

US EPA ARCHIVE DOCUMENT

RISK ASSESSMENT OF *Burkholderia cepacia* BASED BIOPESTICIDES, AND OTHER BACTERIA RELATED TO OPPORTUNISTIC HUMAN PATHOGENS

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Introduction:

The purpose of this paper is to explain the basis for the risk assessment conducted for microbial pesticide products containing *Burkholderia cepacia* strain RAL-3, and to discuss the scientific issues that have informed this risk assessment. More broadly, by considering the specific case of *B. cepacia* (Bc) strain RAL-3, we hope to address inadequacies in the current risk assessment process for Bc, and to the extent possible, for other biocontrol organisms which may have affinities to opportunistic human pathogens.

Although Bc can occasionally infect noncompromised humans and is a serious pathogen of people with the rare congenital disorder, chronic granulomatous disease, this review will confine itself to the consideration of pathogenicity to cystic fibrosis patients as an example of the human risk issues. Considerable research on the involvement of Bc pathogenicity in CF, which can be applied to risk assessment, is available compared to pathogenesis of Bc in other patients, where relatively little data is available. Most importantly, we believe that important risk issues to non-CF patients can be addressed through consideration of CF as an example.

Biopesticides include microorganisms which are used commercially for the control of pests and must be registered by EPA (FIFRA sections 2 (u) and 3, and 40 CFR sections 152.3(i) and 158.65). General risk assessment data requirements for the registration of biopesticides are listed in 40 CFR section 158.740. Data requirements are further elaborated in guidelines of the 885 series.

Bc is a gram negative bacterium belonging to pseudomonas rRNA homology group II, and was first described in 1949. Previously named *Pseudomonas cepacia*, it was renamed *Burkholderia*

cepacia in 1992 (63) based on further taxonomic investigation. The species name is based on the onion pathogenicity of the originally described strains (“*cepacia*”, derived from the Latin for “onion”). Onion pathogenic strains cause a maceration and water soaking of the bulb, and usually require wounding for entry. *Bc* is a common soil bacterium, found at the highest concentrations in association with plant roots, and in moist environments.

In recent years strains of *Bc* were discovered that reduced soilborne plant diseases and nematode infection, and were subsequently developed as biopesticides. Beginning in the early 1970s, some isolates of *Bc* were also recognized as rare human pathogens. Due to its metabolic versatility and resistance to many antibiotics and disinfectants, *Bc* caused several pseudo epidemics due to contamination of hospital solutions (1,40). Also beginning in the late 1970s and early 1980s, *Bc* was identified as a colonizer of the lungs of cystic fibrosis (CF) (25,52) and chronic granulomatous disease patients (38). By the late 1980s, sufficient correlative evidence was acquired to indicate that *Bc* caused serious disease and death in these patients (30). The emergence of *Bc* coincided with the formation or increase in CF social organizations. The occurrence of several epidemic strains in the late 1980s and early 1990s, causing high mortality and apparently passed from patient to patient, led to restrictions on the social interaction of CF patients. Therefore, in addition to the more obvious impact from the disease itself, the social restrictions imposed for the sake of prevention have added an additional hardship for CF patients.

In the late 1980s several strains of *Bc* biopesticides were submitted to EPA for registration. While recognizing that some strains of *Bc* were implicated in human disease, taxonomic data submitted to the Agency at that time indicated that human clinical and biological control strains (*Bc* type Wisconsin) submitted for registration, differed (10). Biochemical tests and tier I acute toxicity/pathogenicity tests (see below) were performed. In addition, serotyping, ribotyping, and bacteriocin testing, described in the scientific literature, were performed to distinguish the *Bc* “type Wisconsin” from human pathogenic strains (15, 35, 21). Several biopesticides containing any of three strains of *Bc* were registered on this basis in 1992 and 1996.

