RESEARCH PAPER

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Evidence for horizontal gene transfer between *Chlamydophila pneumoniae* and Chlamydia phage

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Abbreviations: BLAST, Basic Local Alignment Search Tool; EB, Elementary body; MEGA, Molecular Evolution Genetic Analysis; MUSCLE, Multiple Sequence Comparison by Log-Expectation; PRIP, Putative Replication Initiation Protein; POG, Phage Orthologous Group; RB, Reticulate body; WebACT, Web-based Artemis Comparison Tool.

Chlamydia-infecting bacteriophages, members of the *Microviridae* family, specifically the *Gokushovirinae* subfamily, are small (4.5–5 kb) single-stranded circles with 8–10 open-reading frames similar to *E. coli* phage ϕX174. Using sequence information found in GenBank, we examined related genes in *Chlamydophila pneumoniae* and Chlamydia-infecting bacteriophages. The 5 completely sequenced *C. pneumoniae* strains contain a gene orthologous to a phage gene annotated as the putative replication initiation protein (PRIP, also called VP4), which is not found in any other members of the *Chlamydiaceae* family sequenced to date. The *C. pneumoniae* strain infecting koalas, LPCoLN, in addition contains another region orthologous to phage sequences derived from the minor capsid protein gene, VP3. Phylogenetically, the phage PRIP sequences are more diverse than the bacterial PRIP sequences; nevertheless, the bacterial sequences and the phage sequences each cluster together in their own clade. Finally, we found evidence for another *Microviridae* phage-related gene, the major capsid protein gene, VP1 in a number of other bacterial species and 2 eukaryotes, the woodland strawberry and a nematode. Thus, we find considerable evidence for DNA sequences related to genes found in bacteriophages of the *Microviridae* family not only in a variety of prokaryotic but also eukaryotic species.

Introduction

Chlamydophila pneumoniae, an obligate intracellular Gram-negative bacterium, is a major cause of community-acquired pneumonia in humans.[1] Mounting evidence suggests roles in several chronic diseases of humans as well, including atherosclerosis,[2] osteoporosis,[3] multiple sclerosis,[4] and Alzheimer disease.[5] *C. pneumoniae* has a wide host range, infecting reptiles, amphibians, birds, and a variety of mammals including horses, koalas, and bandicoots, in addition to humans.[6] In mammals, *C. pneumoniae* infects a number of different tissues, including lung epithelia, peripheral blood mononuclear cells, and endothelial cells,[6,7] which may account for the range of diseases with which it is associated.

Unfortunately, studies of *C. pneumoniae* are hampered by the fact that at present there are no routine methods to genetically manipulate the organism,[8] although a recent paper demonstrated that *C. pneumoniae* can be transformed by dendrimer-mediated DNA delivery.[9] However, with the advent of high-throughput next-generation sequencing techniques, more can be learned about the bacterium and its role in these diseases even in the absence of genetic methods. Specifically, in this paper, we examined the *C. pneumoniae* sequences currently available in GenBank.

C. pneumoniae is a member of the order *Chlamydiales*, specifically the *Chlamydiaceae* family.[10] This family has 2 lineages: the Chlamydia lineage, which includes 3 species, *C. trachomatis*, *C. muridarum*, and *C. suis*; and the Chlamydophila lineage, which includes 6 species, *C. abortus*, *C. caviae*, *C. felis*, *C. pecorum*, and *C. psittaci*, in addition to *C. pneumoniae*.[10,11] A number of different members of the *Chlamydiaceae* family have been completely sequenced and finished to a high level, including 5 *C. pneumoniae* isolates: 4 isolated from humans (strains AR39, CWL029, J138, and TW183) and one isolated from koala (strain LPCoLN). The human-infecting strains are closely related (though not identical, since there are major inversions and rearrangements observed

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among these 4 strains; for example, see ref. 12), but the LPCoLN genome is larger and includes a number of additional genes not found in the human-infecting strains.[13]

