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Microbial Recognition of Antibiotics: Ecological, Physiological, and Therapeutic Implications

Although humans use antibiotics to kill microbes, these diverse compounds serve many other ecological and physiological roles

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Microbes release innumerable products into their environment, including many antibiotics. This fact has captivated scientists and clinicians for more than a century, and many of the details of the early history of antibiotics are familiar even to beginning students of microbiology and pharmacology. For example, the discovery in the late 1800s that microorganisms can cause disease was made concurrently with the finding that microbes can also prevent infections. After Robert Koch demonstrated that *Bacillus anthracis* is the causative agent of anthrax, Louis Pasteur showed that *B. anthracis* infection of animals could be prevented by simultaneous inoculation with soil bacteria. Other researchers later showed that cell-free preparations of *Pseudomonas aeruginosa* also conferred protection against anthrax infection, and this preparation was used to treat a variety of infectious diseases (with mixed results).

Proof of the clinical efficacy of penicillin, a triumph in medical history, was built on Alexander Fleming's discovery in 1926 that fungi produce a substance—later purified and named penicillin—that blocks growth of bacteria in the genus *Staphylococcus* (Fig. 1). The discovery and development of this antibiotic helped to trigger a broader search for antimicrobials among natural products of microbes. One pioneer in this field was Selman Waksman, who systematically searched for microbial products that inhibit the growth of disease-causing bacteria. Using this approach, Waksman and colleagues discovered streptomycin, a product of *Streptomyces griseus* and the first antibiotic treatment for tuberculosis (Fig. 1). He and others unearthed a treasure trove of other natural compounds, some of which became clinical antibiotics, revolutionizing the treatment of infectious diseases in the 20th century.

Though nearly all of the antibiotics currently in clinical use were discovered in microbial cultures and have been widely used in medicine for decades, the physiological roles of these compounds within the microbial communities that produce them are largely unknown. Waksman popularized

Summary

- Although humans use antibiotics to kill microbes, these diverse compounds likely serve additional roles in nature, including signaling within and among microbial species.
- In some cases, microbial pathogens respond to specific antibiotics by changing expression of virulence factors, thus modifying the course of an infectious disease.
- The PhoPQ regulatory system, much like innate immunity in mammals, enables bacterial pathogens such as Salmonella to withstand host compounds with antibiotic properties.
- Because an antibiotic produced by one bacterium can damage or protect other bacterial species, community interactions could have unexpected effects on infectious diseases.

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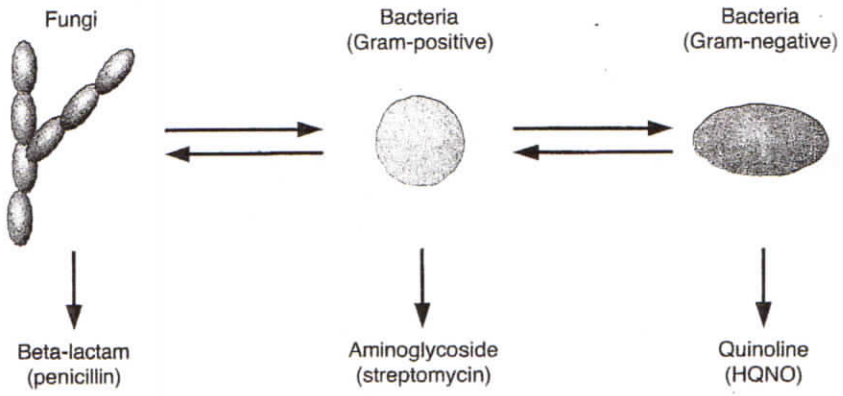
FIGURE 1



Three examples of antibiotic classes produced by microbes, with their source organisms.

the term "antibiotic" in the 1940s, using it to refer to chemical compounds of microbial origin that inhibit the growth or metabolism of other microbes, a definition which continues to be used in modern dictionaries. In part because of the broad scope of this definition, there is as yet no unifying description of the physiological function of antibiotics. Furthermore, the microbes that produce almost all of the currently used antibiotics inhabit the soil. This observation suggests that such microbes are routinely exposed to antibiotics, and that the ecological and physiological consequences of these interactions are likely to have acquired considerable complexity over evolutionary time, perhaps extending beyond simple microbial competition.

