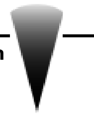

Biofilms in chronic wounds

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ABSTRACT

Chronic wounds including diabetic foot ulcers, pressure ulcers, and venous leg ulcers are a worldwide health problem. It has been speculated that bacteria colonizing chronic wounds exist as highly persistent biofilm communities. This research examined chronic and acute wounds for biofilms and characterized microorganisms inhabiting these wounds. Chronic wound specimens were obtained from 77 subjects and acute wound specimens were obtained from 16 subjects. Culture data were collected using standard clinical techniques. Light and scanning electron microscopy techniques were used to analyze 50 of the chronic wound specimens and the 16 acute wound specimens. Molecular analyses were performed on the remaining 27 chronic wound specimens using denaturing gradient gel electrophoresis and sequence analysis. Of the 50 chronic wound specimens evaluated by microscopy, 30 were characterized as containing biofilm (60%), whereas only one of the 16 acute wound specimens was characterized as containing biofilm (6%). This was a statistically significant difference ($p < 0.001$). Molecular analyses of chronic wound specimens revealed diverse polymicrobial communities and the presence of bacteria, including strictly anaerobic bacteria, not revealed by culture. Bacterial biofilm prevalence in specimens from chronic wounds relative to acute wounds observed in this study provides evidence that biofilms may be abundant in chronic wounds.

Chronic wounds, including diabetic foot ulcers (DFU), pressure ulcers (PU), and venous leg ulcers (VLU), are a grave worldwide problem. It has been estimated that 15% of individuals with diabetes mellitus will develop lower extremity ulcers[1] and 14–24% of diabetic patients with foot ulcers will eventually undergo amputation.[2] Approximately 100,000 limb amputations are performed in diabetic patients each year in the United States. Lower extremity ulcers were the cause of amputation in 67 of 80 patients (84%) in a study carried out by Pecoraro et al.[3] PU are a common and expensive problem in acute care, rehabilitation unit, nursing home, and home care populations. VLU are often painful inflictions that have been estimated to affect 1% of the world's population.[4]

The deleterious effect of microbial infection on wound healing has been recognized for decades and the control of bioburden is recognized as an important aspect of wound management. Historically, it has been presumed that the properties of bacteria that cause chronic infections were similar to those of bacteria grown suspended in liquid growth media. However, research over the past 20 years has indicated that many chronic infections are the result of the biofilm mode of microbial growth.[5–7] Biofilm-related diseases are typically persistent infections that develop slowly, seem to be rarely resolved by immune defenses, and respond transiently to antimicrobial therapy.[7] Chronic wound infections share these characteristics and it has been hypothesized that biofilms play a role in the prevention of wound healing.[8–11] However, direct evidence of biofilm involvement in chronic wound infections is scarce.

The research presented herein consisted of a microscopic evaluation of specimens from 50 chronic and 16 acute wounds for the presence of biofilm. Specimens from chronic wounds were also analyzed using molecular microbiological methods to evaluate bacterial diversity and species composition of the biofilms.

MATERIALS AND METHODS

Patient population

Institutional review board approval was obtained from Montana State University. Subjects were invited to participate in the study upon presentation to the Southwest Regional Wound Care Center for routine wound care treatments. Written informed consent was obtained from all subjects included in the study. Chronic wounds were defined as being 30 days or older and failing to progress through normal wound healing trajectories. For the microscopic analysis, 50 consecutive subjects who were 18 years of age or older and required sharp debridement of their chronic wound(s) were included in the study. Sixteen consecutive subjects who were 18 years of age or older and consented to a biopsy procedure of their acute traumatic wounds were also included in the study. Subjects with chronic wounds underwent standard sharp debridements of their wounds as part of the normal course of their wound care management. The debrided material was fixed with preservative, rather than being discarded as per

standard protocol. For the acute wound biopsy specimens, the area to be biopsied was infiltrated with 1 cm^3 of marcaine 0.5%. After adequate anesthesia was delivered, a 5-mm punch biopsy was obtained from the base of the wound. These specimens were preserved in the same manner as the debridement specimens. Specimens for molecular analysis were collected, as described above, from 27 subjects with chronic wounds. The debridement materials were placed in sterile containers immediately after collection and stored in a refrigerator at 4 °C until extraction of the DNA was performed. Extraction was performed within 48 hours of collection and then the DNA samples were immediately frozen.

