

ORIGINAL ARTICLE

The diversity and host interactions of *Propionibacterium acnes* bacteriophages on human skin

This paper has been corrected and a corrigendum also appears in this issue

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The viral population, including bacteriophages, is an important component of the human microbiota, yet is poorly understood. We aim to determine whether bacteriophages modulate the composition of the bacterial populations, thus potentially playing a role in health or disease. We investigated the diversity and host interactions of the bacteriophages of *Propionibacterium acnes*, a major human skin commensal implicated in acne pathogenesis. By sequencing 48 *P. acnes* phages isolated from acne patients and healthy individuals and by analyzing the *P. acnes* phage populations in healthy skin metagenomes, we revealed that *P. acnes* phage populations in the skin microbial community are often dominated by one strain. We also found phage strains shared among both related and unrelated individuals, suggesting that a pool of common phages exists in the human population and that transmission of phages may occur between individuals. To better understand the bacterium–phage interactions in the skin microbiota, we determined the outcomes of 74 genetically defined *Propionibacterium* strains challenged by 15 sequenced phages. Depending on the *Propionibacterium* lineage, phage infection can result in lysis, pseudolysogeny, or resistance. In type II *P. acnes* strains, we found that encoding matching clustered regularly interspaced short palindromic repeat spacers is insufficient to confer phage resistance. Overall, our findings suggest that the prey–predator relationship between bacteria and phages may have a role in modulating the composition of the microbiota. Our study also suggests that the microbiome structure of an individual may be an important factor in the design of phage-based therapy.

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Introduction

The human skin is inhabited by hundreds of microbial species, including bacteria, fungi and viruses. The homeostasis of this ecosystem is important to its function as a barrier against infection and colonization of pathogens on the skin surface. Bacteriophages are important components of the human microbiota. They are a reservoir of diversity-generating elements

(Rohwer and Thurber, 2009) and regulate both the abundance and diversity of their bacterial hosts by predation (Suttle *et al.*, 1990; Waterbury and Valois, 1993; Rohwer, 2003; Rodriguez-Valera *et al.*, 2009). Although the skin bacterial community has been studied by several groups (Gao *et al.*, 2007; Costello *et al.*, 2009; Grice *et al.*, 2009; Kong *et al.*, 2012; The Human Microbiome Project Consortium, 2012; Blaser *et al.*, 2013; Fitz-Gibbon *et al.*, 2013; Nakatsuji *et al.*, 2013), relatively few studies have characterized the skin viral community (Foulongne *et al.*, 2012; Ma *et al.*, 2014; Wylie *et al.*, 2014). In particular, the composition and dynamics of bacteriophages and their interactions with bacterial hosts on the skin are not well understood.

The microbial community in the skin pilosebaceous unit is dominated by *Propionibacterium acnes*, which accounts for nearly 90% of the microbiota

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(Fitz-Gibbon *et al.*, 2013). Although *P. acnes* is a major skin commensal, it has been considered a pathogenic factor for acne vulgaris (Leyden, 2001; Bojar and Holland, 2004), one of the most common skin diseases affecting over 80% of adolescents and young adults (White, 1998; Bergler-Czop and Brzezińska-Wcisło, 2013). Our previous 16S ribosomal RNA metagenomic study demonstrated that *P. acnes* strain population structure in pilosebaceous units differs significantly between acne patients and healthy individuals (Fitz-Gibbon *et al.*, 2013). Certain strains are highly associated with the disease (Lomholt and Kilian, 2010; McDowell *et al.*, 2012; Fitz-Gibbon *et al.*, 2013; Tomida *et al.*, 2013), while some strains are enriched in healthy skin (Fitz-Gibbon *et al.*, 2013).

In parallel, *P. acnes* phages are dominant bacteriophages in the pilosebaceous unit (Fitz-Gibbon *et al.*, 2013). It has been known for over 50 years that *P. acnes* phages exist on the human skin (Brzin, 1964). They have the morphology of siphoviruses, consisting of a ~50 nm icosahedral head and a ~150 nm flexible tail (Farrar *et al.*, 2007). Zierdt *et al.*, (1968) isolated phage 174 from spontaneous plaques of a *P. acnes* isolate. Phage 174 was able to lyse nearly all *P. acnes* strains tested in the study. Subsequently, more *P. acnes* phages were isolated, which exhibited lytic as well as pseudolysogenic behavior (Lood and Collin, 2011). However, in the past decades, the study of *P. acnes* phages had been limited to the development of phage typing systems to distinguish different serotypes of *P. acnes* (Jong *et al.*, 1975; Webster and Cummins, 1978). Recent sequencing of 14 *P. acnes* phages (Farrar *et al.*, 2007; Lood and Collin, 2011; Marinelli *et al.*, 2012) suggested that they have limited genetic diversity with over 85% nucleotide identity in the genome. All sequenced genomes are similar in size and structure with 45–47 genes encoded in two oppositely transcribed regions named the left arm and right arm (Farrar *et al.*, 2007; Lood and Collin, 2011; Marinelli *et al.*, 2012).

Much is to be learned about whether bacteriophages drive the diversity and dynamics of the skin bacterial community. The ratio between *P. acnes* phages and *P. acnes* was ~1:20 in pilosebaceous units, based on a pooled healthy skin sample that we analyzed previously (Fitz-Gibbon *et al.*, 2013), but can vary in a large range among individuals and over time. *P. acnes* phages do not encode integrases in their genomes (Farrar *et al.*, 2007; Lood and Collin, 2011), suggesting their inability to stably integrate into the host chromosome. They can kill the host bacteria through cell lysis or can enter a pseudolysogenic state in the host strain (Lood and Collin, 2011), in which the phage DNA persists in infected cells without lysing the host or integrating into its genome. Whether *P. acnes* phages modulate the relative abundances of different *P. acnes* strains by selectively killing specific strains of *P. acnes* and thus play a role in skin health and disease is unknown.

On the other hand, bacterial hosts can influence phage populations through antiviral mechanisms, such as the restriction modification mechanism and the bacterial adaptive immune system utilizing clustered regularly interspaced short palindromic repeat (CRISPR) sequence arrays (Horvath and Barrangou, 2010). In our effort to characterize the strain diversity of *P. acnes* in the pilosebaceous unit, we discovered that all sequenced type II *P. acnes* strains harbor Type I-E CRISPR and CRISPR-associated (Cas) proteins (Fitz-Gibbon *et al.*, 2013; Tomida *et al.*, 2013). Marinelli *et al.* (2012) suggested that the CRISPR mechanism explains the resistance of certain *P. acnes* strains to phage infection, yet noting that some of their observations were inconsistent with this theory.