Antibiotics can function as components of a community language within microbial groups. An improved understanding of the specific responses of microbes to antibiotics could permit their clinical use in a more appropriate and effective fashion, and possibly lead to the development of new antibiotics. Furthermore, these studies can reveal unanticipated consequences of polymicrobial community interactions for health and disease. These points are illustrated by the following examples, in which we consider the effects of antibiotics on microbial cells, and provide cases in which microbes respond to individual antibiotic classes with specific sensing mechanisms. Each example offers clues to the essential questions regarding the physiologic functions of antibiotics and their effects within microbial communities, both in the environment and in eukaryotic hosts.

Antibiotics May Have Important Physiological Roles Other Than Antibiosis

A long-used technique in microbiology is auxanography, or the study of the growth requirements of individual microbes. Importantly, some compounds that are a food source at a low concentration can be toxic at higher concentrations. Similarly, the effects of antibiotics are also highly dependent on concentration.

At clinically used concentrations, which typically are higher than those found in natural environments, antibiotics can kill microbial cells or halt their growth. Reducing those concentrations can prevent such killing. Even at concentrations that do not inhibit growth, antibiotics can still induce complex changes in gene expression that are specific for individual antibiotic classes, and that could contribute to the clinical effects observed with some antibiotics.

For example, treatment with the macrolide antibiotic azithromycin often improves lung function in patients with airway infections by *Pseudomonas aeruginosa* without substantially lowering the bacterial density. Azithromycin alters the expression of genes related to virulence and biofilm formation in *P. aeruginosa*, and this activity is believed to play a role in the clinical effects of macrolides. Such sublethal effects are probably more reflective of the types of interactions among bacteria encountered in their environmental niches.

Bacterial Signaling Molecules Can Share Traits of Antibiotics

In considering both the natural roles and the precise definition of antibiotics, it is instructive to consider bacterial cell-to-cell signal molecules. Many bacteria produce small molecules that coordinate the behavior of groups of cells. One class of signaling molecules is called autoinducers because cells express genes that both produce and respond to those signals. N-acylhomoserine lactones, one class of autoinducer signals, are produced by a wide variety of species of gram-negative bacteria.

Although one might expect autoinducers to be readily distinguishable from antibiotics, that

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Miller at First Resisted Pursuing a Career as a Physician-Scientist

Hoping to avoid being compared to his physician-scientist father, Sam Miller deliberately chose not to become a doctor or scientist. His father, who died when Sam was two, had been an expert on heart disease, hypertension, and the blood-brain barrier. "My father was quite popular, and I would often meet people who had been his patient or knew him, and they always asked if I would become a doctor like my father," Miller recalls.

"When I went to college, I performed psychology research on visual perception and worked with the head of child psychiatry at Johns Hopkins Hospital performing play therapy on disturbed children," he says. "As a junior, I fell in love with chemistry and wanted to be a chemist. However, my teachers told me that graduate school in chemistry was hopeless because there were no jobs and suggested I go to medical school and perform biochemical research."

Miller did not immediately take that advice. However, he grew frustrated after working for a year with high-IQ, institutionalized children and applied to medical school. "I considered also getting a Ph.D., but decided to finish medical school first," he recalls. "I was influenced there by infectious disease professors in particular."

Today Miller, 55, is professor of medicine, microbiology, and genome sciences at the University of Washington in Seattle. "I enjoy the fact that I am lucky enough to perform research in a wide variety of areas and with different technologies," he says. "I think that is why I like microbial pathogenesis research. Infectious diseases are quite diverse, and have different outcomes in different individuals. I believe the future of this type of research will involve unraveling the basis of this diversity."

Gradually he shifted almost exclusively to basic research. "I was very involved with clinical medicine when I was younger, but discovered that I liked basic science research and the molecular basis of how things work," he says.

Miller, who grew up in Houston as a fifth-generation Texan, says he enjoyed playing football as a child. After their father died, he and his younger brother were raised by their mother, a former economics history teacher at Hunter College in New York, N.Y., who later worked at the Rice University library.

