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Within-Host Evolution of Bacterial Pathogens in Acute and Chronic Infection

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Abstract

Bacterial pathogens undergo remarkable adaptive change in response to the selective forces they encounter during host colonization and infection. Studies performed over the past few decades have demonstrated that many general evolutionary processes can be discerned during the course of host adaptation, including genetic diversification of lineages, clonal succession events, convergent evolution, and balanced fitness trade-offs. In some cases, elevated mutation rates resulting from mismatch repair or proofreading deficiencies accelerate evolution, and active mobile genetic elements or phages may facilitate genome plasticity. The host immune response provides another critical component of the fitness landscapes guiding adaptation, and selection operating on pathogens at this level may lead to immune evasion and the establishment of chronic infection. This review summarizes recent advances in this field, with a special focus on different forms of bacterial genome plasticity in the context of infection, and considers clinical consequences of adaptive changes for the host.

INTRODUCTION

Fitness landscapes: models used to visualize the relationship between genotype, selection, and fitness, usually measured in terms of replication rate

Mismatch repair: a highly conserved system in bacteria that corrects base pairing errors introduced during DNA replication; the process involves mismatch identification, strand excision, and strand resynthesis; the presence of methylation is used to distinguish the parental strand during error correction

Insertion sequence (IS) elements: small self-replicating fragments of DNA that are able to move within and between genomes and plasmids using encoded recombinases; IS elements are usually less than 2,500 bases in length and are bounded by inverted sequence repeats

Transposons: self-replicating units typically larger than IS elements that may carry cargo genes including antimicrobial resistance genes

Bacteriophages: self-replicating DNA- or RNA-based viruses that infect bacteria as part of the replication cycle

In vitro and in vivo studies over the past few decades have revealed that bacterial pathogens may undergo substantial adaptive change in the context of host colonization and infection. Early work based on careful phenotypic characterization revealed sometimes striking modifications, including alterations in colony size, pigmentation, capsule production, and motility, that hinted at potentially more extensive underlying genetic changes. The clinical importance of such evolution within the host was perhaps no more readily understood than in the context of antimicrobial resistance, where heritable phenotypic changes were observed to emerge rapidly under antibiotic selection with well-understood clinical and epidemiological consequences (1–3). The immune response of the host was appreciated to provide another critical component of the fitness landscapes guiding adaptation, and selection operating on pathogens at this level was observed to lead to changes important to immune evasion and the establishment of chronic infection (4).

Subsequent developments in whole genome sequencing and the ability to analyze the genomes of large numbers of isolates and populations have facilitated rapid progress in the understanding of the genetic basis of many of these adaptive changes. Detailed genomic analysis has revealed that many general evolutionary processes can be discerned in the context of host adaptation, including genetic diversification of lineages, clonal transmission and succession events, convergent evolution, and balanced fitness trade-offs (5–8). Theoretical and experimental work has demonstrated that elevated global mutation rates, for instance due to deficiencies in DNA mismatch repair or replicative proofreading, can accelerate adaptive evolution over time frames relevant to colonization and infection, even if deleterious to genome stability over longer periods of time (9–14). In some cases, active insertion sequence (IS) elements, transposons, and bacteriophages can drive genomic plasticity through intra- and intergenic insertions that result in gene disruption or alteration of gene expression through transplantation of *cis*-acting promoters (15–17). Dramatic examples of genome and proteome compaction or degradation have been noted to occur due to individual gene disruptions as well as large recombination-driven chromosomal deletions (18–23). In some cases, genomic modifications may increase pathogenicity, facilitate long-term persistence, or generate resistance to antimicrobials (15, 24–29), or conversely, they may result in the attenuation of virulence and the evolution of apparently commensal pathogen–host relationships (18, 19). An important insight from these studies has been that evolution that occurs in the context of colonization and infection may parallel evolution underlying the emergence of human-restricted pathogens from broad-host-range generalists over longer periods (18, 30).

More recently, in vivo clinical studies complimented by in vitro transcriptomic, proteomic, metabolomic, and genomic analyses have provided a more complete picture of bacterial adaptive evolution in the context of host infection. This review summarizes recent advances in our understanding of the changes that occur during host adaptation and the underlying mechanisms, with emphasis on the roles of different forms of bacterial genome plasticity. We begin with consideration of general mechanisms that generate genetic diversity in bacterial populations, followed by principles of selection dynamics and host-associated fitness landscapes on which these populations evolve. We conclude with detailed consideration of a series of selected cases that illustrate examples of how these key principles and mechanisms operate in the context of host adaptation in clinical infection. Through these examples, we explore the clinical consequences of these adaptations for the host where they have been characterized. As this review focuses primarily on a set of recent works, there are many excellent older studies that have necessarily been left out due to space limitations.

MECHANISMS THAT GENERATE GENETIC DIVERSITY IN BACTERIAL POPULATIONS

Base excision repair (BER): a glycosylase-based system that removes nucleotide bases to repair lesions resulting from oxidation, deamination, and alkylation of DNA bases

Bacterial species of clinical importance have relatively small (~1–8 × 10^6 bases) haploid genomes with wild-type (WT) baseline spontaneous mutation rates on the order of approximately 10^{-10} per position per generation (31, 32). Due to the large sizes that bacterial populations may attain [$>10^7$ colony-forming units (CFU)/mL] and their short generation times during nonrestricted growth (30–120 min), even these relatively low mutation rates have the potential to generate substantial genetic diversity at the population level over intervals relevant to the time course of acute and chronic infection, setting the stage for a variety of evolutionary processes to unfold. A number of important secondary mechanisms may supervene to generate substantial additional diversity. As we see in the case studies that follow, these secondary mechanisms may materially modify the course of evolution in the context of host adaptation and include generalized (genome-wide) and localized (locus-specific) hypermutation; recombination-dependent and -independent mechanisms that generate structural variation; and mobile genetic element and phage insertions. The goal in this section is to summarize briefly the mechanistic principles rather than to review them in depth. Each of these processes has been covered in detail in excellent previous reviews to which the reader is directed. It is important to emphasize that these processes are general and not specific to intrahost evolution, though they may exhibit special properties in the context of selection dynamics encountered in host infection.

Generalized Hypermutation

The fidelity of genome replication in bacteria is ensured by a set of highly conserved mechanisms operating at different steps of the replicative process. Many bacterial DNA polymerases can select and incorporate correct (template-specified) bases by a factor of 10^3–10^6 over incorrect bases, explained by a mixture of thermodynamic variables and kinetic selection (33–35). The underlying fidelity of polymerase action is improved by orders of magnitude by secondary processes including proofreading (36, 37), DNA mismatch repair (MMR) (13, 14), and base excision repair (BER) (38, 39) to an overall base substitution rate of approximately 10^{-10} per position per generation as measured in *Escherichia coli* and *Pseudomonas aeruginosa* (31, 32). Deficiencies in any of these systems can lead to elevations in mutational rates of orders of magnitude with characteristic alterations in mutational spectra (40). Specific mutagens, including reactive oxygen and nitrogen species, may additionally generate oxidative stress mutagenesis with distinct mutational signatures (41). Finally, the SOS system, a conserved DNA damage- and stress-induced response that leads to the excision of DNA lesions followed by strand resynthesis by error-prone polymerases, may generate secondary mutations during the repair process with complex properties (42, 43). Each of these factors may contribute genome variation on which selection may act and are dealt with individually in this section.

Replicative proofreading occurs following base incorporation by a primary DNA polymerase and is essential for genome stability. This process is accomplished both by proofreading activities intrinsic to the polymerases (for instance Pol δ and Pol ε) and by separately encoded enzymes extrinsic to the primary polymerases (35). In this latter category, the DNA polymerase III ε-subunit, encoded by the *dnaQ* gene (also annotated as *mutD*), is particularly important and provides mismatch-triggered 3′-5′ exonuclease activity during DNA replication, allowing removal and correction of mismatched base pairs (44). Mutations of the *dnaQ* gene may result in defective proofreading, with elevations in genome-wide mutation rates >1,000-fold with characteristic spectra measured with different mutations in different systems (36, 44, 45).

