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Release and Behavior of Recombinant Bacteria in Field Studies

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Numerous opportunities exist for the utilization of genetically engineered microorganisms for useful purposes in agriculture and in waste management. An extremely diverse array of microorganisms is likely to be considered for such processes as biological control of plant pests, degradation of toxic wastes, reclamation of rare metals and other processes. For some purposes, such as in the degradation of toxic materials, it may be possible to make biologically compromised microorganisms that will exist only in the presence of the toxic chemicals that they were designed to transform. A better understanding of the genetics, biology and physiology of microorgansims which is being gained by biotechnological techniques will allow the development of environmentally "safe" microorganisms which would have a limited duration or dispersal potential in natural environments. Other applications, including many agricultural uses, will require environmentally competent microorganisms that actively grow in association with crop plants, for example. Such organisms cannot safely be assumed to have a limited duration in the environment in which they are released or dispersal restricted only to that localized area of application. Most genetically engineered microorganisms will not represent the introduction of strains with greatly different genetic backgrounds, and thus ecological adaptivities, than already existing microbes. For the foreseeable future, the most likely targets of genetic engineering will be endemic strains to which a unique gene or genes are added or deleted. Predictions of the behavior of such strains reintroduced into environments similar to the original source are simplified because of considerable knowledge of the natural history of the native organism. Comparative behavior of modified compared to natural microbial strains can be approached experimentally in contained conditions and should describe the expected behavior in natural situations.
INDEX DESCRIPTIONS: bioethics, biotechnology, genetic engineering, microorganisms.

This is a large topic to address, and, as both of the previous speakers have already pointed out, there are very important questions that can be asked about how we go about releasing recombinant microorganisms and how we might expect them to behave after release. Many eminent scientists have addressed this problem, and they have asked questions such as: Do we want an organism to survive? Do these organisms exchange genes with one another? How do they survive? In what situations would they survive? How do they move around? These are all very valid questions that we might want to ask about a microorganism that we would want to release for any individual reason. We now have many techniques with which we can address some of these very important questions. I think, however, that we really have to stop and take a very careful look at what questions are most pertinent and why we are asking these questions in the first place.

I return to my first question: Do we want that microorganism to disappear? This seems like a very straightforward question, but I don't think it has a simple answer. I think the answer that we're going to arrive at is a very quantitative one and not a qualitative one at all. For example, we could anticipate many experiments, or many practical uses of modified or unmodified organisms in which we would not want the microorganism to disappear. For example, it would be disadvantageous for *Bacillus thuringiensis* to disappear after its release into the field. It might actually be desirable in some instances that organisms would not disappear. Many important agricultural uses of microorganisms will not involve microorganisms that we would expect to, or want to, disappear. For example, we may want to use them as delivery agents for toxic chemicals to kill insects on roots of plants, or in the control of important foliar diseases or pest organisms. In such cases, we would want them to be dynamic; to grow and be around whenever the plants are at risk of pests. It wouldn't be expected or desirable that such agents would disappear. Could we ever envision situations where we wouldn't want a microorganism to disappear? Why would we ever want to absolutely insist that a microorganism disappear? What traits would such a microorganism have that would make it so terrible that we would want every individual cell to disappear? This needs more careful thought, and I will return to it shortly.

There is a lot of technology now being developed to address some of the questions raised above. How can we assess how many microorganisms are present? We can get close to showing whether organisms are disappearing by new, very sensitive techniques which can measure as few as one microorganism per kilogram of soil. The question still remains: In what situation would we ever need or want such an answer? I would also like to promote a concept that I have been quite comfortable with, since it is not possible to predict the absolute behavior or absolute disposition of a microorganism once we release it outside the laboratory, because of the complexities of the environment into which it will move. We're not going to ever come up with one set of measurable parameters that can predict absolutely (in any kind of quantitative sense) the behavior of any individual organism. I don't really think that we need such an answer. I think that what is really needed, however, is a sense of the relative behavior of modified microorganisms compared to indigenous organisms for which we already have some information. We've been studying microorganisms, plants, and other organisms for a long period of time. We have domesticated many organisms for years in agriculture, quite successfully in most cases. Plant pathologists and agronomists and others in the biological fields have a long history of describing the occurrence and behavior of microorganisms under natural conditions. I believe that we need to address seriously how this past information will be useful to us in making future decisions as to the disposition of modified strains.

