Biofilms and Inflammation in Chronic Wounds

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Significance: The incidence, cost, morbidity, and mortality associated with non-healing of chronic skin wounds are dramatic. With the increasing numbers of people with obesity, chronic medical conditions, and an increasing life expectancy, the healthcare cost of non-healing ulcers has recently been estimated at \$25 billion annually in the United States. The role played by bacterial biofilm in chronic wounds has been emphasized in recent years, particularly in the context of the prolongation of the inflammatory phase of repair.

Recent Advances: Rapid high-throughput genomic approaches have revolutionized the ability to identify and quantify microbial organisms from wounds. Defining bacterial genomes and using genetic approaches to knock out specific bacterial functions, then studying bacterial survival on cutaneous wounds is a promising strategy for understanding which genes are essential for pathogenicity.

Critical Issues: When an animal sustains a cutaneous wound, understanding mechanisms involved in adaptations by bacteria and adaptations by the host in the struggle for survival is central to development of interventions that favor the host.

Future Directions: Characterization of microbiomes of clinically well characterized chronic human wounds is now under way. The use of *in vivo* models of biofilm-infected cutaneous wounds will permit the study of the mechanisms needed for biofilm formation, persistence, and potential synergistic interactions among bacteria. A more complete understanding of bacterial survival mechanisms and how microbes influence host repair mechanisms are likely to provide targets for chronic wound therapy.

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Abbreviations and Acronyms

AMP = antimicrobial peptide
EPS = extracellular polymeric substances
MMP = matrix metalloproteinase
QS = quorum sensing
TIMP = tissue inhibitors of metalloproteinase
TLR = toll-like receptors
Tn-seq = transposon-sequencing

SCOPE AND SIGNIFICANCE

THIS REVIEW PROVIDES a current overview of how microbial biofilm may participate in the pathogenesis of chronic wounds. It will consider how prolonged host inflammatory responses to microbes may also participate in the pathogenesis of chronic wounds.

Better understanding of microbial biofilm and host responses to biofilm may provide therapeutic targets in the management of chronic wounds.

TRANSLATIONAL RELEVANCE

Understanding mechanisms by which microbial biofilms may prevent cutaneous wounds from healing and finding strategies that control excessive host inflammation are likely to provide therapeutic approaches to improve chronic wound healing. For example, gallium-based agents have anti-*Pseudomonas* biofilm effects *in vitro* and may be an adjunct to antibiotic treatment of biofilms in chronic wounds, where antibiotic resistance is very high.

Combination treatment strategies involving biofilm prevention or disruption, antibiotics use, and targeted host anti-inflammatory therapy could be translationally relevant.

CLINICAL RELEVANCE

If microbial biofilm is determined to be a causal component of chronic human wounds rather than non-pathogenic colonization, then targeted therapies could be developed to improve wound healing such as: early use of antibiotics directed at planktonic forms of bacteria, new strategies to prevent or break up biofilm to make microbes more susceptible to antimicrobials for clearance by the host immune system, or therapy directed at preventing a prolonged inflammatory component of wound healing.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

Microbial biofilm is associated with many human diseases

Research over the past several decades has revealed that bacteria in many environments exist as complex surface-attached communities termed biofilms.¹ Biofilms are composites of bacterial or fungal cells, encased in extracellular matrix composed of hydrated polymers and debris. These bacterial biofilms exist ubiquitously in the environment, including extreme conditions. Biofilms

are widely distributed in nature (soil, rocks in the stream, or plant roots), industrial materials (especially water-based systems), and in the human body (either commensal or pathogenic). (www.hypertextbookshop.com/biofilmbook/v004/r003/)

Many chronic infections in humans are thought to be related to biofilms. Typical biofilm-associated diseases are cystic fibrosis, periodontitis, endocarditis, and chronic wounds (Fig. 1).² Most knowledge of medical biofilms was derived from studies of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The research gradually extended to cover more endogenous biofilm throughout the body (e.g., gastrointestinal tract), and include many other diseases such as persistent otitis media, chronic rhinosinusitis, prostatitis, chronic osteomyelitis, atopic dermatitis, and onychomycosis. Biofilms are also associated with use of biomaterial implants and devices (intravenous central lines, prosthetics, urinary tract catheters, prosthetic heart valves, and contact lenses) oftentimes resulting in infection.

