PNAS CORE CONCEPTS

How persister bacteria evade antibiotics, prolong infections

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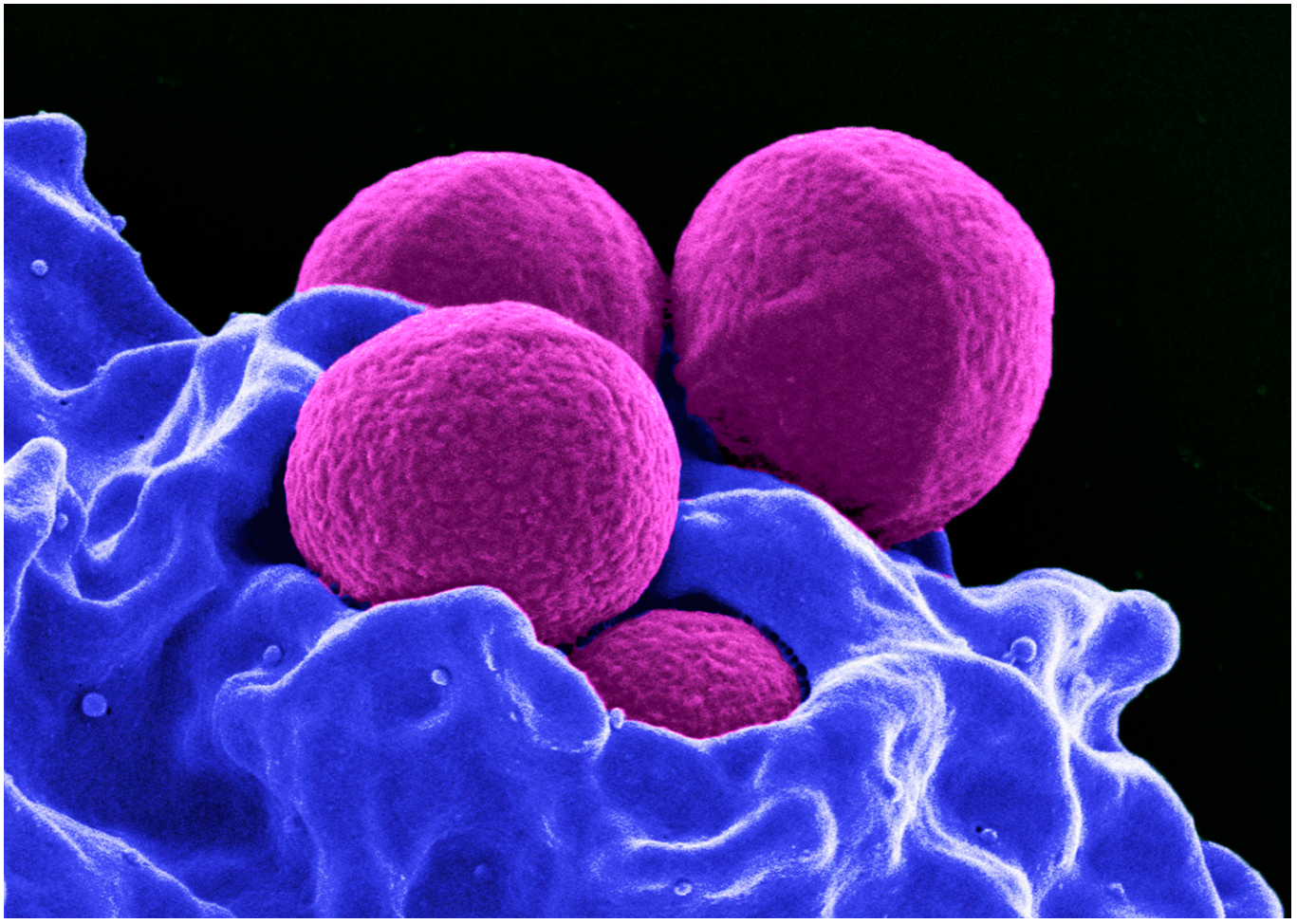

***S. aureus*—as seen in this scanning electron microscopic image in which human white blood cells (blue) are phagocytizing the bacteria (magenta spheres)—is a common source of potentially dangerous bacterial infections. Persister cells may contribute to infection relapses. Image credit: National Institute of Allergy and Infectious Diseases (NIAID).**

When exposed to antibiotics, some elusive bacteria lie low—so low, in fact, that they're actually inaccessible to the drugs. Researchers first identified these unique antibiotic-tolerant cells, also known as persisters, shortly after penicillin was first used to treat patients.

As a clinician, Eleftherios Mylonakis saw first-hand the problems of bacterial persistence: Patients who were treated for infections, particularly those caused by *Staphylococcus aureus*—a common source of potentially dangerous bacterial infections—frequently experienced relapses after they had completed a course of antibiotics. Making matters worse, drug tolerance increases antibiotic use, which is both bad for patients and the ongoing drug resistance crisis. "It's a real clinical problem for us," says Mylonakis, who also studies infectious diseases at Brown University in Providence, RI.

