Within-host evolution of bacterial pathogens

Xavier Didelot[1], A. Sarah Walker[2], Tim E. Peto[2], Derrick W. Crook[2,3], Daniel J. Wilson[2,4]
1 Department of Infectious Disease Epidemiology, Imperial College, London, W2 1PG, United Kingdom
2 Nuffield Department of Medicine, Experimental Medicine Division, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom
3 National Infection Service, Public Health England, London SE1 8UG, United Kingdom
4 Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford, OX3 7BN, United Kingdom

Details for correspondence: X.D. x.didelot@imperial.ac.uk. D.J.W. daniel.wilson@ndm.ox.ac.uk

Abstract

Whole genome sequencing has opened the way to investigating the dynamics and genomic evolution of bacterial pathogens during colonization and infection of humans. The application of this technology to the longitudinal study of adaptation in the infected host — in particular, the evolution of drug resistance and host adaptation in patients chronically infected with opportunistic pathogens — has revealed remarkable patterns of convergent evolution, pointing to an inherent repeatability of evolution. In this Review, we describe how these studies have advanced our understanding of the mechanisms and principles of within-host genome evolution, and we consider the consequences of findings such as a potent adaptive potential for pathogenicity. Finally, we discuss the possibility that genomics may be used in the future to predict the clinical progression of bacterial infections, and to suggest the best treatment option.

Introduction

Until recently the extent of bacterial diversity associated with humans and the ability of these bacteria to evolve in response to drug pressures, immune responses and host environment was presumed limited compared with rapidly evolving viruses such as HIV [1,2]. Long-term estimates of bacterial evolutionary rates (over millions of years), calibrated using known dates of ecological and geological events, were of the order of 10^{-10} to 10^{-9} substitutions per site per year [3–5], which is low enough to suggest that recently diverged within-host populations should be completely monomorphic. However, recent studies have shown that bacterial short-term evolutionary rates (over months or years) are of the order of 10^{-7} to 10^{-5} substitutions per site per year and thus much higher than the aforementioned long-term rates [6–9] — a discrepancy of great consequence that mirrors previous observations in viruses [10,11]. Although bacterial short-term evolutionary rates remain lower than those reported for RNA viruses (10^{-4} to 10^{-3}), bacteria have much longer genomes, which means their per genome evolutionary rates can be comparable or even higher [9,12,13].

Given these short-term per site evolutionary rates and the short timescale of within-host evolution of a few days to a few years, molecular techniques that sample only a small subset of the genome, such as multi-locus sequence typing (MLST [14]), are of limited utility to study within-host diversity . However, whereas higher resolution approaches had previously not been economically feasible for large population studies , the cost and time required to perform whole genome sequencing (WGS) of bacterial isolates has been substantially reduced over the past few years [15,16]. This has led to a flurry of WGS studies revealing previously unsuspected layers of within-host diversity that evolve measurably over time, so much so that bacteria can adapt to the specific conditions in a given host. Analysing the genome evolution of bacterial pathogens in human hosts in these studies requires a wide range of specific methods, including the collection of clinical samples, treatment for isolation and growth of pathogenic bacteria, DNA preparation, genome sequencing, sequence alignment, genome assembly, variant calling and comparative genome analysis (Box 1).

In this Review, we discuss recent work that has advanced our understanding of the genome dynamics of bacterial pathogen populations as they evolve in human hosts. After surveying the various evolutionary processes and forces that affect these populations, we describe how within-host diversity affects epidemiological studies of transmission between individuals. Finally, we consider the contribution of within-host evolution to antibiotic resistance and heightened or reduced virulence phenotypes, as well as to adaptation to the host environment .

Within-host evolutionary dynamics

Until the recent advent of WGS, little was known about the genomic diversity of pathogenic populations of bacteria infecting human hosts. Previous genotyping methods such as pulsed-field gel eletrophoresis (PFGE), variable-number tandem repeats (VNTR), multi-locus enzyme electrophoresis (MLEE) and MLST had comparatively low resolution [17–19], which could only reveal the presence of multiple colonizing or infecting lineages rather than the evolution of each lineage. For example, mixed colonization by several bacterial types has been shown to be relatively common amongst *Staphylococcus aureus* carriers using PFGE [20] and VNTR [21,22]. Such mixed colonization is most likely to be the result of two or more separate transmission events. Alternatively, the different types could have been transmitted simultaneously from the same donor who was also multiply colonized. This would require a relatively loose transmission bottleneck, so that substantial diversity from the donor can be transmitted to the recipient, but little is known about the transmission bottleneck of bacterial pathogens [23]. A noteworthy exception where the within-host evolution of a given lineage can be detected by low-resolution approaches (that is, sequencing only a few genes rather than whole genomes) is the stomach pathogen *Helicobacter pylori*, owing to its unique combination of a long infection time and very high evolutionary rate [24].

Applying WGS to multiple isolates sampled from the same host has allowed us to develop a much more complete picture of the various processes involved in within-host bacterial evolution (for example, for the hospital-associated pathogen *S. aureus*, see Figure 1). Point mutations provide raw material for this evolution, and studies comparing pairs of genomes sampled simultaneously or longitudinally from the same hosts have confirmed the high rate of within-host point mutation in *H. pylori*, with ~30 mutations per year per genome [25,26] . More modest rates of within-host point mutation have been reported for other bacterial pathogens, such as ~10 mutations per year per genome for *Klebsiella pneumoniae* [27], ~8 for *S. aureus* [28], ~2 for *Clostridium difficile* [6,29,30], ~1 for *Escherichia coli* [31] and ~0.5 in both *Mycobacterium tuberculosis* [32,33] and *Mycobacterium abscessus* [34]. These differences between species are partly due to differences in the lengths of the genomes, but also to differences in the per site mutation rates, which are modulated by varying levels of efficacy of the DNA mismatch repair systems (for example, *H. pylori* lacks several genes involved in this process that are found in most other bacteria [35]). A much higher rate of point mutation, known as hypermutation, can also occur when the mismatch repair system becomes disrupted. Hypermutation has long been known to be important for adaptation to new ecological niches in the external environment [36,37] but has recently been shown also to be important within-host, for example generating excess diversity in a *Burkholderia dolosa* infection of the lungs of a cystic fibrosis patient [38]. A related mechanism is that of phase variation, in which loci that are susceptible to high rates of mutation, for example due to slippage of repetitive motifs, can rapidly modulate the expression of phenotypes over very short timescales, often displaying variability even within a colony[39,40]. For example, during long term carriage of *Neisseria meningitidis*, phase variation reduces the expression of certain genes that encode surface proteins in order to avoid detection by the host immune system [41].

Another factor that can lead to faster diversification of the genome than point mutation alone is the acquisition of genetic sequences from unrelated organisms through horizontal gene transfer [42]. One possible outcome is homologous recombination, where a fragment of the chromosomal genome is replaced with a homologous sequence from another cell. This process has an important role in the genomic evolution of many pathogen species, such as *Escherichia coli* [43], *Streptococcus pneumoniae* [44], *Salmonella enterica* [45] and *Campylobacter jejuni* [46]. Recombination is especially potent as a factor in within-host evolution when a mixed infection is present to provide genetic material for import. For example, in *H. pylori* it has been estimated that when multiple strains are present within a host then homologous recombination can accelerate evolution up to a hundred fold [25,26,47]. An alternative outcome is the gain of novel, non-homologous, genetic material, which is counterbalanced by occasional loss of content through genome degradation [48]. For example, both gain and loss of genes have been described during the early stages of adaptation of *Pseudomonas aeruginosa* to the lungs of cystic fibrosis sufferers [49]. The genomic plasticity of gene gain and loss has an important role in adaptation to changes in selective pressures, with antibiotic resistance genes in many pathogens being found on various mobile genetic elements such as plasmids [50], transposons [51] and bacteriophages [52].

Within-host diversity is shaped by random genetic drift [53,54] (Box 2), and by both purifying selection and diversifying selection. The role of genetic drift might seem counterintuitive when the total number of cells infecting a host can be very high, which dampens drift . However, drift is amplified by several factors, such as isolation of distinct populations in different body parts and fluctuations in population size. Indeed, fluctuations in population size have been found to be an important factor for the within-host evolution of *S. aureus* [55] . The effects of selection on within-host diversity is non-random and can take many forms . At longer evolutionary scales, the effect of purifying selection dominates the evolutionary landscape, as evidenced by a paucity of non-synonymous polymorphisms relative to synonymous polymorphisms (measured by the dN/dS ratio). However, purifying selection is expected to be weaker in within-host populations than in other populations , due to stronger genetic drift and little time available to purge slightly deleterious mutations [56]. This may be a factor in the seemingly higher rate of short-term evolution observed in within-host populations since proportionally more mutations will be observed that will get purged in the longer term [8]. Diversifying selection, which favours the evolution of new variants, has been found to be important in several within-host studies [57–59]. For example, certain genes coding for the outer membrane proteins of *H. pylori* have been found to evolve faster than the remainder of the genome, presumably because these proteins would otherwise be targeted by the host immune system [25,47,60]. Diversifying selection can also be driven by the use of antimicrobial drugs such as antibiotics, and may result in an arms race between the bacterial pathogen and its human host [61].

Host-to-host pathogen transmission

Using phylogenies to reconstruct transmission

WGS of bacterial pathogens has allowed the development of a new approach to tracking direct transmission between hosts, based on the simple idea that if a direct transmission event happened, genomes sampled from the donor and the recipient are expected to be highly similar [6,62–64]. Most studies of recent transmission reported to date have been based on single genomes sampled from each host, and have therefore not taken within-host diversity into account . However, depending on the duration of host carriage, incubation and infection compared with the rate of genomic evolution, within-host evolution can make direct transmission events difficult to reconstruct based on a single genome per host [23].

For example, consider single genomes sampled from three individual hosts, A, B and C. The two genomes sampled from B and C may be more closely related to each other than to the genome sampled from A; however, perhaps counter-intuitively, this does not rule out the possibility that A infected both B and C through a lineage that is different from the one that was sampled from A (Figure 2). In other words, the genetic variants are representative of the phylogenetic relationships between the sampled genomes, but these may not directly reflect the order and timing of transmission events from donor to recipient. This problem is similar to the relationship between species trees and gene trees caused by incomplete lineage sorting [65]. Formally establishing the relationship between phylogenetic and transmission trees requires explicit modelling of the transmission bottleneck and within-host dynamics, which increases the accuracy of the results but also reveals much uncertainty in who infected whom based on single genomes per host [2,66,67].

By capturing the diversity within each host — for example, by sequencing multiple genomes per host sampled either simultaneously or longitudinally — within-host evolution can reconstruct transmission events with much more certainty than sampling single genomes . This approach greatly clarifies who infected whom; for example, in cases where host A has infected host B, the diversity of pathogen genomes sampled from B should be a clade contained within the diversity of pathogen genomes sampled from A (Figure 2), a principle that has been known and applied previously in other settings, such as in studies of HIV infection [68,69]. In a pioneering study that applied this approach to bacteria, an outbreak of *B. dolosa* among 14 cystic fibrosis patients over 16 years was investigated by sequencing a total of 112 isolates, clearly revealing likely donors and recipients of transmission events [59]. A similar study used 168 consecutive isolates of *M. abscessus* from 31 patients of an adult cystic fibrosis centre in the UK, and found that 11 patients were likely to have infected one another during hospital visits [34]. Deep sequencing of *S. aureus* was crucial to revealing the likely role of a healthcare worker in spreading infections at an English neonatal intensive care unit [70],

tracing the source of an outbreak in an adult intensive care unit in Thailand to a single individual with high within-host diversity [71] , and reconstructing the transmission network between animal patients and staff at an English veterinary hospital [72] .

Identifying the cause of recurrent infections

A problem related to that of reconstructing transmission networks concerns the ability to distinguish reinfection from relapse in individuals who suffer subsequent episodes of disease. For example, in a study of African patients with recurrent invasive non-typhoidal *Salmonella* infection, 25 out of 32 recurrent episodes were caused by identical or almost identical consecutive strains, suggesting relapse, whereas the remaining seven cases showed many differences between consecutive strains, suggesting reinfection [73]. The same approach was applied to recurring cases of *C. difficile* infection, and relapse was found to be about five times more frequent than reinfection in both the UK and Ireland [74,75]. The question of relapse versus reinfection is especially relevant for *M. tuberculosis* infections, which are more frequent in people living with HIV and can often recur after seemingly successful treatment. In a study focused mostly on HIV-negative patients, 33 clear cases of relapse were found compared with only three reinfections [76]. Another study based on a cohort with higher HIV infection prevalence identified 55 relapsed and 20 re-infected individuals, with the latter being more likely to be HIV-positive [77]. This study also found that lineage-2 was more likely to cause reinfections whereas lineage-3 was more likely to cause relapse, echoing differences in adaptive strategies that have also been observed when comparing transmissibility between lineages of *M. tuberculosis* [78].

Evolution of antibiotic resistance

Natural selection within individual hosts

Following successful transmission, a pathogen must survive in the new host in order to transmit again and continue the life cycle. Survival within the host poses many challenges — including physical barriers to colonization and infection, competition with the native microbiota, containment by the immune system, basic sanitation and medical intervention — and the relative importance of these forces may differ from one host to the next. However, the ability of bacteria to adapt rapidly to selection pressures such as antibiotic treatment, even within a single host [25,26-34], is highlighted by the rapid rise in antibiotic resistance, exacerbated by overuse and misuse [79].