Miller received his B.A. from Johns Hopkins University in 1975 and his M.D. from Baylor College of Medicine in 1979, finishing medical school in three years. "I looked for a research job and was hired by James Field [in Baylor's department of endocrinology], who gave me my start in research," he says. "I worked in his laboratory on thyroid hormone action and biochemistry, and he encouraged my development as a physician-scientist."

Miller next moved to Massachusetts General Hospital and, in 1987, moved across town to work with John Mekalanos. "I spent two years in John's laboratory in the Harvard microbiology department, which was a wonderful place with John Collier, Jon Beckwith, Bernie Fields, and Roberto Kolter," he says. "The environment was incredibly rich, and I would say that those two years have had the greatest impact on my career." He moved from Boston to Seattle in 1995. "I have been very lucky in my career, as it has been a continual expansion of my laboratory, but slowly enough that it was never a big change," he says.

Miller is married to a physician-scientist who studies urinary tract infections and other women's health issues. They have four children, whose ages range from 12 to 18. Outside the lab, he does sports, plays guitar, and is active in his synagogue.

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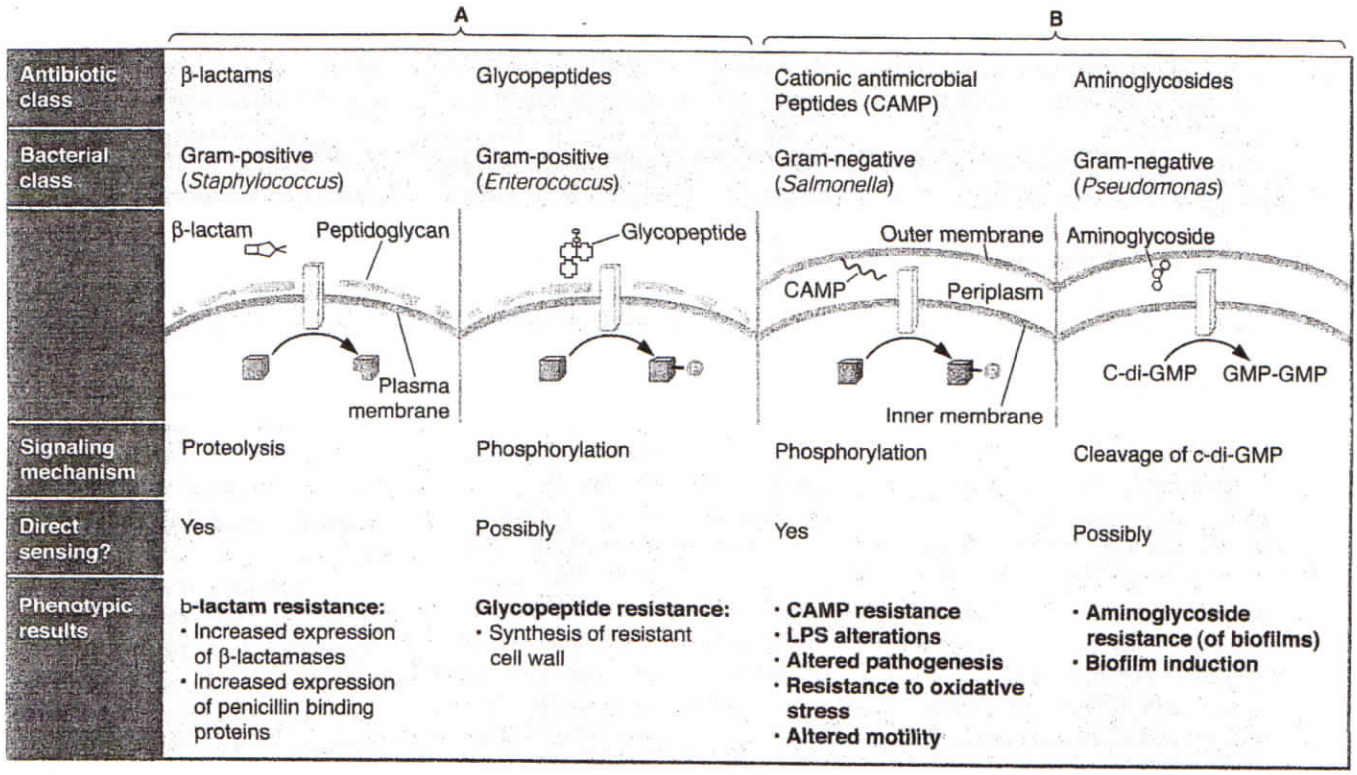
is not always the case. For instance, *Rhizobium leguminosarum* produces an N-acyl-homoserine lactone autoinducer involved in symbiotic interactions with plant hosts that was originally identified as a bacteriocin due to its growth inhibitory effects. Therefore, this autoinducer shares properties with antibiotics in that it halts bacterial growth even though its physiologic role appears to be intercellular communication. Additionally, this molecule acts only upon the source species, apparently by activating cell death, neither of which are characteristics of canonical antibiotics.