The DNA MMR system is highly conserved across prokaryotes and eukaryotes and likewise essential to the fidelity of genome replication (13, 14, 46). In *E. coli*, in which it has been best

Transition: a purine-to-purine or pyrimidine-to-pyrimidine mutation

Transversions: purine-to-pyrimidine or pyrimidine-to-purine mutations

studied, the MMR system comprises a well-characterized set of proteins, including the primary recognition machinery MutS, MutL, and MutH along with a variety of accessory proteins necessary for the execution of different steps in the repair process (MutU, UvrD, exonucleases, SSB, polymerase, and ligase) (46). Together, this system carries out strand-specific repair of base-base mismatches and small indels introduced during replication by making use of dGATC *N*-6-methyladenine sequences to determine the parental strand (46). Mutations in any of the genes composing the MMR system may therefore result in generalized hypermutation, with elevations in baseline mutation rates of orders of magnitude with characteristic mutational spectra (40). Mutations in the *mutS* gene are common causes of MMR-deficient hypermutation and generally result in transition-dominated mutational spectra with enrichment at specific sequence motifs throughout the genome (40).

In addition to the MMR enzymes, a variety of BER mechanisms, including systems encoded by *mutM*, *mutY*, *mutT*, and others, are particularly important in the response to a variety of environmental stresses (39). Reactive oxygen and nitrogen radicals may directly oxidize, or form covalent adducts with, nucleotides, and when incorporated into DNA, these modified nucleotides can drive genome-wide mutagenesis. A commonly observed signature of oxidative mutagenesis is an enrichment in G:C to T:A transversions, resulting from the oxidation of guanine to 8-oxoguanine. Guanine has the lowest redox potential among the nucleotide bases and consequently oxidizes rapidly in response to reactive oxidative stress (47). One of the resulting products is 8-oxoguanine, which mis-pairs with adenine in a manner that may evade proofreading (48). The MutT protein works to clear the free nucleotide pool of 8-oxoguanine nucleotides by hydrolyzing 8-oxoguanine triphosphate to 8-oxoguanine monophosphate, and genomic 8-oxoguanine lesions are excised by the 8-oxoguanine glycosylase MutM, or the mis-paired opposing adenine is excised by MutY (39, 49). Environmental oxidative stress in combination with *mutM*, *mutY*, or *mutT* mutations may result in strongly elevated mutation rates that are highly enriched for G:C to T:A transversions with corresponding amino acid substitution spectra (45).

In response to DNA damage, bacterial cells may activate a global DNA damage response system, originally described as the SOS response in *E. coli* but subsequently understood to be present in diverse bacterial genera with a variety of modifications (43, 50, 51). This response proceeds through a series of defined steps, beginning with proteolysis of the LexA repressor protein by activated RecA, usually in combination with ssDNA. This results in derepression of a complex transcriptional program leading to double-stranded cleavage and base excision, followed by repair of the resulting lesion by dedicated polymerases (50, 51). Importantly, many of the SOS polymerases are error prone, and thus, induction of this system results in mutagenesis (42).

All of the mechanisms described above can drive global mutagenesis with different rates, spectra, and sequence specificities, which have important consequences for subsequent evolutionary processes. Bacterial genomes are haploid, and while recombination between the chromosomes of different lineages does occur, bacteria lack the efficient mechanisms that sexually reproducing diploid organisms employ to segregate beneficial from deleterious mutations under selection. Elevated global mutational rates have been demonstrated to be adaptive in a variety of contexts over shorter time frames, including those relevant to acute and even chronic infection, but when mutation rates are elevated for prolonged periods of time, deleterious mutational loads may accumulate that outweigh adaptive benefits, resulting in degradation of genome stability (9–12). In the individual cases that follow at the end of this review, we see examples of how global hypermutation may diversify bacterial populations, facilitate genome degradation, and accelerate the evolution of antimicrobial resistance in the context of infection.

Localized Hypermutation and Recombination-Dependent Changes

Elevated rates of genetic change may also be confined to specific loci in a sequence-specific manner. These changes may involve small genetic lesions or larger recombination-dependent deletions, insertions, inversions, or translocations. This section reviews some of the principal mechanisms of these changes and focuses on a few well-studied examples. Hypermutable sequences that drive localized genetic changes have been referred to as contingency loci, and in a number of cases there is strong evidence for specific functional roles they play (5, 52–55). As such, it appears likely that many contingency loci have been evolutionarily selected for the adaptive heterogeneity they generate, including, importantly, in the context of infection and host adaptation.

The most well-studied examples of localized hypermutation involve inter- or intragenic simple sequence repeats (SSRs) mediating high-frequency, reversible genetic switching events (52). Stochastic DNA polymerase slippage events occur at SSRs with reproducible probabilities over time to generate deletions or insertions of one or more repeat sequences. When these repeats are located in promoter regions, they may change critical distances between elements required for transcription initiation, and when they are located in genes, they may delete or add codons in the reading frame if the underlying repeat is a multiple of three, or shift reading frames if not. A well-studied example involving an SSR embedded in a promoter is an AT tract located between the fimbria genes *bifA/B* in *Haemophilus influenza* (55, 56). Slippage during replication that deletes or adds AT dinucleotide repeats changes the relative spacing between the −10 and −35 sites (and angular phasing along the helix), interfering with the structural assembly of the complex required for transcription initiation. Slippage at this SSR occurring with defined probabilities thus generates diversity in expression of fimbria genes on which selection can act in the context of host infection. A well-studied example of an SSR embedded in a coding sequence involves CAAT repeats located in the 5′ end of the *lic2* gene in *H. influenzae* responsible for synthesis of a core saccharide component of lipopolysaccharide (LPS) (55, 57, 58). As the repeat length is four, single deletions or insertions shift the reading frame by +1 or −1, resulting in a nonfunctional protein and altered LPS structure (58). As with the fimbria *bifA/B* gene expression example above, polymerase slippage at this SSR occurring stochastically generates high-frequency variation in LPS structure within the population on which selection can act in the context of host infection.

Localized changes that result from recombination may be dependent on, or independent of, RecA and often occur at sites of homology, introducing recurrent structural variation in a sequence-specific manner (59). Other mechanisms involve nonhomologous end joining and site-specific recombination mediated by mobile genetic element (MGE)-encoded recombinases or integrases. Any of these mechanisms may delete, duplicate, invert, or translocate segments of genome that can range in size from hundreds of bases to more than a megabase. A specific example of inversion is found in invertible promoters. Invertible promoters may be located between two divergently arranged genes, and an invertase can catalyze inversion of the promoter sequence with the consequence that expression of one gene is switched off and the divergently oriented gene is turned on (60). Expression of the invertase may occur stochastically in minor subpopulations, resulting in the generation of transcriptional heterogeneity of the involved genes. *Bacteroides fragilis* group species make particularly extensive use of invertible promoters, and the functions include control of antimicrobial resistance genes (61, 62).

Genetic change may also be driven by MGE insertions. Such insertions may involve small IS elements or larger transposons carrying cargo genes. Insertions of MGEs may disrupt native promoters and coding sequences of genes, and they may deliver new strong promoters to downstream genes, resulting in constitutive activation, or in some cases confer new transcriptional regulatory dependencies (63). They may also form sites of homology driving translocations (59).

Transposons carrying genes may form repeat arrays that vary in size and regulate the dosage of the carried gene cargo (55, 64, 65). One well-studied example involves the chromosomal *cap* locus in *H. influenza* that is located within a composite transposon flanked by IS*1016* sequences (66–68). This arrangement facilitates changes in copy numbers by intramolecular homologous recombination, modifying gene dosage of the *cap* locus with functional consequences in capsule production that undergo selection in the context of host infection.

Founder effects: situations in which a population is descended from one individual or a small group of individuals (the "founder" population), resulting in limited genetic diversity

Genetic drift: changes in relative frequency of different polymorphisms in a population due to random chance, independent of selection

SELECTION DYNAMICS

Colonization or infection of a new host begins with a transmission event involving transfer of an inoculum from another living host or fomite. Founder effects can occur at this stage due to the physical means that underlie such transmission, often involving small droplets or particles, or direct physical surface contact, where subsampling of a larger population occurs. Theoretical and experimental studies have often made simplifying assumptions about limiting genetic homogeneity of transmission events with implications for reconstruction of transmission networks (69). However, the actual population structure and genetic diversity transmitted in studied cases has been difficult to measure directly or quantitatively. Whether the initial population structure is relatively homogeneous or very complex, the various processes considered in the previous sections will generate different forms of diversity with characteristic rates, and selection will act on this diversity to modify population structure during the course of adaptation. The sections below consider some features of the underlying dynamics of this process.

