The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods

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ABSTRACT

The bacterial microbiota plays an important role in the prolonged healing of chronic venous leg ulcers. The present study compared the bacterial diversity within ulcer material from 14 skin graft operations of chronic venous leg ulcers using culture-based methods and molecular biological methods, such as 16S rRNA gene sequencing, fingerprinting, quantitative polymerase chain reaction, and fluorescence in situ hybridization. Each wound contained an average of 5.4 species but the actual species varied between wounds. The diversity determined by culture-based methods and the molecular biological methods was different. All the wounds contained *Staphylococcus aureus*, whereas *Pseudomonas aeruginosa* was in six out of 14 wounds. Molecular methods detected anaerobic pathogens in four ulcers that were not detected with anaerobic culture methods. Quantitative polymerase chain reaction was used to compare the abundance of *S. aureus* and *P. aeruginosa* at different locations in the ulcers and their numbers varied greatly between samples taken at different locations in the same ulcer. This should be considered when ulcers are investigated in routine clinical care. The differences between the results obtained with culture-based and molecular-based approaches demonstrate that the use of one approach alone is not able to identify all of the bacteria present in the wounds.

Chronic venous leg ulcers (CVLU) are a debilitating and often painful disease that affects approximately 1% of the world's population.[1,2] Apart from the human consequences, the treatment of wounds is expensive; in Denmark alone, wound treatment has been estimated to cost approximately two billion Danish kroner per year (~US$360 million),[2] and in the United Kingdom, France, and Germany an estimated 1.5–2% of the annual healthcare budget.[3,4]

The conditions leading to a CVLU are not fully understood; however, the primary cause is most likely insufficient valvular function of the veins in the legs causing increased hydrostatic pressure leading to edema of the subcutaneous tissue, which predispose to ulceration. This is linked to old age, obesity, height of the person, hereditary increased risk, number of births (more births lead to increased risk) and occupations in which the person is mainly standing. By removing edema with compression therapy, most CVLU will heal, but a number of ulcers will not despite effective treatment. In these cases, a well-documented and effective treatment is surgical debridement and split skin transplant.[2] Other treatments like topical negative pressure therapy have been found useful. Maggot debridement therapy have also proved promising, which involves having larvae from the fly *Lucilia sericata* removing necrotic tissue and bacteria from the wound, and in this way aiding the wound healing process.[5]

One of the factors affecting the effectiveness of wound healing therapies is the specific microorganisms that colonize the CVLU.[6] For example, the presence of *Pseudomonas aeruginosa* can retard the healing of wounds due to their ability to form biofilms.[6] Many studies describe biofilm as an important factor for the chronic behavior of chronic wounds,[6–10] and the spatial organization of these biofilms in a wound might be complex due to, for example, variations in environmental conditions and population composition.[11] Initial experiments by Bjarnsholt et al.[6] showed that *P. aeruginosa* in CVLU were assembled in microcolony-based structures unevenly distributed across the wound surface, and this uneven distribution might lead to insufficient sampling and wrong diagnosis.[6]

Until recently, the bacteria associated with CVLU have only been examined by culture-dependent methods by taking a swab or biopsy from the wound and using it as inoculate for various bacterial cultures. The emergence of molecular biology methods has illustrated that culture-dependent methods often underestimate the bacteria present, and especially ulcers with slow growing, fastidious, or anaerobic microbes.[9,12–14] Davies et al.[15] found that 40% of the organisms identified from CVLU by molecular biological methods could not be identified by culture-dependent methods, although most were species that are normally considered culturable.

The purpose of this study was to investigate the microbial diversity of chronic ulcers with molecular biological

Table 1. Summary of patient data*

Wound	Age of patient	Sex	Treatment of sample before extraction	Antibiotic treatment	Dressing at time of sampling	Duration of ulcer	Additional information
A	85	Male	DNA extracted from the entire wound	None	Nonsilver	12 months	
B	76	Male	DNA extracted from the entire wound	None	Nonsilver	6 months	Diabetic
C	54	Male	DNA extracted from the entire wound	None	Aquacell Ag	Years	
D	87	Female	DNA extracted from the entire wound	None	Nonsilver	4 months	
E	85	Female	Wound was cut into five parts and DNA extracted separately	None	Biatain AG	7 months	
F	71	Female	Wound was cut into five parts and DNA extracted separately	Sulfametizole due to urinary tract infect	Biatain AG	5 months	Diabetic
G	88	Female	DNA was extracted from six biopsies across the wound	None	Biatain AG	4 years	
H	82	Male	DNA was extracted from six biopsies across the wound	None	Nonsilver	6 months	Diabetic
I	81	Female	DNA was extracted from six biopsies across the wound	Phenoxymethylpenicillin until 2 months before sampling	Nonsilver	4 years	
J	78	Female	DNA was extracted from six biopsies across the wound	Phenoxymethyl-penicillin	Nonsilver	6 months	
K	65	Male	DNA was extracted from four biopsies across the wound	None	Nonsilver	6 months	Diabetic, impetigo
L	85	Female	DNA was extracted from four biopsies across the wound	None	Biatain AG	7 months	
M	69	Female	DNA was extracted from four biopsies across the wound	None	Nonsilver	6 months	
N	46	Male	DNA was extracted from four biopsies across the wound	None	Nonsilver	3 years	Sample from Achilles tendon
Average age	75.2						

*All DNA extractions were done using a DNeasy Blood and tissue kit except for the samples from wound F and wound E (center), which was extracted with an E.Z.N.A. Tissue DNA kit due to their greater size. Registered antibiotic treatment 3 months before sampling is mentioned.

methods and to compare these results with the conventional culture-dependent techniques. Furthermore, the spatial organization of bacteria in CVLU was examined.