But decades after their discovery, researchers remain divided about the origins of persister cells and the nature of their evasive strategies. Nearly all bacteria can undergo physical changes to grow tolerant to antibiotics. Unlike the case of antibiotic resistance, however, some changes are not encoded in any unique genes; tolerance is not a heritable trait that can be transmitted between bacteria or over generations. Instead, changes associated with tolerance are ephemeral, and they are initiated by any number of genetic pathways. These changes can typically be reversed by tweaking the microbes' environment. Some consider

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the switch to tolerance a stochastic process; others believe the underlying regulatory mechanisms simply remain undiscovered.

Lacking specific genes of interest, researchers haven't had much to go on—and tools such as knocking out genes to study a trait don't always work. Subtle differences in experimental conditions can result in cellular stress rather than true antibiotic tolerance, according to Thomas Wood, a biotechnology researcher at Pennsylvania State University in State College. "The environment has a lot of influence on this population," adds biomedical engineer Dacheng Ren of Syracuse University, NY. "If you do an experiment without consistent environmental controls, people can find differences in the phenomena they observe."

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But in recent years, researchers have begun to reach a consensus on the nature of these unique cells and to highlight ways of eradicating them. Some researchers aim to resensitize the cells to antibiotics, whereas others are seeking ways to eliminate the cells while they're in their dormant state. And still others are exploring how host immune responses can drive the formation of persisters. Given the prevalence of bacterial persistence, these efforts are likely to prove crucial for curing recalcitrant infections. "There couldn't be a more important state for understanding bacterial cells, because almost all cells have to go into this state and stay there," Wood says.

Slow-Growing Support

In 1942, the year that doctors first used penicillin in the United States, microbiologist Gladys Hobby, then at the Columbia Medical School in New York, reported that the drug only killed *actively multiplying* cells in streptococcal cultures. Approximately 1% of bacteria appeared to survive the treatment (1). Two years later, microbiologist Joseph Bigger, then at Dublin University in Ireland, replicated Hobby's experiments and named these surviving cells persisters. But like the cells themselves, research on the phenomenon of persistence lay dormant for several decades.

In 1983, researchers Harris Moyed and Kevin Bertrand of the University of California, Irvine, reported that certain mutations in the *Escherichia coli hipA* gene, which produces a toxin that forces dormancy, increased the frequency at which persister cells were formed nearly 1,000-fold (2). But reducing expression of the *hipA* gene didn't eradicate persister cells completely, a phenomenon that led researchers to explore how other pairs of toxin and antitoxin molecules made by bacteria could trigger dormancy and drug tolerance, says microbiologist Kim Lewis of Northeastern University in Boston, MA. The quest to understand drug resistance also led researchers to study cells in biofilms, a complex matrix of proteins and sugars that surround bacteria and make them hard to kill. "The prevailing wisdom at the time was that there's some special mechanisms of resistance that are turned on once cells form a biofilm," Lewis says.

In 2010, Lewis and his colleagues reported that *TisB*, another toxin gene in *E. coli*, could also trigger tolerance. *TisB* produces a small antimicrobial peptide that enters the bacterial cell membrane and depletes its membrane potential as well as ATP reserves, nudging the cell to dormancy (3). When drained of energy stores, bacteria shut down the synthesis of proteins and peptidoglycans as well as other metabolic processes—all of which are the targets of various antibiotics. That same year, Wood and his team identified another toxin in *E. coli* that, when deleted, reduced persistence (4). The toxin, named MqsR, also halted protein synthesis, but by destroying mRNA rather than depleting energy. "There's nothing special about this toxin system," Wood says. "The key is stopping protein translation."

Other studies revealed another potential player in the process: the signaling molecule guanosine (penta) tetraphosphate [(p)ppGpp], which activated some of these toxins. Although several studies suggest that stopping transcription or depleting ATP are hallmarks of persister cell formation, other mechanisms are at work too. In 2019, Eduardo Groisman and Mauricio Pontes of Yale University in New Haven, CT, found that an acidic environment and low levels of magnesium ions could drive *Salmonella* to form persister cells by slowing down bacterial growth (5).

Collectively, these strands of evidence reveal a complex picture: Many metabolic pathways can lead cells to a persister state. Although the pathways themselves may have little in common, they all drive cells to a slow-growing physiological state. And because most antibiotics target bacterial processes associated with growth, silencing these mechanisms effectively stymies an antibiotic's ability to kill bacteria.

Slaying Sleeping Cells

Despite the uncertainty, researchers are working on potential treatments based on the physical traits of drug-tolerant cells. Some approaches rely on reviving dormant cells to resensitize them to antibiotics, whereas others aim to find molecules that can kill cells while in the persister state. "Chemicals that are used to kill them while they're sleeping have to be able to passively diffuse into the cell," Wood says. "Once they're awake, they are the same as a regular cell. There's no genetic change and they're easily killed by traditional antibiotics."