Biofilms differ from planktonic bacteria in their structure, gene expression, antibiotic resistance, and interaction with the host. Microbial cells residing in extracellular polymeric substances (EPS) typically occupy 5%–30% of the volume of the biofilm. The thickness or dimension of cell clusters in a biofilm can range from a few microns to a few millimeters. Nutrients and metabolic waste either diffuse directly through the biofilm or are transported through open water channels.^{3,4} Bacteria reversibly attach to a solid surface, multiply, form microcolonies, and produce EPS. The cluster of bacteria is referred to as biofilm. Biofilms grow through contiguous spreading or shedding of planktonic bacteria.^{2,5} A clump of biofilm may detach from the original cluster and then seed onto surrounding surfaces, resulting in infection dissemination. Though some infectious biofilms are dominated by a single species, many other natural biofilms are polymicrobial.¹

Biofilm bacteria are not only phenotypically distinct from their planktonic counterparts, but are also far more tolerant to antibiotics and biocides than their planktonic forms.^{3,6} The mechanisms of antibiotic tolerance in biofilm appear to be distinct from those that are responsible for conventional antibiotic resistance. Older, mature biofilms are more tolerant to antibiotics than younger, less organized biofilms, and in most cases reduced antibiotic susceptibility of the biofilm bacteria can be reversed by resuspending the microbes into liquid culture.⁷ In biofilms, poor antibiotic penetration, nutrient limitation, slow growth, adaptive stress responses, and formation

Figure 1. Bacterial biofilm. Bacteria can attach to the tissue surface and form biofilm colonies, which have a different phenotype than planktonic bacteria. Tissue section of a diabetic mouse wound stained using Brown and Bren methods, which identifies both Gram-negative and Gram-positive bacteria shows formation of aggressive *Pseudomonas aeruginosa* biofilm. Scale bar = 10 μm . ECM, extracellular matrix.

of phenotypic variants are hypothesized to mediate resistance to antibiotics and biocides.⁸ In certain conditions, biofilm bacteria have a 10-fold higher intracellular survival rate than planktonic bacteria.⁹

Biofilms and chronic wounds

The importance of biofilms in chronic wounds has been recently reviewed in detail.^{10–12} Chronic wounds are an ideal environment for biofilm formation. The necrotic tissue and debris allow bacterial attachment, and wounds are susceptible to infection due to impaired host immune response.^{13,14} Kennedy *et al.* were able to visualize a mixture of clusters of microorganisms from ulcerated burn wounds 7–31 days post injury.¹⁵ Recent microscopic analysis of human wounds revealed the presence of densely aggregated colonies of bacteria surrounded by an extracellular matrix.¹⁶ Sixty percent of chronic wound specimens were characterized as containing biofilm, whereas only 6% of acute wounds contained biofilm, indicating biofilms were prevalent in chronic wound samples and relatively rare in samples from acute wounds.¹⁶ Clinicians have initiated studies to identify biofilm formation on chronic ulcers to guide wound management.¹⁷ We have developed an animal model of delayed wound healing employing inoculation of biofilm into wounds in diabetic mice. While observations from this model showed that more than 99% of bacterial counts localized in the scab covering the chronic wound, rather than residing on the wound bed directly, biofilm-challenged wounds still resulted in a delay in wound healing (Figs. 2 and 3).^{18,19}

Identification of commensal microbial flora on normal skin is contingent upon detection technique. Culturing methods of disease-free skin identified normal flora *Corynebacterium* spp., *Propionibacterium acnes*, and coagulase-negative *Staphylococci*.²⁰ Genomic analysis additionally found stable polymicrobial communities consisting predominantly of *Pseudomonas* and *Janthinobacterium*.

