PULMONOLOGY

Microbiome Diversity and Biofilm Formation in Acne Vulgaris: A Comparative Study of Non-Inflammatory and Inflammatory Lesions

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ABSTRACT

Background: The ability of *C. acnes* strains to form biofilms has been correlated with their virulence.

Objective: This study examined biofilm and skin microbiota in acne patients in order to understand their role in the development of acne lesions.

Methods: Thin sections of punch biopsy specimens of (1) uninflamed comedones, (2) inflammatory lesions, and (3) uninvolved adjacent skin of acne patients were examined. Epiflourescence and confocal laser scanning microscopy were used for biofilm detection, and pyrosequencing with taxonomic classification of 16s rRNA gene amplicons was used for microbiota analysis.

Results: Of the 39 skin specimens from patients with mild-moderate acne (n=13) that were studied, 9 (23%) contained biofilm. Among these specimens, biofilm was most frequently detected in comedones (55.6%) and less frequently in inflammatory papules (22.2%) and uninvolved skin (22.2%). Comedones demonstrated the highest mean alpha diversity of all the lesion subtypes. The relative abundance of *Staphylococcus* was significantly higher in comedones (11.400% ±12.242%) compared to uninvolved skin (0.073% ±0.185%, p=0.024).

Conclusions: The microenvironment of the comedone differs from that of inflammatory lesions and unaffected skin. The increased frequency of biofilm in comedones may account for the lack of host inflammatory response to these lesions.

MESH KEY WORDS

- Acne vulgaris
- Biofilm
- Biofilms
- Microbiome
- Microbiota

INTRODUCTION

Acne pathogenesis has historically been attributed to the overgrowth of *Cutibacterium acnes* in areas of increased sebum production, leading to inflammation and follicular occlusion.[1] Recent studies of the microbiome have challenged the role of *C. acnes* in skin health and disease.[2] Sequencing studies of the skin microbiome in healthy participants have found that, while considerable interpersonal variability exists, microbial community composition is primarily determined by body site, with sebaceous regions being dominated by *Propionibacterium*, now *Cutibacterium*, species.[3-4] C. acnes is the dominant bacterial species in the pilosebaceous unit both in acne patients and controls.[5]

C. acnes acts as a major commensal in sebaceous regions, breaking down sebum into antimicrobial free fatty acids that protect the skin against pathogenic colonization.[6] Specific lineages of *C. acnes* are associated with skin health, while others have been implicated as etiologic agents of acne.[7-8] This finding may in part explain why, although *C. acnes* is a ubiquitous skin commensal, only some individuals will develop acne. It remains unclear, however, why in individuals with acne, a given follicle develops an acne lesion while another does not.

The skin microbiome is composed of bacteria existing in two states—a free-floating planktonic state and a biofilm state.[9] Biofilms are complex sessile communities, often polymicrobial, that are enveloped in a self-secreted extracellular polysaccharide matrix.[9] The biofilm matrix promotes optimal nutrient use and protects microbes from host defenses and antibiotics.[10] Within this protective biofilm matrix, *C. acnes* cells are more resistant to harsh environments than they are as free-floating planktonic *C. acnes* cells.[11]

Matrix-encased macro-colonies of *C. acnes*, which may represent biofilms, have been identified with greater frequency in sebaceous follicles of acne patients compared to acne-free controls.[12-13] A recent study identified lipase, a *C. acnes* virulence factor detected in biofilm, within subclinical microcomedones.[14] An adhesive biofilm of *C. acnes* may therefore promote the keratinocyte retention and accumulation that precipitates microcomedone formation, leading to an inflammatory response.[15]

We hypothesize that the ability of *C. acnes* to form biofilms in the pilosebaceous unit facilitates the development and progression of acne lesions. In this study, we identify and characterize biofilm of individuals with acne, comparing unaffected skin, uninflamed comedones, and inflammatory lesions.

