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Nature knows best: employing whole microbial strategies to tackle antibiotic resistant pathogens

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The 2010s have seen a full recognition of the scourge of antimicrobial resistance (AMR) where antibiotic-resistance genes, many transferred from environmental bacteria, spread rapidly through hospital and farm populations of pathogens when selected for by applied antibiotics. Many of these resistance traits allow survival of bacteria in the very soil environments where other organisms naturally produce antibiotics. In a hospital or farm setting, they nullify the therapeutic effects of prescribed antibiotics.

Along with this realization has come a return to natural environments to seek different new solutions. Although these settings are both the source of genes encoding the antibiotics we have relied upon and of the resistance genes that circumvent individual antibiotic agents or their actions, Nature offers much more than just that. Diverse molecular tactics are employed, between different micro-organisms, in natural environments, in a dazzling array of changing combinations to gain advantage in conflicts for territory and food. The war strategist in these processes is evolution and the armies and vigilantes are bacteria, bacteriophage, protozoa and fungi.

The iChip, a novel method for growing previously-unculturable bacteria, in their natural environment, developed by Kim Lewis and colleagues (Nichols *et al.*, 2010) has been used to access untapped environmental bacteria for potential antibiotics (Ling *et al.*, 2015). Indeed this may lead to the next generation of antibiotics that can be downstream processed into the pharmaceuticals of tomorrow, and combined with better stewardship may cause AMR to recede for future decades. In these new antibiotic discovery approaches, we humans pick up the individual weapons of micro-organisms, from their battlefields, and fire them ourselves, against pathogens. Certainly this will rearm humans to fight infection using the principles first applied by Fleming, Florey and Chain.

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Armed with advances in microscopy, 3rd generation sequencing technologies and greater understanding of the microbiota of different environments, we are now able to ask, more holistically, how do whole micro-organisms kill each other? From this fundamental knowledge we will be able to employ them whole in the fight against AMR infection. The advantage of this is that multiple weapons may be used in a regulated way, by whole micro-organisms, against their foes. This more complex approach may be hard to resist by single gene mutations in the pathogens. Elegant microscopic studies by Melanie Blokesch and others (Borgeaud *et al.*, 2015) studying the Type VI secretion apparatus have shown how environmental bacteria, such as *Vibrio cholerae*, use that system to stab and poison adjacent bacteria in a contact dependent manner wherein the killer senses the 'prey'.

The Type VI secretion apparatus shares conserved structures with the injective machinery of the larger bacteriophage viruses of bacteria. Using phage allows a multi-enzyme approach to bacterial killing but because a single receptor is often the portal of phage attack, selection for phage-resistant mutants is possible. On the plus side, given the enormous number and diversity of phage on earth, cocktails of assorted phage can be useful to delay the effect of such single genetic events (Merabishvili *et al.*, 2009).

Another, but very different, whole organism approach to bacterial killing, comes in the shape of predatory bacteria, including *Bdellovibrio bacteriovorus*. This invasive predatory bacterium enters and kills a wide range of Gram-negative bacteria and was, (akin to the discovery of penicillin by Alexander Fleming), isolated in an environmentally 'infiltrated' experiment that went unexpectedly. Stolp and Petzold isolated such predatory *Bdellovibrio* from plaques on a bacterial lawn in an experiment where they were studying phage. In fact, miniature predatory soil bacteria, too small to cause opacity were also able to invade and kill the larger bacteria on the lawn and produce plaques. (Stolp and Petzold, 1962; Stolp and Starr, 1963).

Bacterial killing, by their predator 'cousins', does not rely on receptor binding, and so does not select for simple prey-resistance; but involves outer-membrane contacts and Type IV pilus activity for prey-invasion.

After this comes peptidoglycan modification, allowing colonization of the prey periplasm; death of the invaded bacterium and its consumption by waves of secreted predator products. Recently, transcriptomics and transposon mutagenesis studies of predatory bacteria have shown the arsenal of some 200 bacterially destructive enzymes that predators employ to kill prey and transporters involved in their secretion or prey-derived nutrient uptake (Medina et al., 2008; Tudor *et al*. 2008; Lambert *et al*., 2010). This sometimes makes the *Bdellovibrio* scientist think that using a single 'molecular weapon' to combat pathogens might seem like a single 'pop-gun' compared to the '1812 Overture' crescendo of multiple enzymes that living predators employ to dismember their prey! As a study in 2011 showed that *Bdellovibrio* are safe orally ingested by animals, the way was open for more work using whole live predatory bacteria as anti-bacterials (Atterbury *et al*., 2011). Also the observation by Iebba and colleagues (2013) that children have DNA indicative of predatory bacteria in their gut microbiota suggested that live predators, possibly ingested accidentally in 'a peck of muck', (soil), during play in the outdoor environment, may be not harmful in the normal microbiota.