Six different Chlamydia-infecting bacteriophages have also been sequenced (ϕChp1 [refs. 14-16], ϕChp2 [refs. 14, 15, 17-19], ϕChp3 [refs. 20, 21], ϕChp4 [ref. 22], fCPAR39 [ref. 20], and ϕCPG1 [see ref. 15]). Members of the *Microviridae* family,[23] specifically the sub-group infecting obligate intracellular parasites, the *Gokushovirinae*,[24,25] these single-stranded DNA phages are distantly related to ϕX-174.[19] Each of the phage genomes encodes ~8–10 open-reading frames (ORFs), but it is currently unclear whether all encode functional proteins; some are quite small and overlap other larger ORFs. However, the annotation information available suggests that each genome encodes several proteins that contribute to the structure of the phage capsid and a protein annotated as putative replication initiation protein or PRIP (also called VP4), represented by Phage Orthologous Group (POG) 2233.[26]

Horizontal gene transfer between bacteria and their phages is an important mechanism for generating bacterial diversity.[27] Previous reports called attention to the fact that orthologs of Chlamydia phage genes are found in *C. pneumoniae*.[13,15,28] With the availability of additional phage and bacterial sequences, we evaluated the extent to which horizontal gene transfer between Chlamydia bacteriophages and Chlamydia species might have occurred. Here we report findings from our bioinformatics analysis, which are consistent with the hypothesis that first, genes could have been acquired by *C. pneumoniae* from the phage; and second, that this transfer event may have occurred in the ancestor of the existing *C. pneumoniae* strains, since we find no evidence for the phage sequences in other Chlamydia or Chlamydophila species, however from this analysis we can't rule out the possibility that the bacterial and the phage PRIP sequences evolved independently. In addition, we found that a different Chlamydia phage gene, the VP1 gene, encoding the major capsid protein (POG 2234) has orthologs in non-Chlamydia species and in 2 eukaryotes.

Materials and Methods

All sequence data were gathered from the NCBI's GenBank database (May 2014) (http://www.ncbi.nlm.nih.gov/genbank/; see **Tables 1–3**). Orthologs were identified using the Basic Local Alignment Search Tool (BLAST; ref. 29) at NCBI. For BLASTN, sequences were considered to be homologous if the expect- (e-) value was $<1 \times 10^{-5}$. For BLASTP, sequences were considered to be homologous if the e-value was $<1 \times 10^{-20}$.

Multiple sequence alignments were performed with Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (http://www.ebi.ac.uk/Tools/msa/muscle/; ref. 30). Phylogenies were constructed using Molecular Evolution Genetic Analysis, version 5.2.2 (MEGA 5; ref. 31). The best substitution models for both DNA and protein phylogenies were determined by calculation of the BIC statistic[31] as described in the appropriate figure legends. Additionally, all trees were constructed using the maximum-likelihood tree building method with bootstrapping of 1000 iterations.

Comparison of synteny in regions where PRIP genes are found in the different bacterial strains was performed using the web-based version of the Artemis Comparison Tool, WebACT.[32]

Table 1. *Chlamydia pneumoniae* sequences used in this study

Strain	Host	DNA Sequence	Accession Number
AR39	Human	NC_002179	ref. 28
CWL029	Human	NC_000922	ref. 44
J138	Human	NC_002491	ref. 12
TW-183	Human	NC_005043	(unpublished)
LPCoLN	Koala	CP001713	ref. 45

Table 2. Other Chlamydia spp. sequences used in this study

Strain	DNA Sequence	Accession Number
Chlamydia muridarum Nigg	NC_002620	ref. 46
Chlamydia trachomatis D/UW-3/CX	NC_000117	ref. 47
Chlamydia trachomatis L2/434/BU	NC_010287	ref. 48
Chlamydophila abortus S26/3	NC_004552	ref. 49
Chlamydophila caviae GPIC	NC_003361	ref. 50
Chlamydophila felis Fe/C-56	NC_003361	ref. 51
Chlamydiophila pecorum E58	NC_015408	ref. 52
Chlamydophila psittaci 6BC	NC_015470	ref. 53

Note – no complete sequence of *Chlamydia suis* is currently available at GenBank.

Results and Discussion

Chlamydia species are difficult to study in vivo since they are intracellular pathogens and there is no system at present for genetic manipulations. Therefore, mining of data via bioinformatics may prove useful to enhance our understanding of these bacteria. In this study, we examined the relationship between genes found in Chlamydia family members, specifically in *Chlamydophila pneumoniae* and Chlamydia-infecting bacteriophages.