I also think we have to flavor our discussions on the potential and hazards of releasing microorganisms with the realization that we're really not as advanced in our ability to modify organisms as many people (in the lay public at least) have been brought to believe. That is, only modest changes in microorganisms or plants are possible in the foreseeable future. For example, a couple of genes can be added or a couple of genes deleted, or perhaps their expression may be changed. We're not talking about massive or unpredictable changes but rather modest, specific changes in organisms for which we already have some considerable information. These organisms are going to be ones that we already have some experience with, such as pathogens or pest control agents. I think that will make our task simpler, since we need look only at how our modified organism might differ from its parental srrains for which we know something already. That was basically the task which we faced when we modified our bacteria for frost control to plants. Since this was the first test of predicting behavior of modified bacteria, we had to look very closely at the fate and predicted effects of

our organism. I proposed to EPA that a deductive approach to the study of risk assessment be taken. For example, a comparative analysis of the organism with its parental strain should be made, and we should ask: (a) What is the expected behavior of the parental strain in situations that we would anticipate in our field site? and (b) In what ways do our modified organisms differ from the parental strain? We can deduce, within those environmental restrictions at least, what we might expect the modified microorganism to do in the future. I am not as comfortable with the prospects of coming up with any one set of guidelines or questions, such as: What is its likelihood of exchanging any one gene with any other microorganism?, as being predictive of any or all experiments, because we have to think of each microorganism in its unique setting and its use in a very particular environment. Microorganisms will actually occupy microhabitats, and we have to ask the questions that are most pertinent to those sites.

I would now like to address a little bit of deductive reasoning that we went through in the first release of recombinant microorganisms. I'll show you how we were quite successful in predicting what actually has happened now that we have released these microorganisms. I hope it also leaves you with some comfort that this approach will be at least partially applicable to most other organisms that we would anticipate releasing in the future. I don't want to dwell on our experiment *per se*, but instead to illustrate the reasoning that we used and the types of studies that we have done, and how these should be applicable to almost any microorganism. If you have not been away from the country for the last five years, you probably have heard at least something about our experiment in the popular press. We have tested recombinant bacteria for the biological control of frost injury to plants, which constitutes an obvious agricultural problem.

What we are interested in is the interaction of microorganisms with those plants. This should be a classic situation for which we can anticipate a lot of future experimentation. Many organisms live happily on plants, even on healthy plants. Microorganisms are very common and numerous in most sites, even on healthy green plants. We therefore addressed how we can change the balance of these microorganisms to achieve a reduction in those species that trigger ice formation and thereby cause frost injury. A lot of future experiments probably will pose a similar question of how we could eliminate potential pathogens from the leaf surface by preventing their growth in the presence of large numbers of competitive organisms. Plant epiphytes could be used to produce useful chemicals, plant growth regulators, insect toxins, and many other products that might be useful to the plant indirectly. So it is a habitat that is very important.

Our particular experiment was in itself rather straightforward. I won't go into great detail on the laboratory aspects of our study. We identified a single gene that is required by *Pseudomonas syringae* to form ice on plants. Over the years, we were able to determine that this one gene is responsible for the trait of ice nucleation, and we subsequently made a modification of this gene (created an internal deletion) that eliminated its ability to form ice. The modified gene that we constructed in the laboratory was reintroduced into a number of different strains that we had known to be very good at colonizing plants under field conditions. This was accomplished by a number of very specific genetic techniques whose use is not important for this forum. The process that we used was very specific and led to changes only in this one gene.

Similar laboratory work and the construction of potentially useful organisms is being done widely throughout the country. Their testing under field conditions has not been as widely pursued. In our case, we wanted to test the usefulness of our organism and to test ecological theory by using it under field conditions at a site in northern California (near the California/Oregon border). We obviously were dealing with a recombinant microorganism and, as such, had to obtain several federal permits to test our modified bacteria outside of the physical containment of our laboratory or greenhouse.

Dr. Dean has done a very good job of pointing out a number of the questions that agencies would ask when confronted with requests for field releases. Tests for fate, product performance, and purity — all of which we were required to address — are only some of the questions regulatory agencies asked to estimate the likelihood of hazard which might be encountered with the use of recombinant organisms under natural conditions. To return to our deductive approach, we said that we could never predict absolute numbers of organisms in any one place at any one time, but instead what we are going to show is that our organism would not be demonstrably different from its natural counterparts. If we could show that it would not differ significantly from the parental strain, or if it would differ in a predictable way, then we would feel safe in using it under those conditions. We therefore compared the strains in a large number of laboratory and greenhouse tests. I will not list all the tests, but the most important tests were designed to show that the modified organism's ability to grow, take up those places on the leaf, and compete for where it wanted to live on a leaf did not differ in any way from the parental strain. Mutant and parental strains did not differ in their ability to grow or compete on leaves or other habitats. The modified strains were shown not to be more aggressive in occupying a leaf in our voluminous data. Three hundred pages of data were generated to demonstrate, for example, that on all the myriad of different plant species (some 65 plant species that we examined that the microorganisms might likely encounter in the area around northern California where the test would be performed), the numbers of the mutant and parental strains survived similarly. The plants differed as hosts for *P. syringae,* but we were able to show that the modified bacteria and their parental strains did not differ in their preference for plants or in their ability to survive on any one plant. Our tests showed that the modified strains did not seem to have any ecological differences from the natural strains, that neither the modified nor the original strains survive in the soil, and that their numbers dropped dramatically after we put them into the soil. We therefore had a good feeling for how the microorganisms might behave after we introduce them. It was suggested by Martin Alexander that these organisms would be forever outside of our control. I only agree partially with such a statement. We had a fairly good indication of the numbers of these organisms that would be encountered in areas around the plot and, because of the information presented previously, what their disposition might be expected to be. We performed a number of preliminary studies in which the parental strains were released under conditions very similar to what we were actually going to do with the mutant strains in the field. We examined the numbers of our parental strains that were found at areas located around our plot, and, as expected, found highest numbers near where we released them and then decreasing numbers as we got further away from the test plots.