To better understand how bacteriophages modulate the bacterial composition of the skin microbiota and their potential roles in skin health and disease, in this study, we determined the diversity of *P. acnes* phages and their interactions with bacterial hosts in the skin of acne patients and healthy individuals. We sequenced the genomes of 48 *P. acnes* phages isolated from 37 individuals and investigated whether certain phage strains dominate the skin microbiota. By analyzing the skin metagenome data from the Human Microbiome Project (HMP), we further verified our conclusions from analyzing sequenced phage isolates. We also challenged a panel of 74 genetically defined *Propionibacterium* strains against 15 of the sequenced phages to determine the outcome and mechanisms of their interactions.

Materials and methods

Phage isolation and DNA extraction

Skin follicle samples were previously collected from the nose of acne patients and individuals with healthy skin as reported in the study by Fitz-Gibbon *et al.*, (2013). To best represent the diversity of populations and history of medical care, the subjects were recruited from private practice, managed care and public hospital settings, as well as outside of dermatology clinics in Southern California. Written informed consent was provided by all study subjects.

The follicle contents collected on the surface of the nose strip were mashed using a sterile loop (Fisherbrand, Pittsburgh, PA, USA), and plated onto a blood agar plate (Teknova Brucella Agar Plate with Hemin and Vitamin K, Teknova, Hollister, CA, USA). The sample plates were incubated at 37 °C for 5–7 days anaerobically using the AnaeroPack System (Mitsubishi Gas Chemical Company, Tokyo, Japan) (Fitz-Gibbon *et al.*, 2013).

Phage plaques observed on the culture plates were isolated by puncturing the agar with a sterile pipette tip and resuspending each tip in 50 µl SM buffer (0.1 M sodium chloride, 8 mM magnesium sulfate

heptahydrate, 1M Tris-HCl, pH 7.5, 2% gelatin and 1 mM calcium chloride). Each phage resuspension was spread onto A-media plates (12 g l⁻¹ pancreatic digest of casein, 12 g l⁻¹ yeast extract, 22.2 mM D-glucose, 29.4 mM g l⁻¹ potassium phosphate monobasic, 8 mM magnesium sulfate heptahydrate and 20 g l⁻¹ agar) with top agar containing *P. acnes* strain ATCC6919. After incubation at 37 °C for 2 days, single plaques were selected and propagated using the same host strain, medium and incubation conditions. Suspensions of each phage isolate were prepared by eluting plates with 8 ml SM buffer at room temperature, filtering with a 0.22-µm PES filter (Millipore, Billerica, MA, USA) to remove bacterial cells, and storing at 4 °C. Phage titers were determined by plaque assay.

Phage DNA extraction was performed using the Lambda Mini Kit (Qiagen, Valencia, CA, USA) with the following modifications. Phage particles were precipitated in Buffer L2 by centrifugation at 20 000 g at 4 °C for 1 h. Extracted DNA was precipitated overnight at -20 °C before centrifugation.

Phage electron microscopy

Copper grids (400 mesh formvar per carbon film) (Ted Pella, Redding, CA, USA) were glow discharged. Phage cultures were applied, followed by a wash with 0.22-µm-filtered water. The samples were stained with 1% uranyl acetate and examined under a JEOL JEM-1200EX electron microscope (JEOL, Peabody, MA, USA) with an accelerating voltage of 80 kV.

Phage genome sequencing, assembly and annotation

Phage genomes were sequenced in multiplex using the Roche GS FLX Titanium (Roche, Branford, CT, USA) or the Illumina MiSeq (Illumina, San Diego, CA, USA) platform. Sequence reads were initially assembled using MIRA 3.2.1 (Chevreux *et al.*, 1999), and the resulting contigs were manually finished in Consed 23.0 (Gordon *et al.*, 1998). Some phage genomes required additional PCRs and amplicon sequencing to fill the gaps between contigs. Fully assembled phage genomes were annotated using Genemark.hmm (Lukashin and Borodovsky, 1998) and Glimmer v3.02 (Delcher *et al.*, 1999) with manual corrections. All phage genome sequences have been deposited in GenBank under BioProject PRJNA173665 with accession numbers JX570702-JX570714, KJ578758-KJ578792.

Genome analysis and phylogenetic tree construction

Sequences present in all 62 phage genomes were defined as core regions of the phage genome. To identify these core regions, we first generated alignments between the PA6 genome and each of the other 61 phage genomes using Nucmer (Kurtz *et al.*, 2004). This yielded 61 sets of starting and ending coordinates describing intervals within the PA6 genome that align with a given phage genome. We then calculated the core regions for all phages by

determining the overlapping intervals between all of the 61 coordinate sets. The core region sequences were concatenated for the subsequent multiple sequence alignments. Single nucleotide polymorphisms (SNPs) in the core regions were identified by using the 'show-snps' option of Nucmer with the default setting. In addition, the set of non-synonymous SNPs was obtained by masking the third codon positions in the coding regions. Using MEGA5 (Tamura *et al.*, 2011), phylogenetic trees were constructed by the Neighbor Joining method from p-distances based on all SNP sites in the core regions or only the non-synonymous SNPs. Bootstrapping was performed on 1000 replicates.

Analysis of nucleotide polymorphism within single phage strains

The genetic variation within a phage strain was measured by the number of SNPs found in the sequencing data from a clonal phage population. The SNPs were identified as sites in a strain's genome assembly that were covered by ≥30 reads with Phred quality score ≥30 and with ≥10% of these reads differing from the consensus sequence.

Analysis of metagenomic shotgun sequencing data of the skin microbiota

Phage diversity was analyzed in the metagenomic shotgun sequencing data from 27 retroauricular crease samples collected in the HMP (The Human Microbiome Project Consortium, 2012). The SRA accessions of these samples are SRS013261, SRS024598, SRS013258, SRS024596, SRS019016, SRS019015, SRS019033, SRS019063, SRS019064, SRS019081, SRS024655, SRS024620, SRS020263, SRS020261, SRS017851, SRS017849, SRS057083, SRS024482, SRS045606, SRS058221, SRS018978, SRS058182, SRS016944, SRS046688, SRS015381, SRS052988 and SRS019116. Access to the phenotype data for this study (phs000228.v3.p1) was obtained from dbGaP. MIRA (Chevreux *et al.*, 1999) was used to identify phage reads in each data set by mapping against the *P. acnes* phage PA6 genome. Parameters similar to the default were used: ≥20 nt overlap and ≥60% identity. To estimate the number of *P. acnes* phage strains in each data set, we first performed a *de novo* assembly using the extracted phage reads from the metagenomic data. We then aligned all the phage reads to the resulting contigs to identify SNPs in the core genome regions. The assemblies and alignments were manually inspected using Consed (Gordon *et al.*, 1998). The same criteria used for nucleotide polymorphism identification in single phage strains were applied as described above.

