Bacterial Strain Diversity Within Wounds

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Significance: Rare bacterial taxa (taxa of low relative frequency) are numerous and ubiquitous in virtually any sample—including wound samples. In addition, even the high-frequency genera and species contain multiple strains. These strains, individually, are each only a small fraction of the total bacterial population. Against the view that wounds contain relatively few kinds of bacteria, this newly recognized diversity implies a relatively high rate of migration into the wound and the potential for diversification during infection. Understanding the biological and medical importance of these numerous taxa is an important new element of wound microbiology.

Recent Advances: Only recently have these numerous strains been discovered; the technology to detect, identify, and characterize them is still in its infancy. Multiple strains of both gram-negative and gram-positive bacteria have been found in a single wound. In the few cases studied, the distribution of the bacteria suggests microhabitats and biological interactions.

Critical Issues: The distribution of the strains, their phenotypic diversity, and their interactions are still largely uncharacterized. The technologies to investigate this level of genomic detail are still developing and have not been largely deployed to investigate wounds.

Future Directions: As advanced metagenomics, single-cell genomics, and advanced microscopy develop, the study of wound microbiology will better address the complex interplay of numerous individually rare strains with both the host and each other.

SCOPE AND SIGNIFICANCE

RARE BACTERIAL TAXA (taxa of low relative frequency) are numerous and ubiquitous in virtually any sample. The discovery of numerous unexpected genera in each sample has occasioned considerable discussion among scientists and physicians. The biological importance of seemingly few cells is often dismissed. Even more ignored is the degree to which the relatively abundant taxa decompose into diverse strains when examined closely. How can rare taxa strains be unimportant when the common taxa they compose are important? This review discusses the existence and significance of the

strains within high-frequency higher taxa.

TRANSLATIONAL RELEVANCE

To adequately address the microbial component of wound infections, scientists must first identify the bacteria actively encouraging or preventing wound healing. Unfortunately, these bacteria may be very closely related to one another and aggregating them inappropriately can confound an otherwise robust analysis. As a result, tracking and accounting for the diverse strains within a single genus or species in each wound sample is a

relevant and immediate challenge for wound microbiologists.

CLINICAL RELEVANCE

Wound care clinicians have been less than enthusiastic about advances in wound microbiology because the data generated often had limited impact on care. In the case of culture-based clinical microbiology, the bacteria identified were generally just a limited subset of the bacteria in the sample. Conversely, molecular methods, including sequencing, promise to identify all the bacteria in the sample, but the overwhelming diversity of bacteria revealed is difficult to act upon. Attempts to simplify this data by aggregating at higher taxonomic levels offer a mirage of easy comprehension; however, it threatens to undermine the predictive utility of more precise microbiological characterization.

BACKGROUND

Rare taxa

The long tail of rare bacterial taxa uncovered by high-throughput amplicon sequencing poses an enormous challenge to microbiology, particularly medical microbiology. Rare taxa are relatively unpredictable, frequently uncharacterized, and so numerous as to defy inspection. Failed attempts to dismiss the rare taxa have denied their importance, viability, and even existence; however, rare taxa can be more active than abundant taxa on a per cell basis,¹ can quickly increase in abundance when the tide turns,^{2,3} and can have outsized impacts on human health.⁴

To cope with this bacterial diversity, the bacteria are aggregated at higher taxonomic levels for analysis. Unfortunately, phylogeny does not mirror ecology.^{5–7} Very closely related *Staphylococcus aureus*, *Escherichia coli*, *Clostridium difficile*, *Vibrio cholera*, and *Bacillus cereus* can have different temperature optima and nutrient requirements,^{1,8} different toxin production capacity,^{9–11} different adhesins,¹² different tolerances for drying, different antibiotic resistances,¹³ and ultimately, completely different implications for human health.^{8,14} Strains that share the exact same alleles at each of several housekeeping genes can be ecologically as different as members of separate families and even form distinct coexisting¹⁵ or codependent populations.¹⁶