Specimen preparation

Immediately after collection, specimens for microscopic analysis were placed in tissue cassettes and fixed in phosphate-buffered saline containing 10% formalin buffered at pH 7.0 for 4–6 hours. The specimens were then transferred to a 70% ethanol solution, and shipped overnight to Center for Biofilm Engineering (CBE). For molecular biological analysis, specimens were homogenized and wound DNA was extracted using the Bio101 FastDNA® Spin for Soil Kit (MP BioMedicals, Solon, OH) and a Savant 101 bead beater (Fast Prep, Aurora, OH). Tissue samples with masses of 0.2–0.5 g were placed in the Lysing Matrix Tube as supplied in the kit (i.e., prefilled with beads), which was subsequently filled with sodium phosphate and extraction buffers. The tubes were processed in the bead beater for 45 seconds at a speed of 6.5. Extraction and cleaning of DNA was accomplished following kit protocols with no further changes. Extracted DNA from the specimens was immediately frozen at −70 °C and then placed in dry ice and transported to CBE.

Microscopy

Upon arriving at CBE, debridement specimens were divided into two equal pieces. One piece was embedded for light microscopy (LM) while the other piece was prepared for scanning electron microscopy (SEM). The acute wound specimens were too small for subdivision and each specimen was examined by either LM or SEM. For imbedding, the specimens were placed in cassettes and immersed in an ethanol gradient consisting of 70%, 80%, three changes of 95%, and three changes of 100% ethanol to dehydrate the tissue. The ethanol was cleared from the tissue with a xylene wash, the specimens were infiltrated with liquid paraffin at 60 °C, and cooled to solidify the paraffin. A microtome was used to cut 5 μm thick sections from the tissue in the paraffin blocks. Each section was placed on a microscope slide for staining and analysis. At least five sections of each tissue specimen were cut and placed on slides.

Before tissue staining, paraffin was cleared from the slide using Safeclear© (Fisher Diagnostics, Waltham, MA) and the sections were rehydrated with a reverse ethanol gradient, consisting of 5-minute washes using absolute, 95%, 80%, and 70% ethanol, ending in deionized water. The sections were then stained using the Chromaview© tissue Gram stain kit (Richard Allan Scientific, Kalamazoo, MI) and examined with LM using a Nikon Eclipse E800 microscope with a ×100 oil immersion objective (Nikon, Melville, NY). Color images were taken with an Olympus Q-color 5 camera (Olympus, Center Valley, PA).

For examination with SEM, tissue specimens were dehydrated with 95% ethanol. Double-sided carbon tape was used to adhere the tissue to 50-mm disk mounts. A colloidal graphite coating was used to secure the specimens and prevent charging in the SEM chamber. The mounted tissue specimens were then placed in a coating chamber and sputter coated with gold/palladium ions under a vacuum of 80 torr. The coated specimens were examined with a SEM Jeol JSM 6100 scanning electron microscope (Jeol, Tokyo, Japan) using an electron beam generated by a LaB_6 cathode with a voltage of 12 kV.

Culture analysis

For culture analysis, debridement specimens were placed in tubes of thioglycolate broth with an indicator (Hardy Diagnostics, Santa Maria, CA) and incubated at 37 °C for up to 24 hours and then transported to a CLIA certified microbiology laboratory for aerobic and anaerobic culture. Bacteria were identified using Gram stains, nonselective and selective/differential media, and biochemical tests.

Polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and cloning

An assessment of bacterial diversity in chronic wound specimens was performed using the molecular techniques of PCR and DGGE. Primer reactions and DNA amplification were performed using a PTC-100 Programmable Thermal Controller (MJ Research, Waltham, MA) and universal Eubacterial primers, with a reverse primer containing a GC clamp. Specifically, the primers used were 518R (5′GTA TTA CCG CGG CTG CTG G 3′) and 357F (5′ CGC CCG CCG CGC CCC GCG CCC GGC CCG CCGC CCC CGC CCC C CTA CGG GAG GCA GCA G 3′) or 1070F (5′-ATG GCT GTC GTC AGC T-3′) and 1392R (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAC GGG ACG GGC GGT GTG TAC-3′) (Integrated DNA Technologies, Coralville, IA). Primer reactions and DNA amplification were performed using the following parameters: 94 °C for 2 minutes, 15 cycles of 94 °C for 45 seconds, 55 °C for 45 seconds, 72 °C for 45 seconds with a final extension step of 72 °C for 7 minutes. Verification of the presence of DNA was assessed in 1.5% agarose gels before analysis by DGGE. Positive controls for PCR were clinical isolates including *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *S. aureus*, and the negative control was sterile water.

Amplified DNA was separated by DGGE using a 40–70% denaturing gradient in 8–12% polyacrylamide gels following recommended manufacturer protocols (BioRad, Hercules, CA).

For bacterial identification, PCR products of approximately 200 bp were directly cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), following the manufacturer's directions. Plasmid preparation was performed using the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, WI), following the manufacturer's directions. The plasmid DNA was sequenced by Laragen Inc. Sequence data were analyzed

using BLAST sequence searches (www.ncbi.nlm.nih.gov) to identify bacterial species. Sequences for 60 clones were analyzed in both the forward and reverse direction. The sequences were checked for chimeras using the Ribosomal Database Project Check Program (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su = SSU).

Image interpretation and statistical analysis

Specimens for microscopic analysis were examined (E. S.) and representative images (n=2–6 for each LM and SEM analysis) were collected (E. S.). The representative images were examined by three independent observers (E. S., G. J., J. W. C.) for the presence of biofilm. Criteria for biofilm classification included the presence of dense bacterial aggregates (LM and SEM) and the presence of extracellular polymeric substance (EPS) matrix (SEM). If specimens from either LM or SEM were considered to contain biofilm by all three investigators, the specimen was considered positive for biofilm presence. Based on these analyses, Fisher's Exact Test was utilized to examine the statistical significance of the results using Minitab (http://minitab.com) software.

RESULTS

Chronic wound specimens were obtained from four wound types: DFU, PU, VLU, and other (O). Acute wound samples were collected from blisters (B), skin tears (ST), and other (O). Other wounds included surgical site infections and traumatic wounds. Information on the subjects of this study and their wounds is presented in Table 1.

Microscopic analysis

The predominant type of bacteria observed in all wound specimens using LM was Gram-positive cocci. These cells were often observed in large aggregates in chronic wound specimens (Figure 1A). In the acute wound specimens, coccoid bacteria were more often observed as individual cells and small microcolonies. SEM examination of the specimens confirmed the predominance of coccoid cells, which often appeared to be coated with EPS (Figure 1B). Biofilms composed of Gram-negative rods (Figure 1C) were also observed in some samples, and mixed-species biofilms (Figure 1D) were also observed.

Results of the microscopic analysis are summarized in Table 1. Overall, 30 of 50 chronic wounds and one of 16 acute wounds were characterized as containing biofilm. Using Fisher's exact test, this was a statistically significant difference ($p < 0.001$) and indicates that biofilms were prevalent in the chronic wound specimens and rare in the acute wound specimens. Pairs of the four chronic wound types were also tested using Fisher's exact test. No wound type was more likely to have biofilm than any other wound type.