Subsequent concern prompted a reevaluation of the initial risk assessment. In addition, several genetic markers were subsequently discovered that correlated with human virulence. One of these markers, dubbed BCESM (*Burkholderia cepacia* Epidemic Strain Marker), is found in a high proportion of epidemic *Bc* strains and a low proportion of environmental strains or nonepidemic strains (31). This marker is being used to screen *Bc* strains seeking registration.

Bc pesticide labels originally allowed the use of the registered strains of *Bc* on crops and turf for the control of various soil fungal pathogens and nematodes. Treatment of seeds or plants through drip irrigation or chemigation was permitted. In 1998, tests performed for the registrant of the *Bc* biopesticides determined that BCESM was present in one of the strains. With the cooperation of the registrant, this strain was removed from use. Use on turf by the remaining strains was also eliminated to prevent possible aerosolization and pulmonary exposure.

EPA has participated in several recent scientific meetings in an effort to gather new data on the risk posed by Bc based biopesticides. This involvement was also used to communicate to the agricultural research community the need for caution regarding the possible human pathogenic potential of some biological control agents. The biological control community consists primarily of agricultural scientists not necessarily aware of medical concerns. In March 1998, a notice was published in the newsletter of the American Phytopathological Society, which contains a substantial component of the biological control community, cautioning about the medical issues surrounding Bc (20). In November of 1998, a half day symposium examining Bc was cosponsored by EPA and the American Phytopathological Society (APS), and presented at the annual meeting of APS and the Entomological Society of America. The symposium brought together for the first time members of the biological control and medical communities to discuss issues concerning Bc. A subsequent interagency meeting involving EPA, NIH, NIEHS, USDA, CDC, and the Pest Management Regulatory Agency (PMRA), Canada, was held in March 1999 to further discuss risk assessment for Bc.

EPA and its Canadian counterpart, PMRA, have participated in three previous meetings of the *Burkholderia cepacia* International Working Group, most recently on April 9-11, 1999, in Banff, Canada. EPA communicated risk assessment needs and requirements, and discussed ongoing research.

A critical need of risk assessment for registration and ongoing evaluation, is that important biological control strains be included in research on the biology, and especially the pathogenicity, of Bc strains. Several of the studies reviewed below were conducted prior to the awareness of this need. However, with the cooperation of the research community, registered and submitted biocontrol strains are being included in many research programs. A further purpose of this paper is to review the current state of knowledge about the pathogenicity of Bc in general, and the biological control strains in particular.

Questions addressed to the SAP are intended to explore the current risk assessment process for Bc to determine whether and how it might be improved. In addition, other microbes related to opportunistic human pathogens are used for biological control (see Appendix). Some of these microbes have been, and others may be, submitted for registration. It is hoped that examination of the risk assessment of Bc will contribute to the broader issue of risk assessment of opportunistic biological control organisms in general.

Summary of the Interim Risk Assessment of *B. cepacia* Strain RAL-3:

Aspects of the following summary are discussed in subsequent sections in the context of the current understanding of the biology of Bc pathogenicity and epidemiology.

A new strain, Bc RAL-3 has been proposed for use as a seed and seedling treatment for conifers, by a different registrant than the currently registered strains. It has been the subject of a pilot joint review process by the U.S. EPA and PMRA, Canada, under the North American Free trade Agreement, Technical Working Group on Pesticides. RAL-3 has been subjected to acute oral,

pulmonary (intratracheal), and intravenous toxicity and pathogenicity testing in immunocompetent rats. These tests, carried out under EPA and PMRA standard protocols, did not reveal indications of toxicity or pathogenicity. Test bacteria were cleared from the animals or, in a few animals, were nearly cleared at the end of the 14 day test period, satisfying EPA and PMRA guideline test requirements (EPA Guidelines 885.3050, 885.3150, and 885.3200).