Increasingly the mechanistic underpinning of this phenotypic diversity is becoming clear. Studies of the diversity within a species or even sequence type are able to leverage hundreds or thousands

of whole microbial genomes. This inspection has revealed substantial subspecies diversity, including both intermediate frequency and infrequently present genes,^{17,18} complex regulation,¹⁹ and poor linkage among diverse alleles.¹ Ecologically meaningful allelic diversity within an operational taxonomic unit creates an abundance of rare taxa from each abundant taxon. These new rare taxa are, like all rare taxa, relatively difficult to detect; because they are present with close relatives, they are also difficult to differentiate. Despite potentially rampant gene flow at conserved loci, which would otherwise be expected to homogenize the populations, these taxa can exist as distinct entities for medically meaningful periods of time and thus represent independent entities that must be accounted for individually. As a result, revised diversity measures reveal underestimates of migration, mutation, recombination, and drift; indicate a misunderstanding of selection pressures; and have enormous implications for microbial interactions and ecological differentiation.

Understudied diversity

Many studies examine genus-level diversity in wounds,^{20–22} but very few studies report species and subspecies diversity within traumatic or comorbid chronic wounds. There are two major reasons.

1. Surveillance and research programs do not seek to characterize the diversity.
2. Low-abundance diversity is technically difficult to assess.

Surveillance and research programs are typically founded on the basis of medical dogma that presumes a single pathogen that is relatively frequent in the microbial community and is established in a patient either through acquisition from another infected individual or from colonization at another body site (*i.e.*, nasal carriage or gastrointestinal carriage). Dogma is that individuals are dominated by a single strain of each species at a given body site—*E. coli* in the intestines, *S. aureus* in the nose, so on. This question has only been explored in detail for a few organisms; the actual strain diversity is certainly species specific. In the example of *E. coli*, the hypothesis of colonization by only a single strain has been falsified for as long as it has been questioned,²³ but the results from antigenic typing were such as to suggest “dominance” by a single strain in combination with other “transient” strains—by implication, unimportant. In additional studies, multiple strains were detected when individual samples were thoroughly characterized but

ad hoc reasons were given for effectively reducing the depth of sampling to a single strain per sample.²⁴ In some large surveys, only a single strain was characterized per individual studied.²⁵ The increasing focus on antibiotic-resistant bacteria may also have encouraged the dogma of one strain per patient. Many patients are sampled after antibiotic treatment failure, in pursuit of the resistant strain. However, some of the multidrug-resistant strains are of recent origin and effectively clonal.^{26,27} As a result, when the patient is sampled, a clonal-resistant population is recovered. Also, the dogma surrounding environmental surveillance in hospitals is that within single species colonizing or infecting patients, single clones dominate a ward. “Polyclonal outbreaks” are noted as exceptional. As a result, surveillance and research typically relies on a single isolate from each sample in which the relevant-named species is found (incidence).

The perception that samples can be reduced in complexity to single isolates is partly driven by two methodological deficiencies, which can be classified into undersampling and undercharacterization (Fig. 1). Culture-based methods preserve the isolates for effectively boundless characterization. However, the labor and cost of further characterization encourages aggressive reduction in the number of isolates to be thoroughly examined. This leads directly to undersampling. The most extreme case of undersampling, typical in both the clinical

Figure 1. Undersampling and undercharacterization. The upper left figure represents a population of bacteria, each with three characters. Through undersampling (upper right) the population is characterized as entirely uniform despite containing relatively high diversity. Despite exhaustive sampling, the same misconception is arrived at by limiting characterization to a relatively conserved character (lower left). The error is multiplied when both undersampling and undercharacterization occur, in whichever order (lower right). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

lab and in the literature, is the selection of only a single isolate per sample. While a single isolate is certainly more informative than no isolates, it by definition cannot address diversity at all, even in low-diversity communities.²⁸ Further, the undersampling is compounded by sampling biases. First, despite an ancient status as a “gold standard,” culture-based methods have extreme deficiencies and biases in chronic wounds^{29,30} and also in other clinical and environmental samples.³¹ Second, and less widely recognized, if one strain is consistently less abundant than another (a common phenomenon), then undersampling will amplify this bias toward failing to consistently detect incidence of the low-abundance strains; chains of transmission will be broken, and temporal patterns will appear discontinuous (Fig. 2).