Evolution of antibiotic resistance within individual hosts epitomizes the evolutionary principle of a selective sweep, as the survival advantage gained from the resistance-conferring gene (or mutation within a gene) in the presence of antibiotic treatment increases the frequency of the gene (or mutation) in the within-host population . Furthermore, where resistance-conferring genes are carried by plasmids or other mobile elements, horizontal gene transfer can accelerate the rate of spread .

Until the advent of WGS, the characterization of bacterial microevolution was the purview of experimental studies [80]. WGS captures this process in extraordinary detail, and as a result has now provided valuable insights into many examples of the evolution of antibiotic resistance within-host . As a result, the occurrence and spread of individual point mutations that increase resistance and contribute to treatment failure can often be pinpointed , as in the evolution of vancomycin-resistant *S. aureus* in an initially vancomycin-susceptible bloodstream infection [81]. Rifampicin resistance-associated mutations in *rpoB*, a β-lactam resistance-associated mutation in *blaR1*, and mutations in several genes associated with reduced vancomycin susceptibility were identified in bacteria isolated from the patient only after treatment with the respective antibiotic . Mutations progressively accumulated over time, concomitant with a stepwise decrease in vancomycin susceptibility [81].

The sheer potency of bacterial evolution was revealed by the evolution of extensively drug resistant (XDR) *M. tuberculosis* from a wholly drug-sensitive ancestor in a single patient over just 3.5 years [82,83]. Resistance evolved to seven drugs to which the patient was exposed: rifampicin (to which resistance was conferred by mutations arising in *rpoB*), isoniazid (*katG*), ethambutol (*embB*), streptomycin (*rss* and *gid*), ofloxacin (*gyrB*), ethionamide (*mshA*) and amikacin (*rss*). In most cases, resistance-conferring mutations arose in one or more lineages in the within-patient population only after several months of treatment with the respective antibiotic. Ultimately, in an extreme example of clonal interference, a single multidrug resistant lineage

prevailed, displacing the others. The spread of advantageous resistance mutations elevated the measured substitution rate 14-fold , and propelled the substitution of numerous synonymous mutations on the same genetic background as the advantageous mutations, in an example of a process known as hitchhiking.

Some naturally evolved resistance mutations appear to be pleiotropic, leading to multifaceted changes in bacterial phenotype, including resistance to drugs to which the population has not been exposed [81,84,85]. In five patients, *S. aureus* with reduced vancomycin susceptibility evolved from initially susceptible strains following fewer than six weeks of vancomycin treatment. Non-synonymous mutations in the WalKR two-component regulator [84], which has a central role in controlling cell wall metabolism[86], were found in four of these cases, as well as in six of eight unrelated isolates with reduced vancomycin susceptibility . The majority of isolates with reduced vancomycin susceptibility also demonstrated reduced daptomycin susceptibility, despite no exposure to this second antibiotic [84]. Allelic exchange experiments showed that mutations in *walKR* led to morphological and transcriptional remodelling, including reduced autolytic activity, increased cell wall thickness, reduced biofilm formation and attenuated virulence in a wax moth model of infection [84]. This incidental pre-adaptation to other antibiotics, a phenomenon known as cross-resistance, presents a particular cause for concern by further limiting treatment options.

Revealing the adaptive potential of bacterial pathogens

The ability to study the evolution of antibiotic resistance in forensic detail using WGS has enabled the discovery of novel mutations and mechanisms of resistance that may help predict treatment failure and guide the development of new drugs [87–93] . These *in vivo* surveys of within-host evolution present a complementary approach to experimental studies . Although it is not possible to control for all variables, observing natural evolution in within-host populations has the advantage of incorporating complexities such as strain differences, the host environment and realistic fitness trade-offs, which has highlighted the formidable adaptive potential of bacterial pathogens .

Certain genes have been identified repeatedly as undergoing adaptive evolution in response to antibiotic treatment, with independently arising mutations often hitting different sites in the same gene. Common targets of selection include RNA polymerase gene *rpoB* in response to treatment with rifampicin (which inhibits RNA polymerase) in several species [85,94–96], DNA gyrase gene *gyrA* [38,96–99] and DNA topoisomerase IV gene *grlA* [100] in response to fluoroquinolones (which target topoisomerases) in various species, and cell wall metabolism genes *walKR* and *vraRS* in response to treatment with vancomycin (which inhibits cell wall synthesis) in *S. aureus* [81,84,101].

Reports of convergent evolution in independent patients exposed to the same drugs offer the strongest evidence for adaptation. A study investigated the evolution of resistance to a wide range of drugs in 123 *M. tuberculosis* strains representing transmission clusters and epidemiologically unrelated cases. Resistance evolved independently up to 20 times to the first line drugs isoniazid, pyrazinamide, ethambutol and rifampicin by substitutions in *katG* and *inhA* (isoniazid), *pncA* (pyrazinamide), *embB* (ethambutol) and *rpoB* (rifampicin). Identical, independent substitutions were detected at two sites in *rpoB* and one in *embB* [87]. The observation that resistance to common antibiotics has evolved not once, but many times in parallel, underlines the adaptive potential and repeatability of bacterial evolution.

Given the rapidity with which bacteria can respond to selection pressures by within-host evolution , it may seem surprising that antibiotic resistance has not spread yet more quickly. It has been suggested that the fitness costs of resistance might explain this discrepancy, because resistance-conferring substitutions in key enzymes may reduce the efficiency of replication and transcription, while specialist resistance-conferring proteins may be costly to produce [88,102]. However, compensatory mutations may arise that counteract fitness costs of antibiotic resistance (Figure 3). WGS allowed a joint investigation of naturally evolving rifampicin resistance-conferring and compensatory mutations in tuberculosis [88]. Putative compensatory mutations were detected in 38 genes, with a particular enrichment of mutations affecting the interface between RNA polymerase subunits α and β′. High *in vitro* fitness was demonstrated in strains bearing compensatory mutations subject to strong convergent evolution across lineages, demonstrating that a cost to resistance is not inevitable.

Even when fitness costs are inevitable, bacteria can evolve adaptability. A well-studied form of adaptability is the previously mentioned phase variation mechanism, in which frequent, reversible, genetic changes determine expression of particular genes, such as those encoding surface antigens . Rapid mutation allows expression of these genes to be quickly adapted to changing selection pressures. Another form of adaptability that is increasingly recognized is heteroresistance . For example, population analysis profiles (PAPs) reveal that, remarkably, most methicillin-resistant *S. aureus* (MRSA) strains are heteroresistant, meaning that the vast majority of cells have only low- or moderate-level resistance in the absence of exposure, whereas cells exhibiting several hundred-fold increased resistance are present only at very low frequency [95]. Upon exposure to methicillin, the rare, highly resistant cells can rapidly sweep through the population. Genome sequencing has found that high-level resistance can be conferred by any one of a large number of mutations across the *S. aureus* genome, many of which affect gene expression . Although this indicates a large target for selection, the over-representation of mutations in genes involved in transcription and stringent stress response suggests a degree of parallel evolution [95].

Heteroresistance demonstrates the capacity for large bacterial populations in the host to mutate and harbour potentially advantageous mutations, facilitating adaptability. The phenomenon of heteroresistance in a range of antibiotics across several pathogens including *S. aureus, M. tuberculosis*, *S. pneumoniae* and enterococci [103] indicates that within-host evolution is not the exception, but the norm, in bacteria and may be a leading contributor to monotherapy treatment failure [100]. As the potential for adaptability seems to vary between bacterial strains, capacity for within-host evolution might even explain differences in the success of global lineages [104] .

Adaptation to the host environment

In the absence of antibiotic pressure, bacterial pathogens must still overcome a variety of challenges in the host, carving out a successful niche by securing nutrient sources and evading killing by other microorganisms and the innate and adaptive immune system. Such challenges represent opportunities for adaptation if mutations arise that confer survival or reproductive advantages. WGS is uncovering diverse signals of adaptation to the host environment, and this is shedding new light on the main forces that shape bacterial populations in the host.

Opportunities for adaptation are particularly abundant in infections of immunocompromised hosts by opportunistic pathogens that are adapted to normally living elsewhere. Infections of cystic fibrosis patients with bacteria that are not usually pathogenic in humans have been particularly well studied (Figure 4). *In vitro* experiments have described an initial period of rapid adaptation to new environments that slows down as the population improves in fitness [105]. Similar patterns of rapid adaptation have been detected in opportunistic infections of patients with cystic fibrosis in the form of signals of parallel evolution. In an outbreak of *B. dolosa* among patients with cystic fibrosis, signatures of selection in the form of convergent evolution were detected in genes responsible for O-antigen repeat expression in the lipopolysaccharide coat, oxygen-dependent regulation and resistance to ciprofloxacin [59]. O-antigens elicit strong immune responses and typically exhibit high variability that probably reflects selection for immune evasion. It is thought that changes in oxygen-dependent regulation may be crucial for adaptation of free-living organisms to the cystic fibrosis lung, where the build up of mucus and biofilms has reduced the availability of oxygen [59].

Patterns of convergent evolution have been used to identify pathoadaptive mutations in *P. aeruginosa* infections in cystic fibrosis lungs, highlighting the roles of genes encoding transcriptional regulators, lipopolysaccharide biosynthetic protein, outer membrane antigen and antibiotic resistance in pathogenicity [58,96,99]. In some cases, within-host adaptation may be facilitated by hypermutators [96,97,99,106], which accelerate the rate of adaptation by increasing the supply of genetic novelty, a greater proportion of which is likely to be beneficial in a novel environment [107]. Selection for changes to transcriptional regulators such as sigma factors and two-component systems indicate that transcriptional reprogramming may be a key element of adaptation to the host environment in opportunistic pathogens [96,99]. Such changes to gene expression can cause rapid phenotypic change with little evolution in terms of genetic mutation. In general, dissecting the molecular mechanisms that drive signals of within-host adaptation is difficult because, unlike in the case of antibiotic resistance, the phenotypes are difficult to recreate *in vitro*, owing to the complexity of the within-host environment and the potential role for host–pathogen interactions . However, an experimental CF lung, which may negate some of these difficulties, has been used to study one candidate for adaptive

transcriptional reprogramming, the iron-scavenging *Pseudomonas* haem utilization system. The increased expression of this system induced by promoter mutations was demonstrated to be advantageous to *P. aeruginosa* growth in the presence of haemoglobin [108]. Although such experiments are challenging, they provide useful validation of the findings of observational studies.

Within-host population structure is likely to be an important modifier of adaptation to the host environment. In homogeneous environments, drift and positive selection are expected to lead to the evolution of one prevailing lineage (Box 2). However, within-host heterogeneity can facilitate the existence of multiple coexisting lineages, either through neutral processes such as genetic isolation of subpopulations, or adaptive processes such as niche differentiation (Figure 4). An investigation of within-host population structure in five *B. dolosa*-infected CF patients found stable coexistence of multiple lineages for more than five years and evidence of parallel evolution even in individual patients, manifest as multiple substitutions in genes with functions in antibiotic resistance, outer-membrane synthesis, iron scavenging and oxygen sensing. These results uncover previously unsuspected population structure in the infected lung [38]. Sequencing of a *P. aeruginosa* infection sampled over a 32-year period revealed that the infecting strain had rapidly diverged into distinct sublineages, with unique functional signatures and threefold variation in the rate of adaptation [97]. Differences in the frequency of these sublineages in the sinuses, upper airways and lower airways, and their stable coexistence over several decades, suggested specialization to distinct within-host niches. Sublineages differed in growth rates, mucoidy and production of proteases, with one of the clusters displaying a phenotypic signature characteristic of chronic infections, namely longer doubling times, mucoidy and the loss of protease production [97].

Notwithstanding the numerous examples of within-host adaptation revealed by WGS , most of the genome nevertheless remains subject to purifying selection. Even within individual hosts, the genome-wide dN/dS ratio typically lies below its neutral expectation of one and thus is indicative of purifying selection . In a *B. dolosa* outbreak in CF patients, singly mutated genes showed a dN/dS = 0.63, significantly below one [38], similar to the dN/dS = 0.56 and 0.66 reported in *P. aeroginosa* outbreaks in CF patients [96,97] and dN/dS = 0.55 reported for *S. aureus* nasal populations colonizing individual hosts [55]. Taken together, these figures indicate that the prevailing effect of natural selection in the host is to conserve functionality for the majority of genes, with adaptation acting only on some genomic positions in a subset of genes.

Within-host adaptation might be less frequent in common bacterial pathogens of humans that have already had many generations over which to adapt to an infectious lifestyle. A study of asymptomatic *S. aureus* nasal carriage in healthy adults concluded that adaptive evolution was rare, revealing only a weak signal of convergent evolution across thirteen carriers. Notably, multiple non-synonymous or protein-truncating mutations were observed in surface anchored proteins Ebh and SasC and secreted enterotoxin Seg [55] . Indeed, surface and secreted proteins are common antigens that interact directly with the adaptive immune system leading diversifying selection to favour novel epitopes that can evade recognition. In another example, a longitudinal study of asymptomatic *H. pylori* carriers reported excess horizontal gene transfer in the Hop family of outer membrane proteins [25]. It may be that most within-host adaptation in common human pathogens occurs within antigenic loci, although well-known examples of recurrent evolution in other genes, such as loss-of-function *agrC* and *lasR* mutants that knock out quorum sensing in *S. aureus* and *P. aeruginosa*, respectively, suggest this will not be the full story [109] .

