNAs were found in either of the two pneumophages. Still, the codon usage frequency was investigated for the phage genomes and compared with the codon usage of *S. pneumoniae* and *S. mitis* (S4 Table). The codon usage of pneumophages mostly corresponds to the codons most frequently used by these two bacteria, although the codon usage of the phages was closer to *S. pneumoniae*, suggesting that both phages are more adapted to this bacterial species. In a few cases, some codons were over-represented in the phages as compared to the two bacterial hosts, likely to favor phage multiplication (S4 Table).

Comparative genomics

Protein-primed DNA replication was reported for phage Cp-1 [44]. Few other phages of the *Podoviridae* family replicate using this mechanism of DNA replication [44,45,46] and, in general, initiation of replication arises at the 3' nucleotide of the DNA [44]. The genomes of phages SOCP and Cp-1 were aligned with the genomes of five phages infecting other Gram-positive bacteria, namely *Bacillus subtilis* phage phi29, *Lactococcus lactis* phage asccphi28, *Weissella cibaria* phage phiYS6 as well as *Bacillus* sp. phages GA-1 and Nf (Fig. 3). The genome organization of the two pneumophages is somewhat different from the other phages due to alternate orientations of some genes, although a few annotated proteins are related (Fig. 3). The genes coding for the DNA polymerase and terminal proteins are on the positive strand in one group (phage Cp-1, SOCP, phiYS61 and asccphi28) and on the opposite strand for another group (phage GA-1, Nf and phi29). Moreover, the genes for structural proteins in the lactococcal phage asccphi28 are in the opposite direction, compared to the other podophages shown in Fig. 3. Overall, a low level of identity was found between phages Cp-1/SOCP and these podophages, which is likely due to their distinct ecological niches and host strains.
**Discussion**

*S. mitis* is a species closely related to *S. pneumoniae* that colonizes the human oral cavity. Both species have emerged as multidrug-resistant pathogens due to their ability to acquire foreign DNA by natural competence. The genome of *S. mitis* has significant similarity to the genome of *S. pneumoniae* and shares a core of 900 genes [18]. Since the publication of the *S. mitis* B6 genome, studies have been carried out to understand this relationship and a number of studies support the hypothesis of evolution of *S. pneumoniae* from *S. mitis* by acquisition of virulence.
factors and numerous sugar-related transport systems [18,47]. Other studies have proposed that another possible concept is the evolution from a pathogenic bacterium, *S. pneumoniae*, to a commensal, *S. mitis*, through loss of virulence genes [18,40]. Virulence genes could also have been acquired through horizontal gene transfer between other related species [48]. Many virulence factors are cell surface proteins that play a role in the interaction with host cells [49]. Other pneumococcal cell surface components such as choline-containing teichoic acid, are components of the Dp-1 phage receptors [50].

Here, we report that two pneumophages can efficiently replicate on a *S. mitis* strain. These two virulent phages were initially isolated from throat swabs of patients with upper respiratory infections—the natural habitat of *S. mitis*. The ability of phage Dp-1 to infect *S. mitis* may be related to the choline-binding proteins found in *S. mitis* [50]. Homologues of pneumococcal surface choline-binding proteins are present in *S. mitis* but their role in this bacterium remains unknown [5]. The host autolysin system, involved in pneumophage virion release [51], is also found in both *S. pneumoniae* and *S. mitis* [52]. The adsorption of phages Dp-1 and SOCP was slightly higher on the *S. pneumoniae* host compared to *S. mitis* and this may be due to the availability of phage receptors on the cell surface or differences in receptor sequences. It was previously reported that phage Cp-1 adsorbed very poorly to the host cell surface [52]. Phage SOCP adsorbed very well to its host as well as the *S. mitis* strain. This could be due to the mutations observed in the genome of SOCP or the experimental conditions used in our study.

