SAP Report No. 99-04
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REPORT

FIFRA Scientific Advisory Panel Meeting, July 20-23, 1999, held at the Sheraton Crystal City Hotel, Arlington, Virginia

Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

***Session I - Burkholderia cepacia*: Risk Assessment of a Biopesticide with Affinities to a Human Opportunistic Pathogen**

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Session I - A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

Burkholderia cepacia: Risk Assessment of a Biopesticide with Affinities to a Human Opportunistic Pathogen

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Designated Federal Official
FIFRA/Scientific Advisory Panel
Date:_____

Ronald J. Kendall, Ph.D
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FIFRA/Scientific Advisory Panel
Date:_____

Federal Insecticide, Fungicide, and Rodenticide Act

Scientific Advisory Panel Meeting
July 20, 1999

Session I: *Burkholderia cepacia*: Risk Assessment of a Biopesticide with Affinities to a Human Opportunistic Pathogen

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INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed its review of the set of scientific issues being considered by the Agency regarding *Burkholderia cepacia*: Risk Assessment of a Biopesticide with Affinities to a Human Opportunistic Pathogen. Advance public notice of the meeting was published in the Federal Register on July 6, 1999. The review was conducted in an open Panel meeting held in Arlington, VA, on July 20, 1999. The meeting was chaired by Ronald J. Kendall, Ph.D, The Institute of Environmental and Human Health, Texas Tech University/Texas Tech University Health Sciences Center, Lubbock, Texas. Mr. Paul Lewis served as the Designated Federal Official.

Some microorganisms used for controlling pests may be opportunistic human pathogens, or closely related to opportunistic pathogens. Opportunistic pathogens are microbes that are capable of causing disease only in people who are immunocompromized or are otherwise especially susceptible. A critical issue concerns the pathogenic strains proposed for registration as biopesticides, since these strains are typically isolated from the environment, for example agricultural fields, rather than as clinical specimens. As such, these strains have no history of actually causing disease and may not be able to do so. Criteria for relatedness between clinical strains and biocontrol strains and the ability to predict pathogenicity of the biocontrol strains is therefore vital.

The Agency requested the SAP to address the sufficiency of current tests used to consider the risk from opportunistic pathogens to immunocompromized populations. *Burkholderia cepacia*, a biopesticide which may cause fatal infections with cystic fibrosis and chronic granulomatous disease, was used as a test case to examine the adequacy of animal models, taxonomic criteria, and criteria using known virulence genes as predictors of the pathogenic potential of individual strains, as well as issues related to the importance of levels of exposure and the nature of susceptible populations.

Douglas Gurian-Sherman, Ph.D. (Office of Pesticide Programs, EPA) opened the session providing an introduction on the risk assessment of *B. cepacia* strain RAL-3 and other biopesticidial bacteria related to human opportunistic pathogens. Chris Wozniak, Ph.D. (Office of Pesticide Programs, EPA) discussed taxonomy as a possible means of distinguishing human pathogenic from biological control strains of *B. cepacia*. Douglas Gurian-Sherman, Ph.D. (Office of Pesticide Programs, EPA) reviewed the use of virulence and pathogenicity traits in the risk assessment of *B. cepacia*. William Schneider, Ph.D. (Office of Pesticide Programs, EPA) summarized human host susceptibility and the use of animal models to distinguish opportunistic pathogen *B. cepacia* from non-pathogenic strains. Douglas Gurian-Sherman, Ph.D. (Office of Pesticide Programs, EPA) closed the Agency's presentation discussing requirements for strain markers of microbial biopesticides - monitoring fate in the environment prior to and after registration.

CHARGE

The specific issues to be addressed by the Panel are keyed to the background document "Risk Assessment of *Burkholderia cepacia* Based BioPesticides, and Other Bacteria Related to Opportunistic Pathogens" and are presented as follows:

1) Does the Panel agree that exposure determinations should be the key component of current risk assessment for biopesticidal use of *Burkholderia cepacia* (Bc)?

2) If so, what parameters should be included in the study designs, and what factors should be considered to conclude that there is a reasonable certainty of no harm to Cystic Fibrosis (Cf) patients from exposure to Bc based biopesticides? How much baseline data are needed to determine typical background populations of Bc? How should the fate of Bc be monitored after test applications, to determine whether undue exposure to CF patients will occur?

Are available assay and strain identification methods adequate for monitoring the fate of these strains?

Can data from studies addressed in the following questions (e.g. genomovar data) be used to set acceptable levels of Bc in the soil/rhizosphere or on the crop?

3) How can current knowledge of Bc taxonomy, genetics, and pathogenicity factors of clinical isolates be used in risk assessment of biological control isolates?

a) Genomovar I strains appear to have a low propensity to cause infection in CF patients, as reflected in the very low proportion of CF clinical strains identified as belonging to genomovar I. What additional data would allow EPA to use genomovar analysis for determining pathogenicity of biological control strains to CF patients? Could better characterization of the clinical outcomes from strains subjected to genomovar analysis allow this process to be used more fruitfully in risk assessment?

b) Are there identified virulence factors that may be used to determine the pathogenic potential of biocontrol strains of Bc? Is there a role for animal models in the identification of virulence factors? What is the best method or combination of methods to determine whether putative virulence or pathogenicity traits are important in the infection of CF patients?

c) Concern has been expressed that even if biological control strains of *Burkholderia cepacia* were found to be nonpathogenic, they might become CF pathogens or contribute to the pathogenicity of CF strains. Such concerns have been largely undefined, which makes them difficult to incorporate in the risk assessment process. What specific, currently available properties or traits can EPA use to evaluate the potential for biological control strains, even if nonpathogenic, to adversely influence pathogenicity to CF patients? [Traits that do not directly effect pathogenicity, but could allow higher exposure of CF patients, e.g. improved environmental fitness, may also be relevant].

d) Are any of the current animal models adequate and sufficiently validated (that is, not likely to give false positive or false negative results), for use in testing biological control strains of Bc for CF pathogenicity?

5) There are currently no specific criteria for strain markers for biopesticides. Such markers are useful or necessary to monitor biopesticides after registration. What might be the role of specific genetic markers, such as RAPDs or AFLPs, that can be used to quickly identify RAL-3 or other biological control strains related to opportunistic pathogens? What markers might be useful, and how should they be validated for sufficient specificity (e.g. how many strains should be used to develop confidence in their specificity)?

6) Is the CF patient**:**Bc, host**:**parasite relationship typical of what is commonly considered as infection by an opportunistic pathogen? Should any of the criteria or methods used to assess risk from Bc be applied to other biocontrol organisms which are related to opportunistic pathogens, if they are submitted for registration?

7) Does the SAP have any additional advise for BPPD in regulating microorganisms where some strains can be opportunistic pathogens?

PANEL RECOMMENDATION

In reviewing the available data regarding the ecology, taxonomy, and microbiology of *Burkholderia cepacia* (Bc), as well as the epidemiology and pathology of human infection due to

Bc, it is clear that many important questions remain unanswered. Such deficits in our understanding make definitive answers to the questions posed to the Panel impossible at this time.

Specifically, a majority of Panel members identified several areas in which lack of information significantly impedes the risk assessment of Bc as a biocontrol agent. These include: (i) background environmental levels of the various genomovars of the Bc complex; (ii) the fate of biocontrol strains after application; (iii) pathogenic mechanisms of Bc responsible for human infection; (iv) clinical outcomes data of Cystic Fibrosis patients relative to pulmonary colonization with Bc complex; and (v) interaction of introduced biocontrol strains with background Bc and clinical strains.

In light of these uncertainties, the majority view of the Panel was one that encourages a very conservative approach to the use of Bc as a biocontrol agent. Until more is known about the above issues, the majority of Panel members agreed that use of Bc in biocontrol programs is ill advised. A minority view held that the risk to CF patients of limited (below ground) usage of strains lacking putative pathogenicity traits is low.

DETAILED RESPONSE TO THE CHARGE

1) Does the Panel agree that exposure determinations should be the key component of current risk assessment for biopesticidal use of *Burkholderia cepacia* (Bc)?

