EVOLUTIONARY BIOLOGY AS A TOOL TO COMBAT ANTIMICROBIAL RESISTANCE

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Fundamental scientific investigations into how bacteria grow, and how they adapt to the development of resistance could have far reaching, translational applications in our attempts to combat antimicrobial resistance. Acquired resistance is usually the result of a mutation in the bacterial genome or the acquisition of DNA containing resistance genes from outside the cell; both of which can affect the fitness of the now resistant bacteria. Here we outline how this knowledge, and that of the related phenomena of epistasis and collateral sensitivity, can be used to preserve the efficacy of existing antibiotics by optimising treatment regimens and stewardship programmes to prevent the emergence and persistence of resistance within bacteria populations.

Why study fundamental cellular and evolutionary processes?

Basic science has a lot to offer in terms of combating AMR and as we scramble to come up with new and inventive solutions and fight for the limited funding available to implement them, it is worth carefully analysing the therapeutic possibilities, and opportunities, presented by increased understanding of the biology of the microbial pathogens themselves. Investigations into the fundamental nature of bacterial growth and evolution are central to our understanding of AMR mechanisms at the molecular level. This understanding is also central to drug design and target identification. There has been, excitingly, an increasing awareness over the last few years that knowledge of evolutionary relationships between resistance acquisition, and how "fit" resistant bacteria are, can be utilized in rationally designed antimicrobial stewardship programmes and treatment options which we will explore below.

Emergence of resistance; mutation and acquisition

Bacteria are remarkably adaptive, which is why they are so successful and have colonised every conceivable environment on earth. It is this adaptive nature that has resulted in bacteria being extremely proficient at evolving mechanisms of resistance to every antibiotic we have ever found, developed or invented.

The adaptation and subsequent resistance occurs at the DNA level within the bacterial cell and is selected for by the enormous quantities of antibiotics used annually for medical, veterinary and agricultural use. Rapid adaptation to stress, such as the emergence of resistance to an antibiotic, is a result of short generation times and two fundamental properties of DNA; mutation and horizontal gene transfer (HGT).

Mutations occur when mistakes are made during the replication of the DNA molecule. Most of these errors are corrected by the cellular replication machinery, but some are not. Of these, most will either not affect the survivability of the cell or will be detrimental, therefore the cell and its descendants will be uncompetitive and its lineage will die out. There are times, however, where a single base-pair mutation in the DNA leads to an amino acid difference in the protein product of the gene which gives that cell an advantage as that protein (or sometimes the RNA) may no longer be a suitable target for a specific antibiotic. An example is a mutation in the dihydrofolate reductase (DHFR) gene in *Staphylococcus aureus* which confers trimethoprim resistance. The DHFR protein plays an essential role in DNA synthesis, however, if the trimethoprim antibiotic molecule is bound to it, DHFR will no longer work and the cell will be unable to produce DNA and will therefore be unable to grow. The mutation in this gene changes a single amino acid in the DHFR protein which means a hydrogen bond which normally locks the DHFR and trimethoprim molecules together will not form, so the antibiotic can no longer bind to its target, resulting in resistance to trimethoprim *(1)*. Similarly, a mutation in a regulatory region of DNA such as a promoter, which drives gene expression, can alter the cellular biology enough to resist antibiotics. A good

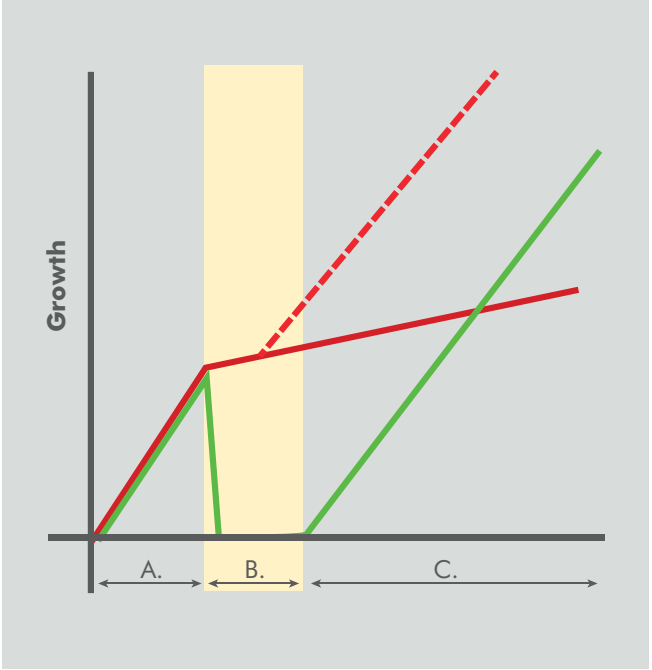
example of this is a single base-pair mutation in the promoter of the *ampC* gene in *Escherichia coli*, which confers resistance to a range of β-lactams, including ampicillin and penicillin. One base-pair change can result in a six-fold increase in expression because the mutation makes it more efficient *(2)*.