Culture analysis

From the subjects involved in the biofilm study, described above, culture data were available for 37 of the chronic wounds and five of the acute wounds. Based on these data, bacteria from eight genera were frequently ($> 10\%$) isolated from wound specimens (Table 2). In agreement with microscopic analysis, bacterial genera that form coccoid cells (*Staphylococcus*, *Enterococcus*) predominated in the wounds, although genera that form bacilli (*Pseudomonas*, *Proteus*) were also present. Additional culture results are described below, in comparison with molecular (cloning) analyses.

Molecular analysis

The diversity of bacterial communities inhabiting chronic wounds was evaluated by DGGE of prokaryotic rDNA extracted from debridement samples and amplified using PCR. Initially two PCR primer sets containing a GC clamp were used; 1070F and 1392R or 357F and 518R (Integrated DNA Technologies), yielding DNA fragments of approximately 200 bp. The combination of 518R and 375F primers resulted in the highest number of bands (data not shown) and was selected for subsequent amplifications.

The number of distinct bands in DGGE gels ranged from two to 13, with the majority of specimens yielding more than four bands. Although some common bands were found in most of the specimens, no two specimens showed exactly the same banding pattern. In addition, specimens that showed similar banding patterns or themes did not group according to wound type. A typical DGGE gel with 17 specimens is shown in Figure 2. The DGGE results (approximate number of bands) are summarized in Table 3.

Clone libraries were constructed for eight chronic wound specimens using 16S rDNA, extracted from the wound debridement samples. BLAST search results of sequenced PCR products identified 15 genera and/or species of bacteria. Three genera of strictly anaerobic bacteria were identified (*Prevotella*, *Clostridium*, and *Porphorymonas*). Results of BLAST searches, along with DGGE results for some of the specimens, are shown in Table 3. The clinical laboratories used by SWRWC were unsuccessful at culturing any anaerobes from any of the samples that were tested.

DISCUSSION

Microscopic analysis of chronic wound specimens revealed the presence of densely aggregated colonies of bacteria often surrounded by an extracellular matrix. These morphological observations are characteristics of biofilms, and provide evidence that biofilms are present in chronic wounds. Similar biofilms were not observed in acute wound specimens. Based on the test criteria used in this study, biofilms were significantly more likely to be present in chronic wounds. The role of biofilms in preventing wound healing was not addressed in this study and there is considerable debate as to the general role of microorganisms as a barrier to wound healing. This may be due in part to the traditional view that a specific etiological agent must be identified as the cause of a particular infection. Bowler et al.[12] have emphasized the polymicrobial nature of wound infections and the presence of anaerobic bacteria that are seldom targeted in clinical microbiological analysis of wounds. During this study, several anaerobic genera were identified using molecular techniques, while

