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RESEARCH ARTICLE

Propionibacterium acnes Bacteriophages Display Limited Genetic Diversity and Broad Killing Activity against Bacterial Skin Isolates

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ABSTRACT **Investigation of the human microbiome has revealed diverse and complex microbial communities at distinct anatomic sites. The microbiome of the human sebaceous follicle provides a tractable model in which to study its dominant bacterial inhabitant, *Propionibacterium acnes*, which is thought to contribute to the pathogenesis of the human disease acne. To explore the diversity of the bacteriophages that infect *P. acnes*, 11 *P. acnes* phages were isolated from the sebaceous follicles of donors with healthy skin or acne and their genomes were sequenced. Comparative genomic analysis of the *P. acnes* phage population, which spans a 30-year temporal period and a broad geographic range, reveals striking similarity in terms of genome length, percent GC content, nucleotide identity (>85%), and gene content. This was unexpected, given the far-ranging diversity observed in virtually all other phage populations. Although the *P. acnes* phages display a broad host range against clinical isolates of *P. acnes*, two bacterial isolates were resistant to many of these phages. Moreover, the patterns of phage resistance correlate closely with the presence of clustered regularly interspaced short palindromic repeat elements in the bacteria that target a specific subset of phages, conferring a system of prokaryotic innate immunity. The limited diversity of the *P. acnes* bacteriophages, which may relate to the unique evolutionary constraints imposed by the lipid-rich anaerobic environment in which their bacterial hosts reside, points to the potential utility of phage-based antimicrobial therapy for acne.**

IMPORTANCE ***Propionibacterium acnes* is a dominant member of the skin microflora and has also been implicated in the pathogenesis of acne; however, little is known about the bacteriophages that coexist with and infect this bacterium. Here we present the novel genome sequences of 11 *P. acnes* phages, thereby substantially increasing the amount of available genomic information for this phage population. Surprisingly, we find that, unlike other well-studied bacteriophages, *P. acnes* phages are highly homogeneous and show a striking lack of genetic diversity, which is perhaps related to their unique and restricted habitat. They also share a broad ability to kill clinical isolates of *P. acnes*; phage resistance is not prevalent, but when detected, it appears to be conferred by chromosomally encoded immunity elements within the host genome. We believe that these phages display numerous features that would make them ideal candidates for the development of a phage-based therapy for acne.**

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The investigation of the microbiome at specific anatomic sites is essential toward understanding the pathogenesis of disease and the development of targeted therapies. In such microbial communities, there is an interaction between bacterial populations and the bacteriophages that infect them. These viruses influence bacterial community structure and function by several mechanisms, including their ability to kill their bacterial hosts and/or mediate genetic exchange that can increase diversity and potentially disseminate virulence genes. Virtually all of the bacteriophage populations that have been studied display a wide range of genetic diversity (1), including those that infect *Mycobacterium* (2–5), *Staphylococcus* (6, 7) and *Pseudomonas* (8, 9) species.

The Gram-positive skin commensal *Propionibacterium acnes* is the dominant inhabitant of the human pilosebaceous unit (10), an invagination within the human epidermis containing a hair follicle, hair shaft, erector pili muscle, and associated sebaceous glands, which produce sebum. Despite its ubiquitous presence on the skin, *P. acnes* is also thought to play a major role in the pathogenesis of acne vulgaris, in part by eliciting a host inflammatory response (11). There is a significant increase in *P. acnes* colonization at puberty, the time during which acne commonly develops, and teenagers with acne can have as many as 100-fold more *P. acnes* bacteria present on their skin than healthy, age-matched counterparts (12). The efficacy of antibiotics for acne treatment is related to the reduction of the number of *P. acnes* bacteria on the skin, as well as to direct anti-inflammatory properties (13), which

TABLE 1 *P. acnes* bacteriophages characterized in this study

Phage	Method of isolation or source	Donor	Date	Accession no.
P1.1	Direct plating of pore strip sample	Acne	06/2007	JX262223
P9.1	Supernatant of pore strip culture plated on ATCC 6919	Acne	01/2008	JX262215
P14.4	Pore strip sample plated on ATCC 6919	Acne	01/2008	JX262216
P100A	FS[a] pore strip sample plated on ATCC 6919	No acne	09/2010	JX262221
P100D	Supernatant of FS pore strip culture plated on ATCC 6919	No acne	09/2010	JX262220
P100.1	Supernatant from uninduced B100.1 plated on B100.4[b]	No acne	11/2010	JX262222
P101A	FS pore strip sample plated on ATCC 6919	No acne	10/2010	JX262217
P104A	FS pore strip sample plated on ATCC 6919	Acne	12/2010	JX262218
P105	FS pore strip sample plated on ATCC 29399	No acne	12/2010	JX262219
ATCC 29399B_C	ATCC	Acne	1978[c]	JX262225
ATCC 29399B_T	ATCC	Acne	1978[c]	JX262224

[a] FS, filter sterilized.
[b] *P. acnes* clinical isolate obtained from same donor as P100A, P100D, and P100.1.
[c] Webster and Cummins (18).

reflects the multifactorial etiology of acne. Nevertheless, the emergence of antibiotic-resistant strains of *P. acnes*, as measured in up to 60% of clinical isolates (14–16), has resulted in clinical challenges and highlights the need for improved therapeutics (13).

P. acnes bacteriophages are a common component of the acne microbiome. In early studies, these phages were used to type *P. acnes* strains, and some were found to exhibit a broad host range against *P. acnes* clinical subtypes (17–19). However, despite the relative ease with which *P. acnes* bacteriophages can be isolated from human skin, the complete genome sequences of only three *P. acnes* phages have been reported (20, 21) and little is known about either their genetic diversity or the molecular basis of their relationships with their bacterial hosts (20, 21). We therefore sequenced 11 novel *P. acnes* phages from healthy individuals and those with acne and present their complete genome sequences, as well as a detailed comparative genomic analysis and phenotypic characterization of these phages. We find that these phages possess a striking lack of genetic diversity, which contrasts with what has been observed in other phage populations. Furthermore, we show that the *P. acnes* phages in this study infect a broad range of clinical isolates, and phage immunity, when present, appears to be conferred by the presence of chromosomally encoded elements.

RESULTS

Isolation of *P. acnes* bacteriophages. To investigate the diversity present within the population of bacteriophages that infect *P. acnes*, we isolated phages and bacterial isolates from microcomedones (the sebaceous follicle content) obtained from the nasal skin of donors either with or without acne. Nine *P. acnes* phages were isolated either by direct culture of the microcomedone material, with or without indicator strains of host bacteria, or from the supernatant of cultures inoculated with microcomedones and grown to saturation (Table 1). Five of these were obtained from healthy donors, and four were isolated from donors with acne (Table 1). Additionally, using *P. acnes* ATCC 6919 as a host, we recovered two phages from a phage stock obtained from the American Type Culture Collection (ATCC), ATCC 29399B, which we named ATCC 29399B_C and ATCC 29399B_T to reflect their distinct plaque morphotypes, clear and turbid, respectively. Because ATCC 29399B was obtained over 30 years ago from an acne patient in Philadelphia, PA (18), the two phages present in this stock are temporally and geographically separated from the other phages isolated in our study.

Morphology of *P. acnes* phage virions. *P. acnes* strain ATCC 6919 is susceptible to infection by all of the phages isolated here and was used as a host to prepare high-titer lysates for all 11 phages. These were analyzed by electron microscopy, and all phages were observed to possess a siphoviral morphology, with an isometric head, ~50 nm in diameter, and a long flexible tail, ~150 nm in length (Fig. 1). Morphologically, these resemble the *P. acnes* phages reported previously (18, 20–23) and thus lack the diversity of forms observed in some other well-characterized phage populations. For example, mycobacteriophages possessing either contractile tails (*Myoviridae*) or prolate heads have been observed (3, 5), and both the *Staphylococcus* and *Pseudomonas* phage populations have members with podoviral morphologies (i.e., with short, stubby tails) (7, 8).

