



FUNGI at the frontline

BY **DAVE SAMMUT** AND **CHANTELLE CRAIG**

The arsenal we have against bacterial outbreaks is dwindling, but there are opportunities to develop new defences.

World Antibiotic Awareness Week came and went in November 2019, with barely a mention outside medical media to remind us of the importance of these drugs in the fight against bacteria. Just five weeks later, a pneumonia of unknown cause was reported in Wuhan, China. Now, the world has no choice but to pay attention to another type of microbe: coronavirus. COVID-19 is a viral rather than a bacterial disease, but the damage it does to the lining of the lungs can make them more susceptible to invasion by bacteria and thus to secondary

infections. In fighting bacteria – sometimes indiscriminately – we are seeing greater resistance to antibiotics as time goes on.

The first commercial* antibiotic chemotherapeutic drug was developed at the Bayer Laboratories of I.G. Farbenindustrie in Germany. Prontosil (1932) was a sulfonamide used with broad effect against certain gram-positive cocci. German chemist and pathologist Gerhard Domagk received the 1939 Nobel Prize in Physiology or Medicine for its development as part of his research into the antimicrobial activity of azo dyes.

The first antibiotic resistance was identified in *Staphylococcus* in 1940. In 1945, Alexander Fleming warned that ‘The overuse of antibiotics clearly drives the evolution of resistance’. Since then, resistance has been observed for nearly all antibiotic drugs that have been developed.

The 1960s through to the 1980s were the halcyon days for new antibiotic discovery. Most of the antibiotics in clinical use today derived from natural defence chemicals (natural products) produced in a laboratory setting by bacteria and fungi to defend against attack from other microbes. These substances

*Alexander Fleming discovered penicillin in 1928, but it wasn’t until after Howard Florey and Ernst Chain developed penicillin for use as medicine that the first patient was successfully treated in 1942. The prominence of penicillin as ‘the first antibiotic’ is a combination of its subsequent extensive use and the geopolitics of the era, but the first synthetic antibiotic actually used for widespread clinical purposes was prontosil.

were then replicated and often modified to amplify their antimicrobial activity.

By late last century, the low hanging fruit of readily accessible microbial natural products with antibiotic potential had been deemed exhausted, prompting the pharma industry to look elsewhere for inspiration. Some 20 or so years on, and confronted with a severely depleted antibiotic drug discovery pipeline, and increasing levels of antibiotic resistance, the need to be inspired is unrequited and more urgent than ever.

In their article 'Seven ways to preserve the miracle of antibiotics' (doi.org/10.1093/cid/cit070), John G. Bartlett and colleagues at Johns Hopkins University School of Medicine described this stagnation bluntly:

Pharmaceutical development that previously kept us ahead of resistance is now stalled due to economic and regulatory barriers. Fifteen of 18 large pharmaceutical companies have totally left the antibiotic field, and there has been no new class of antibiotics for gram-negative bacilli in 4 decades; only 2 drugs with new microbial targets (linezolid and daptomycin) have been introduced since 1998. The pipeline is sparse, the problem is global, and the prognosis is poor.

The impact of this sparse pipeline is equally sobering. In their Action Plan on Antimicrobial Resistance 2016–2020, the UN Food and Agriculture Organization stated:

In humans, [anti-microbial resistance, which includes bacteria, fungi, viruses and parasites] also threatens to undo decades of improvements in human health care outcomes, with direct impacts on the ability of people to live full and productive lives.

Up to 99% of bacteria can't be readily cultured in the lab, which severely limits our ability to observe the chemicals they produce under attack. Although there have been minor breakthroughs in technologies

to help culture recalcitrant bacteria, and although researchers have cast a wider net to samples from marine bacteria, tropical rainforests and other frontiers, the basic numerical problem remains. And while some pharmaceutical companies are going back over old screening with newer, more sensitive techniques, the return on investment for this is dropping markedly.

Given these problems, new drug discovery this century has tended to move away from 'natural products' research, and more towards genomics, proteomics and other 'omics' research. These studies have looked more closely at alternative mechanisms for inhibiting infection (see box, p. 27), and new approaches to activate 'silent genes' that code for antimicrobial weapons.