Table 1. Subject/wound information and results of microscopic analysis

	Subject / Wound Information			Biofilm			
Specimen No.	Sex (age)	Wound type	Wound location	Wound size (L×W×D cm)	Gram stain	SEM	Either
1	F (82)	PU	Sa	2.0×2.0×0.5	+	+	+
2	F (87)	VLU	R Le	15.0×5.0	+	+	+
3	M (32)	PU	R I	4.0×3.5×2.5	+	–	+
4	F (83)	VLU	L Le	4.5×1.5	–	+	+
5	M (80)	O	L Le	0.5×0.5×0.25	–	–	–
6	M (56)	DFU	R Gt	2.0×2.0×0.5	–	–	–
7	M (78)	DFU	L He	5.0×3.0×2.0	+	+	+
8	F (81)	PU	L I	1.5×1.0	+	+	+
9	F (89)	PU	R I	8.0×6.0×6.0	–	–	–
10	F (84)	PU	L He	2.0×2.0×0.25	+	+	+
11	F (83)	VLU	R Le	2.5×1.5×0.5	+	+	+
12	M (49)	DFU	L He	3.5×4.0×2.0	+	+	+
13	F (85)	O	R Sh	0.5×0.5	+	+	+
14	F (81)	O	L Le	4.0×0.5×0.25	+	+	+
15	M (47)	VLU	R Le	6.0×5.5×0.5	+	+	+
16	M (67)	O	L Le	2.0×0.5×0.5	–	+	+
17	F (60)	DFU	R F	13×5.5×0.5	–	+	+
18	M (35)	PU	Sa	8.0×6.0×7.5	–	–	–
19	F (82)	PU	R Le	2.5×2.5×0.5	+	+	+
20	F (81)	PU	L ARM	2.0×2.0	+	+	+
21	M (23)	PU	R HIP	10.0×10.0×1.0	–	+	+
22	F (49)	O	R E	2.5×3.5× 5	–	+	+
23	M (78)	DFU	R Gt	3.0×3.0×0.5	+	+	+
24	M (66)	PU	R Le	6.0×4.0×1.0	+	+	+
25	F (59)	VLU	R Le	5.0×3.0×0.5	–	nd	–
26	M (91)	PU	R He	1.5×1.5×0.5	+	+	+
27	M (59)	PU	R Le	3.5×3.0×0.5	–	–	–
28	F (92)	PU	R He	5.0×3.0	+	+	+
29	F (33)	PU	Sa	3.0×2.0×1.0	–	–	–
30	F (84)	PU	Sa	5.0×1.0×0.5	–	–	–
31	F (35)	PU	R I	2.5×2.0×1.0	–	–	–
32	F (78)	DFU	L A	4.0×2.0×0.25	–	–	–
33	M (70)	PU	R I	5.0×3.5×3.0	–	+	+
34	F (82)	DFU	R F	4.0×4.0×1.0	+	+	+
35	M (56)	DFU	L F	5.0×1.0×0.5	+	+	+
36	M (82)	DFU	R F	1.5×1.5	–	+	+
37	M (63)	DFU	R Gt	3.0×3.0×0.5	+	nd	+
38	F (96)	O	R F	2.0×2.0	+	+	+
39	F (66)	O	L F	10.5×5.0×0.5	–	+	+
40	F (69)	DFU	R He	9.0×5.0×0.5	+	+	+
41	F (69)	DFU	L Le	1.5×1.0	–	nd	–
42	F (78)	VLU	R Le	0.5×0.5	–	nd	–
43	M (44)	PU	L I	7.0×3.0×3.0	–	–	–
44	M (66)	VLU	R Le	3.0×2.0	–	–	–
45	F (82)	VLU	R Le	15.0×11.0×1.0	–	nd	–
46	F (37)	PU	R T	4.5×7.5×0.25	–	–	–
47	M (63)	DFU	R F	3.0×3.0×0.5	–	nd	–

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Table 1. Continued.

	Subject/Wound Information				Biofilm		
Specimen No.	Sex (age)	Wound type	Wound location	Wound size (L×W×D cm)	Gram stain	SEM	Either
48	F (80)	O	R Le	5.0×4.0	–	nd	–
49	M (85)	PU	R I	2.0×2.5×0.5	–	nd	–
50	M (68)	PU	L HIP	2.5×3.0×1.0	–	nd	–
51	M (82)	B	R F	0.5×0.5×0.5	–	nd	–
52	M (63)	B	L K	0.5×0.5×0.5	–	nd	–
53	M (57)	B	L Sh	1.0×1.0×1.0	–	nd	–
54	M (78)	St	L Ue	3.0×2.0	–	nd	–
55	F (51)	B	L Gt	2.0×1.5×0.25	–	nd	–
56	F (64)	B	R Le	1.0×1.0	nd	–	–
57	M (56)	B	L F	5.0×1.0×0.5	nd	–	–
58	F (67)	St	L Ue	1.0×1.0×0.5	nd	–	–
59	F (20)	St	L F	1.0×1.0×0.25	nd	–	–
60	F (96)	St	R Le	4.0×2.0×0.5	nd	–	–
61	F (95)	St	L. Ha	5.5×1.5×0.25	nd	+	+
62	M (70)	O	L. ARM	3×2.5×0.5	nd	–	–
63	F (88)	St	L ARM	7×9×0.5	nd	–	–
64	F (96)	St	R ARM	3.5×1.5×0.25	nd	–	–
65	F (82)	St	L ARM	3.5×2.5×0.5	nd	–	–
66	M (71)	B	R Ha	1×1×0.25	nd	–	–