Genomic characterization of *P. acnes* bacteriophages. To gain a more comprehensive understanding of the genomic diversity present within the *P. acnes* bacteriophage population, total genomic DNA was prepared from each of the 11 phages, and their genomes were sequenced. Upon assembly and annotation, all were found to be similar in size and structure to the three previously reported *P. acnes* phages, PA6, PAD20, and PAS50 (20, 21). The 14 *P. acnes* phages range in size from 29,017 to 29,739 bp, with 45 to 47 predicted protein-coding genes (Table 2); no tRNA genes were identified. Our data further suggest that all of these phage genomes possess the same 11-base single-stranded 3′ extension (5′-TCGTACGGCTT), which is shorter by 2 bases than that reported in the genomes of the three *P. acnes* phages published previously (5′-CCTCGTACGGCTT). For each of our 11 phages, reads from both the 454 and the Illumina data either terminate at the 3′ end with the 5′-CC or read through to the other end of the genome via the 11-base putative single-strand region, suggesting that our interpretation is correct.

There are two notable features of the percent GC content of the *P. acnes* phage genomes. First, there is very little variation in percent GC content from genome to genome, as they all fall within a narrow range of 53.76 to 54.17% (Fig. 2; Table 2). Second, their average percent GC content (54.1%) is substantially different from that of their *P. acnes* hosts, which is ~60.0%. The genomes of few other *Propionibacterium* species have been sequenced, but we note that *P. freudenreichii* is considerably more GC rich (67.3%; Fig. 2). The restricted percent GC content of the *P. acnes* phages is in contrast to the considerable variation observed in phages of other hosts. For example, the percent GC content varies by as

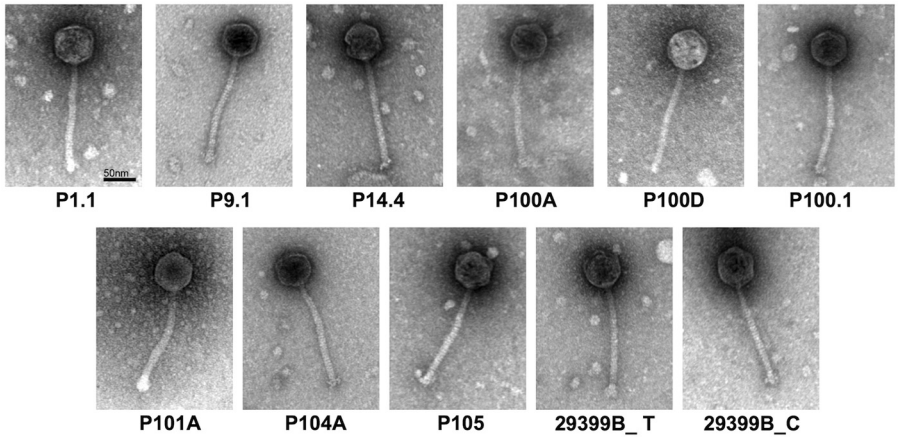

FIG 1 *P. acnes* phage virion morphologies. Negatively stained electron micrographs of the 11 *P. acnes* phages characterized in this study reveal that all have the same siphoviral virion morphotype. All of these phages have similarly sized isometric heads (~50 nm in diameter) and long, flexible tails (~150 nm in length).

much as 18 to 28% within each of the large collections of phages infecting *Mycobacterium smegmatis*, *Staphylococcus* sp., and *Pseudomonas* sp. (Fig. 2). A consequence of this is that most of these phages have percent GC contents that are distinct from those of their known hosts and close relatives therof, which may serve as potential hosts, as is also the case with the *P. acnes* phages (Fig. 2). However, unlike the *P. acnes* phages, each of these other three groups of phages exhibits a range of percent GC contents that overlaps that of the host, and the average percent GC content is similar to the host value, which is to be expected because of the propensity for genetic exchange between a phage and its host.

There are two plausible explanations for these observations. First, the *P. acnes* phages may have sufficiently broad host ranges that although they were isolated on *P. acnes*, in their natural environment, they were most recently associated with another host organism(s). Second, the phages may have adapted for growth in the known host relatively recently, such that their percent GC content has yet to fully ameliorate toward that of their bacterial host (24). Collectively, the limited range of genome size and percent GC content observed in the *P. acnes* phages suggests the possibility of limited diversity in this bacteriophage repertoire compared to that of other well-characterized phage populations.

Limited nucleotide sequence diversity of *P. acnes* phages. To determine whether the limited percent GC content variation of the *P. acnes* phages is reflective of limited nucleotide sequence diversity, we performed dot plot comparisons (Fig. 3A), which clearly demonstrate a high level of nucleotide sequence similarity extending across the lengths of all 14 genomes. This is illustrated by the nearly solid line of "dots" along the diagonal of each pairwise comparison, with each dot representing a nucleotide segment that is shared by two phages. The lack of diversity observed among the *P. acnes* phages is in contrast to that seen in *Mycobacterium*, *Staphylococcus*, and *Pseudomonas* phages (Fig. 3B), where although some genomes exhibit similarity, most pairwise comparisons do not. The comparative lack of diversity observed among the *P. acnes* phages versus other phage populations is unlikely to be a consequence of sample size or bias, because six randomly chosen sets of 14 mycobacteriophages from the total of 221 each show a diversity considerably greater than that of the *P. acnes* phages (see Fig. S1 in the supplemental material).

Alignment of the *P. acnes* phage genome maps and display of pairwise nucleotide sequence similarities, as indicated by the shading between the genomes, further reveals the striking similarity shown by these phages but also identifies regions of sequence variation (Fig. 4). The greatest variation occurs close to the right genome ends, with some additional variation in the central portions. In comparison, genome maps depicting a subcluster of related mycobacteriophages (see Fig. S2A in the supplemental material) show a lesser degree of nucleotide identity, and genome maps from a selection of mycobacteriophages chosen to reflect their maximal diversity (see Fig. S2B) display almost no shared nucleotide sequence identity.

Organization of the *P. acnes* phage genomes. The limited genetic variation among the *P. acnes* phage genomes is also reflected in their genome organizations. The DNA packaging and virion structure and assembly genes (*1* to *19*) occupy approximately half of the genome (coordinates 1 to ~15 kbp) and are organized into an operon that is presumably transcribed rightward, with few intergenic gaps, as is common among phages with a siphoviral morphotype, such as the λ-like coliphages and many of the mycobacteriophages (1, 3, 5) (Fig. 4 and 5; colored boxes represent predicted protein coding genes). These genes are all highly conserved at the nucleotide level, with the notable exception of the last of the putative minor tail protein genes (P100D gene *19* and its

TABLE 2 *P. acnes* bacteriophage genome features

Phage	Length (bp)	% GC	No. of ORFs
PAS50	29,017	53.97	46
PAD20	29,074	54.10	45
P105	29,202	54.17	45
P9.1	29,214	54.12	45
P1.1	29,348	54.37	45
P104A	29,371	53.98	45
P100A	29,505	53.83	45
P100D	29,506	53.76	47
ATCC 29399B_T	29,516	54.01	46
ATCC 29399B_C	29,516	54.00	46
P101A	29,574	54.14	47
P100.1	29,612	54.07	47
P14.4	29,729	54.08	47
PA6	29,739	54.02	45

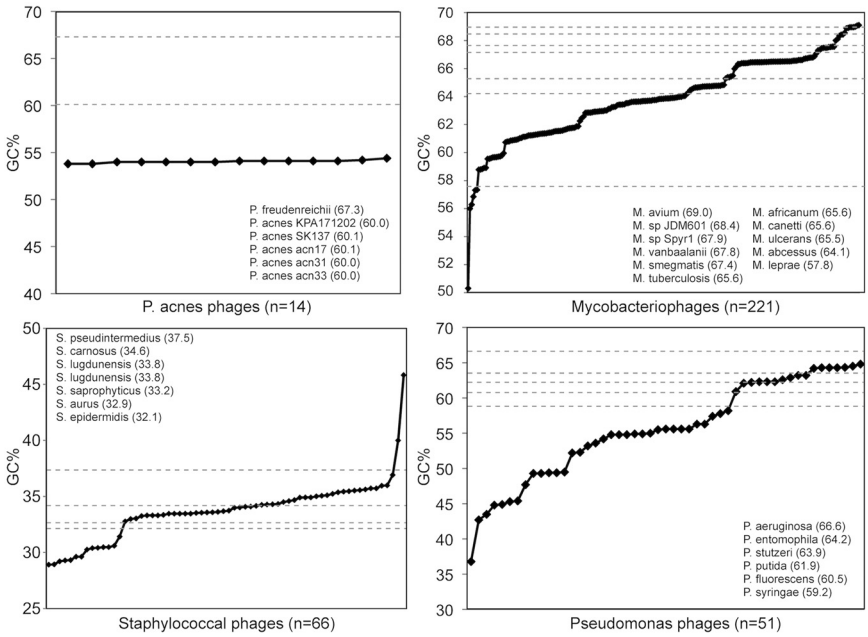

FIG 2 Percent GC contents of bacteriophages and their hosts. The percent GC contents of the genomes of individual bacteriophages that infect *P. acnes*, *M. smegmatis*, *Staphylococcus* sp., or *Pseudomonas* sp. are plotted in order, from the lowest value to the highest. The analysis includes 14 phages of *P. acnes*, 221 phages of *M. smegmatis*, 66 phages of *Staphylococcus* sp., and 51 phages of *Pseudomonas* sp. (see Materials and Methods). The percent GC contents of host bacteria and their close relatives, which may serve as potential hosts, were calculated from published genomes; these are shown as dotted lines and listed with percent GC contents in parentheses.

relatives), as noted previously (21). We have found that the variation is greatest within the 3′ halves of these genes, and the protein products vary substantially in the corresponding C-terminal parts. The high glycine content (~20%) of these proteins and the presence of variable numbers of collagen-like GXXG repeats (from 17 to 42) support their likely role as tail fibers, and the observed variation is consistent with the possibility that these play roles in host recognition and thus in host range determination. A lysis cassette that encodes an endolysin (gp20) and a putative holin (gp21) is situated immediately to the right of the tail gene and is also transcribed rightward.

