

# BEHIND ENEMY LINES

A close look at  
the inner workings  
of microbes in this  
era of escalating  
**ANTIBIOTIC RESISTANCE**  
is offering new  
strategies for  
designing drugs

by **K. C. Nicolaou and Christopher N. C. Boddy** • Photographs by Eric O'Connell



**CHEMISTS ARE SEEKING** to overcome bacteria such as vancomycin-resistant enterococci (*petri dish at right*) that now grow easily in the presence of vancomycin (*left*).

## In the celebrated movie *Crouching Tiger, Hidden Dragon*, two warriors face each other in a closed courtyard whose walls are lined with a fantastic array of martial-arts weaponry, including iron rods, knives, spears and swords.

The older, more experienced warrior grabs one instrument after another from the arsenal and battles energetically and fluidly with them. But one after another, the weapons prove useless. Each, in turn, is broken or thrown aside, the shards of an era that can hold little contest against a young, triumphant, upstart warrior who has learned not only the old ways but some that are new.

One of the foundations of the modern medical system is being similarly overcome. Health care workers are increasingly finding that nearly every weapon in their arsenal of more than 150 antibiotics is becoming useless. Bacteria that have survived attack by antibiotics have learned from the enemy and have grown stronger; some that have not had skirmishes themselves have learned from others that have. The result is a rising number of antibiotic-resistant strains. Infections—including tuberculosis, meningitis and pneumonia—that would once have been easily treated with an antibiotic are no longer so readily thwarted. More and more bacterial infections are proving deadly.

Bacteria are wily warriors, but even so, we have given them—and continue to give them—exactly what they need for their stunning success. By misusing and overusing antibiotics, we have encouraged super-races of bacteria to evolve. We don't finish a course of antibiotics. Or we use them for viral and other inappropriate infections—in fact, researchers estimate that one third to one half of all antibiotic prescriptions are unnecessary. We put 70 percent of the antibiotics we produce in the U.S. each year into our livestock. We add antibiotics to our dishwashing liquid and our hand soap. In all these ways, we en-

courage the weak to die and the strong to become stronger [see “The Challenge of Antibiotic Resistance,” by Stuart B. Levy; SCIENTIFIC AMERICAN, March 1998].

Yet even absent the massive societal and medical misuse of these medications, the unavoidable destiny of any antibiotic is obsolescence. Bacteria—which grow quickly through many cell divisions a day—will always learn something new; some of the strongest will always survive and thrive. So we have had to become ever more wily ourselves.

In the past 10 years, long-standing complacency about vanquishing infection has been replaced by a dramatic increase in antibacterial research in academic, government and industrial laboratories. Scientists the world over are finding imaginative strategies to attack bacteria. Although they will have a limited life span, new antibiotics are being developed using information gleaned from genome and protein studies. This exciting research and drug development is no panacea, but if combined with the responsible use of antibiotics, it can offer some hope. Indeed, in April 2000 the Food and Drug Administration approved the first new kind of clinical antibiotic in 35 years—linezolid—and several agents are already in the pharmaceutical pipeline.

### Dismantling the Wall

ALMOST ALL THE ANTIBIOTICS that have been developed so far have come from nature. Scientists have identified them and improved on them, but they certainly did not invent them. Since the beginning of life on this planet, organisms have fought over limited resources. These battles resulted in the evolution of antibiotics. The ability to produce such powerful compounds gives an organism—a fungus or plant or even another species of bacteria—an advantage over bacteria susceptible to the antibiotic. This selective pressure is the force driving the development of antibiotics in nature.

Our window onto this biological arms race first opened with the discovery of penicillin in 1928. Alexander Fleming of St. Mary's Hospital Medical School at London University noticed that the mold *Penicillium notatum* was able to kill nearby *Staphylococcus* bacteria growing in agar in a petri dish. Thus was the field of antibiotics born. By randomly testing com-

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pounds, such as other molds, to see if they could kill bacteria or retard their growth, later researchers were able to identify a whole suite of antibiotics.

One of the most successful of these has been vancomycin, first identified by Eli Lilly and Company in 1956. Understanding how it works—a feat that has taken three decades to accomplish—has allowed us insight into the mechanism behind a class of antibiotics called the glycopeptides, one of the seven or so major kinds of antibiotics. This insight is proving important because vancomycin has become the antibiotic of last resort, the only remaining drug effective against the most deadly of all hospital-acquired infections: methicillin-resistant *Staphylococcus aureus*. And yet vancomycin’s power—like that of the great, experienced warrior—is itself in jeopardy.