MATERIALS AND METHODS

Patient population, sampling, and DNA extraction

The excision of biopsies and swabs of the wounds for culture-dependent and -independent experiments was performed by Copenhagen Wound Healing Center, Bispebjerg Hospital (Copenhagen, Denmark). Samples were obtained from patients diagnosed with chronic venous leg wounds just before surgical debridement and split skin transplant. In total, chronic wounds from 14 patients were investigated (named as wound A–N). The patients' age, sex, antibiotic treatment, dressings at the time of sampling, and additional information are described in Table 1. Patients with wound B, F, H, and K were also diagnosed with diabetes mellitus.

All ulcers were chronic and nonhealing despite optimal wound care and compression therapy. The duration of the ulcers are shown in Table 1. The patients were not receiving antibiotic treatment during the three months before sampling with three exceptions: Patient F was receiving sulfonametizole at the time of sampling, and patients J and I had received phenoxymethylpenicillin up until 3 days and 2 months before sampling, respectively. Five of the patients' wounds had been dressed with a silver-releasing dressing in the period before sampling (patient C, E, F, G, and L). The samples were collected with the patients' acceptance and in accordance with the biomedical project protocol (KA-20051011) approved by the Danish scientific ethical board.

On the day of surgery, the area surrounding the ulcer was swabbed with chlorhexidine in 70% alcohol but the surface of the ulcer was not disturbed. The excised wound material from the patient was transferred to a sterile Greiner tube and stored at $-20\,^{\circ}C$ until DNA extraction.

Before DNA extraction, the frozen wounds were thawed and cut to smaller pieces using sterile disposable scalpels. The total DNA content of wound F and E was extracted using an E.Z.N.A Tissue DNA kit (Omega Bio-Tek, VWR, Herlev, Denmark). Other wounds were cut to smaller pieces and were extracted using a DNeasy Blood and Tissue kit from Qiagen (Hilden, Germany). Both kits are based on proteinase K digestion for 2–4 hours.

Culture analysis

Identification of bacteria from the wounds by culturing was performed by the Department of Clinical Microbiology, Hvidovre Hospital, according to their standard protocols. Tissue samples were transported in sterile containers and swabs were transported in Stuart medium. Anaerobe culturing was performed on anaerobe plates (Statens Serum Institute [SSI], Copenhagen, Denmark) in a CO_2 atmosphere at $37\,^{\circ}C$ for 2 and 4 days. Aerobe culturing was performed on horse-blood agar (SSI) and Blue plates (SSI) for 1 and 2 days, respectively.

16S rRNA gene amplification

The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using *Taq* DNA polymerase with primers targeting conserved domains. The primers were 8F[16] and 1390R[17] and the samples were amplified according to Thomsen et al.[18] Negative controls including water and PCR mix were included for every five samples and were always negative indicating that there was no contamination of the reagents. Stringent procedures were generally used to avoid contamination, e.g., by using a PCR cabinet with UV light and all DNA handling was carried out with aerosol filter pipette tips to avoid cross contamination.

Cloning, sequencing, and phylogenetic studies

The amplified 16S rRNA gene products were purified with a Qiaquick PCR purification kit (Qiagen), according to the manufacturer's instructions. Cloning was performed using a TOPO TA Cloning® kit (Invitrogen, Taastrup, Denmark) for sequencing. Plasmids were purified using a Fastplasmid mini kit (Eppendorf, Horsholm, Denmark) and purified plasmids were amplified using M13 primers to test for inserts with the correct length. The plasmids were sequenced by Macrogen Inc. (Seoul, South Korea) using the M13F primer. The closest relative of the clones were identified by performing a BLAST search of the sequences at http://www.ncbi.nlm.nih.gov/blast. At least one representative clone from each species was additionally sequenced using the M13R primer, in order to obtain consensus sequences covering the entire length of the DNA fragments. Checks for chimeric sequences were conducted using the program BELLEROPHON.[19]

The ARB software[20] was used for the alignment of imported sequences with the FastAligner tool, and alignments were subsequently refined manually and phylogenetic analysis was performed. Only unambiguously aligned sequences were used for the calculation of trees using distance matrix, parsimony, and maximum likelihood approaches using default settings in the ARB software. The *Bacteria* sequence conservation filter of the ssu_jan04_corr_opt ARB database [available at http://www.arb-home.de]) in addition was applied. Phylogenetic trees were initially constructed using the consensus sequences representing the different groups of bacteria. Subsequently, partial sequences were added to the existing consensus trees by the "add species to existing tree" function in the ARB software. Priorly, a filter was carried out to define which positions to be used in adding the partial sequences (data not shown). Generally, the results obtained by the NCBI Blast Search corresponded well to the phylogenetic identifications. The coverage ratio (C) for each of the clone libraries were calculated with $C = (1 - (n1 \cdot N^{-1})) \cdot 100\%$ where $n1$ is the number of operational taxonomic units (OTUs) containing only one sequence and N is the total number of clones analyzed.[21]

Denaturant gradient gel electrophoresis (DGGE) fingerprinting

Amplification of samples for DGGE was performed using primers 341F-GC[22] and 907R.[17] The PCR products were

run on 8% polyacrylamide gels containing denaturant gradients of 30–70%, in 1×TAE buffer at 100 V overnight using the D-GENE™ gel system (Biorad) and stained with SYBR Gold (Invitrogen). The most intensive DGGE bands were excised and prepared for sequencing. The excised bands were reamplified with PCR, and the PCR products were thereafter purified using a NucleoSpin Extract II Machery Nagel and sequenced commercially by Macrogen Inc.