A, ankle; B, blister; DFU, diabetic foot ulcer; E, elbow; F, foot; Gt, great toe; Ha, hand; He, heel; I, ischium; K, knee; L, left; Le, lower extremity; PU, pressure ulcer; R, right; St, skin tear; Sa, sacrum; Sh, shin; T, thigh; Ue, upper extremity; VLU, venous leg ulcer; O, other included surgical site infections and traumatic wounds; nd, not determined; SEM, scanning electron microscopy.

anaerobes were not detected using standard clinical culture methods. However, the specimen collection and processing methods used were the standard procedures used in the clinic at the time of the study and the culture techniques involved standard clinical laboratory procedures. The use of more specialized techniques for anaerobic culture would have likely resulted in the isolation of strict anaerobes, as these species are believed to play an important role in chronic wounds.[12]

The polymicrobial nature and potential involvement of biofilms in chronic wound infections may be analogous to periodontal disease, where a diverse community of microorganisms acting in consort over time results in a chronic infection.[13] Although the role of polymicrobial biofilm communities in preventing wound healing remains unclear, this study provides evidence that these biofilms are prevalent in chronic wounds.

Various morphotypes of bacteria were observed within chronic wound biofilms, in agreement with culture and molecular analysis, indicating these biofilms were polymicrobial. Previous studies using both culture-based[14–16] and molecular-based[17–19] approaches have also indicated the presence of polymicrobial communities. The most common bacteria observed in this study were Gram-positive cocci, which agrees with culture data indicating the predominance of *Staphylococcus* and *Enterococcus* in specimens from these subjects. Staphylococci and *Staphylococcus aureus* in particular have been implicated in delayed wound healing, although a clear correlation between the presence of *S. aureus* and wound infection has not been shown.[12,20] The staphylococci are also well-known biofilm formers.[21]

DGGE analysis of the wounds revealed patterns ranging from two bands to 13 distinct bands, with the majority of the specimens having more than four bands. Theoretically, each band in the DGGE gel represents a different bacterial species, although in practice DNA amplified from a single species may produce more than one band. In addition, a single band may represent more than one species. The limitations of DGGE are reviewed by Muyzer and Smalla.[22] Nonetheless, the number of bands has been used as a measure of species diversity in wounds.[19] The banding pattern of each wound (n=21) was unique, in agreement with an earlier DGGE analysis of VLU.[19] Overall, these results confirm the polymicrobial nature of many chronic wounds and underscore the utility of molecular methods for examining complex microbial communities.

Cloning and sequencing of the16S RNA gene fragment, followed by a BLAST search, revealed the presence of genera commonly cultured from chronic wounds (*Staphylococcus*, *Pseudomonas*) as well as genera of strictly anaerobic bacteria (*Prevotella*, *Porphorymonas*). In this study, strict anaerobes were not cultured using standard clinical techniques. Again these results underscore the diversity of wound microbial communities and the limitations of

