A Shared Strategy for Virulence

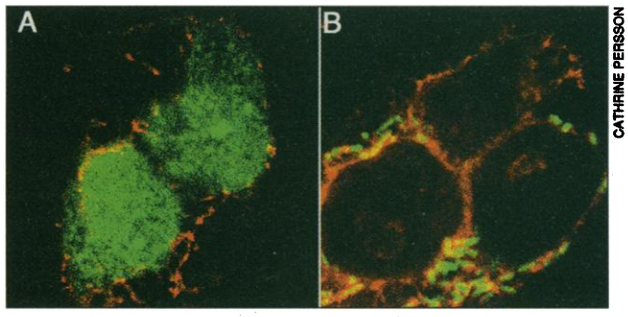
A versatile bacterial weapon that fires harmful proteins directly into cells has turned up in many unrelated pathogens, and offers insight into diseases ranging from bubonic plague to tree blight

Five years ago, when related genes for a specialized secretion system began popping up in a wide range of pathogenic bacteria, microbiologists realized that this was no ordinary case of bacterial gene-swapping. Wily bacteria are known to trade useful genes among themselves like baseball cards, a trick that allows traits such as antibiotic resistance to spread rapidly to unrelated pathogens. But these microbes had acquired not just an individual gene, but an entire set of genes that codes for an elaborate protein delivery system. This recently discovered system acts as a versatile weapon in the bacterial arsenal, a biochemical missile that can fire a variety of damaging proteins directly into the host's cells—and cause diseases that range from bubonic plague in humans to fire blight in fruit trees.

"It is one of the most exciting recent developments in bacterial pathogenesis," says Jorge Galán, a microbiologist at the State University of New York, Stony Brook, who organized a symposium on the new findings at last week's meeting of the American Society for Microbiology (ASM) in New Orleans. "It is exciting to think of pathogens of plants and animals sharing so much in terms of the way they interact with eukaryotic cells." To microbiologists, the system—called the type III secretion system—promises new insights into how different kinds of bacteria cause disease, and so a standing-room-only crowd packed the symposium hall at last week's meeting. What they heard was the results of a deluge of papers that has poured forth in recent years, exploring the biochemical details of the launching device and its disease-causing missiles.

Indeed, researchers keep finding new avenues of interest in this sophisticated machinery. Unlike other bacterial secretion systems, the type III system is triggered specifically by contact with host cells, and delivers its payload directly into those cells, a move that sidesteps host defenses. "These bugs are making potent virulence determinants and sending them straight to their target, without being accessible to neutralization by the immune system," marvels microbiologist Sue Straley of the University of Kentucky College of Medicine in Lexington. Because the delivery apparatus is shared by many bacteria and specializes in delivering virulence proteins, the system may be an ideal focus for antibiotic development, says Harvard University microbiologist John Mekalanos.

The virulence proteins that this system delivers are also the focus of intense scrutiny, because they manipulate the host in cunning and subtle ways, for example by biochemically hobbling white blood cells. "These are the magic molecules of pathogenesis," says microbiologist Dan Portnoy of the University of Pennsylvania. Because the bacteria are apparently homing in on key host processes, he says, "finding out the precise targets [of the virulence molecules] will tell us interesting things" about how plants and animals normally fight infection.



CATHRINE PERSSON

On target. Normal *Yersinia* shoot harmful protein *(green)* into a cell *(A)*; in bacteria with a mutant type III gene *(B)*, the protein is stuck outside the cell membrane *(yellow)*.

Strange secretions

Although the importance of the type III system was recognized only in the past few years, the seeds of these discoveries were sown in the 1980s, when researchers in several labs were studying *Yersinia*, a genus of gram-negative bacteria that includes several species that cause human diseases such as bubonic plague and intestinal infections. The scientists found that disease-causing species of *Yersinia* produce large amounts of proteins that seemed to be associated with the outer membrane of the bacteria.