Mutation, Recombination Rates, and Effective Population Size

Single-nucleotide variant (SNV) mutations and small indels have been commonly characterized as the main drivers of intrahost evolution in bacterial populations, with recombination events and mobile genetic element insertion events playing a less frequent, if not less important, role. When discussing mutation rates in bacterial populations, there are at least two relevant measures, the first involving the intrinsic spontaneous mutational rates measured per site per genome division in vitro under standardized conditions and the second involving the substitution rates observed over longer evolutionary periods in isolates sampled at different times from natural populations. As noted above, intrinsic WT spontaneous mutation rates have been measured carefully in vitro to be approximately 10^{-10} per position per generation in the model organisms *E. coli* and *P. aeruginosa* (31, 32).

The rates of mutational fixation measured in vivo over much longer evolutionary periods, on the other hand, appear to be orders of magnitude lower than these spontaneous in vitro mutation rates, implying that the vast majority of spontaneous mutations are not retained over time in the population. Estimates over a timescale of millions of years suggested substitution rates of 10^{-9} to 10^{-10} per site per year, during which time thousands of replications of the underlying genome may occur (5, 70, 71). Analyses have highlighted the dependence of such estimates on the underlying timescale of the measurement itself, with shorter timescales of months to years revealing microevolutionary processes with much higher substitution rates (72, 73). Measurement of these microevolutionary rates for a variety of bacteria has revealed general rates on the order of 10^{-5} to 10^{-7} per site per year, translating into approximately 0.5–30 mutations per genome per year depending on the species (74–78). For instance, in a study by Didelot and colleagues (75) of *Helicobacter pylori* isolated from individuals in South Africa, the synonymous mutation rate was estimated at 1.38×10^{-5} per site per year, representing the higher end of reported rates. Another important related measure is effective population size, or $N(e)$, which has been defined in terms of the rate of change in the composition of a population caused by genetic drift (79). Didelot and

colleagues (75) estimated $N(e) = 1{,}318$ for the *H. pylori* isolates in the South African study cited above. Genetic drift is considered in further detail below.

Recombination, resulting in the exchange of sometimes large segments of genomes between genetically dissimilar isolates, can be another important source of diversity. In the study by Didelot and colleagues (75) mentioned above, it was found that most *H. pylori* isolates demonstrated low overall rates of recombination in the context of host colonization, but a subset of isolates demonstrated higher rates, introducing 100 times as many substitutions as would otherwise have resulted from mutagenesis alone, broadly consistent with previous reports on recombination in *H. pylori* (80). An important earlier study in *H. pylori* isolates derived from four chronically infected individuals and one human infection volunteer demonstrated that recombination was not evenly distributed throughout the genome but rather was concentrated in certain classes of genes, including the outer membrane proteins (74). Outer membrane proteins are major antigens in bacteria, and mechanisms that increase antigenic diversity may facilitate immune escape during infection. This study found evidence suggestive of diversifying selection in the *hop* family of outer membrane proteins, consistent with a role in generating antigenic diversity (74).

Purifying selection: stringent selection that sharply reduces population diversity by permitting the survival of only certain lineages containing a specific beneficial trait; antibiotic selection may often be purifying

Population bottlenecks: selection conditions or events that reduce the size of a population by permitting survival only of a subgroup, resulting in reduction of genetic diversity

Genetic Drift and Signatures of Selection

Above, we considered mechanisms generating genetic diversity, rates of spontaneous mutation, and rates of mutational fixation. Classic work by Kimura and others (81–83) has argued that most mutations are either severely deleterious and strongly selected against or close enough to neutral fitness relative to the power of selection that they fix in populations due to random drift alone. The implication is that many of the mutations that are observed within bacterial populations, including during host adaptation, are likely accounted for by genetic drift, not selection (84). This has consequences for the analysis of whole-genome mutational data in the context of adaptive evolution, where evidence should be sought for any individual mutation to which fitness effects are ascribed. Indirect evidence includes the identification of signatures of positive or negative selection. A classical approach for investigating positive selection is the calculation of ratios of nonsynonymous substitution rates to synonymous substitution rates (dN/dS), where values greater than 1 are taken to indicate potential evidence of positive selection. It should be noted that dN/dS calculations on small, relatively clonal populations often require subtle interpretation and can be misleading for a variety of reasons, including insufficient time for purifying selection to act, and selection on synonymous codons (85–87). Direct evidence for the functional or fitness consequences of a putatively selected allele usually involves genetic engineering of defined mutants, deletions, knockdowns, or WT complementation strategies.

Founder Effects, Population Bottlenecks, Selective Sweeps, and Clonal Succession

As noted above, the inoculum that initiates a new colonization or infection may subsample the diversity of the original population from which it is drawn, resulting in founder effects. However, such founder effects that occur during transmission have been very difficult to measure in practice, particularly for more rapidly diversifying bacteria (5, 69, 80, 88). Subsequent purifying selection may lead to population bottlenecks, as commonly occurs with antibiotic treatment (**Figure 1*a***) (89). Recurrent subsampling of diversity may also result in founder effects on smaller scales as occurs, for instance, during movement between different compartments within the host, as seen in the transition from nasal colonization to invasive infection with *Staphylococcus aureus* (24, 25). This has also been observed to occur in the context of geographic segregation within a compartment by *P. aeruginosa* in the lungs of individuals with cystic fibrosis (90).

