Discovering Microbes with a Taste for PCBs

Microbial ecologists and microbiologists are finding new organisms in the environment with unexpected abilities to degrade toxic chemicals

MICROBIAL ecologists and microbiologists are beginning to unearth a startling array of microorganisms with unexpected abilities to biodegrade some of the toughest and most recalcitrant environmental chemicals. In the past few years they have found entirely new types of bacteria that can carry out reactions previously thought to be impossible. Efforts are now under way to harness those natural abilities and use them in cleaning up toxic chemicals, first by enhancing the performance of nonengineered microorganisms and later by endowing them with new capabilities through genetic engineering.

Although many questions remain about how to turn these findings into practical, cost-effective systems for pollution control, work is rapidly moving from the laboratory to the field. For instance, this summer General Electric scientists began the first field test of a biological approach to degradation of polychlorinated biphenyls (PCBs), at a drag strip some 35 miles from the corporate research laboratory in Schenectady, New York.

Projects such as this one, and the basic research behind them, were discussed at a recent meeting in Seattle on biotechnology and pollution control.* Talks ranged from descriptions of bench-scale tests and field tests to actual use of nonengineered microbes to clean up Superfund sites.

Exploitation of natural biodegradative processes is not new; indeed, biological approaches have been used for years to treat industrial and municipal wastewaters. But most of these applications have occurred above ground, where the processes can be fairly easily controlled. Now the goal is to modify those techniques to work in soil and ground water, often on exceedingly recalcitrant chemicals that are biodegraded only slowly, if at all.

But before these newly found microorganisms can be harnessed, researchers must first figure out how to make them work in the right place, and at sufficient speed, on the appropriate chemicals. And that, in turns, depends on understanding the "mesmerizing complexity" of microbial ecosystems, says Rita Colwell of the University of Maryland, as well as the genetics and biochemistry of particular organisms. Fundamental knowledge is lacking, she says, because microbial ecology research has been so poorly funded. "We've had a couple of decades of intense molecular biology, but ecology and systematics have been left behind."

"We will find lots of surprises when we start prying into the corners of soil and water ecosystems."

That changed with the advent of genetic engineering. The possibility of manipulating genes to create a "superbug" with new biodegradative abilities illuminated how little is known about what microorganisms already exist, how they function, and what effect modifying them might have on natural ecosystems. In response, the National Science Foundation, which until a few years ago did not even have a separate program in microbial ecology, has dramatically increased its funding. Although genetic engineering applications remain years away, the upsurge in basic research has led to the discovery of these fascinating new microbes, which may not need any engineering at all.

James Tiedje, for instance, has isolated an entirely new anaerobic bacterium. It is able to do what was considered impossible only a few years ago: to remove chlorine from aromatic compounds, a key step in breaking down these compounds, which include such major pollutants as PCBs, dioxins, chlorinated phenols, and chlorinated benzenes.

Not only are chlorinated aromatics toxic, they are also very refractory, in part because of the aromatic ring structure but mostly because of the chlorination. If the chlorine can be removed, the remaining compound is often less toxic and more easily degraded, says Tiedje, who is a microbiologist at Michigan State University. However, although it was known that some compounds can be dechlorinated by microorganisms, until recently it was believed that aromatic compounds could not. That changed in 1982, when Tiedje observed dechlorination occurring in sewage sludge and realized the reaction was being carried out by indigenous microorganisms.

Two years later he isolated this microorganism, which he has dubbed DCB1, that can dechlorinate aromatic compounds—in this case, chlorobenzoate. "Our present knowledge suggests that this microorganism has no known close relatives that have been previously studied," says Tiedje. It has a distinctive collar around every cell that seems to be involved in cell division. "This is a very unusual morphological structure, not previously known in microbiology. That points out the unknown nature of this organism."

Since then Tiedje has found that this new organism works in concert with two others, which act in continuous sequence to completely degrade chlorobenzoate. (Chlorobenzoate is not an important pollutant, but it provides a model system for detailed, basic studies.) DCB1 performs only one step: it removes the chlorine from chlorobenzoate to produce benzoate. Then a second organism, a benzoate oxidizer, takes over and transforms benzoate to acetate, hydrogen, and carbon dioxide. Finally a methanogen, a bacterium that produces methane, finishes off the process by converting hydrogen and carbon dioxide to methane.

Tiedje is now probing the details of the interactions of the microbial consortium, and specifically, how the key dechlorination step works at both the genetic and enzymatic levels. Another critical question is, where does DCB1 get its energy? In this way, too, DCB1 appears to be unique. Tiedje believes that the energy source may involve the dechlorination process itself. "There are only a limited number of ways an organism can make energy," Tiedje says, "and this represents a new one."

