



The importance of a multifaceted approach to characterizing the microbial flora of chronic wounds

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ABSTRACT

Chronic wounds contain complex polymicrobial communities of sessile organisms that have been underappreciated because of limitations of standard culture techniques. The aim of this work was to combine recently developed next-generation investigative techniques to comprehensively describe the microbial characteristics of chronic wounds. Tissue samples were obtained from 15 patients with chronic wounds presenting to the Johns Hopkins Wound Center. Standard bacteriological cultures demonstrated an average of three common bacterial species in wound samples. By contrast, high-throughput pyrosequencing revealed increased bacterial diversity with an average of 17 genera in each wound. Data from microbial community profiling of chronic wounds were compared with published sequenced analyses of bacteria from normal skin. Increased proportions of anaerobes, Gram-negative rods and Gram-positive cocci were found in chronic wounds. In addition, chronic wounds had significantly lower populations of *Propionibacterium* compared with normal skin. Using epifluorescence microscopy, wound bacteria were visualized in highly organized thick confluent biofilms or as scattered individual bacterial cells. Fluorescent in situ hybridization allowed for the visualization of *Staphylococcus aureus* cells in a wound sample. Quorum-sensing molecules were measured by bioassay to evaluate signaling patterns among bacteria in the wounds. A range of autoinducer-2 activities was detected in the wound samples. Collectively, these data provide new insights into the identity, organization, and behavior of bacteria in chronic wounds. Such information may provide important clues to effective future strategies in wound healing.

INTRODUCTION

Chronic wounds are a significant public health burden and cost the American health system approximately \$25 billion a year.¹ A large percentage of this expenditure is spent on costly antimicrobial agents. Yet the relationship between bacteria and delayed wound healing remains poorly understood. With the advent of new and improved molecular techniques and technologies, there may be an opportunity to explore new microbial targets to facilitate wound healing. Such an opportunity could be thoroughly explored using a systematic, multifaceted approach to better characterize the microbial flora in chronic wounds.

Chronicity in wounds is associated with an elevated concentration of bacteria in the affected tissue.² As bacterial load increases, wounds appear to take longer to heal. Inflammation is a normal part of the wound-healing process, but healing can be significantly delayed if the inflammatory response becomes excessive. Chronic wounds induce a dysfunctional response characterized by a continuing influx of

neutrophils that release cytotoxic enzymes, free oxygen radicals, and inflammatory mediators that cause extensive collateral damage to the host tissue. Bacterial species play a critical and active role in chronic wounds.³ Many common wound bacteria (such as members of the genera *Staphylococcus*, *Streptococcus*, and *Pseudomonas*) produce exotoxins that cause broad damage to the host by destroying cells and disrupting normal cellular metabolism producing further tissue necrosis. Polymicrobial interactions may well play a crucial role. For example, mixed aerobic and anaerobic bacteria that are capable of working in synergy can have a greater net pathogenic effect.⁴ At the same time, it has been shown that the total number of different species present, rather than one particular bacterial species, correlates positively with impaired healing.⁵ Multispecies biofilm development is common in chronic wounds because of the moist adherent environment where bacteria aggregate and become embedded in a self-secreted exopolysaccharide matrix. The presence of such biofilms results in inefficient eradication of bacteria by antibiotic treatment and host

defense mechanisms. Finally, benign colonizers in normal skin flora may protect the wound from pathogenic bacteria and their ill effects on wound healing.

Traditional views of bacteria as free living cells in a planktonic state have been replaced by the understanding that bacteria frequently attach to exposed surfaces and form a biofilm. Bacteria in these two cell states differ significantly in their morphology, mode of communication, and metabolism. Much clinical microbiology is still based on the assessment of bacteria in a planktonic state. The general theory of biofilm predominance was not well promulgated until 1978.⁶ Direct recovery techniques and microscopic observations from the natural environment demonstrated that more than 99.9% of bacteria grow in biofilms attached to a wide variety of surfaces. Biofilm predominance was established in all natural ecosystems except in very harsh environments in the ocean and deep groundwater.⁷

Biofilms are complex sessile polymicrobial communities embedded in a self-secreted exopolysaccharide matrix and typically exist at interfaces.⁸ The basic structural unit of a biofilm is the microcolony. The cells are located in matrix-enclosed clusters forming complex structures that can resemble towers and mushrooms. Biofilms are well hydrated, and approximately 15% of their volume is composed of cells and 85% is composed of matrix material. These elements are heterogenous in time and space, and some biofilms do not have such pronounced structures. Biofilms provide a unique environment to facilitate bacterial cell-cell signaling by the production and detection of quorum-sensing molecules, which promote the collective behavior of biofilm bacteria.⁹ Quorum sensing has been shown to play a role in biofilm formation and the regulation of virulence factors.¹⁰ Two classes of quorum-sensing molecules are the acylated homoserine lactone (AHL) autoinducers and a family of inter-convertible molecules derived from 4,5-dihydroxy-2,3-pentanedione that is collectively called autoinducer-2 (AI-2). While AHLs are produced solely by Gram-negative bacteria, AI-2, is produced by many Gram-positive and Gram-negative bacteria. Evidence suggests that AI-2 can mediate intra- and interspecies communication that allows bacteria to signal to one another in biofilms.^{11,12}