Selection pressures within the host are difficult to dissect. Conflicts arise not only with the host, owing to the need for nutrient acquisition, immune evasion and onward transmission, but also with other bacteria, owing to direct competition for resources. Complex selective forces may also be at work as a result of social dynamics within a bacterial community. For example, whereas loss-of-function mutations might in many cases signal host adaptation[110], the loss of siderophore production in *P. aeruginosa* during long-term infections in CF patients can instead be driven by cheating behaviour[111] . In some patients, cheats that no longer synthesize the iron scavenging molecule pyoverdine still retain the pyoverdine receptor, allowing them to uptake iron scavenged by pyoverdine produced by co-operative bacteria. Only when the co-operators are lost from the population do the cheats lose the pyoverdine receptor.

Evolution of disease severity

Does within-host evolution increase virulence?

A key question that follows from the discovery that bacteria rapidly evolve on timescales relevant to colonization and infection is what the effect of this within-host bacterial evolution is on disease manifestation. In other words, does bacterial evolution in the human body increase virulence? Dramatic phenotypic changes and global transcriptional remodelling in the host support the feasibility of such a notion [84,85,108,112,113]. For example, a single spontaneous mutation in *relA* in a persistent *S. aureus* infection was sufficient for permanent activation of the stringent response, which diverts cellular resources towards survival during nutrient limitation by instigating widespread regulatory changes, including upregulation of amino acid synthesis and protease production. In this pathogen, the stringent response induced multifactorial phenotypic changes including reduced growth, smaller colony size, reduced attachment to human cells and attenuated virulence in a wax moth model of infection[85] . WGS provides an unprecedented opportunity to reveal how within-host bacterial evolution is associated with the progression of disease in a wide variety of bacterial pathogens.

Many clinically important bacterial pathogens are predominantly commensals of humans, including *S. aureus*, *N. meningitidis*, *S. pneumoniae*, *H. pylori* and *E. coli*. As carriage is common and invasive disease is not an obligate part of the lifecycle, most transmission may be asymptomatic, with disease being caused by previously carried bacteria rather than transmission. In the case of *S. aureus*, 82% of serious infections of deep tissue are self-infections, in the sense that the invasive strain matches the nasally carried strain [114]. Although the triggers for invasive disease are likely to be complex and multifactorial, and include the general health of the patient and host genetics, an important question in light of rapid within-host evolution is whether the carried bacteria can evolve to become more virulent.

In one long-term *S. aureus* nasal carrier who developed a severe bloodstream infection, WGS charted the genomic changes that accompanied the transition from asymptomatic carriage to invasive disease [28]. Over thirteen months, multiple isolates were sequenced from each of a longitudinal series of samples, revealed 30 point mutations and four insertions or deletions. The population evolved at a steady rate except for a cluster of mutations preceding the transition to disease. Eight mutations differentiated the original nasal population from the bloodstream population, of which half were protein-truncating, including a mutation in *rsp*, an *AraC*-family transcriptional regulator (AFTR) previously implicated in pathogenicity [115]. AFTRs are regulators of carbon metabolism, stress response and virulence that respond to changing environmental conditions such as antibiotic use and oxidative stress[115]. In *N. meningitidis*, a loss-of-function mutation in the AFTR *mpeR* is associated with the hypervirulent ST 32 complex[116]. A statistically significant excess of protein-truncating mutations accompanied the progression of *S. aureus* to invasive disease in this patient. However, both for this case and more generally, whether disease progression is driven by bacterial evolution (or whether, conversely, bacterial evolution is driven by the worsening health of the patient) remains an open question [28,117].

Attenuation of virulence as an evolutionary strategy

Rather than exacerbating virulence, there is evidence that bacterial evolution in the host can attenuate virulence [57,118]. *Burkholderia pseudomallei* causes the potentially life-threatening disease melioidosis, and is not considered a commensal. However, one patient out of 707 survivors in a 23-year study in Darwin, Australia, developed persistent asymptomatic carriage [57]. Two isolates sampled 11.5 years apart differed by 23 point mutations, including a protein-truncating mutation in the gene encoding universal stress response sigma factor RpoS. Four deletions in chromosome two removed 221 genes during this time, including some involved in metabolism, survival outside the host and pathogenesis. Both isolates showed loss-of-function mutations in the essential virulence factor *wcbR*, a component of the capsular polysaccharide I locus, suggesting early attenuation of virulence may have promoted long-term persistence in this patient.

A recent study on *H. pylori* virulence sought to explain why two Columbian regions separated by only 200km and with similarly high prevalence of infection showed a 25-fold difference in the incidence rates of gastric cancer [119]. In the region where disease was rare, most individuals were of African descent and colonized asymptomatically with African lineages of *H. pylori*, whereas in the region with high disease rate, African ancestry was low in the human population (~3%) but higher in the bacterial population (~20%). The

highest risk of disease was found in individuals of non-African descent who were infected by *H. pylori* with substantial African ancestry. This suggests that, in Africa, *H. pylori* and its human host have coevolved towards lower virulence, which could also explain why the high prevalence of *H. pylori* in Africa does not seem to correspond to similarly high levels of disease [120]. According to the adaptive trade-off hypothesis, host–pathogen coevolution can lead to the evolution of disease with reduced severity [121–123]. *H. pylori* is often transmitted in families or communities of closely related individuals [26,124,125], and the virulence optimum is expected to be particularly low for pathogens transmitted in this way because their long-term survival is linked with that of the host [126].

Strains that have evolved attenuated virulence may even provide a therapeutic tool. Urinary tract infection (UTI) by *E. coli* is a severe and potentially life-threatening condition. However, *E. coli* can also be carried asymptomatically in the bladder in a state known as asymptomatic bacteriuria (ABU). ABU strains have been extensively used for therapeutic urinary bladder colonization in chronic UTI patients[127]. Previous work showed that 50% of long-term ABU strains evolved from uropathogenic strains in which genome reduction and inactivating mutations attenuated virulence [128]. By studying six patients colonized with a single prototypic ABU strain, WGS revealed further evolution of the strain following therapeutic inoculation that suggested within-host adaptation specific to each individual host. In some patients who required repeated therapeutic inoculations with the prototypic strain, mutations were observed repeatedly in loci involved in iron uptake (*fecIR* promoter), oxidative stress response (*frmR*) and osmoregulated periplasmic glucan synthesis (*mdoH*). The repeatability of evolution within individual hosts suggested the existence of a characteristic signature of adaptation to individual hosts sometimes known as host imprinting . Ongoing loss of gene function suggested that progressive evolution towards commensalism rather than virulence was favoured in these ABU strains[127].

Conclusion

By enabling the study of microbial evolution right inside our own bodies, WGS has revealed a remarkable degree of adaptability of bacteria in the human host . Even the notoriously slow-growing and slowly evolving [32] *M. tuberculosis* is capable of extraordinarily rapid adaptation in response to antibiotics, with one infection evolving resistance to seven antibiotics over a 42-month period [82]. The numerous examples in which resistance has evolved in response to antibiotic therapy (not only in *M. tuberculosis* [82,88] but also *S. aureus* [85,95], *E. coli* [129], *K. pneumoniae* [92,130] and *P. aeruginosa* [96,99]), together with the examples of convergent evolution in opportunistic pathogens such as *P. aeruginosa* [96,97] and *B. dolosa* [59], shows that rapid within-host evolution is common across disparate species. This raises some important questions. First, what are the requirements for rapid within-host adaptation? Second, is within-host adaptation of bacterial pathogens sufficiently rapid to influence disease outcome? If so, which conditions favour increased virulence in the host, and which conditions favour attenuated virulence?

Rapid within-host adaptation depends on a range of factors, notably the mutation rate of potentially beneficial mutations, the effective population size and the fitness advantage of mutants [131]. The larger these quantities are, the faster is the rate of adaptation of the population as a whole. In *M. tuberculosis*, recent studies have shown that both the mutation rate and effective population size within the host are very small. The genome-wide mutation rate barely registers one mutation every two years [32,33,82], and diversity is so low that isolates sampled at the same time differ by an average of only 0.5 mutations [33]. Assuming, for illustrative purposes, a generation length of 24 hours[132], this is equivalent to an effective population size of fewer than 200 reproductively viable cells. Target sizes for selection, in terms of the number of possible beneficial mutations, differ among drugs but commonly involve around a dozen sites within a particular gene. To observe adaptation, in the presence of antibiotics, in a timescale of months then requires a thousand-fold or greater daily replicative advantage of resistant over susceptible cells. This is plausible for antibiotic-induced selection, where the advantage of resistance over susceptibility can in some cases be of this order of magnitude . However, these values suggest that *M. tuberculosis* is unlikely to evolve within-host adaptations during the course of a single infection unless there are many potentially beneficial mutations or unless adaptation is a matter of life or death .

For within-host adaptation to influence the outcome of a single infection, selection must therefore be relatively strong, although other pathogens are likely to be more adaptable than *M. tuberculosis*. For example, *S. aureus* exhibits approximately ten-fold higher rates of mutation and within-host diversity than

M. tuberculosis, at least during asymptomatic carriage [42], suggesting it has greater capacity for adaptation during the course of infection — not just to antibiotic treatment, but also to the host immune response . Although clear-cut examples of adaptation to the host have come mainly from opportunistic infections of *P. aeruginosa* and *B. dolosa*, several studies**[Au: Ref?]** of *S. aureus* infection show that even single point mutations can lead to widespread changes in gene expression that may radically alter phenotypes, including virulence in model systems [84,85]. These studies strongly suggest that within-host bacterial adaptation has the potential to influence disease progression. However, disentangling cause from effect is crucial for drawing firm conclusions from such studies.

Whereas the adaptive potential of bacteria in the host is striking, less clear is whether we should expect bacteria to evolve to become more or less harmful. Adaptive trade-off theory predicts that when the long-term survival of the pathogen depends on the well being of the host, the pathogen will tend to evolve reduced virulence [121–123]. A number of longitudinal studies of within-host evolution have reported the attenuation of virulence over time [85,127]. However, adaptive trade-off theory concerns selection acting on transmission at the population level, and not during colonization or infection of individual hosts. Where trade-offs exist between onward transmission and short-term survival, we should expect within-host evolution to favour immediate reward at the expense of long-term success.

The years ahead promise further insights as studies continue to investigate within-host evolution in an increasingly diverse array of major pathogens . Fully capitalizing on the potential of WGS will require the development of new analysis methods for detecting recent transmission and adaptation, untangling gene expression and elucidating phenotype-to-genotype relationships . For example, studies of transmission stand to gain in accuracy by sampling within-host diversity (Figure 2), but existing analysis methods do not currently exploit this information to its full potential. Our current knowledge of the size of transmission bottlenecks is very limited, and so this is an area where studies of diversity within donors and recipients could provide valuable new information . Predicting bacterial phenotypes from genotypes is likely to grow in importance, particularly in translational settings for genome-based antibiotic resistance prediction . Already, high levels of accuracy can be achieved [133,134], and the quality of prediction is set to increase as we unravel the genetic architecture of bacterial phenotypes. As sequencing technologies continue to improve, real-time WGS-based diagnostics will provide clinicians with deeper insights into the pathogen population and its evolutionary potential to respond to different treatments, and therefore more information on which to base clinical decisions.

Acknowledgements
XD is funded by the BBSRC (Grant BB/L023458/1) and the NIHR Health Protection Research Unit on Modelling Methodology (Grant HPRU-2012-10080). TEP and DWC are NIHR Senior Investigators. DJW is a Sir Henry Dale Fellow, jointly funded by the Wellcome Trust and the Royal Society (Grant 101237/Z/13/Z). This study was supported by the Health Innovation Challenge Fund, a parallel funding partnership between the Wellcome Trust (Grant WT098615) and the Department of Health (Grant HICF-T5-358), the NIHR Health Protection Research Unit on Healthcare Associated Infection and Antimicrobial Resistance (Grant HPRU-2012-10041) and the NIHR Oxford Biomedical Research Centre. The views expressed in this publication are those of the authors and not necessarily those of the funders.

References

59. Lieberman et al (2011) Nat Genet
Study of a *B. dolosa* outbreak in cystic fibrosis patients revealed evidence for adaptation to the host in the form of convergent evolution across multiple patients at genes encoding antibiotic resistance and bacterial membrane composition.

97. Markussen et al (2014) mBio
Investigation of a 32-year persistent *P. aeruginosa* DK1 infection showed diversification and co-existence of sub-lineages with distinct functional and genomic signatures and rates of evolution that may occupy different niches within the cystic fibrosis airway.

96. Marvig et al (2013) PLoS Genet
Evolutionary analysis of the *P. aeruginosa* DK2 lineage over 38 years identified pathoadaptive mutations occurring independently in multiple patients in genes relating to antibiotic resistance, cell envelope and regulatory functions.