Figure 2. Illustration shows presence of biofilm and leukocytes in a biofilm-challenged wound covered with a scab at 28 days post-wounding. (Adapted from Zhao *et al.*¹⁹)

Figure 3. Micrographs of *P. aeruginosa* on mouse wound. Light (a) and transmission electron microscopy (b) images show presence of biofilm containing rod-shaped *P. aeruginosa* (P) embedded in an extracellular matrix. (Adapted from Zhao *et al.*¹⁹)

bacterium. *Staphylococcus epidermidis* and *P. acnes* constitute <5% of microbiota.²¹ Multiple species of aerobic and anaerobic bacteria have been isolated from both chronic and acute wounds by conventional culture techniques,^{22–25} but traditional culturing methodology only identifies ~1% of the bacteria in a chronic wound.¹² More recent studies using molecular techniques have shown that microbial communities in chronic wounds are more diverse than indicated by culture-based techniques.^{26,27} Multiple bacterial species, usually two to five species, concurrently reside on a single ulcer.^{24,28,29} The chronicity of unhealed wounds is associated with a higher proportion of anaerobic bacterial colonization and a greater variety of aerobic species.²²

Although more observations have confirmed the presence of biofilms on chronic wounds, causality remains an evolving research topic. One hypothesis is that biofilms simply form on the chronic wound over time with no pathogenic relationship, because chronic wounds often have no typical signs of active infection, such as redness, warmth, or purulence and do not respond to antibiotic treatment. To investigate whether biofilms inhibit wound healing, recent investigations have focused on two primary areas: wound healing mechanisms

using *in vitro* keratinocyte culture or *in vivo* chronic animal wound models.²⁵ These studies support the idea that chronic wounds are the result of wound colonization that evolves into biofilm formation. Several factors influence the magnitude of bacterial proliferation and persistence, including wound care management, virulence, and other characteristics of the pathogen, and ability of the host to control and eliminate the infection.

Chronic wounds and prolonged inflammatory phase

Cutaneous wound healing is a dynamic process mediated by interactive reactions of parenchymal cells, soluble mediators, blood elements, and extracellular matrix. During normal wound healing, there is an orderly transition from the inflammatory phase lasting only a few days, with progression to tissue regeneration including epithelialization, granulation tissue formation, and angiogenesis, followed by tissue reorganization.³⁰ A prolonged inflammatory phase alters the progression of skin wound healing. The major players in the inflammatory phase include the release and resolution of a plethora of cytokines as discussed in another review in this journal issue, blood components, especially platelets, neutrophils, and macrophages, changes in oxygen concentration, and pH in the wound. The presence of bacterial biofilm can target many of these major inflammatory players (Fig. 4). The prolonged inflammatory phase of healing in chronic wounds and the interaction between the innate immune response and the wound microbiome has been recently reviewed.¹¹

Host inflammatory response to biofilm

The presence and persistence of biofilms on chronic skin wounds can affect cellular (leukocytes, keratinocytes, endothelial cells, and fibroblasts) function, the inflammatory cellular response, cutaneous innate immune response, and the repair phase of wound healing (angiogenesis and fibrogenesis). These infections develop gradually and may be slow to produce overt symptoms. Once established, however, biofilm infections often persist.

Neutrophils. Neutrophils are among the first inflammatory cells to populate the initial wound site and contribute to wound healing by removing bacteria, foreign material, necrotic tissue, and releasing cytokines to promote revascularization and fibrosis. However, prolonged presence of neutrophils delays wound healing through release of inflammatory factors, oxygen species, and proteinases (elastase and cathepsin G) that degrade extracellular matrix and key proteins involved in the wound healing cascade

Figure 4. Normal and delayed wound healing of cutaneous wounds. Illustration shows differences between normal healing of an excisional wound (a, b) compared to the delayed healing of an excisional wound in the presence of bacterial biofilm (c).