With the exception of one or two genes at the extreme right ends of the genomes, the remainders of the genes are transcribed leftward; these are closely spaced and are presumably cotranscribed. As observed in phages of other hosts, only a small number are related to proteins with known functions (Fig. 4 and 5). The few genes for which putative functions can be predicted are implicated in DNA metabolism and regulation, including P100D genes *30*/*31*, *33*, and *36* (and their relatives), which encode DNA primases, DnaB-like helicases, and RecB-like exonucleases, respectively (Fig. 4 and 5). P100D gene *23* and its relatives encode likely transcriptional regulators and contain strongly predicted helix-turn-helix DNA-binding motifs; HHPred also predicts a 95.9% probability of structural similarity of P100D gp23 to the DNA-binding domain of the phage 186 repressor. Presumably, these proteins act as the phage repressor and/or as modulators of lytic gene expression. Interestingly, the *P. acnes* genomes lack either integration or partitioning systems that are common to temperate bacteriophages. It is unlikely that these functions have been lost during recent evolutionary history—as has been reported for mycobacteriophage TM4 (4)—but rather it is likely that their absence reflects a core feature of these genome architectures. In agreement with these observations, we were unable to isolate stable lysogens for any of the phages that we tested (data not shown).

A notable genomic feature is the presence of a large (>1-kbp) noncoding region near the right end of the *P. acnes* phage genomes (Fig. 4 and 5). This region shows the greatest variation among the genomes (Fig. 4) and is also notable for its unusual base composition. The left side of the noncoding region has a large drop in percent GC content, below 25% at the greatest extreme (Fig. 5). Throughout the region are several surprisingly well-conserved low-complexity runs (see Fig. S3 and S4 in the supplemental material). Additionally, plots of GC content skew summed over all 14 genomes show a clear excess of G's on the forward strand over most of the genome, switching to an excess of C's within the noncoding region (see Fig. S3 and S4). Transcription appears to have a minimal effect on the GC content skew, with no clear difference evident across the primary region where coding switches from one strand to the other (position 17500 of the multiple alignment) (see Fig. S3). This indicates that the switch in GC content skew is related to replication rather than transcription and suggests an origin of replication within the noncoding region.

Relationship of *P. acnes* phages to other phages. BLASTP searches of predicted *P. acnes* phage gene products against the complete protein database show that almost 50% match only predicted proteins from other *P. acnes* phages or putative *P. acnes* prophages. Many of those that do have matches outside the *P. acnes* phages/prophages are related to phages of other *Actinomycetales* hosts and their prophages, including mycobacteriophages, *Streptomyces* phages, and *Rhodococcus* phages; however, none of these protein relatives show greater than 50% amino acid identity. To examine these relationships further, a gene content map was constructed from the *P. acnes* phages and phages of other *Actinomycetales* hosts, including subsets of those that infect *Mycobacterium*, *Rhodococcus*, *Streptomyces*, *Gordonia*, *Corynebacterium*, and *Tsukamurella* (Fig. 6A). There are two striking observations that emerge from this analysis, the first of which is the limited diversity of the *P. acnes* phages, especially in comparison with that of the mycobacteriophages. Like the *P. acnes* phages, the mycobacteriophages (with the notable exception of DS6A) have the ability to infect a single bacterial host strain (in this case, *M. smegmatis*), yet the diversity of their gene contents is dramatically different. The second is the observation that although ~20% of the *P. acnes* phage genes show >30% amino acid sequence identity to mycobacteriophage gene products, the homologues are distributed broadly among the mycobacteriophages, such that the *P. acnes* phages are not more closely related to any particular group of mycobacteriophages than to the others. This is in con-

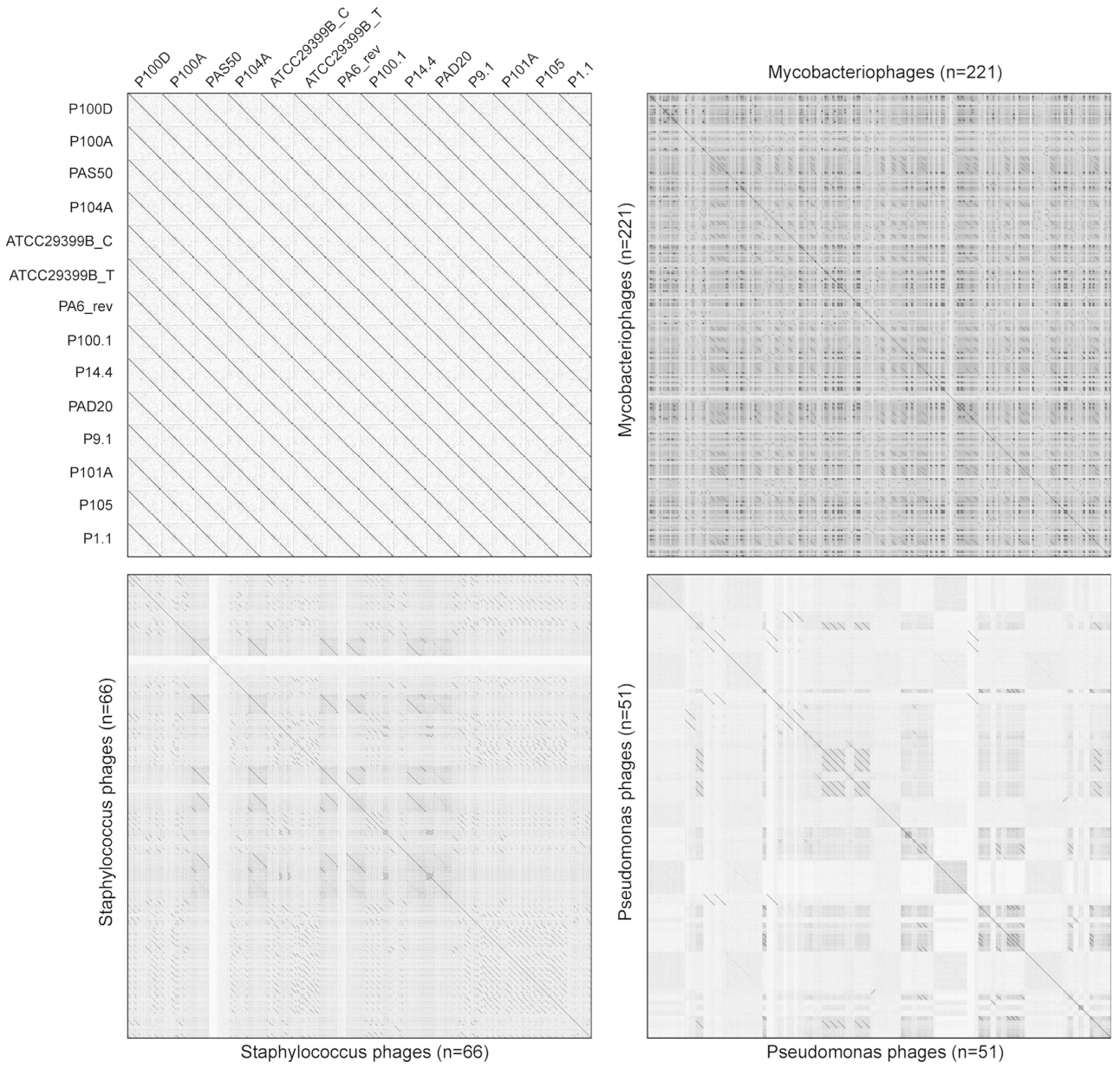

FIG 3 Dot plot nucleotide sequence comparisons of *P. acnes* phages, mycobacteriophages, *Staphylococcus* phages, and *Pseudomonas* phages. The genome sequences of 14 *P. acnes* phages, 221 mycobacteriophages, 66 *Staphylococcus* phages, and 51 *Pseudomonas* phages were concatenated to form four compiled sequences of 0.41, 15.46, 3.3, and 3.88 Mbp, respectively. Each was then compared with itself using the dot plot program Gepard (48).

trast to *Rhodococcus* phage RequPine5 and *Tsukamurella* phage TPA2, both of which are more closely related to the cluster B mycobacteriophages (PG1, Colbert, Nigel, Cooper, Qyrzula, Rosebush, Phlyer, and Phaedrus) than to those of other clusters (Fig. 6A).