Quantitative PCR (qPCR)

Pure culture DNA was extracted using a FastDNA® Spin Kit for Soil (MP Biomedicals, LLC, Illkirch, France), according to the manufacturer's instructions. qPCR targeting the *nuc* gene[23] and *oprL* gene[24] was used to measure the amount of *Staphylococcus aureus* and *P. aeruginosa*, respectively. For each determination, triplicates of 20 µL reactions were run with each containing: 12.5 µL Brilliant® II SYBR® Green qPCR Master mix (Stratagene, AH Diagnostics, Aarhus, Denmark), 25 µg BSA, 10 µM of each primer, and 0.75 µM reference dye and 5 µL of template or standard. Reactions were run on an Mx3005P (Stratagene) for 10 minutes at 95 °C, 40 cycles of 30 seconds at 95 °C, 30 seconds at 62 °C (*nuc*)/ 62 °C (*oprL*), 60 seconds at 72 °C and 15 seconds, and SYBR data capture at 80 °C (*nuc*)/ 82 °C (*oprL*). For *S. aureus,* the specific product was separated at 79 °C and for *P. aeruginosa* at 90 °C. The specificity of the PCR reactions performed for each run was confirmed by the melting curve analysis and gel electrophoresis. Standard curves were prepared from serial dilutions of *S. aureus* (DSM 6148) and *P. aeruginosa* (DSM 1253) genomic DNA (5×10^6–5×10^1) in AE buffer (Qiagen). The limit of detection was 100 gene copies per PCR.

Fluorescence in situ hybridization (FISH)

After removal from the patient, the tissue sample was transferred to 4% neutral formaldehyde buffer and embedded in paraffin wax, cut into 4-µm–thick slides, and stored at room temperature. Before the hybridization, the paraffin was removed by xylene. The slides were treated using a Histology FISH Accessory Kit from DAKO cytomation according to the protocol. Hybridization was performed by covering the slide with 20 µL of hybridization buffer containing 0.9 M NaCl, 0.02 M Tris/HCl, 0.01% SDS, and formamide, depending on the requirement of the probes and probe mix (5 ng/µL). The probes used were an EUB mix (EUB-338,[25] EUB II-338,[26] and EUB III-338[26]) targeting most *Bacteria*; BET42a with GAM42a competitor[27] targeting most *Betaproteobacteria*; a mix of LGC354b, LGC354A, and LGC354C[28] targeting the *Firmicutes*, and probe Sau[29] targeting *S. aureus*. For more information about the probes, consult probeBase.[30] Lastly, the slides where treated with Vectashield hardset mounting medium with DAPI (4′,6-diamidino-2-phenylindole). Unspecific binding was examined by applying Non-EUB probes on a slide as described above. This revealed sporadic nonspecific binding but only with little signal intensity, and hence it was possible to use probes to examine CVLU. PNA FISH was performed as described previously.[10]

Nucleotide accession numbers

GenBank accession numbers for 16S rRNA gene consensus sequences determined in this study are EU931393-EU931450.

RESULTS

Culture analysis

Culture analysis of the 14 wounds (A–N) showed the presence of more than one species in all but one of the wounds (Tables 2 and 3). Although a diversity of other bacteria were isolated, *S. aureus* was detected in 13 wounds, *P. aeruginosa* in six, *Klebsiella oxytoca* in three, and *Enterococcus* sp. in three wounds. No obligate anaerobic species were detected in any of the wounds.

DGGE fingerprinting

The results of DGGE fingerprinting are shown in Tables 2 and 3, indicated by an "S." DGGE detected *S. aureus* in all of the wounds except wound C, despite *S. aureus* being detected by the culture methods. Wound E and F showed the presence of additional uncultured bacteria. DGGE showed that the wounds also contained a variety of anaerobic bacteria with multiple findings of species such as *Finegoldia magna, Anaerococcus vaginalis, Peptoniphilus asaccharolyticus, Peptoniphlus harei,* and *Peptostreptococcus anaerobius,* often with several of these species in the same wound. *P. aeruginosa* was detected in only one wound with DGGE fingerprinting despite its detection in six wounds using the culture methods. An average of 3.2 species per wound were detected using DGGE fingerprinting and 3.0 species per wound were detected using culture methods. In combination, DGGE and culture identified 5.4 species per wound.

Clone library and sequence analysis

To elucidate the bacterial diversity in the samples, clone libraries were constructed where the amplified 16S rRNA genes were inserted into cloning vectors, thereby a separation of the different fragments and its subsequent sequencing were possible. The sequences from the two clone libraries (clone library 1 from wounds A–F and clone library 2 from wounds G–N) were divided into OTUs using a similarity level of $> 97\%$. A total of 60 clones were sequenced for clone library 1 and 94 clones for library 2. Table 4 shows the name and accession number of the closest relative for each OTU as identified by the phylogenetic analysis.

Clone library 1 showed many *S. aureus* and some *Alcaligenes* sp., *Anaerococcus* sp., *Stenotrophomonas* sp., *Enterococcus faecalis,* and *P. aeruginosa*. Clone library 2 showed a large amount of *S. aureus* and *P. aeruginosa*. Almost all OTUs have a similarity of $> 97\%$ with their closest relatives. Only OTU 9 (uncultured *Anaerococcus*) in clone library 1 and OTU 10 *Helcococcus kunzii* in clone library 2 had a smaller similarity than 97% indicating that these OTUs had a lower phylogenetic resolution. The coverage ratio for the clone library 1 was 87.7% and for clone library 2 was 93.5%.

