Experimental evolution can unravel the complex causes of natural selection in clinical infections

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It is increasingly clear that rapid evolutionary dynamics are an important process in microbial ecology. Experimental evolution, wherein microbial evolution is observed in real-time, has revealed many instances of appreciable evolutionary change occurring on very short timescales of a few days or weeks in response to a variety of biotic and abiotic selection pressures. From clinical infections, including the chronic bacterial lung infections associated with cystic fibrosis that form a focus of my research, there is now abundant evidence suggesting that rapid evolution by infecting microbes contributes to host adaptation, treatment failure and worsening patient prognosis. However, disentangling the drivers of natural selection in complex infection environments is extremely challenging and limits our understanding of the selective pressures acting upon microbes in infections. Controlled evolution experiments can make a vital contribution to this by determining the causal links between predicted drivers of natural selection and the evolutionary responses of microbes. Integration of experimental evolution into studies of clinical infections is a key next step towards a better understanding of the causes and consequences of rapid microbial evolution in infections, and discovering how these evolutionary processes might be influenced to improve patient health.

A video of this Prize Lecture, presented at the Society for General Microbiology Annual Conference 2015, can be viewed via this link: https://www.youtube.com/watch?v=N1bodVSl27E

THE EXPERIMENTAL EVOLUTION ADVANTAGE

The large population sizes and short generation times of microbes can potentiate fast evolutionary responses to selection. This simple observation underpins the logic of microbial experimental evolution, wherein this ability to observe evolution happening in real-time has allowed evolutionary biologists to test a wide variety of the predictions of evolutionary theory (Barrick et al., 2013; Buckling et al., 2009; Elena & Lenski, 2003). Unlike observational approaches to the study of evolution, experimental evolution allows unequivocal determination of the causal relationship between the selective force applied and the evolutionary response because of the precise control over experimental conditions, treatments and the genetic composition of the starting populations (Buckling et al., 2009). Moreover, due to the ability for most microbes to be stored cryogenically, it is possible to directly compare the traits of evolved lineages to their evolutionary ancestor (or intermediates) and even to estimate the relative fitness of evolved lineages against the ancestor using head-to-head competition assays (Elena & Lenski, 2003).

Experimental evolution is commonly used to examine the tempo and mode of evolutionary adaptation to abiotic environments or stressors. Such studies typically reveal a diminishing rate of increasing fitness over time, with the rate of adaptation slowing as populations approach the optimum phenotype (Barrick et al., 2009; Buckling et al., 2009). Studies of biotic selection, and in particular biotic conflicts between bacteria and phages, reveal contrasting evolutionary dynamics (Brockhurst & Koskella, 2013; Koskella & Brockhurst, 2014). Here, recurrent coevolutionary cycles of adaptation and counter-adaptation between bacterium and phage mean that the selective force acting on each species is itself evolving, and therefore continual evolutionary change is required simply to maintain current levels of fitness – an evolutionary concept known as the Red Queen hypothesis (Brockhurst et al., 2014; Van Valen, 1973). Experiments comparing lytic phage populations evolving against either genetically fixed or evolving populations of Pseudomonas fluorescens showed that allowing coevolution more than doubled the rate of molecular evolution in the phage (Paterson et al., 2010). Similarly, P. fluorescens coevolving with lytic phage gained 10 times more non-synonymous substitutions than control populations evolving without phage and there was little overlap in the sets of mutations selected under these treatments (Scanlan et al., 2015). Indeed,
coevolving appears to limit the capacity for abiotic adaptation in both the bacterium (Scanlan et al., 2015) and phage (Zhang & Buckling, 2011).

Using carefully designed factorial experiments it is also possible to determine the relative importance of multiple sources of natural selection. For example, in P. fluorescens populations, phages reduce the evolution of diversity in bacterial colony morphology in spatially structured environments, but increase diversity in spatially unstructured environments (Brockhurst et al., 2004). Thus, the effects of biotic and abiotic selection interact: although competition in spatially structured environments selects for high diversity in the absence of phages, phage killing weakens competition and thus weakens diversifying selection (Brockhurst et al., 2004; Buckling & Rainey, 2002). Conversely, in spatially unstructured environments that would not support diversity without phages, phages select for multiple, resistant colony morphologies, thus increasing diversity (Brockhurst et al., 2004; Brockhurst, 2007). Using experimental evolution we can therefore disentangle the causes of natural selection operating in multidimensional selective environments to understand their effects on microbial evolutionary responses.