The limited diversity of *P. acnes* phage gene content is shown in finer detail in the network map in Fig. 6B. It should be noted that the diversity of the *P. acnes* phages on this gene content map is apparent only because the scale has been magnified 100 times compared to the map in Fig. 6A. Despite their limited diversity, *P. acnes* phages from the same general source, such as the ATCC phages and the two phages from Sweden, PAD20 and PAS50, group together. However, phages arising from the same donor (e.g., P100A, P100D, and P100.1) do not necessarily appear to be more related to one another than they are to the other *P. acnes* phages. However, we recognize that the variation of *P. acnes* phage gene content is very restricted, and therefore, these representations are sensitive to minor differences in genome annotation.

***P. acnes* bacteriophage endolysins.** The bacteriophage endolysin protein functions at the completion of the lytic cycle. With the assistance of the holin protein, the endolysin gains access to and degrades the bacterial cell wall peptidoglycan, leading to host cell lysis and release of progeny phage (25). Endolysins can display a number of different enzymatic activities, including amidase, endopeptidase, glucosaminidase and lytic transglycosylase activities, which target various covalent bonds within the host cell wall (25).

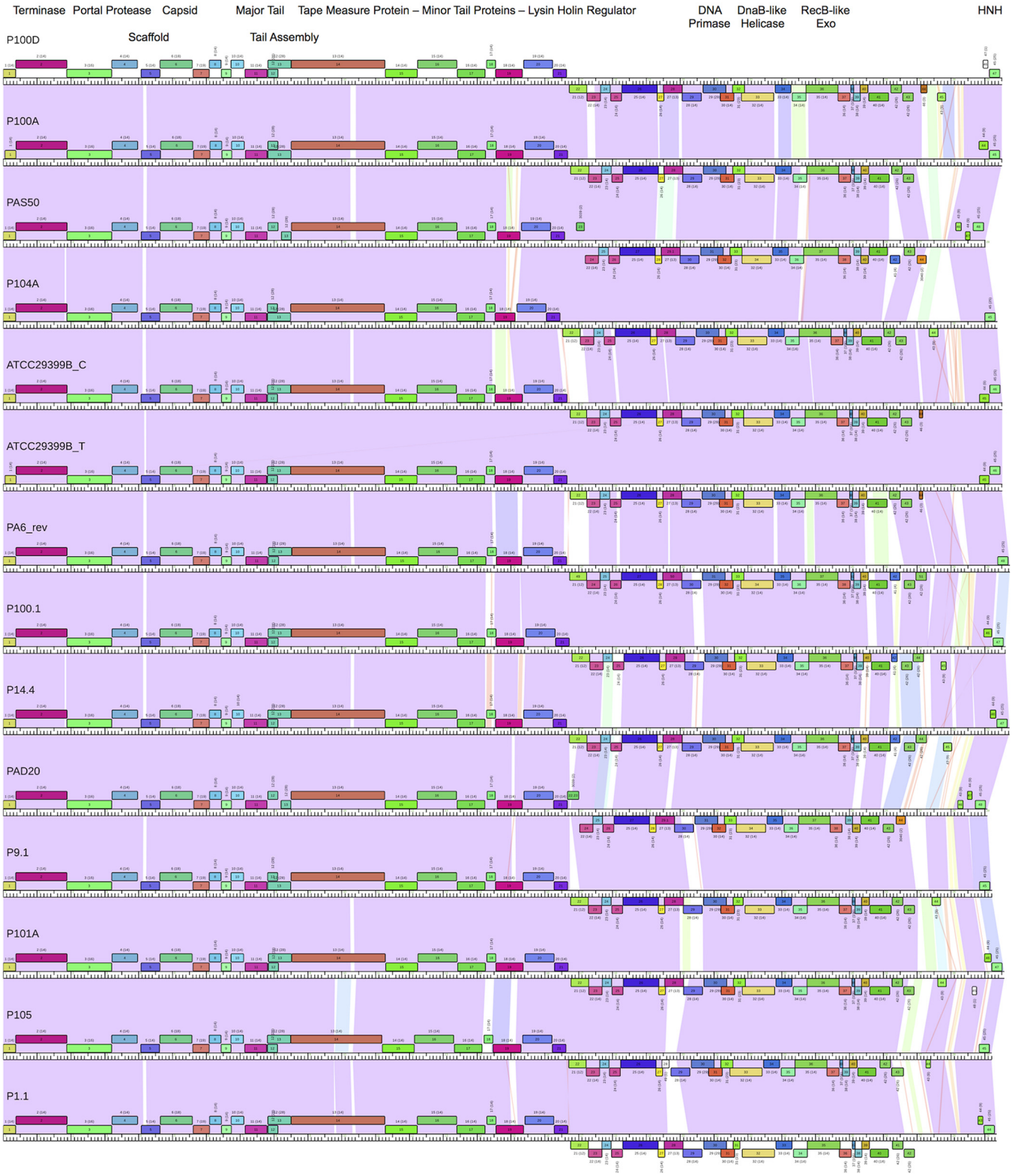

FIG 4 Whole-genome comparisons of *P. acnes* phages. The genome maps of the 14 completely sequenced *P. acnes* phages are shown with the pairwise nucleotide sequence similarities displayed as colored segments between the genomes; the strength of sequence similarity is represented according to a color spectrum in which violet is the most similar and red is the least. The positions of predicted genes are shown as boxes either above (transcribed rightward) or below (transcribed leftward) each genome, with gene numbers shown within the boxes; putative gene functions are noted at the top. The maps were generated using the program Phamerator (49) and a database named acnes_myco30 (see text).