Vancomycin works by targeting the bacterial cell wall, which surrounds the cell and its membrane, imparting structure and support. Because human and other mammalian cells lack such a wall (instead their cells are held up by an internal structure called a cytoskeleton), vancomycin and related drugs are not dangerous to them. This bacterial wall is composed mostly of peptidoglycan, a material that contains both peptides and sugars (hence its name). As the cell assembles this material—a constant process, because old peptidoglycan needs to be replaced as it breaks down—sugar units are linked together by an enzyme called transglycosidase to form a structural core. Every other sugar unit along this core has a short peptide chain attached to it. Each peptide chain is composed of five amino acids, the last three being an L-lysine and two D-alanines. An enzyme called transpeptidase then hooks these peptide chains together, removing the final D-alanine and attaching the penul-

timate D-alanine to an L-lysine from a different sugar chain. As a result, the sugar chains are crocheted together through their peptide chains. All this linking and cross-linking creates a thickly woven material essential for the cell’s survival: without it, the cell would burst from its own internal pressure.

Vancomycin meddles in the formation of this essential material. The antibiotic is perfectly suited to bind to the peptide chains before they are linked to one another by transpeptidase. The drug fastens onto the terminal D-alanines, preventing the enzyme from doing its work. Without the thicket of cross-linking connections, peptidoglycan becomes weak, like an ill-woven fabric. The cell wall rends, and cell death rapidly occurs.

## Resisting Resistance

VANCOMYCIN’S LOVELY FIT at the end of the peptide chain is the key to its effectiveness as an antibiotic. Unfortunately, its peptide connection is also the key to resistance on the part of bacteria. In 1998 vancomycin-resistant *S. aureus* emerged in three geographic locations. Physicians and hospital workers are increasingly worried that these strains will become widespread, leaving them with no treatment for lethal staph infections.

Understanding resistance offers the possibility of overcoming it, and so scientists have focused on another bacterium that has been known to be resistant to the powerful drug since the late 1980s: vancomycin-resistant enterococci (VRE). In most enterococci bacteria, vancomycin does what it does best: it binds to the terminal two D-alanines. At a molecular level, this binding entails five hydrogen bonds—think of them as five fingers clapping a ball. But in VRE, the peptide chain is slightly different. Its final D-alanine is altered by a simple substitution: an oxy-

## RISING RESISTANCE

**MANY ANTIBIOTICS** are no longer effective against certain strains of bacteria, as these examples—collected from different hospitals in the late 1990s—show. One strain of *Staphylococcus aureus* found in Korea, for instance, is 98 percent resistant to penicillin (*top left*); another, found in the U.S., is 32 percent resistant to methicillin (*bottom left*). All these strains are not resistant to vancomycin, for now.

STAPHYLOCOCCUS AUREUS  
VS. PENICILLIN



ENTEROCOCCUS FAECIUM  
VS. CIPROFLOXACIN (CIPRO)



STREPTOCOCCUS PNEUMONIAE  
VS. TETRACYCLINE



STAPHYLOCOCCUS AUREUS  
VS. METHICILLIN



ENTEROCOCCUS FAECIUM  
VS. AMPICILLIN



STREPTOCOCCUS PNEUMONIAE  
VS. PENICILLIN





**COMPOUNDS** are mixed in the chemist's workspace under the fume hood. In the setup to the left, organic molecules are being purified. In the two to the right, reactions occur in the presence of argon gas, which is in the balloons and which protects sensitive molecules from oxygen and water in air.

# New methods in combinatorial chemistry facilitate the **RAPID DESIGN** of huge libraries of compounds.



gen replaces a pair of atoms consisting of a nitrogen bonded to a hydrogen. In molecular terms, this one substitution means that vancomycin can bind to the peptide chain with only four hydrogen bonds. The loss of that one bond makes all the difference. With only four fingers grasping the ball, the drug cannot hold on as well, and enzymes pry it off, allowing the peptide chains to link up and the peptidoglycan to become tightly woven once again. One atomic substitution reduces the drug's activity by a factor of 1,000.