causing collateral damage to neighboring healthy host tissue. Keratinocyte migration is reduced in the presence of neutrophils.³¹ A study of human chronic venous leg ulcers showed that the *P. aeruginosa*-containing wounds had significantly higher number of neutrophils compared with *S. aureus*-containing wounds, but the functions of these neutrophils were not analyzed.³² In cystic fibrosis lungs, massive neutrophilic infiltration is also detected. Despite massive neutrophil accumulation, the biofilm infection persists. Observation of biofilms in both cystic fibrosis lungs and chronic wounds suggests that biofilms are surrounded or overlaid with neutrophils but not penetrated and actively killed by the neutrophils. One explanation is that the circulating neutrophils are constantly being recruited by the biofilm. *Pseudomonas*, through their quorum sensing (QS) system factors can then paralyze and lyse neutrophils found

in close contact.³³ It should be noted that many *P. aeruginosa* in chronic cystic fibrosis are QS mutants, suggesting the role of the QS system is complicated and still unclear. Bacteria in biofilms may protect themselves from being phagocytized by neutrophils through their rhamnolipid protective shield.²⁴ Other biofilm matrix components, including bacterial DNA and alginate were also reported to stimulate neutrophils. The hypothesis that the presence of bacterial biofilm induces excessive neutrophil accumulation and arrests the wound in a persistent inflammatory stage needs to be confirmed in prospective studies.

Macrophages. The role of macrophages in wound healing is complicated. Although macrophages may not be required in embryonic or neonatal wound healing,³⁴ they are important in mediating wound healing in adult animals, as shown in transgenic mouse models.^{35–37} Macrophages are functionally identified as activated proinflammatory M1 or reparative M2 macrophages dependent on the wound microenvironment in which they are located.³⁸ Proinflammatory M1 macrophages phagocytize neutrophils, debris, and bacteria and release multiple proinflammatory mediators. Most *in vivo* investigations of macrophage response to biofilms are performed in foreign body-associated infection, in which macrophages are the key component. One study using a mouse model of catheter-associated biofilm infection showed that *S. aureus* biofilm exaggerated macrophage accumulation, but changed macrophage function by suppressing microbicidal activity, altering gene expression toward M2 phenotype, decreasing migration, and increasing cell death.^{39,40} Macrophages are plentiful in chronic wounds, presumably, continuing to be attracted by the presence of bacteria.¹¹

Innate immune response. The innate immune system in the skin involves Toll-like receptors (TLRs), Nod-like receptors, antimicrobial peptides (AMPs), chemokines, and cytokines. TLRs belong to pattern recognition receptors, which are part of the innate immune system that recognize conservative microbial molecular patterns and activate the immune system. There is increasing evidence that TLRs play an important role in wound healing. All TLRs modulate the innate immune response to wounding, although they differ in time after wounding and cellular localization.⁴¹ For example, when TLR2 and TLR4 expression is increased, proinflammatory cytokine secretion is increased resulting in impaired wound healing.⁴¹

Other studies in biofilm-associated diseases, especially cystic fibrosis, suggest that TLRs mediate host response to biofilm matrix and bacterial products. TLR5 expression was increased in neutrophils in cystic fibrosis lung and the TLR5 binds to the bacterial lipoprotein.⁴² The effects of other TLRs are not clear. Although biofilms are associated with higher levels of TLR2 in patients with chronic rhinosinusitis,⁴³ and ligands of TLR2 and TLR9 are present within *S. aureus* biofilms, some studies suggest these receptors do not necessarily impact biofilm growth.⁴⁴ MyD88, a converging pathway of both TLR and IL-8, regulates fibrosis and activates M2 macrophages.⁴⁴ To our knowledge, TLR response in biofilm-infected chronic wounds has not been studied.

AMPs are an important component of the innate immune system that includes defensins (hBD-1, -2 and -3) and cathelicidins (LL-37).⁴⁵ AMPs contribute to innate defense against infection. How host AMPs respond to biofilm in chronic wounds is unclear. Some components of *S. aureus* have been reported to induce production of beta-defensin peptides. Recent studies focus on developing AMPs that directly target biofilm as a topical treatment for biofilm-associated infection.⁴⁶