Horizontal gene transfer is the second major mechanism of adaptation to antibiotics and is the process whereby bacteria can acquire genes, by one or more of three main mechanisms. These processes are the acquisition of free DNA from their environment, usually originating from dead cells (a process known as transformation), being the recipient in a DNA transfer process directly from a live donor cell (called conjugation), or being infected with a bacterial virus (a bacteriophage) containing its previous host's DNA (a process known as transduction). Each of these, not mutually exclusive, mechanisms of HGT enable bacteria to acquire large sections of DNA containing many genes, often on discrete sections of DNA capable of catalysing their own movement and called mobile genetic elements (e.g., plasmids and transposons). As large regions of DNA containing many genes can be acquired in a single event, HGT can lead to the acquisition of more complex resistance genotypes which require multiple proteins to work such as the eight membered *vanG* gene cluster conferring vancomycin resistance in *Enterococcus* species *(3)*.

Fitness, compensatory mutation and collateral sensitivity

The ability of a bacterium to grow in any environment is referred to as its fitness. Fit bacteria grow well and replicate faster relative to unfit bacteria. When a bacterium becomes resistant to an antibiotic by one or more of the above mechanisms there is usually a fitness cost (also known as a biological cost). This refers to the phenomenon where the bacterium in which the mutation has happened, or which has acquired DNA from an exogenous source, is no longer as fit as it was before the mutation, compared to the ancestral, precursor strain (Figure 1, A). This can be measured by comparing their growth rates in the laboratory. The reasons for these fitness costs vary and may be due to, for example, the bacterial protein responsible for resistance being slightly changed and no longer working as efficiently as it did before, or newly produced, or differentially expressed, proteins being metabolically expensive to produce and/or interacting negatively with other cellular proteins or processes. In the presence of a selection pressure as strong as antibiotics this biological cost is not significant as without the resistance mechanism the cells do not grow or they die (Figure 1, B). However, in the absence of antibiotics, for example when treatment finishes, the impact of fitness on bacteria is fundamental to its survival and persistence within an environment because without the selective pressure of antibiotics, unfit resistant strains will be outcompeted by sensitive, more fit bacterial strains (Figure 1, C).

Figure 1: The growth of two identical bacterial populations are represented by the red and green lines. A: In the absence of antibiotic selection both populations display identical growth. B: Under the selective pressure of antibiotic (shaded region) the "red" bacterial population develop resistance quickly, which also has a fitness cost, indicated by a lower rate of growth. The susceptible green population are rapidly killed. The red dotted line represents a sub-population of the red population which, having undergone compensatory mutations expands rapidly. C: After removal of the antibiotic selective pressure, as would happen once therapy has finished, any remaining susceptible green population rapidly expands and soon exceeds that of the less-fit resistant red population. Note the population which have undergone compensatory mutations are now resistant and able to compete with the susceptible green population as they are of similar fitness. This means that this resistant population will be very difficult to displace



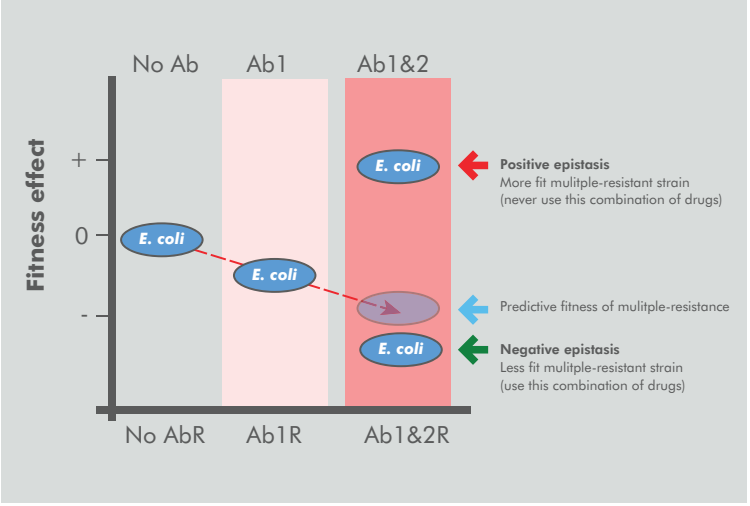
Examples of fitness costs associated with antibiotic resistance acquisition, either by mutation or HGT, are many and include the varied relative change in fitness of *Enterococcus faecium* following acquisition of one of several different plasmids conferring vancomycin resistance compared to the ancestral, plasmid-free strain. The fitness costs determined in these experiments ranged from a fitness cost of 27% to an actual fitness benefit of 10% (meaning the strain with the plasmid grew 10% faster than the ancestral strain) depending on the plasmid that was acquired *(4)*. Fitness costs also arise following mutation, for example mutations resulting in the overexpression of efflux pumps in antibiotic resistant *Pseudomonas aeruginosa (5)*.