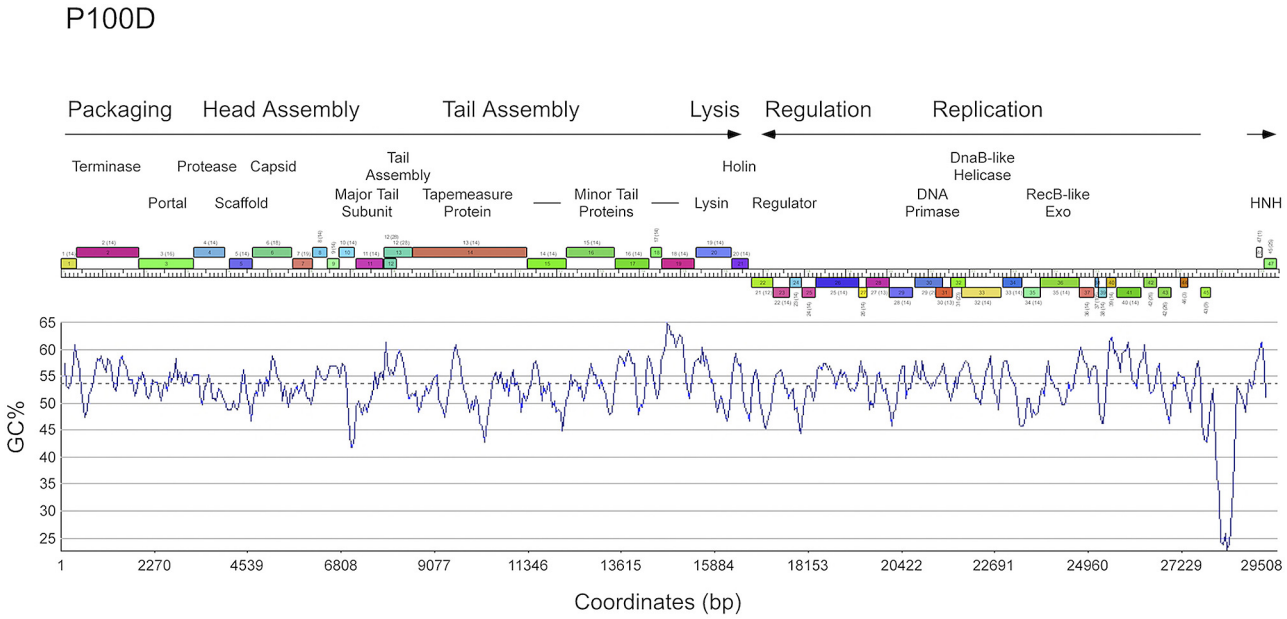

FIG 5 Organization of the P100D genome and its percent GC content variation. A genome map of *P. acnes* phage P100D is shown with predicted genes represented as colored boxes either above (transcribed rightward) or below (transcribed leftward) the genome. The gene number is shown within each box, and the "phamily" to which that the gene corresponds is displayed above, with the number of "phamily" members shown in parentheses. Putative gene functions are noted with arrows indicating the orientation of transcription. All of the other genomes described here have the same basic organization. At the bottom is a scan of percent GC contents across the P100D genome done with window and step sizes of 100 and 50 bp, respectively. The sharp deviation of percent GC content at the right end of the genome occurs in all of the *P. acnes* phages.

Gene *20* is predicted to encode the *P. acnes* phage endolysin, and this protein is highly conserved in all sequenced *P. acnes* phages (Fig. 7A). Partial sequences are available for a number of other *P. acnes* phages, and these also demonstrate a high degree of conservation in this gene family (22). Database comparisons predict that the *P. acnes* endolysin proteins contain a highly conserved N-terminal domain associated with *N*-acetylmuramoyl-l-alanine amidase activity, coupled to a 110-residue C-terminal extension whose function is unknown but which likely mediates cell wall binding. The most closely related phage endolysins are those encoded by *Rhodococcus* phages ReqiDoc7 and REQ2 and a large group of cluster B mycobacteriophages (26). Although all of these have related amidase domains, the C-terminal regions are quite different, likely reflecting their proposed role in conferring cell wall binding specificity. The majority of the differences among *P. acnes* phage endolysins are within the small linker region separating the two domains and, to a lesser degree, within the C-terminal putative cell wall-binding region (Fig. 7B). Collectively, these data show that *P. acnes* phage endolysins are highly conserved; the encoded proteins likely target essential elements within the *P. acnes* cell wall and are predicted to kill a broad range of *P. acnes* isolates.

***P. acnes* phage host preferences.** The limited diversity of the *P. acnes* phages raises the question of whether they have similar host preferences. We thus tested the phage sensitivities of a panel of 27 *P. acnes* strains (Fig. 8), including 23 isolates recovered from the donors screened for *P. acnes* phages (the strains and their sources are described in Table S1 in the supplemental material); 16S rRNA genotyping confirms that these are all *P. acnes* (27). The 11 phages tested display a broad array of host preferences, and only ATCC 29399B_T infects the entire set of host strains with equal efficiencies of plating (Fig. 8). Three of the ATCC strains and 12 donor isolates derived from five different donors—and thus at least 8 distinct isolates total—show no discrimination and are infected equivalently by all 11 phages. The other 11 strains from six different donors, plus one from the ATCC, have a variety of phage sensitivities (Fig. 8). In most strains where variable phage sensitivities are observed, this occurs against a small number of phages and the reductions in plating efficiency range from 10- to 1,000-fold, relative to that of reference strain ATCC 6919. However, in comparison, two strains—B66.8 and B101.9—are distinct from the others in that they are strongly resistant to 9 and 10 of the phages, respectively, with plating efficiencies reduced by at least 1,000,000-fold as compared to those observed on ATCC 6919 (Fig. 8).

The role of *P. acnes* CRISPR (clustered regularly interspaced short palindromic repeat) elements in phage resistance. Variations in phage sensitivity could arise from a variety of mechanisms, including receptor variation, restriction-modification, abortive infection, lysogenic immunity, or innate immunity conferred by CRISPRs (28). Because a subset of *P. acnes* isolates for which genomic sequence information is available in the NCBI database contain putative CRISPR loci belonging to the type I-E subfamily and that appear to confer resistance to mobile genetic elements (29), we examined if there is any correlation between phage sensitivities and CRISPR elements in the 27 *P. acnes* strains. The *P. acnes* CRISPR loci contain eight conserved *cas* genes (29), and PCR amplification of two of these, *cas3* and *csc4* (*cas7* in the nomenclature system proposed by Makarova and colleagues [30, 31]), identified CRISPR elements in six of our *P. acnes* isolates, i.e., B66.8, B69.7, B101.9, B102.1, B102.8, and B102.10. Sequencing of the associated spacer/repeat loci showed that all have the same

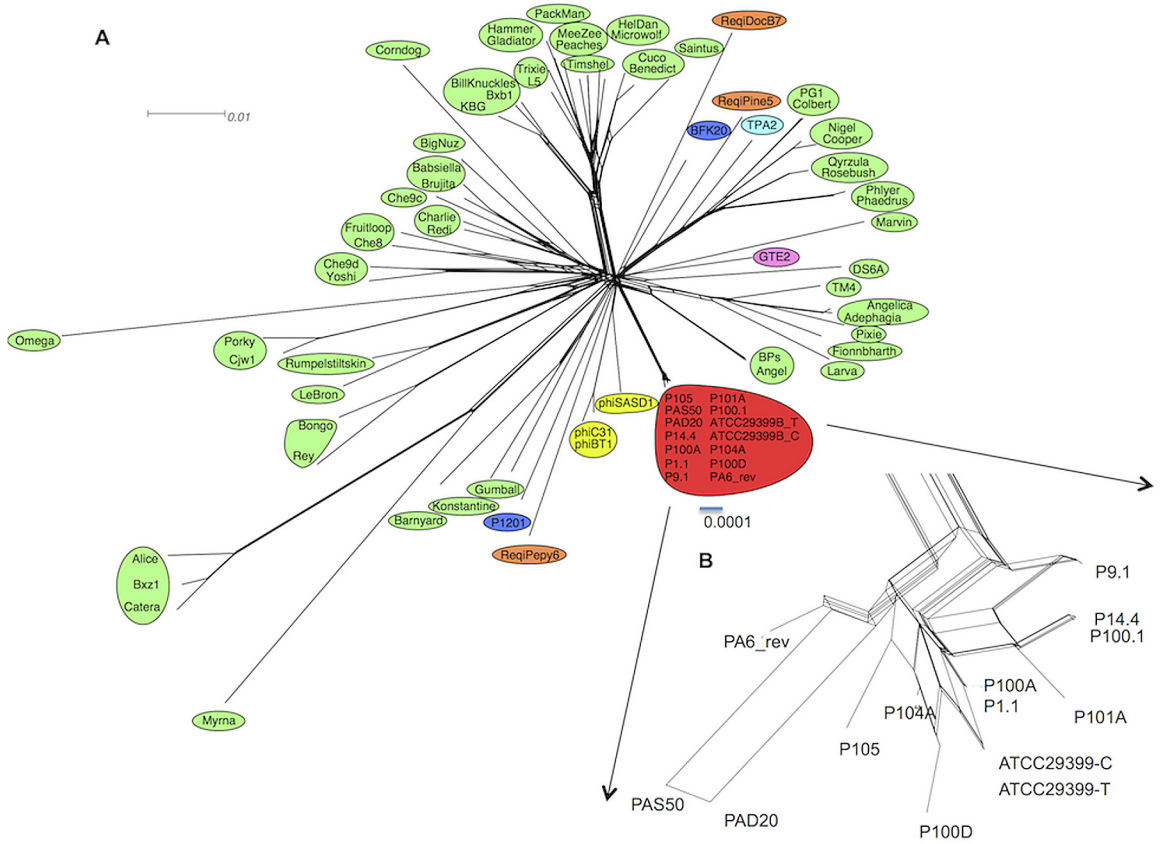

FIG 6 Gene content relationships between *P. acnes* phages and phages of related hosts. (A) The relationships between the 14 *P. acnes* phages and a selection of phages that infect other bacteria of the order *Actinomycetales* were obtained by using the NeighborNet function in Splitstree4 (51). The *P. acnes*, *Mycobacterium*, *Rhodococcus*, *Corynebacterium*. *Streptomyces*, *Tsukamurella*, and *Gordonia* phages are shown in the red, green, orange, blue, yellow, aqua, and mauve circles, respectively. (B) Detailed view of the display in panel A showing the *P. acnes* phages.