Burkholderia cepacia (Bc) is a naturally-occurring bacterium which is present in relatively high populations in the soil, and in particular, the rhizosphere. Bc does not pose a risk to healthy individuals. The central question in evaluating risk is whether use of Bc as a biopesticide will result in increased exposure of CF patients to Bc. The question might be refined further to address whether the exposure of CF patients to pathogenic, or potentially pathogenic, strains of Bc is increased by biopesticidal use. The definition of pathogenic Bc remains problematic, however (see below).

Therefore, it is appropriate to base risk assessment on criteria that ensure, to a reasonable level of certainty, that exposure of such individuals to pathogenic strains of Bc will not be enhanced through pesticidal use. However, several concerns were expressed by the Panel regarding whether exposure should be the key component of risk assessment.

Intuitively, if commercial use of Bc does not increase exposure of susceptible persons above natural levels (page 19 of the Agency's background document), then there should be no increased risk, unless the introduced strain has greater pathogenic potential for susceptible hosts than other naturally occurring strains. However, it seems that inherent in commercial use is the production and application of large concentrations of certain Bc strains that will significantly increase levels above background, albeit for relatively short periods of time (several days) and in prescribed areas. In the view of some Panel members, the extent of this increase is difficult to

address given current uncertainties regarding levels following commercial application as well as natural background levels. In contrast, other Panel members expressed the view that Bc is common on root surfaces, and that the population sizes established by applied Bc strains have been studied quite extensively. These arguments are presented in some detail later in this report.

The notion that Bc are ubiquitous in the environment and therefore readily encountered by the CF patient is not accepted by all Panel members. Sampling of vegetables and soil that are likely encountered in daily activities has not resulted in high rates of recovery in some studies (Mortensen, et al, 1995; Butler et al, 1995).

Furthermore, LD_{50}s or ID_{50}s, which allow quantitation of risk of illness after exposure to a pathogen, (e.g. risk of gastroenteritis after exposure to *Salmonella* spp. verses *Shigella* spp.) are not known for Bc, so risk associated with various levels of exposure is not yet possible to quantify. Therefore, a safe level of exposure cannot be determined.

Several parameters will need to be explored further before exposure can be used as the key component of risk assessment:

1. The strain proposed for use. All strains and genomovars appear capable of causing infection in patients with CF, although some (i.e., genomovar III and *B. multivorans*) likely pose a greater risk.

2. Quantification of the level of exposure. If the level of exposure will not be above natural background levels, then it may not be considered a risk. However, natural levels of Bc are not clearly defined. In addition, recent changes in the taxonomy of this species require reinterpretation of previous studies of environmental prevalence of Bc. Additional systematic studies that examine a large number of isolates from various environmental sources and use methods to determine species within the Bc complex will have to be carried out in order to determine background levels of specific genomovars.

3. The level of exposure required to cause infection. This is not known, particularly for non-epidemic isolates in the natural environment. There is a better understanding of the risk associated with patient-to-patient spread of epidemic CF strains, but this is not known for non-epidemic strains, which currently account for the majority of colonization with Bc. Quantification of the natural exposure required to cause infection in patients with CF will be difficult.

4. Minimizing the exposure during use of biocontrol agents. With appropriate package labeling and application methods, it may be possible to reduce the risk of exposure to the concentrated biocontrol products. However, after application there will always be a transient increase in the level of Bc where the products are applied. Quantifying what risk this exposure constitutes will be very difficult if not entirely impossible.

Other questions that will need to be addressed include: How will exposure determinations

be made? How accurate are these? Will these be made on an ongoing basis? And, perhaps most importantly, will commercial strains with enhanced survival be developed or selected?

Also, consideration of exposure should not be limited to that between bacteria and susceptible human hosts, but also to that between different bacterial populations and natural mutagens present in the bacterial ecosystem that may indirectly increase risk of human infection (i.e., through genetic exchange between natural, biocontrol, and CF strains and displacement of natural populations).

In summary, a majority of the Panel believes that although exposure determinations should have a key role in risk assessment, several unanswered questions (particularly those pertaining to natural background levels and the level of exposure needed for infection) severely limit this approach.

2) If so, what parameters should be included in the study designs, and what factors should be considered to conclude that there is a reasonable certainty of no harm to Cystic Fibrosis (Cf) patients from exposure to Bc based biopesticides? How much baseline data are needed to determine typical background populations of Bc? How should the fate of Bc be monitored after test applications, to determine whether undue exposure to CF patients will occur?

The Panel agreed that a number of studies are needed to define background levels and to determine the period of time during which the population size of introduced biocontrol strains exceeds that of indigenous strains. Such studies are prerequisites for use of exposure determinations in risk assessment. These must be complemented, however, with tests evaluating the pathogenic potential of the biocontrol strain of Bc to be introduced into the environment (i.e., since strains likely vary with respect to virulence in CF, densities of biocontrol strains relative to indigenous populations alone are insufficient for risk assessment).

Studies that should be performed include:

1. Identify biopesticidal strains according to the state-of-the-art taxonomy of this group (determine species/genomovar by polyphasic tests, including biochemical tests, PCR, DNA-DNA hybridization, *recA* RFLP/sequence).
2. Document lack of currently known putative pathogenic factors: BCESM and cable pili
3. Determine PFGE fingerprint.
4. Monitor fate of introduced bacteria and of indigenous Bc in field studies using selective media (PCAT, TBT, BCSA), in combination with a stable genetic marker, or use a PCR-based approach on extracted soil DNA
5. Determine thev time required for introduced bacteria to fall below the limit of detection, and determine time until total Bc population returns to background levels.

It is well recognized that populations of bacteria in many natural substrates, including soil, are underestimated by conventional culturing methods typically used to estimate such populations. A number of investigators have estimated soil populations of Bc by conventional spread plate techniques, and these studies provide most of our current knowledge of background population sizes of Bc in soils. It is likely that populations of indigenous Bc are higher than have been estimated by culturing methods. In the rhizosphere, population sizes of gram negative bacteria estimated from direct counts are also somewhat larger than those estimated from culturing methods, but it appears that a greater proportion of viable bacteria can be cultured from the rhizosphere than from the bulk soil (Troxler et al. 1997).

The literature contains discrepancies regarding the ubiquity of Bc in nature, and the presence of bacteria in a viable-non-culturable state in soil and water may be one reason for this. Furthermore, studies have been done using different methods and various selective and non-selective media. Systematic studies estimating the population size of Bc by various methods are urgently needed to establish background populations and the composition of those populations with respect to genomovars. Methods based upon direct extraction of DNA from soil followed by PCR amplification with genomovar-specific rRNA primers should be compared to culturing methods for the detection and estimation of Bc. Bulk and rhizosphere soil should be sampled by accepted methods from a large number of locations (no less than 50) in agricultural fields, forest nurseries, and representative natural ecosystems.

Some data on indigenous Bc populations already exist for soil (Hagedorn et al., 1987; MacArthur et al., 1988, 1992), and water (Wise et al., 1995). Considerable data is available on Bc populations associated with plant rhizospheres, specifically peas (King and Parke, 1996), maize (Di Cello et al., 1997; Nacamulli et al., 1997), and grass (Nijhuis et al., 1993). At a minimum, populations of total indigenous Bc should be determined at the beginning and the end of the growing season for each crop and location by plating onto Bc selective media (PCAT, TBT, or BCSA).

Available data indicate that the introduction of specific biopesticidal strains of Bc do not change the total Bc population in these habitats for more than a few weeks. Instead, they tend to temporarily displace the indigenous Bc. Populations of biocontrol strains of Bc generally achieve a maximum within the several days after planting, and then generally decline rapidly over a period of a few weeks. This is a typical pattern for root-colonizing pseudomonads (Kluepfel, 1993).

Due to the presence of nutrients in root exudates, the rhizosphere provides a habitat for Bc and other rhizosphere bacteria. Populations of both introduced and indigenous strains of Bc will be higher in the rhizosphere than in bulk soil, so the best estimates of potential human exposure can be obtained by estimating rhizosphere populations.