Table 2. A condensed overview of the bacteria found in wound A–F[1]

Species	Clone lib. 1	A	B	C	D	E	F
Staphylococcus aureus	+	S, C, 220 ± 6%	S, ND	C, ND	S, C	S,C,*	S,C,*
Pseudomonas aeruginosa	+	ND	ND	C, ND		C,*	C,*
Staphylococcus sp.	+	S			S	S,C	S,C
Stenotrophomonas maltophilia	+			S			
Alcaligenes sp.	+			S			
Enterococcus sp.	+	C					
Enterococcus faecalis	+		C			S	
Actinobaclulum schaalii	+					S	
Helcococcus kunzii	+						S
Finegoldia magna	+					S	
Staphylococcus cohnnii			S				
Corynebacterium amycolatum			S				
Achromobacter xylosoxidans				S			
Unidentified Gram-negative rod		C					
Proteus sp.			C				
Morganella morganii			C				
Klebsiella oxytoca					C		
Enterobacter cloacae							C
Peptoniphilus sp.						S	
Uncultured *Clostridia*						S	
Uncultured *Clostridia*							S
Uncultured *Porphyromonas*						S	
Uncultured *Bacterium*							S

[1]Bacteria identified from wounds A–F using culture-based methods (C) and sequencing of DGGE bands (S). Quantitative PCR data are presented for *S. aureus* and *P. aeruginosa* (copies/ng DNA ± standard error of the mean, n=3).
*The spatial orientation of bacteria was examined in wound D, E, and F revealing a diverse microbiota in wound E and F. Data for these two wounds are described in Table 5. Sequences also found in Clone library 1 are indicated with ''+''.
ND, not detected.

The consensus sequences in clone library 1 and 2 were used to produce phylogenetic trees to determine the detailed phylogenetic relationship of the 16S rRNA gene of the clones. A neighbor joining tree, a maximum parsimony tree, and a maximum likelihood tree all showed congruent phylogenetic relationships, and the maximum likelihood tree is shown in Figure 1. The locations on the tree confirm the BLAST identification of the sequences. The sequences are distributed into five phyla: *Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria,* and *Actinobacteria*. Similar bacteria were identified in the two clone libraries, although clone library 1 did not detect any bacteria from the phylum *Fusobacteria* and clone library 2 did not detect any *Bacteriodetes*. The clone libraries were dominated by sequences related to *S. aureus* and *P. aeruginosa*, but also contained many sequences from *E. faecalis, Alcaligenes faecalis,* and *Stenotrophomonas maltophilia*.

All 110 partial 16S rRNA gene sequences obtained from DGGE were added to the consensus maximum likelihood tree (data not shown) to confirm the result of the BLAST search. While the BLAST result was confirmed for most of the sequences, the phylogenetic analysis showed that it was not possible to distinguish the sequences identified as different *Alcaligenes* and *Ahcromobacter* species and no *Peptoniphilus* could be differentiated to more than the genus level. It also showed that the DGGE fingerprinting sequences most related to *Fusobacterium equinum* according to the BLAST were located closer to *Finegoldia gonidiaformans* on the tree. *F. gonidiaformans* was also found in clone library 2.

Quantitative PCR

The abundance of *S. aureus* and *P. aeruginosa* was found to vary considerably between the different wounds (Tables 2 and 3). While *S. aureus* could be detected by DGGE and by culturing in most samples, they were only above the limit of detection using the qPCR approach in four of the 14 ulcers investigated. *P. aeruginosa* could be quantified in three of the ulcers investigated.

Spatial location

To determine whether the bacterial composition varied throughout the wound, three wounds (D–F) were each

Table 3. A condesed overview of the bacteria found in wounds G–N*

Species	Clone lib. 2	Wounds G	H	I	J	K	L	M	N
Staphylococcus aureus	+	S, C, ND	S, C, 120 ± 14%	S, C, 5600 ± 13%	S, C, NT	S, C, NT	S, NT	S, C, 100 ± 5%	C, ND
Pseudomonas aeruginosa	+	C, 1400 ± 18%	C, ND	ND	NT	S, C, NT	NT	ND	ND
Alcaligenes sp.	+		S						
Proteus mirabillis	+			C					
Alcaligenes faecalis	+			C					
Enterococcus sp.	+			C					
Coagulase negative staphylococci	+				C	C			C
Staphylococcus epidermidis						S			
Peptoniphilus harei		S							S
Finegoldia magna		S					S		S
Fusobacerium equinum		S							
Peptostreptococcus anaerobius		S							
Peptoniphilus asaccharolyticus			S	S					S
Uncultured *Clostridia*				S					
Anaerococcus vaginalis							S	S	
Peptostreptococcus micros								S	
Corynebacterium sp.								S	C
Brevibacterium casei					S				
Gram-negative rod		C						C	
Morganella morganii		C							
Escherichia coli-like rod			C						
Hemolytic *Streptococcus*			C					C	
Klebsiella-like rod				C					
Klebsiella oxytoca				C					
Bacillus sp.					C				
Enterobacter cloacae								C	

*Bacteria identified from wounds G–N using culture-based methods (C) and sequencing of DGGE bands (S). Quantitative PCR data are presented for *S. aureus* and *P. aeruginosa* (copies/ng DNA ± standard error of the mean, n=3). Sequences also found in Clone library 2 are indicated with ''+''.
ND, not detected, NT, not tested.

divided in five parts and DNA was extracted from each of them. Each part was separately examined by DGGE fingerprinting and by subsequent sequencing of bands (Table 5). In wound D, only *S. aureus* could be detected by DGGE fingerprinting and it was present in all examined parts of the wound (data are not included in Table 5). Wound E was dominated by the aerobe *S. aureus*, the facultative aerobe *E. faecalis,* and the two anaerobes *Actinobaculum schaalii* and *F. magna,* and wound F was dominated by *S. aureus* and an uncultured *Clostridia* bacterium.