This example shows how a functional defini-

FIGURE 2



Summary of four antibiotic sensing mechanisms discussed in the text. (A) Two such mechanisms found in gram-positive bacteria. (B) Two mechanisms found in gram-negative bacteria.

tion of antibiotics, one based on the laboratory observation of growth-inhibitory effects, can lead to assumptions about the ecological and physiological roles played by microbial compounds which may not reflect a natural situation.

Bacteria Can Specifically Sense and Respond to Distinct Classes of Antibiotics to Induce Antimicrobial Resistance

Microbial communities composed of diverse species are commonly found in soil and water and on eukaryotic host surfaces. However, the effects of antibiotics (whether at natural concentrations or at those used for therapy) on such communities remain largely unknown. These interactions could be clinically important. For instance, do antibiotics have unanticipated effects on polymicrobial infections, and do polymicrobial community interactions alter how patients respond to antibiotics? And what are the effects of the antibiotics that the bacterial community members themselves produce, including the commensal microbes that inhabit all of us?

Such interactions cannot be measured by in vitro tests of antibiotics using single bacterial isolates removed from a multispecies community. The cases presented below begin to address these questions. We begin with four antibiotic sensing mechanisms, each leading to antibiotic resistance, and each specific for one class of antibiotics (Fig. 2). Such specificity suggests that these signaling pathways are the end products of natural selection, and that the physiological consequences are of biological relevance.

Staphylococcus and β-Lactams: Antibiotic Sensing by Sequential Proteolysis to Promote Antibiotic Resistance

The β-lactam class of antibiotics is produced by fungi and some bacteria, and members of this class target enzymes involved in cell wall biosynthesis. These antibiotics are the first clinical line

of defense against infections with staphylococci, gram-positive bacteria that are among the most common pathogens of humans. It has long been known that exposure of bacteria to antibiotics can lead to inducible antibiotic resistance, but the specific response illustrated below is an example of exquisite specificity and recognition that likely reflects millennia of coevolution.

Staphylococci can directly sense β-lactams, and respond with two mechanisms of resistance (Fig. 2A). Upon binding to β-lactams, specific transmembrane receptor proteins are self-activated by proteolytic cleavage, and the activated receptors then cleave repressor proteins. The result of this proteolytic cascade is derepressed expression both of a β-lactamase (an enzyme that cleaves and inactivates β-lactams) and of a cell wall biosynthetic enzyme (penicillin-binding protein) that is relatively resistant to inhibition by β-lactams.

In *Staphylococcus*, the β-lactam sensors themselves and their regulatory targets are encoded by genetic elements that can be acquired by horizontal transfer. Given that proteolytic transmembrane signaling is such an elegant system for sensing and responding to antibiotics, it is likely that additional examples in diverse bacteria await discovery.

Enterococcus and Vancomycin: Antibiotic Sensing by Sequential Phosphorylation

The glycopeptide vancomycin (derived from the soil-dwelling bacterium *Streptomyces orientalis*) is one of the most useful antibiotics in the treatment of gram-positive bacterial infections, such as those caused by the staphylococci and enterococci. Vancomycin, like β-lactam antibiotics, targets the bacterial cell wall, and enterococci are frequently inducibly resistant to vancomycin due to a regulatory system that alters cell wall structure.