The registrant, independent of EPA and PMRA requirements, also tested pathogenicity in an immunocompromised cyclophosphamide mouse model using three CF strains as a control. Leukopenia was induced by intraperitoneal (IP) injection of cyclophosphamide. Mice were challenged by IP injection of bacteria in saline four days after cyclophosphamide treatment. The test did not adequately disclose the methods involved (C. Schaffer, Memorandum, "BPPD Review of Data Submitted in Joint Review with Canadian Pest Management Regulatory Agency (PMRA) by Agrium U.S. Inc. for the Registration of Agrium Microbial Inoculant (*Burkholderia cepacia* strain RAL-3). May 1, 1998). No deaths occurred from strain RAL-3 at the highest dose tested (5×10^7 CFU/ml). The LD₅₀ for the three CF Bc strains ranged from 1.4×10^5 to 7.4×10^6 CFU/ml. This mouse model is not considered similar enough to the CF lung phenotype, nor adequately validated, to serve as basis for risk assessment decisions.

RAL-3 was found to be resistant to: amikacin, amoxicillin K, carbenicillin, cefamandole, defazolin, cefoperazone, cefotaxime, ceftriaxone, defurozime, gentamicin, piperacillin, and tobramycin. Intermediate reactions were found for ceftazidime and imipenem. RAL-3 was sensitive to: ampicillin, chloramphenicol, ciproflaxacin, and trimethoprim-sulfamethoxazole.

In addition, the registrant performed PCR ribotyping, RAPD analysis, and testing for the presence of cable pili and the Bc epidemic strain marker (BCESM). The latter two markers have been associated with epidemic causing strains of Bc. Several strains of Bc isolated from CF patients were used as controls. Typing studies revealed a unique "fingerprint" for the RAL-3 strain. Both cable pili and BCESM were absent from strain RAL-3.

The unique typing fingerprint pattern is not adequate to determine CF pathogenicity of a Bc strain. Many CF strains have unique fingerprints.

On the basis of the negative results for the presence of cable pili and BCESM, it was concluded that RAL-3 was a "low risk" strain. However, non-epidemic strains of Bc that are associated with disease in CF are known. Therefore, while the absence of these markers might argue for a lower likelihood of epidemic spread, they are inconclusive for determining pathogenicity (see the sections on pathogenicity and virulence below for a more complete discussion of these issues for opportunistic pathogens and their hosts).

The registrant was required to perform genomovar testing (see taxonomy section below) of RAL-3, but results from these tests have not been supplied to PMRA or USEPA as of this date. It is currently concluded that sufficient evidence of the safety of RAL-3 to CF patients has not been provided.

Biological Control Using *Burkholderia cepacia* and Fate of Bc in the Soil:

Control of plant diseases, insects and nematodes by bacteria and fungi have been proposed as alternatives or supplements to chemical pesticides. In general, use of introduced biological controls have been lauded as a safer and environmentally benign means of controlling pests compared to chemical pesticides. However, despite several decades of research and promising results under controlled conditions, use of biological controls has remained a small fraction of pest control practices. Use of biocontrols has rarely been high enough to be reported in U.S.D.A. data on pesticide treatment of crops in the U.S.

Several reasons have been suggested for this low level of use. Production and long term storage of viable propagules may be difficult. Cost of treatment may be higher than for traditional pesticides. Most important has been inconsistent efficacy in the field compared to traditional pesticides. This may be due to several factors including sensitivity to variation in temperature, pH, or moisture (5). For example, Bc has been reported to be generally more effective at higher soil temperatures (32). At least some strains are reported to produce more anti-fungal pyrrolnitrin with increasing growth temperature above 18 °C, with a maximum at 37 °C (53).

Bc has not only shown promise in greenhouse testing, but also in field tests (3,27,32,64). In several of these tests, Bc has performed comparably with standard fungicide treatments such as captan. In other tests, Bc did not perform as well as fungicides, but did significantly increase crop growth compared to no treatment.

Public concern about the use of chemical pesticides makes biological controls with naturally occurring organisms more attractive. Bc is most often used to control seedling and root diseases. Common chemical alternatives include captan, thiram, PCNB, benlate, and thiobendazole.