Bacteria can often overcome the fitness cost of resistance development by a process known as compensatory mutation. This happens when one or more, often unrelated, mutations occur within the bacterial genome which restores fitness to the cell following acquisition of resistance by mutation or HGT. A globally important and clinically relevant example of this is compensation for the costs associated with rifampicin

resistance in *Mycobacterium tuberculosis*. Following mutations in the gene encoding the RNA polymerase that lead to rifampicin resistance, further mutations elsewhere within the genome have the effect of bringing to the fitness of the rifampicin-resistant strain back up to the levels of the ancestral strain (6). There are also specific instances where compensatory mutations result in a resistant strain which is more fit than the ancestral strain, for example, following acquisition of vancomycin resistance encoding plasmids in *Enterococcus faecium (4)*.

The acquisition of resistance, and indeed these compensatory mutations which can follow, can lead to another phenomenon known as collateral sensitivity. Collateral sensitivity can be defined as a change in susceptibility to one antibiotic upon becoming resistant to another. Collateral sensitivity is a translational phenomenon in that it could be used to design rationale combinatorial therapy where the emergence of resistance to one antibiotic will sensitise the cell to the other, leading to less chance of multiple-resistant strains emerging. An interesting example of collateral sensitivity networks being used to recommend combinatorial therapy is demonstrated with the experimentally determined synergy of a meropenem-piperacillin-tazobactam combination which supresses the evolution of resistance during the treatment of MRSA *(7)*.

Figure 2: Representation of epistasis with a susceptible *Escherichia coli* (No AbR) under no antibiotic selective pressure (No Ab) with a fitness starting point at zero. When the *E. coli* has evolved resistance (Ab1R) to the first antibiotic (Ab1) there is a fitness cost of minus one. This is predicted to change to minus two when resistance to the second antibiotic (Ab2) develops (Ab1&2R). However, sometimes the actual fitness cost is more (negative epistasis) or less than predicted (positive epistasis).



Epistasis and the management of AMR

Another layer of complexity which is being increasingly investigated with respect to AMR is the relationship between mutation or acquisition of resistance, and the genetic background of the host cell. These interactions are known as epistasis and occur when the same mutation, which is responsible for resistance, can have different effects on the fitness of the host cell depending on previous mutations and other differences in the genome (reviewed in *(8)*).

The hypothesis of translatable epistatic control of resistance is that if resistance emerges, or is acquired, by a cell which already has a pre-existing resistance genotype the effect on fitness of the second resistance may be different than if it would have emerged or been acquired in a susceptible cell (Figure 2). This has implications for the choice of antibiotics clinicians use as first, second and even third-line therapy. If resistance is taken as an inevitable consequence of treatment then we should aim for maximising the fitness cost of these resistances to the pathogens.

Predictable epistatic interactions give us an intriguing possibility to force pathogens down an evolutionary route which will maximise the fitness costs associated with multiple antibiotic resistances. If combinations and/or the order in which antibiotics are used give rise to multiple resistance phenotypes which have a greater than predicted fitness cost (negative epistasis) then it is possible that the use of these combinations in the clinic would prevent the emergence of fit multiple-resistant strains. Likewise, if combinations and/or the order of antibiotics is found to lead to multiple resistance phenotypes with less of a predicted fitness cost than the sum of the individual fitness costs then these combinations should not be used in the clinic as they may promote the emergence of fit multiple-resistant strains. Examples of both types of interactions have been previously reported in a wide range of different bacteria demonstrating that this is a common evolutionary phenomenon *(8)*. If a pathogen emerges with multiple resistances and is fitter than the ancestral strains from which it derived there is very little chance of it disappearing from the environment following the removal of the selective pressures of antibiotics. This problem is exacerbated in LMICs where there is less choice of available antibiotics and the access to and quality of antibiotics are less stringently controlled.

Conclusions

Understanding the evolutionary trajectories of AMR in clinically relevant bacteria will present us with a unique opportunity to be able to tailor antibiotic therapy to bacterial isolates with certain resistant profiles. The strategy of harnessing natural selection to suit our clinical requirements has the potential to prevent the emergence of resistant lineages in the population by specifically selecting for fitter, antibiotic-sensitive ones. This will extend the useful lifetime of antibiotics, both old and

new, concomitantly increasing the window of opportunity to discover new antibiotics and therapies. ■

Dr Alasdair Hubbard graduated with a BSc (Hons) in Microbiology from the University of Leeds in 2007 and a PhD from St George's, University of London in 2015. Post PhD, he worked as a Post-Doctoral Scientist at a molecular diagnostic company, Micropathology Ltd, and as a Research Assistant at the University of Oxford. He is currently a Post-Doctoral Research Associate at the Liverpool School of Tropical Medicine with research interests based around AMR, including the evolution of resistance in clinically relevant bacteria, the emergence and spread of AMR in different environments and identifying novel antimicrobials with activity to clinically relevant bacteria.

Dr Adam P Roberts has a BSc (Hons) in Applied Biology from Coventry University (1995) and a PhD from the University of London (2002). He worked as a Post-Doctoral Research Fellow, Lecturer and Senior Lecturer at University College London until joining the Liverpool School of Tropical Medicine as head of AMR research in 2017. His research focuses on the molecular biology of transferable antibiotic resistance in a variety of bacteria and the use of evolutionary biology to combat it. He also leads a citizen science drug discovery programme called Swab and Send; founded and runs the International Transposon Registry; and is an adviser to the Longitude Prize.

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