82. Elholm et al (2014) Genome Biol
First documented case of extensively drug resistant (XDR) *M. tuberculosis* strain evolved from a susceptible ancestor within a single patient. Resistance for most drugs evolved multiple times, with ultimately one lineage prevailing.

25. Kennemann et al (2011) PNAS
Examination of five longitudinally sampled patients infected with *H. pylori*, detailing extensive mutation and recombination within individual hosts.

88. Comas et al (2012) Nat Genet
Using a number of approaches, this study identifies high-confidence compensatory mutations associated with rifampicin resistance-conferring mutations in *M. tuberculosis*, noting an enrichment in the rpoA and rpoC genes encoding subunits of RNA polymerase.

71. Tong et al (2015) Genome Res
In this study, all patients from two intensive care units in a Thai hospital were repeatedly screened for carriage of MRSA over a period of three months. Whole genome sequencing of patients and staff allowed the reconstruction of transmission within and between wards.

117. Young et al (2012) PNAS
Charts the genetic changes associated with the transition from long-term asymptomatic carriage of *S. aureus* to invasive bloodstream infection in one patient, identifying an excess of loss-of-function mutations separating carried from invasive isolates including the transcriptional regulator *rsp*.

95. Dordel et al (2014) mBio
Reveals that most methicillin resistant *S. aureus* (MRSA) exhibit heteroresistance, where the majority of isolates are methicillin-sensitive. Low-frequency mutants possess several-hundred-fold higher resistance, allowing rapid population adaptation upon antibiotic exposure, while avoiding constitutive expression of resistance genes.

1. Fraser, C. *et al.* Virulence and pathogenesis of HIV-1 infection: an evolutionary perspective. *Science* **343,** 1243727 (2014).

2. Pybus, O. G. & Rambaut, A. Evolutionary analysis of the dynamics of viral infectious disease. *Nat. Rev. Genet.* **10,** 540–50 (2009).

3. Wilson, A., Ochman, H. & Prager, E. M. Molecular time scale for evolution. *Trends Genet.* **3,** 241–247 (1987).

4. Ochman, H., Elwyn, S. & Moran, N. A. Calibrating bacterial evolution. *Proc Natl Acad Sci USA* **96,** 12638–12643 (1999).

5. Ochman, H. & Wilson, A. C. Evolution in bacteria: Evidence for a universal substitution rate in cellular genomes. *J. Mol. Evol.* **26,** 74–86 (1987).

6. Didelot, X. *et al.* Microevolutionary analysis of Clostridium difficile genomes to investigate transmission. *Genome Biol* **13,** R118 (2012).

7. Wilson, D. J. *et al.* Rapid evolution and the importance of recombination to the gastroenteric pathogen Campylobacter jejuni. *Mol. Biol. Evol.* **26,** 385–97 (2009).

8. Morelli, G. *et al.* Microevolution of Helicobacter pylori during prolonged infection of single hosts and within families. *PLoS Genet* **6,** e1001036 (2010).

9. Biek, R., Pybus, O. G., Lloyd-Smith, J. O. & Didelot, X. Measurably evolving pathogens in the genomic era. *Trends Ecol. Evol.* **30,** 306–313 (2015).

10. Ho, S. Y. W. *et al.* Time-dependent rates of molecular evolution. *Molecular Ecology* **20,** 3087–3101 (2011).

11. Ho, S. Y. W. The changing face of the molecular evolutionary clock. *Trends Ecol. Evol.* **29,** 496–503 (2014).

12. Linz, B. *et al.* A mutation burst during the acute phase of Helicobacter pylori infection in humans and rhesus macaques. *Nat. Commun.* **5,** 4165 (2014).

13. Drake, J. W., Charlesworth, B., Charlesworth, D. & Crow, J. F. Rates of Spontaneous Mutation. *Genetics* **148,** 1667–1686 (1998).

14. Maiden, M. C. *et al.* Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci USA* **95,** 3140–5 (1998).

15. Loman, N. J. *et al.* High-throughput bacterial genome sequencing: an embarrassment of choice, a world of opportunity. *Nat. Rev. Microbiol.* **10,** 599–606 (2012).

16. Loman, N. J. *et al.* Performance comparison of benchtop high-throughput sequencing platforms. *Nat. Biotechnol.* **30,** 434–9 (2012).

17. Didelot, X., Bowden, R., Wilson, D. J., Peto, T. E. A. & Crook, D. W. Transforming clinical microbiology with bacterial genome sequencing. *Nat. Rev. Genet.* **13,** 601–612 (2012).

18. Köser, C. U. *et al.* Routine Use of Microbial Whole Genome Sequencing in Diagnostic and Public

Health Microbiology. *PLoS Pathog* **8,** e1002824 (2012).

19. Wilson, D. J. Insights from genomics into bacterial pathogen populations. *PLoS Pathog* **8,** e1002874 (2012).

20. Cespedes, C. *et al.* The clonality of Staphylococcus aureus nasal carriage. *J. Infect. Dis.* **191,** 444–52 (2005).

21. Mongkolrattanothai, K. *et al.* Simultaneous carriage of multiple genotypes of Staphylococcus aureus in children. *J. Med. Microbiol.* **60,** 317–22 (2011).

22. Votintseva, A. A. *et al.* Multiple-strain colonization in nasal carriers of Staphylococcus aureus. *J. Clin. Microbiol.* **52,** 1192–1200 (2014).

23. Worby, C. J., Lipsitch, M. & Hanage, W. P. Within-Host Bacterial Diversity Hinders Accurate Reconstruction of Transmission Networks from Genomic Distance Data. *PLoS Comput. Biol.* **10,** e1003549 (2014).

24. Falush, D. *et al.* Recombination and mutation during long-term gastric colonization by Helicobacter pylori: estimates of clock rates, recombination size, and minimal age. *Proc Natl Acad Sci USA* **98,** 15056–15061 (2001).

25. Kennemann, L. *et al.* Helicobacter pylori genome evolution during human infection. *Proc Natl Acad Sci USA* **108,** 5033–5038 (2011).

26. Didelot, X. *et al.* Genomic evolution and transmission of Helicobacter pylori in two South African families. *Proc Natl Acad Sci USA* **110,** 13880–13885 (2013).

27. Mathers, A. J. *et al.* Klebsiella pneumoniae carbapenemase (KPC) producing K. pneumoniae at a Single Institution: Insights into Endemicity from Whole Genome Sequencing. *Antimicrob. Agents Chemother.* **59,** 656–1663 (2015).

28. Young, B. C. *et al.* Evolutionary dynamics of Staphylococcus aureus during progression from carriage to disease. *Proc Natl Acad Sci USA* **109,** 4550–4555 (2012).

29. Eyre, D. W. *et al.* Diverse Sources of C. difficile Infection Identified on Whole-Genome Sequencing. *N. Engl. J. Med.* **369,** 1195–1205 (2013).

30. He, M. *et al.* Emergence and global spread of epidemic healthcare-associated Clostridium difficile. *Nat. Genet.* **45,** 109–113 (2013).

31. Reeves, P. R. *et al.* Rates of Mutation and Host Transmission for an Escherichia coli Clone over 3 Years. *PLoS One* **6,** e26907 (2011).

32. Ford, C. B. *et al.* Use of whole genome sequencing to estimate the mutation rate of Mycobacterium tuberculosis during latent infection. *Nat. Genet.* **43,** 482–6 (2011).

33. Walker, T. M. *et al.* Whole-genome sequencing to delineate Mycobacterium tuberculosis outbreaks: a retrospective observational study. *Lancet Infect. Dis.* **13,** 137–146 (2013).

34. Bryant, J. M. *et al.* Whole-genome sequencing to identify transmission of Mycobacterium abscessus between patients with cystic fibrosis: a retrospective cohort study. *Lancet* **381,** 1551–1560 (2013).

35. Tomb, J. F. *et al.* The complete genome sequence of the gastric pathogen Helicobacter pylori. *Nature* **388,** 539–47 (1997).

36. LeClerc, J., Li, B., Payne, W. & Cebula, T. High mutation frequencies among Escherichia coli and Salmonella Pathogens. *Science* **274,** 1208–1211 (1996).

37. Taddei, F. *et al.* Role of mutator alleles in adaptive evolution. *Nature* **387,** 700–702 (1997).

38. Lieberman, T. D. *et al.* Genetic variation of a bacterial pathogen within individuals with cystic fibrosis provides a record of selective pressures. *Nat. Genet.* **46,** 82–7 (2014).

39. Moxon, E. R., Rainey, P. B., Nowak, M. A. & Lenski, R. E. Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr. Biol.* **4,** 24–33 (1994).

40. Moxon, R., Bayliss, C. & Hood, D. Bacterial contingency loci: the role of simple sequence DNA repeats in bacterial adaptation. *Annu. Rev. Genet.* **40,** 307–333 (2006).

41. Alamro, M. *et al.* Phase variation mediates reductions in expression of surface proteins during persistent meningococcal carriage. *Infect. Immun.* **82,** 2472–84 (2014).

42. Ochman, H., Lawrence, J. G. & Groisman, E. A. Lateral gene transfer and the nature of bacterial innovation. *Nature* **405,** 299–304 (2000).

43. Didelot, X., Méric, G., Falush, D. & Darling, A. E. Impact of homologous and non-homologous recombination in the genomic evolution of Escherichia coli. *BMC Genomics* **13,** 256 (2012).

44. Croucher, N. J. *et al.* Rapid pneumococcal evolution in response to clinical interventions. *Science* **331,** 430–434 (2011).

45. Didelot, X., Achtman, M., Parkhill, J., Thomson, N. R. & Falush, D. A bimodal pattern of relatedness between the Salmonella Paratyphi A and Typhi genomes: Convergence or divergence by homologous recombination? *Genome Res.* **17,** 61–8 (2007).

46. Sheppard, S. K. *et al.* Progressive genome-wide introgression in agricultural Campylobacter coli. *Mol. Ecol.* **22,** 1051–1064 (2013).

47. Cao, Q. *et al.* Progressive genomic convergence of two Helicobacter pylori strains during mixed infection of a patient with chronic gastritis. *Gut* **64,** 554–61 (2015).

48. Andersson, J. O. & Andersson, S. G. Insights into the evolutionary process of genome degradation.

Curr. Opin. Genet. Dev. **9,** 664–671 (1999).

49. Rau, M. H., Marvig, R. L., Ehrlich, G. D., Molin, S. & Jelsbak, L. Deletion and acquisition of genomic content during early stage adaptation of Pseudomonas aeruginosa to a human host environment. *Environ. Microbiol.* **14,** 2200–11 (2012).

50. Rankin, D. J., Rocha, E. P. C. & Brown, S. P. What traits are carried on mobile genetic elements, and why? *Heredity (Edinb).* **106,** 1–10 (2011).

51. Dingle, K. E. *et al.* Evolutionary History of the Clostridium difficile Pathogenicity Locus. *Genome Biol. Evol.* **6,** 36–52 (2014).

52. Stanczak-Mrozek, K. I. *et al.* Within-host diversity of MRSA antimicrobial resistances. *J. Antimicrob. Chemother.* 11–13 (2015). doi:10.1093/jac/dkv119

53. Charlesworth, B. Fundamental concepts in genetics: effective population size and patterns of molecular evolution and variation. *Nat. Rev. Genet.* **10,** 195–205 (2009).

54. Kuo, C., Moran, N. & Ochman, H. The consequences of genetic drift for bacterial genome complexity. *Genome Res* **19,** 1450–1454 (2009).

55. Golubchik, T. *et al.* Within-Host Evolution of Staphylococcus aureus during Asymptomatic Carriage. *PLoS One* **8,** e61319 (2013).

56. Rocha, E. P. C. *et al.* Comparisons of dN/dS are time dependent for closely related bacterial genomes. *J Theor Biol* **239,** 226–35 (2006).

57. Price, E. P. *et al.* Within-host evolution of Burkholderia pseudomallei over a twelve-year chronic carriage infection. *MBio* **4,** e00388–13 (2013).

58. Marvig, R. L., Sommer, L. M., Molin, S. & Johansen, H. K. Convergent evolution and adaptation of Pseudomonas aeruginosa within patients with cystic fibrosis. *Nat. Genet.* **47,** 57–65 (2015).

59. Lieberman, T. D. *et al.* Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes. *Nat. Genet.* **43,** 1275–80 (2011).

60. Krebes, J., Didelot, X., Kennemann, L. & Suerbaum, S. Bidirectional genomic exchange between Helicobacter pylori strains from a family in Coventry, United Kingdom. *Int. J. Med. Microbiol.* **304,** 1135–1146 (2014).

61. Palmer, A. C. & Kishony, R. Understanding, predicting and manipulating the genotypic evolution of antibiotic resistance. *Nat. Rev. Genet.* **14,** 243–8 (2013).

62. Gardy, J. L. *et al.* Whole-genome sequencing and social-network analysis of a tuberculosis outbreak. *N. Engl. J. Med.* **364,** 730–9 (2011).

63. Snitkin, E. S. *et al.* Tracking a hospital outbreak of carbapenem-resistant Klebsiella pneumoniae with whole-genome sequencing. *Sci. Transl. Med.* **4,** 148ra116 (2012).

64. Croucher, N. J. & Didelot, X. The application of genomics to tracing bacterial pathogen transmission. *Curr. Opin. Microbiol.* **23,** 62–67 (2015).