These proteins came to be known as Yops, for *Yersinia* outer proteins. But while the name stuck, it soon became clear that it didn't quite fit. It turned out that the Yops weren't outer membrane proteins at all, but were actually secreted by *Yersinia* into the surrounding medium. That was surprising, because the Yops lack the classic hallmark of secreted proteins, a so-called signal sequence that threads the protein through the membrane and then is chopped off. The Yops' lack of a signal sequence means that "they are secreted by a mechanism that is different from any known secretion mechanism," says Guy Cornelis at the Catholic University of Louvain in Brussels, Belgium, whose lab contributed to these early findings. As there were already two well-known secretion systems by which gram-negative bacteria ferry proteins across their unique double membrane, this new secretion system was called type III.

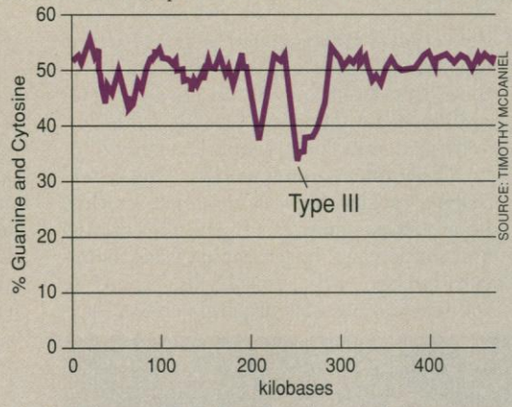
Soon researchers began identifying the genes for this mysterious new biochemical machine. In 1991, Cornelis's group reported finding a set of genes needed for Yop secretion, and within a year, researchers discovered similar genes in the gram-negative animal pathogens *Shigella flexneri* and *Salmonella typhimurium*. These bacteria cause human gastrointestinal infections, but are not close relatives of *Yersinia*. More amazingly, the genes also turned up in even more distantly related bacteria such as *Pseudomonas*, *Xanthomonas*, and *Erwinia*, which cause disease in plants rather than animals. And the number of pathogens—so far all gram-negative—known to carry the genes is still growing. Last summer microbiologist James Kaper of the University of Maryland School of Medicine in Baltimore and his colleagues reported finding the system in enteropathogenic *Escherichia coli*, a strain of the common human intestinal bacteria that causes diarrhea in infants.

The genes appear more closely related to each other than do the bacteria in which they were found, suggesting that they originated in an as-yet-unknown source and then were picked up by many different microbes (see box on p. 1262). What's more, the genes are similar to those that code for a specialized set of proteins that assemble the molecular components of flagella, the hairlike structures that propel bacteria. Flagellar assembly requires ferrying proteins across bacterial membranes, and "it is possible that through some sort of gene duplication event, flagellar-assembly genes evolved to secrete something else," says Kentucky's Straley, who studies type III secretion in *Yersinia*.

When the dust had settled after the first whirlwind of gene cloning, more than 20 proteins appeared to be part of the type III secretion system in *Yersinia*, and homologs of many of those genes had been found in

The "Alien DNA" of Virulence Genes

How did so many unrelated pathogens come to acquire the same complex weapon? Diverse bacteria possess the same machinery, called the type III secretion system, which allows them to fire virulence proteins at their host cells. Researchers are quite sure these microbes didn't inherit the system from the primordial mother of all bacteria, because type III genes stand out from the rest of the bacterial genome "like alien creatures," according to Stanford microbiologist Stanley Falkow. Instead, scientists think bacteria picked up the block of 15 or more genes from other bacteria. And although the identity of the genes' original owners remains a mystery, researchers are finding clues by scanning the type III genes and surrounding DNA.



Foreign fingerprint. G-C pairs make up half of most bacterial DNA, but less than 40% of pathogenicity islands.