Strains that occur as 10% of the abundance require characterization of more than 20 colonies from each sample for routine incidence detection across multiple samples; as such, methods that involve selecting 3 colonies, for example,³² should not be expected to reveal even substantial diversity within a taxa. This creates problems at higher levels of study (*i.e.*, healthcare facility and hospital ward) because undersampling subsidiary units can lead to ineffective surveillance at the higher organizational level. An even more sophisticated form

Figure 2. Probability of detecting outbreaks with low-abundance strains. This graph represents the results of a simulation in which a variable number of isolates are characterized from five samples; the probability of detecting a strain that composes some fraction of the actual population at least once in each of the samples is charted. By characterizing one isolate per sample, even a strain that is 90% of the population is detected only half the time in all five of the samples. Thus, its ubiquity would be unacknowledged. As the number of strains characterized per sample increases, the probability of detecting strains of even lower abundance increases substantially. With 20 isolates per sample, even a strain that is only 10% of the population can be detected at least once in all five samples. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

of undersampling involves the selection of one strain of each phenotype from the primary isolation plate, which engages undercharacterization (see next paragraph) in the service of undersampling. Unfortunately, thorough downstream characterization methods are quite labor intensive. To achieve realistic levels of effort both in the clinical laboratory and the research laboratory, restrictive sampling strategies must be used. Thus, the apparent choice is between thorough sampling and thorough characterization.

Undercharacterization occurs when the diversity is not represented in the traits characterized. Undercharacterization typically results in the combining of strains into a single group. One outcome of this confusion is the illusion of outbreaks and patient-to-patient transfers.³³ It can also result in unexplained variations in virulence or antibiotic resistance. Finally, some undercharacterization leads to the overinterpretation of sporadic variation, such as a single-nucleotide polymorphism in a diagnostic allele. The discrete nature of these mutations creates the illusion of proportional diversity elsewhere in the genome.

Biochemical tests are often by definition uninformative at the level of the named species; the tests are expected to be consistent across the entire species or even higher phylogenetic units (genera, families). Morphology is similarly uninformative, whether cell morphology or colony morphology? Colony morphology masks significant genomic diversity even when consistent with phylogeny.^{34,35}

Molecular methods can also result in undercharacterization. Pulsed-field gel electrophoresis (PFGE) is particularly notable for undercharacterization.^{36,37} Characterizing both microheterogeneity and clone diversity requires additional methods beyond PFGE.³⁸ 16S rRNA or housekeeping genes are also often uninformative below the level of the genus, or with some additional effort, the species.^{39,40} Unfortunately, readily accessed loci are typically the least informative because of the requirement for conserved amplification sites (for amplicon-based sequencing) or reliable mapping to a reference genome (for draft genome sequencing and metagenomics).

Shotgun metagenomics can also result in substantial undercharacterization (Fig. 3). While the low-frequency genes representing ecological diversity are not theoretically excluded from detection, in practice the diversity may go undetected. The presence of human DNA in samples drowns out the microbial DNA, lowering the effective depth of sampling. Within the microbial fraction, high-abundance organisms are often present at several

Figure 3. Detecting low-frequency strains via shotgun metagenomics. In a normal wound sample, over 90% of the DNA recovered is human DNA. The human-DNA-depletion kits typically remove 90% of the human DNA. This creates a sample that is 91% bacterial DNA. In a sample dominated by a single genus, that genus may contribute 50% of the total bacterial DNA, and a species within that genus may thus represent ~2% of the original DNA sample. Given that the sample is run alone on a MiSeq (an extremely conservative estimate), 6 M reads may be recovered from that one species. Using a marker gene present in all the members of the species to assess diversity, ~2,400 reads will map to that gene. Given the length of an average gene and an average read, any given location along the length of the gene will be covered a median of 100-fold. This could be adequate for genotyping a single strain, but for detecting subpopulations, it is not. Most reads include errors, making detection of minority strains within the most-frequent species difficult at best. A potential solution is the use of even higher sequencing depths, but this carries a great expense, not only for sequencing but during data analysis and computation. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

orders of magnitude higher relative frequency than the rare taxa. In addition, high-frequency genes are better represented across all taxa, masking the presence of the low-frequency genes that delineate ecotypes. The high-frequency genes are also more likely to be present in reference data sets and to be functionally annotated.