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One approach, proposed by Bigger shortly after the discovery of persisters, relies on a "pulsed" dosing of antibiotics, which entails treatment punctuated by pauses to nudge cells out of dormancy. This, however, has "potential drawbacks because it invites the development of resistance," Lewis says, because the repeated exposures can give antibiotic resistant bacteria the opportunity to thrive.

Instead, Mylonakis, Wood, and others induce persistence in bacterial cultures and use these tolerant cells in

high-throughput screens to identify potential drugs. Wood and his team have screened more than 10,000 compounds to home in on certain compounds that could work either by awakening cells, so that they're susceptible to antibiotics (6), or killing them in the persister state (7).

In a search for compounds that could kill persister cells without awakening them, Ren and his team sought out approved antibiotics such as eravocycline and minocycline. Actively growing cells produce proteins that expel these drugs from bacteria, thus protecting the microbes from the drugs' killing actions. But in dormant *E. coli* cells, these proteins are inactive because they require energy to work. As a result, the antibiotic accumulated at much higher concentrations inside these cells. Then the team removed the drugs and allowed the dormant cells to regrow. But the accumulated antibiotics killed these bacteria when they began to resume normal metabolism (8).

Seeking out drugs that work in a similar way might be one route to finding new means of killing persister cells, Ren says. Such drugs must be able to cross cells' lipid membrane and bind strongly to an intracellular target. "It won't work if the drug target has disappeared due to dormancy," Ren says. And these antibiotics might fail if cells are able to expel persisters quickly once they awaken.

Traditional drug discovery can overlook molecules that work this way, Ren says, because researchers typically select candidate drugs for their ability to kill or inhibit actively growing cells, not for their capacity to slay sleepy persisters. "If you apply this logic to screen for drugs," Ren says, "we might find a lot of new candidates that we might have ignored before."

Interacting in Infections

So far, researchers studying antibiotic tolerance have focused on bacteria grown in laboratory cultures, not on animal models of infections. But interactions between host immune responses and bacteria can sway how, where, and when drug tolerance develops, says Brian Conlon, a microbiologist at the University of North Carolina at Chapel Hill. "It's far more complex in the environment of an infection," he says. "The energy state of the bacteria is really going to be driven by its interaction with the host."

Those interactions can differ from one patient to another, or between sites of infection in the same individual. Whether a bacterial cell survives antibiotic treatment depends on its interactions with immune cells, the stage of infection, availability of nutrients and sugars, and several other factors. Until recently, "this was really unexplored territory," Conlon says.

Researchers have recently made some inroads into understanding these dynamics, however. In 2018, microbiologist Sophie Helaine of Harvard Medical School in Cambridge, MA, and her colleagues found that in a mouse model of *Salmonella* infections, persister cells were not entirely dormant. They actively secreted toxins that suppressed inflammatory responses, creating an environment where the bacteria could regrow once antibiotics were stopped (9).

Paradoxically, host immune responses can also drive the formation of tolerant cells. Immune cells known as macrophages trap bacteria within intracellular vesicles, then produce a burst of reactive oxygen and nitrogen species to kill the microbes by attacking their respiratory cycle. But in 2020, Conlon and his colleagues found that this respiratory burst—meant to kill bacteria—could simply force dormancy by stopping bacterial energy production (10).

In another recent preprint, the team reports that macrophages consume more glucose when mounting an inflammatory response. *S. aureus*' energy levels are largely determined by its ability to access glucose. So when immune cells compete for the same foods, bacteria lose their access to them—and could resort to a dormant, antibiotic-tolerant state (11).

Identifying these immune drivers of tolerance will be key to understanding the phenomenon and developing treatments that account for host responses, Conlon says. "It is really important to find out where these cells are during infection and what's driving that tolerance," he says. "In vivo, we're not talking about a homogenous artificial environment, so it could be something very specific, orchestrated by the host."

In ongoing studies, Conlon and his colleagues are exploring whether tweaks to the infection microenvironment can reduce the formation of tolerant cells. "We don't want to knock the immune response out, obviously," he says, "but there might be nuanced ways to target it to curtail the pathogen but also allow antibiotics to work better."

Demonstrating clinical utility will require many lines of research to converge, Conlon adds. Typically, studies of antimicrobial chemotherapy focus on in vitro studies of a potential drug, followed by preclinical and clinical studies. Research on host–pathogen interactions tends to be a distinct field that relies on different techniques, such as animal infection models, to understand how immune cells interact with bacteria vis-à-vis changes in gene expression, toxin production, or other physiological factors. "The two rarely meet, and that's left us really lacking in our understanding of how antibiotics work in vivo," Conlon says. "It's kind of created a void and left a lot of questions to be answered."

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