In recent years, microbial genomes have been shown to be rich in genes that code for many more defence molecules (potential antibiotics) than detected in lab cultures. These genes are under tight regulatory control, and are only activated in response to unknown environmental cues. But how to access this untapped molecular reservoir?

Enter Professor Rob Capon, RACI Fellow and a group leader at the University of Queensland, Institute for Molecular Bioscience, who describes the silent capacity of genes as the holy grail of antimicrobial research.

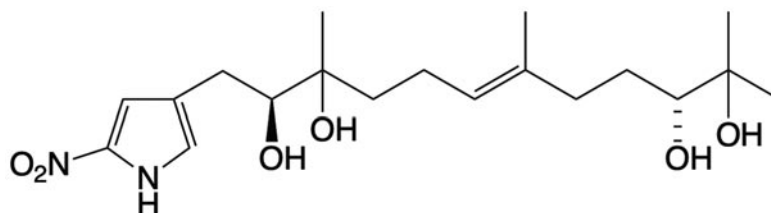
While pursuing basic research into bacteria and fungi isolated from beach sand on Queensland's Heron Island, Capon and his team identified complex inter-kingdom chemical communication that he believes sheds light on a future path to antibiotic discovery.

Natural products chemists change the media for a bacterial culture to challenge the bacteria. Or, if they are being more elegant, they use a 24-well plate and rapidly challenge the organism through different culture media. But this doesn't necessarily produce the chemical cue(s) to activate the genes.

By contrast, Capon said that the 'gene jockeys' chop the gene into bits and try to express these in another organism. But this is time consuming, with accessibility issues and high costs.

Starting with a beach sand-derived bacteria, a *Streptomyces* species, Capon and his team identified a new class of natural products featuring an unprecedented 2-nitropyrrole, which they named the heronapyrroles. The heronapyrroles exhibited promising antibacterial activity against pathogenic gram-positive bacteria, which was interesting, but not patentable, despite similarity to synthetic nitroimidazoles with anti-tuberculosis properties. It's at this point that the story takes an unexpected turn.

In an effort to study heronapyrrole antibiotic properties, Capon attempted a simple experiment. He added a synthetic nitric oxide synthase inhibitor (aminoguanidine) to the live *Streptomyces* culture, with the aim of inhibiting the biosynthesis of the nitro functionality. What was to be a quick 2–3-week study led to a more than five-year adventure, which involved synthetic chemistry, genomics and transcriptomics, and imagination, to decode an intricate cycle of attack and counter-attack between two warring microbes.



Heronapyrrole B is showing promise as an antifungal agent. Zeinab Khalil

There's a lot of technical detail from here, so we'll jump to the big discovery. Capon and his team found a chemical conversation going on between two species in the beach sand: the *Streptomyces* bacteria and a fungus. Each tries to outcompete the other.

The Heron Island *Streptomyces* only produced the heronapyrroles when in co-culture with a fungus co-isolated from the same sample of beach sand. The researchers found that an antibacterial chemical defence (diketopiperazine) produced by this fungus stimulated the *Streptomyces* to produce nitric oxide, which in turn proved to be the secret regulator of the silent genes responsible for producing heronapyrroles. The heronapyrroles were in turn uniquely antifungal. Remarkably, this battle had a twist. When confronted by antifungal heronapyrroles, the fungus responded by diverting all its energy into producing and secreting more diketopiperazine, only to further antagonise the *Streptomyces* into making more heronapyrroles.

Eventually the fungus succumbed, and as its cell count slipped below the level needed to stimulate the *Streptomyces*, a very lopsided armistice ensued.

This is a cycle of attack, defence and counter-attack. And now, knowing what to look for, the researchers have found the same behaviour in other organisms.

But how does this help guide the future discovery of new antibiotics? Capon reasoned that, because it was too difficult to find and identify all the secret chemical cues that regulate so many of the silent genes hidden in so many microbial genomes, why not bypass the cues altogether, and just add nitric oxide to *Streptomyces* cultures?