Encouraged by a US Government Defence Advanced Research Projects Agency (DARPA) 'Pathogen Predators Research Programme', the vision of Dr. Barry Pallotta and colleagues, researchers have taken, and published, the next steps with therapeutic tests, using whole live predatory bacteria. Shatzkes and colleagues have shown that repeatedly-applied aerosols of predatory *Bdellovibrio* restrict the bacterial burden of *Klebsiella pneumoniae* in rat lungs (Shatzkes *et al*., 2016). Willis and colleagues (2016) showed that injected *Bdellovibrio* kill injected *Shigella* pathogens in the hindbrain of zebrafish embryos and that this treatment significantly improves the survival of the zebrafish from c30% to 67% at 72 h post infection. Moreover, the innate immune system of the zebrafish synergises with the action of the predators in clearing the pathogen (Willis *et al*., 2016).

Again this observation may relate back to complex actions of protozoa and predatory bacteria in soil environment. On the early earth, before evolution of higher eukaryotes, simpler mechanisms of such eukaryotic–prokaryotic cooperativity in soil may have contributed to the evolution of the white blood cell (WBC)-predator interactions seen in the zebrafish treatment experiments. Although, as the zebrafish studies show, the immune system will eventually clear live therapeutic predatory bacteria, they may dwell in the bodily environment long enough to be beneficial as an applied emergency dose to an infected wound or surgery site (Willis *et al*., 2016). Also the work of several laboratories shows that experimental injection/ingestion/application of *Bdellovibrio* alone to live animals or tissues, while causing some inflammation, are not pathogenic (Atterbury *et al*., 2011; Shatzkes et al., 2015; Gupta *et al*., 2016; Monnappa *et al*., 2016; Willis *et al*., 2016).

These experiments make us think that natural live predatory bacterial remedies may be beneficial treatments for AMR infections, even though the idea of introducing more bacteria into an already pathogen-infected host may sound unconventional.

The need for research into new antibacterial treatments is great; a speech by John Rex MD, [at a meeting convened by The Pew Charitable Trust, The Wellcome trust and American Society for Microbiology, following publication of The Pew Charitable Trusts report 'A Scientific Roadmap for Antibiotic Discovery' (June 16, 2016)], put this into sharp relief. John Rex suggested we think of antibiotics as fire extinguishers (Rex and Outterson, 2016). Fire extinguishers are present in certified buildings and available at all times. You may never have had to use one, but they are always there. In the event of a fire, there's no time to start to build a fire service. In the same way anti-bacterials need to be in reserve, ready and available in hospitals, even if rarely used, except for new AMR infections. Scientific challenges need to be addressed now to ensure that the supply of such new anti-bacterials is there in advance of their requirement as AMR infections increase.

We feel that whole predatory bacteria may be the contents of future 'extinguishers', discharged into infected compartments of the body to extinguish Gram-negative infections, with bacteriophage additives to combat Gram-positive co-infectors.

Predatory bacteria may not be suitable for systemic blood stream infections; but they may play a role decolonizing lungs, guts or wounds from problematic pathogens that resist conventional antibiotics. This approach would bring with it a new challenges; production of whole organisms rather than chemically defined drugs for treatment will need to draw upon the food and brewing industry as much as the pharmaceutical. Supporting the patient's physiology and immune status, during the administration of the predators, and monitoring predator clearance at the end of treatment will be vital.

Modern microscopic methods now make it more possible to follow and enumerate the predators in the new environments of the living animal or human body. How to assure the safety of a living predatory bacterium with over 3,500 gene products, rather than a single chemical entity like an antibiotic will be new and difficult legislative – and safety – territory, but the advance of untreatable AMR will certainly focus the minds of clinicians, scientists, patients and legislators on relative risks. Further animal studies will be very important and helpful. Indeed test-treating farm livestock with predators for their conventionally AMR infections and monitoring, (in contained

conditions), the longevity and environmental fate of the predators and pathogens (Ibrahim *et al*., 2016), as well as the treatment outcome, will be a useful way ahead.

So in summary, although some of the genes encoding AMR have come from soil bacteria, returning to Nature to source solutions using the multi-faceted approaches of live micro-organisms, including predatory bacteria, will give new possibilities that take longer for pathogens to resist. The natural properties of prokaryotic predators, studied and applied by environmental as well as clinical and fundamental microbiologists working together, can bring new solutions to AMR.

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