Analysis of phage genes under diversification

Multiple sequence alignments of Group VI and Group VIII phages and their related phages (PHL037M02 and PHL073M02) were generated using MAFFT (Kato *et al.*, 2002). The positions of

all mismatches and gaps were recorded. Sites of discrepancy were plotted in Artemis (Rutherford *et al.*, 2000).

Propionibacterium culture

P. acnes, *P. humerusii*, *P. granulosum* and *P. avidum* strains were cultured under anaerobic conditions in Clostridial Reinforced medium (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 4–6 days. *Propionibacterium* cultures were used to prepare top agar overlays for phage culture on A-media plates.

Phage resistance test

The resistance/susceptibility of *Propionibacterium* strains against phages was determined using a modified cross-streak assay. Fifteen of the 48 newly sequenced phages were randomly chosen for the analysis. Two sets of phages that each belongs to the same group, PHL010M04 and PHL066M04 in Group VIII and PHL115M02, PHL085N00, and PHL085M01 in Group VI, were included. The bacterial strains were streaked across in A-media plates, along with ATCC6919 on the same plate as a control. Approximately 5 µl of 10⁶ PFU ml⁻¹ phage suspension was spotted onto each bacterial streak. The plates were incubated at 37 °C anaerobically for 2 days. At least five replicates of each cross-streak experiment were performed to determine whether the strains were susceptible or resistant.

For the strains that showed resistance in the modified cross-streak experiment, we further quantitatively determined the resistance by assaying the efficiency of plaquing of the phages relative to *P. acnes* strain ATCC6919, calculated as the following:

$$\text{Resistance} = \frac{1}{\text{Efficiency of plaquing}} = \frac{\text{Titer of phage strain X on ATCC6919}}{\text{Titer of phage strain X on bacterial strain Y}}$$

We considered a 100-fold or greater increase in efficiency of plaquing to be evidence of resistance.

The plaques on cross-streak plates were visually inspected by one person and scored for turbidity based on the re-growth of the bacteria after plaque formation using the following scale: 0 = clear, 1 = little to no re-growth, 2 = mild re-growth, 3 = moderate re-growth and 4 = heavy re-growth. The average plaque turbidity score of all the strains from the same *P. acnes* clade was calculated for each of the tested phages and was compared among different clades.

Pseudolysogeny characterization

PCR was performed on phage suspensions using the primers annealing to the ends of the phage genomes (Forward 5'-CCGAAGCCGACCACATCACACC-3', Reverse 5'-TCATCCAACACCTGCTGCTGCC-3') to determine whether phage genomes are circularized.

The PCR was run under the following conditions: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 53 °C for 35 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min.

P. acnes ATCC6919 cultures, which were re-grown after lytic infection with specific phages, were tested for superinfection immunity by passaging sequentially two to four times without further phage infection. Phage resistance was assayed using the same cross-streak method described above. The presence of phage DNA in re-grown cultures was determined by PCR using the primers targeting the phage *gp11* gene (Forward 5'-GGCTGGAACACGTAAAGCG-3', Reverse 5'-CACGATCGATCAACTCAACC-3'). The PCR was run under the following conditions: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 95 °C for 45 s, annealing at 58 °C for 35 s and extension 72 °C for 1 min, with a final extension at 72 °C for 10 min.

CRISPR analysis

CRISPR spacer sequences were previously identified in *P. acnes* genomes (Fitz-Gibbon *et al.*, 2013; Tomida *et al.*, 2013). The spacer sequences were aligned against all phage genomes using BLASTn. Protospacers with up to two mismatches were identified.

Results

Phage isolation and genome features

In an effort to determine the diversity of the skin microbiota, we previously collected 203 skin samples from 179 individuals, including 94 samples from acne patients and 109 samples from healthy individuals with clear skin (Li, 2010; Fitz-Gibbon *et al.*, 2013). Twenty-four individuals were sampled twice over a 4–6 month period. When we cultured the skin samples for bacteria, we observed phage plaques in 49 of these samples: 14 from acne patients and 35 from healthy individuals. *P. acnes* phages were found more frequently in samples from healthy individuals than from acne patients. Our rate of phage detection (24% of investigated samples) is similar to those previously reported, ranging from 26 to 30% (Marples *et al.*, 1973; Puhvel and Amirian, 1979). The phages that we isolated have the morphology of siphoviruses as previously described (Farrar *et al.*, 2007). A representative electron micrograph is shown in Supplementary Figure S1. Among the phage isolates obtained from these samples, we selected 21 phages from acne patients and 27 from healthy individuals for whole genome sequencing using 454 or MiSeq platforms (Supplementary Table S1). In some samples, multiple phage plaques were isolated and selected for sequencing. All phage genomes were assembled, completed and annotated (Supplementary Figure S2). A representative phage genome is shown in Figure 1.

data set. Seven samples (25.9% of total samples) had a modest to high sequencing coverage of *P. acnes* phages ($17\times$ – $2060\times$) (Supplementary Table S3), which allowed metagenomic assembly. We performed the SNP analysis on the assembled phage genomes using the same criteria as in the analysis of complete phage genomes described above. To evaluate the effect of sequencing depth on the detection rate of SNPs, for samples with $>30\times$ phage coverage, we repeated the phage genome assembly and SNP analysis using only portions of phage reads at various coverages ($20\times$, $100\times$, $250\times$, $500\times$, $1000\times$ and $1500\times$), as applicable. One of the samples, HMP20, had >1000 SNPs in the core regions of the assembled phage genome, which leveled off to 1353 sites when all the sequence reads ($538\times$) were used in the assembly (Figure 3, Supplementary Table S4). This suggests that the *P. acnes* phage pool in HMP20 was adequately sampled and that it likely consists of two dominant phage strains based on phage genome comparison (Supplementary Materials).

On the other hand, three other samples, HMP04, HMP09 and HMP15, each had six or fewer SNPs in the core genome regions, despite having substantially higher sequencing coverages than HMP20. This suggests that these samples each harbor only one dominant *P. acnes* phage strain. This result supports our earlier conclusion that an individual skin microbiota is often dominated by one *P. acnes* phage strain. We were able to assemble high-quality draft genomes of *P. acnes* phages from these three metagenomic data sets, which cover 96.4–96.7% of the core genome regions. They are typical of the *P. acnes* phage strains, with high similarities to the 62 sequenced genomes. A phylogenetic tree including these three new genomes with the 62 isolated phage genomes is shown in Supplementary Figure S4. Samples HMP04 and HMP09 were collected from the left and right retroauricular crease of the same individual, and the phage genomes assembled from these two samples are highly similar, potentially originating from the same strain.