Bacteria do a brisk trade in foreign genes, which can be passed among species by loops of DNA called plasmids, or by viruses called phages. In some species of bacteria, type III genes are on plasmids. In other species, the genes are on the bacteria's main chromosome, but they stand out because they're clumped together in regions called "pathogenicity islands"—stretches of DNA that contain a variety of virulence genes. These islands are marked as foreign by their DNA composition, which differs from the rest of the bacterial chromosome.

Because DNA codes for protein, the identity of the nucleotides (A, T, G, or C) at many positions on the chromosome is fixed. However, a redundancy in the genetic code allows some nucleotides to vary without altering protein structure, and nucleotides in noncoding positions can vary as well. Certain species-specific factors in DNA processing influence this variation. The result is that the DNA of each species has a characteristic percentage of guanine and cytosine (G and C) pairs.

And the G-C content of pathogenicity islands doesn't match that of other bacterial DNA. For example, says James Kaper of the University of Maryland, Baltimore, the human intestinal bacterium *Escherichia coli* has an average G-C content of 51%. But the type III secretion genes in the diarrhea-causing enteropathogenic strain of *E. coli* are on a pathogenicity island with a G-C content of 38% (see graph).

In another sign of their alien origin, pathogenicity islands are sometimes present in one strain of bacteria but not in its relatives. For example, *E. coli* strain K12 and *Salmonella typhimurium* are close enough relatives to have a similar arrangement of genes on their chromosomes. But *Salmonella* has a pathogenicity island that *E. coli* lacks. "Forty kilobases [of DNA] magically appear there, and all the genes surrounding it are the same in K12 and *typhimurium*," says microbiologist Samuel Miller of the University of Washington.

Acquiring a pathogenicity island would be "a major evolutionary step" in the life of a pathogen, says microbiologist Jorge Galán, of the State University of New York, Stony Brook, perhaps transforming a benign species into a pathogenic one. The acquisition of type III genes more than 4 million years ago, for example, was likely a key step in the evolution of pathogenic strains of *E. coli*.

But no one knows where these genes originally came from. The only clue is that they seem to have evolved from genes that code for proteins that assemble flagella (see main text). Using the G-C content of the type III genes as a starting point, evolutionary biologist Thomas Whittam of Pennsylvania State University and others are seeking relatives of the mysterious bacterial ancestor in which the type III genes evolved. Says Whittam: "We haven't detected any close relatives yet"—but they're still looking.

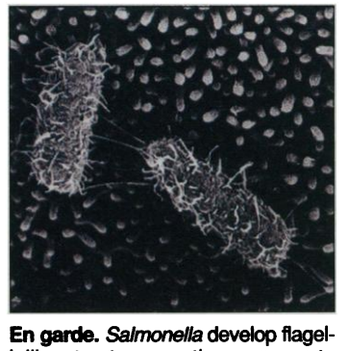
–M.B.

other bacteria as well. The genes provide only a few clues to the biochemical functions of the proteins, although one protein seems to be an adenosine triphosphatase (ATPase), which presumably powers the secretion process by cleaving energy-rich molecules of ATP. Microscopy and protein sequence analysis suggest that some of the proteins span the inner bacterial membrane, some the outer membrane (which is unique to gram-negative bacteria), and some bridge the two. All this might be expected for a secretion machinery. But why are there so many proteins? "You can get proteins out of the bacterial cell through both membranes with three novel proteins," says Straley. The complexity of the system, she says, "implies that there are extra functions associated with this mechanism."

A bacterial launch pad

Precisely what those functions are is still an unfolding story. But there are plenty of tantalizing clues from studies of bacteria with mutations that affect the secretion process. Back in the late 1980s—before it was known that the type III system was so widespread—Hans Wolf-Watz and his colleagues at the University of Umeå, Sweden, reported that of the dozen or so proteins secreted by the type III system in *Yersinia*, two, called YopH and YopE, prevent *Yersinia* from being gobbled up by macrophages and other cells. For YopH and YopE to do their job, they require the action of another protein, YopD, but the researchers could circumvent the need for YopD by injecting YopH and YopE directly into their target cells.