Biofilms are particularly relevant to chronic wounds. Wounds provide a moist surface on which polymicrobial biofilms easily form. Several properties of biofilm predispose them to confer greater resistance to traditional antibiotics and to be less susceptible to host defenses. These include slow penetration of an antimicrobial agent through the matrix-embedded biofilm, giving bacteria a chance to initiate stress responses, the expression of efflux pumps by the biofilm bacteria, metabolic heterogeneity of biofilm cells, and the enhanced presence of "persister cells."¹³ The latter is important for antibiotics where bactericidal activity is dependent on cell growth and multiplication. Antimicrobial susceptibility also is state-dependent, and 10–1,000 time increases in the minimum inhibitory concentration (MIC) are not uncommon for biofilm bacteria when compared with their planktonic state.¹⁴

Chronic wound biofilms create an environment that is very different from the planktonic environment, because many species may be harbored within these biofilms, which possess very different nutritional requirements in order to grow. Thus, routine culture techniques are inadequate for studying the microbial flora in chronic wounds. With the advent and/or improvement of new research techniques including 16S rRNA pyrosequencing, epifluorescence microscopy, fluores-

cent in situ hybridization, and quorum-sensing analysis, we now have the tools to identify the full spectrum of bacterial species, visualize biofilm morphology, and measure levels of cell-cell signaling in wound biofilms. A systematic, multifaceted approach is outlined herein that will enable us to begin to characterize the microbiologically complex nature of chronic wounds.

MATERIALS AND METHODS

Sample collection

Chronic wounds from 15 patients presenting to the Johns Hopkins Wound Center were sampled between July and December 2009. Specimens were collected at two sites by wedge tissue biopsy and curettage of the leading edge of each wound. Wedge tissue biopsies were processed for epifluorescence microscopy and fluorescent in situ hybridization. The curette samples were processed for quantitative culture, bacterial community profiling using 16S rRNA gene pyrosequencing, and bacterial signaling detection. Clinical data were collected at the time of sample procurement. Institutional Review Board approval was obtained.

Quantitative cultures

Quantitative cultures were processed in duplicates within 4 hours of tissue collection using previously described culturing techniques with minor modifications.^{15,16} Briefly, specimens were weighed (20–50 mg), homogenized in 5 mL of saline in a sterile tissue grinder, and the resulting tissue homogenate serially diluted in sterile saline. Ten and 100 μ L of the undiluted tissue homogenate, as well as 10 μ L of four different dilutions, were plated on selective media (TSA, chocolate, MacConkey agar, BD, Franklin Lakes, NJ), incubated aerobically at 37 °C for 24 hours, and the colonies counted to determine the colony-forming units per gram of tissue. Speciation and MIC for each bacterial isolate were determined on the MicroScan Walk-Away[®] (DADE BEHRING INC., West Sacramento, CA). Anaerobic cultures were carried out directly from the undiluted tissue homogenate by plating on colimycin nalidixic acid (CNA), laked-blood Kanamycin agar (LKA), and CDC anaerobic blood agar (CDC) media and incubated anaerobically for 7 days. Speciation of anaerobic organisms was carried out by standard biochemical procedures.

Pyrosequencing and taxonomic classification of 16S rRNA gene amplicons from wound samples

Wound samples were initially frozen at –70°C without any medium added for storage. During processing, they were thawed on ice without any medium added initially, and vortexed vigorously for 5 min. One mL of phosphate-buffered saline was then added to the sample. Cell lysis was initiated by adding 50 μ L of lysozyme (10 mg/mL), 6 μ L of mutanolysin (25,000 U/mL; Sigma-Aldrich, St. Louis, MO) and 3 μ L of lysostaphin (4,000 U/mL in sodium acetate; Sigma-Aldrich). After a 1-hour incubation at 37 °C, the samples were further lysed by adding 10 μ L Proteinase K and 50 μ L 10% sodium dodecyl sulfate, followed by an incubation at 55°C for

45 minutes and mechanical disruption by bead beating in a FastPrep instrument FP120 at 6.0 m/s for 40 s using 0.1 mm silica spheres (QBiogen Lysis Matrix B). Total genomic DNA was then purified using the ZYMO Fecal DNA Kit from Zymogen according to the manufacturer's recommendations.