Diversity at the high-frequency loci within the high-abundance taxa could theoretically reveal some ecotype diversity and certain polyclonal infections, but even then the sequencing error rate prevents the effective recognition of this diversity at relatively conserved sites. Finally, primary sequence diversity at defined loci is not necessarily the most ecologically (and thus medically) significant form of variation. In many bacteria, it is becoming clear that recombination outpaces single-nucleotide variation many fold. Inherent in the process of recombination is the possibility for deletions, or even gene amplification. However, variations in gene copy number, even with sequence divergence among the copies, are unlikely to be recognized in metagenomic samples, masked by

the algorithms meant to correct for sequencing biases, such that copy number variation is typically filtered out during the assembly process. This bias toward single-copy genes does not stop at shotgun metagenomes; it also limits the utility of draft assemblies.

Exemplar hypotheses

If one was able to characterize the rare taxa effectively, then a number of critical ecological hypotheses could be addressed at the appropriate phylogenetic, spatial, and temporal scales. Using actual ecotypes, rather than confused aggregates, hypotheses about migration, selection, mutation, and mating could be addressed (Fig. 4).

Hypotheses about migration are particularly important for infection control. They include the characteristic dispersal range and routes of bacteria through the community or medical facility, the role of persistent colonization at other body sites, and the number of different equivalent strains that can persist in a single wound simply because the spread of a clone across the wound is too slow and other clones become established as they migrate in from the environment (spatial autocorrelation within the wound beyond the autocorrelation of underlying physiological variables; Fig. 5).

Hypotheses about selection include the number of distinct habitats within a single wound and the differences among patients, such as clinical presentation, wound characteristics, antibiotic use, and

Figure 4. Decomposition of taxa. As frequent higher taxa are decomposed into multiple less-frequent taxa, the number of rare taxa above the threshold of detection increases, but each taxa has a lower effective population size. This has population genetic implications. First, drift is a more significant factor in each of the populations, and this will tend to reduce the impact of selection. The addition of numerous taxa into a relatively short-lived habitat (the wound) suggests a relatively high rate of migration into the wound. The diversity may also be the result of relevant mutational processes. Finally, increased diversity implies greater opportunity for mating/recombination within the wound. The population genetic processes themselves have an impact on epidemiology, infection control, the rate of pathoadaptation, and bacterial response to antibiotic administration. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

Figure 5. Spatial variance. Community composition is shown on a spatial grid in the context of three environmental variables. Each variable has its own internal spatial autocorrelation as variables change continuously over space. The correlation between the collected environmental variables and the community composition is perceived as the impact of the environment on the community. The degree of autocorrelation within the environmental variables that overlaps with autocorrelation within the community explains some fraction of the autocorrelation within the community composition. The excess autocorrelation within the community composition is the degree to which migration limits the distribution of organisms in the wound. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

immune status, that may favor different clones. Migration and selection together are assessed in the traditional ecological debate over neutrality (spatial autocorrelation) and adaptation (local habitat selection). The debates over antibiotic heterogeneity are also an expression of the balance between environmental selection and migration; they must be informed by knowledge of the migration and selection of virulent and antibiotic-resistant clones in the healthcare facility.