The key question is how long do populations of the introduced strain exceed those of indigenous strains following seed treatment? This interval represents the only period of time following planting during which human exposure to Bc could conceivably be enhanced by the use

of the biopesticide. Various methods can be used to assess the relative population sizes of the biological control agent and indigenous strains, including spread plate techniques with selective media and PCR methods based upon direct isolation of DNA from soil. The relative detection limits of methods based upon culturing of viable cells and PCR amplification of DNA must be compared. Signals from PCR primers that amplify DNA from a specific biological control agent (with a marker gene, for example), specific genomovars of Bc, *Burkholderia* and *Ralstonia* spp., and all Gram-negative bacteria can be compared to standardize PCR reactions from different rhizosphere samples. Such comparisons will provide perspective on the relative contribution of the introduced strain to the total population of Bc in the rhizosphere. During the period of time in which the population size of the introduced strain exceeds that of indigenous strains (usually a period of 3-6 weeks), existing culturing methods, backed up with biochemical and physiological tests performed on representative colonies, are likely to be adequate for assessing environmental fate of the biological control strain. It is essential to a risk assessment strategy based upon exposure determinations to define this time interval.

Are available assay and strain identification methods adequate for monitoring the fate of these strains?

It should be feasible to track the biocontrol strain following field treatment by a combination of biochemical tests including substrate utilization and antibiotic resistance patterns. Selective media are available for strain recovery. A number of DNA based procedures are available for more rigorous strain identification. For example, PCR amplification of genes specifying 16S and 23S RNA followed by analysis of fragments generated by treatment of the PCR product with restriction enzymes that recognize 4-bp sequences has been used successfully in a number of labs for identification of clinical isolates (Mahenthiralingam et al 1999; Segonds et al 1999; van Pelt et al 1999). Also genomovar-specific primers are available for strain identification (Bauernfeind et al 1999).

Macrorestriction fragment analysis using PFGE would provide unambiguous identification of biocontrol strains. Consideration might be given to introduction of markers that would facilitate strain tracking. For example the *gfp* gene, which specifies green-fluorescent protein, might be a useful marker/reporter gene. The *gfp* gene has been introduced into the genome of a biocontrol strain of *B. vietnamiensis* on a mini-Tn*5* transposon (H.W. Zhou and T.G.Lessie, unpublished data) (Xi et al 1999). Transposants were detected readily on the basis of bright green fluorescence of the colonies when examined under uv light (365nm).

Can data from studies addressed in the following questions (e.g. genomovar data) be used to set acceptable levels of Bc in the soil/rhizosphere or on the crop?

It is not clear how acceptable levels for populations can be set. The one certainty is that levels of Bc in soil, the rhizosphere, or on the crop cannot be lower than what already exist in nature. Therefore, data on levels of indigenous Bc in soil and the rhizosphere are pertinent.

Reports on the natural abundance of Bc in the environment vary from infrequent (Mortensen et al., 1995; Butler et al., 1995) to very common (Hagedorn et al., 1987; King and Parke, 1996; Di Cello et al., 1997; Nacamulli et al., 1997). The discrepancy among researchers may result from the different selective media used to recover the Bc population as well as the habitats selected for sampling. Selective media used for isolation of clinical strains is likely to result in an underestimate of environmental isolates (Wigley and Burton, 1999). This is because media developed for isolation of clinical strains contain high levels of antibiotics to which environmental strains are susceptible.

Some data on indigenous Bc populations already exist for soil (Hagedorn et al., 1987; MacArthur et al., 1988) and water (Wise et al., 1995). Although Bc has been recovered from these and other habitats, it appears to reach the highest populations in the rhizosphere, or root zone of plants.

Bc is one of the most prevalent bacteria in the rhizosphere of perennial ryegrass (Nijhuis et al., 1993). Bc is also found at high population densities (10^4-10^5 cfu per cm root) in the rhizosphere of 4-6 wk-old pea plants (King and Parke, 1996). Maize appears to sustain particularly large rhizosphere populations of Bc (DiCello et al., 1997); it can comprise 4-35% of the total culturable bacteria from the maize rhizosphere (Hebbar et al., 1994).

Background levels of Bc in the soil environment range from 10^2-10^4 cfu/g soil. The addition of specific strains of Bc to seeds and root systems results in a temporary boost needed to protect these localized sites from pathogenic fungi. Some of these fungi, such as *Pythium*, infect seeds within 18 hr after planting (Parke, 1990). High populations of these bacteria during the first hours and days after planting are effective in preventing infection. In some cases the protective effect results from antifungal metabolites produced by the bacterium. After augmentation of the Bc population with artificially high levels of an introduced strain, the population declines to the normal carrying capacity of the seed or root (Parke, 1990; King and Parke, 1996). This is because exudates from the seed or root are not sufficient to sustain the higher population. Populations greater than the carrying capacity of the plant typically fall within hours, days, or weeks after application (Kluepfel, 1993).

Several investigations have focused on the fate of introduced populations of specific biocontrol strains of Bc in the rhizosphere. Strain 526 (ATCC 53267) applied to maize seed reached a maximum population at 15 days after planting (10^6 cfu/g dry wt root), and then declined steadily during the 60-day experiment to 10^4 cfu/g dry wt root (Hebbar et al., 1992). Similar results were found for strain MCI7. Introduction of MCI7 increased the rhizosphere population of total Bc only at the first sampling date (14 days after planting) but thereafter no difference in rhizosphere populations between treated and nontreated plants was detected. The population of MCI7 declined from an initial population of 10^7 cfu/g root to 10^3 cfu/g root 76 days after planting. King and Parke (1996) compared the population density of Bc in the pea rhizosphere among peas treated or not treated with the biocontrol strain Bc AMMDR1. The population of Bc fell to

background levels within 6 weeks after planting for 3 of the 4 cultivars studied. In contrast, populations of an introduced Bc strain (P2), selected specifically for its ability to colonize *Lolium perenne*, a perennial grass, maintained a stable population level for the 10-week duration of an experiment (Nijhuis et al., 1993).

Substantial data on indigenous population density of Bc in the rhizosphere and of the fate of specific, introduced strains allows estimation of the total number of Bc bacteria per hectare, either with or without augmentation with biocontrol strains. For peas, the population of Bc is approximately 1.5×10^{11} cfu/ha. For corn, the population estimate is 8.6×10^{9} cfu/ha. This is in addition to soil populations of indigenous Bc, estimated at approximately 7.5×10^{12} cfu/ha. Calculations are based on the following assumptions: **Peas:** 2500 pea seeds/lb x 250 lbs seeds planted/acre = 625,000 plants/acre x 2.47 acres/ha = 1.5×10^{6}plants/ha x 10^{5} cfu/plant = 1.5×10^{11} cfu/ha. **Maize:** 2500 seeds/lb x 14 lbs/acre = 35,000 plants/acre x 2.47 acres/ha = 8.6×10^{4} plants/ha x 10^{5} cfu/plant = 8.6×10^{9} cfu/ha. **Soil:** Assume 10^{3} cfu/g soil x 1.5 g soil/cm3 soil = 1.5×10^{3} cfu/cm3 x 10^{6} cm3/1m3 = 1.5×10^{9} cfu/m3. 1 ha=10,000m2 x 0.5 m deep = 5000 m3 x 1.5×10^{9} cfu/m3 = 7.5×10^{12} cfu/ha (top 0.5 m). **Deny:** 8.8×10^{9} cfu/29.6 ml x 500 ml = 1.5×10^{11} cfu/acre x 2.47 acre/ha.= 3.7×10^{11} cfu/ha.

Treatment of soil with the only registered product containing Bc (Deny) applied at the highest labeled rate would result in the addition of approximately 3.7×10^{11} cfu/ha, or less than 5% of the Bc population that is already present in soil. It is likely that the total population would decline to the normal carrying capacity within days or weeks.