Genomic DNA was used for polymerase chain reaction (PCR) amplification of the V1–V2 hypervariable region of the 16S rRNA gene, using the bacterial universal primers 27F and 338R. The 338R primer included a unique sequence tag to barcode each sample. The primers were as follows: 27F-5'-GCCTTGCCAGCCCGCTCAGTCAGAGTTTGATCCTG GCTCAG-3' and 338R-5'-GCCTCCCTCGCGCCATCAGN NNNNNNCATGCTGCCCTCCCGTAGGAGT-3', where the underlined sequences are the 454 Life Sciences FLX sequencing primers B and A in 27F and 338R, respectively, and the bold letters denote the universal 16S rRNA primers 27F and 338R. The 8-bp barcode within primer 338R is denoted by 8 Ns. Using 96 barcoded 338R primers¹⁷ the V1–V2 regions of 16S rRNA genes were amplified in 96-well microtiter plates using AmpliTaq Gold DNA polymerase (Applied Biosystems) and 50 ng of template DNA in a total reaction volume of 50 μ L, using the following cycling parameters: 5 minutes of denaturation at 95 °C, followed by 20 cycles of 30 seconds at 95 °C (denaturing), 30 seconds at 56 °C (annealing), and 90 seconds at 72 °C (elongation), with a final extension at 72 °C for 7 minutes. Negative controls without a template were included for each bar-coded primer pair. After confirming the presence of PCR amplicons by gel electrophoresis, PCR products were quantified using a GelDoc quantification system (Bio-Rad) and the Quant-iT PicoGreen dsDNA assay, and equimolar amounts (100 ng) of the PCR amplicons were mixed in a single tube. Amplification primers and reaction buffer were removed using the AMPure Kit (Agencourt). The 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.

Sequences were then binned by samples using the sample-specific barcode sequences and trimmed by removal of the barcode and primer sequences. Criteria previously described¹⁸ were used to assess the quality of sequence reads. 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 genus using the Ribosomal Database Project (RDP) Naïve Bayesian Classifier¹⁹ using the recommended quality score filtering of 0.5.

Epifluorescence microscopy

Fifteen wound biopsies were fixed in 4% paraformaldehyde for 1 hour for each millimeter (thickness) of tissue, transferred to a 30% sucrose solution in phosphate-buffered saline and shipped to the Center for Biofilm Engineering. Upon receipt, the samples were embedded in optimum cutting temperature compound (Sakura Finetek, Torrance, CA) and frozen on dry ice. Thin sections (5 μ m) 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. Sections were examined using Eclipse E-800 epifluorescence microscope (Nikon, Melville, NY). Each section was scored based on the amount of bacteria/biofilm observed using five-point scale. 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).

Peptide-nucleic acid fluorescent in situ hybridization (PNA-FISH) and confocal microscopy

Infected tissue from chronic wounds were obtained and immediately fixed in 100% ethanol. Samples were cryosectioned and then hybridized with species-specific PNA-FISH probes according to the manufacturer's directions (Advandx, Woburn, MA). *Staphylococcus aureus* probes were coupled to Cy2 (green) while general eukaryotic probes were labeled with Cy3 (red). Fluorescence was captured with a Zeiss 510 Meta (Carl Zeiss, Thornwood, NY) by confocal scanning laser microscopy (CSLM) using a combination of differential interference contrast (DIC) and/or Cy2/Cy3 filter sets. Additionally, standard light microscopy utilizing DIC was used to image and to enumerate microbial species.

Quorum-sensing molecule detection

In order to infer the presence of cell–cell signaling molecules in chronic wound samples, agar plate-based AHL screens and bioluminescence-based AI-2 detection assays were performed using the approach of Rickard et al.²⁰ Briefly, using a modified method of Bassler et al.,²¹ the bioluminescent bacterium *Vibrio harveyi* BB170 was used to detect AI-2 in filtered wound samples. Cell-free culture supernatants from *V. harveyi* BB152 were used as positive controls as this strain produces AI-2. Bioluminescence induction from wound samples were compared with that from PBS (pH 7.4) and the difference was calculated as fold inductions using the approach of Blehert et al.²² Inductions greater than 10-fold were considered positive for signal activity. This represents a typical fold-induction of between 1–2% of the signal detected in the *V. harveyi* BB152 positive controls. *Agrobacterium tumefaciens* A136, which hydrolyzes chromogenic X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) because of the expression of β -galactosidase upon exposure to various AHLs^{23,24} was used in agar plate-based studies.²⁰ *A. tumefaciens* A136 detects N-3-(oxo-octanoyl) homoserine lactone as well as a broad range of AHLs.^{23,24} *A. tumefaciens* KYC6 was used as a positive control as it is a producer of a variety of AHL molecules.²³

RESULTS

Bacterial diversity

Standard culture demonstrated an average of three common bacterial species in a sample. By contrast, high-throughput pyrosequencing of the 16S rRNA gene revealed an average of 17 bacterial genera, most of which were anaerobic organisms (Figure 1).