The burst size and latent period reported here are rather different from previous studies for both pneumophages [23,53]. Again, this could be due to the difference in experimental conditions and media used. For phage Dp-1, the host bacterial strain used by Lopez et al. [53] was *S. pneumoniae* R36A and in our study we used the *S. pneumoniae* R6, a derivative of R36A. Likewise for phage SOCP, its burst size was much higher than reported for Cp-1 [41]. It is not known if this difference is due to the experimental conditions or the genomic variations. Of note, it was previously reported that phage Cp-1 can infect some *Streptococcus oralis* strains [54]. SOCP can also infect a few *S. oralis* strains (data not shown).

In addition to the observations that pneumophages can replicate on *S. mitis*, the other most striking finding was the difference (0.16%) in the genome sequences between SOCP and Cp-1. The nature of such discrepancies remains unclear. Phage SOCP was retrieved from the Félix d’Hérelle Reference Center for Bacterial Viruses and was initially believed to be Cp-1. Either sequencing errors occurred in the original deposited sequence (GenBank no. Z47794) or a very efficient pneumophage was recovered. The latter could be due to phage host-adaptation, giving SOCP the ability to efficiently infect its hosts [55]. It has been reported that viral adaptation occurs through numerous mutations in the genome at low frequency that increases fitness of the phage [55,56]. It will thus be interesting to assess whether some of the nucleotide variations detected in SOCP are enabling such increased fitness.

This study on virulent phages provides another example of the relatedness of *S. pneumonia* and *S. mitis*. Both species also carry numerous temperate phages in their genomes that are probably involved in their evolution. Further study is needed to identify most of the hypothetical genes in phage SOCP as well as to increase our understanding of phage-host interactions [57], with a goal of eventually using virulent streptococcal phages in medical applications.

**Supporting Information**

S1 Table. Primer sequences of four housekeeping genes. F, forward; R, reverse. (DOCX)
S2 Table. Primers used in this study to confirm variations within the genome of phage SOCP. F, forward; R, reverse.

S3 Table. Differences between the genomes of phage SOCP and Cp-1. relative to the genome of phage Cp-1.—absence of the nucleotide.

S4 Table. Codon usage of pneumophages Dp-1 and SOCP for selected amino acids compared to the hosts S. pneumoniae and S. mitis. The values represent the frequency of codon usage per thousand. The phage preferentially used codons for each amino acid are displayed in bold.

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Author Contributions
Conceived and designed the experiments: SO PL SM. Performed the experiments: SO PL SM. Analyzed the data: SO PL SM. Contributed reagents/materials/analysis tools: SO PL SM. Wrote the paper: SO PL SM.