Introduction of Bc strains to the rhizosphere results in a temporary, highly localized increase in the total Bc population. Thus, the exposure of CF patients to Bc applied to below-ground plant parts should not increase by the introduction of Bc in these specific habitats (soil, seeds, or roots). It is reasonable to require data from the registrant that demonstrates that Bc populations fall to background levels within a certain period after treatment. In contrast to the considerable body of knowledge on fate of introduced Bc in the rhizosphere, the data on persistence of Bc applied to the phyllosphere is currently insufficient. Determination of exposure resulting from application and drift of aerosols of Bc is completely lacking. Until studies of this kind are conducted, biopesticidal uses of Bc which involve application to above-ground plant parts should not be permitted.

Data obtained from further taxonomic, genetic, and pathogenicity studies of clinical isolates most certainly will add to our knowledge of Bc. However, these studies must also include soil and plant isolates to be valid. Such studies are important to determine if a safe isolate of Bc can be defined. One Panel member raised the question of common pathogenicity mechanisms between clinical and plant Bc and posed the following questions: Do plant pathogens and human pathogens share common pathogenicity genes? Can pathogens found in soil or plants serve as a reservoir for pathogenicity genes? This Panel member cited evidence that common pathogenicity factors exist among plant and clinical bacteria**.** *Pseudomonas aeruginosa* has been found to be infectious in an *Arabidopsis thaliana* leaf infiltration model and in a full-thickness skin

burn model (Rahme et al 1995). Mutations in several genes resulted in significant reduction in the pathogenicity of *P. aeruginosa* in both hosts. Another Panel member cautioned that the data of Rahme et al. (1995) do not indicate that plant and human pathogens are separated by few genes or characteristics. An alternate interpretation is that pathogenicity is intricately enmeshed in the physiology of the bacterial cell, such that the global regulatory and secretion genes identified by Rahme et al. (1995) are involved in multiple functions in *P. aeruginosa*.

Expanded coordinated studies are needed to address unanswered questions about Bc. Criteria that define pathogenicity are necessary before a safe strain can be defined, and only then can a determination be made of an acceptable level to introduce into the environment. However, once a safe strain is defined (if possible) then the question of gene transfer in the environment becomes a consideration.

The question of an acceptable biocontrol strain must be answered, before one can address the question of acceptable levels. Is there an acceptable manner in which to determine the potential pathogenicity of any Bc strain? The answer at this time is unfortunately, no. At present, there are no acceptable animal models and all known genomovars have been isolated from CF patients. No one study has defined pathogenicity for Bc. At present, traits associated with isolates from CF patients (e.g. BCESM, and cable pilus) have been identified. Genomovar I may pose the least risk but this information is based on only limited data. What data does exist suggest bacterial adaptation to microgeographical environments and extensive recombination in Bc (Wise et al 1995). Studies by Knudsen et al. (1988) using a Bc strain that contained a transmissible plasmid reported that when donor and recipient populations were 10^6 to 10^8 CFU/g on plant tissue or in soil, transconjugants in a range of 10^1 to 10^4 CFU/g were observed. This raises the question of levels to be introduced and survival of the introduced isolate in a given environment and the potential for gene transfer given the high levels that may be introduced. It is therefore imperative that we understand the ecology and population genetics of Bc in natural environments. Using organic soils obtained from onion fields under non-selective conditions, a total bacterial population of 10^8/gm of soil and a Bc population that ranges from 5 X10^2 to 1.5 X 10^3 using selective plating has been found (Gonzalez, unpublished). Of interest is that a bacteriophage to a genomiovar III isolate from soil samples has been identified. What are the implications? Until we do host range studies on this and other phages isolated, we can only speculate. One possible implication would be that soils may be a reservoir for genomovar III isolates.

The question of acceptable levels is a double-edged sword. One Panel member argues that acceptable levels should include those that would prevent plant disease and those that would prevent any chance that a non-pathogenic strain could become a pathogen given the opportunity. Also the question of how the bacteria will be applied is important. This same Panel member believes that many published studies using a Bc inoculum to compare its activity to known antifungals looked only at the end result without examining the fate of the microorganism in the natural environment. To address this question we must know something about the ecology of Bc and the long-term fate of the introduced isolates. To answer the question of what levels are necessary to prevent plant disease we must look at studies in which the treated seeds have been

challenged with a soil pathogen. Studies by Hebbar et al. (1992) using Bc to suppress disease in maize indicate that at least 10^7 CFU/seed are needed to suppress soilborne disease. Mao et al. (1997) reported high levels of protection at levels of 10^8 - 10^9 CFU of Bc /seed using maize seed and a combination of *Pythium ultimum* and *P. arrhenomanes*, and *Fusarium graminearum* as the challenge inoculum. Parke (1990) has stated that in pea seed, an initial inoculum level of 10^8 Bc /seed is necessary to effectively control disease caused by *Pythium*. Given the reported need for a high initial population of Bc to control disease, there does exist the opportunity for gene transfer based on the study of Knudsen et al.(1988).

Population sizes of Bc exceeding background levels are not acceptable on the harvested crop. We can not hope to decrease populations of either introduced or indigenous populations of Bc below those present naturally, unless fields are left fallow (in which case indigenous populations will not increase in the rhizosphere). Acceptable levels of the biological control agent should not exceed background levels of Bc on food products or, perhaps, in the rhizosphere at the end of a growing season.

The question of acceptable levels of Bc may also consider the threat posed to CF patients by naturally occurring background levels of Bc. This risk is unknown at present. One Panel member argued that iIndigenous populations of Bc in the soil and rhizosphere are quite high. This fact is not appreciated by many people who are concerned about the release of biopesticidal strains. Bc is already present in the environment, and in high numbers - far greater than would be added to soil through intentional release of biopesticidal strains. The only way to reduce these indigenous populations is to eliminate the rhizosphere by applying herbicides to agricultural fields, ornamental plantings, and native vegetation, something that cannot be done for obvious reasons. If we really perceive that environmental sources of Bc pose a danger to CF patients, then we should advise them not to encounter soil or plant rhizospheres. Yet the CF Trust Guidelines (UK) state that "vegetable and typical garden soils are not considered important sources of Bc. It is not necessary to avoid contact with onions and other vegetables." This is because these activities are perceived by medical epidemiologists as representing a low risk for acquisition of human pathogenic forms of Bc. Every child that plays on a lawn or digs in the garden is repeatedly exposed to high populations of Bc. Despite this, only a small percentage (<5%) of CF patients acquire Bc, and of these, only 1/3 die from Bc. Far fewer individuals are becoming infected with Bc now that strict procedures are in place to reduce person-to-person spread. These observations suggest that patient-to-patient transmission of virulent forms of Bc in the UK is a much more likely source of human infection than is acquisition from environmental sources.

Other Panel members counter that although the incidence of Bc infection has dropped in select CF centers after segregation of colonized patients (Thamassen et al 1986, Mahenthiralingam, unpublished), national (U.S.) surveillance data indicate that the incidence of Bc acquisition has remained stable during the past several years despite increasingly stringent infection control practices (CFF National Patient Registry). Furthermore, recent genotyping analyses (LiPuma, unpublished) of clinical isolates indicate that many (if not most) newly acquired isolates are genetically unrelated. These data are consistent with published results indicating that

strains from all genomovars of Bc can be associated with lung infections of CF patients (Vandamme et al 1997). These observations suggest that in addition to person-to-person transmission, acquisition of Bc occurs as independent events from unknown sources (perhaps the environment).

In summary, although data regarding natural levels of Bc in soil and various crop rhizospheres are available, the definition of "acceptable levels" of introduced biocontrol strains is dependent on defining the pathogenic potential of specific strains. Currently, this is not possible. Thus, most Panel members conclude that acceptable (i.e., safe) levels of biocontrol strains cannot be determined at this time. The threat to CF patients posed by background Bc populations is unknown at present.