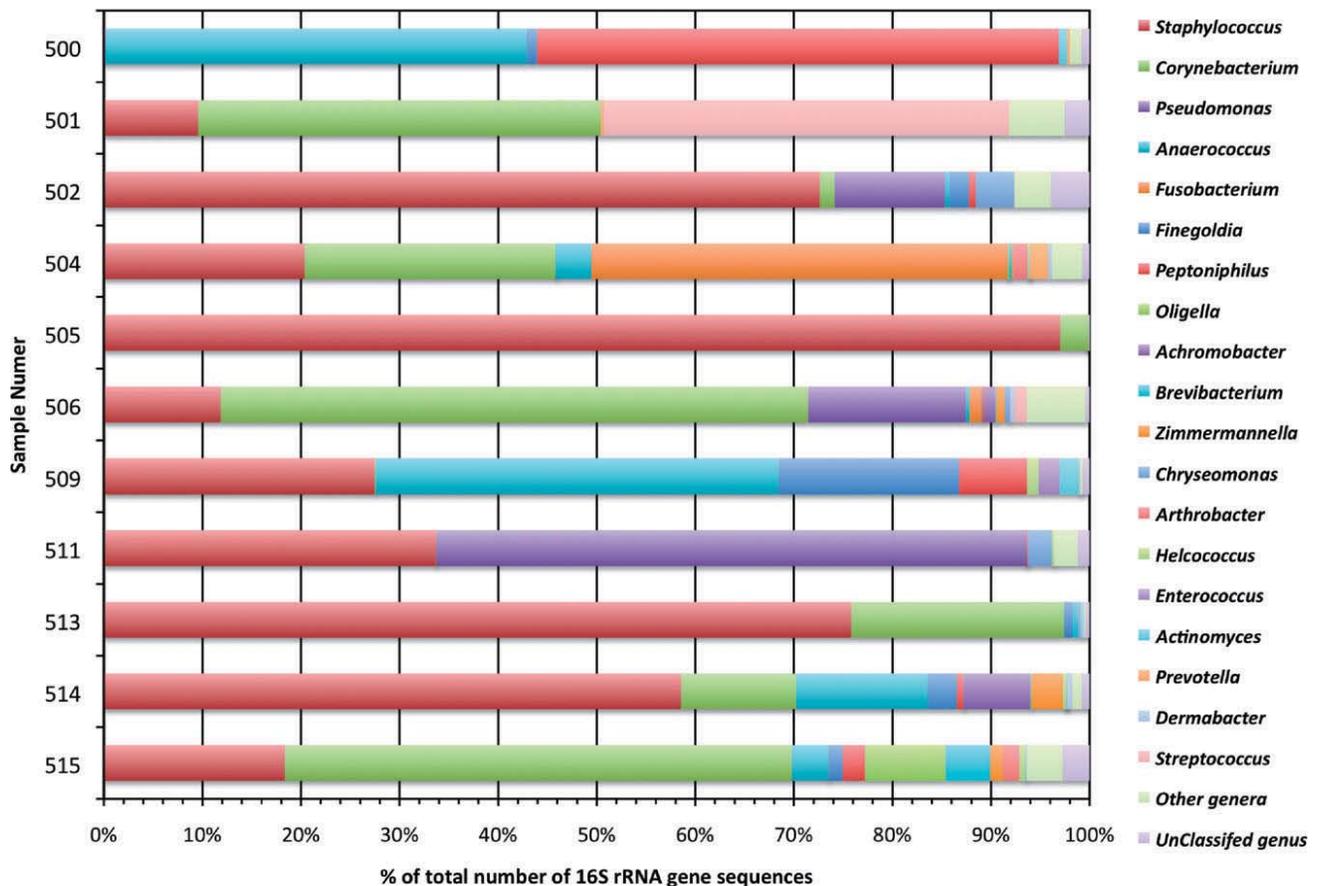


Figure 1. Culture-independent 16S rRNA-based identification of bacteria from curette samples from chronic wounds.

Epifluorescence microscopy

Bacteria or biofilm were detected in 9/15 (60%) of the specimens and these received scores from 1 to 5 (Table 1). Seven of these specimens (47%) received a score of 4 or 5, indicating the detection of significant biofilm coverage (Figure 2A). By contrast, two specimens received a score of 1, signifying only scattered individual bacterial cells were found in the sample (Figure 2B).

PNA-FISH microscopy

Figure 3 shows the image of a sample probed using PNA-FISH. The green labeled *S. aureus* cells exist as discrete multicellular biofilm communities within the host tissue (red) of a wound sample. This patient was also positive for *S. aureus* as detected by molecular techniques and culture.