METHODS

Patient Population

In this small prospective study, participants with mixed comedonal and inflammatory acne were screened and enrolled at the Johns Hopkins Outpatient Center in Baltimore, MD. We enrolled 14 participants between the ages of 18 and 40 years old with a current diagnosis of facial or truncal acne vulgaris and with both comedonal and inflammatory lesions. To avoid confounders, subjects agreed to comply with an acne medication washout period of 4 weeks prior to the study visit for systemic medications and 2 weeks for topical medications. Subjects who were unable to provide informed consent, who had a history of keloids, or who had a bleeding disorder were excluded. Written informed consent was obtained, and the study was conducted in accordance with good clinical practice and approved by the Johns Hopkins Medicine Institutional Review Board.

Sample Collection

Location, description, severity of acne (Leeds Revised Acne Grading system),[16] and biopsy sites were recorded at baseline. Three punch biopsies (3 mm) were obtained from each participant

using standard technique. The biopsy sites included 1 uninflamed comedone, 1 inflammatory papule, and 1 area of adjacent uninvolved skin per participant (Figure 1). Specimens were taken from the head/neck region in 8 subjects and from the trunk in 6 subjects, according to the area of involvement. Of the 14 total uninflamed comedone specimens, these included 13 closed comedones and 1 open comedone.

Laboratory Procedures

Epifluorescence and confocal laser scanning microscopy. Biopsy specimens were fixed in 4% paraformaldehyde (PFA) and transferred to a 30% sucrose solution in phosphate-buffered saline (PBS) prior to shipment to the Center for Biofilm Engineering. Specimens were embedded in optimum cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) and frozen on dry ice. The specimens were then cut in half length-wise and thin sections (5 µm) were cut at -20 °C using a Leica CM1850 cryostat. The sections were placed on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and stained with ViaGram™ Red+ Bacterial Gram-Stain and Viability Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Six sections of each specimen were examined using Eclipse E-800 epifluorescence microscope (Nikon, Melville, NY). Representative images of the biofilms were collected using a CoolSNAP EZ cooled CCD camera (Photometrics, Tucson, AZ) and processed using MetaVue software (Molecular Devices, Sunnyvale, CA).

Epifluorescence imaging was done using a Nikon Eclipse E-800 microscope with a Photometrics MYO cooled CCD camera. The microscope was equipped with FITC (green) and CY3 (red) filter cubes. Universal Imaging Corporation's MetaVue software was used for digital image acquisition. Confocal laser scanning microscopy was done using Leica SP5 upright confocal

laser scanning microscope with excitation using the 488 and 561 nm lasers and detection at 500-550 nm (green) and 600-650 nm (red).

A 6-item scoring system was used to classify the presence of bacteria/biofilm in specimens as previously described: 0=no bacteria observed; 1=small individual cells; 2=small micro-colonies of approximately 10 cells; 3=large micro-colonies of approximately 100 cells; 4=continuous film; and 5=thick continuous film.[17] We only considered a specimen to be positive if biofilm was identified within the follicular shaft, not merely within the stratum corneum.

Pyrosequencing and taxonomic classification of 16S rRNA gene amplicons. Total genomic DNA was purified using the ZYMO Fecal DNA Kit from Zymogen. Polymerase chain reaction (PCR) amplification of the V1–V2 hypervariable regions of the bacterial 16S rRNA gene was performed, using the universal primers 27F and 338R. Negative controls without a template were included for each bar-coded primer pair. Purified amplicon mixtures were sequenced by 454 FLX pyrosequencing using 454 Life Sciences primer A by the Genomics Resource Center at the Institute for Genome Sciences, University of Maryland School of Medicine, using protocols recommended by the manufacturer as amended by the Center.[18] To pass, a sequence read (1) included a perfect match to the sequence tag (barcode) and the 16S rRNA gene primer; (2) was at least 200 bp in length; (3) had no more than two undetermined bases; and (4) had a least 60% match to a previously determined 16S rRNA gene sequence. On average, 4.8% of the sequence reads did not pass this quality control step. Each processed 16S rRNA gene sequence was then classified at the level of the species using the QIIME bioinformatics software package and the Ribosomal Database Project (RDP) Naïve Bayesian Classifier using the recommended quality score filtering of 0.5.[19-20]