A minority of Panel members define "acceptable levels" of introduced biocontrol strains as the background population size of indignenous Bc at some time interval after planting. These Panel members also recognize that the composition and pathogenic potential of indigenous Bc is unknown, but they point out that many existing agricultural practices, most notably planting crops (as described above), influence the size of indigenous Bc populations to a greater extent and for a greater time interval than has been observed following the introduction of biocontrol strains of Bc. In their opinion, basing the risk assessment of biocontrol strains of Bc on their potential influences on the composition of indigenous strains of Bc is inconsistent with existing knowledge of the ecology of this bacterium.

3) How can current knowledge of Bc taxonomy, genetics, and pathogenicity factors of clinical isolates be used in risk assessment of biological control isolates?

Taxonomy can be primarily used to assess potential for transmissibility and potential severity of infection, i.e. some evidence suggests that genomovar III causes more severe infections.

Current knowledge of genetics is insufficient to use in risk assessment. In the view of one Panel member, genome sequencing of at least some of the genomovar strains is critical in order to determine the important differences between these strains. The significance of multiple chromosomes in pathogenicity or biocontrol properties is not known. Although the presence of multiple insertion sequences suggests that there is potential for genetic transfer between the genomovars, the frequency or actual potential is unknown. Pathogenicity islands and phage are beginning to be identified. Genetic transfer appears to be variable between strains and genomovars suggesting that all genomovars/strains are not readily transformable. It is not known if biocontrol strains exchange genetic information with clinical isolates.

As with most opportunistic pathogens, there are a number of potential factors that may contribute to the pathogenesis of Bc infections in CF. These include LPS, cable pili, siderophores, hemolysins, proteases, lipases, BCESM, quorum sensing systems, and potentially

others. There is no clear correlation between the presence or absence of most of these factors and virulence, although BCESM and cable pili have been associated with epidemic strains. These potential virulence factors are present in some strains of all genomovars, environmental and clinical isolates and therefore may not be sufficient in distinguishing between virulent strains and safe biocontrol strains.

Taxonomy, genetic markers (such as the BCESM) and pathogenicity factors such as the cable pilus gene (cblA) can and have been used to identify strains/species of the Bc complex which currently constitute the greatest health risk to patients with CF (as measured by the predominance of certain strains types in CF).

However, taxonomy, genetic markers and pathogenicity factors cannot be used to define a safe strain with no pathogenic potential. All strains of the Bc complex should be considered to be potentially "unsafe" in terms of exposure to susceptible patients such as those with CF. The diversity of strains recovered from the sputum is high and encompasses all currently known groups, thus indicating that all have the capacity to cause infections in patients with CF.

Comments provided to the Panel from Dr. Peter Vandamme, University of Ghent, Belgium, on the use of taxonomy as a possible means of distinguishing human pathogenic strains from biocontrol strains of Bc are as follows:

"All Bc genomovars occur in CF patients and the environment. At this stage we can not define pathogenicity for Bc strains. Therefore presence in CF lungs is the only objective and absolute criterion I see as a risk factor. IF one accepts this then none of the Bc genomovars can be considered safe and taxonomy does not provide a means to separate safe/biocontrol strains from pathogenic Bc strains. The only other way to evaluate the data would be to look at the outcome of colonization/infection in the CF patients for each of the different genomovars. In that respect, one could classify genomovar III as the most dangerous one, followed by *B. multivorans*, and again followed by genomovars I, IV, and V. I don't have data to suggest an order for the last three. But how could the 'least dangerous one' be classified as safe??

In summary, the Panel concludes that although taxonomy may allow the identification of species that are more likely to cause infection in CF, clinical outcomes data are not yet sufficient to allow definitive conclusions about risk of specific species / strains. The current understanding of Bc genetics and pathogenic mechanisms is insufficient to be used in a meaningful way in risk assessment.

a) Genomovar I strains appear to have a low propensity to cause infection in CF patients, as reflected in the very low proportion of CF clinical strains identified as belonging to genomovar I. What additional data would allow EPA to use genomovar analysis for determining pathogenicity of biological control strains to CF patients? Could better characterization of the clinical outcomes from strains subjected to genomovar analysis allow this process to be used more fruitfully in risk assessment?

Genomovar I does not seem to colonize CF respiratory tract nearly as frequently as other species; Vandamme reports that a small proportion of strains recovered from CF sputum belong to genomovar I (Vandamme et al 1997). Therefore, it may be reasonable to speculate that genomovar I is less dangerous than the other Bc complex species. However, there are insufficient data correlating clinical outcome with genomovar and, more importantly, in the absence of knowledge regarding pathogenic mechanisms and virulence factors of *Burkholderia* species, it is not possible to determine pathogenicity of biological control strains. Anecdotally, other Bc complex species that are also infrequently encountered in CF patients (i.e., genomovars IV and VI) do seem to be associated with poor outcome (LiPuma, unpublished).

Dr. John Govan of the University of Edinburgh, Scotland, in written comments to the Panel indicated that "one of our Edinburgh patients who died was colonised by a *B. cepacia* belonging to genomovar I. I am sure that other centres have had the same experience." Such clinical outcomes associated with Bc argue against conclusions that genomovar I strains of Bc are safe, but some Panel members caution that these associations can not be interpreted as establishing causality.

Further correlation of genomovar and clinical outcome will provide important insight to possible pathogenic mechanisms, but is unlikely able to provide definitive answers regarding safety of specific species. This is particularly true with respect to closely related species that may readily exchange genetic elements involved in virulence, possibly resulting in strains with enhanced virulence. Sweeping conclusions about the safety of species seem to ignore the possibility of the emergence of virulent strains within species.

Genomovar analysis is useful to roughly predict the likelihood of disease from a particular isolate, but it clearly cannot be used to confirm the absence of risk. Since all genomovars cause disease at some level, this analysis cannot lead to the qualitative statement that a strain is safe. At present, we know little about the mechanisms of pathogenesis or how those mechanisms may vary among genomovars. Strong correlations have been made between clinical outcome or transmissibility and certain determinants such as the cable pilus and BCESM locus that are prevalent in genomovar III. In the absence of data on the function of these determinants, however, their predictive value is limited. For example, the BCESM locus appears to specify a regulator that may control the expression of some as yet unknown virulence factors. A strain without BCESM could conceivably convert to the more virulent form simply by a by-pass mutation that constitutively expressed the relevant genes. In fact, the well-documented presence of IS elements capable of activating adjacent genes makes such mutations quite common in Bc. As more isolates are characterized in greater detail, the number of genomovars is increasing. Therefore, it would take considerable additional data on the basic nature of pathogenicity and the stability of such traits within genomovars.

Better characterization of clinical outcome with analysis of genomovar status would make this analysis more useful; however, such characterization is extremely difficult. Again, our present ignorance of pathogenic mechanisms makes it impossible to delineate the contribution of specific

bacterial and host factors. An additional complication is the documented interplay between Bc and *P. aeruginosa* via cross-regulation of virulence genes and stimulation of host immune functions. Since *P. aeruginosa* colonization rates are quite high, these interactions must be considered.

Koch's postulates cannot be carried out on Bc to demonstrate disease causality by particular strains or genomovars. Virulence factors for Bc are only partially understood. Therefore we must rely heavily on retrospective data which provide correlations between genomovars isolated from CF patients and their clinical outcomes. This correlative data is not perfect because it fails to take into account host factors that influence disease, as well as confounding factors such as infection by multiple species and strains.

Nevertheless, this correlative data provides much-needed information on risks associated with different genomovars. For example, data from Vancouver on the epidemiology of Bc in Canadian CF patients (Henry et al., 1999 IWGBC Abstr.) shows that for 866 isolates of Bc complex recovered from 448 patients, genomovar III was recovered most frequently (82.6% of patients), *B. multivorans* was recovered from 8.2% of the patients, and genomovars I, IV,and *B. vietnamiensis* together were recovered from 4.7% of the patients. Clinical outcomes further established mortality rates for patients colonized by genomovar III (46% mortality) and *B. multivorans* (15% mortality rate), the only patient groups with sufficient individuals to permit statistical analysis. Epidemiological data from Toronto and Philadelphia also indicate that genomovars I, IV, and *B. vietnamiensis* are only rarely encountered in isolations from CF patients. Most biopesticidal strains being considered for commercial development are genomovar I or *B. vietnamiensis*. In assessing risk associated with use of release of biopesticidal strains, it would therefore be extremely useful to have clinical outcome data for CF patients in North America and Europe from whom these genomovars were isolated. It would also be important to know if these patients colonized by genomovar I or *B. vietnamiensis* had other predisposing factors, such as colonization by other Bcc genomovars or *P. aureofaciens*.