References
1. Arbique JC, Poyart C, Trieu-Cuot P, Quesne G, Carvalho Mda G, et al. (2004) Accuracy of phenotypic and genotypic testing for identification of Streptococcus pneumoniae and description of Streptococcus pseudopneumoniae sp. J Clin Microbiol 42: 4686–4696. PMID: 15472328
2. Doern CD, Burnham CA (2010) It’s not easy being green: the viridans group streptococci, with a focus on pediatric clinical manifestations. J Clin Microbiol 48: 3829–3835. doi:10.1128/JCM.01563-10 PMID: 20810781
3. Joyce EA, Popper SJ, Falkow S (2009) Streptococcus pneumoniae nasopharyngeal colonization induces type I interferons and interferon-induced gene expression. BMC Genomics 10: 404. doi:10.1186/1471-2164-10-404 PMID: 19712482
4. Carrascosa M, Perez-Castrillon JL, Sampedro I, Valle R, Cillero L, et al. (1994) Lung abscess due to Streptococcus mitis: case report and review. Clin Infect Dis 19: 781–783. PMID: 7803651
5. Mitchell J (2011) Streptococcus mitis: walking the line between commensalism and pathogenesis. Mol Oral Microbiol 26: 89–98. doi: 10.1111/j.2041-1014.2010.00601.x PMID: 21375700
6. Donkor ES (2013) Understanding the pneumococcus: transmission and evolution. Front Cell Infect Microbiol 3: 7. doi: 10.3389/fcimb.2013.00007 PMID: 23471303
7. Arai J, Hotomi M, Hollingshead SK, Ueno Y, Briies DE, et al. (2011) Streptococcus pneumoniae isolates from middle ear fluid and nasopharynx of children with acute otitis media exhibit phase variation. J Clin Microbiol 49: 1646–1649. doi: 10.1128/JCM.01990-10 PMID: 21346044
8. Rukke HV, Hegna IK, Petersen FC (2012) Identification of a functional capsule locus in Streptococcus mitis. Mol Oral Microbiol 27: 95–108. doi: 10.1111/j.2041-1014.2011.00635.x PMID: 22394468
9. Madhour A, Maurer P, Hakenbeck R (2011) Cell surface proteins in S. pneumoniae, S. mitis and S. oralis. Iran J Microbiol 3: 58–67. PMID: 22347584
10. Sunderkotter C, Becker K (2014) Systemic therapy with antibiotics: Overview of important antibiotics in dermatology. Hautarzt 65: 113–124. doi: 10.1007/s00105-013-2743-6 PMID: 24549482
11. Gutierrez A, Laureti L, Crussard S, Abida H, Rodríguez-Rojas A, et al. (2013) beta-Lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. Nat Commun 4: 1610. doi: 10.1038/ncomms2607 PMID: 23511474
12. Henderson-Begg SK, Livermore DM, Hall LM (2006) Effect of subinhibitory concentrations of antibiotics on mutation frequency in *Streptococcus pneumoniae*. J Antimicrob Chemother 57: 849–854. PMID: 16531433
13. Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys JP (2006) Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science 313: 89–92. PMID: 16825569
14. Serwer P, Hayes SJ, Thomas JA, Hardies SC (2007) Propagating the missing bacteriophages: a large bacteriophage in a new class. Virol J 4: 21. PMID: 17324288
15. Krause RM (1957) Studies on bacteriophages of hemolytic streptococci. I. Factors influencing the interaction of phage and susceptible host cell. J Exp Med 106: 365–384. PMID: 13463248
16. Ramirez M, Severina E, Tomasz A (1999) A high incidence of prophage carriage among natural isolates of *Streptococcus pneumoniae*. J Bacteriol 181: 3618–3625. PMID: 10368133
17. Romero P, Croucher NJ, Hiller NL, Hu FZ, Ehrlich GD, et al. (2009) Comparative genomic analysis of ten *Streptococcus pneumoniae* temperate bacteriophages. J Bacteriol 191: 4854–4862. doi: 10.1128/JB.01272-08 PMID: 19502408
18. Denapaite D, Bruckner R, Nuhn M, Reichmann P, Henrich B, et al. (2010) The genome of *Streptococcus mitis* B6—what is a commensal? PLoS One 5: e9426. doi: 10.1371/journal.pone.0009426 PMID: 20195536
19. Suboo IR, Bensing BA, Sullam PM (2003) Genomic organization and molecular characterization of SM1, a temperate bacteriophage of *Streptococcus mitis*. J Bacteriol 185: 6968–6975. PMID: 14617660
20. Gindreau E, Lopez R, Garcia P (2000) Cp-1, a temperate bacteriophage of *Streptococcus pneumoniae*: structural analysis of the site-specific integration system. J Virol 74: 7803–7813. PMID: 10933687
21. Tiraby JG, Tiraby E, Fox MS (1975) Pneumococcal bacteriophages. Virology 68: 566–569. PMID: 844
22. McDonnell M, Lain R, Tomasz A (1975) "Diplophage": a bacteriophage of Diplococcus pneumoniae. Virology 63: 577–582. PMID: 234654
23. Ronda C, Lopez R, Garcia E (1981) Isolation and characterization of a new bacteriophage, Cp-1, infecting *Streptococcus pneumoniae*. J Virol 40: 551–559. PMID: 6275103
24. Martin AC, Lopez R, Garcia P (1998) Pneumococcal bacteriophage Cp-1 encodes its own protease essential for phage maturation. J Virol 72: 3491–3494. PMID: 9525689
25. Rigvava S, Tchgkonia I, Jgenti D, Ovadize T, Carpio J, et al. (2013) Comparative analysis of the biological and physical properties of *Enterococcus faecalis* bacteriophage vB_EfaS_GEC-EfS_3 and *Streptococcus mitis* bacteriophage vB_SmM_GEC-SmitisM_2. Can J Microbiol 59: 18–21. doi: 10.1139/cjm-2012-0385 PMID: 23391224
26. Djurkovic S, Loeffler JM, Fischetti VA (2005) Synergistic killing of *Streptococcus pneumoniae* with the bacteriophage lytic enzyme Cpl-1 and penicillin or gentamicin depends on the level of penicillin resistance. Antimicrob Agents Chemother 49: 1225–1228. PMID: 15728935
27. Doehn JM, Fischer K, Reppe K, Gutfier B, Tschernig T, et al. (2013) Delivery of the endolysin Cpl-1 by inhalation rescues mice with fatal pneumococcal pneumonia. J Antimicrob Chemother 68: 2111–2117. doi: 10.1093/jac/dkt131 PMID: 23633685
28. Loeffler JM, Djurkovic S, Fischetti VA (2003) Phage lytic enzyme Cpl-1 as a novel antimicrobial for pneumococcal bacteremia. Infect Immun 71: 6199–6204. PMID: 14573637
29. Loeffler JM, Fischetti VA (2003) Synergistic lethal effect of a combination of phage lytic enzymes with different activities on penicillin-sensitive and -resistant *Streptococcus pneumoniae* strains. Antimicrob Agents Chemother 47: 375–377. PMID: 12499217
30. McCullers JA, Karlstrom A, Iverson AR, Loeffler JM, Fischetti VA (2007) Novel strategy to prevent otitis media caused by colonizing *Streptococcus pneumoniae*. PLoS Pathog 3: e28. PMID: 17381239
31. Nelson D, Loomis L, Fischetti VA (2001) Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. Proc Natl Acad Sci U S A 98: 4107–4112. PMID: 11259652
32. Witzenrath M, Schmeck B, Doehn JM, Tschernig T, Zahllen J, et al. (2009) Systemic use of the endolysin Cpl-1 rescues mice with fatal pneumococcal pneumonia. Crit Care Med 37: 642–649. doi: 10.1097/CCM.0b013e31819586a6 PMID: 19114881
33. Moineau S, Durmaz E, Pandian S, Klaenhammer TR (1993) Differentiation of Two Abortive Mechanisms by Using Monoclonal Antibodies Directed toward Lactococcal Bacteriophage Capsid Proteins. Appl Environ Microbiol 59: 208–212. PMID: 16348844
34. Duplessis M, Moineau S (2001) Identification of a genetic determinant responsible for host specificity in *Streptococcus thermophilus* bacteriophages. Mol Microbiol 41: 325–336. PMID: 11489121
35. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y: CSH Protoc. 3v p.