Cytokines and growth factors. Inflammatory cytokines and growth factors, including epidermal growth factor, platelet derived growth factor, vascular endothelial growth factor, tumor necrosis factor- α (TNF- α), transforming growth factor- β , basic fibroblast growth factor, keratinocyte growth factor, granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1 β , IL-6, and the chemokine CXCL8/IL-8 are significantly increased in chronic wounds.⁴⁷ The elevation of these cytokines and growth factors is often associated with the presence of bacteria and their endotoxins. Chronic *S. aureus* biofilm infection increased levels of IL-2, IL-4, IL-6, IL-12, IL-17, and TNF- α , indicating a predominantly Th1 and Th17 type response.⁴⁸ When compared with planktonic bacterial infection, biofilm-induced inflammatory response is modest, which may contribute to persistent infection and delayed wound healing. Using an *in vitro* model, Secor *et al.* showed that *S. aureus* biofilm upregulated genes associated with inflammation, apoptosis, chemotaxis and signal transduction in human keratinocytes, and induced low levels of IL-1 β , IL-6, CXCL-8, CXCL-1, and GM-CSF production.⁴⁹ This is supported by an *in vivo* biofilm wound model, which also showed that sustained low-grade inflammatory response correlated with impaired epithelial

migration and granulation tissue growth.⁵⁰ If the host circulation and immune response are intact, the host is capable of stabilizing the bacterial burden over time. Therefore, the bacterial burden, including biofilm, is decreased and inflammatory cytokine levels return to baseline, resulting in wound healing.

Matrix metalloproteinases and tissue inhibitors of metalloproteinases. A balance between proteinases and their inhibitors is involved in tissue remodeling and wound repair. Matrix metalloproteinases (MMPs) are one class of proteinases that digest extracellular material and allow an influx of reparative keratinocytes, fibroblasts, and endothelial cells.⁵¹ With normal wound healing progression, tissue inhibitors of metalloproteinases (TIMPs) levels begin to increase and concomitantly downregulate levels of proinflammatory cytokines that stimulate MMP production. In chronic wounds, the balance between MMPs and TIMPs is altered.⁵¹ Trengove *et al.* found levels of MMP 2 and MMP 9 elevated in nonhealing chronic wounds and levels of their associated TIMPs reduced.⁵² MMPs not only degrade the extracellular matrix but also reduce levels of growth factors necessary for repair.⁵² In the presence of biofilm, keratinocytes produced more MMP-1 and MMP-3 in *in vitro* culture,⁵³ and MMP-10 gene expression was elevated in chronic biofilm-challenged *in vivo* wounds.¹⁹ The role of MMPs and TIMPs in biofilm chronic wound has not been explored in depth.

Potential therapeutic targets based on understanding biofilm

Quorum sensing. QS is a density dependent form of cell-cell communication in which bacterial cells synthesize and react to small signaling molecules. There are two major groups of signaling molecules of QS. Gram-positive bacteria use peptide derivatives such as autoinducing peptides. Gram-negative bacteria use fatty acid derivatives such as N-acylhomoserine lactones and gamma-butyrolactones.^{54,55} Furanosyl diester (also known as autoinducer-2) is a signaling molecule that is expressed and recognized by both Gram-positive and negative bacteria. *P. aeruginosa* does not express autoinducer-2 but responds to it.

Many of the bacterial genes involved in biofilm formation are controlled by the same regulatory systems that control virulence factors.⁵⁶⁻⁵⁸ However, the role of QS in biofilm-associated infection is controversial. QS may or may not affect biofilm formation under different conditions. One hypoth-

esis is that QS coordinates bacterial activities so that they can operate in groups, for example, by delaying exo-product synthesis until population density is sufficient to produce effective concentrations.⁵⁹ An alternative view is that QS signals represent the turnover of other secreted products rather than being associated with bacterial group or social activities.⁶⁰

Antibiotic resistance. The persistent infections of biofilm are not cleared by the host immune system and are resistant to systemic and topical antimicrobial agents. Antibiotic resistance is an important mechanism for biofilm survival. Effective antibiotic concentrations against biofilm may be many times higher than the minimum inhibitory concentration against planktonic bacterial cells.⁶ Biofilm infections respond only transiently to antibiotic therapy, because the bacterial counts are generally only temporarily suppressed. It was thought that EPS and other extracellular materials protect the bacteria in biofilm by preventing antibiotic penetration. However, there is evidence that antibiotics still diffuse through biofilm efficiently.⁶¹ Therefore, antibiotic resistance may be related to intrinsic features of biofilm bacteria in addition to slow diffusion. One mechanism of antibiotic resistance is alternation of activity status. Since most antibiotics kill or inhibit bacteria through disrupting metabolic activity and proliferation, dormant bacteria in biofilm are less likely to be the target of antibiotics. Also, the change of environmental stress, bacterial density, nutrition supply, and oxidative stress may trigger mutation and gene expression. A recent study found that antibiotic tolerance of *P. aeruginosa* biofilm is mediated by active responses to starvation rather than passive effects of growth arrest. Inactivation of this protective mechanism sensitized biofilms to antibiotics and markedly enhanced the efficacy of antibiotic treatment.⁶²