Mutation in wounds provides opportunities for immune avoidance, pathoadaptation, and *de novo* antibiotic resistance. High migration and low mutation favor replacement of antibiotic-sensitive strains with antibiotic-resistant strains from the environment; high mutation and low migration will favor the selective sweep of personalized mutant strains. Mutators significantly change the pathoadaptation dynamics of infection.^{41,42} Mutations need not be advantageous to create local diversity or create problems for the host; they can accumulate faster than purifying selection removes them, producing diverse local clones in a source-sink dynamic.⁴³ In addition, this diversity can initiate frequency-dependent selection, selecting for higher mutation rates⁴⁴ and directly impacting virulence.⁴⁵ Frequency-dependent selection is a particularly tricky phenomenon, confounding traditional population genetics⁴⁶ and requiring special attention as a result.

Mating also can change the course of patho-adaptation, allowing key genomic islands to sweep through an otherwise heterogeneous population. High rates of horizontal gene transfer via mobile genetic elements create a completely different pattern of diversity within the wound, allowing genes to sweep across multiple ecotypes^{47–49} without necessarily disturbing local adaptation to microenvironments, symbioses, and other optimizations within the wound microbiome.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

Existing wound ecology studies

Several studies have approached microbial biogeography within a single wound, but these microgeographic^{50–54} and macrogeographic studies⁵⁵ lose resolution at phylogenetic levels above the species. The existence of microcolonies⁵² and sovereign monospecies biofilms⁵⁰ in wounds suggests that at that spatial scale, mixing and migration are slower than bacterial growth. In the study of macrogeographic ecology, the clinical question was raised about how many samples, spatially, would be required to characterize a wound.⁵⁵ Certainly there were established depth profiles in the wounds, with distinct communities,⁵⁶ but laterally, the degree of diversity was unknown. Multiple samples from the same wound, inspected via 16S rRNA sampling, yielded coherent UniFrac clusters. This coherence supports the conclusion that a single sample from a wound is sufficient to distinguish it from other wounds. However, after examining the data, substantial community composition variation was evident laterally across each wound, suggesting either migration-limited patchiness or a range of microhabitats being combined in varying proportions with each of the samples taken (or both, Fig. 6). Studies that combine lateral sampling with local physiological measurements will be required to disentangle migration limitation and local adaptation (Fig. 5).

Temporal studies have demonstrated successional patterns in wounds. The degree to which these are driven by microbial interactions⁵⁷ as opposed to changes in the host⁵⁸ is unclear. Certainly the structural changes in the wound, including progression to a shallow wound, impact the microbiome. The relative proportion of anaerobes is expected to decline as the wound becomes less deep, for example. A small study demonstrated that (in different wounds) *Staphylococcus* resides more shallowly than does *Pseudomonas*.⁵⁹ In addition, correlations exist among the relative frequency of staphylococci,

Figure 6. Sampling microhabitats. The process of sampling a wound may lead to the recovery of several different habitats in a sample, each represented in varying proportion. In the figure, the wound biofilm is simplified to four distinct communities. The two samples represent capturing all four communities in one sample but only one community in the other. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

the depth of the wound, and the time to healing; more staphylococci and a relatively shallow wound are indicative of a shorter time to heal.⁶⁰ The progression from deep to shallow with the restoration of surface microbiology is somewhat analogous to the traditional succession during pond infill. However, the bacteria during healing have not been characterized at a fine phylogenetic scale, leaving room for undercharacterization and perhaps misunderstanding of both pattern and process.

Staphylococcus

In a pioneering study of large wounds (epidermolysis bullosa) numerous diverse *S. aureus* strains were found distributed across the surface of the wound rather than organized into large patches.⁶¹ The mere existence of this diversity overturns some dogma. It has been persistently assumed that individuals are colonized by at most a single strain of *S. aureus*⁶² and that wounds are infected by a single clone, likely associated with nasal carriage or spread in a nosocomial outbreak. Dogma leads to assumptions that colonization events are rare, pathogenic strains are discrete, and thus founder effects dominate the population dynamics. These assumptions are practically important because the prevention of wound colonization by methicillin-resistant *Staphylococcus aureus* becomes an infection control priority, to include radical decolonization therapies and hospital cleaning. The dogma has been deprecated by several studies of carriage and cystic fibrosis infections.^{35,63–67}