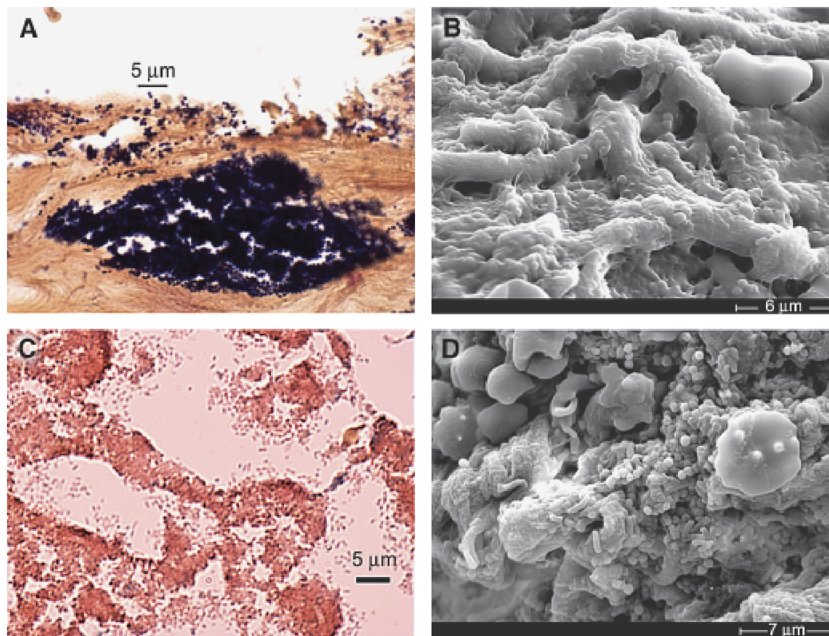

Figure 1. Microscopic images from chronic wounds. (A) Photomicrograph of a Gram-stained thin section from a pressure ulcer specimen showing a biofilm formed by Gram-positive cocci near the tissue surface. Gram-positive cocci were commonly observed in samples examined using light microscopy, which agreed with culture analysis that revealed the predominance of *Staphylococcus* and *Enterococcus*. (B) Scanning electron micrograph of pressure ulcer specimen showing a biofilm of coccoid bacterial cells colonizing collagen bundles within the wound. The bacterial cells are blanketed in extracellular polymeric substance, which had collapsed onto the cells during the dehydration steps of specimen preparation. (C) Photomicrograph of a Gram-stained thin section from a diabetic foot ulcer specimen showing a biofilm formed by Gram-negative rods near the tissue surface. (D) Scanning electron micrograph of pressure ulcer specimen showing bacteria of different morphotypes (rods and cocci) colonizing the wound within close proximity.

standard clinical culture techniques. The potential role of strictly anaerobic bacteria in wound infections has been under investigated because these organisms are difficult to culture.[12] One genus of bacteria commonly isolated from chronic wounds, *Enterococcus*, was not detected in the cloning and sequencing analysis. This is surprising because enterococci were routinely isolated from patients at the clinic (Table 2). These results may indicate that the methods used failed to extract or amplify *Enterococcus* DNA from the wound samples. Further molecular characterization of chronic wound biofilms will be necessary to fully elucidate the diversity and structure of these complex communities.

Table 2. Bacteria isolated from wounds of subjects that provided specimens for microscopic analysis

Genus	Chronic %	Acute %
Staphylococcus	65	60
Enterococcus	62	80
Pseudomonas	35	20
Proteus	24	20
Citrobacter	24	20
Enterobacter	24	20
Streptococcus	22	0
Escherichia	14	0
Morganella	8	0
Klebsiella	5	0
Acinetobacter	5	0
Serratia	3	0
Xanthomonas	3	0

Overall, this study provides evidence that biofilms are prevalent in chronic wounds and rare in acute wounds. These biofilms were often diverse polymicrobial communities. Although the role of biofilms in delayed healing of chronic wounds remains to be elucidated, the presence of biofilms and similarities between chronic wounds and