Detection of cell-cell signal molecules in wound samples

Fourteen cell-free samples from five different wound types were analyzed for the presence of quorum-sensing molecules (AHLs and AI-2). Testing the samples with *A. tumefaciens* A136 inferred the presence of AHLs in three of the 14

samples (21%, Table 1). These were WS505 (VU), WS506 (NHW), and WS507 (PU). All three positive results were weak, compared with the positive control, and yielded a light blue coloration of agar plates because of the expression of β -galactosidase (and the resulting hydrolysis of X-gal) by the AHL reporter *A. tumefaciens* A136. Conversely, bioluminescence assays using *V. harveyi* BB170, indicated the presence of AI-2 in 12 of 14 of the samples (86%). The range of average fold-induction values, which may indicate the approximate amount of AI-2 in wounds varied from 1.4 (PU, WS510) to 128.7 (VU, WS505). No relationship between average fold-induction value and wound type could be determined. Furthermore, no relationship between average fold-induction value and biofilm quantification by epifluorescence microscopy could be ascertained (Table 1).

DISCUSSION

A cross-section of a variety of different types of chronic wounds was examined in this study (Table 2). Common wounds such as venous stasis, diabetic neuropathic, and decubitus ulcers were included, as well as less common types such as traumatic wounds and skin popping ulcers caused by illicit intradermal drug injections. Clinical parameters were recorded to account for individual patient and wound variables

Table 1. Presence of biofilm, as determined by microscopic evaluation of wedge tissue biopsies, and inference of quorum-sensing signal molecules from curette samples from wounds

Sample	Wound type	Biofilm score*	Observations	<i>Agrobacterium tumefaciens</i> A136	<i>Vibrio harveyi</i> BB170
WS 500	PU	5	Thick continuous film	–	11.3
WS 501	VU	1	Single individual cells	–	12.9
WS 502	LV	4	Continuous film	–	17.5
WS 503	PU	4	Continuous film	–	36
WS 504	PU	0	No bacteria detected	–	15.1
WS 505	VU	1	Single individual cells	+	128.7
WS 506	NHW	0	No bacteria detected	+	45.8
WS 507	PU	4	Continuous film	+	71.6
WS 509	DFU	4	Continuous film	–	16.3
WS 510	PU	0	No bacteria detected	–	1.4
WS 511	NHW	5	Thick continuous film	–	16.9
WS 512	DFU	0	No bacteria detected	–	11.5
WS 513	DFU	0	No bacteria detected	–	8.1
WS 514	VU	0	No bacteria detected	–	22
WS 515	DFU	5	Thick continuous film	Nd	nd

Biofilm score system relates to the amount of bacteria/biofilm present. *A. tumefaciens* A136 and *V. harveyi* BB170 were used to detect AHLs and AI-2, respectively. Bold numbers represent those samples that were considered positive for cell–cell signal molecules. Samples were taken from DFUs, LV, NHWs, PUs, and VLU.

*Scoring system used to classify wound specimens based on the amount of bacteria/biofilm present. Score 0, no bacteria observed; 1, single individual cells; 2, small micro-colonies (~10 cells); 3, large micro-colonies (~100 cells); 4, continuous film; 5, thick continuous film.

DFU, diabetic foot ulcer; LV, livedo vasculitis; NHWS, nonhealing wounds; PU, pressure ulcers; VLU, venous leg ulcers.

such as age, sex, comorbidities, ulcer location, and duration. Clinical signs and symptoms of infection in addition to the use of both oral and topical antibiotics were noted because they could influence the bacterial flora in the samples. A systematic,

multifaceted approach was developed using state-of-the-art techniques in order to characterize the complexity of the microbial flora in these wound samples. Because of the presence of multiple variables, confounding factors existed that

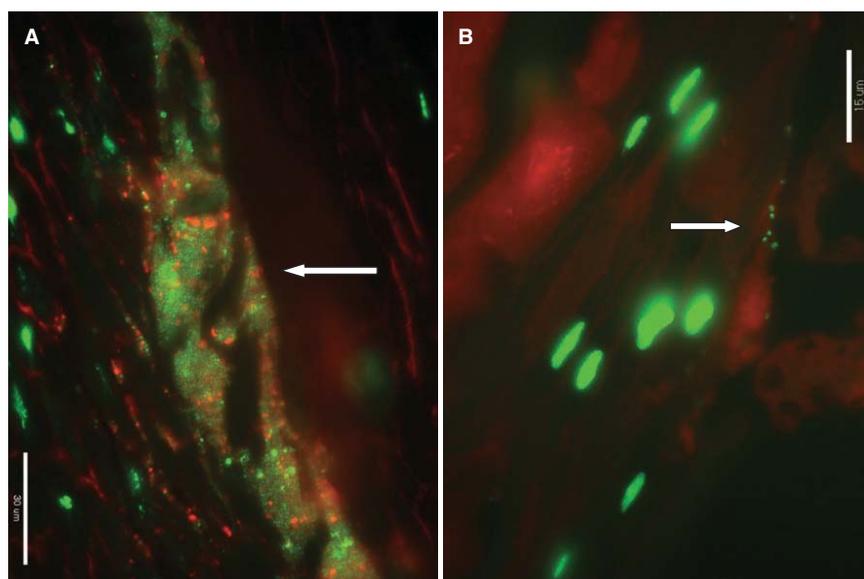


Figure 2. Comparison of two representative epifluorescence micrographs. One wound sample received a score of 5, indicating extensive biofilm formation (left arrow = thick continuous biofilm). By contrast, the other sample received a score of 1, indicating scattered individual cells (right arrow = single individual bacteria). (A) Scale bar is 30 μm . (B) Scale bar is 15 μm .