Each study of *S. aureus* infection uncovers diversity beyond the expected single clone, but the

results of the studies are not entirely consistent and may differ by the depth of sampling. Transitions over time between high-frequency clones are universally acknowledged, in part because such transitions are readily detected despite undersampling as low as one isolate per sample.⁶⁷ In addition, transient cocolonizations are detected at transitions.⁶⁶ In a study of cystic fibrosis that disclosed elevated *S. aureus* diversity, an attempt was made to differentiate the fraction of the diversity generated within the population by mutation from that generated by migration, but the results were not so readily interpreted.⁶⁴

Mutation of *S. aureus* within a patient is also widely recognized, largely because it can lead to *de novo* antibiotic resistance, which has a notable clinical phenotype and thus attracts specific attention, unlike most mutations, which would be missed due to undercharacterization. *In vivo* mutation of *S. aureus* exhibits an immune evasion function and may represent pathoadaptation.^{41,68–70} Phenotypically and clinically meaningful variation within *S. aureus* blood stream infections has been observed via whole-genome sequencing, and there is no reason to believe that wound infections will not also contain such variability.⁷¹ Concordant observations of *S. aureus* antagonistic mechanisms suggest regular strain–strain competition; these mechanisms, though potentially intraspecific, may also be interspecific because of constant coexistence and interactions with other bacteria, including *Pseudomonas*.^{72–74} Further study of the distribution and range of antagonistic mechanisms is required before a quantitative assessment of *S. aureus* intraspecific competition can be made.

It bears mentioning that though many studies focus on *S. aureus*, there are numerous other species of *Staphylococcus* in wounds. With some effort, these can be distinguished based on 16S rRNA alone. These staphylococci have been demonstrated to have divergent association with dermatological disorders at the species level. However, most high-throughput sequencing studies ultimately aggregate the genus, if not the family or phyla. Aggregation of constituent diversity is more likely to cloud the question than provide clear understanding.

The demonstration of *S. aureus* diversity in epidermolysis bullosa wounds is particularly significant because the pattern of dispersal suggests that the individual clones are adapted to separate interspersed microhabitats in the wound rather than occupying coherent patches, as would be predicted by an autocorrelation (habitat neutral) model. In addition, the interspersed pattern sug-

gests ample opportunity for genetic exchange among the clones, as opposed to patterns that interpose spatial segregation among the clones. In fact, if mating would disturb coadapted gene complexes, which allow habitat utilization, then *S. aureus* might benefit from accelerated sequence diversification^{75,76} and antagonistic interactions.^{63,77} Further study of epidermolysis bullosa *S. aureus* populations may allow independent estimates of mutation, mating, and migration within the wound community—at least, of *S. aureus*. Generalizing across genera or families is inappropriate without further study.⁷⁸ Epidermolysis bullosa creates persistent wounds over very long periods of time; the dynamics in these wounds are likely to involve more mutation than those in a traumatic wound, for example. As a result, generalizing across wound types is also difficult.

Pseudomonas

Pseudomonas is the signature organism of burn wounds, though it is also of concern in other wounds, chronic and traumatic. Studies of *Pseudomonas* diversity exhibit the same characteristic deficiencies of other studies—for example, taking a single isolate per sample and a single sample per time point. However, even these studies hint at strain diversity in the hospital environment^{79,80} during the establishment of infections, and as the result of diversifying selection during the course of the infection. *Pseudomonas* infections in cystic fibrosis are frequently complex mixtures of interacting strains.^{81,82} In one of the few studies to examine multiple strains at a time, *Pseudomonas* colonization in wounds was frequently polyclonal.⁸³ This result, unfortunately, casts doubt on the conclusions of many other studies due to implicit undersampling.

Acinetobacter

Acinetobacter as a genus is well-known for polyclonal outbreaks across a hospital or ward.^{84–87} The diversity and plasticity of *Acinetobacter* has made whole-genome sequencing a vital epidemiological tool.^{88,89} *Acinetobacter* is highly capable of recombination, which has an impact on virulence.⁹⁰ The genus has a number of named species that occur in wounds, including *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, and *Acinetobacter pittii*. Scattered reports have identified more than one species or genotype recovered for a single patient (Hauck *et al.*⁸⁴ identifies four such patients), even in a single wound sample, much like *Staphylococcus*, and have repeatedly identified mutations to antibiotic resistance during the course of infection.⁹¹ However, systematic published data

about strain diversity within a single species within single wounds do not appear to be available, and, given the methodological difficulties, suggestive unpublished data require extensive analysis and peer review before it can be given credence.