Evolutionary relationships among the Chlamydia bacteriophages

Six Chlamydia phage genomes are currently available for study, although the number of other *Microviridae* family members in GenBank is increasing markedly.[23-25] The Chlamydia phages include ϕCPAR39, which was isolated directly from a human-infecting strain of *C. pneumoniae*, AR39;[20] ϕChp1, isolated from *C. psittaci*,[14] ϕChp2[18] and ϕChp4[22] from *C. abortus*; ϕChp3 from *C. pecorum*,[21] and ϕCPG1 from *C. caviae*.[33] Several of the phages have host ranges that include more than the

Table 3. Chlamydia phage sequences used in this study

Name	Isolated From	DNA Sequence	Accession Number
ϕChp1	*Chlamydia psittaci*	NC_001741	ref. 16
ϕChp2	*Chlamydia psittaci*	NC_002194	ref. 14
ϕChp3	*Chlamydia pecorum*	NC_008355	ref. 21
ϕChp4	*Chlamydophila abortus*	NC_007461	ref. 22
ϕCPAR39	*Chlamydia pneumoniae AR39*	NC_002180	ref. 15
ϕCPG1	*Chlamydia psittaci*	NC_001998	(unpublished)

species from which they were isolated, including ϕCPAR39, which can infect *C. abortus*, *C. caviae*, and *C. pecorum*.[18] From the literature currently available, it appears that none of these phages infect the Chlamydia lineage (i.e., *C. muridarum*, *C. suis*, or *C. trachomatis*) but only members of the Chlamydophila lineage (see **Table 4**).

We first examined the relatedness among the 6 bacteriophage genomes by constructing sequence alignments with MUSCLE.[30,34] Percent sequence identity between pairs of phage is shown in **Figure 1A**. Subsequently, we constructed a phylogeny of these sequences with MEGA5[31] (**Fig. 1B**). Based on this tree, ϕChp1 is the most distantly related of the Chlamydia phages. However, in general these sequences are fairly divergent from one other, as shown by the relatively weak bootstrap support for this branching pattern.

Table 4. Host ranges of the six Chlamydia phages

	Chp1	Chp2	Chp3	Chp4	CPAR39	CPG1
Chlamydia muridarum		no	no			
Chlamydia suis		no	no			
Chlamydia trachomatis		no	no			
Chlamydophila abortus		**YES**	yes	**YES**	yes	
Chlamydophila caviae		no	yes		yes	**YES**
Chlamydophila felis		yes	yes		no	
Chlamydophila pecorum		yes	**YES**		yes	
Chlamydophila pneumoniae		no	no		**YES**	
Chlamydophila psitticai	**YES**	yes	no		no	

Information gleaned from the literature about host ranges of the 6 Chlamydia bacteriophages. **YES** indicates the species from which the phage was isolated; yes indicates that the phage can infect that species; no indicates that the phage does not infect that species; no entry means was no information available. Information about the following phages was obtained from: ϕChp1;[16] ϕChp2;[14,18] ϕChp3;[20,21] ϕChp4;[22] ϕCPAR39;[15,20,28] and ϕCPG1.[50]

C. pneumoniae strains contain an ortholog of Chlamydia bacteriophage PRIP

Each of the 6 phages contains a gene annotated "putative replication initiation protein" or PRIP (also called VP4, represented by POG 2233[26]), which matches a conserved domain, PHA00330 that only occurs in members of the bacteriophage *Microviridae* family.[35] No structure or function has yet been associated with this domain.