Tiedje's eventual goal is to use DCB1 and other dechlorinating microorganisms, once they are isolated, in a practical system to clean up hazardous waste. But first he needs to determine which pollutants these organisms will transform and how the reactions can be enhanced.

To Colwell, Tiedje's work hints at what lies ahead. "We will find lots of surprises when we

*The meeting, "Reducing Risks from Environmental Chemicals Through Biotechnology," was held 19 to 22 July at the University of Washington, Seattle.

start prying into the corners of soil and water ecosystems. As we start taking these communities apart and looking at the components, we will find the kinds of organisms that Jim did—strange creatures that have a mix of characteristics that are quite unexpected."

Perry McCarty, a civil engineer at Stanford University, has detected two microorganisms that can also do the unexpected—in this case, biodegrade trichloroethylene (TCE) and trichloroethane (TCA), which are major ground water contaminants. TCE and TCA belong to the broader class of halogenated aliphatics, which were thought to be completely refractory to biodegradation until McCarty found out otherwise a few years ago.

But, as McCarty's work reveals, adapting either of these microorganisms for practical use (or for that matter, Tiedje's DCB1 or the slew of other microorganisms yet to be detected) will be tricky. "We need to learn how to create the right environment for these organisms to carry out the transformation," McCarty says. "We need to understand the organisms and their growth needs, then we need to learn how to optimize the system—to get nutrients to them and make the reaction run faster."

McCarty wants to use these microbes to treat contaminated ground water in situ, but little is known about microbial processes in ground water and simply gaining access to deep aquifers is a major obstacle. Another problem is toxic intermediates. In some microbial reactions the hazardous chemical is not completely degraded, or mineralized, but is transformed to intermediates. For some chemicals, transformation is sufficient to detoxify them. But for others, it makes the problem worse. For example, methanogens, the anaerobic bacteria McCarty has detected in ground water, transform TCE to organic intermediates, one of which—vinyl chloride—is more harmful to human health than is TCE. The trick, says McCarty, is to figure out how to prod the reaction along to complete mineralization.

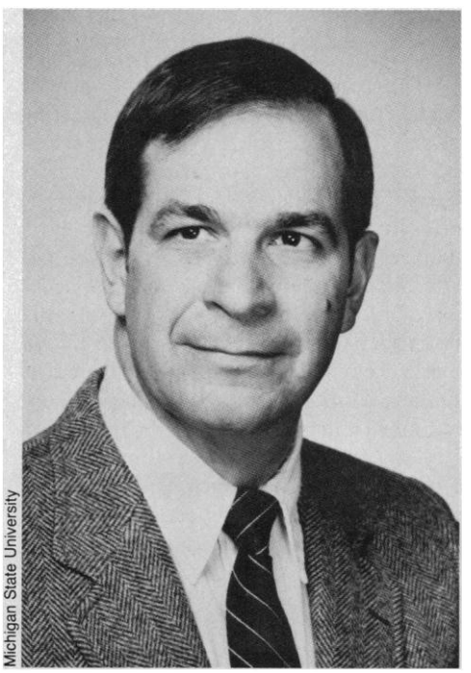
The other TCE degraders McCarty and his colleagues have found, aerobic soil bacteria known as methanotrophs, do mineralize TCE to harmless, inorganic components, which holds great promise for in situ treatment of contaminated soils. But if McCarty is to use methanotrophs in ground water, oxygen would have to be injected into the aquifer along with all the other substances necessary for bacterial growth.

But the biggest obstacle, at least for the compounds McCarty is working with, is a poorly understood process known as cometabolism. Biodegradation occurs fairly readily, even in ground water, if the microorganism can use the hazardous chemical as its primary energy source. But for many if not all halogenated aliphatics, this is not the case. Instead, the microorganism requires a second compound as its energy source and, in the process of metabolizing that energy source, degrades the "target" compound in a fortuitous reaction. This biochemical piggybacking is known as cometabolism.

"That is the process we are trying to capture," McCarty says, "but we know very little about it and how to optimize it." Methanogens must have either methanol or acetate to grow and to degrade TCE. Methanotrophs require methane. Finding the right balance can be tricky, as McCarty has discovered. Although methanotrophs cannot degrade TCE without methane, too much inhibits the degradation.

Researchers at the Environmental Protection Agency's Gulf Breeze laboratory in Florida are also encountering problems with cometabolism. Michael Nelson and his colleagues recently isolated another new bacterium, which they call strain G4, that also degrades TCE. The catch is that its energy source is phenol, a highly toxic aromatic compound. "You don't want to dump it in the ground water," says Nelson.