Klebsiella

A survey of rapidly mutating repetitive polymerase chain reaction regions in conjunction with known antibiotic resistance genes has demonstrated that *Klebsiella* also causes polyclonal outbreaks in healthcare facilities.⁹² However, once again, patients have not been sampled sufficiently to determine how often infections of individuals are polyclonal. Circumstantial evidence based on observed horizontal gene transmission suggests that the various *Klebsiella* are in close proximity in the hospital environment and observed environmental *Klebsiella* diversity follows the same pattern as that of *Acinetobacter*, suggesting that *Klebsiella* infections may also be frequently polyclonal.

Aeromonas

Aeromonas spp. have been known to coinfect catheters and other recurrent sites of infection.⁹³ *Aeromonas hydrophilia* has been reported polyclonally from an individual wound,⁹⁴ but no systematic study of diversity within wounds has been conducted. Certainly many other bacteria (and some fungi) deserve to be discussed due to their unacknowledged importance to wound pathology, but relatively few have been studied in sufficient detail within the context of wounds to receive such notice. It is hoped that within a few years, a revisitation of the topic will go into much greater depth.

FUTURE DIRECTIONS

Technologies that will likely impact our understanding of strain diversity within wounds are advanced metagenomics, optical mapping, single-cell genomics, and advanced microscopy. Advanced metagenomics with both high throughput and very high processivity (*i.e.*, long reads) would theoretically permit the characterization of strain diversity from a sample. Individual genomes could be assembled and variation within a population could be characterized. Though it is unlikely that amplification could be inserted into the sample processing, several long-read, amplification-free methods may potentially be applied, including nanopore sequencing and sequencing by synthesis. However, these new sequencing methods are less developed than the current generation of high-throughput sequencing and are limited by error rates, throughput, and cost, even for research applications. Even mod-

est error rates prevent the assembly of minimally divergent genomes; throughput limitations prevent detection of rare variants in minority taxa; and high per-sample cost limits spatial and temporal resolution.

Optical mapping typically has other functions, but some of its current implementations have the potential to develop into a substitute for shotgun metagenome sequencing. These methods process extremely long fragments of DNA (from 15% to 70% of a bacterial chromosome) from the sample, fixing them to a surface or in a channel during processing. Instead of directly sequencing, microscopy is used to locate particular short sequences, often six or eight basepairs along the fragment, and measure the DNA between them. At this time, the number of target sequences that are sought in any given sample is one or a very few. The data returned is an extremely sparse characterization of the DNA fragments, useful for genome assembly ("scaffolding") or strain barcoding, but not equivalent to sequencing. However, if the density of target sequences was greatly increased, then optical mapping could provide an alternative to long-read sequencing directly from a sample. It would be particularly suited to detecting copy number and gene content variation on a single chromosome. Nevertheless, optical mapping from a clinical sample would not be able to identify the coexistence of multiple plasmids or chromosomes in a single cell, and single-nucleotide mutations would also be difficult to detect reliably.

Single-cell genomics retain the cells as individual entities during sample processing. This bypasses many assembly challenges and allows plasmids and second chromosomes to be associated properly. Typically, microfluidics is used to segregate the individual cells during sample preparation. Single-cell genomics is challenging because of the limited starting material that enters the DNA purification process. Methods of overcoming the low starting material, such as rolling-circle amplification, introduce replication errors. Elevated error rates lead to only ~70% of the genome being assembled, undermining the advantages of the method, and it is likely that the most variable, interesting regions are the very ones that cannot be interrogated. Single-cell genomics also require segregation of sequencing runs from each individual cell, either by barcode or some other mechanism. Even large single-cell studies typically examine fewer than 100 cells, treating each as an individual sample. The goal of detecting strain diversity precludes the expedient of selecting a representative from each species for sequencing. The cost per clinical sample would be, for the foreseeable future, prohibitive.