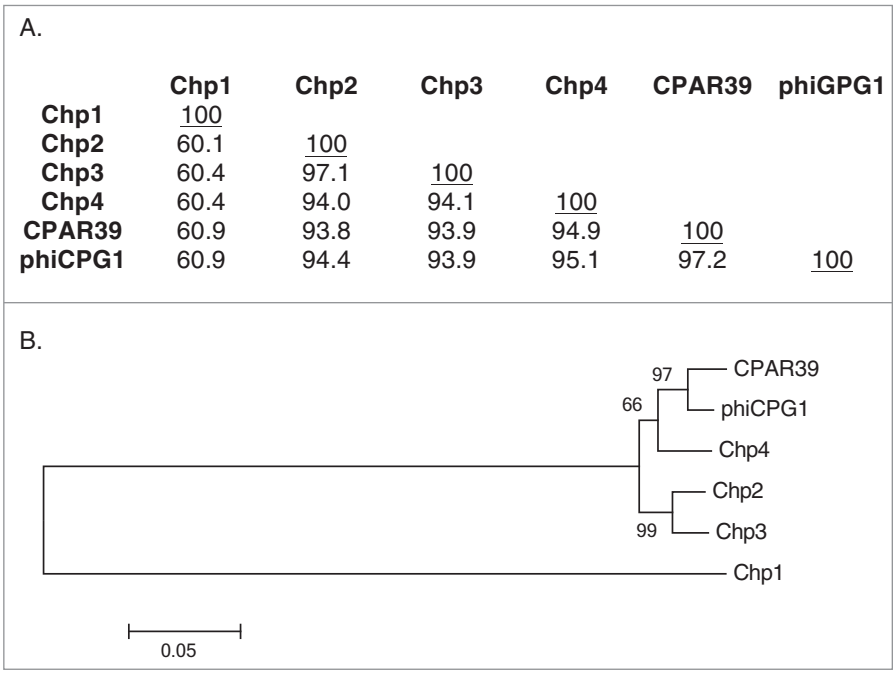

Figure 1. Comparison among Chlamydia bacteriophages. (**A**) Matrix derived from MUSCLE analysis of the phage DNA sequences. The matrix shows the extent of identity between 2 phage sequences in pairwise alignment. (Chp1 shows ~60% identity to the other phages, whereas the others show >90% identity to one another. (**B**) Phylogenetic analysis of the 6 phage genomes. The evolutionary history was inferred by using the maximum likelihood method based on the Hasegawa-Kishino-Yano model using 1000 bootstraps.[42] The tree with the highest log likelihood (−11567.9705) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIO neighbor joining method with maximum composite likelihood distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 5.9605)). The analysis involved 6 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 3729 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.[31]

In addition, each of the 5 sequenced *C. pneumoniae* strains also contains an open-reading frame that is homologous to the core region of PRIP.[13,15] PRIP from the 4 human-infecting strains is 100% identical at the DNA sequence level, while the one found in the koala-infecting LPCoLN strain is slightly different. It is thought DNA sequences shared between related organisms but absent in close relatives are likely acquired via horizontal gene transfer.[36,37] Given that we found no evidence of PRIP or other genes orthologous to genes from *Microviridae* in any of the sister species of *C. pneumoniae* within the *Chlamydiaceae* family, this result is consistent with acquisition of PRIP through this mechanism but is not the only explanation for these results. However, given that these phages are not thought to be lysogenic or temperate, that is, do not in insert themselves into the host genome as part of their life cycles as the *E. coli* phage l does, the mechanism by which transfer may have occurred is unclear.[25] Nevertheless, it has been suggested that bacterial genes are usually quickly deleted[32] unless retained for some specific purpose,[38] although at present the function provided to *C. pneumoniae* by PRIP is unknown. Microarray data suggest that PRIP is expressed at low levels, but is not differentially expressed with time of infection.[39] Similarly, RNA-Seq data also

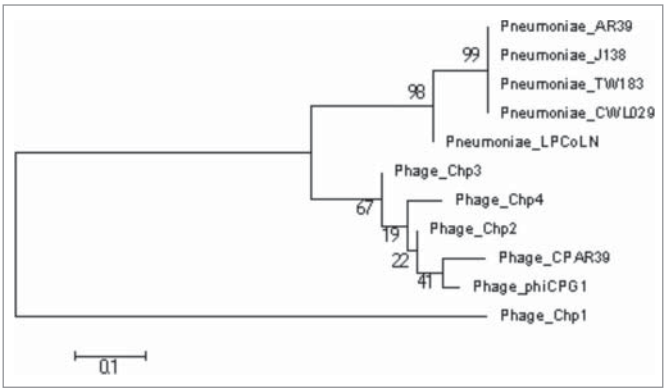

Figure 2. Phylogenetic Analysis of the 11 PRIP Proteins. The evolutionary history was inferred by using the maximum likelihood method based on the Whelan and Goldman model.[43] The tree with the highest log likelihood (−713.8725) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 11 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 88 positions in the final data set. Evolutionary analyses were conducted in MEGA5.[31]

suggest that PRIP is expressed at low levels, but is not differentially expressed in elementary bodies (EBs) compared to reticulate bodies (RBs).[40]