What cometabolism means in a practical sense, McCarty says, is that huge quantities of the energy source must be made available to the microorganisms. On the basis of preliminary studies, McCarty says, it looks as if the primary energy source must be present in quantities 100 to 1000 times greater than the hazardous chemical if it is to be transformed. In other words, in order to transform 1 kilogram of TCE you would need to add 100 to 1000 kilograms of the energy source. "This horrendous ratio means that large quantities of chemicals would need to be injected into an aquifer system for even a relatively small contamination." In addition, the carbon, nitrogen, and phosphorus needed for cellular growth all must be present and in proper balance for the reaction to proceed. "If it weren't for the high cost of the alternatives, it wouldn't be worth considering this at all," McCarty says. "It is very expensive."



James Tiedje *has isolated a new bacterium that can do what was previously thought to be impossible.*

As these basic studies proceed, other researchers are moving toward application in the field. "We are finding, eureka, what we are seeing in the lab *does* work in the field," Colwell says. "But what we are also finding, not unexpectedly, is that the precise extrapolation does not work. Parameters like temperature and nutrient concentration in a given system are important and not always entirely controllable."

General Electric's current assault on PCBs is a good example. The company has much at stake in this research, as it was a major user of PCBs for some 50 years and now is faced with a hefty clean-up task. At the drag strip, where PCBs were sprayed to hold down dust, the soil is contaminated with roughly 525 parts per million of Aroclor 1242, one type of PCB. PCBs are a "tough nut," says Ronald Unterman, the chief scientist on the project. There are more than 200 different forms, and what works on one will not necessarily work on another. Unterman and his colleagues are testing a strain of *Pseudomonas putida*, LB400, one of two dozen bacterial strains they have isolated that can grow on biphenyls and transform PCBs.

In the laboratory, LB400 works superbly, Unterman says. In what he calls a "shake and bake" procedure, they inoculated soil from the site with LB400, mixed it, and put it into an incubator. Within 3 days, 51% of the PCBs were transformed. But when they tried it under simulated field conditions—they inoculated a few kilograms of soil with LB400 and left it at ambient temperatures in the laboratory without shaking—nothing happened. Thirty days brought a "hint" of activity, Unterman says, and by 100 days they had achieved 50% degradation.

They are now testing LB400 on a test plot at the drag strip. "It's very low tech, we just spray them on," says Unterman. He expects transformation to occur even more slowly in the field, where the bacterial concentration is more dilute and temperature and moisture content cannot be precisely controlled. At the time of the meeting, 23 days into the test, there was, not unexpectedly, no sign of activity.

In other work, Ronald Crawford of the

University of Idaho and Thomas Frick of the University of Minnesota have developed a microbial consortium to degrade pentachlorophenol, a wood preservative and increasingly common ground and surface water contaminant. In a demonstration project, BioTrol Corporation of Minnesota is using that consortium in bioreactors to clean penta- and creosote-contaminated ground water at Superfund sites. And Ecova Corporation of Washington State has used biodegradation in combination with physical processes to clean up contaminated soils at an abandoned refinery on the Gulf Coast.

"I'm very impressed with the translation of basic microbiology into field work," said Alan Bull of the University of Kent, England, at the end of these talks. "Europe and the U.K. are a good deal behind." But as Bull pointed out, these early applications were picked precisely because they stood a good chance of success. "We're not hearing about aromatic compounds or heavy metals," he said.

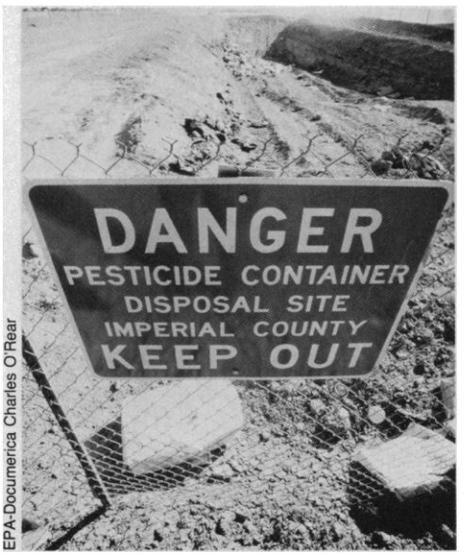
Similarly, while several of the speakers predicted that these processes will be cost-competitive, and eventually cheaper than the alternatives, that potential has yet to be realized. "The only thing that makes a lot of these technologies possible is the Superfund requirement that sites have to be cleaned up," McCarty said. "Otherwise, we couldn't afford it."