36. Rousseau GM, Moineau S (2009) Evolution of Lactococcus lactis Phages within a Cheese Factory. Appl Environ Microbiol 75: 5336–5344. doi: 10.1128/AEM.00761-09 PMID: 19542338

37. Staden R, Beal KF, Bonfield JK (2000) The Staden package, 1998. Methods Mol Biol 132: 115–130. PMID: 10547834

38. Kraemer L, Beszteri B, Gabler-Schwarz S, Held C, Leese F, et al. (2009) STAMP: Extensions to the STADEN sequence analysis package for high throughput interactive microsatellite marker design. BMC Bioinformatics 10: 41. doi: 10.1186/1471-2105-10-41 PMID: 19183437

39. Lowe TM, Eddy SR (1997) tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res 25: 955–964. PMID: 9023104

40. Kilian M, Poulsen K, Blomqvist T, Havarstein LS, Bek-Thomsen M, et al. (2008) Evolution of Streptococcus pneumoniae and its close commensals relatives. PLoS One 3: e2683. doi: 10.1371/journal.pone.0002683 PMID: 18628950

41. Sabri M, Haüser R, Ouellette M, Liu J, Dehbi M, et al. (2011) Genome annotation and intraviral interaction of Virulent Pneumophages Infect Streptococcus mitis and its close commensal relatives. PLoS One 6: e25124. doi:10.1371/journal.pone.0025124 PMID: 21966432