We examined the 11 predicted PRIP proteins, from the 6 phages and the 5 *C. pneumoniae* strains by phylogenetic analysis. By MUSCLE alignment, the phage PRIP proteins are more diverse, and are larger, having extensions on both the N- and C-termini compared to the orthologs found in the bacterial strains (Figure S1). When a phylogenetic tree based on the alignments was constructed (which only uses amino acids present in all 11 sequences, thus ignoring the N- and C-terminal extensions in the phage PRIP proteins), the phage-derived PRIPs fell into a single clade (with ϕChp1 PRIP as the most divergent protein) while the bacterially derived PRIP proteins clustered in another clade (**Fig. 2**). The PRIP proteins found in the bacterial strains are most closely related to phage ϕChp4 by BLASTP analysis, which was initially isolated from *C. abortus*.[22] BLASTP of the ϕChp4-predicted PRIP protein identifies PRIP of the *C. pneumoniae* strains as the top bacterial hits, while reverse BLASTP of the bacterial PRIP sequences identifies the Chlamydia phages as the top hits, rather than other *Microviridae* sequences currently in GenBank., We also compared the GC content of the phages, the phage-related sequences in the bacteria, and the rest of the bacterial chromosome, to examine the possibility of amelioration, a process that takes place over time on horizontally acquired DNA sequences, in which they lose the properties of the source, while becoming more similar to the new host.[36] However, this particular analysis was unrevealing, as the GC content of all 3 DNA sequences was ~36%.