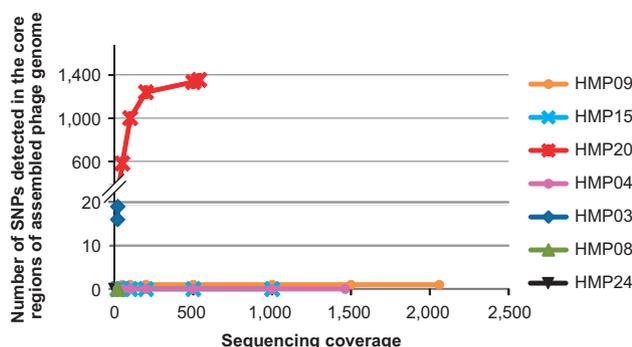


Figure 3 *P. acnes* phage population in the skin microbiota is often dominated by a single strain. The numbers of SNPs identified in the core regions of *P. acnes* phage genomes are shown at different sequencing coverages. The phage genomes were assembled from the HMP metagenomic shotgun sequencing data.

The assembled phage genomes in the remaining three samples, HMP03, HMP08 and HMP24, contained <20 SNPs, which are in the range of the SNPs found in a single phage strain. Due to their lower phage coverages, it is yet inconclusive whether single or multiple dominant phage strains were present in these communities.

Our analysis of the phage diversity from the HMP metagenomic data showed that among the skin communities that had detectable *P. acnes* phages, three harbored only one dominant strain while one harbored two different strains. These findings are consistent with our conclusions based on the isolated phages from our study cohort.

Phage genes under diversification

Phylogenetically related strains can reflect phage diversification under selection. Two phages, PHL037M02 and PHL073M02, are highly related to the members of Groups VI and VIII, respectively (Figure 2). However, they contain many more nucleotide variations than 11 bp, and thus are considered separate strains based on our criterion. Nonetheless, their high degree of similarity to the members of these groups may reflect recent selective pressures driving phage diversification. We identified 160 sites of nucleotide variations between PHL037M02 and the Group VI members, 50 of which are non-synonymous. All but one of these genetic differences is located in a region encoding Gp16, Gp17 and Gp18, as annotated in the genome of phage PA6 (Supplementary Figure S5A). The exact functions of these genes are unknown, but their location near the 3' end of the left arm between structural protein genes and lysis protein genes suggests that they could encode late-acting proteins. Based on the McDonald–Kreitman test (Egea *et al.*, 2008), gene *gp17* is under selection ($P=0.011$).

We identified 81 sites of nucleotide variations between PHL073M02 and the Group VIII members. The sequence differences lie primarily within the region encoding an endolysin and a putative type II holin (Gp20 and Gp21, Supplementary Figure S5B). These lytic cycle proteins permeabilize the cell membrane and degrade the extracellular peptidoglycan layer to release new phage particles from the bacterial host. As previously mentioned, PHL073M02 and two Group VIII members, PHL010M04 and PHL066M04, were isolated from three siblings living in the same household. Thus, they likely originated from the same ancestral phage strain. This suggests that the endolysin and holin genes, which are essential for phage multiplication, are under rapid evolution. Consistent with this finding, in all sequenced phages, we found frequent amino acid variations in these two gene products. This suggests that mechanisms determining host bacterium lysis specificity and kinetics may be under selection in these phages. The sequence variation sites in these two genes among the phages may be

potential targets for phage engineering to manipulate their lytic activities against bacterial hosts.

The two highly similar phage genomes assembled from the HMP samples, HMP04 and HMP09, which were collected from the same individual, differed by 222 nucleotides. Most of these variations are centered at the 5' end of the right arm of the genome, which encodes putative regulatory or DNA-binding proteins of largely unknown functions.

Range and specificity of Propionibacteria–phage interactions

To determine whether *P. acnes* phages modulate the relative abundances of different *P. acnes* strains in the skin microbiota by selective killing, we characterized the host range and specificity of *P. acnes* phages. We tested 15 of the 48 sequenced phages against a panel of 74 *Propionibacterium* strains, including 67 *P. acnes* strains, 3 *P. humerusii* strains, 1 *P. granulosum* strain and 3 *P. avidum* strains. Except for the *P. acnes* strains KPA171202, ATCC11828, HL201PA1 and HL202PA1, all of these *Propionibacterium* strains were isolated from the same cohort of subjects sampled for phages. The genomes of all 67 *P. acnes* strains and 3 *P. humerusii* strains have been sequenced (Fitz-Gibbon *et al.*, 2013; Tomida *et al.*, 2013). Our bacterial collection included all major lineages of *P. acnes* found on the human skin, with multiple strains representing each of the major clades, IA-1, IA-2, IB-1, IB-2, IB-3 and II, as well as one type III strain. We constructed a phylogenetic tree of the 67 *P. acnes* strains based on the SNPs in their core genomic regions (Figure 4a) (Tomida *et al.*, 2013). Using a modified cross-streak method, we determined the resistance/susceptibility of each of the 74 bacterial strains against the 15 phages. In total, 1110 bacterium–phage interactions were measured. Each experiment was repeated a minimum of five times. For the bacterial strains that showed resistance to phages, we determined the fold increase in resistance by measuring efficiency of plaquing (EOP) relative to the *P. acnes* strain ATCC6919, which is known to be susceptible to all tested phages.

We found that the outcome of the bacterium–phage interactions is *P. acnes* lineage dependent. All type I *P. acnes* strains (clades IA-1, IA-2, IB-1 and IB-2) except clade IB-3 were susceptible to all tested phages (Figure 4a). Among them, the phages often formed turbid plaques on *P. acnes* strains of clade IA-1, but clear plaques on strains of clades IB-1 and IB-2, as summarized in Figure 5. This suggests that these phages engage in two different states depending on the host strains: a pseudolysogenic response in clade IA-1 strains, and a lytic cycle in clade IB-1 and IB-2 strains.

Certain *P. acnes* strains of clades IB-3, II and III are highly resistant to multiple phages. Two strains of clade IB-3 (KPA171202 and HL030PA1) were highly resistant to most of the tested phages with a ≥ 100 -fold increase in resistance. The genomes of clade

IB-3 show that these strains encode components of a restriction modification system (genes PPA1611 and PPA1612 in KPA171202). This may explain their resistance to phages. Among the nine type II strains, two strains, HL001PA1 and HL042PA3, were highly resistant to some of the phages. This is consistent with previous observations that strains of this type were more frequently resistant to phages (Webster and Cummins, 1978). The resistance to phages observed in type II strains could be partially attributed to the CRISPR mechanism encoded in their genomes, which is addressed below. The only type III strain tested, HL201PA1, was resistant to all 15 phages. Since type III strains are not commonly found on the skin of the face, it is possible that these *P. acnes* phages isolated from the face have not yet evolved a mechanism to infect type III strains.