S. aureus and *P. aeruginosa* qPCR detected these species in all parts of wound E and F, except in subsample 3 in wound E (E3) (Table 5). The abundance of *S. aureus* and *P. aeruginosa* was, however, found to vary significantly depending on the location in the wound. This was particularly apparent for *P. aeruginosa,* which varied by three orders of magnitude in the various samples from wound F. Thus, not only the bacterial diversity but also the abundance of organisms were found to vary throughout the wound. To examine further the spatial organization of the CVLU, thin histological slides of wound H and another CVLU known to contain *P. aeruginosa* were produced and examined with FISH and PNA-FISH (Figure 2). It was found that the bacteria on the histological slides known to contain *P. aeruginosa* were located very locally (areas of

Table 4. Closest relatives of the bacterial OTUs in clone libraries

OTU	Number*	Species (BLAST)	Acc. number	Similarity (%)
Clone library 1				
1	[8/28]	*Staphylococcus aureus*	BX571856	97.1–100
2	[2/6]	*Alcaligenes* sp.	AY331576	99–100
3	[2/4]	*Anaerococcus* sp.	AM176522	99
4	[4/4]	*Stenotrophomonas* sp.	AM402950	99–100
5	[1/3]	Uncultured *Porphyromonas*	DQ130022	99–100
6	[2/3]	*Enterococcus faecalis*	DQ239694	99–100
7	[1/2]	*Pseudomonas aeruginosa*	EF064786	99–99.6
8	[1/1]	*Anaerococcus vaginalis*	AF542229	98
9	[0/1]	Uncultured *Anaerococcus*	DQ029049	95
10	[1/1]	*Enterobacter* sp.	EF088367	99
11	[1/1]	*Bacteroides tectus*	AB200228	99
12	[1/1]	*Actinobaculum schalli*	AF487680	98
13	[1/1]	*Helcococcus kunzii*	X69837	97
14	[1/1]	*Finegoldia magna*	AB109772	99
Total	57			
Clone library 2				
1	[7/46]	*Staphylococcus aureus*	DQ997837	98.8–99.9
2	[6/14]	*Pseudomonas* sp.	AY914070	98.7–99.0
3	[3/3]	Uncultured bacterium	EF511972	99.7–99.9
4	[1/3]	*Fusobacterium gonidoformans*	M58679	98.6–99.8
5	[2/2]	*Enterococcus faecalis*	DQ239694	99.8–100
6	[2/2]	*Acinetobacter junii*	AB101444	99.9
7	[1/2]	*Proteus mirabilis*	AF008582	98.6–99.8
8	[1/1]	*Actinobaculum schaalii*	AY957507	98.4
9	[1/1]	*Alcaligenes faecalis*	AY548384	97.2
10	[1/1]	*Helcococcus kunzii*	X69837	96.7
11	[1/1]	Uncultured bacterium	AM697030	98.2
12	[1/1]	Uncultured Clostridia	AY383733	99.7
Total	77			

approximately 150 μm) and nowhere else. This made it difficult to locate the area of infection if present. Wound H was examined to see if the bacteria found with DGGE fingerprinting could be located. It was possible to find small populations of *S. aureus* and *Alcaligenes* sp. using specific probes, thus confirming their presence but no large area of infection could be located.

DISCUSSION

There is an emerging body of evidence that bacteria play an important role in the persistence of chronic wounds. Using culture-based methods, the most frequently observed bacteria in CVLUs are *S. aureus*, *P. aeruginosa,* and *E. faecalis,* but the diversity is generally polymicrobial and heterogeneous.[31] To improve treatment of CVLUs, it is necessary to identify whether the most frequently detected bacteria are the critical causative agents or if other bacteria may also contribute to wound persistence. The choice of the analytical method, mode of sampling and the compositional variety of the wounds all play an important role in the results obtained from bacteriological studies. Some studies have been conducted to identify the important bacteria in wounds, however, the conclusions from the studies differ. Stephens et al.[8] focused on anaerobic bacteria and concluded that anaerobic bacteria play an important role in mediating the chronicity of CVLU. Gjodsbol et al.[32] in comparison suggested that *P. aeruginosa* is most important, rather than anaerobes, as it is *P. aeruginosa* that induces ulcer enlargement and delays healing. In the present study, it was examined how molecular methods could contribute to the characterization of the bacteria in CVLUs. As has been reported previously, the molecular biological methods uncovered a different and more diverse microbiota than the culture-based methods. Bacteria were detected that had not previously been identified from wounds but the potential virulence of these bacteria and their impacts on wound healing needs further investigation. Ultimately, the eventual significance of the different wound bacteria requires the determination of their pathogenesis and in order to do this, all of the bacteria that are present must be identified. The differences