The Panel concludes that genomovar data alone is currently insufficient to allow the definition of safe strains. Additional information about pathogenic mechanisms and clinical outcomes associated with specific species/strains is needed.

b) Are there identified virulence factors that may be used to determine the pathogenic potential of biocontrol strains of Bc? Is there a role for animal models in the identification of virulence factors? What is the best method or combination of methods to determine whether putative virulence or pathogenicity traits are important in the infection of CF patients?

There is likely not a presence or absense of identified virulence factors in biocontrol vs. pathogenic strains. There may be differences in gene expression and regulation that account for increased virulence. Little is currently known about virulence factor genes and their regulation in Bc. There are also host factors that influence patients susceptibility to infection.

Although there are no natural animal models of Bc infection, there is a role for animal models in the identification of virulence factors. Models could be used to assess the potential virulence of biocontrol strains compared to genomovar III or other clinical strains to see if there is a difference in colonization, persistence and pathology. This has not really been assessed. For example, not many genomovar I strains have been tested for virulence in animal models. Most strains of known origin with reported testing in animal models are genomovar III or genomovar II.

The major role for animal models may be to assess contributions of specific virulence factors in virulence using genetically defined strains. The other major use is to test the efficacy of specific anti-Bc therapies, such as antibiotic treatment, anti-inflammatories, protease inhibitors and vaccines.

It is important to use a combination of methods to determine the role of putative virulence markers in CF infections. Defined genetic mutants and wild type strains should be compared to assess virulence. Virulent clinical isolates should be used as wild type or parental strains. It is also necessary to use genetically manipulatable strains and many strains of Bc are not easy to genetically manipulate. Other approaches to identify virulence factors include antibody studies, passive and active immunotherapy. *In vivo* expression technology (IVET) or signature tagged mutagenesis studies (STM) may also be useful tools in identifying potential virulence factors.

One Panel member suggests that in addition to animal models, plant models may also be useful for assessing the virulence of Bc. For example, the water soaking model assesses the ability to cause electrolyte leakage. Genomovar III strains have been found to have this characteristic. It is possible that the electrolyte leakage could also be a contributing factor to disease in CF patients.

c) Concern has been expressed that even if biological control strains of *Burkholderia cepacia* were found to be nonpathogenic, they might become CF pathogens or contribute to the pathogenicity of CF strains. Such concerns have been largely undefined, which makes them difficult to incorporate in the risk assessment process. What specific, currently available properties or traits can EPA use to evaluate the potential for biological control strains, even if nonpathogenic, to adversely influence pathogenicity to CF patients? [Traits that do not directly effect pathogenicity, but could allow higher exposure of CF patients, e.g. improved environmental fitness, may also be relevant].

The Panel disagreed about the degree to which biocontrol strains may be able to influence the pathogenicity of strains that infect CF patients. Most members agreed that because the definition of any Bc strain as nonpathogenic in a susceptible host is not yet possible, this question is very difficult to answer. Nevertheless, features relating to the potential for horizontal genetic transfer are a cause for concern.

One Panel member presented an interpretation of what is and what is not known about Bc

as follows:

WHAT IS KNOWN:

a. Bc harbor mobile genetic elements - phages, plasmids, transposons and insertion sequences. These have the ability to transfer between strains/species and potentially contribute to virulence/pathogenicity.

b. Biocontrol strains have been isolated as a direct result of their "environmental fitness". Their ability to colonize crop rhizospheres, survive and protect crops has been enriched by the selection procedures used in their isolation. Biocontrol strains are generally good rhizosphere colonizers and this is integral to the way biocontrol is mediated.

c. Bc biocontrol strains are innately resistant to several antibiotic classes (a basic phenotype shared by all members of the Bc complex). Pathogens with multidrug resistance are considered highly virulent since treatment of disease becomes very problematic.

d. Some Bc strains/genomovars appear to have very plastic genomes and hence may be more likely to alter rapidly in the natural environment. eg. *B. multivorans*. The multiple replicon genome structure may enable unusual genomic rearrangements to occur.

e. Genomovar conversion as a result of such genomic alterations has not been documented and is unlikely to occur.

WHAT IS NOT KNOWN:

a. The pathogenic capacity of biocontrol strains in susceptible hosts or animal models.

b. The extent to which DNA is exchanged by Bc in the natural environment to enable transfer of pathogenic traits.

c. Whether the mechanisms and factors required for rhizosphere colonization are also required for colonization of the CF lung. Could there be an overlap in the factors expressed for colonization of both environments? These would be considered virulence or pathogenicity factors in relation to human infection.

Bc is found in the rhizosphere and not within plant tissue. An analogy to the CF lung may be drawn. In the CF lung, Bc colonizes an "out of body cavity" and yields nutrients from surrounding tissues and dying inflammatory cells. Hence the possibility of some overlap in the factors required to colonize and survive in both lung and rhizosphere may still be raised. Studies to demonstrate this have not been published, but studies that indicate overlap between *P. aeruginosa* plant and human virulence factors have been documented (Tan et al. 1999; Rahme et al 1997)

d. Whether artificially increasing the levels of a highly antibiotic resistant organism in the natural environment contribute to the emergence of antibiotic resistance in other known or opportunistic pathogens.

e. The exact degree of genetic relatedness between biocontrol strains and commonly encountered CF strains.

f. The potential for high concentrations of biocontrol isolates to alter their pathogenicity once in contact with the natural environment and naturally occurring mutagens.

Although most Panel members agreed that these unanswered questions are worrisome and warrant a conservative approach, other members do not support these interpretations and provide the following arguments:

Genetic instability. The genetic instability of Bc in culture has been well documented, but the genetic instability of biological control strains has not been evaluated, nor has the genetic instability of any strain been evaluated in a natural environment. Clearly, environmental factors influence the physiology of bacteria, with consequent effects on processes such as plasmid transfer that bring about genetic change. Furthermore, the environment poses a major factor in selection for or against bacterial variants resulting from insertions, deletions, or rearrangements of DNA in the genome of Bc. The diversity of environmental strains in nature indicates that Bc changes genetically over an evolutionary time scale. The question with respect to risk assessment is whether genetic change of an introduced biological control strain during the limited period of time in which localized populations on seed and root surfaces exceed background levels is significant, given the genetic change that already occurs within much larger sizes and distributions of indigenous populations of the bacterium. Given the wide distribution of Bc in the rhizosphere, water, and bulk soil, a transient and localized population of a naturally-occurring biological control strain is not likely to contribute to the genetic diversity of this bacterium in nature.

Transfer of DNA between introduced and indigenous strains of Bc. Numerous studies have demonstrated that DNA transfer can occur between bacteria in the rhizosphere. Studies evaluating genetic structure of Bc provide further evidence that DNA transfer has occurred over evolutionary time. The frequency of such transfer is influenced primarily by the relative population sizes, spatial proximity, and intrinsic conjugal characteristics of donor and recipient strains; as well as the selective advantage conferred upon exconjugants relative to parental strains. Most studies evaluating the frequency of DNA transfer between two strains have been done in systems designed to optimize this frequency. For example, donor and recipient strains typically are coinoculated onto the same sites on seed or root surfaces in roughly equivalent populations, which promotes plasmid exchange. Such experiments overestimate the frequency of plasmid transfer that may occur between an introduced strain of Bc and other components of the microflora. Nevertheless, it is reasonable to expect that DNA transfer will occur at some frequency among biocontrol strains and other components of the rhizosphere microflora. Again, our concerns regarding such transfer should focus on the discrete space and period of time in

which populations of the biological control strain exceed a background level of Bc. Once populations of the introduced strain reach an equilibrium with other members of the species, its contribution to DNA exchange is unlikely to differ from that of indigenous strains in the rhizosphere. Again, given the wide distribution of Bc in the rhizosphere, water, and bulk soil of many ecosystems (not just agricultural fields or forest nurseries), a transient and localized population of a naturally-occurring biological control strain is likely to contribute minimally to the genetic diversity of this bacterium in nature.