In a process Capon and his team refer to as nitric oxide mediated transcriptional activation, or NOMETA, they add a small amount of the vintage angina medication sodium nitroprusside to the cultures of both bacteria and fungi. Sodium nitroprusside slowly breaks down to release nitric oxide and, presto, many cultures respond by producing new

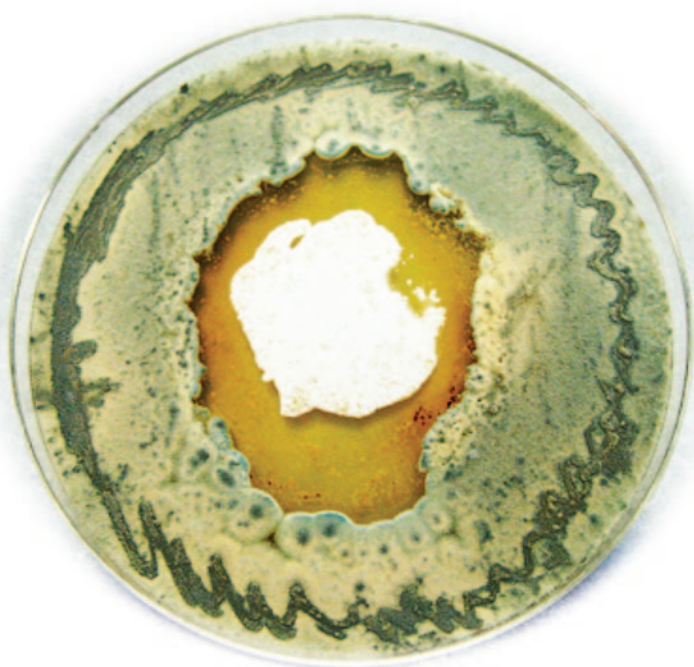
defensive chemicals.

'The reason we've missed these genes in the past is that we've been growing our microbes in monoculture – there's no threats, there's lots of food and no competition, so a bacterium won't turn on its armoury because it doesn't feel threatened,' Capon said.

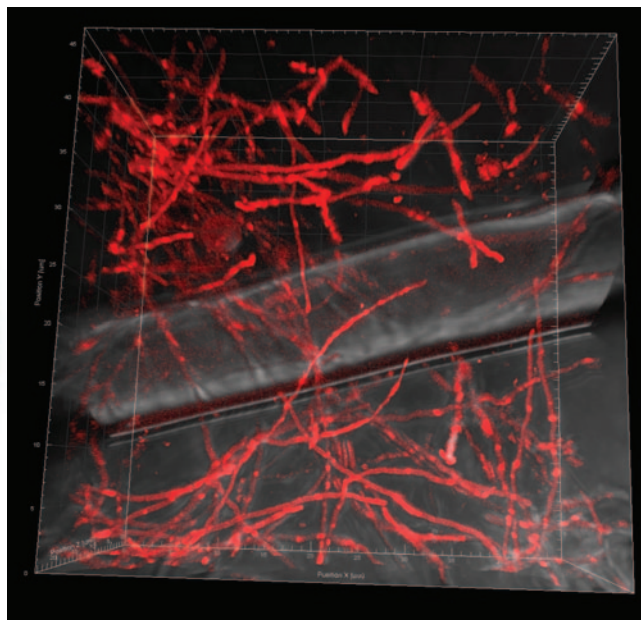
Capon reflected that 'Nature's use of toxic gas in an inter-kingdom beach warfare between bacteria and fungi was a revelation to us. We can only hope that our discovery provides an opening salvo in another war – the one being waged against infectious pathogens that are growing ever more resistant to our current arsenal of vintage antibiotics.'

This is a hope that we all share. Because this is a war that will never end – microbes evolve too swiftly. All we can do is seek to slow the battles until the political will builds to improve our currently poor antibiotic practices.

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A co-cultivation agar plate of *Streptomyces* sp. (bacteria), shown in the centre, and *Aspergillus* sp. (fungi), shown on the outside.