Additionally, biological controls such as *Bacillus thuringiensis* have been accepted for use on organically grown foods, which have experienced steadily increasing demand in recent years. The higher prices often paid for these foods may allow somewhat lower efficacy by pest control agents. Therefore, development of promising biological control organisms is considered generally desirable.

Bc has been used experimentally and commercially for biocontrol either by inoculation as single or mixed Bc strains, or in combination with other biological control organisms. Bc has been used to treat seeds or as a soil drench or inoculum. In particular, Bc is most useful in controlling plant pathogenic soil fungi and nematodes (microscopic plant infecting roundworms). It has also recently shown promise in controlling some above ground plant diseases.

A petition for the use of a new strain, Bc AMMD, on an experimental basis (Experimental Use Permit (EUP) petition) was considered in 1997, and contained several proposed foliar uses (Federal Register, Feb. 20, 1997, 62: 7780-7782). The Applicant reports that Bc strain AMMD could not be detected on the foliar parts of the plant after four days. This result is consistent with the general susceptibility of Bc to dessication. Bc is not typically found as an inhabitant of the

above ground parts of plants. However, EPA must also be concerned about exposure of susceptible populations during arial application (e.g. “spray drift”), or during the period that the organism remains viable (e.g. exposure after proposed use on turf). This EUP has not been approved as of the current date.

An important consideration concerning possible human exposure to Bc is the fate of the biocontrol organism in the soil. In the case of naturally occurring organisms such as Bc, it is important to compare the population of the introduced strains to indigenous strains over time to determine whether the biological control strain is likely to increase the overall amount of the biocontrol species in the environment.

Experiments with Bc strain AMMDR1 in the rhizosphere of four pea cultivars demonstrate that within about 6 weeks after application, the total Bc population falls to levels present without application (27). The authors of this work suggest that the rhizosphere has a carrying capacity, or maximum population under given conditions, that apply to bacteria in general and species in particular. Results from other similar research using other biological control bacteria show similar results. For example, survival of the registered biological control bacterium, *Agrobacterium radiobacter* strain K84, was followed in the cherry rhizosphere after inoculation of roots at a rate of 10^8 CFU/ml (10^{6-7} CFU/g root initial population after treatment) (50). Populations fell to levels typically reported for *A. radiobacter* (about 10^{4-5} CFU/g root) within 5 to 10 weeks. In another study, population levels of introduced Bc strain P2 remained stable for 10 weeks (37).

Concern has been expressed that because biological control strains may be particularly good rhizosphere colonizers, they may develop larger populations than other strains. By this means, potential exposure of susceptible human populations, such as CF patients, to a possible pathogen like Bc, might be increased. Results such as those discussed above do not support this concern. It may be more likely that the total population of a species in the rhizosphere is determined by the availability of necessary resources, including nutrients. In general, traits that are important for rhizosphere colonization are not well known. Among the traits that have been associated with efficient colonization by pseudomonads are: antibiotic production, lipopolysaccharide O-antigenic side chains, amino acid synthesis, siderophore production and uptake, and utilization of root exudates (9). Good colonizers may not generally be able to increase the total population of the species in the rhizosphere by significant amounts, but instead displace strains that are not as competitive, at similar population levels.

However, the results above do show elevated populations for several weeks after treatment, and this may be considered in the registration process. For example, preharvest intervals (PHI), which restrict application to dates prior to the interval, are often used to reduce exposure of the susceptible population of humans or non-target organisms to conventional pesticides. PHIs are required where it has been determined that levels of the pesticide applied close to harvest would translate to unacceptably high exposure to the susceptible populations. PHIs could be applied where necessary to biological control microbes to prevent undesirable exposure. It should be

noted that Bc is usually used to control seedling diseases, and is therefore typically applied very early in the growth of the crop, leaving most of the season for the population of the applied strain to fall to background levels.

Fate of naturally occurring biological control strains is typically considered to be an upper tier test (see discussion below), but is often included as a first tier test, and may be used to waive some tier I studies. For potentially opportunistic pathogens, these studies may be required along with tier I tests.