65. Maddison, W. P. & Knowles, L. L. Inferring phylogeny despite incomplete lineage sorting. *Syst. Biol.* **55,** 21–30 (2006).

66. Ypma, R., van Ballegooijen, W. M. & Wallinga, J. Relating Phylogenetic Trees to Transmission Trees of Infectious Disease Outbreaks. *Genetics* **195,** 1055–1062 (2013).

67. Didelot, X., Gardy, J. & Colijn, C. Bayesian inference of infectious disease transmission from whole genome sequence data. *Mol. Biol. Evol.* **31,** 1869–1879 (2014).

68. Ou, C. Y. *et al.* Molecular epidemiology of HIV transmission in a dental practice. *Science* **256,** 1165–71 (1992).

69. Metzker, M. L. *et al.* Molecular evidence of HIV-1 transmission in a criminal case. *Proc Natl Acad Sci USA* **99,** 14292–7 (2002).

70. Harris, S. R. *et al.* Whole-genome sequencing for analysis of an outbreak of meticillin-resistant Staphylococcus aureus: a descriptive study. *Lancet Infect. Dis.* **13,** 130–136 (2013).

71. Tong, S. Y. C. *et al.* Genome sequencing defines phylogeny and spread of methicillin-resistant Staphylococcus aureus in a high transmission setting. *Genome Res.* **25,** 111–118 (2015).

72. Paterson, G. K. *et al.* Capturing the cloud of diversity reveals complexity and heterogeneity of MRSA carriage, infection and transmission. *Nat. Commun.* **6,** 6560 (2015).

73. Okoro, C. K. *et al.* High-resolution single nucleotide polymorphism analysis distinguishes recrudescence and reinfection in recurrent invasive nontyphoidal Salmonella typhimurium disease. *Clin. Infect. Dis.* **54,** 955–63 (2012).

74. Eyre, D. W. *et al.* Whole-genome sequencing demonstrates that fidaxomicin is superior to vancomycin for preventing reinfection and relapse of infection with clostridium difficile. *J. Infect. Dis.* **209,** 1446–1451 (2014).

75. Mac Aogáin, M. *et al.* Whole-genome sequencing improves discrimination of relapse from reinfection and identifies transmission events among patients with recurrent Clostridium difficile infections. *J. Hosp. Infect.* **90,** 108–16 (2015).

76. Bryant, J. M. *et al.* Whole-genome sequencing to establish relapse or re-infection with Mycobacterium tuberculosis: a retrospective observational study. *Lancet. Respir. Med.* **1,** 786–92 (2013).

77. Guerra-Assunção, J. A. *et al.* Relapse or reinfection with tuberculosis: a whole genome sequencing

approach in a large population-based cohort with high HIV prevalence and active follow-up. *J. Infect. Dis.* **211,** 1154–1163 (2015).

78. Guerra-Assunção, J. *et al.* Large scale population-based whole genome sequencing of Mycobacterium tuberculosis provides insights into transmission in a high prevalence area. *Elife* **4,** e05166 (2015).

79. WHO. *Antimicrobial Resistance Global Report on Surveillance 2014*. *World Health Organization* (2014).

80. Elena, S. F. & Lenski, R. E. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nat. Rev. Genet.* **4,** 457–469 (2003).

81. Mwangi, M. M. *et al.* Tracking the in vivo evolution of multidrug resistance in Staphylococcus aureus by whole-genome sequencing. *Proc Natl Acad Sci USA* **104,** 9451–6 (2007).

82. Eldholm, V. *et al.* Evolution of extensively drug-resistant Mycobacterium tuberculosis from a susceptible ancestor in a single patient. *Genome Biol.* **15,** 490 (2014).

83. Koch, A. & Wilkinson, R. J. The road to drug resistance in Mycobacterium tuberculosis. *Genome Biol.* **15,** 520 (2014).

84. Howden, B. P. *et al.* Evolution of multidrug resistance during staphylococcus aureus infection involves mutation of the essential two component regulator WalKR. *PLoS Pathog.* **7,** e1002359 (2011).

85. Gao, W. *et al.* Two novel point mutations in clinical Staphylococcus aureus reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. *PLoS Pathog.* **6,** e1000944 (2010).

86. Delauné, A. *et al.* The WalKR system controls major staphylococcal virulence genes and is involved in triggering the host inflammatory response. *Infect. Immun.* **80,** 3438–3453 (2012).

87. Farhat, M. R. *et al.* Genomic analysis identifies targets of convergent positive selection in drug-resistant Mycobacterium tuberculosis. *Nat. Genet.* **45,** 1183–9 (2013).

88. Comas, I. *et al.* Whole-genome sequencing of rifampicin-resistant Mycobacterium tuberculosis strains identifies compensatory mutations in RNA polymerase genes. *Nat. Genet.* **44,** 106–110 (2011).

89. Peleg, A. Y. *et al.* Whole genome characterization of the mechanisms of daptomycin resistance in clinical and laboratory derived isolates of Staphylococcus aureus. *PLoS One* **7,** e28316 (2012).

90. Arias, C. a *et al.* Genetic basis for in vivo daptomycin resistance in enterococci. *N. Engl. J. Med.* **365,** 892–900 (2011).

91. Sydenham, T. V., Sóki, J., Hasman, H., Wang, M. & Justesen, U. S. Identification of antimicrobial

resistance genes in multidrug-resistant clinical Bacteroides fragilis isolates by whole genome shotgun sequencing. *Anaerobe* **31,** 59–64 (2014).

92. Cannatelli, A. *et al.* In vivo evolution to colistin resistance by PmrB sensor kinase mutation in KPC-producing Klebsiella pneumoniae is associated with low-dosage colistin treatment. *Antimicrob. Agents Chemother.* **58,** 4399–403 (2014).

93. Ba, X. *et al.* Novel mutations in penicillin-binding protein genes in clinical Staphylococcus aureus isolates that are methicillin resistant on susceptibility testing, but lack the mec gene. *J. Antimicrob. Chemother.* **69,** 594–597 (2014).

94. Saunders, N. J. *et al.* Deep resequencing of serial sputum isolates of Mycobacterium tuberculosis during therapeutic failure due to poor compliance reveals stepwise mutation of key resistance genes on an otherwise stable genetic background. *J. Infect.* **62,** 212–217 (2011).

95. Dordel, J. *et al.* Novel determinants of antibiotic resistance: Identification of mutated Loci in Highly methicillin-resistant subpopulations of methicillin-resistant Staphylococcus aureus. *MBio* **5,** e01000 (2014).

96. Marvig, R. L., Johansen, H. K., Molin, S. & Jelsbak, L. Genome analysis of a transmissible lineage of pseudomonas aeruginosa reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. *PLoS Genet.* **9,** e1003741 (2013).

97. Markussen, T. *et al.* Environmental heterogeneity drives within-host diversification and evolution of Pseudomonas aeruginosa. *MBio* **5,** e01592–14 (2014).

98. Wong, A. & Kassen, R. Parallel evolution and local differentiation in quinolone resistance in Pseudomonas aeruginosa. *Microbiology* **157,** 937–944 (2011).

99. Yang, L. *et al.* Evolutionary dynamics of bacteria in a human host environment. *Proc Natl Acad Sci USA* **108,** 7481–6 (2011).

100. Kim, S., Lieberman, T. D. & Kishony, R. Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance. *Proc. Natl. Acad. Sci.* **111,** 14494–14499 (2014).

101. van Hal, S. J. *et al.* In vivo evolution of antimicrobial resistance in a series of Staphylococcus aureus patient isolates: the entire picture or a cautionary tale? *J. Antimicrob. Chemother.* **69,** 363–7 (2014).

102. Sun, G. *et al.* Dynamic population changes in mycobacterium tuberculosis during acquisition and fixation of drug resistance in patients. *J. Infect. Dis.* **206,** 1724–1733 (2012).

103. Morand, B. & Mühlemann, K. Heteroresistance to penicillin in Streptococcus pneumoniae. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 14098–14103 (2007).

104. Ford, C. B. *et al.* Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat. Genet.* **45,** 784–790 (2013).

105. Barrick, J. E. *et al.* Genome evolution and adaptation in a long-term experiment with Escherichia coli. *Nature* **461,** 1243–1247 (2009).

106. Feliziani, S. *et al.* Coexistence and Within-Host Evolution of Diversified Lineages of Hypermutable Pseudomonas aeruginosa in Long-term Cystic Fibrosis Infections. *PLoS Genet.* **10,** e1004651 (2014).

107. Montanari, S. *et al.* Biological cost of hypermutation in Pseudomonas aeruginosa strains from patients with cystic fibrosis. *Microbiology* **153,** 1445–1454 (2007).

108. Marvig, R. L. *et al.* Within-host evolution of Pseudomonas aeruginosa reveals adaptation toward iron acquisition from hemoglobin. *MBio* **5,** e00966–14 (2014).

109. Allen, R. C., Popat, R., Diggle, S. P. & Brown, S. P. Targeting virulence: can we make evolution-proof drugs? *Nat. Rev. Microbiol.* **12,** 300–308 (2014).

110. Weinert, L. A. *et al.* Genomic signatures of human and animal disease in the zoonotic pathogen Streptococcus suis. *Nat. Commun.* **6,** 6740 (2015).

111. Andersen, S. B., Marvig, R. L., Molin, S., Krogh Johansen, H. & Griffin, A. S. Long-term social dynamics drive loss of function in pathogenic bacteria. *Proc. Natl. Acad. Sci.* **112,** 10756–10761 (2015).

112. Croucher, N. J. *et al.* Population genomics of post-vaccine changes in pneumococcal epidemiology. *Nat. Genet.* **45,** 656–663 (2013).

113. Damkiær, S., Yang, L., Molin, S. & Jelsbak, L. Evolutionary remodeling of global regulatory networks during long-term bacterial adaptation to human hosts. *Proc Natl Acad Sci USA* **110,** 7766–71 (2013).

114. von Eiff, C., Becker, K., Machka, K., Stammer, H. & Peters, G. Nasal carriage as a source of Staphylococcus aureus bacteremia. Study Group. *N. Engl. J. Med.* **344,** 11–16 (2001).

115. Yang, J., Tauschek, M. & Robins-Browne, R. M. Control of bacterial virulence by AraC-like regulators that respond to chemical signals. *Trends Microbiol.* **19,** 128–135 (2011).

116. Fantappiè, L., Scarlato, V. & Delany, I. Identification of the in vitro target of an iron-responsive AraC-like protein from neisseria meningitidis that is in a regulatory cascade with Fur. *Microbiology* **157,** 2235–2247 (2011).

117. Young, B. C. & Wilson, D. J. On the evolution of virulence during Staphylococcus aureus nasal carriage. *Virulence* **3,** 454–6 (2012).

118. Smith, E. E. *et al.* Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA* **103,** 8487–92 (2006).

119. Kodaman, N. *et al.* Human and Helicobacter pylori coevolution shapes the risk of gastric disease. *Proc Natl Acad Sci USA* **111,** 1455–60 (2014).

120. Campbell, D. I. *et al.* The african enigma: Low prevalence of gastric atrophy, high prevalence of chronic inflammation in West African adults and children. *Helicobacter* **6,** 263–267 (2001).

121. Anderson, R. M. & May, R. M. Coevolution of hosts and parasites. *Parasitology* **85,** 411–426 (1982).

122. Ewald, P. W. Host-Parasite Relations, Vectors, and the Evolution of Disease Severity. *Annu. Rev. Ecol. Syst.* **14,** 465–485 (1983).

123. Alizon, S., Hurford, A., Mideo, N. & Van Baalen, M. Virulence evolution and the trade-off hypothesis: History, current state of affairs and the future. *J. Evol. Biol.* **22,** 245–259 (2009).

124. Suerbaum, S. & Josenhans, C. Helicobacter pylori evolution and phenotypic diversification in a changing host. *Nat. Rev. Microbiol.* **5,** 441–52 (2007).

125. Schwarz, S. *et al.* Horizontal versus familial transmission of Helicobacter pylori. *PLoS Pathog* **4,** e1000180 (2008).

126. Agnew, P. & Koella, J. C. Virulence, parasite mode of transmission, and host fluctuating asymmetry. *Proc. R. Soc. B Biol. Sci.* **264,** 9–15 (1997).

127. Zdziarski, J. *et al.* Host imprints on bacterial genomes-rapid, divergent evolution in individual patients. *PLoS Pathog.* **6,** 95–96 (2010).

128. Klemm, P., Roos, V., Ulett, G. C., Schembri, M. A. & Svanborg, C. Molecular Characterization of the Escherichia coli Asymptomatic Bacteriuria Strain 83972 : the Taming of a Pathogen Molecular Characterization of the Escherichia coli Asymptomatic Bacteriuria Strain 83972 : the Taming of a Pathogen. *Infect. Immun.* **74,** 781–785 (2006).

129. Toprak, E. *et al.* Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat. Genet.* **44,** 101–105 (2011).

130. Espedido, B. A. *et al.* Whole genome sequence analysis of the first Australian OXA-48-producing outbreak-associated Klebsiella pneumoniae isolates: the resistome and in vivo evolution. *PLoS One* **8,** e59920 (2013).