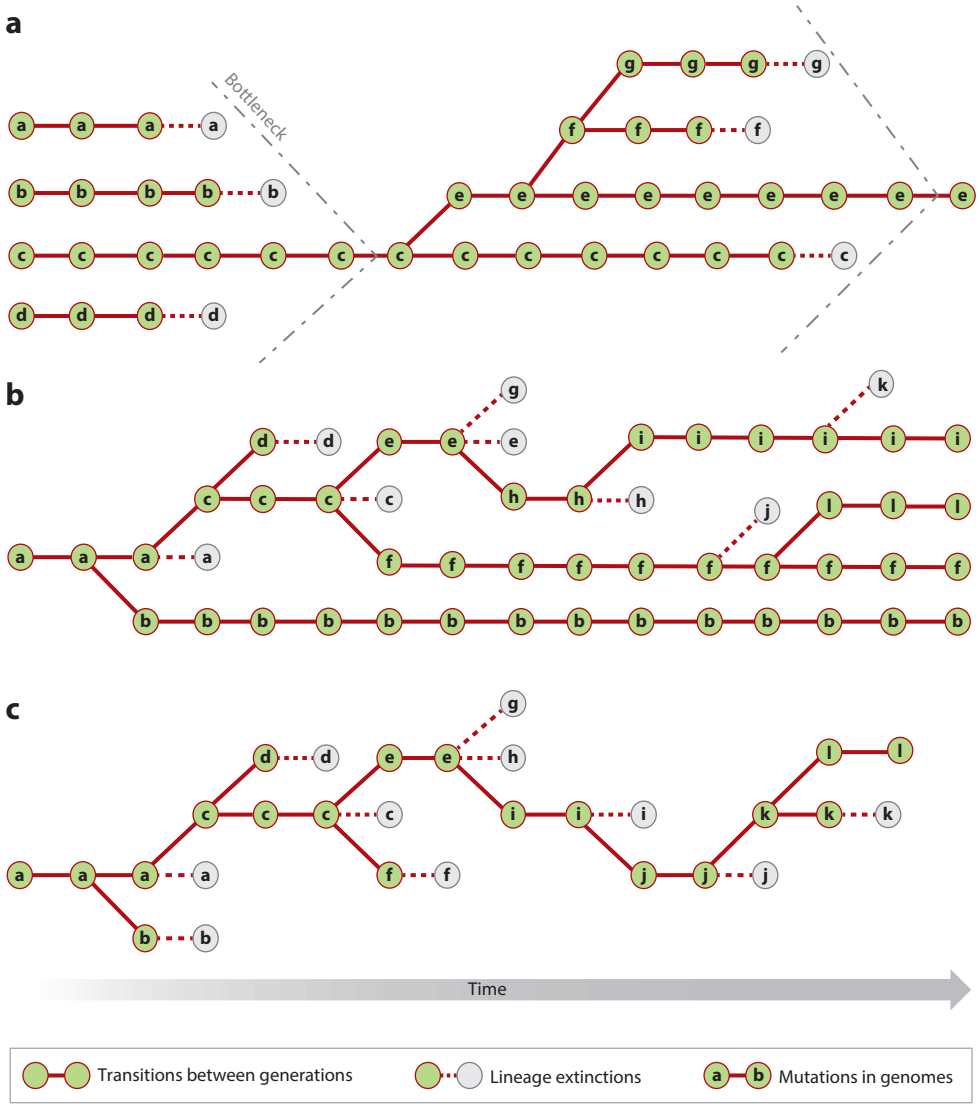

Figure 1

Bottlenecks, population diversity, and clonal succession. Lineages at successive points in time are represented schematically as lettered nodes. Time flows from left to right, and solid lines connecting nodes represent transitions between generations. Mutations in genomes are represented by a change in the letter assignment of the lineage, and dotted lines indicate lineage extinction. (*a*) Illustration of the effect of two successive population bottlenecks on an evolving set of lineages. Bottlenecks are illustrated schematically as dashed wedges. Lineage c survives the first bottleneck, and lineage e survives the second bottleneck. (*b*) Illustration of a population in which multiple lineages evolve and coexist. (*c*) Illustration of a population demonstrating clonal succession, in which clones a, c, e, i, j, k, and l succeed one another.

Within the resulting populations, advantageous mutations may arise that sweep to fixation, resulting in the emergence of dominant clones with a reduction in population diversity. In some cases, there may be stable coexistence of multiple diverse clones that are maintained in low or moderate frequencies over long periods of time (**Figure 1*b***). Alternatively, selection may result

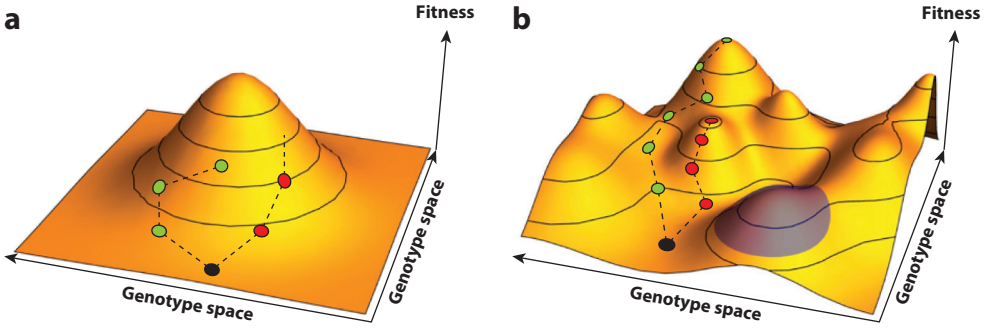

Figure 2

Smooth and rugged fitness landscapes. The horizontal planes in each plot represent different possible genotypes for a given individual schematically. The vertical axis represents the fitness associated with each genotype and defines a surface on which evolution is represented by a process of hill climbing. (*a*) Smooth, single-peaked fitness landscape, with many paths to the global optimum, two of which are represented by the red and green circles. (*b*) Rugged fitness landscape with multiple local optima, valleys, and ridges. The green circles represent a path to the global optimum, whereas the red circles represent a path that gets trapped at a local (nonglobal) optimum. Figure adapted from Reference 94; copyright 2015 National Academy of Sciences.

in clonal succession of a series of dominant lineages (**Figure 1*c***) (91). In sexually reproducing diploid organisms, advantageous mutations that occur in different lineages of a population may be combined in one lineage. In contrast, beneficial mutations arising within different lineages of a clone of largely asexual bacteria compete with each other, resulting in an evolutionary process called clonal interference (92, 93).

CASE STUDIES ILLUSTRATING PRINCIPLES AND MECHANISMS OF WITHIN-HOST ADAPTATION

Above, we reviewed mechanisms generating diversity in bacterial populations and some general principles of selection dynamics. Evolution is usefully conceptualized as structured by fitness landscapes that reflect the interactions of selective forces encountered by a biological population, and their interplay with mutagenesis and genetic drift (**Figure 2**) (94). In the context of colonization and infection, dominant features of this selection landscape may include the innate and adaptive immune systems, oxidative stress generated by phagocytes, antibiotics, and metabolite or metal restriction by the host, among many others. Below, we explore a series of recent case studies that lend insight into the host fitness landscapes and strategies used by bacterial pathogens evolving in response to them. As noted above, this review focuses on recent work and does not cover the great body of prior excellent studies that have been published.

Clonal succession: a process in which individual clones are selected to dominance in succession either as a consequence of progressive increases in fitness, or by changes in the underlying fitness landscape and selective forces

Clonal interference: a situation in which beneficial mutations arising within different lineages of a clone of largely asexual bacteria compete with each other as a consequence of the infrequency of recombination

Evolution of a Zoonotic Pathogen in an Immunodeficient Host Driven by a Proofreading Deficiency

Launay et al. (45) studied the mechanisms driving evolution of the emerging zoonotic pathogen *Bordetella hinzii* following infection in an individual with interleukin-12 receptor β1 deficiency. Unlike *Bordetella pertussis*, its better-known human-restricted genetic relative, *B. hinzii* is primarily an animal pathogen that was originally characterized in the avian respiratory tract (95–98). More recently, *B. hinzii* has been recognized to be capable of crossing the species barrier and causing serious infection in human hosts, including bacteremia, endocarditis, meningitis, and respiratory tract disease. As such, this case of persistent infection provided an opportunity to study how a zoonotic bacterial pathogen adapted after a presumed jump to a human host. Genomic sequencing