Whilst it is feasible to conduct very long-term evolution experiments with microbes lasting many thousands of generations, a great many studies report appreciable evolutionary responses to selection over much shorter timescales of tens or hundreds of generations, i.e. over durations of several days or weeks, such that ecological and evolutionary timescales can be considered convergent (Buckling et al., 2009). Remarkably, bacteria are capable of evolving new functions over a weekend, as shown in a recent study of P. fluorescens where engineered immotile strains re-evolved flagellar motility in <96 h. Starvation on solid agar imposed strong selection for motility in the immotile bacteria which responded by evolutionary rewiring of their nitrogen gene regulatory network, switching its specificity towards regulation of flagellar genes instead, thereby allowing the cells to regain the ability to build flagella and swim towards nutrients (Taylor et al., 2015). My own experimental evolution studies of bacterial populations reveal the potential for similarly rapid evolutionary gain or loss of a wide variety of ecologically important traits, including colony morphology (Brockhurst et al., 2004, 2005, 2007b; Vogwill et al., 2011), biofilm production (Brockhurst et al., 2007a), resistance to drugs (Habets & Brockhurst, 2012) or phages (Brockhurst et al., 2003, 2005), motility (Brockhurst et al., 2005; Taylor et al., 2015) and loss of social secretions such as siderophores (Brockhurst et al., 2008).

**RAPID MICROBIAL EVOLUTION IN THE CLINIC**

Many of these same bacterial traits are also known to evolve rapidly in clinical infections and this evolution may in some cases contribute to important changes in patient prognosis or the response of the infection to treatment (e.g. Fitzgerald, 2014; Folkesson et al., 2012). The evolutionary dynamics of chronic bacterial lung infections associated with cystic fibrosis (CF) have been particularly well-described (Folkesson et al., 2012). CF is a genetic disorder common among Caucasians and affects ~10 000 people in the UK (http://www.cysticfibrosis.org.uk). Patients produce thickened mucus that makes them prone to lifelong lung infections, which cause morbidity and mortality. The predominant cause of chronic lung infection in CF is the opportunistic bacterial pathogen *Pseudomonas aeruginosa* (Williams & Davies, 2012), which can be acquired from the environment although transmissible strains capable of spreading between patients have become increasingly common (Fothergill et al., 2012). Once established in the CF lung, *P. aeruginosa* can persist for decades due to the poor efficacy of antibiotic treatment against these infections, offering substantial opportunity for evolutionary adaptation (Folkesson et al., 2012).

The CF lung is likely to offer a challenging environment for colonizing *P. aeruginosa*, comprising attack by the host immune system and constant exposure to antibiotics, competition with co-colonizing microbes, infection by phages, as well as osmotic, oxidative and nitrosative stresses (Folkesson et al., 2012; Harrison, 2007). In response, *P. aeruginosa* populations rapidly adapt and diversify. Lineages accumulate mutations over time (Smith et al., 2006; Yang et al., 2011), sometimes at highly variable rates between lineages due to the frequent evolution of hypermutability in CF lung infections (Mena et al., 2008; Oliver et al., 2000). Across patients, independently evolving *P. aeruginosa* lineages appear to converge upon a suite of phenotypic adaptations to life in the CF lung (Folkesson et al., 2012; Marvig et al., 2015). These adaptations often include the evolution of mucoidy (Govan & Deretic, 1996), altered motility/attachment (Mahenthiralingam et al., 1994), gain of multiple antibiotic resistances (Lopez-Causapé et al., 2013), and altered social behaviours and secretions, including signals, siderophores and toxins (Fothergill et al., 2007; Jirincny et al., 2014). In common with many experimental evolution studies (Barrick & Lenski, 2013), evolutionary adaptation to the CF lung is often underpinned by regulatory mutations affecting the expression of (many) other genes (Folkesson et al., 2012; Hoffman et al., 2009).