Other anti-biofilm strategies. It has been demonstrated in an *in vivo* biofilm infected wound model that the vast majority of bacteria reside in the eschar above the wound bed.¹⁸ Hence, wound debridement remains a proven cornerstone for wound management, perhaps, in part, because microbial biofilm is removed.

Iron metabolism is essential for pathogen growth and function. Gallium, an FDA-approved agent for treating hypercalcemia, has been found to disrupt *P. aeruginosa* biofilm formation *in vitro* through inhibiting iron uptake and metabolism.⁵⁴ Lactoferrin is an iron-chelating protein of the

innate immune system found in certain bodily fluids. It has been shown to have an inhibitory effect on biofilm formation in clinical isolates from *P. aeruginosa*.⁶³

An alternative approach to improving biofilm control is to target the EPS that hold the biofilm together rather than just bacteria within the biofilm. Weakening or dispersing microorganisms from the biofilm should render them more susceptible to the host defenses and to conventional antimicrobial agents. Potential means of biofilm disruption include surfactants and enzymes. For example, an enzyme that degrades the poly-N-acetylglucosamine polymer that many bacteria synthesize as a matrix material has been shown to both remove biofilm and reduce biofilm antimicrobial tolerance. Many biofilms contain extracellular DNA in the EPS; thus, it may be possible to reduce biofilm by applying DNAase.

The natural processes by which bacteria disperse from biofilms are a current subject of investigation and these mechanisms, once better understood, could open the door to new therapies for reversing biofilm recalcitrance.

Because killing microorganisms in a biofilm typically requires much higher antimicrobial concentrations than are needed to control planktonic cells, one simple strategy for improving efficacy against a biofilm is to use topically applied antiseptics and antibiotics. These agents, perhaps delivered via a gel or cream, would need to be formulated at the highest concentrations that are not detrimental to host tissues. Local delivery of antibiotics has the added benefit of minimizing the chances of adverse systemic effects such as organ toxicity and disturbance of gut microflora. Another general strategy that makes sense when targeting a biofilm is to use combinations of antimicrobials. Because wound biofilms are polymicrobial, it is unlikely that a single antibiotic can control across the spectrum of microbial diversity. In addition, the physiological heterogeneity that is characteristic of biofilms, even within a species, may require a combination of agents for effective control. An additional benefit of combination therapies is that they may reduce the potential for development of resistance. New anti-infective agents targeting biofilm are also being explored, but all of the biofilm strategies described in this section remain to be demonstrated to speed healing of chronic wounds in a prospective study.

TAKE-HOME MESSAGES

- Microbes on chronic wound surfaces commonly live in phenotypic communities called biofilms. Biofilm differs from planktonic microbes in its structure, dynamics, gene expression, communication, and interaction with the host. Microbial cells residing in EPSs typically occupy 5%–30% of the volume of the biofilm.
- Chronic wounds have a prolonged inflammatory phase and microbes appear to contribute to the prolongation of this inflammation. The bacteria thus gain access to nutrients from the host for a prolonged period.
- Bacteria living in a biofilm are many times more resistant to antibiotics than planktonic bacteria and the biofilms are usually polymicrobial. This may explain why antibiotics are of limited use in treating chronic wounds.
- New strategies targeting biofilm disruption or prevention of biofilm formation may be important new approaches in the management of chronic cutaneous wounds.
- Rapid high-throughput genomic approaches have revolutionized the ability to identify and quantify microbial organisms from *in vivo* wounds.
- Characterization of microbiomes of clinically well-characterized chronic human wounds may suggest future therapies.