28-bp direct repeat sequence, separated by variable numbers of 33-bp spacers (Fig. 9; see Table S2 in the supplemental material), ranging from 1 spacer in B69.7 to 10 in B101.9. Four of these CRISPRs (in B69.7, B102.10, B102.1, and B102.8) contain only one or two spacers each, none of which have corresponding sequences in any of the phage genomes (see Table S2). Accordingly, these were sensitive to the panel of phages assayed as described above. In contrast, the two strains with the most spacers—B66.8 and B101.9, with 9 and 10, respectively—are also those that show extensive phage resistance (Fig. 8), implicating CRISPRs in this phenotype.

In strain B101.9, five of the spacers have sequence-related segments (known as protospacers) in the *P. acnes* phage genomes, with spacers 1 to 5 corresponding to genes *9*, *2*, *3*, *7*, and *29*, respectively (Fig. 9). However, the matches to spacers 2 and 5 are poor and the numbers of mismatches likely preclude any role in phage resistance. However, spacers 1, 3, and 4 have either a perfect match or a small number of mismatches in each of the phage genomes, with the notable exception of ATCC 29399B_T (Fig. 9), the only one of these phages to which this strain is sensitive.

A similar pattern was found in strain B66.8. Three of the five spacers (spacers 3, 4, and 5) are the same as those in B101.9, but as noted above, spacer 5 has no close phage matches. Spacer 2 of B66.8 has a related sequence in phage gene *2* but at a location different from that of spacer 2 of B101.9 (Fig. 9). It also has five mismatches in all of the phages and thus is unlikely to contribute to resistance. In contrast, spacer 1 of B66.8 matches gene *16* of the phages, with only two to four mismatches (Fig. 9). Like strain 101.9, B66.8 is sensitive to phage ATCC 29399_T, and all of the CRISPR spacers have three or more mismatches. However, B66.8 is also sensitive to phage ATCC 29399_C, suggesting that the CRISPR spacers—including spacers 3 and 1—are ineffective at conferring resistance, even though they have 0 and 2 mismatches, respectively (Fig. 8). This discrepancy cannot be explained by mutations in the protospacer-adjacent motifs (PAMs) in this phage, as the 10 bases downstream of the protospacers found in all 14 *P. acnes* phage genomes follow the expected CRISPR type I consensus motif, TGGC/TGAAGA/CG/AT/G. In spite of this unexplained observation, the strong overall correlation between the presence of CRISPR elements and spacer matches with the phage genomes suggests strongly that these play a role in the phage resistance profiles observed.

DISCUSSION

Genomic analysis of bacteriophages has revealed a remarkable diversity within populations that target specific host bacteria, providing insights into how they interact with and kill their hosts, as well as the evolution of microbial communities. Here, the analysis of 14 fully sequenced *P. acnes* bacteriophages reveals an unexpected degree of homogeneity in terms of percent GC content,

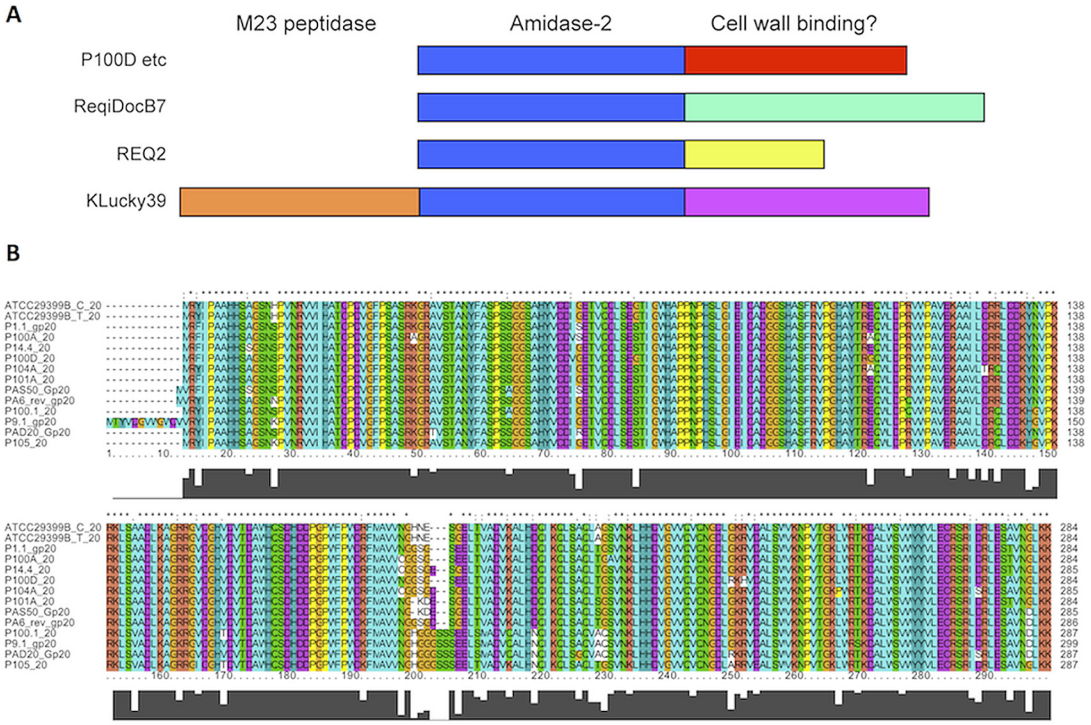

FIG 7 Organization of *P. acnes* phage endolysins. (A) Organization of the *P. acnes* endolysins and their closest homologues present in *Rhodococcus* phages ReqiDocB7 and REQ2 and a group of subcluster B1 mycobacteriophages of which KLucky39 is representative. All have an amidase-2 domain that cleaves peptidoglycan but have different and unrelated C-terminal domains, which are presumed to confer cell wall binding specificity. The KLucky39 endolysin—like many of the mycobacteriophage endolysins—contains three domains, including an N-terminal M23 peptidase (26). (B) Alignment of the *P. acnes* phage endolysins shows that they are closely related and display the greatest amino acid sequence differences at around position 200, which likely corresponds to a glycine-rich interdomain linker.

nucleotide sequence identity, and gene content. This is particularly evident when these phages are compared with other groups of well-characterized bacteriophage populations. Phylogenetic relationships based on gene content show that the *P. acnes* phages cluster together on a single node, unlike the mycobacteriophages, which show multiple nodes with extensive branching indicative of gene content diversity. Strikingly, the differences between the *P. acnes* phages are revealed only when their node is magnified 100-fold relative to that of the other bacteriophage populations. The lack of *P. acnes* phage diversity is further reflected by their broad host ranges against clinical isolates. This suggests the presence of evolutionary constraints imposed upon this population of phages and their bacterial hosts that function to maintain a single phage type and prevent significant diversification of *P. acnes* and the widespread dispersal of phage resistance.

It is noteworthy that the lack of genetic diversity among *P. acnes* phages is maintained despite their collection over a varied geographic and temporal range, spanning two continents and more than 30 years. One explanation for this is the relatively isolated niche in which they and their host bacteria are found. *P. acnes* is the dominant microbial inhabitant of the human pilosebaceous unit (10), in part related to the unique nature of this anaerobic microenvironment, which is characterized by high lipid content. Therefore, the lack of other microbes—and presumably their phages—in this environment diminishes the possibility of recovering more distantly related phages that may prefer alternative hosts but are still able to form plaques on *P. acnes* bacterial lawns. It also provides fewer opportunities for lateral gene transfer and recombination. This is in contrast to the diverse phage populations that are found in complex microbial communities, such as those in aquatic environments or soil and even in other regions of the human body, including the oral mucosa (32) and the gut (33). In addition to the occupation of a specialized niche, the *P. acnes* phage homogeneity, combined with an average percent GC content that is so distinct from that of the host bacteria, might suggest that these phages arose from a common ancestor that only recently acquired the ability to infect *P. acnes*. It is also possible that a genetic bottleneck eliminated much of the *P. acnes* bacteriophage diversity, leaving the one dominant genotype present today.