Researchers have turned to other members of the glycopeptide class of antibiotics to see if some have a strategy that vancomycin could adopt against VRE. It turns out that some members of the group have long, hydrophobic—that is, oily—chains attached to them that have proved useful. These chains prefer to be surrounded by other hydrophobic molecules, such as those that make up the cell membrane, which is hidden behind the protective peptidoglycan shield. Researchers at Eli Lilly have borrowed this idea and attached hydrophobic chains to vancomycin, creating an analogue called LY333328. The drug connects to the cell membrane in high concentrations, allowing it more purchase and, as a consequence, more power against peptidoglycan. This analogue is effective against VRE and is now in clinical trials.

Other glycopeptide antibiotics use a different strategy: dimerization. This process occurs when two molecules bind to each other to form a single complex. By creating couples, or dimers, of vancomycin, researchers can enhance the drug's strength. One vancomycin binds to peptidoglycan, bringing the other half of the pair—the other molecule of vancomycin—into proximity as well. The drug is more effective because more of it is present. One of the aims of our laboratory is to alter vancomycin so it pairs up more readily, and we have recently developed a number of dimeric vancomycin molecules with exceptional activity against VRE.

Even so, the good news may be short-lived. A second mechanism by which VRE foils vancomycin has recently been discovered. Rather than substituting an atom in the final D-alanine, the bacterium adds an amino acid that is much larger than D-alanine to the very end of the peptide chain. Like a muscular bouncer blocking a doorway, the amino acid prevents vancomycin from reaching its destination.

One method by which the deadly *S. aureus* gains resistance is becoming clear as well. The bacterium thickens the peptidoglycan layer but simultaneously reduces the linking between the peptide fragments. So it makes no difference if vancomycin

binds to D-alanine: thickness has replaced interweaving as the source of the peptidoglycan's strength. Vancomycin's meddling has no effect.

## The Cutting Edge

AS THE STORY of vancomycin shows, tiny molecular alterations can make all the difference, and bacteria find myriad strategies to outwit drugs. Obviously, the need for new, improved or even revived antibiotics is enormous. Historically, the drug discovery process identified candidates using whole-cell screening, in which molecules of interest were applied to living bacterial cells. This approach has been very successful and underlies the discovery of many drugs, including vancomycin. Its advantage lies in its simplicity and in the fact that every possible drug target in the cell is screened. But screening such a large number of targets also has a drawback. Various targets are shared by both bacteria and humans; compounds that act against those are toxic to people. Furthermore, researchers gain no information about the mechanism of action: chemists know that an agent worked, but they have no information about how. Without this critical information it is virtually impossible to bring a new drug all the way to the clinic.

Molecular-level assays provide a powerful alternative. This form of screen identifies only those compounds that have a specified mechanism of action. For instance, one such screen would look specifically for inhibitors of the transpeptidase enzyme. Although these assays are difficult to design, they yield potential drugs with known modes of action. The trouble is that only one enzyme is usually investigated at a time. It would be a vast improvement in the drug discovery process if researchers could review more than one target simultaneously, as they do in the whole-cell process, but also retain the implicit knowledge of the way the drug works. Scientists have accomplished this feat by figuring out how to assemble the many-enzyme pathway of a certain bacterium in a test tube. Using this system, they can identify molecules that either strongly disrupt one of the enzymes or subtly disrupt many of them.

Automation and miniaturization have also significantly improved the rate at which compounds can be screened. Robotics in so-called high-throughput machines allow scientists to review thousands of compounds per week. At the same time, miniaturization has cut the cost of the process by using ever smaller amounts of reagents. In the new ultrahigh-throughput screening systems, hundreds of thousands of compounds can

be looked at cost-effectively in a single day. Accordingly, chemists have to work hard to keep up with the demand for molecules. Their work is made possible by new methods in combinatorial chemistry, which allows them to design huge libraries of compounds quickly [see “Combinatorial Chemistry and New Drugs,” by Matthew J. Plunkett and Jonathan A. Ellman; *SCIENTIFIC AMERICAN*, April 1997]. In the future, some of these new molecules will most likely come from bacteria themselves. By understanding the way these organisms produce antibiotics, scientists can genetically engineer them to produce new related molecules.