Influence on gene expression through quorum sensing. It is possible that populations of a biological control strain of Bc on seed and root surfaces could cause a transient alteration in gene expression by other bacteria (including indigenous Bc) due to the process of quorum sensing. Bc is known to produce *N*-acyl-homoserine lactones that function as inducers of certain genes expressed as a function of cell density, and cross-feeding of *N*-acyl-homoserine lactones is known to occur among bacteria in the rhizosphere (Pierson et al. 1998. Molecular Plant-Microbe Interactions 11:1078-1084). Mediating factors that should be considered in evaluating this possibility include the following: 1) Bc appears to recognize autoinducers produced by other rhizosphere organisms, so an influence of a biological control strain on gene expression by indigenous Bc will occur against the background contributed by indigenous bacteria of different bacterial genera; 2) *N*-acyl-homoserine lactones induce gene expression only when they are present at critical densities. They are not known to have a persistent or permanent influence on the phenotype of a bacterium.

Factors contributing to the fitness of saprophytic strains of gram-negative bacteria, such as those used for biological control of plant disease, in the rhizosphere have been identified primarily by comparing the population size and dynamics of mutants deficient in a specific phenotype to those of the parental, wildtype bacterial strain. For example, antibiotic production (Mazzola et al. 1992. AEM 58:2616-2624), and siderophore utilization capability (Raaijmakers et al. 1995. Can J. Microbiol. 41:126-135) can positively influence the population size established by fluorescent pseudomonads in the rhizosphere. The capacity to survive exposure to environmental stress, notably oxidative stress (Kim et al. 1998. Phytopathology 88:S48; Sarniguet et al. 1995. PNAS 92:12255-12259) and desiccation (Stockwell et al. 1998. Phytopathology 88:S85), also contribute to the fitness of fluorescent pseudomonads in the rhizosphere. The capacity to utilize a nutrient that is present in the rhizosphere but is not commonly utilized by other components of the rhizosphere microflora can also contribute to the population size of a bacterium in the rhizosphere (Colbert et al. 1993. AEM 59:2064-2070; Savka and Farrand. 1997. Nat. Biotechnol. 15:363-368). Successful root colonization by a biological control strain of *Pseudomonas fluorescens* involves amino acid biosynthesis, rapid growth rate, utilization of organic acids, lipopolysaccaride, and a NADPH::ubiquinone oxidoreductase activity (Simons et al. 1997. Molecular Plant-Microbe Interactions 10:102-106; Dekker et al. 1998. Molecular Plant-Microbe Interactions 11:763-771). The capacity to utilize amino acids is also a factor allowing biocontrol strains of *Enterobacter cloacae* to grow on seed surfaces (Roberts et al. 1996. Soil Biology & Biochemistry 28: 1015-1020).

The Bc genome is highly plastic, so genetic rearrangements involving IS elements or lateral transfer of pathogenicity islands could conceivably occur if a human pathogenic strain could grow in soil at a high density, close to a biopesticidal strain. The modified soil strain would then have to maintain this genetic change while continuing to dwell in soil, where there would be no selective advantage in retaining genes for human pathogenicity. It would then need to find its way back into the lungs of a CF patient. This scenario seems unlikely.

Bc in soil and water is very diverse genetically; apparently more so than human clinical strains (Wigley and Burton, 1999; MacArthur et al., ; Butler et al., 1995; Wise et al, 1996; Mahenthiralingam et al, 1996). For example, among 217 Bc isolates collected from a 5 km stretch of blackwater stream, Wise et al. (1996) detected 65 unique electropherotypes based on multilocus enzyme electrophoresis. The genetic diversity of Bc in the maize rhizosphere also appears to be extremely high. Of 83 Bc isolates recovered from maize, 68 distinct RAPD haplotypes were found (Di Cello et al., 1997). Twenty-one environmental strains collected from South Wales were found to each have a unique PFGE profile and ribotype. In a comparison of RAPD fingerprinting of 627 Bc isolates, Mahenthiralingam et al. (1996) found that only 58 of the 525 isolates from CF patients had unique fingerprints. This is in contrast to 16 unique fingerprints among 44 Bc isolates from non-CF patients, and 21 unique fingerprints among 58 environmental isolates. In certain CF treatment centers, the majority of Bc isolates belong to a limited number of clones. For example, of 866 isolates (448 patients) examined in a Vancouver study, genomovar III was recovered from 82.6% of the patients, and of these, 74.9% were colonized by a single strain, type 02 (based on RAPD and PFGE). Recovery of single strain types from multiple individuals suggest that patient-to-patient spread of virulent, highly transmissable strains is responsible for most CF infections, rather than adaptation of diverse environmental strains to colonize human lungs. The relative homogeneity among clinical isolates in comparison with the greater diversity among environmental isolates argues that populations occupying these two different habitats are separate and genetically distinct despite high populations of indigenous Bc in the environment. Therefore the potential for biocontrol strains to contribute genes important for increased pathogenicity or fitness in the human lung is remote.

Many traits have been shown, through mutant analysis, to contribute to environmental fitness of rhizosphere bacteria, especially pseudomonads. These include: adhesion, antibiotic production (and resistance to antibiotics), siderophores (particularly the ability to utilize siderophores from other microbes), ability to utilize unique rhizosphere substrates, motility (perhaps), stress tolerance, and quorum sensing. However, transfer of traits from biocontrol strains to human pathogenic strains would require that they co-occur at populations high enough, and long enough, for the frequency of genetic exchange to be significant. It is unlikely that these two distinct populations could both proliferate in either the lung or the rhizosphere long enough for this to happen.

In summary, while most Panel members expressed serious concern about how introduced biocontrol strains may influence other Bc strains, a minority held that interaction between strains resulting in increased risk to CF patients was not likely to be enhanced appreciably due to the proposed introduction of biocontrol strains of Bc. The Panel recognizes that because studies to

more precisely define the degree to which gene transfer might occur between introduced biocontrol strains, background strains and strains that infect CF patients are lacking, at present we can only speculate about the likelihood of such events.

Animal Models

There are a number of animal models that have been used to study the virulence of Bc. The agar bead model of chronic infections has been used in both rats and mice (Cash, 1979; Starke, 1987; Sokol ,1988). Acute and chronic histopathology changes in lungs from infected animals resembles that seen in CF patients in this model. This model has also been widely used with *P. aeruginosa*. Long term chronic infections can be established in this model. Pathology, inflammatory markers, antibody response as well as bacterial numbers can be monitored in this model. Studies with genetic mutants have been used to identify virulence factors and have shown absence of some factors result in reduced virulence. These studies have been done with clinical strains but biocontrol strains or environmental isolates have not been tested to see if the model could be used to test virulence. One of the drawbacks of this model is that it bypasses the normal route of colonization since the bacteria are introduced directly into the lung. If there are differences between genomovars in terms of colonization, these may not be apparent in the agar bead models.

Pulmonary infections have also been induced in neutropenic mice pre-treated with cyclophosphamide. Yamagishi (1993) described aerosol and transtracheal inoculation with Bc but only transtracheal inoculation of Bc resulted in pneumonia. Aerosol inoculation of Bc was rapidly cleared from the lungs. The genomovar type of strain used is not known. Sokol et al (1999) compared aerosol inoculation of an ornibactin biosynthesis mutant and parent strain in neutropenic mice and determined that the ornibactin mutant was unable to colonize lungs compared to the parent genomovar III strain. Could this model be used to assess colonization ability of biocontrol strains? This has not been adequately assessed, but the model does have potential to differentiate between strains that can colonize and strains that are poor colonizers of mouse lungs.