Microbiome data analysis. Alpha diversity, which measures microbial diversity within a given sample, was calculated using the Shannon diversity metric. The Shannon diversity metric takes into account both the richness (number) and evenness (distribution) of each bacterial taxon in a sample. Differences in the mean alpha diversity between sample groups were assessed with analysis of variance (ANOVA) using Tukey's honest significance difference (HSD) post-hoc test. Beta diversity evaluates microbial diversity between sample groups. Principal coordinates analysis (PCoA) of weighted and unweighted UniFrac similarity measures was calculated, and significance was determined using analysis of similarity (ANOSIM). Relative abundance of bacterial taxa was calculated for each sample from phylum to species levels. The significance of differences in relative abundance was evaluated with independent samples t-tests using SPSS-25 software (IBM Corp, Armonk, NY). For all comparisons, $p<0.05$ was considered statistically significant.

RESULTS

Study Population

The study population included 14 individuals with mild-to-moderate acne (mean Leeds RAGS 2.23): 8 men and 6 women; 6 Caucasian, 6 African American, and 2 Asian; ages 18-40. Prior to enrollment, 57.1% of participants reported having ever used acne medications. Of those individuals, 28.6% had used oral antibiotics, 28.6% had used topical retinoids, and 57.1% had used over-the-counter treatments.

Of the 14 participants initially enrolled, specimens from 1 participant were excluded due to sample processing error. The 39 specimens from the remaining 13 participants were examined for the presence of biofilm. 30 specimens from 10 of these participants next underwent pyrosequencing for microbiome analysis. Samples from 3 participants were completely cut through for quality assessment during the preparation for microscopy and did not undergo pyrosequencing.

Detection of Biofilm

Biofilm, defined as a score of 3 (>100 bacterial cells) or greater, was present in 9 (23%) of the 39 skin specimens studied. The remaining skin specimens either displayed no organisms (72%) or small micro-colonies (5%). Of the 9 samples containing biofilm, 7 (77.8%) were taken from acne lesions, and the remaining 2 (22.2%) were from unaffected skin. Of the 7 biofilm-containing acne lesions, the majority were taken from comedones (5/7, 71.4%), followed by inflammatory papules (2/7, 28.6%).

No statistically significant differences in biofilm presence were observed between normal and lesional skin or comedonal and inflammatory lesions. Figure 2 demonstrates representative epifluorescence and confocal laser scanning (CLS) micrographs of both a comedone and inflammatory papule that received scores of 4 and 5, respectively. The biofilm scores and characteristics of each sample can be found in the Supplemental Materials.

Microbial Diversity

Of the 30 specimens that underwent pyrosequencing, 2 specimens from 2 different participants (1 normal skin and 1 inflammatory papule) were expended during protocol optimization, leading to 8 complete sets of 3 samples for comparison. The mean microbial alpha diversity did not vary to a statistically significant degree between sample groups (Figure 3). However, the mean alpha diversity of comedone samples (2.360 ±0.752) was higher than that of inflammatory lesion samples (1.988 ±0.724, p=0.545) and unaffected skin samples (1.889 ±0.597, p=0.411).

Beta diversity was evaluated using principal coordinates analysis (PCoA) plots of weighted and unweighted UniFrac similarity measures. We did not observe any significant clustering of samples on the PCoA plot (Figure 3). Likewise, ANOSIM demonstrated no significant

similarities in bacterial community composition between samples from each lesion subtype (weighted R= -0.015, p=0.514; unweighted R= -0.056, p=0.790).