Despite a signature of parallel evolution between patients, an important feature of *P. aeruginosa* populations infecting CF lungs is that they frequently exhibit exceptionally high levels of intra-host diversity and rapid turnover (Ashish et al., 2013; Fothergill et al., 2010; Mowat et al., 2011; Workentine et al., 2013). Whilst diversity at some traits may be relatively neutral with respect to patient health, for other traits the observed diversity is likely to have important clinical consequences. For example, high within-population diversity in antibiotic resistance can make it hard to diagnose which drug to prescribe (Mowat et al., 2011; Workentine et al., 2013), whereas changes over time in the frequency of overproducers of the toxin pyocyanin correlate with exacerbations in patient symptoms (Mowat et al., 2011). Taking a population-level perspective has also revealed the coexistence of highly genetically diverged lineages within
CF patients and sharing of lineages among multiple patients, indicating ongoing patient-to-patient transmission within the cohort of patients attending the clinic (Williams et al., 2015).

UNRAVELLING COMPLEX CLINICAL SELECTIVE ENVIRONMENTS

Whilst we have a good understanding of how *P. aeruginosa* populations adapt to life in the CF lung, it is very challenging to link these evolutionary responses to particular selective causes. Causal arguments made in the literature linking, for example, loss of motility structures to immune selection, although interesting, are mere speculation: whilst it is possible that host immunity selects against motility structures, it is equally possible that motility is simply not useful in this environment, or that phages may select for motility loss, or that all of these factors play a role. This gap in knowledge limits the scope for designing treatment interventions that alter the selective environment in infections to prevent (or promote) particular trajectories of pathogen evolution (e.g. to limit the evolution of drug resistance). How then can we begin to unravel this multidimensional selective environment to understand why bacterial populations evolve as they do in clinical infections? One answer is to use experimental evolution to empirically test the evolutionary hypotheses that emerge from analyses of clinical data, allowing predicted causal links between evolutionary responses and selective causes to be validated. Integrating experimental evolution into clinical investigations would ideally become an iterative process whereby the findings from each approach would inspire further investigation (Fig. 1).

Exciting advances in developing more realistic laboratory models of CF infection, such as *in vitro* artificial sputum media (ASM) mimicking lung sputum (Sriramulu, 2013), *ex vivo* lung tissue (Harrison et al., 2014) and *in vivo* mouse chronic lung infection (Fothergill et al., 2014), are allowing clinically relevant experimental evolution studies to be designed and performed. Although most existing studies have been of relatively short duration, they suggest enormous potential for experimental evolution to be a valuable tool in advancing our understanding of the selective drivers of real-time evolution in infections. For example, experimental evolution of *P. aeruginosa* in ASM...
recapitulates aspects of bacterial adaptation to the CF lung, including mutations associated with living in a biofilm (Wong et al., 2012). Exposure to antibiotics in ASM reveals resistance evolution via stereotypical genetic mechanisms, compensatory evolution to reduce costs of resistance (Wong et al., 2012) and the evolution of greater phenotypic diversity in response to subinhibitory doses of some clinically relevant drugs (Wright et al., 2013). Evolution experiments in an in vivo mouse model of chronic lung infection demonstrate that P. aeruginosa establishment in the upper respiratory tract is necessary for invasion of the lung (Fothergill et al., 2014), as hypothesized in CF (Hansen et al., 2012). Evolved bacteria are better able to establish lung infections than the ancestor, suggesting evolutionary adaptation to the host environment (Fothergill et al., 2014). Mutations detected in multiple evolved clones are likely to have caused enhanced resistance to host immune peptides (Fothergill et al., 2014) and similar mutations have been observed in P. aeruginosa isolated from human CF lung infections (Moskowitz et al., 2012).

CONCLUDING REMARKS

Current data on rapid microbial evolution in infections is largely descriptive, providing understanding of what evolutionary changes occur, but little insight into why they occur. Better understanding of the causal link between microbial evolutionary responses and the selective causes can be gained through carefully designed experimental evolution studies, which allow the multidimensional clinical selective environment to be unravelled. This is a vital step towards development of new treatment strategies that manipulate the in vivo selective environment to reduce selection for undesirable microbial traits, such as drug resistance, and thereby alter pathogen evolutionary trajectories towards better outcomes for patient health. Achieving this will require cross-disciplinary dialogue and multidisciplinary collaboration between evolutionary biologists, microbiologists, sociologists and clinicians.

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