FUTURE DEVELOPMENTS

Recent technological advances in high-throughput sequencing have provided us with two unprecedented strategies to investigate the molecular mechanisms attendant on host–microbe interaction leading to and sustaining chronic wounds. To elucidate the molecular mechanisms underlying host–microbe interaction, the longitudinal dynamics of diabetic mouse wound microbiome was examined by sequencing the bacterial 16S ribosomal RNA genes present in the wounds and integrating with the mouse tissue gene expression examined by microarray analysis of mouse gene transcripts. Integration of the two global datasets demonstrated that the wound microbiota longitudinally shifts with the host gene expression, particularly genes associated with immune response. In addition, the shift in microbial composition also correlated with impaired wound healing in diabetic mice.^{11,21}

In a parallel but distinct approach to investigate host–pathogen interaction, the contribution of individual bacterial genes for bacterial fitness in wounds can be interrogated by employing a recently developed transposon-sequencing (Tn-seq) methodology.^{64–67} In this approach, the genome-scale genetic Tn-seq approach is utilized to directly assess each bacterial gene's contribution to the microbial fitness and survival of microbes in wounds. Specifically, transposon mutagenesis is used to generate bacterial pools consisting of mutants with nearly every non-essential gene inactivated by a transposon insertion. Then, by

Figure 5. Experimental overview of *in vivo* mouse wound model coupled with genome-wide mutant analysis (Tn-seq). **(a)** A pool of genome-wide transposon bacterial mutants is inoculated onto diabetic mouse wounds that are harvested at selected duration(s) of infection. Bacterial mutants lacking functions required for initiating infection are lost early during infection, and mutants unable to persist in wounds will be lost after a longer duration. Only bacterial mutants that did not lose an essential function were able to survive into the harvest pool of bacteria. **(b)** Genomic DNA isolated from the wound harvest is processed and examined using Tn-seq methodology to map the exact location of disrupted bacterial genes and quantify the relative abundance of individual mutants. The chromosome-transposon junctions are used as unique barcodes to identify each bacterial mutant. The relative abundance of individual bacterial mutants corresponding to specific Tn insertions is shown in the bar graph (height of blue and red columns). Whereas transposon insertion mutants in Gene B (orange shade) were abundant in the inoculum (blue columns), they were not detected in the harvest pool of bacteria. The encoded product of Gene B represents bacterial functions required for survival on the harvested wound. These bacterial functions inactivated by transposon insertion in their corresponding genes are potential novel therapeutic targets for chronic wound infections. Tn-seq, transposon-sequencing.

utilizing high-throughput sequencing, the Tn-seq methodology allows identification of the exact location of the chromosome-transposon junctions in the bacterial genome, which serve as unique barcodes to identify each bacterial mutant. Moreover, the number of sequence reads corresponding to each unique barcode is imputed to calculate the relative abundance of each bacterial mutant in the pool. Due to the selective amplification of junctional regions, Tn-seq can be applied to samples containing both bacterial and host genetic material.

The combination of the novel Tn-seq methodology and recently developed diabetic mouse wound model allows genome-wide identification of bacterial functions required to produce wound infection contributing to delayed wound closure.¹⁸ In this two-part experimental approach, a bacterial pool composed of mutants spanning the entire genome is used to infect an acute wound, and the relative abundance of individual insertion bacterial mutants in the pool is determined before and throughout the infection (Fig. 5). Bacterial mutants that are present in the inoculum, but either

eliminated or decreased (in abundance) mutants in the harvest represent bacterial functions that are required for initiating infections and/or persisting in wounds, thereby delaying healing. These functions are potential targets for anti-infection therapeutics, as drugs that interfere with their functions might block the ability of bacteria to produce infection.

As evidenced by these studies, approaches coupling novel animal models with advances in genome-wide techniques allow the possibility to directly determine the *in vivo* contribution of individual bacterial genes for survival and pathogenesis of chronic wounds, and to examine the interplay between the host and microbiome at a molecular level. These innovative strategies may set the foundation for accurate diagnostics and effective treatment of chronic wounds.

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