Another interesting feature of the *P. acnes* phages is that none encode any recognizable proteins predicted to be involved in the establishment or maintenance of lysogeny, such as integrases or putative partitioning functions. We note that these phages do contain a possible candidate for the phage repressor, P100D gene *23* (and its relatives), which encodes a likely transcriptional regulator predicted to contain a helix-turn-helix DNA-binding motif and has predicted structural similarity to the DNA-binding domain of the enterobacterial phage 186 repressor. However, in the absence of other genes required for lysogeny, and because of our inability to isolate stable lysogens, it is more likely that this protein functions to regulate the transcription of lysis genes. These observations do not preclude the possibility that these phages engage in an unstable "pseudolysogenic" relationship with their hosts (21). This is supported by our observation that many of our phages form turbid plaques on some *P. acnes* strains and the detection of

	Phage										
Isolate	P1	P9	P14	P100A	P100D	P100.1	P101A	P104A	P105	ATCC_C	ATCC_T
6919	1	1	1	1	1	1	1	1	1	1	1
6921	1	1	1	1	1	1	1	1	1	1	1
11827	1	1	1	1	0.1	0.1	0.1	1	0.1	1	1
29399	1	1	1	1	1	1	1	1	1	1	1
B66.8	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	0.01	1
B101.9	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	1
B69.7	1	1	0.01	1	1	1	0.1	1	1	1	1
B102.1	1	1	0.01	1	1	1	1	0.1	1	1	1
B102.8	1	1	0.01	1	1	1	1	1	1	1	1
B102.10	1	0.1	0.01	0.1	1	1	1	1	1	1	1
B101.3	1	1	1	1	1	1	1	1	1	1	1
B101.8	1	1	1	1	1	1	1	1	1	1	1
B102.3	1	1	1	1	1	1	1	1	1	1	1
B102.9	1	1	1	1	1	1	1	1	1	1	1
B103.3	1	1	1	1	1	1	1	1	1	1	1
B103.6	1	1	1	1	1	1	1	1	1	1	1
B103.7	1	1	1	1	1	1	1	1	1	1	1
B103.8	1	1	1	1	1	1	1	1	1	1	1
B104.4	1	1	1	1	1	1	1	1	1	1	1
B104.5	1	1	1	1	1	1	0.1	1	1	1	1
B104.6	1	1	1	1	1	1	1	1	1	1	1
B104.7	1	1	1	1	1	1	1	1	1	1	1
B104.8	1	1	1	1	0.1	1	0.1	1	1	1	1
B105.1	1	1	0.001	1	1	1	1	1	1	1	1
B105.3	1	1	1	1	1	1	0.1	1	1	1	1
B105.8	1	1	1	1	1	1	1	1	1	1	1
B105.9	1	1	0.01	1	1	1	1	1	1	1	1

FIG 8 Host preferences of *P. acnes* phages. The efficiency of plating of each of the 11 *P. acnes* phages was calculated for 27 bacterial isolates, including 23 donor isolates and four ATCC strains; ATCC 6919 was used as a reference for normalization. Eleven isolates and ATCC 11827 have variable degrees of phage sensitivity; most differences result from modest reductions in plating efficiency (10- to 1,000-fold relative to reference strain ATCC 6919), but two isolates, B66.8 and B101.9, are strongly resistant to 9 and 10 of the phages, respectively (plating efficiencies are reduced by at least 1,000,000-fold). The remaining 12 isolates and three ATCC strains are infected equivalently by all of the phages.

nonintegrated bacteriophage sequences within sequenced *P. acnes* bacterial isolates that have been deposited in GenBank (see Text S1 in the supplemental material).

The lack of genetic diversity within the *P. acnes* phage population, combined with a broad host range and an inability to form stable lysogens within the host bacteria, makes these phages ideal candidates for the development of a phage-based topical anti-acne therapy. The potential therapeutic use of phages has been known for over 90 years, with a recent resurgent interest brought about by the emergence of antibiotic resistance in many pathogenic bacteria (34). Phages have been administered to humans without toxicity (35, 36), and the efficacy of phage therapy has been demonstrated in a number of animal models for infections with *Shigella dysenteriae* (37), *Escherichia coli* (38, 39), and cutaneous *Staphylococcus aureus* (40, 41). The FDA has also approved the use of *Listeria* phage LMP-102 to control contamination of meat (34).

One possible issue with phage therapy for acne is a potential for the emergence of resistant strains. We have found that when broad phage resistance is observed, it is generally correlated with, and therefore likely attributable to, the presence of a CRISPR element. Clearly, definitive experiments demonstrating the role of *P. acnes* CRISPR elements in providing immunity to phage lysis and elucidating the rate at which *P. acnes* strains may acquire CRISPR-mediated resistance are needed. There are, however, potential strategies to circumvent CRISPR-mediated resistance, including the possibility of treatment with a cocktail of phages, selecting for escape mutants with alterations in their protospacers and/or PAMs, or using engineered phages in which potential matches to the known spacers has been eliminated.

An alternative approach to topical phage therapy for acne would be to develop the *P. acnes* phage endolysin as an anti-acne therapeutic. The endolysin is a cell wall hydrolase predicted to function in bacterial peptidoglycan hydrolysis at the completion of the lytic cycle (25). The *P. acnes* phage endolysin, a predicted amidase, is encoded in one of the most conserved regions of the phage genome and was found to be >95% conserved at the amino acid level in all known *P. acnes* phages. As has been demonstrated for other Gram-positive bacteria, *P. acnes* may be sensitive to killing by exogenous application of purified endolysin, with potential antibacterial application (42). The conserved nature of this protein also strongly implies that, regardless of which one is chosen, the endolysin from any *P. acnes* phage should have activity against most, if not all, isolates of *P. acnes* bacteria. Importantly, in other phage-host systems, resistance to purified recombinant endolysin protein has not been observed, even after repeated rounds of exposure, perhaps because they have evolved to target essential components of the cell wall (42).

In summary, by studying the diversity of *P. acnes* bacteriophages, we have gained insight into the microbiome of a unique anatomical niche, the pilosebaceous unit. The unexpected but striking limited diversity of these phages, which can lyse the major bacterial inhabitant of the pilosebaceous unit, *P. acnes*, likely reflects the coevolution of virus and bacteria in a distinct and restricted microenvironment characterized by high lipid content and anaerobic conditions. *P. acnes* is also found on the skin surface as part of a more complex microbiome (43, 44), yet the *P. acnes* bacteriophages isolated from different anatomic sites appear to be similar in terms of the amidase and major tail protein genes