To determine whether *P. acnes* phages modulate the abundance and diversity of other species in addition to *P. acnes* in the skin microbiota, we investigated the host range of *P. acnes* phages in related species. Strains of other human skin-associated *Propionibacteria*, including 3 strains of *P. humerusii*, 1 strain of *P. granulosum* and 3 strains of *P. avidum*, were tested against the 15 phages (Figure 4b). *P. humerusii* is a newly defined species (Butler-Wu *et al.*, 2011). In our previous study, *P. humerusii* was one of the major species found on the skin with a relative abundance of 1.9% in the pilosebaceous unit based on 16S ribosomal RNA analysis (Fitz-Gibbon *et al.*, 2013). It is closely related to *P. acnes* with $>98\%$ identity in the 16S ribosomal RNA gene sequence. *P. granulosum* and *P. avidum* are common skin commensals (Cummins, 1976; Ördögh and Hunyadkürti, 2013). While all tested *P. granulosum* and *P. avidum* strains showed strong resistance to all the phages, two *P. humerusii* strains, HL037PA2 and HL037PA3, were susceptible to all the phages tested. The third *P. humerusii* strain, HL044PA1, was susceptible to 10 of the 15 phages tested. Our results show that the host range of *P. acnes* phages is not limited to *P. acnes* but also includes a closely related *Propionibacterium* species, suggesting that *P. acnes* phages may also be able to modulate *P. humerusii* populations in the skin microbiota.

P. acnes phages can adopt a pseudolysogenic state depending on the host *P. acnes* strains

It has been suggested that *P. acnes* phages may enter pseudolysogeny as an alternative to the lytic cycle (Farrar *et al.*, 2007; Lood and Collin, 2011). As described above, we discovered that *P. acnes* phages often adopt a pseudolysogenic state in clade IA-1 strains, but rarely in clade IB-1 and IB-2 strains, suggesting that their pseudolysogeny is dependent on the host *P. acnes* strains (Figure 5). In support of the existence of pseudolysogeny in *P. acnes* phages and consistent with the result by Marinelli *et al.*, (2012), our genome sequencing data revealed

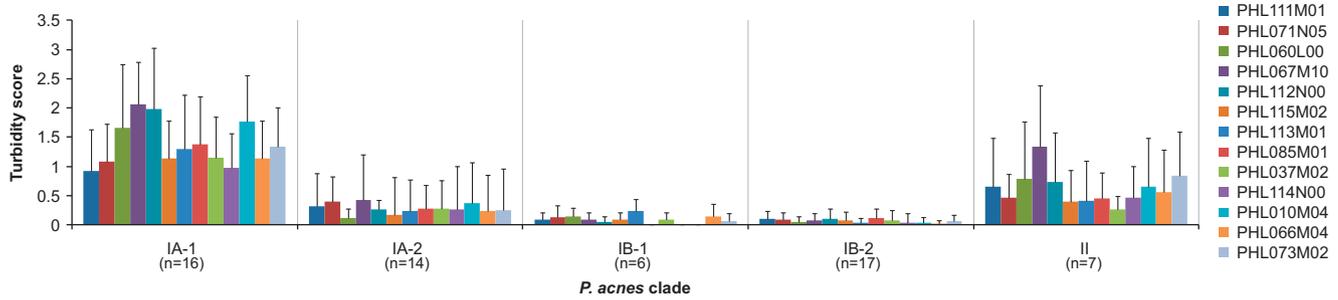


Figure 5 The frequency of phage pseudolysogeny varies among different *P. acnes* strains. The turbidity of the phage plaques formed on *P. acnes* culture plates was examined. Data from 60 *P. acnes* strains challenged by 13 phages were recorded and summarized. The 60 *P. acnes* strains belong to five clades: IA-1 ($n=16$), IA-2 ($n=14$), IB-1 ($n=6$), IB-2 ($n=17$) and II ($n=7$). The average turbidity score of the bacterial strains in each clade challenged by each phage (in colors) is shown. Independent of the phages tested, the plaque turbidity score varied among different *P. acnes* strains: it is high in clade IA-1 and low in clades IB-1 and IB-2. This suggests that the phages tend to enter a pseudolysogenic state in clade IA-1 strains and a lytic cycle in clades IB-1 and IB-2 strains.

the ends of the phage genomes to be flanked by 11-nucleotide single-stranded overhangs. Previous reports suggested that these overhangs may be involved in the circularization of the phage genomes (Farrar *et al.*, 2007; Lood and Collin, 2011). To test this hypothesis, we designed PCR primers annealing to the ends of the phage genomes. A PCR product spanning the two ends with the predicted size (~735 bp) and sequence was amplified from all the phages tested, suggesting that the phage DNA can exist in a circular form mediated by the overhangs at the ends (Supplementary Figure S6A).

To demonstrate the pseudolysogenic properties of *P. acnes* phages, we infected *P. acnes* strain ATCC6919, a clade IA-1 strain (Liu *et al.*, 2014), with five different phages (PHL060L00, PHL112N00, PHL037M02, PHL073M02 and PHL114L00). Following the formation of plaques by each phage, re-growth of the bacteria was observed starting in the center of the plaque regions. The re-grown bacteria showed no evident lysis when challenged by 13 phages that previously could lyse this *P. acnes* strain, including the phages of the initial exposure (Supplementary Figure S6B). This suggests that the bacterial host gained superinfection immunity after infection by these phages.

To further determine whether the phage DNA exists as an episome in the bacterial host after infection, we tested the presence of phage DNA in four distinct colonies of the re-grown ATCC6919 culture that was initially infected with PHL060L00 and subsequently passaged. Two of the four colonies produced an expected 437 bp amplicon in a PCR targeting the *gp11* gene (Supplementary Figure S6C), supporting the presence of phage in a subpopulation of the re-grown *P. acnes* as an episome. In addition, we sequenced the genomic DNA extracted from two ATCC6919 cultures, each of which was passaged from a re-grown culture after phage infection. In both cases, the phage reads obtained from whole genome sequencing were assembled into complete genomes that are separate from host *P. acnes* contigs, supporting earlier evidence that *P. acnes* phage DNA does not integrate into the host genome

(Farrar *et al.*, 2007; Lood and Collin, 2011), but exists as an extrachromosomal element.

In summary, all above results suggest that *P. acnes* phages can adopt a pseudolysogenic state in clade IA-1 strains.