131. Whitlock, M. C. Fixation probability and time in subdivided populations. *Genetics* **164,** 767–779 (2003).

132. Gill, W. P. *et al.* A replication clock for Mycobacterium tuberculosis. *Nat. Med.* **15,** 211–214 (2009).

133. Gordon, N. C. *et al.* Prediction of Staphylococcus aureus antimicrobial resistance by whole-genome sequencing. *J. Clin. Microbiol.* **52,** 1182–91 (2014).

134. Stoesser, N. *et al.* Predicting antimicrobial susceptibilities for Escherichia coli and Klebsiella pneumoniae isolates using whole genomic sequence data. *J. Antimicrob. Chemother.* **68,** 2234–2244 (2013).

135. Eyre, D. W. *et al.* Detection of Mixed Infection from Bacterial Whole Genome Sequence Data Allows Assessment of Its Role in Clostridium difficile Transmission. *PLoS Comput. Biol.* **9,** e1003059 (2013).

136. Bentley, D. R. *et al.* Accurate whole human genome sequencing using reversible terminator chemistry. *Nature* **456,** 53–9 (2008).

137. Eyre, D. W. *et al.* A pilot study of rapid benchtop sequencing of Staphylococcus aureus and Clostridium difficile for outbreak detection and surveillance. *BMJ Open* **2,** e001124 (2012).

138. Reuter, S. *et al.* Rapid bacterial whole-genome sequencing to enhance diagnostic and public health microbiology. *JAMA Intern. Med.* **173,** 1397–404 (2013).

139. Li, H., Ruan, J. & Durbin, R. Mapping short DNA sequencing reads and variants calling using mapping quality scores. *Genome Res.* **18,** 1851–8 (2008).

140. Stoesser, N. *et al.* Genome Sequencing of an Extended Series of NDM-Producing Klebsiella pneumoniae Neonatal Infections in a Nepali Hospital Characterizes the Extent of Community Versus Hospital-associated Transmission in an Endemic Setting. *Antimicrob. Agents Chemother.* **58,** 7347–57 (2014).

141. Nagarajan, N. & Pop, M. Sequence assembly demystified. *Nat. Rev. Genet.* **14,** 157–67 (2013).

142. Treangen, T. J. & Salzberg, S. L. Repetitive DNA and next-generation sequencing: computational challenges and solutions. *Nat. Rev. Genet.* **13,** 36–46 (2011).

143. Foster, T. J., Geoghegan, J. A., Ganesh, V. K. & Höök, M. Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus. *Nat. Rev. Microbiol.* **12,** 49–62 (2013).

144. Walker, T. M. *et al.* Assessment of Mycobacterium tuberculosis transmission in Oxfordshire, UK, 2007–12, with whole pathogen genome sequences: an observational study. *Lancet Respir. Med.* **2,** 285–292 (2014).

145. Drummond, A. J. & Rambaut, A. BEAST : Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* **7,** 214 (2007).

146. Didelot, X. & Wilson, D. J. ClonalFrameML: Efficient Inference of Recombination in Whole Bacterial Genomes. *PLoS Comput. Biol.* **11,** e1004041 (2015).

147. Croucher, N. J. *et al.* Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res.* **43,** e15 (2015).

Text Box 1: methodology for within-host studies.

Isolate collection and whole genome sequencing.

A first approach to capturing within-host diversity is to collect multiple clinical samples, either longitudinally or simultaneously, and either at one or several body sites. A second approach is to use a single clinical sample but sequence several separate genomes, for example by culture on a suitable medium, selection of colonies and independent further sub-culturing [55]. A third approach is to sequence only a single sample, but look for variation in the raw output before assembling the raw sequence data into a genome [135]. It is however important to bear in mind that no approach is guaranteed to fully sample the within-host pathogen population. Once each isolate has been grown long enough to yield sufficient DNA for whole-genome sequencing, the DNA is extracted and purified. The most popular approach to sequencing is currently sequencing-by-synthesis [136], as implemented by the Illumina HiSeq and MiSeq sequencers. Sample preparation for these platforms entails fragmentation of the DNA and multiplexing allows several genomes to be sequenced at the same time by uniquely tagging every fragment from each genome; the typical output is paired-end reads corresponding to the start and ends of the genomic fragments . In the past few years, read lengths have been increasing (up to 300bp), as has the speed and affordability of sequencing protocols. This is especially true of benchtop sequencers, to the point that real-time clinical applications of whole-genome sequencing are emerging [15,16,137,138].

Assembly and variant calling.

The most frequently used approach to assemble sequencing reads into a genome is to use a previously sequenced reference genome as a scaffold. Assuming that the reference and target genome are not too distantly related, each read can be mapped onto the reference genome [139], creating a so-called pileup. The average number of reads mapping to each unique position of the reference genome is called the coverage or depth, and represents an important measure of sequencing reliability. At each position along the reference genome, variant calling is done by determining whether the mapped reads of the target genome are identical or different to the reference genome . If the disagreement between reads is too high, or the site coverage too low, the site may be left uncalled. If no closely related reference genome exists, lower throughput long-read technology can be used to generate one, as in the study of a *Klebsiella pneumoniae* outbreak [140] . Alternatively, *de novo* assembly can be attempted, without the requirement of a reference genome [141], but is typically able to reconstruct only segments of the genome, so called contigs . In both approaches, repetitive regions of the genome are difficult to assemble [142], which is especially problematic for studying highly repetitive genes, such as the ones coding for surface proteins [143].

Comparative genome analysis.

The simplest method for comparative genome analysis is to count the number of positions where two genomes differ. When the two genomes are from the same host, this provides a first idea of the amount of within-host diversity [55]. When the two genomes are from different hosts, the number of differences reflects the likelihood of transmission between the two hosts[144], although it is difficult to define the threshold above which a transmission event can be definitively ruled out . Comparisons of more than two genomes typically involve the reconstruction of their ancestry using phylogenetic methods. When the genomes have been sampled at different times, a popular approach is to use the BEAST software tool to try to infer both the rate of evolution and a time-scaled genealogy [9,145]. Alternatively, the evolutionary rate can be estimated using multiple pairs of sequential genomes from the same hosts [9]. Recombination can disrupt phylogenetic reconstructions, but new methods are emerging that can account for this in whole genome datasets [146,147].

Text Box 2: a primer on genetic drift.

Genetic drift is the process whereby allele frequencies fluctuate over time due to the birth and death of individuals in the population. To illustrate this concept, the evolution of a bacterial population was simulated under the assumptions of the Wright-Fisher model (see the figure). In this example, the population has an initial effective population size of 20 bacteria and each generation (horizontal row) is formed by randomly

selecting parents from the previous generation (parental relationships are shown by lines connecting individuals from one row to the next in the figure). At generation 5, the leftmost individual mutates, as illustrated in red, and the new mutation is inherited by all of its descendants . This mutation slightly increases the fitness of its carriers, so that they have a slightly higher expected number of offspring compared with the wild type bacteria shown in black. For that reason, even though its frequency was only 1 in 20 in generation 5, the red allele sweeps through the population, which means that its frequency progressively increases. At generation 14, the whole population carries the red allele, which means that it has become fixed and the black allele has become extinct. At generation 15, a new allele appears, shown in green, which again has slightly increased fitness compared to the red allele, and its frequency starts to increase progressively. However, at generations 19 and 20, the bacteria undergo a bottleneck, so that the effective population size is reduced to only 6 individuals. Bottlenecks enhance the effect of genetic drift, so that sweeping, fixation and extinction all happen at faster rates . By the the end of the bottleneck in generation 21, the green allele has swept through the population and the red allele has become extinct. Similar loss of variation happens when a new population is initiated from a small number of individuals drawn from a larger population, a phenomena known as the founder effect, which corresponds to the transmission bottleneck in the case of the transmission from a donor to a new host.

Figure legends

***Figure 1. Within-host evolution of* Staphylococcus aureus.**
A *Staphylococcus aureus* infection begins with the transmission of a *S. aureus* population (blue circles) from a donor host to a new host. Owing to the transmission bottleneck, only a subset of the within-host diversity in the donor host is transmitted to the newly infected host. Colonization starts at a given site — typically somewhere on the skin — and the pathogen population initially increases in size, with a new mutation arising occasionally (orange circle). The infection may also spread to other sites in the host, such as from skin to nose. When adaptive mutations (red circles) arise — that is, mutations that confer a selective advantage — they can quickly become fixed in the pathogen population through a selective sweep . When a secondary infection from another donor transmits a second *S. aureus* population (green circles) to the host, the two infecting populations may recombine to form a new genotype (purple circles). Transmission to other hosts may occur at any stage of colonization, as can spread to the blood stream, where the pathogen can cause life-threatening septicaemia.

Figure 2. Effect of within-host evolution on the reconstruction of transmission events .
This example illustrates the benefit of sampling several genomes, rather than a single genome, from each host when reconstructing a transmission network . In this example, three hosts (A, B and C) are infected with a bacterial pathogen, with host A having become infected first and having directly transmitted to B and then to C, as indicated by the vertical arrows. If only a single genome is sampled from each host (see inset), their ancestry is only weakly informative about who infected whom. In the example shown, the ancestry of the genome sampled from host A is such that the genomes sampled from host B and host C are more closely related to one another than with the genome sampled from host A, which might be mistaken as evidence for host B having infected host C (or vice versa) . This error can be avoided if within-host evolution is accounted for, but doing so with only a single genome sampled from each host results in substantial uncertainty in reconstructions of transmission . However, when several genomes are available for each host (coloured circles in the transmission tree), an ancestry analysis becomes much more informative about the transmission events . In the example shown, the three genomes sampled from host B form a clade that is closely related to a clade of genomes sampled in host A, as do the four genomes sampled from host C, correctly suggesting that A infected both B and C . Different colour circles represent genetic variation in the pathogen genome following within-host mutations .

Figure 3. Within-host adaptive potential during antibiotic exposure .
A susceptible bacterial strain (blue circle), when exposed to an antibiotic, will be highly likely to be killed, but may occasionally survive by evolving into a resistant strain (orange circle). Resistance usually has a high fitness cost (inset), so that resistant strains disappear when not exposed to the antibiotic. However, resistant strains can evolve compensatory mutations (green circle) so that they remain resistant without the associated fitness cost. Such compensated strains pose a serious danger to public health, because they do not disappear simply as a result of antibiotic disuse. Alternatively, strains may evolve adaptability (yellow circle), allowing them to quickly switch resistance on or off and therefore avoid the associated fitness cost, presenting a similar risk to public health**[Au: Ok?]** as that presented by compensated strains.

Figure 4. Within-host evolution of pathogens in the lungs of a cystic fibrosis patient.
Infection of the lungs of a cystic fibrosis patient begins with transmission from the environment or the skin of a donor, and progresses with a rapid increase in the size of the pathogen population . Mutations occasionally occur, some of which may be adaptative mutations that spread through the pathogen population in a selective sweep (red circles). Another important mechanism of genome evolution for these pathogens is hypermutation, whereby a strain loses the function of its mismatch repair machinery and thus becomes a hypermutator, with a mutation rate that is increased several fold, thus greatly increasing its evolutionary potential . As the infection progresses, the pathogen colonizes all ecological niches within the cystic fibrosis lungs, and separate adaptation to each niche leads to the coexistence of differentially adapted lineages.

Glossary

Adaptability
The ability to rapidly adapt to a change in selective pressure, such as antibiotic usage.

Adaptive trade-off hypothesis
Hypothesis that the long term evolutionary success of a pathogen requires a balance between duration of infection and virulence, based on the assumption that an increase in virulence decreases the average duration of infection.

Genome assembly
Bioinformatics process in which overlapping sequencing reads are combined into longer, contiguous sequences known as 'contigs', ideally a single contig per chromosome but usually several .

Clonal interference
Evolutionary dynamic in which selectively advantageous alleles at a given locus in one lineage outcompete advantageous alleles at other loci in other lineages , driving them to extinction. In organisms with the capacity for genome recombination , this can be avoided by combining all advantageous mutations in the same genome.

Compensatory mutations
Mutations that redress, possibly only partially, the fitness cost of mutations conferring adaptation to specific selection pressures, such as antibiotic resistance. Without compensatory mutations, adaptations that incur a fitness cost may be lost when the selection pressure is removed.

dN/dS ratio
The ratio of the number of non-synonymous substitutions, which alter the protein sequence, to the number of synonymous substitutions, which do not alter the protein sequence, normalized by the ratio expected under neutrality. dN/dS below one indicates purifying selection and above one positive selection.

Diversifying selection
A form of recurrent positive selection that favours the emergence of new alleles in a population; for example, the selective pressure of the host immune system on antigen evolution in pathogens .

Fitness trade-offs
The existence of some constraint, possibly mechanistic or genetic, that causes adaptations to one selection pressure to be disadvantageous with respect to another.

Fixation
The point at which an allele replaces all alternative alleles of the same locus in the population. Coincides with loss of the other alleles.

Heteroresistance
Varying levels of antibiotic resistance within an extremely closely related population, such as an individual colony.

Hitchhiking
Effect whereby an allele can increase in frequency even though it is not favoured by selection, only because it is found on the same genomes as other alleles of other loci that have a selective advantage.

Homologous recombination
Evolutionary event in which a segment of the genome of a recipient cell is replaced with the homologous segment of the genome from a donor cell.