In this image of a mixed culture of fungus and bacteria, the fungus (the grey tube-like structure) has produced a defensive antibacterial, to which the bacteria responds with nitric oxide (and glows red due to an NO detection agent), stimulating the release of a powerful antifungal that defeats the fungus. Dr Nicholas Condon

The antibiotic arms race

According to Jeff Goldblum's character, Dr Ian Malcolm, in *Jurassic Park* – a film (in)famous for its scientific accuracy – 'Life, uh, finds a way'. When it comes to microbes, they find *lots* of ways.

'Resistance' denotes the ability of a bacterium to survive a specific antibiotic treatment. Some bacteria are naturally resistant. Resistance can occur through spontaneous chromosomal mutation, but it can also be transmitted extra-chromosomally, such as when bacteria exchange plasmids or transposons (horizontal gene transfer).

The 'golden age' natural product antibiotics (1940–70) target structures unique to bacteria and not present in mammals (to minimise side effects) – to destroy the bacterial wall, cell membrane or crucial bacterial enzymes involved in nucleic acid and protein synthesis. Basically, the idea was to disrupt essential bacterial processes, ultimately killing the cell.

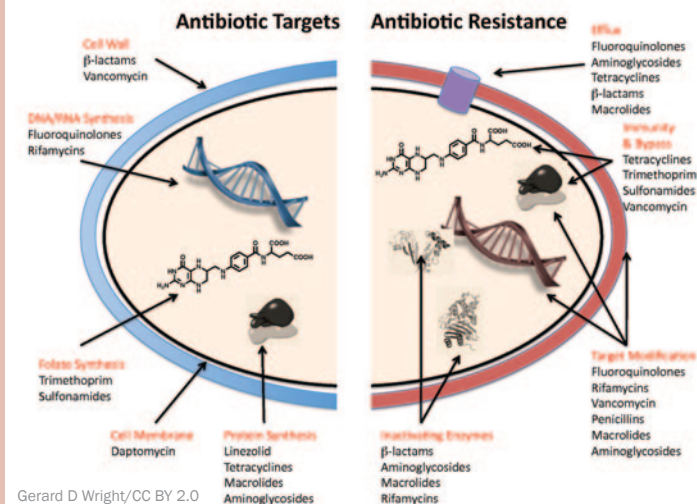
Antibiotic treatment creates substantial adaptation pressure. By removing drug-sensitive competitors, the field is left wide open to the survivors – typically those with greater capacity to produce genetic variability.

Common mechanisms for antibiotic resistance evolved to include the appearance of efflux pumps (which remove antibiotics from the bacterial cell), changes to membrane permeability (keeping the antibiotic out), changes to the bacterial target site(s), or modification or deactivation of the antibiotic itself.

Subsequent generations of antibiotics mostly tried to improve the efficiency of attack on these same target sites, meaning that the resistant bacteria were already well primed to adapt. Researchers then adapted their approach to look for novel, unexploited targets, to seek a molecule that is effective against the target and safe to use.

New drugs have also been created through bioinformatics and rational design. Using detailed knowledge and libraries of how variations to a molecule's structure affect its behaviour, activity and performance (bioinformatics), virtual models can be created to design molecules for specific functionality. For example, a new drug was designed in 2017 to fight resistant bacterial strains by combining three antimicrobial mechanisms: increased potency, increased cell wall permeability and inhibition of cell wall biosynthesis.

Alternatively, targets are now being sought that take advantage of processes that are not critical to bacterial



... virtual models can be created to design molecules for specific functionality.

survival. By leaving the bacteria alive, but 'declawed', this decreases the evolutionary pressure for adaptation, and leaves more competition in place for the remaining harmful organisms.

It would take another article to discuss the chemical mechanisms that bacteria used to adhere to, invade and recode host cells to their purposes; or the chemical communication between bacteria known as 'quorum sensing' (by which bacteria can communicate their numbers to hold off an attack until critical mass has been reached, to avoid triggering an immune response until it is too late); or the formation of defensive structures such as biofilms, which allow individual bacteria to act in concert as multicellular organisms. Suffice to say, bacteria are clever and inventive.

That necessarily means that the design of our response gets equally complicated, equally quickly. We might focus on disarming the pathogens, blocking parthenogenesis, neutralising toxins or interfering with host-pathogen interactions at the protein level. We might even boost the fight by enhancing the host immune system.