Resistance to bacteriophages does not correlate with the presence of matching CRISPR spacers in type II P. acnes strains

Since certain type II *P. acnes* strains are resistant to phages, we next investigated whether the CRISPR/Cas mechanism could explain the resistance of the type II strains against phages. Among the 67 *P. acnes* strains, 9 belong to type II and encode CRISPR/Cas elements. Each of the type II strains has one to nine 33-bp spacers in their CRISPR arrays (Tomida *et al.*, 2013). In total, they encode 36 spacers, 20 of which are unique. We identified 34 unique protospacers in the 15 tested phage genomes that match any of the 20 unique spacer sequences in the 9 type II *P. acnes* strains. Because the CRISPR/Cas system has been shown to tolerate a limited number of mutations in protospacer targets (Semenova *et al.*, 2011; Manica *et al.*, 2013), in our analysis we allowed up to two mismatches for a sequence to be considered a recognizable protospacer. Similar results were obtained when only perfectly matching protospacers were considered. We found that all identified protospacers are located primarily on the left arm of the phage genomes, which is more conserved than the right arm (Supplementary Figure S7). In addition, the locations of the protospacers are generally conserved among all phage genomes that harbor the same protospacer sequences. These suggest that the CRISPR/Cas system tends to target the more conserved regions of the phage genomes.

In contrast to a prior report (Marinelli *et al.*, 2012), we found that the resistance/susceptibility of the nine type II *P. acnes* strains against phages did not correlate with the presence/absence of at least one phage-matching CRISPR spacer ($r=0.39$, Figure 6). There were multiple observations that even though the strain encodes a matching CRISPR spacer, it was

susceptible to the corresponding phage. To determine whether more than one matching spacer is needed to confer phage resistance, we analyzed the correlation between the resistance/susceptibility and the number of matching spacers encoded in the CRISPR array. No strong correlation ($r=0.16$) was found, suggesting that encoding multiple matching spacers does not necessarily protect the strain from phage infection. In addition, no presence of particular spacer sequences can explain the resistance/susceptibility to phages. When we allowed no mismatches or up to five mismatches for a sequence to be considered a recognizable protospacer, the correlations were not much improved. This suggests that having matching CRISPR spacers is insufficient for these strains to be resistant to phages and that other regulation mechanisms are likely to be involved.

Phages can escape the CRISPR/Cas mechanism through mutations at sites involved in protospacer recognition. The short nucleotide motif adjacent to the protospacer, known as the protospacer-adjacent motif (PAM), is highly conserved among targets of CRISPR/Cas systems (Mojica *et al.*, 2009). Mutations in these nucleotides have been found to disrupt CRISPR-mediated resistance despite complete identity in the protospacer sequence (Semenova *et al.*, 2011, 2009; Westra *et al.*, 2012).

To determine whether the lack of correlation between bacterial resistance and the presence of matching spacer sequences is due to mutations in the PAM sequence, we examined the PAM regions of the 137 protospacers identified in the phages from this study and from the study by Marinelli *et al.*, (2012). The protospacers can be separated into two groups depending on the outcomes of the bacterium–phage interaction—resistant or susceptible to the phages (Figure 7). Fifty-three protospacers belong to the first

group, where the bacterial hosts with matching spacer sequences were resistant to the phages. The other 84 protospacers belong to the second group, where lysis of the bacterial hosts occurred despite the presence of a matching spacer. Among the first group of protospacers, we observed a conserved ‘AA’ motif immediately upstream of the protospacer sequence (Figure 7a). The AA motif is also conserved in the PAM sequences of the 84 protospacers from the second group where the bacterial hosts were susceptible to the phages, except in 3 cases, where an A-to-G mutation occurred. These three mutated positions were found in phages PHL060L00 and PHL112N00, which are targeted by spacers encoded in *P. acnes* strain HL042PA3, and in phage PHL113M01, which is targeted by a spacer encoded in strain HL110PA3 (Figure 7b). However, these mutations in the PAM sequence cannot explain the lack of resistance against these phages, as all three phages contain two to four additional protospacers that are recognizable by the spacers encoded in the corresponding bacterial strains. All of these additional protospacers have intact PAMs. Our findings are corroborated by the previous observation of unexplained susceptibility to *P. acnes* phage despite the presence of matching spacers (Marinelli *et al.*, 2012). In summary, our data demonstrate that encoding CRISPR spacers that match against the genome of an invading *P. acnes* phage is often insufficient for an effective defense in *P. acnes* and that other regulation mechanisms are likely to be involved.

Discussion

The skin microbial community has an essential role in defending against the invasion and colonization of pathogens. Although the bacterial and fungal

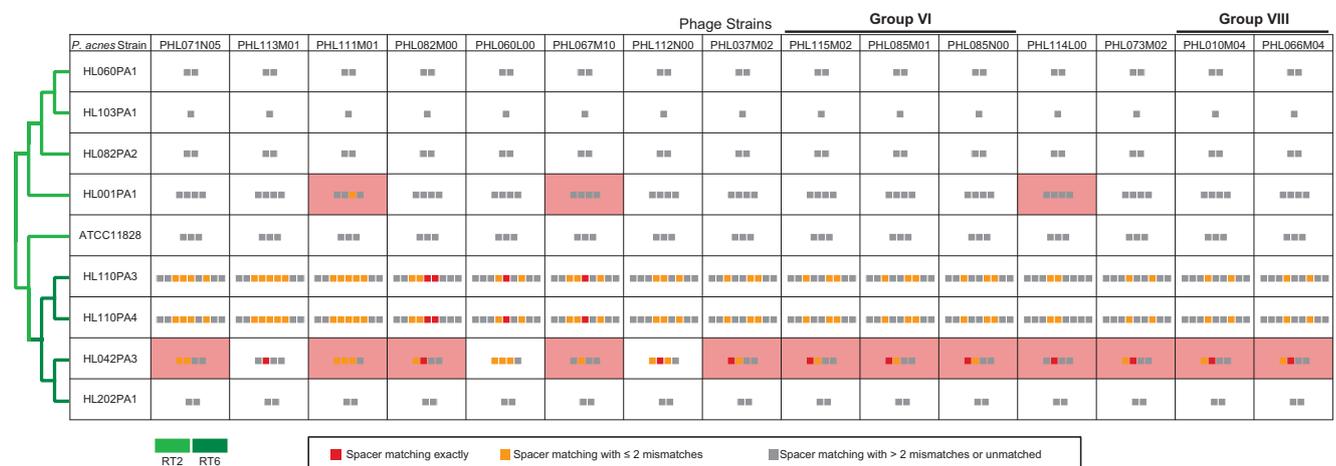


Figure 6 Phage resistance is not correlated with the presence of matching CRISPR spacers in type II *P. acnes* strains. The pattern of resistance/susceptibility of the 9 type II *P. acnes* strains against 15 phages did not correlate with the presence/absence of matching CRISPR spacers, that is, the pink cells do not perfectly overlap with the cells containing red or orange pixels. *P. acnes* type II strains are shown in rows and the phages are shown in columns. Pink cells indicate that the bacterial strains are resistant to the corresponding phages. The colored pixels in each cell represent the CRISPR spacers encoded in each *P. acnes* strain. Each red pixel indicates that this spacer has an exact protospacer match in the corresponding phage. Each orange pixel indicates that this spacer has a partially matched protospacer (with one to two mismatches) in the corresponding phage. Gray pixels indicate no matched protospacers found in the corresponding phage.