Those findings suggested that *Yersinia* senses the presence of eukaryotic cells and then transfers YopE and -H directly into them, relying on YopD for the transfer process. Indeed, in 1993, Wolf-Watz's team reported that when eukaryotic cells are nearby, *Yersinia* discharge their Yops neatly into those cells. Such sophisticated targeting could explain the type III system's complexity. Injecting a secreted protein into specific cells would require many proteins, some acting outside the bacterium itself.



GINOCCHIO *ET AL.*

En garde. *Salmonella* develop flagellalike structures as they prepare to attack cells.

The newest work in *Yersinia* and other microbes supports that interpretation. As-yet-unpublished data from Wolf-Watz's lab suggest that YopB and YopD form a pore in the membrane of eukaryotic cells, through which other proteins can be sent. And recent work shows that YopN, another secreted protein, seems to act as a stopcock on

the secretion apparatus: Mutant *Yersinia* that lack YopN secrete other Yops constantly and in all directions, while normal bacteria secrete Yops only when triggered to do so by contact with eukaryotic cells, says Wolf-Watz. It's as if YopN is a valve that is turned on only by that direct cell-to-cell contact.

Data from other bacteria known to have the type III genes sketch the same functional picture. Last fall Wolf-Watz's group reported that when the *yopE* gene was put into *Salmonella*, the *Salmonella* made the YopE protein and deposited it into eukaryotic cells. "That says *Salmonella* has everything necessary to do the translocation," says Wolf-Watz. And Galán's group has preliminary evidence that a *Salmonella* virulence protein called SptP is also delivered into the host cell cytoplasm. *Shigella* also uses the type III system to deliver the virulence protein IpaA into host cells, according to Philippe Sansonetti of the Pasteur Institute.

Evidence reported last week says that plant pathogens do it too: At the ASM meeting, Cornell University plant pathologist Alan Collmer reported new results from his group and that of Sheng Yang He at Michigan State University. They found that the plant pathogen *Pseudomonas syringae* apparently uses the type III system to introduce a protein called AvrB into plant cells; that protein determines the plant's response to the pathogen. So from a flurry of studies a pattern has emerged: Like a missile with many kinds of warheads, different bacteria rely on the same delivery system to inject different proteins into their hosts.

Multiple warheads

Just how this system delivers its payload remains an unsolved mystery. Given the similarity of the type III proteins to those that assemble flagella, it's tempting to hypothesize that they form a flagellumlike structure that shoots proteins into the host cells. Indeed, Galán's group has electron micrographs showing such appendages all over the outside of *Salmonella* as they infect eukaryotic cells. But the role these appendages play in infection is still unclear. And Galán cautions against viewing the system as an automatic injector, for some virulence proteins secreted by the type III system may act on the outside of cells instead of entering them. "We cannot say the sole purpose of this system is to inject proteins into the cell," he says. "We know it can do that, but it is not necessarily the only thing it does."

One way or another, the system disables the host or makes it more hospitable to the bacterium. *Yersinia* deploys the Yops to keep from being eaten by macrophages, but *Salmonella* and *Shigella* use their type III–secreted proteins for a nearly opposite purpose: to induce non–immune cells to take the bacteria into their cytoplasm. That gives the bacteria a safe haven, "a privileged site where they won't be killed by the immune system," says microbiologist Samuel Miller of the University of Washington. The enteropathogenic *E. coli* have yet another goal: to cozy up as tightly as possible to cells of the intestinal epithelium. To this end, their virulence proteins alter the microfilaments inside intestinal cells, producing a bulge in the cell surface that serves as a pedestal to which the bacteria can bind tightly.