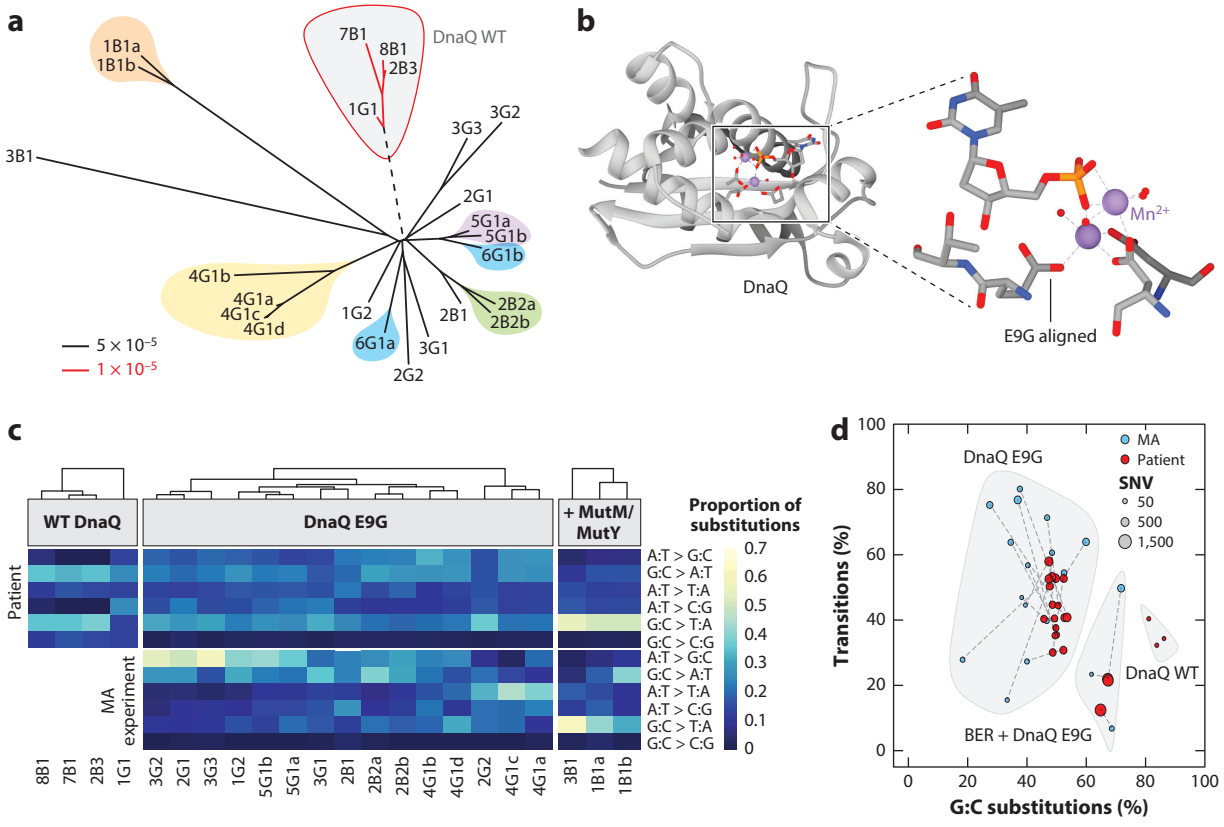

Figure 3

DnaQ E9G substitution disabled proofreading and drove within-host diversification of *Bordetella hinzii* over 45 months. (*a*) Unrooted maximum likelihood tree demonstrating diversification of patient *B. hinzii* isolates collected over 45 months of clinical care. Isolates connected by black branches contain DnaQ E9G, while the isolates connected by red branches carry the WT DnaQ allele. The branch scale represents nucleotide substitutions per site, and the scale of the red branches was magnified five times for clarity of presentation. (*b*) Crystal structure of *Escherichia coli* DnaQ (PDB identifier: 2IDO) demonstrates that the γ-carboxyl group of the aligned DnaQ E9 forms the binding site for one of the two catalytic divalent metal ions (Mn^{2+}, *purple*) in the active site, required for enzymatic function. Substitution of glycine in DnaQ E9G eliminates this critical γ-carboxyl group. (*c*) Heat maps of clustered base-pair substitution spectra in patient isolates (*top*) and measured in MA experiments in vitro (*bottom*) are consistent with a combination of oxidative stress mutagenesis, proofreading deficiency, and BER deficiency. Relative mutation proportions are indicated by color for each of the base-pair substitutions as shown on the color scale legend. (*d*) Relative transition and G:C substitution frequencies in patient isolates (*red dots*) and in MA experiments (*blue dots*). Dot size represents divergence from an ancestor, and matching isolates are connected by dashed lines. MA experiments were performed in vitro. Three distinct mutational patterns are observed as defined by WT DnaQ, DnaQ E9G, and DnaQ + BER (MutM or MutY) mutations. Abbreviations: BER, base excision repair; DnaQ E9G, an E9G substitution in the ε-subunit of DNA polymerase III; MA, mutation accumulation; PDB, Protein Data Bank; SNV, single-nucleotide variant; WT, wild type. Figure adapted from Reference 45 (CC BY 4.0).

performed on 24 *B. hinzii* isolates cultured from blood and stool over the course of 45 months revealed a clonal lineage that had undergone extensive within-host genetic and phenotypic diversification (**Figure 3*a***). During this period, the patient also had a history of *Candida tropicalis* esophagitis but no documented history of either mycobacterial infection or salmonellosis, which are otherwise common infections in patients with interleukin-12 receptor β1 deficiency. The patient additionally had a history of clinical colitis and membranoproliferative glomerulonephropathy with nephrotic syndrome.

Importantly, 20 of 24 cultured isolates shared an E9G substitution in the DNA polymerase III ε-subunit (DnaQ E9G) active site at a position that is conserved across prokaryotes and eukaryotes and forms part of the binding site of one of the catalytic divalent metal ions in the enzyme core (**Figure 3*b***). As described in the section titled Generalized Hypermutation above, the DNA polymerase III ε-subunit is encoded by the *dnaQ* gene and provides 3′-5′ proofreading exonuclease activity during replication. Mutations in *dnaQ* can disrupt proofreading, resulting in proofreading-deficient hypermutation. Launay and colleagues (45) characterized the mutational rate and spectra of the DnaQ E9G lineages in vitro (**Figure 3*c*,*d***), which confirmed that the isolates were phenotypic hypermutators with mutation rates elevated by 100- to 1,000-fold relative to the isolates with WT DnaQ. The authors hypothesized that the lineage of the original infecting strain was represented by the cultured isolates with WT DnaQ and intact proofreading and that this ancestor had given rise to the DnaQ E9G lineages in which proofreading was disrupted, driving diversification during the course of the infection. Interestingly, after the second culture day (day 139), the WT DnaQ lineage isolates disappeared from cultures, leaving only DnaQ E9G lineage hypermutators for a period of more than 8 months. During this period, multiple independent lineages derived from a DnaQ E9G ancestor demonstrated clonal succession, with individual lineages accumulating hundreds to more than a thousand mutations relative to the inferred ancestor (**Figure 3*a***). Furthermore, compound hypermutators were observed with additional mutations in the *mutM* and *mutY* genes with measured spontaneous mutation rates greater than one mutation per genome per replication (isolates 1B1a, 1B1b, and 3B1). In addition to the mutations resulting from the proofreading deficiency, an excess of G:C to T:A transversions was observed, suggesting the presence of oxidative stress mutagenesis (see the section titled Generalized Hypermutation) (45).

To assess the fitness consequences of the mutations that occurred in the hypermutators, the authors looked for evidence of selection by calculating d*N*/d*S* ratios and mutational expectation values. These calculations (with assumptions) suggested positive selection on a number of targets, implying that the underlying mutations were adaptive. Primary targets of selection included multiple core metabolic enzymes that were repeatedly mutated, suggesting that metabolic reprogramming may have occurred during adaptation from the avian respiratory tract to the human blood and gastrointestinal (GI) compartments. Additionally, two of the most highly targeted genes were *bvgS* and *risS*, which encode components of master virulence systems in other homologous bordetellae. Many of the mutations that occurred in these targets resulted in nonsynonymous substitutions at conserved positions, suggesting the possibility of functional disruptions in virulence control systems. In this case, the host remarkably demonstrated bloodstream infection intermittently for a period of at least 45 months without manifesting evidence of either frank sepsis or overwhelming systemic infection. Whether this was attributable to attenuation of organism virulence due to these mutations requires further study.

Convergent Metabolic Specialization in *Pseudomonas aeruginosa* in Cystic Fibrosis Chronic Infection

The next case we consider examines a more defined example of convergent longitudinal metabolic adaptation. La Rosa and colleagues (99) studied metabolic specialization in *P. aeruginosa* cultured over a period of 8 years from an individual with cystic fibrosis. Individuals with cystic fibrosis are unable to clear deep lung infections with *P. aeruginosa* and related pathogens over periods of years to decades, providing unique conditions for long-term evolution and adaptation. The individual in this case had persistent infection with three distinct clones of *P. aeruginosa*, designated as DK15, DK53, and DK01. One of these clones (DK01) was highly transmissible and presumably highly adapted to the cystic fibrosis respiratory system, having been isolated from 40 different individuals with cystic fibrosis. DK01 and a second strain (DK53) had also evolved

MMR-deficient hypermutator phenotypes due to mutations in the *mutS* and *mutL* genes, respectively (99). The authors studied longitudinal adaptation in metabolism, oxygen consumption, and growth rates in these isolates as well as the organic composition of cystic fibrosis respiratory sputum for insight into the environment to which the isolates were adapting. Interestingly, the DK53 and DK01 strains appeared to have an auxotrophy for growth in minimal media with both glycolytic and gluconeogenic substrates, and further analysis demonstrated convergent metabolic adaptations to alternative preferences for carbon source assimilation that matched sputum composition. These changes occurred along with reduced arginine and pyruvate fermentation, increased oxygen consumption among the more adapted isolates, and overall slower growth rates (99).

Auxotrophy: the inability of a bacterium to synthesize a specific molecule required for its growth

Evolution of *P. aeruginosa* Driven by Extensive Mobile Genetic Element and Prophage Insertions

In the above two cases, genetic diversity driving adaptive evolution was enhanced by global hypermutation. In the next cases, we consider mobile genetic element movement as a critical source of diversity on which selection can act. Previous work, including that by Song and colleagues (100) on *Burkholderia mallei* and *Burkholderia pseudomallei*, and Parkhill and colleagues (30) on *Bordetella bronchiseptica* and *Bordetella pertussis*, has demonstrated that insertion sequences can mediate dramatic genome compaction in the transition from environmental strains to host-restricted lineages. Attrée and colleagues (16, 101) studied the evolution of a previously characterized exolysin-secreting *P. aeruginosa* that caused a fatal hemorrhagic pneumonia in a human patient with chronic obstructive pulmonary disease. The authors performed genomic, transcriptomic, and proteomic studies on two isolates recovered from this patient. The initial isolate was pansusceptible and the second isolate was highly resistant across multiple classes of antibiotics including cephalosporins and carbapenems (101).