There are other obstacles as well, points out Martin Alexander of Cornell University. Some industrial discharges and Superfund sites are so toxic that they would "pickle" the organism before it had a chance to degrade them. In addition, he said, bacteria have to be able to work on the chemical as it appears in nature—which usually means in a mixture—rather than in isolation in the laboratory. And if the microbe or microbial consortium does not completely degrade a compound but leaves intermediates, how will those be removed?

Only one talk at the Seattle meeting focused on genetic engineering. Kenneth Timmis of the University of Geneva described his laboratory's efforts to draw on the diverse catabolic abilities scattered among soil and water microorganisms. Microorganisms have extraordinary capabilities to evolve pathways to degrade new industrial chemicals, he said. But evolution can be slow, especially when it requires multiple genetic events for which selection pressures are low. (This is especially true for catabolic pathways, which usually require 10 to 15 different enzymes.) Moreover, some chemicals appear to be inherently resistant to biological attack. For those, genetic engineering may be the only approach.

With co-workers Fernando Rojo and Juan Ramos, Timmis is trying to accelerate evolution in the laboratory. They are using two experimental strategies to construct new degradative pathways: restructuring an existing pathway and assembling an entirely new one.

The idea behind the first approach is to modify an existing catabolic pathway so that it will accept a compound that it previously would not, in this case, a model aromatic compound. *P. putida*, for example, degrades methylbenzoate and 3-ethylbenzoate but not 4-ethylbenzoate. Timmis and his colleagues set out to broaden this pathway by identifying the roadblocks to degradation of



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Toxic waste dumps *like this may be cleaned up by nonengineered microorganisms.*

4-ethylbenzoate and then engineering them.

The first obstacle they found is that the protein that stimulates synthesis of the catabolic enzymes in *P. putida* does not recognize 4-ethylbenzoate. But once they engineered that protein to recognize 4-ethylbenzoate, the organism still did not degrade it. The next roadblock turned out to be an intermediate step in the normal catabolic pathway, in which *P. putida* produces an enzyme that cleaves the aromatic ring. Timmis found that the enzyme is indeed produced and functions when 4-ethylbenzoate is present, but it is killed during the reaction. (The intermediate of 4-ethylbenzoate is a "suicide substrate" that kills the enzyme.) They selected a mutant enzyme that does work, cloned its gene, and inserted it into *P. putida*, which now degrades 4-ethylbenzoate in the laboratory.

The second approach comes into play when there is no obvious pathway related to what you want to degrade, Timmis says. The answer, he says, is to "design a new pathway on paper and then go looking in the environment for bacteria that will provide enzymes to construct it."

As their model system, Timmis and his co-workers decided to create a single pathway to degrade two types of aromatic compounds, chloroaromatics and methylaromatics. In nature these are handled by two distinct pathways (an *ortho* and a *meta* pathway). Although soil microorganisms often possess both pathways, only one is usually activated, depending on which substrate is present. When both compounds are present, however, both pathways can be switched on, which results in quite a muddle; intermediates are channeled down the wrong route, and, in the end, neither compound is degraded. Through a series of steps, the Geneva researchers recruited enzymes and assembled a pathway in *P. putida* that accommodates both. "It works," Timmis says. "It can simultaneously degrade mixtures of both types of compounds in the lab." And that, he says, holds promise for dealing with mixtures of toxic chemicals in the environment. The next step is to try both of these approaches on such major pollutants as PCBs and dioxin.

Such applications for major pollutants are thought to be several years away, however, for both scientific and regulatory reasons. Concern about releasing altered organisms into the environment is certainly one obstacle. But as Tiedje pointed out, no U.S. researcher has yet applied for permit to use a genetically altered microbe for pollution control. "There are no well-developed GEMs [genetically modified microorganisms] that people are waiting to test. We aren't there yet." And, as several speakers mentioned, Ananda Chakrabarty's oil-degrading microbe, the first genetically modified microbe to be patented, has not been used in the field, not for regulatory reasons but simply because it doesn't work very well.

For genetic engineering, says Colwell, "a key question is expression at the ecological level. You may get the gene expressed in the organism but not in the field. Factors such as temperature, the amount of nutrients, and the presence of heavy metals may affect the function of the organism you have so carefully engineered." And if the organism does work in the field, she asks, "how do you prime it and keep it primed so it will continually function?"

The difficulty is compounded, Tiedje says, by how little is known about these newly discovered microbes. "Comparatively, getting *E. coli* to produce insulin was far simpler because the biochemistry was well known." McCarty concurs. "The real need now is to understand the diverse abilities of natural organisms if we are to have any hope of improving them through recombinant DNA." ■ LESLIE ROBERTS