Gene clusters encoding the most common types of inducible vancomycin resistance are part of mobile DNA elements (transposons) that can be found on self-transferable plasmids, thereby facilitating the spread of resistance. These gene clusters encode a two-component regulatory system consisting of a sensor kinase, VanS, and response regulator, VanR, as well as cell wall modifying enzymes. According to current models, the VanS transmembrane protein senses either vancomycin directly or vancomycin-induced perturbations of the cell wall, autophosphorylates, and then phosphorylates the cytoplasmic regulator VanR. Once activated, VanR induces the expression of enzymes that produce new cell wall components resistant to the action of vancomycin, and also enzymes that eliminate vancomycin-susceptible components (Fig. 2A).

In some cases, microbial pathogens respond to specific antibiotics not only by inducing resistance to the antibiotic class that was sensed, as with the two previous examples, but also by altering characteristics that have important implications for disease: properties related to virulence are altered. Thus, antibiotic sensing can modify the propensity for and course of infectious disease.

P. aeruginosa and Aminoglycosides: Antibiotic Induction of Biofilm Formation

The aminoglycoside antibiotics are a diverse group of cationic, amino sugar-based molecules produced mostly by the streptomycetes. Like these bacteria, *P. aeruginosa* is also found in the soil, and therefore likely encounters aminoglycosides in its natural environment.

Recently, we showed that subinhibitory concentrations of aminoglycosides but not other antibiotics induce the gram-negative bacteria *P. aeruginosa* and *E. coli* to grow as biofilms, adherent aggregates of cells that are markedly more resistant to many antibiotics and some mammalian immune functions than are planktonic cultures. Biofilms are implicated in many chronic, difficult-to-treat infections. Thus, these and other gram-negative bacteria exposed to sublethal concentrations of aminoglycosides during treatment may adopt a growth phenotype that leads to persistent infection despite a vigorous host immune response and intensive antibiotic therapy.

Aminoglycoside-induced biofilm formation by at least one strain of *P. aeruginosa* requires a gene named *arr* (for aminoglycoside response regulator). Arr is predicted to be a transmembrane protein with a periplasmic loop that could sense a signal, and evidence suggests that the cytoplasmic domain regulates the levels of the dinucleotide second messenger cyclic di-GMP (Fig. 2B).

It remains unknown whether Arr directly senses aminoglycosides or senses metabolic per-

turbations induced by these antibiotics. A narrow range of aminoglycoside concentrations induces biofilm formation. This range is shared even by *P. aeruginosa* clinical isolates with increased resistance to this antibiotic, suggesting that the mechanism by which aminoglycosides are sensed is distinct from the mechanism by which they kill bacterial cells. The *arr* gene is situated on a genomic island in approximately one-quarter of clinical isolates, suggesting that this gene represents a transferable mechanism of inducible resistance, much like those for inducible antibiotic resistance in enterococci and staphylococci described above.

Salmonella Recognition of Cationic Antimicrobial Peptides by a Sensor Kinase Promotes Virulence for Mammals

Diverse organisms, including animals, plants, fungi, and bacteria, produce an array of cationic antimicrobial peptides (CAMPs). Environmental bacteria, such as the gram-positive *Paenibacillus polymyxa*, produce CAMPs, including clinically useful compounds such as polymyxin B and colistin. As befits their ancient origin, CAMPs are components of the mammalian innate immune system, serving as a first line of defense by protecting mucosal surfaces against a variety of infectious agents. To kill bacteria, CAMPs interact with outer membrane anionic molecules, such as lipopolysaccharide (LPS), and some bacteria become inducibly resistant to CAMPs by altering LPS structure.

Gram-negative bacteria such as *Salmonella typhimurium* sense and respond to CAMPs using a two-component regulatory system: the PhoQ transmembrane sensor kinase and the PhoP cytoplasmic response regulator (Fig. 2B). CAMPs displace magnesium ions that bridge the PhoQ periplasmic domain and inner membrane phospholipids, triggering a conformational change that leads to autophosphorylation, followed by phosphorylation of PhoP.

Subsequently, activated PhoP induces the expression of a wide variety of genes, resulting in *Salmonella* bacterial envelope changes that not only lead to resistance to CAMPs but also enhance bacterial survival within host phagocytes. This suggests that direct sensing of animal CAMPs acts as a signal for *Salmonella* of host tissues. Indeed, much like LPS is a pathogen-associated molecular pattern that triggers innate immune responses in mammals, host-produced CAMPs might serve as molecular markers triggering virulence responses in bacterial pathogens.