Microbial Human Health Risk Assessment for Biopesticides:

Microbial pesticides are evaluated for potential human risk by a tiered approach. The first tier consists of single high dose acute exposure of the microbe to animals (usually rats or mice) to assess toxicity and pathogenicity. Tests are conducted using the microbe as grown for commercial purposes, or the formulated product containing it. Test levels are 10^7 or 10^8 CFU (colony forming units, or culturable cells) per animal depending on the test. Adverse reaction in first tier tests may trigger higher tier tests, which may consider likely actual exposure by examining the fate of the applied bacteria in the environment. Such higher tier tests may also examine dose response data and chronic or subchronic effects.

Tier I tests include oral, interperitoneal or intravenous, pulmonary, skin irritation, and eye irritation testing (the latter two tests typically using the formulated product in rabbits). Animals are monitored shortly after dosing and afterwards at least daily. Necropsies are performed at various time points and at the conclusion of the tests. Recovery of the test organism from test animal tissues is also tested.

Applicants for registration may request waiver for some or all of these tests. Granting of waivers depends on what is known about the safety and exposure to the organism from the proposed uses. Where the product is to be used on food, tolerances, or levels of acceptable (safe) exposure, may be set, but if required for microbial pesticides would indicate problems arising from dietary exposure. Therefore, to date, tolerance exemptions have been granted for registered microbial active ingredients, consistent with the lack of adverse effects.

Tier I acute toxicity/pathogenicity tests are intended to screen for toxicity caused by the microbe, fermentation products that may be in the pesticide formulation, and inert ingredients, as well as to test for pathogenicity. Because the host range of pathogens can vary dramatically (and, in particular, between humans and rodents), tests for pathogenicity cannot be considered broadly reliable in determining the potential for causing human disease. Therefore, the taxonomy of the organism must be identified to the degree necessary to determine that it is not pathogenic to humans. For bacteria, these tests typically include physiology (e.g. substrate utilization) and genetic markers such as RFLPs or RAPDs. The maximum growth temperature of the organism can be determined, as one indicator of the potential to infect humans. A survey of the medical literature for the organism is an important component of the evaluation of risk posed by the use

of that organism. This analysis provides substantial information regarding the human pathogenic potential of a biological control organism.

In the case of the originally registered Bc products, tests performed by the registrant included carbohydrate utilization and assays for several enzyme activities. The medical literature disclosed other tests used to determine the identity of clinical strains of Bc, and these were applied to the biocontrol strains. For example, sensitivity to Bc bacteriocins and reaction with immune sera, used to identify clinical Bc isolates, was used to test the biological control strains. Other tests included: ribosomal typing, onion pathogenicity, and root colonization ability. Lack of identity between the registered strains and the clinical strains by these tests led to the conclusion at that time that clinical and registered biological control strains were distinct.

Acute oral, pulmonary, and intravenous toxicity and pathogenicity tests conducted on immunocompetent rats also suggested that the registered strains of Bc were not pathogenic. The bacteria were cleared from the test animals as expected for a nonpathogenic microbe.

Recent taxonomic studies call into question the ability of the original tests to distinguish human pathogenic from non-pathogenic strains. This uncertainty has prompted the current reevaluation of the tests required to determine the potential human pathogenicity of Bc.

Risk Assessment of Opportunistic Pathogens:

Bc has been referred to as an opportunistic human pathogen. However, as might be expected, the strains registered or proposed for use as biopesticides were isolated from the soil or plant roots, rather than from human patients. Therefore, techniques for comparing strains of bacteria must be employed to determine the potential pathogenicity of Bc biocontrol strains.