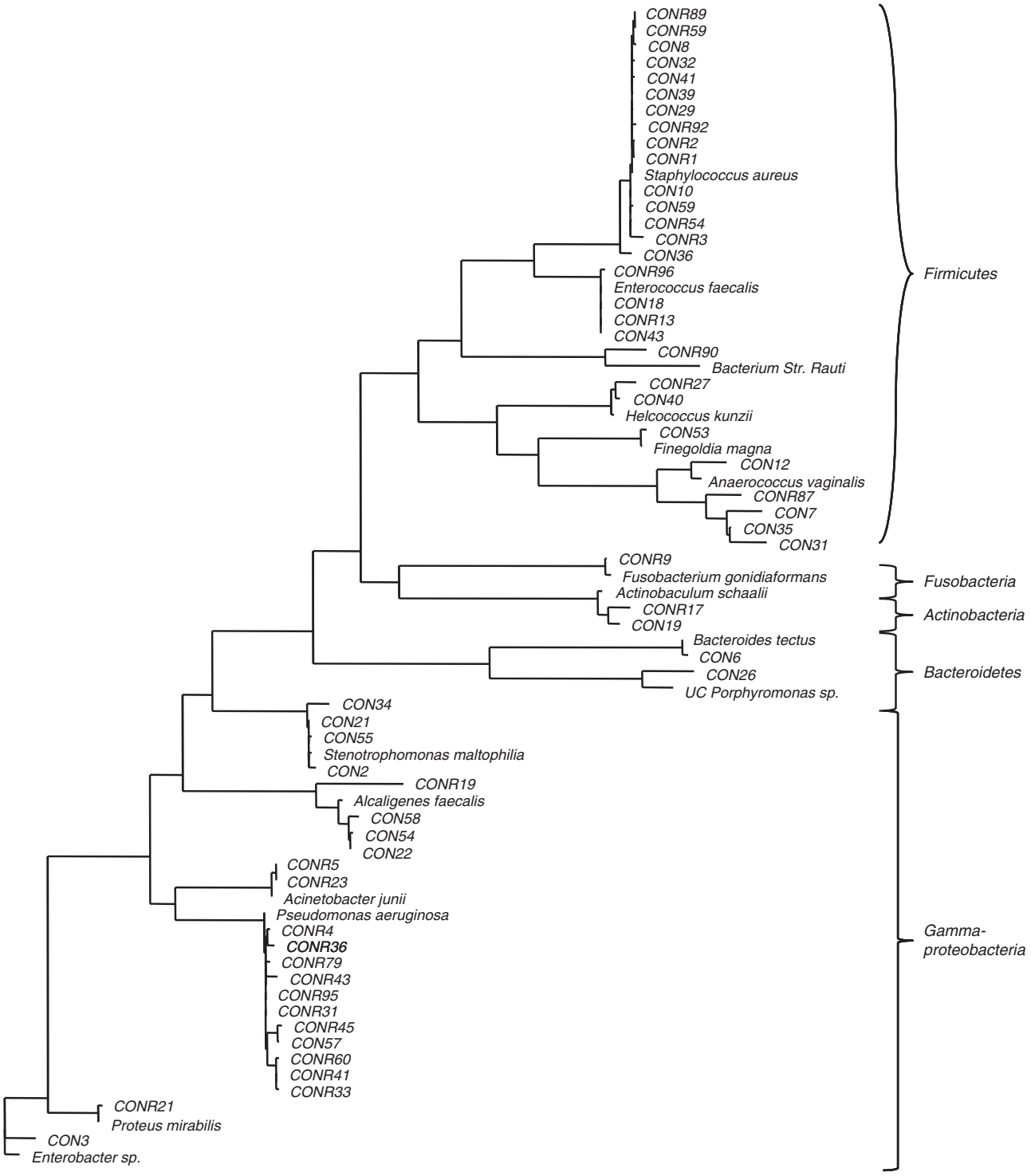

Figure 1. Maximum likelihood (AxML) tree of consensus sequences (1364 nt compared) of consensus sequences from clone library 1 (CON#) and 2 (CONR#). The scale bar represents a 10% deviation of sequence.

Table 5. A condensed overview of the spatial orientation of bacteria found in wounds E and F*

		Wound parts									
Species	Clone lib. 1	E, C	E, 3	E, 6	E, 9	E, 12	F, C	F, 3	F, 6	F, 9	F, 12
Pseudomonas aeruginosa		510 ± 18%	NT	760 ± 7%	47 ± 9%	280 ± 3%	920 ± 9%	300 ± 13%	8200 ± 8%	800 ± 10%	15 ± 5%
Staphylococcus aureus	+	S, 89 ± 11%	B, NT	B, 240 ± 10%	B, 310 ± 13%	S, 180 ± 8%	S, 200 ± 2%	S, 86 ± 8%	B, 290 ± 8%	B, 80 ± 5%	B, 93 ± 12%
Staphylococcus sp.	+	S	B	B	B	S	B	B	S	S	B
Enterococcus faecalis	+	S	S	S	S						
Enterococcus sp.			S							S	
Actinobaculum schaalii	+	S	B	B	B	B					
Helicococcus kunzii	+							S			
Finegoldia magna	+	B	B	B	B	S					
Peptoniphilus sp.				B		S					
Uncultured *Clostridia bacterium*						S					
Uncultured *Clostridia bacterium*							B	S		B	B
Uncultured *Porphyromonas* sp.		B		B		S					
Uncultured bacterium							S	B		B	

Besides wound E and F, clone library 1 represented wounds A–D.
*The spatial orientation of wounds E and F was examined by applying molecular methods on samples taken at the center (C), and at approximately 3, 6, 9, and 12 o'clock around the wounds' periphery. Bacteria were identified by sequencing DGGE bands (S) and putatively identified by comparison of bands to the sequenced bands at the same position on the gel (B). Sequences also found in Clone library 1 are indicated with ''+''. Quantitative PCR data are presented for *S. aureus* and *P. aeruginosa* (copies/ng DNA ± standard error of the mean, n=3). [I]NT, not tested; PCR, polymerase chain reaction.

between the results obtained with the culture-based and the molecular-based approaches demonstrate that the use of one of the methods alone might miss potentially important information about the bacteria present.