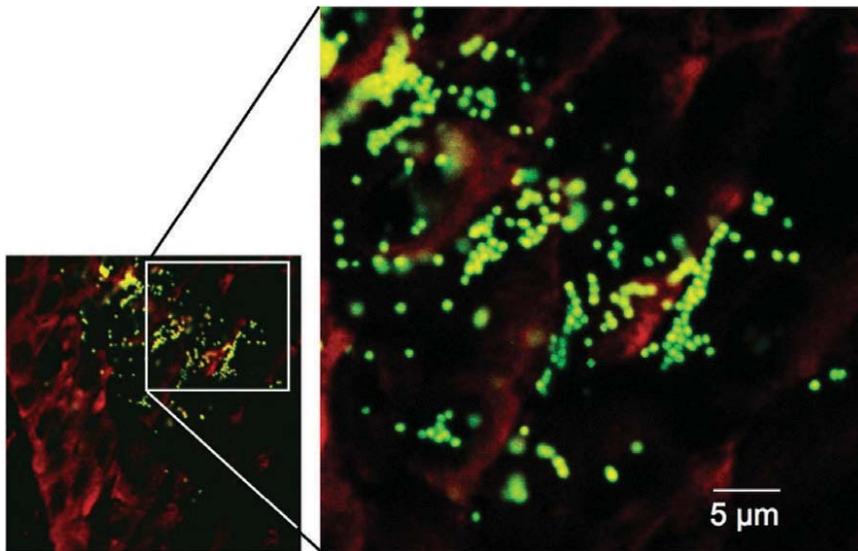


Figure 3. Protein nucleic acid—fluorescent in situ hybridization using *Staphylococcus aureus* probe (Cy2—Green) and a general eukaryotic probe (Cy3—Red) imaged using confocal scanning laser microscopy. Scale bar for enlarged area in Figure 3 is 5 μ m.

complicated the interpretation of data from these studies. However, several general patterns could be elucidated to characterize the complexity of chronic wound microbial flora. Studies using more rigorous inclusion and exclusion criteria as well as modifications on the methods used for sample collection and processing are now in progress.

The identity and relative abundance of bacterial species in the samples were defined using quantitative culture as well as culture-independent 16S rRNA-based analysis. Our culture techniques demonstrated an average of three bacterial species in each sample with the use of manual tissue grinding, although use of an automated tissue grinder may have provided improved bacterial bioburden results. Characterization of the chronic wound microbiome using 16S rRNA gene sequencing demonstrated larger and previously unsuspected numbers of anaerobes compared with results obtained with our quantitative culture technique, confirming prior publications that there are many more anaerobic bacteria in chronic wounds than previously thought and that anaerobes probably play a pathogenic role in wound healing.^{23–25} Our results also show a great deal of bacterial profile variability across chronic wound samples. For example, bacteria belonging to the *Staphylococcus* genus were present in 10 out of 11 samples, although in different proportions: 97% of the 16S sequences belonged to the *Staphylococcus* genus for one sample, but they represented only 9.57% of the sequences in another. Sample # 500 was the only one for which bacteria from the genus *Staphylococcus* were absent: instead, the two major species in this sample were *Anaerococcus* (42.75%) and *Peptoniphilus* (52.91%) bacteria. *Pseudomonas* bacteria, which were identified using cultivation methods, could be identified in the 16S sequence data as well, but at very low levels (0.14%). Although *B. fragilis* bacteria could be isolated using cultivation methods in this sample, only one sequencing read belonging to the *Bacteroides* genus was identified in the sample # 500 dataset, probably because of the very low abundance of *Bacteroides* bacteria in the wound from this patient. Despite efforts to get a successful 16S PCR reaction for each of the samples processed for microbial community profiling, four wound samples failed to produce enough 16S PCR

amplicons for pyrosequencing (WS 503, 507, 510, 512). One possible explanation could be the relative amounts of human and bacterial DNA in these samples (e.g., very high amounts of human DNA and low amounts of bacterial DNA), resulting in very few 16S target sequences in the PCR reaction. A second possibility could be the presence of high levels of blood in the wound samples, resulting in significant amounts of PCR inhibitors such as hemoglobin in the extracted DNA.²⁶

Sequencing data of 45 chronic wound samples (which combined data from 15 samples in this series and 30 additional samples in a previous series conducted at Johns Hopkins Wound Center) were compared with the microbial composition of normal skin flora from two published studies performed by other research groups.^{27,28} Normal skin flora is populated by several major bacterial phyla that include the genera *Propionibacterium*, *Staphylococcus*, *Streptococcus*, and *Bacteroides*. In contrast to normal skin flora, our wound samples demonstrated a significantly larger proportion of anaerobes, large quantities of Gram-negative rods such as *Pseudomonas*, *Proteus*, *Escherichia coli*, and *Klebsiella*, and an increased proportion of *Staphylococcus* and *Streptococcus*. Chronic wounds also had a noticeably decreased proportion of *Propionibacterium*, which may have served as a benign and even protective colonizer in normal skin.