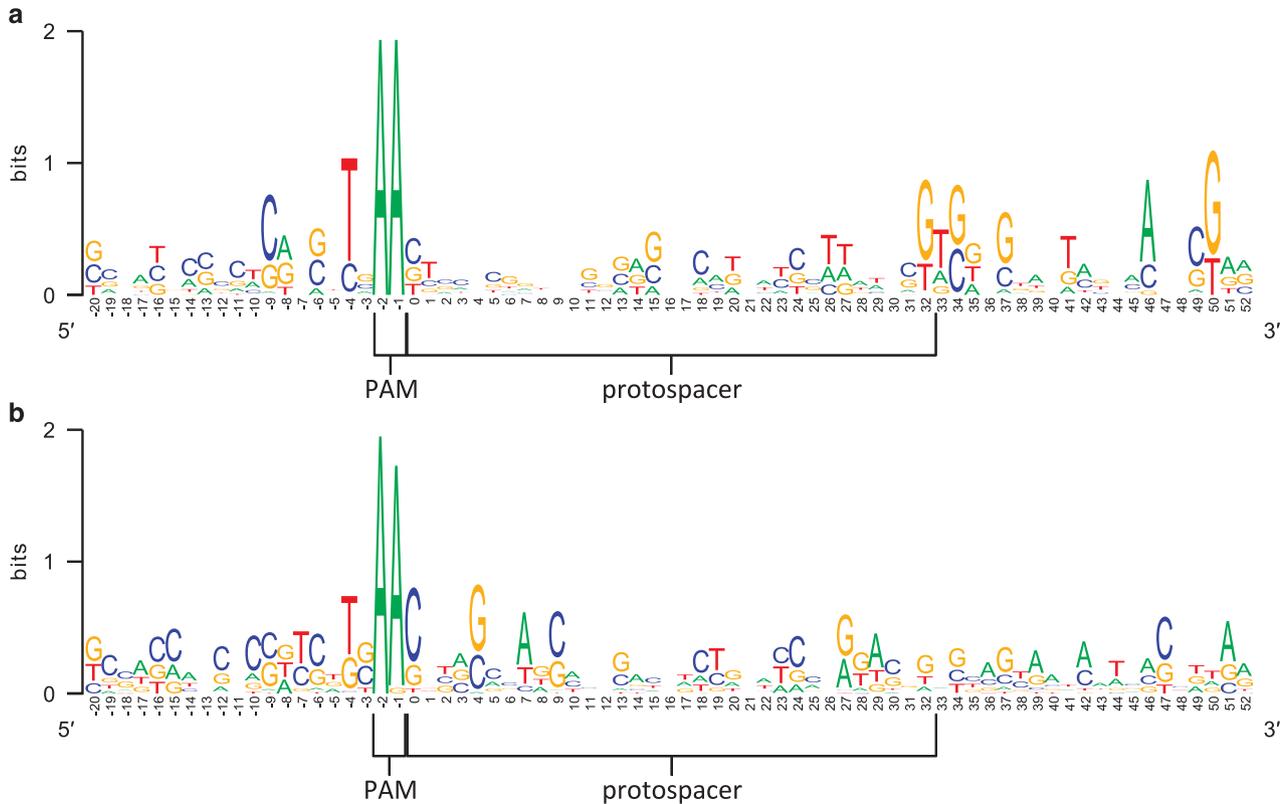


Figure 7 Sequence conservation in protospacers and PAMs. A logo representation of the protospacer sequences that match *P. acnes* CRISPR spacers (within two mismatches) is shown. The PAM sequence of the protospacers is also shown. (a) Sequence conservation among the protospacer motifs from the phages unable to lyse the corresponding bacterial strains. (b) Sequence conservation among the protospacer motifs from the phages able to lyse the corresponding bacterial strains. The PAM sequences are conserved in both cases.

compositions of human skin have been characterized at multiple sites in healthy individuals (The Human Microbiome Project Consortium, 2012; Gao *et al.*, 2007; Paulino *et al.*, 2008; Grice *et al.*, 2009; Gao *et al.*, 2010; Ursell *et al.*, 2012; Blaser *et al.*, 2013; Findley *et al.*, 2013; Fitz-Gibbon *et al.*, 2013; Nakatsuji *et al.*, 2013), we have limited understanding of the skin virome (Foulongne *et al.*, 2012; Ma *et al.*, 2014). Whether bacteriophages have a role in skin health and disease by modulating the diversity and dynamics of the bacterial community on the skin is poorly understood.

The lack of knowledge of the skin virome is partly due to the limited microbial biomass of skin samples, which poses challenges in determining the composition and diversity of skin viruses. As methods are being developed in recent years for high-throughput sequencing from a limited amount of DNA (Duhaime and Sullivan, 2012; Duhaime *et al.*, 2012; Solonenko *et al.*, 2013) and for capturing metagenomic viral sequences in metagenomic sequencing data (DeLong *et al.*, 2006; Mizuno *et al.*, 2013), it is becoming feasible to study lowly abundant viral communities directly from metagenomic samples without enriching the viral portion. In this study, using genome sequencing and metagenomic analysis of the skin microbiome, we characterized a large number of *P. acnes* bacteriophages and their genome

diversity in the skin of healthy individuals and acne patients. This effort significantly increased our knowledge about this viral population and allowed us to investigate its population diversity in the human skin microbiota.

Unlike phages of other species (Nolan *et al.*, 2006; Dorscht *et al.*, 2009; Sepúlveda-Robles *et al.*, 2012), *P. acnes* phages show a high genetic similarity. The phages may have evolved recently under limited selection, as no clear lineages can be found among the phages studied, suggesting that these phages may be relatively new to their hosts (Marinelli *et al.*, 2012). Alternatively, the high similarity among the phages may be attributed to the low genetic diversity of their host strains, as *P. acnes* has limited genome expansion among different lineages (Tomida *et al.*, 2013).