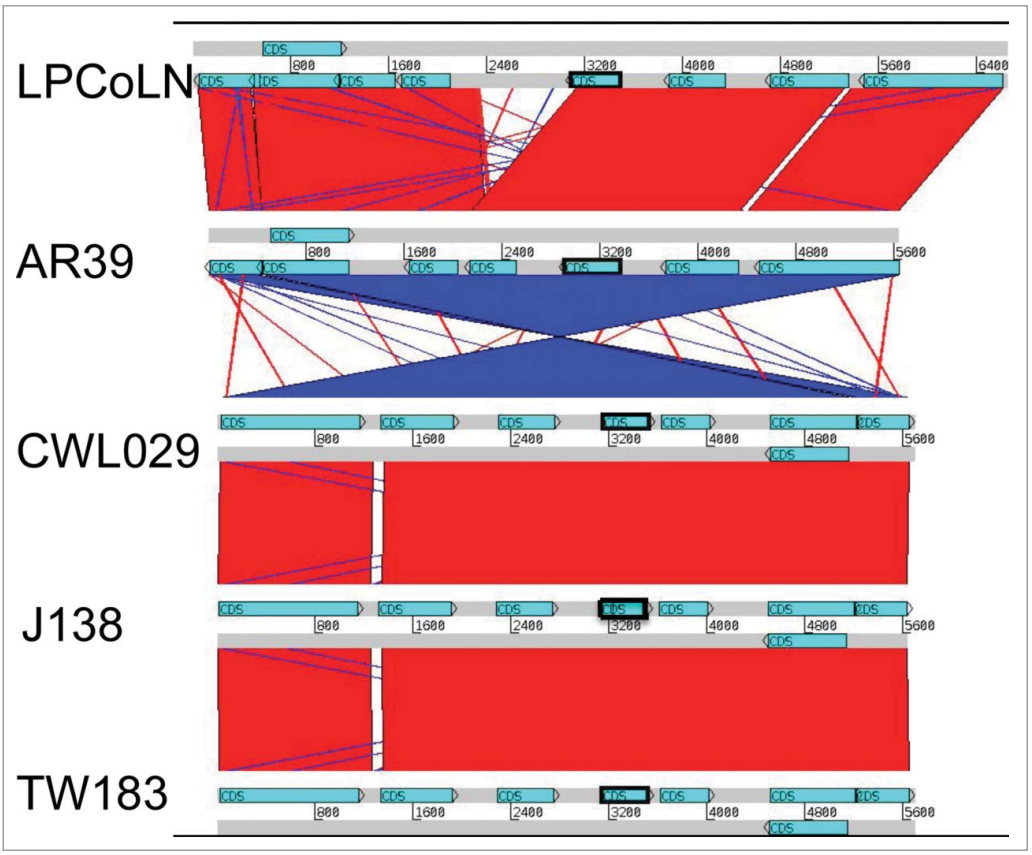

Figure 3. Comparison of the 5 *C. pneumoniae* strains in the region containing the PRIP gene. Ten kb regions centered around the PRIP gene for each of the 5 sequenced *C. pneumoniae* strains were compared to each other using the WebACT (Artemis Comparison Tool).[32] Light blue arrows represent the open-reading frames on each sequence; PRIP is outlined with a heavier black line. The red chords between sequences signify homology in those regions; blue chords show homology to the opposite strand. In LPCoLN and AR39, PRIP is found on the bottom strand, while in CWL029, J138, and TW183, PRIP is on the top strand. The sequence in LPCoLN that does not match anything in the other strains corresponds to the additional phage sequences similar to VP3 not found in the human-infecting strains.

Table 5. VP1 homologs found in non-Chlamydia bacterial and plant species The protein sequence corresponding to VP1 from Chp4 (YP_338238.1) was submitted for BLASTP analysis.[29] The top hits with e-values ~<1e-50 are reported here. Hits to "uncultured bacteria" have been eliminated. Most bacteria are members of the *Bacteroidetes* family (*Parabacteroides* and *Elizabethkingia*) or the Firmicutes family (*Clostridium*) except for Richelia (a cyanobacterium). Surprisingly, 2 eukaryotes also had reasonably high e-values with >50% query coverage: *Fragaria vesca* (woodland strawberry) and *Necator americanus* (a nematode)

Name	Max Score	Total Score	Query Cover	e-value	Max identity	Accession Number
capsid family protein [*Parabacteroides distasonis* str. 3999B T(B) 4]	336	336	97%	2e-104	37%	KDS61824.1
PREDICTED: capsid protein VP1-like [*Fragaria vesca subsp. vesca*]	321	321	64%	3e-100	50%	XP_004309312.1
putative major coat protein [*Clostridium* sp CAG:306]	324	324	96%	5e-100	36%	WP_022246927.1
hypothetical protein [*Parabacteroides distasonis*]	313	313	97%	1e-95	38%	WP_005867318.1
capsid protein VP1 [*Parabacteroides merdae* CAG:48]	309	309	97%	4e-94	36%	WP_022322420.1
conserved hypothetical protein [*Elizabethkingia anophelis*]	293	293	96%	5e-88	34%	CDN73650.1
hypothetical protein [*Elizabethkingia anophelis*]	292	292	96%	9e-88	35%	WP_024568106.1
protein [*Richelia intracellularis*]	271	271	40%	1e-83	59%	WP_008233569.1
capsid family protein [*Parabacteroides distasonis* str. 3999B T(B) 6]	259	259	74%	8e-77	38%	KDS75238.1
capsid family protein [*Parabacteroides distasonis* str. 3999B T(B) 4]	206	206	46%	2e-58	42%	KDS63784.1
putative capsid protein [*Necator americanus*]	188	188	88%	2e-49	33%	ETN80368.1

The koala-infecting bacterial strain, LPCoLN, contains a larger region of phage-homologous sequence

In addition to PRIP-encoding sequences, it was previously found that a sequence related to a second phage-related gene, VP3, the minor capsid protein (POG 2232, ref. 26), is found in the LPCoLN strain.[13] This gene CPK_ORF00730 (nucleotides 580833–581277) is annotated in GenBank as "scaffolding protein, authentic frameshift." This region shows homology to the Chlamydia phage VP3 gene, but is missing 2 nucleotides, resulting in the frameshift (**Fig. S2**). In summary, overall, more phage-related sequences are found in the LPCoLN genome compared to the phage-related sequences found in the human-infecting *C. pneumoniae* strains (**Fig. 3**).