Normal Skin (arm):

- (1) *Actinobacteria* (28–51%): *Propionibacterium* + *Corynebacterium*
- (2) *Proteobacteria* (19–40%): *Betaproteobacteria*
- (3) *Firmicutes* (12–24%): *Staphylococcus* + *Streptococcus*
- (4) *Bacteroidetes* (2–14%): *Bacteroides* + *Prevotella*

Chronic Wounds (45 samples):

- (1) $\uparrow\uparrow$ Anaerobes—*Bacteroidetes*; *Fusobacterium*
- (2) \uparrow *Proteobacteria*: *Gammaproteobacteria* (Gram-negative rods such as *Pseudomonas*, *Proteus*, *E. coli*, *Klebsiella*, etc.)
- (3) \uparrow *Firmicutes*: *Staphylococci* + *Streptococci*
- (4) \downarrow *Actinobacteria*: *Propionibacterium*

Table 2. Clinical data for wound samples

ID	Age	Sex	Primary ulcer type	Location	Weeks	New	S/S	Infxn	Abx within 2 weeks	Topical antimicrobial	Comorbidity
WS500	69	F	Arterial	L lateral ankle	52	No	Yes	Yes	0	Biafine	Livedoid vasculitis
WS501	67	M	Decubitus	Perineal	4	Yes	No	No	0	0	0
WS502	54	F	Skin popping	R lower extremity	600	No	Yes	Yes	0	0	Venous
WS503	43	F	Decubitus	Coccyx	12	Yes	No	No	0	Aquacel Ag	Diabetes
WS504	19	M	Decubitus	Coccyx	3	Yes	No	No	Vanc, Cipro, Fluc	0	0
WS505	48	F	Venous	R gaiter	24	Yes	No	No	Vanc, Bactrim	0	Diabetes
WS506	54	F	Skin popping	R lateral ankle	520	No	No	No	0	0	0
WS507	23	F	Decubitus	R heel	12	Yes	No	No	0	Aquacel Ag	0
WS509	43	F	Diabetic foot ulcer	L plantar foot	24	Yes	No	No	0	Iodosorb	Diabetes
WS510	42	M	Decubitus	Sacrum	8	No	No	No	0	Aquacel Ag	0
WS511	47	M	Traumatic	L anterior shin	200	Yes	No	No	0	Mepilex Ag	Malnourished, substance abuse, smoking
WS512	79	M	Diabetic foot ulcer	L trans metatarsal	50	No	No	No	Cipro	Iodosorb	Diabetes, atherosclerosis
WS513	85	F	Diabetic foot ulcer	R plantar trans-metatarsal	20	Yes	No	No	0	Iodosorb	Diabetes
WS514	60	M	Venous	L medial ankle	200	No	No	No	Levo	0	Coagulation disorder, rectal carcinoma with chemotherapy + radiation; DVT/PE
WS515	61	F	Diabetic foot ulcer	L plantar foot	44	Yes	Yes	Yes	0	Select Silver, Iodosorb	Diabetes

S/S Infxn, signs and symptoms of infection (pain, pus, erythema, swelling, induration, warmth or wound breakdown); Abx, antibiotics taken within 2 weeks; Vanc, vancomycin; Cipro, ciprofloxacin; Fluc, fluconazole; Levo, levofloxacin; DVT/PE, history of deep venous thrombosis/pulmonary embolus; R, right; L, left.

The spatial arrangement of bacteria in chronic wounds was visualized with epifluorescence microscopy of tissue thin-sections. The bacteria were arranged in aggregated communities of varying densities. Biofilm was quantified based on a 0–5 scale. We found biofilms ranging in size from scattered single individual cells to thick continuous films in nine of 15 samples. No bacteria were observed in the analysis of the remaining six samples. It is important to note that microscopic analysis of thin sections was limited to very small specimens relative to the size of a typical wound. Thus, biofilm may have also been present in wounds where bacteria were not observed and wounds where biofilm was detected were not necessarily entirely covered with biofilm. As a result, biofilm morphology visualized using epifluorescence microscopy in a single sample cannot be used to accurately quantify the amount of bacteria in the entire wound. New technologies are required to study the global biofilm density of wounds.