Pulmonary infections have also been induced in CF Mice (Davidson 1995). These mice had an impaired capacity to clear Bc compared to CFTR + mice. CF mice, however, as is true for other rodents, are not readily susceptible to infection with Bc or *P. aeruginosa*. This may be due to the presence of an alternate chloride channel expressed in the lung epithelial cells. These mice are expensive, not readily available and difficult to maintain. Also, there may be differences in susceptibility between different *cftr* mutations.

A burn model developed by Stover et al. (1993) was used to show that Bc could persist in mice with burn wounds. This model may be useful for testing persistence of Bc but its relevance to CF would be questionable.

An ip injection-spleen persistence model in C57/Bl or BalbC mice has been described by

Speert (1999), however, only *B. multivorans* persists in this model, not genomovar III. Although this model may be relevant to CGD, its relevance to CF infections may be questionable.

4) There are currently no specific criteria for strain markers for biopesticides. Such markers are useful or necessary to monitor biopesticides after registration. What might be the role of specific genetic markers, such as RAPDs or AFLPs, that can be used to quickly identify RAL-3 or other biological control strains related to opportunistic pathogens? What markers might be useful, and how should they be validated for sufficient specificity (e.g. how many strains should be used to develop confidence in their specificity)?

Specific genetic markers would be essential in monitoring the levels of the biocontrol agent and well as its long-term persistence in the environment. If the organism remains in the environment at high levels for extended time, the possibilities for mutation, adaptation, genetic exchange with indigenous organisms, etc. are vastly increased. Selectable markers such as drug resistance and catabolic functions would be the easiest to monitor, but it is unclear whether such markers could be developed for this strain to the exclusion of related soil bacteria. The high level of drug resistance in Bc and the highly plastic genome make it unlikely that selection based on drug markers alone would be sufficient. A combination of drugs and biochemical functions similar to that recently developed for identification of Bc likely could be developed for a given strain, but the problem then becomes detection in soil samples. For monitoring purposes, it is essential that the organisms in the soil can be quantitatively identified even if they are relatively dormant. Production of drugs may be turned off after prolonged culture in the soil or rhizoshpere, and drug resistance may not be readily reactivated.

Organisms obtained in culture can be subjected to RAPD analysis or genomic RFLP using PFGE. These analyses would be especially useful for short term monitoring. They would also provide some indication of the strain's genomic stability during growth in the environment. For long term monitoring, the likelihood of rearrangements and large deletions would result in shifting patterns. At what point is a pattern considered a mutation or a new strain? RFLPs and AFLPs might be difficult in distinguishing the organism from the background population, depending on the strain. On the other hand, some PCR methods of Bc strain identification are becoming well developed. Ribosomal genes generated by PCR are analyzed by RFLP of the 16s gene or the spacer region between the 16s and 23s genes. The inherent problem with such analysis is the paucity of changes when comparing closely related strains. The spacer region would provide the highest discrimination. Comparison of coding regions, such as the *recA* gene provide more statistically relevant data. The *gyrB* gene has also been used to distinguish strains of *P.aeruginosa*. The genes can be amplified using highly conserved primers. RFLP analysis of the products may prove sufficient for monitoring. If not, it may be necessary to develop primers that are relatively specific for the strain. Ultimately, the introduction of one or, better yet several, specific DNA markers would be the simplest, clearest method to follow the strain both short and long term. An inert, short DNA sequence could be introduced that would allow positive identification both in the field and to monitor potential transfer to humans. Such a marker should be introduced at multiple locations to essentially eliminate the possibility that it would be deleted.

The largest practical reference strain set should be used to confirm detection of the biocontrol strain regardless of the marker chosen. Such strain sets are being developed. Unless a unique marker is used, it will not be possible to make an absolute determination of strain identity in the field.

5) Is the CF patient:Bc, host:parasite relationship typical of what is commonly considered as infection by an opportunistic pathogen? Should any of the criteria or methods used to assess risk from Bc be applied to other biocontrol organisms which are related to opportunistic pathogens, if they are submitted for registration?

There may not be a consensus definition of a typical opportunistic pathogen. There are, however, common themes that apply to opportunists as discussed in the Agency's background document (e.g., that opportunists are able to infect their host only under certain conditions and/or only infect a small subset of the total host population [pp. 8 & 19]). Opportunistic pathogens typically are nonpathogenic or have low virulence for normal, i.e., healthy, hosts. These features apply to infection of CF patients by Bc. Bc is generally not pathogenic for healthy humans. Humans do not seem to be colonized with Bc for any appreciable time following encounter, nor do they seem to become infected. Persons with CF do become colonized and many, *but not all*, will manifest infection, marked by pathology and an inflammatory response. The observation that CF host response to Bc colonization (and perhaps to encounter per se) is markedly variable may distinguish this host:pathogen interaction from that of some other opportunists where clinical outcome may be more predictable. This also confounds identification of critical virulence determinants.

Perhaps the most relevant feature of opportunists is the inability to predict when and how they will emerge (or when a new vulnerable host population will emerge). Therefore, it seems unreasonable to indelibly define species as nonpathogenic. This is at best a relative definition and, as it pertains to any given species, should remain open to revision as circumstances warrant.

Criteria to assess Bc as well as other potential opportunists should include ongoing surveillance for emerging vulnerable human populations and/or emergence of strains with enhanced capacity for causing human infection.

The identification of virulence factors in opportunistic pathogens is very difficult because by definition the susceptible host is not able to mount a normal response, and Koch's postulates do not apply to disease caused by such organisms.

Another typical aspect of many opportunistic pathogens is that they are closely related to other species within the same genera which are primary pathogens. For example also within the *Burkholderia* genus are the primary pathogens *Burkholderia pseudomallei* and *Burkholderia mallei*. Other genera that consist of both primary and opportunistic pathogens include Mycobacterium species, *Neisseria* species and *Streptococcus* species.

6) Does the SAP have any additional advise for BPPD in regulating microorganisms where

some strains can be opportunistic pathogens?

EPA should consider the risk of exposure by individuals who handle large concentrated quantities of biopesticides such as those who ferment, blend, package and handle Bc preparations.

Pesticide labeling is an accepted method to reduce human exposure to many types of herbicides, fungicides, rodenticides, and insecticides. Some of these products are potentially quite dangerous (e.g. snail and slug killer, rat poison); not just to compromised individuals, but to all individuals. They are widely available to the general public. Warning labels on these products instruct the user to apply the product in a safe way. Society is willing to accept the risks associated with broad target pesticides as long as the products are used according to label directions. Explicit cautionary labeling on biopesticide products, such as the language on the Deny label, should be adequate to minimize exposure of CF patients to Bc, during application of these products.

Many people equate biological control and biopesticides with the introduction of an exotic organism into an environment where it was not found previously. A common perception is that a biological control organism, once introduced, will establish a persistent population in environments where it was never found before. Indeed, certain organisms, notably predators and parasites of insect pests, have been introduced into new environments for biological control. This approach is called classical biological control, achieved by importing a pest's natural enemy from a remote location. Importation is only one of three general categories of biological control, with the other two categories being augmentation and conservation of biological control organisms. These latter two categories are far more important than importation for biological control of plant pathogens, and both approaches are based upon biological control organisms that already exist in the environment of interest. The use of Bc falls into the category of augmentation, in which populations of biological control organisms that already exist in the environment are augmented by adding more of these organisms to the plant surface at a specific time. Augmentation achieves the purpose of placing indigenous biological control agents on the plant at the right time and place needed to protect the plant from disease. Unlike the more commonly known importation approach, biological control microorganisms introduced by augmentation typically decline to background levels within a discrete period of time, because these microorganisms are subject to the same competitive pressures that keep other microbial populations in check in natural systems. Valid risk assessment can not consider all biopesticides in the same way. Instead, risk assessment must consider the ecology of the biological control organism in the target environment, as the type of biological control must certainly influence most, if not all, answers to the questions posed above.

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