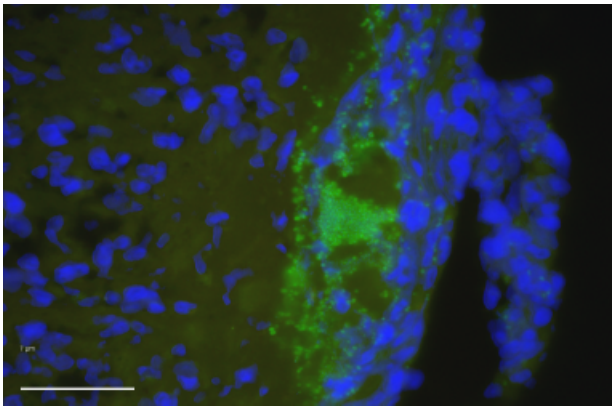

Figure 2. A PNA-FISH micrograph. The green color is a general probe for all bacteria and the picture was counter stained with DAPI, a DNA stain to visualize the localization of the host cells (blue).

Comparison of culture and molecular biological methods

All of the examined wounds contained a unique microbiota. The DGGE fingerprinting and culture method identified an average of 3.1 and 3.0 bacterial species per wound, respectively. Combined, 5.4 species were identified per wound. In accordance with previous reports, e.g.,[13] separate bands were observed in some lanes in the DGGE gels representing the same species. This may be due to more than one type of active 16S rRNA genes in the same species or the presence of different sub strains of the identified microorganisms differing in only one or a few base pairs. The presence of several species in the same wound complicates the task of determining which bacteria are mainly involved with infection. There might also be synergy between some species, e.g., predisposing or additive polymicrobial infections. For instance, species living in immunocompromised pockets created by different microorganisms are capable of killing leukocytes (like *P. aeruginosa*[6]).

The results of the culture experiments showed the presence of 12 different species in the analyzed wounds compared with 33 species found with molecular methods. None of the species found using culture methods were anaerobic. DGGE fingerprinting showed the presence of

anaerobic bacteria in wound G, H, I, M, and N. The anaerobic species are often overlooked by culture methods because they require longer culture times and previously lacked a valid identification scheme.[8] Many of the bacteria identified by DGGE fingerprinting have close relatives identified previously by culture experiments, and are therefore likely themselves to be culturable to some degree.

Some of the observed differences between the results obtained by the culture and the molecular methods could be due to the inability to differentiate species on the culture plates or that some specimens were collected as a biopsy and others using a swab. The differences may also be attributable to a fraction of the bacteria being dead or in a viable but unculturable state. This can be caused by the use of antibiotics[15]; however, only wound F was receiving antibiotics and indeed this wound showed the presence of only one species. The other two wounds, which had been treated with antibiotics until a short time before the study, both showed a diverse microbiota detected by culture methods. Based on these findings, there is no evidence of large amounts of residual genetic material from organisms no longer colonizing the ulcer bed.

Cultivation techniques have some limitations, but the molecular biological methods also have biases. These include amplification of naked DNA, unknown DNA recovery yields from extraction, differential amplification due to PCR primer bias, 16S rRNA copy number, and heterogeneity and co-migration of bands on DGGE fingerprinting. Some of the biases associated with, e.g., the DGGE approach were compensated by using the cloning approach, in which different primers were used with different specificities.

Diversity of CVLU bacteria

The clone library and DGGE analysis revealed a large diversity of bacteria of which some have not been associated previously with wounds: *Brevibacterium casei, Corynebacterium simulans*, *Corynebacterium amycolatum*. *A. schaalii, P. harei, F. gonidiaformans, Bacteroides tectus, Achromobacter xylosoxidans*, *A. faecalis,* and some uncultured bacteria. *B. casei* has been identified as an opportunistic pathogen in immunocompromised patients. The case reports by Reinert et al.[33] and Brazzola et al.[34] are examples, describing that *B. casei* needs a host with reduced immune system in order to initiate infection. Two other bacteria from phylum *Actinobacteria* (*C. simulans* and *C. amycolatum*) were also identified. The *Corynebacteria* are known as an aerobe and ubiquitous on human skin and are all opportunistic pathogens. *C. amycolatum* is frequently isolated from clinical specimens and infected wounds and it is resistant to most antibiotics[35] whereas *C. simulans* is a rare species found previously in blood and bile samples.[36] *A. schaalii* is a Gram-positive bacterium resembling normal skin flora and it is often overlooked by culture methods due to its slow growth in ambient air. Recently *A. schaalii* has been found as a pathogen in 10 cases of urinary infection.[37] *P. harei* belongs to the anaerobic Gram-positive family *Peptostreptococcaceae,* which is a heterogeneous family of opportunistic pathogens colonizing the skin and the mucosal surfaces of humans.[35] *H. kunzii* has been isolated previously from human skin and from diabetic foot wounds. It is mainly identified as a part of a polymicrobial community[38] but it has also been seen as the sole pathogen in a foot abscess.[39] The *Fusobacteria* are Gram-negative anaerobes found in the human gastrointestinal tract. Here, they are a part of the polymicrobial flora but they are also involved in a variety of different diseases.[40] The phylum *Fusobacterium* is often associated with chronic wounds.[41] *F. gonidiaformans* is a rare type of *Fusobacterium* species isolated previously from infected dog bites[42] and from skin infections.[43] In both surveys, the *F. gonidiaformans* constituted a very small percentage of the isolated bacteria. *A. xylosoxidans* and *A. faecalis* are both aerobe Gram-negative *Betaproteobacteria* from the *Alcaligenaceae* family. They are ubiquitous in the environment but rarely involved with human disease. They have been isolated from blood cultures of various immunosuppressed patients[44] and also appeared in a recent study of chronic wounds by Dowd et al.[12] The uncultured *Porphyromonas* (DQ130022) was identified previously from the forearm of a healthy human[45] and the uncultured bacterium (AY958901) was identified from the vaginal epithelium of a healthy woman.[46]