By comparing the phages isolated from different individuals (Figure 2), we found that in individuals harboring *P. acnes* phages, the skin community is often dominated by one *P. acnes* phage strain. Our metagenomic data analysis of the skin samples from the HMP cohort confirmed this finding (Figure 3), despite the differences between the two analyses in methodology, study cohorts and skin sites that were sampled. We also observed the same phages in the skin microbiota when the individuals were sampled 14–21 weeks later (Supplementary Table S2). Our

findings are consistent with the intra-individual temporal stability found in the human oral and gut viromes (Pride *et al.*, 2012; Minot *et al.*, 2013). Further studies at multiple time points are needed to determine the dynamics of *P. acnes* phage populations in the skin microbiota. In addition, we found that while many individuals harbor unique phage strains (Figure 2), some individuals share the same phage strains in the skin microbiota, suggesting that a pool of common phages exists among the human population. Lastly, we isolated identical phage strains from siblings (Supplementary Table S2), suggesting possible transmission of skin viruses between related individuals.

Pseudolysogeny occurs abundantly in natural environments and is an important part of bacteria–phage interactions (Ripp and Miller, 1997). It has been suggested to influence phage survival (Ripp and Miller, 1997), phage-dependent bacterial mortality (Ripp and Miller, 1998) and the virulence of certain bacterial strains (Sakaguchi *et al.*, 2005). Although it has been observed that *P. acnes* phages can adopt pseudolysogeny (Lood and Collin, 2011), for the first time we revealed that this behavior is largely dependent on the lineages of the host strains and is not a property of the phages. All tested *P. acnes* phages more frequently become pseudolysogenic when the host *P. acnes* strains are from clade IA-1, compared with other lineages (Figure 5). We further determined several pseudolysogenic characteristics of *P. acnes* phage infection (Supplementary Figure S6). These include the ability of the phage genome to exist as a circular episome in the host cells, superinfection immunity of the host bacteria gained from prior phage infection and phage DNA persistence in a subpopulation of the host cells following infection. While the term ‘pseudolysogeny’ is used here and in the previous studies (Lood and Collin, 2011; Marinelli *et al.*, 2012) to describe this behavior of *P. acnes* phages, further mechanistic definitions are needed to determine whether this infection state represents phages undergoing extra-chromosomal lysogeny, inefficient lysis or other potential mechanisms.

We found that although *P. acnes* phages can lyse *P. acnes* strains from most lineages, certain strains from clades IB-3, II and III are resistant (Figure 4a). This bacterial lineage-dependent resistance to phages suggests that antiviral mechanisms may have a role in defining the *P. acnes* strain populations in the presence of phages. Based on the genomes of *Propionibacterium* strains, we identified two antiviral mechanisms that may explain phage resistance in different *P. acnes* lineages, restriction modification and CRISPRs. Both of these systems act against phage DNA that has successfully entered the host. It is possible that these *P. acnes* lineages employ additional mechanisms that act at other stages of infection, such as receptor binding or DNA injection, which are commonly responsible for phage resistance in mycobacteria (Jacobs-Sera *et al.*, 2012).

We observed that phage DNA recognition by *P. acnes* CRISPRs often did not protect the tested bacterial strains from phage lysis (Figure 6). This result contrasts with a previous study reporting a correlation between matching spacers and phage resistance (Marinelli *et al.*, 2012). While this earlier study similarly reported 1 unexplained lytic outcome out of 20 tested interactions, where 2 matched spacers failed to confer phage resistance, in our study we observed a much higher proportion of lytic outcomes, 15 out of 25 non-redundant interactions. The contrasting conclusions from the two studies may be attributed to the differences in the bacterial host strains and phage strains tested. To demonstrate the robustness of our results, we included redundant bacterial host strains and phage strains in our experiments. Both RT2 and RT6 strains, which belong to two different lineages of *P. acnes* that encode CRISPR/Cas systems, and several members of the same phage strains were tested in the analysis and yielded consistent results.

Several possible reasons may explain the low correlation between spacer matching and phage resistance. The CRISPR/Cas system in *P. acnes* may be used for purposes other than defense against foreign DNA, as suggested for the Type I-E CRISPR/Cas system of *Escherichia coli* (Touchon and Rocha, 2010; Touchon *et al.*, 2011). Alternatively, other molecular regulations employed by bacterial host or phage may be important for CRISPR-mediated resistance and may contribute to the infection outcome. Such regulation mechanisms potentially include transcriptional and/or translational regulation of CRISPR RNA and Cas genes and anti-CRISPR genes encoded by phages (Bondy-Denomy *et al.*, 2013; Pawluk *et al.*, 2014). Interactions between *P. acnes* and phages may also depend on additional mechanisms involved in phage binding, entry, replication or release.

To our knowledge, this study is the first large-scale investigation of bacterium–phage interaction in the human skin microbiota using both genetically defined bacterial strains and phage strains. Our study suggests that the prey–predator relationship between *P. acnes* and its phages may have a role in modulating the composition of the skin microbiota. The finding that the infection outcome of *P. acnes* phages varies depending on *P. acnes* lineages suggests that the presence of phages may alter the *P. acnes* population structure by targeting particular subsets of strains. Based on evidence of different propensities among *P. acnes* lineages to be lysed, to resist or to sustain a pseudolysogenic infection, it is plausible that in the presence of phages various *P. acnes* strains may populate at different rates in the community. On the other hand, the *Propionibacterium* strain composition may determine the abundance and dynamics of the phages in the community. Phage bursts may occur in specific skin community structures. Future studies utilizing recently developed techniques, such as microfluidic digital PCR (Tadmor *et al.*, 2011), phageFISH (Allers *et al.*, 2013) and viral

tagging (Deng *et al.*, 2012), will provide further insight on the diversity and dynamics of the phage populations in the human microbiome and the prey–predator relationship between the bacterial hosts and phages.

P. acnes phages may hold therapeutic potential for modifying the skin microbiota based on the individual bacterial community structure to restore a healthy microbial composition. We previously demonstrated that *P. acnes* strain population structure differed significantly between acne patients and healthy individuals (Fitz-Gibbon *et al.*, 2013). Certain strains were highly associated with the disease, while some strains were highly associated with healthy skin. The interactions between genetically defined *P. acnes* strains and phages that we characterized in this study may be able to guide the selection of phage strains to inhibit specific *P. acnes* population types associated with acne or other skin diseases. For example, phage therapy may not be effective if the host microbiota contains phage-resistant pathogenic strains. Potential phage-based therapy should consider the microbiome structure of the individual to be effective and personalized. The host strain specificity of *P. acnes* phages may prove advantageous for the selective suppression of pathogenic *P. acnes* strains and preservation of commensal or beneficial strains in the skin microbial community.

Conflict of Interest

The Regents of the University of California is the owner of a patent application based on some of the results presented, which names ST and HL as inventors.

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Author contributions

JL and ST analyzed the data. RY, QZ, SN, NJB, TL and LN performed the experiments. ML helped perform the experiments. MCE collected the clinical samples. NC maintained the IRB protocol and collected the clinical samples. HL conceived and directed the project and analyzed the data. JL and HL wrote the paper.

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