The biochemistry used to accomplish these diverse tasks is eerily unbacterial in nature. For example, YopH is a tyrosine phosphatase that apparently removes phosphate from signaling proteins inside macrophages, thus disrupting the signals that cause the macrophages to scarf up bacteria. But where would a bacterium get a gene for an enzyme that removes phosphate from tyrosine? That function is typical of eukaryotes, not bacteria, says Straley. And YopH is not alone. At least two other *Yersinia* virulence proteins are eukaryotic-type kinases—enzymes that add phosphate to proteins—and Miller's and Galán's labs recently found evidence for a tyrosine phosphatase in a *Salmonella* protein. "This is a common theme of virulence determinants, that they target biochemistry that is uniquely eukaryotic," says Straley. And that, she says, has led some people to speculate that these genes were stolen at some time in the past from eukaryotic cells.

While researchers puzzle over the origins of the system, they continue to search for the type III system in other bacteria. So far, 10 or so bacterial species are known to have type III systems, but the story certainly won't end there. Harvard's Mekalanos says his lab is using polymerase chain reaction techniques to search systematically for the package of secretion genes in other gram-negative pathogens such as *Vibrio cholerae*. Other groups are looking for ways to use their knowledge of the system to disarm bacteria, perhaps by causing them to "prematurely ejaculate" their virulence proteins before they deliver them to their target cells, says Sansonetti. Researchers are also searching for host proteins targeted by the virulence molecules, to learn more about the cellular processes that the bacteria have chosen to sabotage.

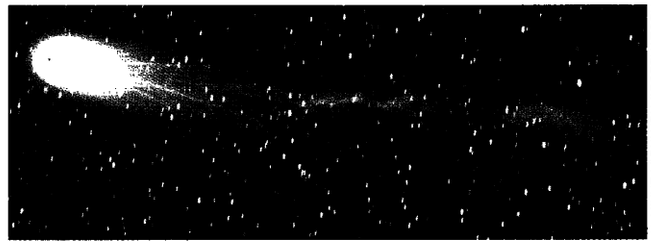
The search is on for a practical use for the new knowledge, but biologists are also marveling at the fact that such diverse bacteria have put this common machinery to use for their own individual ends. It's a fascinating habit of biological systems, says Mekalanos, "that once a problem is solved, it will be used over and over again for different purposes." And even if those purposes are the nefarious ones of disease-causing bacteria, one has to admire nature's ingenuity nevertheless.

–Marcia Barinaga

COMET CHEMISTRY

Hyakutake Produces Another Surprise

When comet Hyakutake sped past Earth last March, it did more than put on a breathtaking light show. It also delivered a series of scientific surprises, beginning with the first x-rays ever detected from a comet (*Science*, 12 April, p. 194). Now, as a paper in this issue reports, the surprises are continuing with a revelation about Hyakutake's composition that is hard to reconcile with standard explanations of how comets originated.



H. MIKUZ / CRNI VRH OBSERVATORY

Unexpected element. Analysis of comet Hyakutake reveals ethane.

In this case, "surprise is more than a mild understatement—it was serendipity," says Michael DiSanti, an astronomer at Catholic University of America in Washington, D.C. On page 1310, DiSanti and his colleagues report that Hyakutake contains abundant ethane and methane, compounds never before confirmed in comets. The ethane was a shock because astronomers had assumed it wasn't present in the primordial cloud of material that gave rise to the solar system. The new findings "really require us to rethink entirely our ideas about how comets formed," says Michael Mumma of the Goddard Space Flight Center in Greenbelt, Maryland, who led the collaboration.

Analyzing the composition of comets is a challenge, because they are small and cool, emitting most of their radiation in the infrared region of the spectrum, which is largely blocked by Earth's atmosphere. But Mumma and his colleagues thought Hyakutake's close approach—to within 10% of the Earth-sun distance at one point—might give them a better look at comet composition. Using an infrared spectrometer at NASA's Infrared Telescope Facility atop Mauna Kea, Hawaii, they set out to find methane, which Mumma describes as "one of the Holy Grail species that astronomers have been trying to find." Because