These preliminary studies gave us an indication of where the organisms would reside after release and therefore what plants they might encounter and what effect they might have on such plants. All of these considerations condensed to the same answer. We eventually convinced all the regulatory agencies and our peers in the scientific community that the organism did not pose any differential environmental behavior compared to natural strains. I think this often is going to be the case with modified microbes. The simple changes we are going to be making in the microorganisms are not going to confer either large differences or unexpected differences in their behavior. Those behavioral traits that we had identified in the laboratory were used in the design of an experiment in which we could have some good control over where the majority of the organisms would reside after release, and over how many would remain in the area after the test was complete.

Our experimental site, which was initiated in April 1987, consisted of a small plot of about a half-acre in size surrounded by a large bare-soil area (no plant material). Since we knew plants were the major

habitat for these organisms, we could ensure that there wouldn't be any place for the bacteria to grow if we surrounded the release site by a large buffer area.

We were required in EPA permits obtained for our experiment to generate much new data in support of future experiments by measuring the presence of our strains at many sites in and around the plot. This was done in order to obtain sufficient background information to reveal whether the predicted ecological scenario that we had developed was indeed indicative of what actually happened. We therefore did extensive monitoring of this plot.

The experimental plot itself was surrounded by a large buffer zone which we kept bare of plants to prevent organisms landing there during spray inoculation from having any place to grow. We used one liter of suspension containing about 3×10^9 bacteria per milliliter (a total of 3×10^{12} bacteria of each of two strains) that was first applied to potato seed pieces which were planted in the soil. After the seed pieces had sprouted and plants emerged, we then sprayed the plant surfaces. The EPA had a number of sampling devices in place to measure the physical environmental conditions at the time, and also to measure the numbers of microorganisms in the air around the plot and generate new data on the behavior of these organisms in the field. We also performed very extensive monitoring, to ascertain how our organisms were behaving and to compare with how they behaved in our greenhouse tests. Some 80,000 samples were taken during the summer of 1987 to measure the numbers of organisms at various places, at various times, and on various types of materials. I want to stress the very sensitive identification of our strains made possible by the various techniques that we used. We synthesized unique sequences of DNA which would recognize (were homologous to chromosomal DNA contained by the mutant strains) only the mutant strains that we had produced. Therefore techniques that have developed from the technology by which the strains were made were very helpful with the measuring and predictive ecology of the strains when field-tested.

A summary containing about 400 pages of data was submitted to the agency in late 1988 for their independent evaluation of the behavior of these strains. When we inoculated the plot, large numbers of microorganisms were dispersed around the plot. However, while there were large numbers of bacteria found in, and within about 10 meters of, the plot, very few bacteria were encountered more than 10 meters from the plot. The mutant bacterial strains didn't behave any differently in our actual field tests than parental strains or mutant strains had in our laboratory and greenhouse simulations. We were never able to measure the occurrence of our recombinant bacteria on plants and objects outside the plot. This was due to two mechanisms indicated in preliminary studies: (a) the bacteria were very strongly held in check because of the competition with all the other bacteria that were already present on the plants around the plot when we sprayed in late May, and (b) only very small numbers of mutant strains actually moved out to these surrounding areas. Ice$^-$mutant *P. syringae* strains did survive well on treated potato plants, however, as predicted. The numbers of the recombinant bacteria on treated potato plants were high for several weeks. Because of the high numbers of Ice$^-$mutant *P. syringae* on potatoes, a reduction in the incidence of plants that were damaged during several field frosts during 1987 was obtained. We were able to demonstrate (basically for demonstrational purposes only, and not because of any inherent risk) that the mutant bacteria could be largely eliminated at the end of the growing season from the experimental plot. Since both the natural and mutant strains occur exclusively on leaf surfaces, and since they don't occur in the soil, they could be eliminated by burning off the tops of the plants when they died and by removing all the potato tubers by digging and sterilizing them with steam at the end of the experiment. Bacteria were undetectable in the experimental site after removal of plant material.

The conclusion that I make, not only from our experiment but also from two other experimental releases of recombinant bacteria that were conducted during 1987 (one very similar to mine and an introduction of a modified soil microorganism) is that the laboratory measurements emphasizing particular ecological characteristics of the microorganism and the habitat to which it is to be reintroduced are largely predictive of what actually occurs after release in the environment. We shouldn't be interested so much in knowing the answer to many of the global questions asked previously, but instead seek better knowledge of pertinent questions applicable to individual strains. There are going to be overriding special questions that we're going to be asking of individual bacterial species and particular uses that are going to require much attention. Experience should permit later solution of many of these bigger global questions. We must always keep these larger questions in our minds, but they may not be applicable to each individual case.