42. Samson JE, Moineau S (2010) Characterization of Lactococcus lactis phage 949 and comparison with other lactococcal phages. Appl Environ Microbiol 76: 6843–6852. doi: 10.1128/AEM.00796-10 PMID: 20802084

43. Mark DF, Richardson CC (1976) Escherichia coli thioredoxin: a subunit of bacteriophage T7 DNA polymerase. Proc Natl Acad Sci U S A 73: 780–784. PMID: 768986

44. Martin AC, Blanco L, Garcia P, Salas M, Mendez J (1996) In vitro protein-primed initiation of pneumococcal phage Cp-1 DNA replication occurs at the third 3' nucleotide of the linear template: a stepwise sliding-back mechanism. J Mol Biol 260: 369–377. PMID: 8757800

45. Longas E, Villar L, Lazaro JM, de Vega M, Salas M (2008) Phage phi29 and Nif terminal protein-priming domain specifies the internal template nucleotide to initiate DNA replication. Proc Natl Acad Sci U S A 105: 18290–18295. doi:10.1073/pnas.0809882105 PMID: 19011105

46. Kotsonis SE, Powell IB, Pillidge CJ, Limsworth GK, Hillier AJ, et al. (2008) Characterization and genomic analysis of phage ascphi28, a phage of the family Podoviridae infecting Lactococcus lactis. Appl Environ Microbiol 74: 3453–3460. doi: 10.1128/AEM.02379-07 PMID: 18390678

47. Raskin DM, Seshadri R, Pukatzki SU, Mekalanos JJ (2006) Bacterial genomics and pathogen evolution. Cell 124: 703–714. PMID: 16497582

48. Zahner D, Gandhi AR, Yi H, Stephens DS (2011) Milis group streptococci express variable pilus islet 2 pilus. PLoS One 6: e25124. doi: 10.1371/journal.pone.0025124 PMID: 21966432

49. Ilyarnikova LN, Filimonova AV, Malakhova MV, Savinova T, Filimonova O, et al. (2013) Discrimination between Streptococcus pneumoniae and Streptococcus mitis based on sorting of their MALDI mass spectra. Clin Microbiol Infect 19: 1066–1071. doi: 10.1111/cmi.12113 PMID: 23931578

50. Lopez R, Garcia E, Garcia P, Ronda C, Tomasz A (1982) Choline-containing bacteriophage receptors in Streptococcus pneumoniae. J Bacteriol 151: 1581–1590. PMID: 7107560

51. Ronda-Lain C, Lopez R, Tapia A, Tomasz A (1977) Role of the pneumococcal autolysin (murein hydrolase) in the release of progeny bacteriophage and in the bacteriophage-induced lysis of the host cells. J Virol 21: 366–374. PMID: 13229

52. Sheppard CL, Harrison TG, Morris R, Hogan A, George RC (2004) Autolysin-targeted LightCycler assay including internal process control for detection of Streptococcus pneumoniae DNA in clinical samples. J Med Microbiol 53: 189–195. PMID: 14970243

53. Lopez R, Ronda C, Tomasz A (1977) Properties of “diplophage”: a lipid-containing bacteriophage. J Virol 24: 201–210. PMID: 20516

54. Ronda C, Garcia JL, Lopez R (1989) Infection of Streptococcus oralis NCTC 11427 by pneumococcal phages. FEMS Microbiol Lett 53: 187–192. PMID: 2612885

55. Bennmayor R, Hodgson DJ, Perron GG, Buckling A (2009) Host mixing and disease emergence. Curr Biol 19: 764–767. doi: 10.1016/j.cub.2009.03.023 PMID: 19375316

56. Hall JP, Harrison E, Brockhurst MA (2013) Viral host-adaptation: insights from evolution experiments with phages. Curr Opin Virol 3: 572–577. doi: 10.1016/j.coviro.2013.07.001 PMID: 23890845

57. Häuser R, Sabri M, Moineau S, Uetz P (2011) The proteome and interactome of Streptococcus pneumoniae phage Cp-1. J Bacteriol 193: 3135–3138. doi: 10.1128/JB.00481-10 PMID: 21515781