If single cells can be used to generate isolated microcolonies, then the relatively low error rate of natural genome replication can be maintained and the starting quantity of DNA for sequencing can be increased. However, this introduces many of the standard challenges inherent in culture methods; for instance, the initial cell population must be closely monitored to assess the sampling bias inherent during culture.

Advanced microscopy characterizes intact cells; however, like optical mapping, it does not generate sequence. Instead, marker sequences are detected indirectly through probe hybridization or a similar strategy. Advanced microscopy can detect dozens, possibly hundreds, of probes simultaneously in thousands or millions of cells. Probe targets can also be RNA (to reveal transcription and regulation), protein (to reveal expression levels), carbohydrate (for serotyping), or other substances, including metabolites and toxins. Flow cytometry allows the cells to be streamed through a detector for high throughput and reproducibility while more traditional methods retain the spatial localization of the cells. Hybrid methods, such as those pioneered by Accelerate Diagnostics, dissociate the cells but bind them to a surface so that cell behavior can be observed directly. The challenge of extracting strain-level phylogenetic information from microscopy is substantial and unaddressed; however, the methods do have the potential to reveal strain diversity in minority populations at an acceptable cost per sample.

SUMMARY

Microbial diversity in wounds includes not only diverse bacterial and fungal genera, but also multiple strains within a given species in a single wound. This kind of diversity is very difficult to study and greatly complicates the microbiological evaluation of wounds because it breaks abundant taxa into a series of relatively rare taxa and creates additional opportunity for confusion among distinct ecotypes, including pathogens. Successful studies of complex populations have been conducted via

TAKE HOME MESSAGES

- Strains within a bacterial species vary greatly in their clinical impact.
- Multiple strains of a bacterial species often coexist in a wound.
- Strains within a wound can coexist because of diversification within the wound or because of repeated bacterial introductions from outside the wound.
- Currently available methods, even in the research laboratory, are insufficient for characterizing the full suite of strains from wound tissue samples.

whole-genome sequencing of multiple isolates from relatively small samples. Multimodal studies of wound ecology that include evaluation of local host physiology and differentiate among closely related clones will be required to ultimately understand microbial pathogenesis and the ecology of healing and nonhealing wounds.

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Abbreviations and Acronyms

PFGE = pulsed-field gel electrophoresis
 16S rRNA = 16-Svedberg ribosomal ribonucleic acid gene
 UniFrac = while appearing an acronym, actually a name

Glossary

Allele: One of the alternate sequences of a gene.

Amplicon: A unit of nucleotide sequence generated by replication during a laboratory polymerase reaction.

Horizontal Gene Transfer: The process of moving DNA from one cell to another and incorporating it into the genome of the recipient. Contrasted with vertical gene transmission during reproduction.

Locus (plural, loci): A genomic segment that is the physical or logical position of a gene by definition; the various alternative alleles occupy the same locus.

Metagenomics: The study of the several, aggregate genomes present in a biological assemblage or community.

Molecular Methods: Laboratory strategies for characterizing organisms that do not involve observing behavior or intact-organism morphology but instead observe the composition of biological polymers (nucleic acids, proteins, and polysaccharides).

Operational Taxonomic Unit: A taxa recognized for the purposes of a specific scientific, engineering, or medical purpose. For the sake of consistency, the units in a single context are typically at the same taxonomic level.

Pathoadaptation: The process by which an infecting organism becomes better able to conduct a pathogenic lifestyle during the course of infection.

Phylogeny: The relationship among organisms from a history of reproduction.

Taxon (plural, taxa): A group of organisms related by common descent and potentially given some collective name or identity.

Taxonomic Level: A hierarchical position in a taxonomy. Examples include genus, species, and family.

UniFrac: A mathematical method of collecting communities of organisms into a hierarchical clustering for comparison and analysis.