The local environment of PRIP in *C. pneumoniae* strains

In each of the 5 bacterial genomes, PRIP is found near the tgt gene, which encodes queuine tRNA-ribosyl transferase, an enzyme responsible for a post-transcriptional modification of some tRNAs. Additionally, this region contains a set of genes annotated as "hypothetical proteins" that are homologous to one another and are only found in Chlamydia species.[41] According to Albrecht et al., the PRIP gene (annotated Cpn0222) in CWL029 is the 2nd gene in a 2-gene operon with Cpn0221.[40] We compared this region among all 5 *C. pneumoniae* strains using WebACT (Artemis Comparison Tool) (**Fig. 3**). As can be seen, these regions are all highly syntenic with one another. The exception is the region in LPCoLN that corresponds to the additional phage sequences homologous to VP3, the minor capsid protein gene remnant. We also compared the region containing the tgt gene between *C. pneumoniae* AR39 and the other *Chlamydophila* and Chlamydia species and saw little homology, save at tgt itself (data not shown). Thus the region containing PRIP and tgt appears to be unique in the *C. pneumoniae* lineage, again suggesting that since PRIP is uniquely present in *C. pneumoniae*, but not in other members of the *Chlamydiaceae* family, acquisition of these sequences by horizontal gene transfer is a reasonable hypothesis.

Orthologs of the major capsid protein are found in several other species

We next examined all of the ORFs in ϕChp4 by BLASTP against the non-redundant (nr) GenBank CDS translation database to determine whether we could uncover other instances of phage gene orthologs in bacteria. As expected, the minor capsid protein gene, VP3 identified an ortholog in the LPCoLN strain, while PRIP (VP4) identified orthologs in all 5 *C. pneumoniae* strains. For 5 of the other predicted proteins, the only hits were to other members of the *Microviridae* family. However, BLASTP with the major capsid protein (VP1, also called the Phage F protein, represented by POG 2234[26]), identified proteins encoded by the genomes of several other bacterial species, members of the *Bacteroidetes* (*Parabacteroides* and *Elizabethkingia* spp.) and *Firmicutes* (*Clostridium* spp) families. Others have observed the presence of phage sequences in *Bacteroidetes* previously.[25] *Richelia intracellularis*, a cyanobacterium, was also found to contain a gene encoding a homolog of VP1 (**Table 5**). Interestingly, we also found evidence for a VP1-like protein encoded by the genomes of the woodland strawberry and a nematode. Thus, we found phage gene orthologs in completely different bacterial clades as well as in eukaryotes. By reverse BLASTP, the closest phage homologs of some of the bacterial or eukaryotic genes are in fact the Chlamydia phages, but for others, the closest phage homologs are from uncultured marine *Gokushoviruses* (data not shown), raising questions as to the ultimate source of these sequences in some of these organisms. Interestingly, in exploring this aspect in more detail, we found nearly all of ϕX174 contained as a block within *E. coli* strain 0.1288.

Conclusions

The results presented here are consistent with the hypothesis that a block of Chlamydia phage sequence, containing the minor capsid protein gene (VP3) and the PRIP gene (VP4), was transferred into the ancestor of the *C. pneumoniae* strains, and the koala-infecting strain, LPCoLN retained a larger portion of the block, while the VP3 orthologous sequences were lost from the ancestor of the human-infecting strains. This

seems to be the most parsimonious explanation for the observed results, although this is not the only explanation. For example, it is possible that the PRIP sequences arose independently in the bacteria and the phages. At present, since there are no complete sequences for *C. pneumoniae* strains that infect animals other than mammals in GenBank, we cannot determine whether PRIP is found in *C. pneumoniae* strains that infect birds and reptiles as well, or whether PRIP is found exclusively in the *C. pneumoniae* strains that infect mammals. Further, our analyses are consistent with the work demonstrating the existence of *Microviridae*-related genes in diverse bacterial families,[13,15,24,25,28] in keeping with the notion that horizontal gene transfer between phages and their bacterial hosts is an important driver for bacterial evolution.[27,37]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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