Extending the comparison, the PhoPQ regulatory system could be viewed as a component of "bacterial innate immunity." Such a system could have been modified over evolutionary time to enable bacterial pathogens such as *Salmonella* to withstand host compounds with antibiotic properties that are closely related to those produced by other microbes.

The Physiological Roles of Antibiotics May Involve Microbial Community Interactions

Since antibiotics are used by humans to kill microbes, it is frequently assumed that these molecules serve naturally as weapons in microbial interspecies warfare. However, the examples provided above share a common theme that may suggest another role for antibiotics: in each case, molecules produced by one microbial species generate a response in another (Fig. 1). Furthermore, the responses cannot uniformly be identified as "defensive," which would be expected if antibiotics serve as nothing more than artillery.

Such a paradigm, taken at face value, may appear to be more characteristic of a system for signaling than of one for competition. In reality, the natural roles of antibiotics may include both of these possibilities. The diversity of compounds with antibiotic activity suggests that they likely serve other natural roles, as well. Regardless, the molecules we use as antibiotics probably serve as important mediators of microbial community structure and function, and these natural roles can have unanticipated effects in the clinical setting as well as the environment. In the following example, an antibiotic produced by one bacterium has both adverse and protective effects on another bacterial species, demonstrating how microbial community interactions can unexpectedly affect the course of infectious disease.

HQNO: an Antibiotic with Paradoxical Effects

Among the antibiotics produced by the bacterium *P. aeruginosa* is 4-hydroxy-2-heptylquino-

line *N*-oxide (HQNO), which inhibits the respiration of diverse organisms, including gram-positive bacteria as well as mammalian cells (Fig. 1).

Because its target range is so broad, the natural function of HQNO is not readily apparent. Adding further complexity, HQNO can have apparently contradictory effects on a single organism. HQNO not only can inhibit the growth of *Staphylococcus aureus*, but it also can protect *S. aureus* from killing by aminoglycoside antibiotics.

This paradox is explained by the fact that HQNO inhibits cytochrome-mediated electron transport in gram-positive bacteria, but only enough to limit growth. Aminoglycosides, which are highly charged, must be actively taken up through an electron transport-dependent mechanism before they can kill bacteria. HQNO inhibits aminoglycoside uptake, thereby exerting a protective effect, by the same mechanism that it restricts bacterial growth.

The potential for such complexity exists in any multispecies bacterial community, either those encountered in the soil or those causing opportunistic human infections in which *P. aeruginosa* and *S. aureus* are commonly found together. In the latter case, unanticipated protection of staphylococci by a *Pseudomonas* antibiotic could hamper treatment in a fashion that could not be predicted by routine laboratory testing.

Microbial Recognition of Antibiotics Impacts Health and Disease

The study of interspecies microbial interactions mediated by antibiotics is as old as the science of microbiology itself. Such studies remain particularly relevant today because of our current struggles to deal with failures of antibiotic therapies. By integrating new technologies and scientific concepts, such as metagenomics (the study of genomic diversity of natural samples) and sociomicrobiology (a synthesis incorporating ideas from sociology about communication and group behavior) into microbiology, we are learning how to define the structure and behavior of microbial communities in a way that was not previously possible.

The study of microbial interspecies interactions can thus shed light not only on how microorganisms colonize hosts or particular environmental niches, but also on what roles polymicrobial communities play in health and disease. From such a perspective, antibiotics are likely to play multiple natural roles, ranging from communication to manipulation to warfare (Fig. 1).

An improved understanding of these natural roles can inform the therapeutic use of antibiotics, with the hope of explaining or even predicting clinical treatment failures. For instance, microbes are able to sense and respond to structurally diverse antibiotics through specific signaling pathways, leading to resistance that is often transient (Fig. 2) and thus not detected by routine testing. Thanks in part to genome sequencing, there is increasing recognition that many signaling mechanisms by which microbial cells sense and respond to antibiotics are highly conserved. Despite such conservation, these systems are integrated into the physiologies of particular organisms in myriad ways, contributing to the extraordinary diversity of the microbial world.

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