Phylogenetic analysis showed that the 33 different species belonged to six phyla. Both in terms of the number of different species and the number of identified clones, the *Proteobacteria* and the *Firmicutes* (Clostridia) were the dominating phyla. Gao et al.[45] examined the skin flora of healthy forearms in a large molecular biological study. They found that the dominating phylum was the *Actinobacteria,* although the *Firmicutes* and *Proteobacteria* were also present in high numbers. Healthy skin seems to be the only human environment where *Actinobacteria* are dominating.[45] In comparison, the inner mucosal surfaces of humans (e.g., colon and oral cavity) are dominated by *Firmicutes* and *Proteobacteria*.[45] This difference is probably due to environmental changes such as humidity and changes in pH value.

Eleven of the species were confirmed with both the cloning approach and DGGE fingerprinting. There was not a complete overlap between the findings of the two molecular methods and a reason for this might be that the DNA from the wounds was pooled before cloning on basis of the intensities of the bands on a gel. Another explanation might be that the primers used in the two methods had different affinity. Differences between the findings of the applied methods were also seen by Dowd et al.[12]

This study also indicated the presence of a varied anaerobic flora dominated by *F. magna* and *P. asaccharolyticus,* which were found in three wounds each. Table 3 (representing wound G, M, and N) also shows that the anaerobic species were often located in the same wound. This suggests that anaerobic pockets were present in the wound and that there is a possible synergistic effect between them. Stephens et al.[8] tested the effects of *P. vaginalis, F. magna,* and *P. asaccharolyticus* on cellular wound healing responses and found that they caused delayed reepitheliazation and defective extracellular matrix reorganization and angiogenesis in vitro. These are all important steps in wound healing. They also compared this with the effect of *P. aeruginosa* and found that this had less detrimental effect compared with the anaerobes.

Spatial orientation of bacteria in CVLU

The results from the DGGE approach investigating the spatial orientation of the bacteria in three wounds

illustrated that if only one biopsy from a wound was analyzed it would most likely not represent the bacterial composition of the entire wound. The qPCR results demonstrated that the abundance of *S. aureus* and *P. aeruginosa* also varied depending on the different locations in the wound. The technique is rapid and has recently been used to determinate *Pseudomonas* in a chronic wound within few hours, enabling fast decisions on treatment.[47] In addition, multiple biopsies from the same wound can also indicate which species of bacteria are most important for the infection as these are probably present in large numbers all over the wound. Furthermore, it supports the claim that the bacteria found in wounds are located in niches, which covers their needs. Using FISH, we detected bacteria in microcolonies also known as biofilms (Figure 2), which might explain how the bacteria survive inside the wound bed. This correlates with the finding that in some CVLU, *P. aeruginosa* live in large biofilms underneath the wound surface.[6] Antibacterial dressings, e.g., silver containing dressings are likely to influence the bacterial flora on the surface of the wounds. However, as the PNA-FISH pictures show that the bacteria reside deep in the tissue, it is not likely that bacteria will be influenced by the dressings. Furthermore, all swabs were taken after thoroughly surgical revision far away from local antimicrobial dressings. This indicates that the diversity was probably not influenced by the dressing, but by other factors such as antibiotics and difference in skin flora. The FISH technology increases the understanding of the pathology of bacteria in chronic wounds and how it might impact therapies.

This study compared the bacterial flora of different types of wound material from 14 skin graft operations of CVLU. Results from the culture methods were compared with the results from the molecular biological methods, which showed that the flora of the wounds varied, as did the number of *S. aureus* and *P. aeruginosa* investigated by qPCR. Each wound contained multiple species but apart from that the methods detected rather different floras. An average of 5.4 species were found in each wound by the methods combined. All of the wounds contained *S. aureus* but *P. aeruginosa* was also frequent. The molecular biological methods detected a varied anaerobic flora in four of the wounds and species not found previously in CVLU were identified. All of these were known pathogens. No anaerobes or new species were detected with culture methods. It was also found that the wound flora was different and that the number of the pathogens *S. aureus* and *P. aeruginosa* varied, depending on which location and depth of the wound was examined. Three wounds were examined and they showed that some species were present all over while some were only present in parts of the wounds. This emphasizes the need for multiple samplings when examining wounds, and swabs and biopsies each have specific advantages as sampling technologies.

qPCR is a promising fast method for fast characterization of the bacteria present in ulcers, and importantly the running cost is comparable with the cultivation techniques. The next important step is to elucidate the bacteria that contribute to the pathogenicity of these chronic wounds. This information could be used to develop the optimal sampling, identification, and treatment regimes.

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