Genomic analysis revealed extensive movement of IS*L3* family insertion sequences throughout the genome, resulting in disruptions of a variety of genes, including type IV pili, flagellar components, and O-specific antigens involved in immune recognition. Additionally, there were interruptions of the *oprD* gene encoding the outer membrane porin required for entry of beta lactams into the cell and the *ampD* gene encoding the negative regulator of the chromosomal *Pseudomonas*-derived cephalosporinase (PDC) beta lactamase, conferring resistance to beta lactams. The authors found further that the later isolate contained many more insertion events and was less virulent in a *Galleria mellonella* model. In summary, this work demonstrated that insertion sequences may drive dramatic modification of immunodominant antigens potentially involved in immune escape, as well as the evolution of antibiotic resistance in the context of infection (16).

Gloag and colleagues (26) studied adaptive evolution among competing *P. aeruginosa* lineages during the course of a chronic infection in a porcine full-thickness thermal wound model. In this model, standardized wounds were inoculated with six *P. aeruginosa* strains, and biopsies were examined at 3, 14, and 28 days following initial infection. Hyperbiofilm-forming rugose small-colony variants (RSCVs) were observed to appear by the earliest sampling time and the lineages producing them took over the population. Small-colony variants of different morphologies are frequently isolated from chronic *P. aeruginosa* infections, including in individuals with cystic fibrosis, and are correlated with persistence and poor outcomes (102, 103). The ability of these variants to persist has been previously attributed in part to deletions of components of the Wsp pathway and overproduction of cyclic di-GMP, leading to overproduction of biofilm (103). The biofilm structure itself is composed of the exopolysaccharides Psl and Pel and facilitated by overproduction of the adhesin CdrA (102, 104–106).

Further analysis of the RSCVs cultured from the wound demonstrated that the majority of these isolates were descended from a single initial strain, and whole-genome sequencing revealed

driver mutations in the Wsp chemosensory system, consistent with expectations. The strong selection for hyperbiofilm-secreting lineages aligns with prior work demonstrating the importance of biofilms in the establishment of chronic infections (107) and importantly demonstrates how an evolutionary race to increase biofilm elaboration may dominate early competition. Interestingly, several of the examined Wsp mutants also demonstrated CRISPR-Cas adaptive immunity to a lysogenic phage that had been carried by one or more of the other six *P. aeruginosa* strains, suggesting that immunity to lysogenic phage may have played an additional role in selection (26).

To understand the genetic basis of the above results (due to a failure of reference-based short-read mapping to reveal the mechanisms explaining RSCV in all lineages), Marshall and colleagues (15) performed a deeper analysis searching for insertions not present in the reference PAO1 genome and discovered extensive prophage gene insertions ranging in length from 7 kb to 70 kb throughout the genome of one of the successful lineages (PAO1). The phages surprisingly had been donated by one or more of the initial, but subsequently overtaken, lineages. Long-read sequencing confirmed that multiple genes interrupted by repeated phage insertions explained convergent evolution of the RSCV phenotype in this lineage, based on previous work. In summary, this case demonstrated how mobile phages may be amplified and transmitted by transient members in a community and then drive adaptive evolution by insertional mutagenesis in the context of an infection (15).

Convergent Evolution in the Transition from Colonization to Invasive Infection in *Staphylococcus aureus*

The case by Gloag and colleagues (26) described above examined the phenotypic and genotypic mechanisms driving *P. aeruginosa* adaptation to a wound environment. It has become clear that different host compartments present distinct fitness landscapes but that similar dynamics of selection and adaptation may prevail. Giulieri and colleagues (25) studied niche-specific adaptations in *S. aureus* occurring during the transition from nose and skin colonization to invasive infection that involved bacteremia, endocarditis, osteomyelitis, and wounds in 2,590 isolate genomes (1,397 invasive and 1,193 colonizing) from 396 independent infection episodes. Importantly, the collection was representative of global *S. aureus* diversity with colonizing and invasive isolates represented across the major clades (25). Based on much prior work in the field, the authors assumed that isolates would fall into three groups: nose- and skin-adapted colonizing strains, early infection-adapted strains, and late infection-adapted strains. Within these groups, the authors looked for signatures of convergent adaptation.

The authors had previously demonstrated that genome compaction/degradation occurs in infection-adapted isolates of *S. aureus* represented by an enrichment in gene truncations (24), and they found in this dataset a similar enrichment of truncating mutations in invasive strains relative to colonizing strains (25). A strong signal for gene interruptions caused by insertion sequences was found, particularly by IS*256*, supporting previous observations of the role of this specific insertion sequence in driving genome plasticity involved in the transition from colonizing to invasive strains (24, 108, 109). To identify signatures of adaptation, the authors then looked for common genes with excessive mutations, deletions, or insertion sequences across invasive isolates (25). This analysis identified strong evidence of recurrent, convergent disruptions in the *agrA* and *agrC* genes of the quorum sensing system (previously identified as playing a central role in infection adaptation in *S. aureus*), and three antibiotic resistance genes: *stp1* (vancomycin resistance), *mprF* (daptomycin resistance), and *rpoB* (rifampin resistance). Using a broader approach that integrated genetic variation and insertion sequences in intergenic regions, they identified the *sucAB* (2-oxoglutarate dehydrogenase) and *walKR* (vancomycin resistance-associated two-component system) operons as

significantly enriched for convergent mutations and insertions in the invasive strains. The presence of these convergent signatures identified across this globally diverse set of *S. aureus* isolates argues for distinct adaptive pathways involved in the transition from colonization in the nose and skin, and the invasive phenotype (25).

Selective sweeps: processes by which genomes containing a favored mutation are selected to dominance, resulting in reduction of genetic diversity

Niche-Specific Gastrointestinal Tract Adaptation of *Enterococcus gallinarum* Drives Translocation and Inflammation

Yang and colleagues (27) studied how colonizing *Enterococcus gallinarum* evolves in the GI tract of mice to generate an invasive phenotype. *E. gallinarum* is a component of more than 6% of human gut microbiomes, and while it may exist as a benign commensal in many individuals, it may also translocate into mesenteric lymph nodes and initiate inflammation that is hypothesized to be associated with a variety of autoimmune diseases including lupus, sclerosing cholangitis, and autoimmune hepatitis (110, 111). Yang and colleagues (27) used a combination of powerful in vivo studies performed in monocolonized gnotobiotic mouse gut with a genetically defined starting strain, genomic sequencing, and phenotypic characterization. They found that *E. gallinarum* diverges into at least two independent lineages inhabiting the gut lumen and mucosa, with the latter lineage translocating into the liver. This divergence was driven by distinct patterns of mutagenesis involving selection of nonsynonymous mutations and indels in the *manY*, *lacE*, *ypdA*, and *immR* genes in translocated liver isolates from mucosa and the *walK* and *manX* genes in luminal isolates represented in feces (27). Interestingly, mutant alleles of these genes dominated the populations, indicating that selective sweeps had apparently eliminated the original infecting clone. Corroborating this finding, the fecal isolates with the *walK* and *manX* mutations demonstrated enhanced competitive fitness in the colon when compared with the original infecting strain or the translocated isolates cultured from the liver (27).

The liver isolates demonstrated increased resistance to clearance by phagocytes and to antimicrobial peptides and lysozyme, potentially explaining their ability to persist following translocation. Finally, divergent epithelial transcriptome signatures were observed in an in vitro cultured epithelial cell system in response to exposure to the two different lineages of isolates. In these experiments, the fecal isolates drove increased expression of defense-related immune response genes in epithelial cells, whereas the liver-derived isolates did not elicit these initial defense-related immune responses (possibly facilitating persistence) and, instead, drove inflammatory responses in the lamina propria of ileal mucosa (27). Overall, the findings demonstrated how within-host niche-specific evolution of a commensal may drive the emergence of a pathogen capable of translocating to new compartments and facilitating disease-causing inflammation, while evading host immune clearance mechanisms.