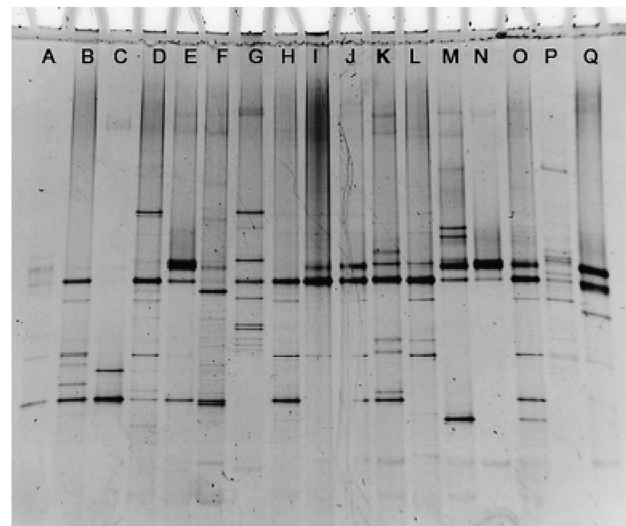

Figure 2. Photograph of a denaturing gradient gel electrophoresis gel showing results from 17 chronic wound specimens. Theoretically, each band represents a distinct bacterial species. Most of the specimens had multiple bands and each specimen had a unique banding pattern. Wound types included diabetic foot ulcers (A, E, I, M, N, P, Q), pressure ulcers (D, G, H, J), venous leg ulcers (C, K, L), and other (B, F, O).

Table 3. Subject/wound information and results of molecular analysis

	Subject/wound information				Molecular analysis	
Specimen No.	Sex (age)	Wound type	Wound location	Wound size (L×W×D cm)	BLAST matches	DGGE bands*
83	F (65)	VLU	A	6×5×0.25	*Pseudomonas* sp. *Pseudomonas putida* *Rhodococcus erythropolis*	4
84	M (54)	O	Le	4×3×0.25	*Actinobacterium* sp.	4
85	F (43)	DFU	F	2×1×0.5	nd	4
86	M (52)	DFU	F	2×1×1	nd	7
87	M (72)	O	Le	8×3×0.25	nd	6
88	F (62)	DFU	F	2×1.5×0.5	nd	2
89	F (52)	DFU	F	1×0.5×0.25	nd	5
90	F (81)	VLU	Le	15×11×1	nd	5
91	M (81)	VLU	Le	10×6×0.5	nd	7
92	F (87)	PU	F	2×2×0.25	nd	4
93	M (75)	DFU	F	3×1×0.5	nd	2
94	M (23)	PU	Hip	3×1.5×2	nd	4
95	M (36)	PU	Hip	8×4×2	nd	6
96	F (26)	O	T	4×2×1	nd	4
97	F (82)	DFU	F	2×1×0.25	nd	3
98	M (31)	PU	Hip	NA	nd	5
99	F (56)	VLU	Le	0.25×0.25×0.25	nd	2
100	M (65)	O	Lb	6×4×2	nd	6
101	M (69)	DFU	F	3×1.5×0.25	nd	5
102	M (77)	VLU	A	2×2×0.5	*Staphylococcus aureus* *Staphylococcus* sp.	nd
103	F (49)	PU	O	2×2×0.25	*Pseudomonas aeruginosa* *Pseudomonas* sp.	nd
104	F (67)	VLU	Le	3×3×0.25	*Staphylococcus aureus*	nd
105	M(34)	PU	Le	8×4×2	*Haemophilus* sp. *Prevotella oralis* Clostridia sp.	9
106	M (80)	O	A	3×4×0.5	*Pseudomonas* sp.	nd
107	M (69)	O	F	3×2×0.25	*Staphylococcus epidermidis*	nd
108	NA	O	NA	NA	*Clostridia* sp. *Streptococcus* sp. *Bacteroidetes* sp.	nd
109	M (60)	PU	F	3×3×0.5	*Porphyromonas somerae* *Streptococcus* sp.	13

A, ankle; B, blister; DFU, diabetic foot ulcer; E, elbow; F, foot; Gt, great toe; Ha, hand; He, heel; I, ischium; K, knee; L, left; Lb, lower back; Le, lower extremity; PU, pressure ulcer; R, right; St, skin tear; Sa, sacrum; Sh, shin; T, thigh; Ue, upper extremity; VLU, Venous leg ulcer; O, other included surgical site infections and traumatic wounds; NA, not available; nd, not determined.
*DGGE bands are an approximate number.

other biofilm diseases suggest that further research in this area is warranted. Furthermore, if biofilms are involved in chronic wounds, treatments that specifically target biofilms may aid in the healing of these wounds.

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