## The Genomic Advantage

THE METHODOLOGY of drug design and screening has benefited tremendously from recent developments in genomics. Information about genes and the synthesis of their proteins has allowed geneticists and chemists to go behind enemy lines and use inside information against the organism itself. This microbial counterintelligence is taking place on several fronts, from sab-

otaging centrally important genes to putting a wrench in the production of a single protein and disrupting a bacterium’s ability to infect an organism or to develop resistance.

Studies have revealed that many of the known targets of antibiotics are essential genes, genes that cause cell death if they are not functioning smoothly. New genetic techniques are making the identification of these essential genes much faster. For instance, researchers are systematically analyzing all 6,000 or so genes of the yeast *Saccharomyces cerevisiae* for essential genes. Every gene can be experimentally disrupted and its effect on yeast determined. This effort will ultimately catalogue all the essential genes and will also provide insight into the action of other genes that could serve as targets for new antibiotics.

The proteins encoded by essential genes are not the only molecular-level targets that can lead to antibiotics. Genes that encode for virulence factors are also important. Virulence factors circumvent the host’s immune response, allowing bacteria to colonize. In the past, it has been quite hard to identify these genes because they are “turned on,” or transcribed, by events in the host’s tissue that are very difficult to reproduce in the laboratory. Now a technique called *in vivo* expression technology (IVET) can insert a unique sequence of DNA, a form of tag that deactivates a gene, into each bacterial gene. Tagged bacteria are then used to infect an organism. The bacteria are later recovered and the tags identified. The disappearance of any tags means that the genes they were attached to were essential for the bacteria’s survival—so essential that the bacteria could not survive in the host without the use of those genes.

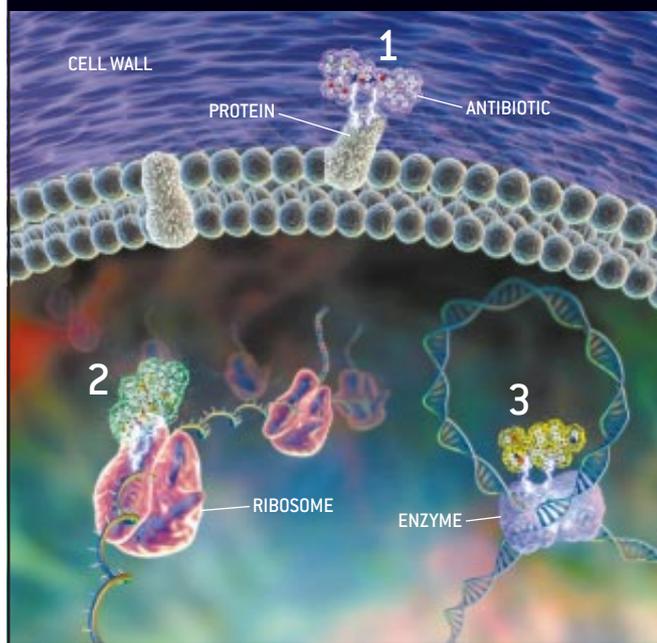
Investigators have long hoped that by identifying and inhibiting these virulence factors, they can allow the body’s immune system to combat pathogenic bacteria before they gain a foothold. And it seems that the hypothesis is bearing fruit. In a recent study, an experimental molecule that inhibited a virulence factor of the dangerous *S. aureus* permitted infected mice to resist and overcome infection.

In addition to identifying essential genes and virulence factors, researchers are discovering which genes confer antibiotic resistance. Targeting them provides a method to rejuvenate previously ineffective antibiotics. This is an approach used with  $\beta$ -lactam antibiotics such as penicillin. The most common mechanism of resistance to  $\beta$ -lactam antibiotics is the bacterial production of an enzyme called  $\beta$ -lactamase, which breaks one of the antibiotic’s chemical bonds, changing its structure and preventing it from inhibiting the enzyme transpeptidase. If  $\beta$ -lactamase is silenced, the antibiotics remain useful. A  $\beta$ -lactamase inhibitor called clavulanic acid does just that and is mixed with amoxicillin to create an antibiotic marketed as Augmentin.