Evolution of an Asymptomatic Carriage State During Host Adaptation of Mismatch Repair (MMR)-Deficient *Salmonella*

In the above examples, we saw how invasive, pathogenic phenotypes of *P. aeruginosa*, *S. aureus*, and *E. gallinarum* may be enhanced during the course of adaptive evolution. It is presumed that the underlying mechanisms mediating invasion and pathogenicity in these cases and others directly confer selective advantages in the context of host infection. Klemm and colleagues (18) studied a fascinating case of the apparent opposite—the evolution of an asymptomatic carrier state in an immunocompromised host chronically infected with pathogenic *Salmonella* Enteritidis. The host was an individual with an interleukin-12 receptor β1 deficiency [similar to the host in the *B. hinzii* case above (45)], who presented with an initially severe systemic infection with nontyphoidal *Salmonella* Enteritidis. The authors studied 11 clonal blood isolates collected during febrile episodes over the course of 15 years. Genome analysis revealed that the isolates differed from each other by as many

as 600 SNVs and from the inferred common ancestor by as many as 1,000 SNVs (18). The authors calculated the mutational clock rate as 10^{-5} per site per year, which was more than an order of magnitude greater than that reported for WT *Salmonella* isolates. Notably, the mutations were transition dominated, and this was consistent with the finding of an in-frame deletion in the *mutS* gene (see the section titled Generalized Hypermutation), which, as the authors demonstrated in vitro, resulted in an MMR deficiency.

Further analysis of the genomes revealed that the SNVs had resulted in extensive pseudogene formation, with each isolate having acquired 158 pseudogenes on average compared with the inferred ancestor (18). Interestingly, a number of pseudogenes were reactivated through reversion mutations (often reversal of frameshifts), suggesting that transient inactivation of genes may have played a role in host adaptation. Many of the acquired pseudogenes were located in central metabolism, with specific disruptions of nitrate, ethanolamine, and B12 pathways. Other pseudogenes occurred in pathways controlling colonization, including fimbria genes, nonfimbrial adhesins, and secreted effector systems. The authors further found that there was statistical overlap with the sets of disrupted genes in human host-restricted *Salmonella* Typhi and Paratyphi A, suggesting convergent evolution and recapitulation of historical evolutionary processes that have occurred in other members of the *Salmonella* genus during host adaptation (18, 112).

Perhaps most interestingly, the authors found that at least two of the patient isolates were unable to invade a cultured Hep-2 cell line as efficiently as a WT isolate. This correlated with substantial attenuation in the ability to cause infection in mice following oral and intravascular inoculation and with the clinical course of the chronic infection of the patient, which was described as asymptomatic. Despite the attenuated virulence in WT mice, one of the patient isolates demonstrated higher levels of colonization in mice deficient in the interferon-gamma (IFN-γ) receptor, suggesting specific adaptation to the immune lesion that was present in the host, as interleukin-12 receptor β1 deficiency results in an inability to produce adequate IFN-γ for normal intracellular immunity (18).

Evolution of Antibiotic Resistance in Acute Infection Driven by an MMR Deficiency in *P. aeruginosa*

The evolution of antibiotic resistance in the context of host adaptation can have important clinical and epidemiologic consequences. Previous studies have demonstrated remarkably rapid and dynamic evolution of antibiotic resistance in populations sampled from primary clinical specimens (113–115). The cases described above demonstrate how proofreading and MMR deficiencies can accelerate the evolution of a variety of traits in the context of chronic infection in *B. hinzii*, *P. aeruginosa*, and *Salmonella*. This next case illustrates how global hypermutation due to an MMR deficiency can operate on shorter timescales, generating resistance to a critical antibiotic over the course of a week, resulting in a treatment failure. Khil and colleagues (116) studied the in vivo evolution of resistance to ceftazidime-avibactam (CZA), a novel cephalosporin/beta lactamase inhibitor combination antibiotic, in *P. aeruginosa* in the context of an acute infection. The patient, who was undergoing treatment for a refractory T cell lymphoblastic leukemia, presented with pneumonia and bacteremia in the context of neutropenia. *P. aeruginosa* isolates initially cultured from blood and sputum were susceptible to CZA with minimum inhibitory concentrations (MICs) of 1 μg/mL, and the patient was treated with CZA in addition to amikacin. The bacteremia transiently cleared but within a week returned with the new isolates resistant to CZA with MICs of >256 μg/mL, resulting in a treatment failure (116). Whole-genome sequencing of all isolates revealed a clonal lineage consisting of ST155 *P. aeruginosa*. The initial susceptible isolates were genetically identical but the resistant isolates had developed 31 mutations in aggregate, with 14 shared among the total set of six isolates. One of the identified

mutations led to a G183D substitution in the PDC chromosomal cephalosporinase, previously demonstrated to confer resistance to CZA (117). Further analysis revealed that an R656H substitution was also present at an absolutely conserved position in the MutS MMR protein, and an MMR-deficient hypermutator phenotype was confirmed in vitro. Interestingly, the mutation underlying the G183D substitution underlying the resistance phenotype was a transition, falling within the spontaneous mutational spectrum that would have been expected for a MutS-deficient hypermutator (see the section titled Generalized Hypermutation). This led to the hypothesis that MMR-deficient hypermutation may have facilitated the discovery of the G183D substitution and CZA resistance, leading to the coselection of MutS R656H and PDC G183D (116).

To confirm that MMR-deficient hypermutation could indeed accelerate acquisition of CZA resistance under selection, replicate in vitro adaptive evolution experiments based on passaging through increasing CZA concentrations were performed in parallel with a laboratory MPAO1 WT and MPAO1 *mutS*^{Tn} strain in which the *mutS* gene was disrupted by a transposon, disabling mismatch repair (116). These experiments demonstrated that rapid MMR-deficient hypermutation facilitated acquisition of CZA resistance with remarkable reproducibility compared with the MPAO1 WT strain (116, 118). The number of theoretically possible single mutations in transition-constrained mutagenesis (in the limiting case including nonviable mutations) that would occur in MMR-deficient hypermutators is equal to genome length, in this case 6.9 Mb. Thus, most viable single mutations may be present in one or more copies in larger population sizes that can occur in heavy-burden infections (10^7–10^9 CFU), as may occur in pneumonia in a neutropenic patient (116). Transition mutations underlying the G183D substitution conferring resistance may have been generated in one or more copies by the hypermutator in this context, resulting in rapid coselection of the combined hypermutator and resistance phenotype, similar to findings by others (119). These results suggest a possibly underappreciated role for MMR deficiency in facilitating rapid adaptive evolution of *P. aeruginosa* in the context of acute infection.

A subsequent study by Dulanto Chiang and colleagues (118) examined the mutations and transcriptional consequences that occurred in the MPAO1 WT and MPAO1 *mutS*^{Tn} strains in the in vitro adaptive evolution experiment described above. The MPAO1 WT isolates developed fixed mutations in genes previously described to be involved in beta lactam resistance, including those encoding MexR, MexAB, PDC beta lactamase, NalC, and OprN, consistent with expectations based on prior literature. Unexpectedly, the MMR-deficient MPAO1 *mutS*^{Tn} lineages developed the very high levels of CZA resistance almost entirely without fixed mutations or transcriptional changes in these previously described resistance genes that were utilized by the WT isolates.

Analysis of the genomic sequences revealed that MPAO1 *mutS*^{Tn} lineages had generated an early mutation in the *mexB* gene of the MexAB-OprM efflux pump, a primary mediator of CZA resistance in *P. aeruginosa*. This mutation paradoxically did not confer resistance but rather inactivated the MexAB efflux pump. The authors proposed that this early inactivation was due to a mutation generated by the MMR deficiency, as it occurred along with 49 other early mutations, all of which were transitions, and that it resulted in an evolutionary search for alternative resistance mechanisms (118). Construction of genetic mutants demonstrated that two mutations in the noncanonical RND efflux pump MexVW (120) in the MPAO1 *mutS*^{Tn} lineages explained a component of the CZA resistance that evolved during the in vitro adaptive evolution experiment (118). Although based on in vitro experiments, these results suggest that MMR-deficient isolates may sometimes alter the set of available evolutionary alternatives by breaking essential cellular machinery, thus forcing evolutionary searches for novel responses to selective pressures. Under antibiotic selection, this may result in rapid switching to alternative and unexpected resistance mechanisms. More work remains to be done to determine the relevance of these findings to clinical in vivo evolution of MMR hypermutators.