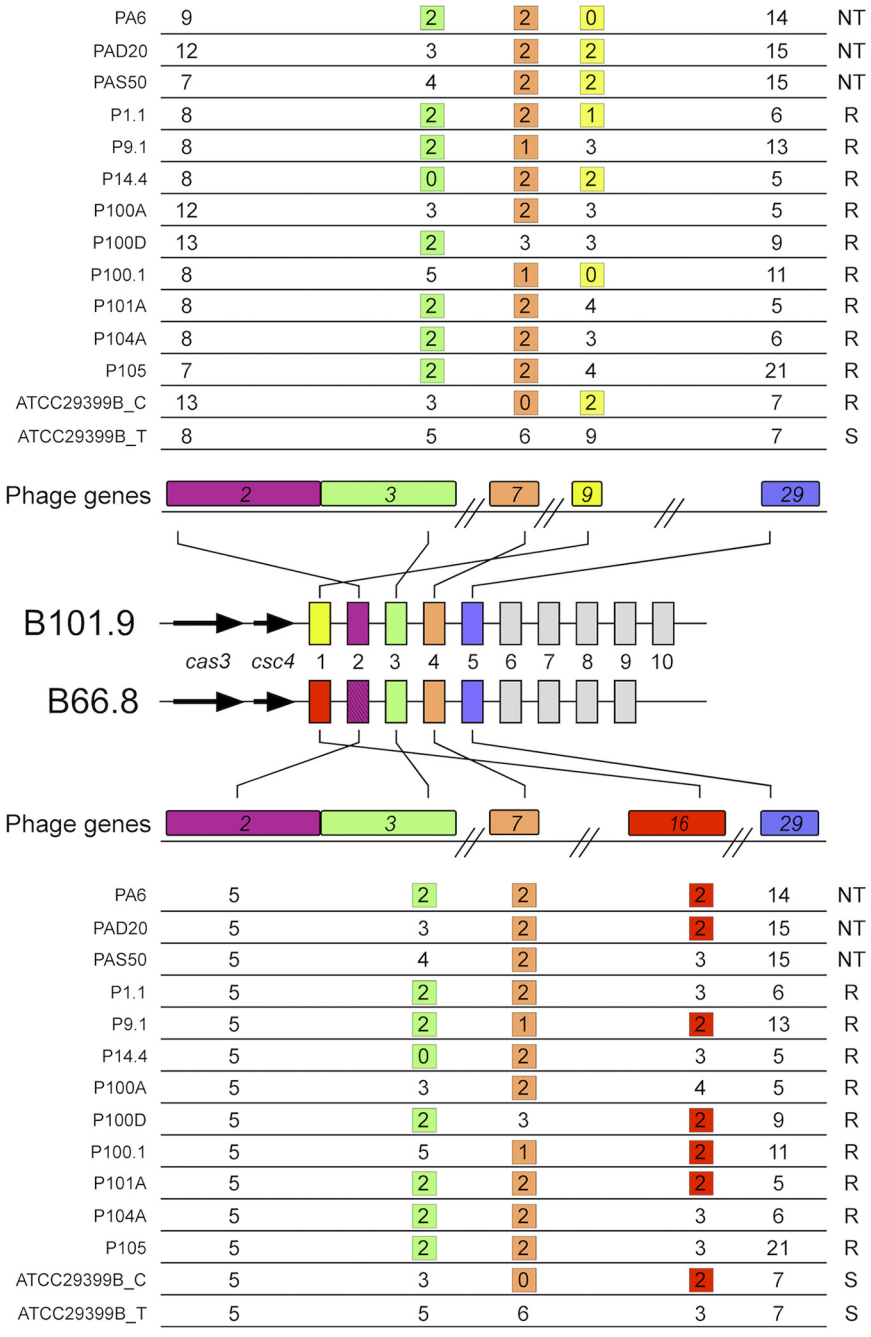

FIG 9 Correspondence of CRISPR spacer sequences and *P. acnes* phages. *P. acnes* strains B101.9 and B66.8 each contain a CRISPR locus containing at least the *cas3* and *csc4* genes flanked by an array of direct repeats separated by 33-bp variable spacer sequences. The spacers are represented by colored boxes, and those numbered 3, 4, and 5 are identical in the two strains. Each of the first five spacers has closely related sequences in the *P. acnes* phages, with the corresponding genes indicated. The numbers of nucleotide sequence deviations from each of the 33-bp spacers in the 14 *P. acnes* phage genomes are represented above (for strain 101.9) and below (strain B66.8) the CRISPRs. Those phage segments that have two or fewer sequence departures from the spacer are boxed in yellow. At the right, the patterns of phage resistance (R) or sensitivity (S) to the two strains are indicated; NT, not tested.

(22). The limited diversity of *P. acnes* bacteriophages is also striking in comparison to the diversity of both viruses and bacteria at other microanatomic sites (33, 32). These studies point the way to further investigation of the *P. acnes* bacteriophage populations in health and disease but also indicate the possibility of using these phages as a targeted approach to acne treatment.

MATERIALS AND METHODS

Bacterial strains and media. *P. acnes* strains used for the propagation of bacteriophages were obtained from the ATCC (Manassas, VA) and include ATCC 6919, ATCC 6921, ATCC 11827, and ATCC 29399. Colonies were grown on brucella agar with 5% sheep blood, hemin, and vitamin K (Thermo Fisher Scientific Remel Products, Lenexa, KS) at 37°C for 3 to 5 days under anaerobic conditions (either in an anaerobic chamber at an $N_2/CO_2/H_2$ ratio of 80:10:10, by volume, or in sealed containers containing oxygen-absorbing, carbon dioxide-generating Anaero Packs [Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan]). Cultures inoculated from single colonies were grown under the same conditions in Reinforced Clostridial Medium (RCM; Oxoid, Basingstoke, England).

Isolation and propagation of *P. acnes* bacteriophages and donor isolates. *P. acnes* bacteriophages and bacterial isolates were obtained from nasal skin microcomedones using Bioré Deep Cleansing Pore Strips collected either from patients attending the Division of Dermatology outpatient clinic at the University of California, Los Angeles, or from donors recruited to the laboratory of Robert L. Modlin at the University of California, Los Angeles. This study was approved by the Institutional Review Board (IRB) at the University of California, Los Angeles (IRB numbers 02-03-083-11 and 10-05-021-01), and all donors gave their written informed consent.

P. acnes bacterial isolates were obtained by streaking donor microcomedones onto brucella agar; individual colonies were selected and repassaged twice to obtain clonal isolates. A subset of *P. acnes* bacteriophages were isolated by plating the pore strip material either alone or with indicator strains of host bacteria as top agar overlays onto A medium plates (Table 1; see Text S1 in the supplemental material). Some were obtained from the filtered supernatant of cultures inoculated with donor microcomedones and grown to saturation under anaerobic conditions at 37°C. All phages were plaque purified at least three times to ensure sample homogeneity, and nine were chosen for sequencing and further analysis (Table 1).

A stock of phage ATCC 29399B was obtained from the ATCC. Upon plating, two plaque morphologies were observed on the host, ATCC 6919, one clear and one turbid. Each phage was picked and propagated and found to plate true (e.g., phages from clear plaques always gave rise to clear plaques). To rule out the possibility of lab-derived contamination, we reordered the phage stock and confirmed the presence of both plaque morphotypes. These were analyzed separately

and are referred to here as ATCC 29399B_C (clear) and ATCC 29399B_T (turbid).

Electron microscopy. Purified phage lysates with titers ranging from 10^9 to 10^{11} PFU/ml were spotted in 5-μl aliquots onto freshly glow-discharged 400-mesh carbon-Formvar-coated copper grids and allowed to sit for approximately 1 min. Grids were washed with distilled water and stained using 1% uranyl acetate. Images were captured using an FEI Morgagni transmission electron microscope at ×56,000 magnification.

Bacteriophage genome sequencing and analysis. High-titer plate lysates of purified phage stocks were prepared in phage buffer, and bacterial cells were removed using 0.22-μm syringe filters. Total genomic DNA was isolated from these by either ammonium sulfate precipitation, followed by phenol-chloroform extraction, or using the phage DNA Isolation Kit (Norgen Bio-Tek Corp., Thorold, Ontario, Canada). Purified genomic DNA was subjected to whole-genome sequencing using either the 454 or the Illumina platform. Raw sequence reads were assembled using Newbler Assembler and Consed or the ABySS software package with manual correction. Finished sequences were analyzed and annotated using the genome editor DNAMaster (http://cobamide2.bio.pitt.edu); additional genome features were identified using tRNA ScanSE (45), Aragorn (46), and a programmed frameshift finder (47). GenBank accession numbers are shown in Table 1. Dot plots were generated in Gepard (48), and comparative genomic analyses were performed using Phamerator (49).

Comparative genome analysis suggests that some revision of the annotation of the previously reported PA6 genome is warranted. A revised annotation of the PA6 genome—designated PA6_rev—was used in the analyses reported here.

Other genomes used for comparative analysis. The phage genomes used in comparative analyses are listed in Text S1 in the supplemental material. The 14 *P. acnes* phages are P100D, P100A, PAS50, P104A, ATCC 29399B_C, ATCC 29399B_T, PA6_rev, P100.1, P14.4, PAD20, P9.1, P101A, P105, and P1.1. The corresponding GenBank accession numbers are listed in Table 1.

Phylogenetic typing of *P. acnes* isolates. The 16S rRNA gene was PCR amplified from purified bacterial isolates using universal primers UFPL (5′-AGTTTGATCCTGGCTCAG-3′) and URPL (5′-GGTTACCTTGTTACGACTT-3′) (50) (see Text S1 in the supplemental material). The 1,455-bp product was purified (Qiagen, Germantown, MD) and sequenced by Sanger sequencing (Laragen, Los Angeles, CA) in both the forward and reverse orientations with the same primers used to generate the product.

Host range determination. Donor isolates of *P. acnes* were inoculated from a single colony into 3 to 5 ml of RCM and grown to early stationary phase under anaerobic conditions. Top agar lawns containing 0.35% A agar were seeded with ~500 μl of each culture and overlaid onto A medium agar plates. Individual phage lysates were serially diluted in phage buffer and spotted onto the freshly poured top agar overlays. From these experiments, efficiencies of plating were calculated for all phages against each strain tested using ATCC 6919 as a reference for normalization.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00279-12/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, PDF file, 1.7 MB.
Figure S2A, PDF file, 0.5 MB.
Figure S2B, PDF file, 0.3 MB.
Figure S3, TIFF file, 0.7 MB.
Figure S4, TIFF file, 1.6 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

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