In the near future, with improvements in the field of DNA transcriptional profiling, it will become routine to identify resistance determinants, such as  $\beta$ -lactamase, and virulence factors. Such profiling allows scientists to identify all the genes that are in use under different growth conditions in the cell. Virulence genes can be determined by identifying bacterial genes whose expression increases on infecting a host. Genes that code for antibiotic resistance can be determined by comparing expression

## ANTIBIOTICS AT WORK

**EXISTING ANTIBIOTICS** fight infections by preventing bacteria from making essential substances. Vancomycin and  $\beta$ -lactam antibiotics interfere with synthesis of the cell wall (1). Erythromycin and tetracycline disrupt ribosomes that make proteins (2). Quinolone antibiotics inhibit enzymes involved in replicating DNA (3), and sulfonamide antibiotics also interfere with DNA synthesis (*not shown*).



# Vancomycin's power—like that of the great, **EXPERIENCED WARRIOR**—is itself in jeopardy.



levels in bacteria treated with the antibiotic and those not treated. Though still in its infancy, this technique monitored tiny changes in the number of transcription events. With DNA transcriptional profiling, researchers should also be able to determine whether certain drugs have entirely new mechanisms of action or cellular targets that could open up new fields of antibiotic research.

## Killing the Messenger

ANOTHER INTERESTING LINE of genomic research entails interfering with bacterial RNA. Most RNA is ribosomal RNA (rRNA), which forms a major structural component of ribosomes, the cellular factories where proteins are assembled. Ribosomal RNA is vulnerable because it has various places where drugs can attach and because it lacks the ability to repair itself. In 1987 scientists determined that antibiotics in the aminoglycoside group—which includes streptomycin—bind to rRNA, causing the ribosome to misread the genetic code for protein assembly. Many of these antibiotics, however, are toxic and have only limited usefulness. Recently scientists at the Scripps Research Institute in La Jolla, Calif., have reported a new synthetic aminoglycoside dimer that may have less toxicity.

Investigators can also interfere with messenger RNA (mRNA), which directs the assembly of proteins and travels between the genetic code and the ribosome. Messenger RNA is created by reading one strand of the DNA, using the same nucleic acid, or base pair, interactions that hold the double helix together. The mRNA molecule then carries its message to the ribosome, where a protein is assembled through the process of translation. Because each mRNA codes for a specific protein and is distinct from other mRNAs, researchers have the opportunity to create interactions between small organic molecules—that is, not proteins—and specific mRNAs. Parke-Davis chemists have been able to use such an approach to combat HIV infection. They identified molecules that bind to a part of an mRNA sequence and prevent it from interacting with a required protein activator, thus inhibiting the replication of HIV. This proof-of-principle experiment should help pave the way for further studies of mRNA as a drug development target.

Scientific interest has been intense in another approach, called antisense therapy. By generating sequences of nucleotides that bind perfectly with a specific mRNA sequence, investigators can essentially straitjacket the mRNA. It cannot free itself from the drug, which either destroys it or inhibits it from acting.

Although the FDA has recently approved the first antisense drug to treat human cytomegalovirus infections, antisense for bacterial infections has not succeeded yet for several reasons, including toxicity and the challenge of getting enough of the drug to the right spot. Nevertheless, the approach holds promise.

As is clear, all these genomic insights are making it possible to identify and evaluate a range of new biological targets against which chemists can direct their small, bulletlike molecules. A number of antibiotics developed in the past century cannot be used, because they harm us. But by comparing a potential target's genetic sequence with the genes found in humans, researchers can identify genes that are unique to bacteria and can focus on those. Similarly, by comparing a target's genetic sequence to those of other bacteria, they are able to evaluate the selectivity of a drug that would be generated from it. A target sequence that appears in all bacteria would very likely generate an antibiotic active against many different bacteria: a broad-spectrum antibiotic. In contrast, a target sequence that appears in only a few bacterial genomes would generate a narrow-spectrum antibiotic.

If physicians can identify early on which strain is causing an infection, they can hone their prescription to a narrow-spectrum antibiotic. Because this drug would affect only a subset of the bacterial population, selective pressure for the development of resistance would be reduced. Advances in the high-speed replication of DNA and transcriptional profiling may soon make identification of bacterial strains a routine medical procedure.

Although the picture looks brighter than it has for several decades, it is crucial that we recognize that the biological arms race is an ancient one. For every creative counterattack we make, bacteria will respond in kind—changing perhaps one atom in one amino acid. There will always be young warriors to challenge the old ones. The hope is that we exercise restraint and that we use our ever expanding arsenal of weapons responsibly, not relegating them so quickly to obsolescence. **SA**

## MORE TO EXPLORE

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