Evolution of Antibiotic Resistance Driven by Mutations in Core Metabolic Genes in *Escherichia coli*

Lopatkin and colleagues (121) investigated the role of metabolic adaptation in antibiotic resistance. To identify potential metabolism-related mutations that occur during the evolution of antibiotic resistance, they performed in vitro adaptation experiments in a lab strain of *E. coli* (BW25113) with the antibiotics streptomycin, carbenicillin, and ciprofloxacin at different temperatures. The authors then identified enrichment of mutations in a variety of metabolic pathways using Gene Ontology classification (121). To assess whether the metabolic mutations identified in these in vitro experiments might be relevant to actual in vivo evolution of antibiotic resistance, the authors focused on 109 identified coding sequence variants and queried a database of 7,243 *E. coli* genomes from the National Center for Biotechnology Information (NCBI) Pathogen Detection database to see how frequently variants in these genes occur in isolates classified as clinical ($n = 3{,}700$) versus those classified as environmental ($n = 3{,}543$). They found that 42 of the identified mutations were present in NCBI genomes, including a number of mutations in metabolic genes that were abundantly present and statistically enriched in clinical isolates. The authors then focused on a set of metabolic genes identified in this analysis (*sucA* encoding 2-oxoglutarate dehydrogenase subunit, *gltD* encoding a glutamine subunit of glutamate-oxoglutarate amidotransferase, *icd* encoding isocitrate dehydrogenase, and *ycgG* and *yidA* encoding a sugar phosphatase) and performed a series of experiments in which they expressed WT and mutated copies of each gene in knockout backgrounds. All of the metabolic mutations were found to confer some degree of resistance to one or more of the three tested antibiotics. Further functional analysis suggested that the *sucA* mutation may confer resistance to carbenicillin by lowering the basal metabolic rate. While these mutations were found using an in vitro adaptation experiment, their abundant enrichment in clinical isolates suggests that they are likewise discovered through within-host adaptation and demonstrates the value of careful in vitro adaptive evolution experiments (121).

Evolution of Antibiotic Resistance Driven by Recurrent Amplification Events in *S. aureus*

Silva et al. (122) used an in vitro adaptive evolution system to study mechanisms of resistance to the fluoroquinolone delafloxacin (DLX) in *S. aureus*. DLX has two distinct targets, DNA gyrase and topoisomerase IV, and individual target mutations do not lead to resistance. The authors found that resistance evolved robustly and reproducibly under DLX selection, and genomic analysis revealed that resistance was largely attributable to recurrent genomic amplifications of the *sdrM* gene, encoding a poorly studied efflux pump (**Figure 4**). Interestingly, the amplified genomic regions included chromosomally adjacent efflux pump genes, resulting in streptomycin cross-resistance. Though this work was based on in vitro selection of a lab strain, the authors found that similar recurrent localized chromosomal amplifications occurred in two diverse clinical isolates, pointing to a general mechanism. From a clinical point of view, this work suggests that bacteria may generate resistance to multitarget antibiotics through overexpression of secondary efflux mechanisms and that localized chromosomal hot spots may exist that facilitate useful recurrent amplification of such efflux mechanisms under such selection (122).

Convergent Within-Host Adaptation Mediated by Intergenic Evolution in *P. aeruginosa*

The above examples focused primarily on changes occurring within coding regions in genomes. Khademi and colleagues (123) studied how variants within intragenic regions may contribute to host adaptation in *P. aeruginosa*. The authors analyzed the genomes of 534 isolates composing 44

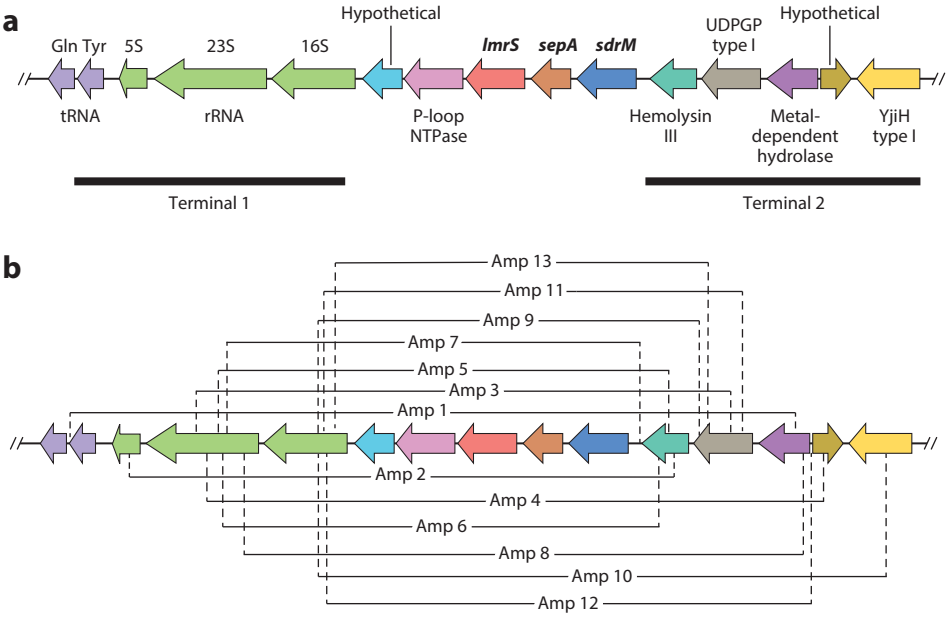

Figure 4

Recurrent genomic amplifications of the *sdrM* efflux pump gene occur under delafloxacin (DLX) selection. (*a*) Schematic of the *sdrM* locus in *Staphylococcus aureus*. (*b*) The junctions of 13 distinct genomic amplifications that occurred under selection with DLX in vitro. These recurrent amplifications resulted in SdrM efflux pump overexpression, mediating resistance to DLX. Figure adapted from Reference 122 (CC BY 4.0).

clonal lineages of *P. aeruginosa* collected from seven prior longitudinal studies of individuals with cystic fibrosis (124–126). These lineages included 22,491 identified mutations that were presumed to have occurred during host adaptation, and 3,866 of these mutations were intergenic. To simplify the analysis, the authors focused on 3,489 mutations (1,465 indels and 2,024 SNVs) occurring in 1,610 intergenic regions that were shared with the PAO1 laboratory strain and employed additional assumptions that allowed them to focus their search for parallel mutations likely affecting transcriptional regulatory regions.

The analysis revealed 77 regions in which likely convergent, or parallel, evolution appeared to have occurred under selection in the host (123). These regions were biased to be located upstream of genes, which the authors interpreted as evidence of adaptive targeting of *cis*-regulatory elements, directly affecting transcriptional control of the immediate downstream gene. Experiments with a luciferase reporter revealed moderate changes in transcription of downstream genes in most of the tested cases, demonstrating that the mutations indeed altered gene expression. They further tested the functional consequences of two identified mutations in the regulatory region between the *ampR* and *ampC* genes. AmpR ordinarily represses transcription of the AmpC beta lactamase through a local interaction with an operator site interposed between the two genes. Introduction of these two mutations into a WT PAO1 background increased resistance to various beta lactams, demonstrating a clear functional consequence that would confer a fitness advantage under antibiotic selection (123). In a final analysis, the authors found that many of the identified intergenic regions were located adjacent to genes known to be essential in *P. aeruginosa*, providing a mechanism by which the expression of these essential genes is targeted during adaptation.

CONCLUSION

Genomic sequencing over the past couple of decades has substantially advanced our understanding of how bacterial populations evolve in the context of host colonization and infection. In this review, we have examined both general principles governing evolution within the host and a set of recent case studies illustrating relevant mechanisms and clinical consequences. Future work integrating genomics and systems-level physiologic studies of evolving microbial populations will likely yield new insights into currently unappreciated metabolic and functional mechanisms underlying successful host adaptation.

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