Several definitions of opportunistic pathogens have been suggested, contrasting them with “frank” or primary, pathogens. For the purpose of this document, an opportunistic pathogen is defined as: “A pathogen requiring hosts having one or more deficiencies in their normal ability to resist infection, or where the severity or nature of the infection and host response differs in the deficient host”. Use of the term normal is problematic because the natural variation in biological populations makes the definition of “normal” somewhat arbitrary. For example, the elderly, or neonates could be considered susceptible to some opportunistic pathogens, although most would not consider these groups to be “abnormal.”

However, it is clear that patients with cystic fibrosis and chronic granulomatous disease are more susceptible to infection by Bc than the general human population, and that these infections are often much more severe when they occur.

Opportunistic pathogens have been described as differing from primary pathogens in their lack of dependence for long term survival and transmission by their host(s) (11). This distinction fits biological control microbes, which typically need to be good colonizers of the target

environment, e.g rhizosphere or other plant surface. Therefore, if pathogenic to humans, biological control microbes are likely to be opportunistic rather than primary pathogens.

Compared to many frank pathogens, many opportunistic pathogens are not as well characterized in most aspects of their biology, in particular their human pathology and epidemiology, making risk assessment more difficult. This may be due in part to the recent emergence of many of these pathogens resulting from changes in population demographics or behavior. For example, there has been a substantial increase in organ transplantations in recent decades, with concomitant use of immunosuppressive drugs to prevent rejection of the organ. Similarly, the percent of the population with chronic diseases that may predispose them to opportunistic infection has also increased. For example, the percent of the U.S. population with diabetes mellitus has increased about six fold since 1935 and three fold since 1960 (data from the Centers for Disease Control and Prevention).

As with several other bacteria, Bc has become an important pathogen, or recognized as such, only in the last two decades, and especially in the last 10-15 years (16). The reasons for this emergence are unclear, but the short time that this organism has been recognized as an important pathogen has limited the amount of information that exists about its biology compared to species such as *E. coli* or *Salmonella*.

Less is known about specific factors that may be used in risk assessment for Bc than may be the case for other better known pathogens. For example, several well characterized animal models have been used to evaluate the biology of *E. coli* and *Salmonella* (12), aided in part by the fact that these pathogens can infect other mammals as well as humans.

In the case of an opportunistic pathogen such as Bc, perhaps because of the need for a compromised host, reliable animal models for risk assessment have not been identified and may be inherently difficult to develop (see below for discussion of CF mouse models).

Similarly, dozens of virulence or pathogenicity traits have been identified in better characterized pathogens (12). In association with a possible opportunistic pathogen proposed for biocontrol, such traits might serve to determine whether a strain has potential to be a human pathogen. In the case of Bc, confirmed virulence traits have not been identified, though several potential traits have been examined in clinical strains (16).

Finally, the degree of relatedness of biocontrol strains to clinical strains may supply important information regarding the pathogenic potential of the biocontrol strains. Important work in this area of research has been accomplished in recent years for Bc (54), but has not resolved the issue. In part, this is due to the ambiguous status of some of the strains used in this work regarding either their clinical importance or their environmental competence (see below). In addition, unambiguous demarcation between biocontrol and human pathogenic strains has not been found.

In summary, several of the important procedures typically used to determine the human risk of a biocontrol microorganism are more difficult to apply to bacteria related to opportunistic pathogens, such as Bc biocontrol strains. Recommendations for the use of these methods in current Bc risk assessment and for other possible opportunistic pathogen biocontrols, is the primary objective of the current SAP.

The more uncertain the information regarding the potential risk of a biopesticide, the broader the precautions that must be taken to ensure that important but uncertain risks are not introduced to the public. Such restrictions may range from denial or revocation of registration at one extreme, to the imposition of use restrictions to prevent public exposure. Such measures might not be necessary if the risks could be characterized more accurately and shown to be negligible.

Taxonomy and Characterization of *B. cepacia*:

Bergey's Manual of Systematic Bacteriology (1984) describes Bc as a member of the genus *Pseudomonas*, part of the group of gram negative aerobic bacteria. At that time the genus was informally subdivided into rRNA and DNA homology groups based on hybridization (39). There were five designated groups, with Bc placed in rRNA homology group II. rRNA homology group II strains are non-fluorescent, as distinguished from homology group I species which include the important opportunistic human pathogen, *P. aeruginosa*. Bc can be distinguished from other closely related species by its ability to grow on *m*-hydroxy benzoate or tryptamine as sole carbon sources.