Relative Abundance of Bacterial Taxa

The relative abundance of bacterial taxa was examined from phylum to species levels. The most abundant phylum (mean ±standard deviation) was *Actinobacteria* followed by *Proteobacteria* in all lesion types, respectively: normal (69.893% ±41.635% and 29.115% ±41.230%), comedone (50.522% ±33.982% and 25.708% ±36.250%), and inflammatory (71.438% ±40.128% and 16.885% ±31.729%). Differences in mean relative abundance of phyla between normal skin, comedones, and inflammatory lesions were evaluated. While the mean relative abundance of phyla varied across lesion types (Figure 4), the only statistically significant difference observed was in mean *Firmicutes* relative abundance between normal (0.362% ±0.618%) and comedonal (15.714% ±14.148%) samples (p=0.012).

At the genus level, *Cutibacterium* was the predominant taxa in all lesion types: normal (69.131% ±42.990%), comedone (49.891% ±34.950%), and inflammatory (70.366% ±40.335%). *Staphylococcus*, a member of the *Firmicutes* phylum, was significantly enriched in comedones (11.400% ±12.242%) compared to normal skin (0.073% ±0.185%, p=0.024). No significant differences were observed in mean *Staphylococcus* relative abundance between normal skin and inflammatory lesions (p=0.110) or comedones and inflammatory lesions (p=0.093). While not statistically significant, various fluctuations in relative abundance of the remaining detected genera between lesion types were observed (Figure 4).

After excluding species present in <0.1% mean relative abundance and species present in ≤1 sample per group, no species were statistically significantly different in relative abundance between lesion types. Likewise, no single *Staphylococcal* species was significantly enriched in comedones compared to normal skin. However, we observed interesting trends in the relative abundance of species across lesion types (Figure 5). The relative abundance of *S. epidermidis*, *Kocuria palustris, S. aureus*, *Paracoccus aminovorans, S. succinus*, *Prevotella melaninogenica*, *Stenotrophomonas geniculata*, *Micrococcus luteus*, *Lactobacillus iners*, *and Petrobacter succinatimandens* peaked in comedones while demonstrating minimal representation in normal skin and in inflammatory lesions. *C. acnes*, while being the most abundant species overall, was least abundant in comedones. The relative abundance of *C. granulosum* steadily increased between normal skin, comedones, and inflammatory lesions. Overall, samples taken from comedones displayed the widest range of species relative abundance compared to any other lesion type.

DISCUSSION

Since the discovery that *C. acnes* is capable of producing biofilm, biofilm has been recognized as an important *C. acnes* virulence factor.[21-23] The ability to form biofilms varies among *C. acnes* isolates, with more invasive isolates being associated with increased biofilm production.[24-25] *C. acnes* biofilms, while detected to some degree in normal skin, are present in significantly greater frequency in the sebaceous follicles of acne patients, suggesting that *C. acnes* biofilm production is involved in the pathogenesis of acne.[12-13] In this study, we examined the skin of individuals with acne for the presence of biofilm in unaffected skin, comedones, and inflammatory papules in order to better understand the association between biofilm and acne progression.

Biofilm was not universally present among acne lesions. We detected biofilm in 9 specimens, including: 2 from unaffected skin, 5 comedones, and 2 inflammatory papules. Microbiota analysis demonstrated no statistically significant differences in alpha or beta diversity across lesion types. Mean alpha diversity was highest in comedonal lesions, while the mean alpha diversity of unaffected skin and inflammatory lesions was quite comparable. Comedones, but not inflammatory lesions, were significantly enriched in *Staphylococcus* compared to unaffected skin. This interesting observation correlates with findings that *C. acnes* has a mutualistic

relationship with some *Staphylococcal* species.[26-27] When grown anaerobically *in vitro*, *Staphylococcus aureus* and *C. acnes* form a dense polymicrobial biofilm mass, from which viable *S. aureus* can be recovered, suggesting that *C. acnes* biofilm may provide an ideal environment for *S. aureus* growth.[28] Acne biofilm may occur as a polymicrobial community, including *Cutibacterium*, *Malassezia*, and *Staphylococcus*.[29-31] In our study, comedones were the lesion type with the greatest frequency of biofilm presence, the highest alpha diversity, and the highest relative abundance of *Staphylococcus*. It remains unclear whether the presence of *Staphylococcus* contributes to biofilm formation, or whether the presence of biofilm creates an environment that allows *Staphylococcus* and potentially other microbes to flourish.