PNA-FISH and CSLM enabled localization of a *S. aureus* with wound tissue. Imaging revealed the characteristic arrangement of *S. aureus* cells in grape-like clusters and these were attached to tissue as part of a biofilm (Figure 3). However, these clinical samples are heterogeneous, difficult to work with, and require complex equipment and highly trained microscopists. Therefore, the routine use of PNA-FISH for screening purposes to confirm the location, number, and morphology of specific bacteria in a wound sample may not be feasible. However, this methodology provides promise to elucidate highly specific questions and is effective in other less complicated clinical samples such as screening for bacteria liquid samples such as blood or wound exudate.

Quorum-sensing molecules have been associated with biofilm formation and the regulation of virulence factors.⁹ Similar to findings by Rickard et al.,²⁰ both AHL and AI-2 activities were detected in the wound samples. What is particularly interesting from our study is that many of the samples from the wounds were inferred to contain AI-2, albeit over a range of concentrations as determined by the bioluminescence assay (Table 1). AI-2 is a proposed quorum-sensing signal molecule used by both Gram-positive and Gram-negative bacteria.²⁹ These data suggest that interspecies communication may be occurring between the bacteria in a broad range of chronic wound types. However, it is important to note that the complex chemical composition of the wounds may have an effect on the *V. harveyi* BB170 bioassay used to detect AI-2. Furthermore, increasing evidence suggests that bacteria can remove AI-2 from their surrounding environment.^{30,31} As such, fold-induction values likely represent approximate relative AI-2 activities in the original wounds. An alternative approach would be to test wound samples using a recently described quantitative approach that uses liquid chromatography-tandem mass spectrometry.³² Using this quantitative approach, AI-2 has been detected in nanomolar quantities in saliva.³²

AHLs, which are used solely by Gram-negative species for quorum sensing,³³ were inferred to be present in low amounts or absent in the samples (Table 1). Reasons for low levels of AHLs include the absence or low cell density of AHL-producing bacteria (such as *Pseudomonas aeruginosa* or *Acinetobacter* species), the degradation (quenching) of AHLs by N-acylhomoserine lactone acylases such as those produced by *P. aeruginosa*³⁴ or the hydrolysis of the lactone ring of AHLs by paraoxonase-like enzymes produced by human cells.³⁵ Also, the detection of AHLs relied upon agar plate A.

tumefaciens A136 bioassays, a more sensitive and quantitative approach could use liquid chromatographic techniques such as those used by Nakagami et al.³⁶ and Chambers et al.³⁷

We found little correlation between AI-2/AHL activities and biofilm morphology, as visualized by epifluorescence microscopy. Samples with high relative activities of AI-2 did not necessarily demonstrate the presence of thick biofilm, and vice versa. Global biofilm quantification may be needed before morphology can be used in comparisons with quorum-sensing data. Furthermore, there was a lack of correlation between AI-2/AHL activities and 16S data. Samples that yielded insufficient 16S PCR product because of possible low bacterial load did not necessarily demonstrate low levels of quorum-sensing molecules, and vice versa. However, as described earlier, the absence of 16S PCR products does not necessarily mean that no bacteria were present as the wounds may have contained PCR inhibitors such as hemoglobin and lactoferrin.²⁶ Additionally, similar to possible reasons for inconsistencies between epifluorescence microscopy and diversity analyses, it is possible that the bacterial load and species diversity varies within the wound. For future studies, samples should be homogenized first, and then divided into subsamples for analysis. This modification in the methods could allow for more meaningful comparisons between AI-2 and 16S results for specimens from the same homogenate in a wound.

Recently developed or improved technologies such as high-throughput 16S rRNA gene sequencing, along with epifluorescence microscopy, fluorescent in situ hybridization, and quorum-sensing analysis have allowed greater in-depth investigation of the complexity of bacteria in chronic wounds. However, bioburden analysis of wound samples is challenging and scientists need to understand the limitation of each technique or methodology. Our results, while preliminary, demonstrate that the bacterial flora in chronic wounds are extremely complex and differ significantly from the microbiome found on normal skin. In particular, pathogenic anaerobes, Gram-negative rods, and Gram-positive cocci were remarkably more abundant in the chronic wounds examined in this study. Microscopic imaging showed that bacteria in these chronic wounds were present in biofilms attached to the wounds. The inferred presence of quorum-sensing molecules in the majority of samples suggests that the different species of bacteria residing in chronic wounds may be actively communicating with each other. Research is needed to correlate bacterial ecological data under more rigorous clinical protocols. We are embarking on strict protocols to minimize sampling errors. Studying the effect of antimicrobial intervention on bacterial ecology and healing rates will provide much needed information on the judicious use of traditional antibiotics in wound healing. Candidate biofilms for in vitro and in vivo experimentation are currently under development and may be used to test a new generation of anti-biofilm strategies. It is very clear that these methodologies offer unique opportunities to reassess the role of microbial organisms in delayed wound healing. Hopefully, data such as these will permit the formulation of evidence-based guidelines for the use of anti-microbial agents in the therapy of chronic wounds.

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