Horizontal gene transfer
Uptake of genetic material by a recipient cell by a variety of mechanisms, commonly transformation of naked DNA, bacteriophage-mediated transduction or plasmid-mediated conjugation.

Hypermutators
An individual or lineage with elevated mutation rate, usually as a result of a loss of functionality of the DNA repair systems.

Incomplete lineage sorting
A phenomenon where a gene tree is discordant with the population or species tree. Occurs when lineages ancestral to several different populations split prior to, and in a different order to, the splitting of the respective populations. For within-host populations, causes discordance between phylogenies and transmission trees.

Melioidosis
Infectious disease caused by *B. pseudomallei*, leading to septisis and pneumonia, and endemic in South-East Asia and Australia.

Mismatch repair systems
Mechanism found in all bacteria to repair the mistakes introduced in the genome when it is replicated to allow clonal reproduction.

Evolutionary rate
The rate at which substitutions arise in a lineage, also known as the molecular clock rate. Population genetics theory predicts a constant rate in a neutrally evolving population with constant mutation rate, irrespective of changes in population size.

Mucoidy
A bacterial phenotype describing the production of glycoproteins resembling mucus.

Multi-locus enzyme electrophoresis (MLEE)
A molecular epidemiology approach in which strains are typed by the electrophoretic properties of a number of proteins.

Multi-locus sequence typing (MLST)
A molecular epidemiology approach in which strains are typed by their nucleotide sequences at a number of loci, typically 400 to 500bp long fragments of seven housekeeping genes.

Effective population size
Also known as N_e. The size of an idealized (neutrally evolving, homogeneous) population that is otherwise equivalent to an observed population. N_e is typically smaller than the number of individuals in the population, owing to population structure and variation in survival or reproductive viability.

Convergent evolution
Also known as parallel evolution. Occurrence of the same mutation at the same site in two or more independently evolving lineages, for example in separate hosts.

Pathoadaptive
An adaptation that confers pathogenicity.

Phase variation
A mechanism that bacteria use to allow rapid evolution of a specific trait in which frequently occurring, reversible mutations control gene expression.

Pleiotropic
The unexpected influence of one locus on multiple, apparently unrelated, phenotypes.

Point mutations
Mutation changing a single nucleotide.

Positive selection
Tendency for an allele that confers a survival or reproductive advantage to increase in frequency and fix at a higher rate. Advantageous alleles may nevertheless become lost, owing to random genetic drift despite positive selection.

Pre-adaptation
Also known as exaptation. Phenomenon where a previously existing trait confers an advantage in an environment to which it was not previously exposed.

Pulsed-field gel electrophoresis (PFGE)
A molecular epidemiology marker that allows strains to be typed by the lengths of the DNA molecules obtained after cutting the genome using a restriction enzyme .

Purifying selection
Tendency for an allele that incurs a survival or reproductive disadvantage to decrease in frequency and become lost. Deleterious alleles may nevertheless become fixed, owing to random genetic drift.

Quorum sensing
Mechanism by which a cell responds to changes in population size or density, classically by the secretion and detection of small peptides (also known as pheromones) .

Random genetic drift
Variations in genetic allele frequencies in a population caused by the random genetic sampling that occurs during the birth and death of individuals .

Selective sweep
Rapid increase in frequency and fixation of an advantageous allele. Selective sweeps are caused by positive selection.

Variable-number tandem repeats (VNTR)
A molecular epidemiology marker that allows strains to be typed by counting the number of copies of a specific pattern, which may be made of one or more nucleotides and is known to occur in a repeated fashion at a given location along the genome .

Variant calling
Bioinformatics process that determines the nucleotide at a given genomic site based on sequencing reads .

Virulence
The quantifiable frequency or severity of disease.

Online only

Key Points

- Whole genome sequencing of multiple isolates from single hosts has revealed previously unsuspected within-host diversity of many bacterial pathogens.
- Within-host bacterial populations are subject to multifarious evolutionary forces including mutation, genetic drift, natural selection and fluctuating population sizes.
- Within-host evolution blurs transmission relationships based on sampling a single genome per host, conferring a benefit to sequencing multiple genomes per host.
- Resistance to some antimicrobials frequently evolves independently in multiple hosts, revealing the enormous potential of bacteria to adapt in the human body.
- Within-host adaptation plays a major role in the evolution of opportunistic infections in immunocompromised patients by otherwise free-living bacteria.

- The study of within-host genomic evolution promises to shed new light on whether pathogens tend to become more or less virulent within the host, and the underlying selective pressures.

Author information

Xavier Didelot

Xavier Didelot is a Lecturer in the Department of Infectious Disease Epidemiology at Imperial College London. He received his D.Phil. in statistical genetics from the University of Oxford, UK, in 2007. He was a research fellow at the University of Warwick, UK, for 3 years before moving back to Oxford to work on the Modernising Medical Microbiology (MMM) project. He is perhaps best known as the author of ClonalFrame, software that reconstructs the genealogy of a sample of bacteria while accounting for the confounding effect of recombination.

A. Sarah Walker

Sarah Walker is a Professor of Medical Statistics and Epidemiology at the University of Oxford and University College London. She received her PhD from University College London in 1999 and has spent the last 20 years designing, managing and analysing large randomised controlled trials and observational studies in infectious diseases. Over the last 10 years she has been a key member of the Modernising Medical Microbiology (MMM) consortia, integrating electronic health records and pathogen whole genome sequencing to investigate transmission epidemiology and improve patient management.

Tim E. Peto

Tim Peto is an infectious diseases physician and a professor of medicine at the Oxford University Hospitals, and a UK National Institute of Health Research (NIHR) senior investigator. He read for a D.Phil. and qualified in medicine at the University of Oxford. As a clinical epidemiologist and clinical trialist for over 20 years, he has been overseeing key large scale trials in HIV, malaria and tuberculosis. Currently he studies the use of whole-genome sequencing for tracking common pathogens and predicting drug resistance in both hospitals and the community.

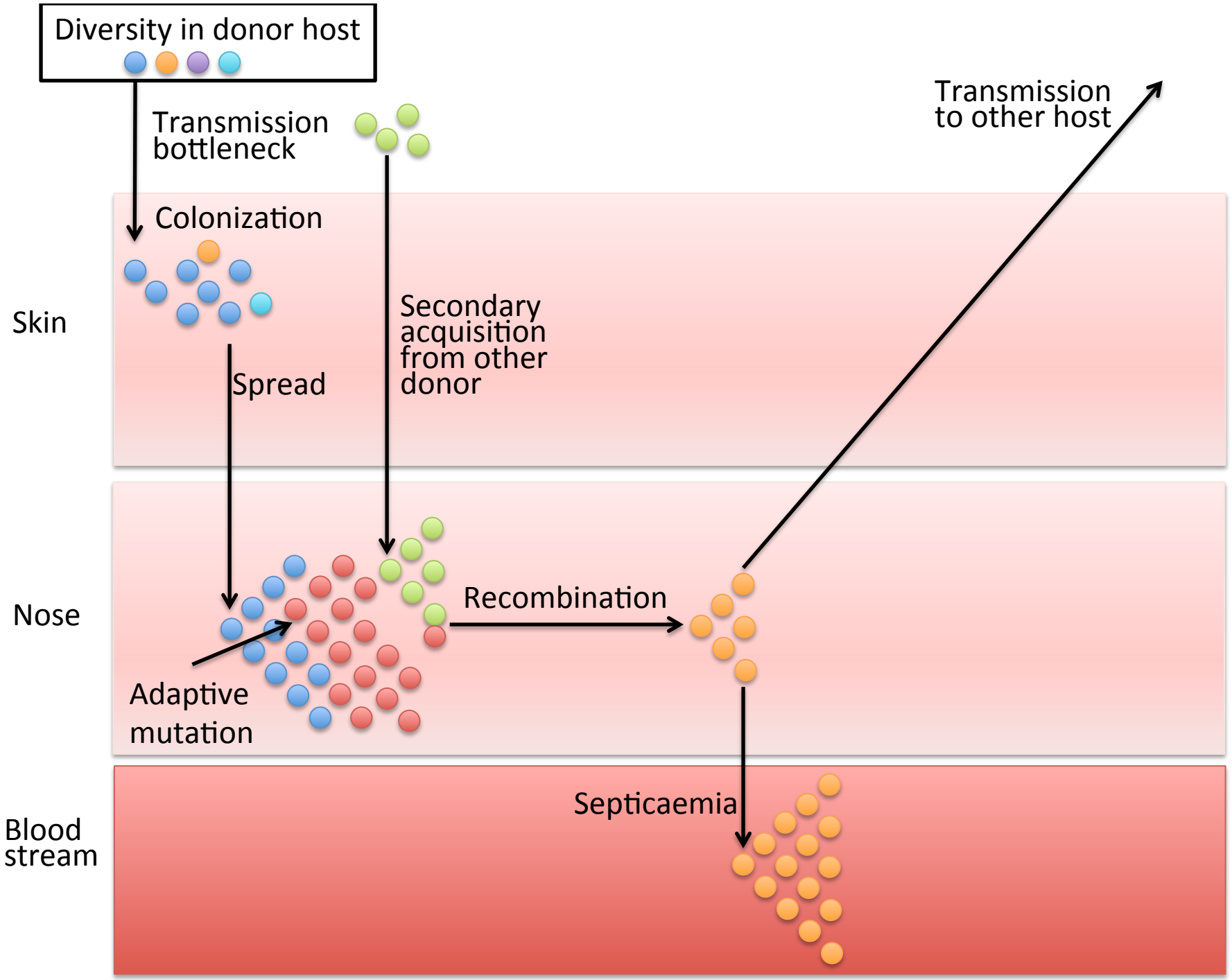
Derrick W. Crook

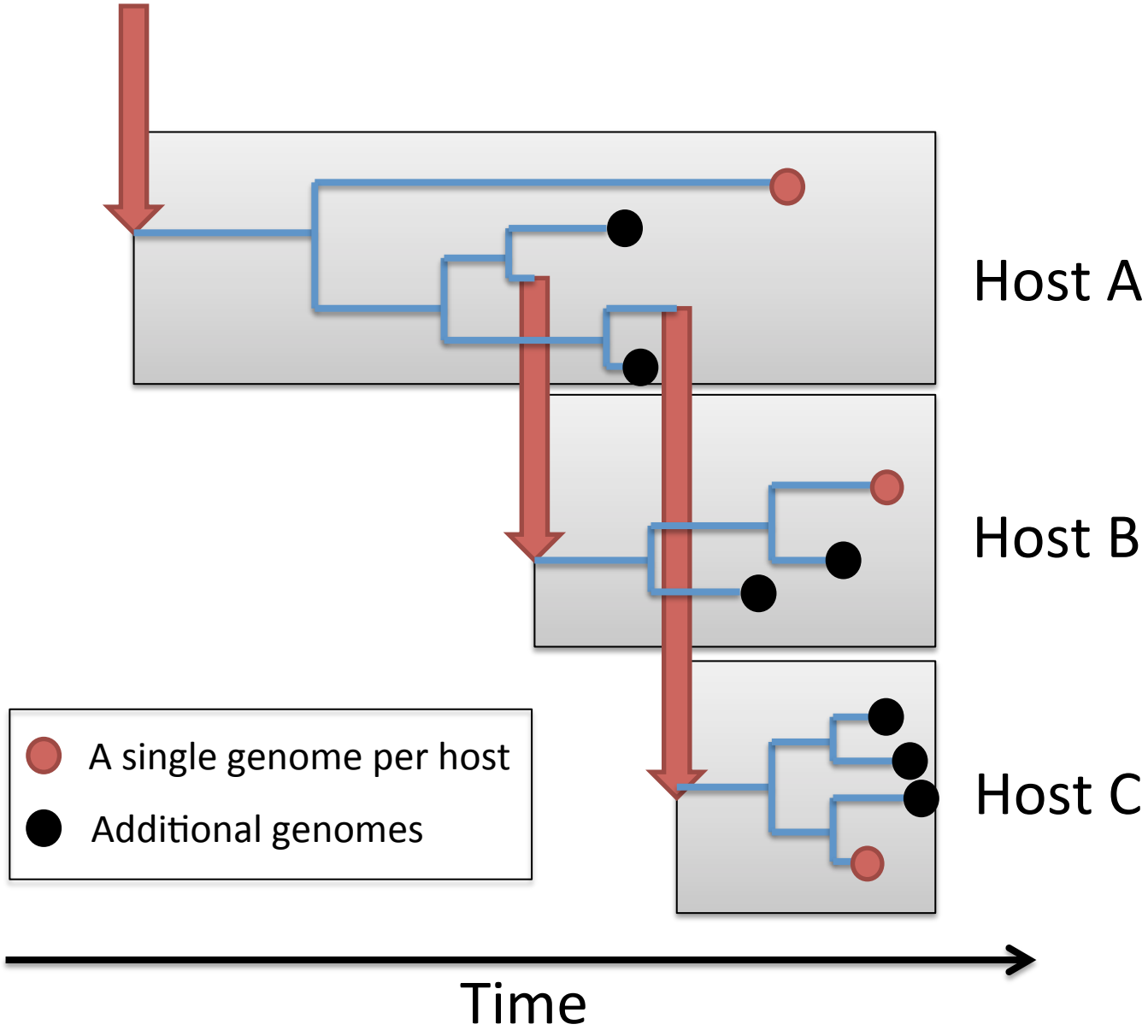
Derrick Crook is a clinical microbiologist, infectious diseases physician, professor of microbiology at the Oxford University Hospitals, an NIHR senior investigator and Director of the National Infection Service at Public Health England. He qualified in medicine at the University of the Witwatersrand, Johannesburg, South Africa, studied at the London School of Tropical Medicine, UK, and trained in the Department of Medicine, University of Virginia, Charlottesville, Virginia, USA, and the Department of Infectious Diseases, New England Medical Centre, Boston, Massachusetts, USA. His research encompasses diagnostics, epidemiology, new sequencing and informatics technologies aimed at improving management of infectious diseases.