Within a biofilm, microbes are relatively protected against host defense.[9] This mechanism may in part explain why the relative abundance of multiple genera, including *Staphylococcus*, *Burkholderia*, and *Micrococcineae*, peaked in the comedone lesions. On the contrary, among all lesion types, *Cutibacterium* was present in the lowest relative abundance in comedones. It has been shown that microbial proliferation and metabolic activity within a biofilm necessarily decrease, as the availability of nutrients decreases towards the center of the biofilm.[9] *Cutibacterium* may be more susceptible to this relative depletion of nutrients than other microbes; or, enrichment of the comedone with other microbes may create a less favorable environment for *Cutibacterium* growth. Additional studies would be needed to further characterize the microbiome within comedonal biofilm.

Interestingly, despite the fluctuations that were observed within comedones, bacterial genera were overall present at similar relative abundances in unaffected skin and inflammatory lesions. Likewise, biofilm was detected at equal frequency in unaffected skin and inflammatory lesions in this small study. This finding suggests that, in contrast to other lesion types, the comedone has a

distinct microbial environment that seems to be related to a greater frequency of biofilm. Perhaps the ability of microbes to escape host detection within biofilm favors the development of a comedonal rather than inflammatory lesion type.

Our study has a number of limitations, including a relatively small sample size. We also did not enroll adolescent patients, whose increased sebum production may result in a different microbial profile and different frequency of biofilm formation than in adults.[32] Our uninflamed comedone samples included primarily closed comedones, so additional studies of open comedones would be useful in further characterizing the comedone microenvironment. Biofilm analysis was limited by conventional vertical sectioning of the biopsy tissue, which permits only a 2D investigation of hair follicles.[29] Since visualization of sebaceous follicles in the tissue section may be variable, the prevalence of biofilm in our specimens may be underestimated. Although the 6-item scoring scale for evaluating biofilm has been commonly used, it is not a validated tool.[17] Additionally, in our microbiome analysis, we did not achieve strain-level resolution. As significant variability occurs between the behavior of strains within the same species, the interpretation of our results is somewhat limited.[25] The study of microbiota through pyrosequencing provides microbial data in terms of relative abundance of different taxa. For a fully quantitative analysis, a targeted qPCR-based method could be used. Also, although every effort was made to decrease handling of the tissue, it was processed twice to allow both microscopy and pyrosequencing to occur, increasing the risk for incidental contamination. Participants were not required to follow a skin prep regimen prior to sampling as previously described,[33] which may affect microbiota analysis. Additionally, samples were taken from either the skin of the participants' head/neck or trunk depending on the area of disease involvement. Because the microbiome is known to vary by body site,[3] some of the differences that we observed in the skin microbiota between groups may

be attributable to body site. Although our inclusion criteria required participants to abstain from oral antibiotics for 4 weeks prior to sampling, studies have indicated that antibiotics may have lasting effects on the skin microbiota beyond this time frame.[34]

In this study, biofilm was not universally present in acne lesions. Biofilm was detected more frequently in non-inflammatory comedones compared with inflammatory lesions or unaffected skin. Comedones also demonstrated significant enrichment in *Staphylococcus* species compared with unaffected skin. The comedone contains a unique microbial environment, including biofilm and *Staphylococcal* enrichment. This unique microbial environment may favor the development of non-inflammatory comedones rather than inflammatory papules.

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FIGURES