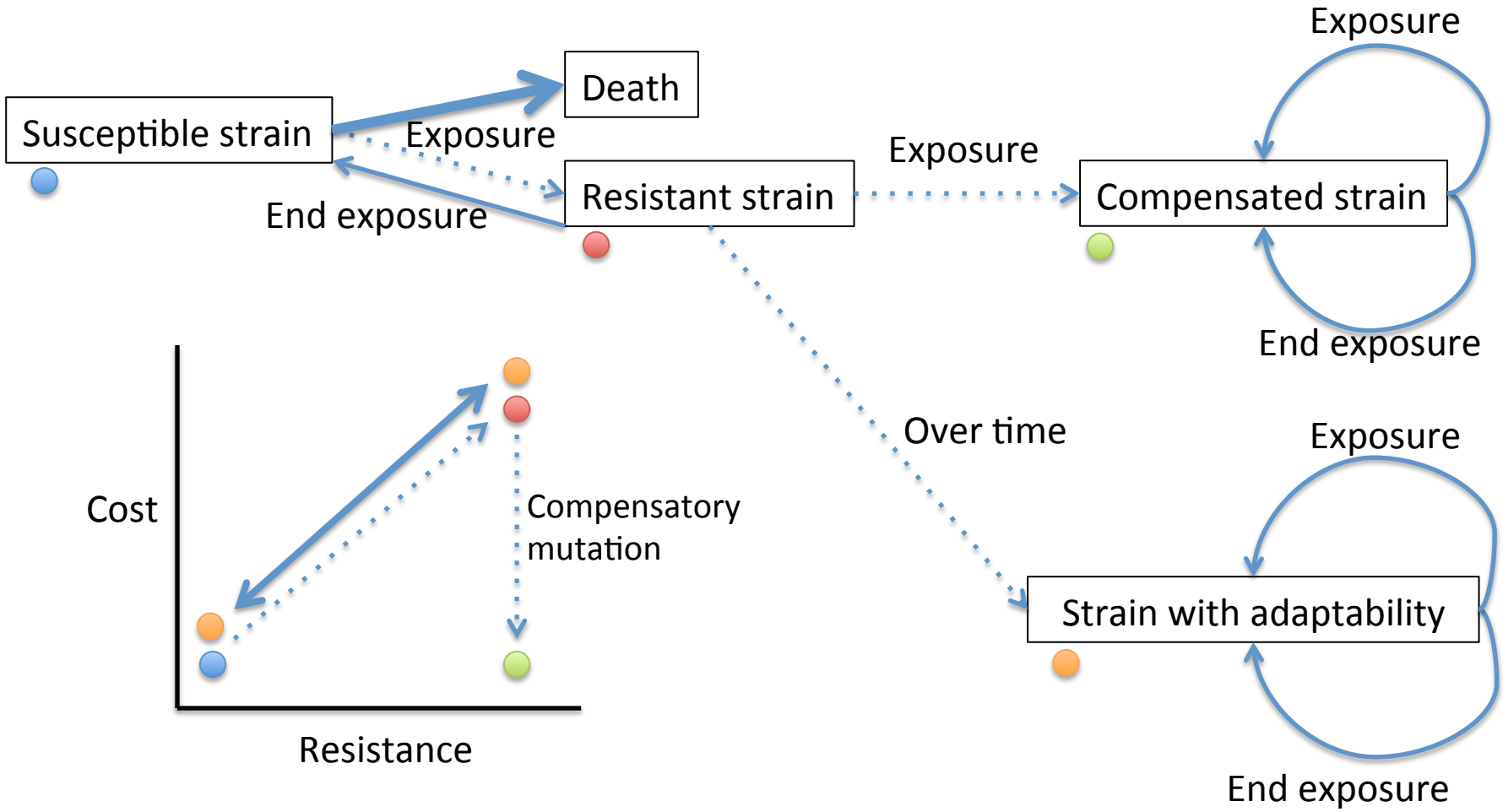
Daniel J. Wilson

Daniel Wilson is a Wellcome Trust and Royal Society Sir Henry Dale Fellow at the University of Oxford, where his laboratory investigates pathogen evolution and epidemiology via whole-genome analysis. Daniel's work currently focuses on the identification of genetic variants in pathogen genomes that explain differences

in the frequency and severity of infections, particularly hospital-associated infections. He read biological sciences and completed a doctorate in pathogen population genetics at the University of Oxford, before pursuing postdoctoral research at Lancaster University and the University of Chicago.

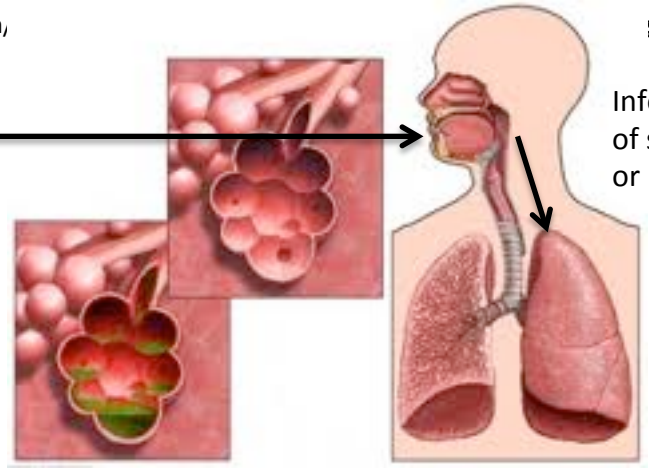

Diversity in donor host
Transmission bottleneck
Transmission to other host
Colonization
Skin
Secondary acquisition from other donor
Spread
Nose
Recombination
Adaptive mutation
Septicaemia
Blood stream


Host A
Host B
Host C
A single genome per host
Additional genomes
Time


Susceptible strain
Death
Exposure
End exposure
Resistant strain
Exposure
Compensated strain
Exposure
End exposure
Over time
Cost
Compensatory mutation
Resistance
Strain with adaptability
Exposure
End exposure

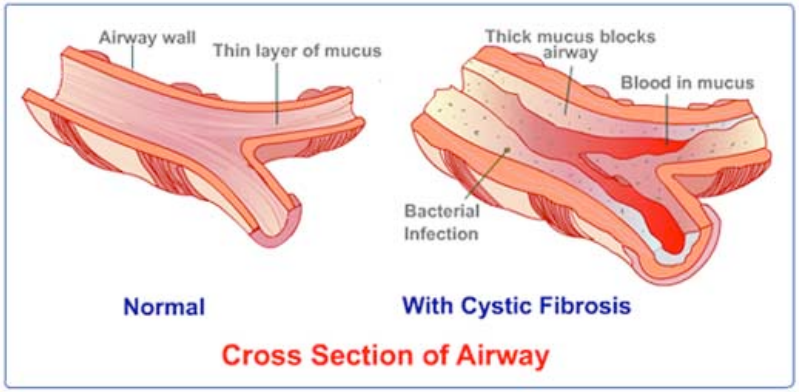
Credit: http://cfdancer.com/

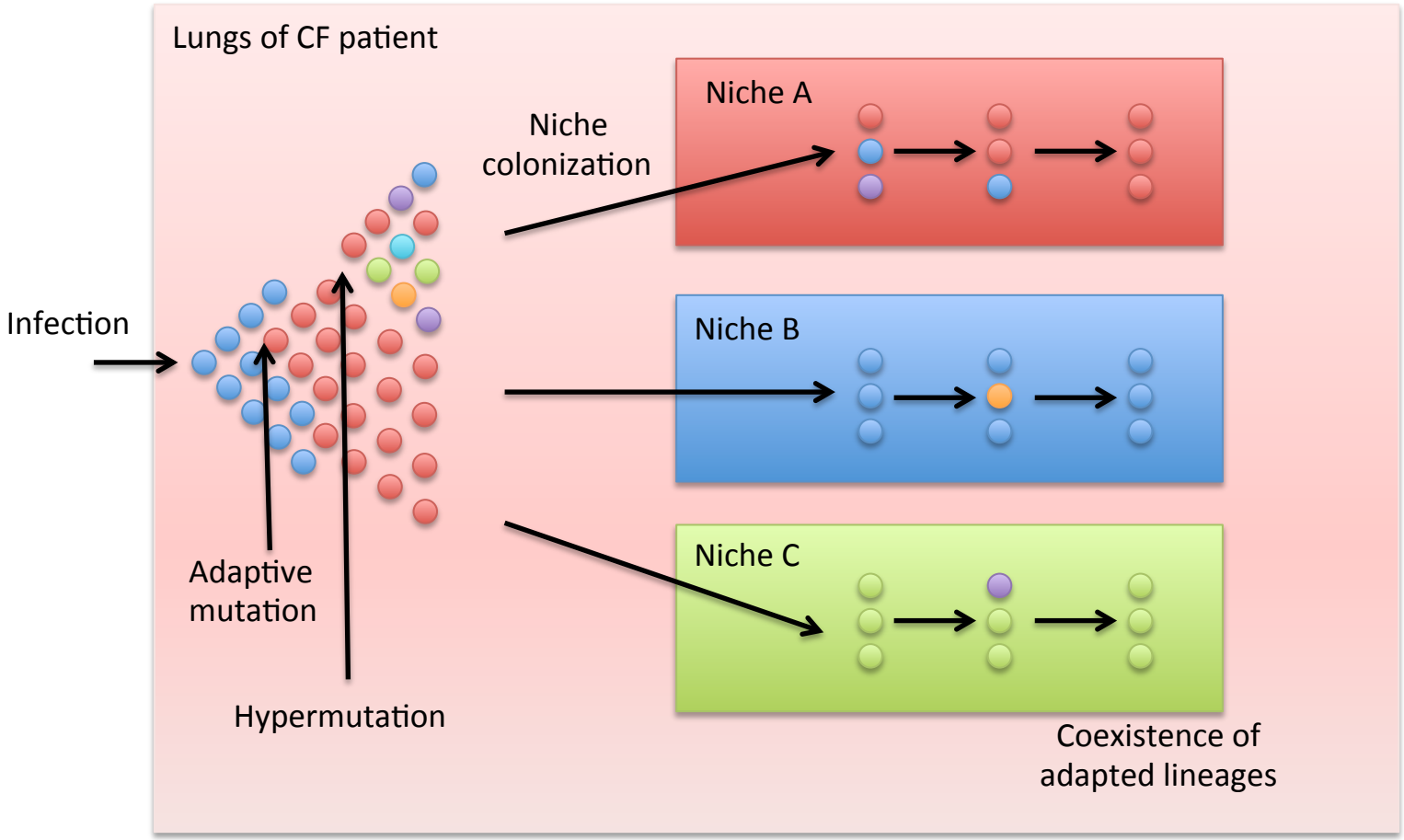
Colonization from:
soil
water
skin

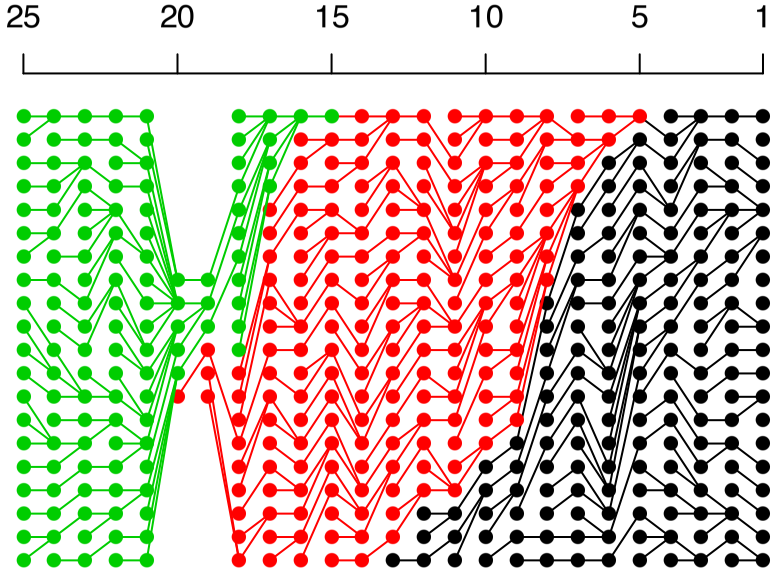


Infection
of sinuses
or lungs

Credit: http://www.e-steroid.com/wp-content/uploads/2010/05/identification-of-molecule-leading-to-destructive-lung-inflammation-300x219.jpg






25
20
15
10
5
1