In 1992, the genus *Burkholderia* was split from the other *Pseudomonads* and consisted of homology group II species, with Bc as the type species (63). In 1997, an extensive polyphasic (genetic and phenotypic) taxonomic evaluation of *Burkholderia* strains was undertaken (54). This work consisted of analysis of 128 strains and resulted in the designation of five genomovars covering the *B. cepacia* strains. The biocontrol strains under consideration for registration were not among those tested, although several of those tested were isolated from the rhizosphere. The designation of genomovar, based on DNA hybridization, is intended to indicate genetic taxonomic distinction sufficient to delineate species, but for which sufficient diagnostic characteristics are not available to assign a separate species eponym. Extensive polyphasic taxonomic analysis, as reported in this paper, is considered to be highly reliable in most cases. In particular, DNA hybridization is considered to be the standard for classification at the species level (57,61). For genomovars II and V, sufficient phenotypic characteristics were known to name them *B. mutlivorans* and *B. vietnamiensis*, respectively. Collectively, these five genomovars have been referred to as the "*B. cepacia* complex".

The strains evaluated in the study above were from several sources. Many were clinical specimens, mostly from cystic fibrosis patients, of various levels of virulence. Other strains were of environmental origin outside of hospital or residential settings, including from soil and the rhizosphere of several plant species.

Of the 25 strains that were assigned to genomovar I, one was isolated from a cystic fibrosis patient. Many were soil or rhizosphere isolates, while some were human isolates not known to be from cystic fibrosis patients. It is interesting to note that while almost all of the identified cystic fibrosis strains are grouped in genomovars II, III, and IV (see below), almost all of the strains infecting non-CF patients were from genomovars I. Though these infections are rare, and the clinical outcomes and possible predisposing conditions are not reported, these observations do suggest that at least some genomovar I strains are potential pathogens.

Almost all of the many genomovar II (*B. multivorans*) and III strains were isolated from cystic fibrosis patients. Most of the epidemic causing strains are located in genomovar III, with at least one in genomovar II. One of the genomovar II strains was isolated from soil. All five of the genomovar IV strains were isolated from cystic fibrosis patients, and the nine *B. vietnamiensis* strains were isolated from several sources, in particular rice plants or rhizosphere or from cystic fibrosis patients. More recently, analysis of additional strains is reported to have blurred the distinction between strains source and genomovar designation, but the details of this analysis have not been published (17). However, data continue to show only a very small percentage of genomovar I strains isolated from CF patients (LiPuma, Sixth Meeting of the International *Burkholderia cepacia* Working Group, April 9-11, 1999, Banff, Canada).

In addition, Bc has been shown to have an unusually complex genome. Examined strains have from two to four (usually three) "chromosomes", as well as numerous insertion sequences (IS) (6, 29). The chromosomes, as well as the total genome size, vary between strains. Even strains placed in the same genomovar differ in these characteristics. Genes which may be duplicated on these chromosomes would supply regions of homology which might allow rearrangement and recombination of these and surrounding genes. In addition, supplying regions of diploidy could allow the more rapid mutation of one of the copies of duplicated genes. IS elements are also well known for causing mutations by insertion in or near genes. IS elements flanking genes can facilitate their movement either within the organism or between organisms if they insert, for example, into transmissible plasmids (extra chromosomal replicating DNA in the bacterial cell).

For example, DNA sequences indicative of IS-like elements are found flanking some "pathogenicity islands" (clustered genes involved in pathogenesis) and other virulence genes in several species of bacteria. Examination of their sequences demonstrates that several of the closely examined events did not occur recently on a human time scale (18,19), and therefore may not be commonplace. The pathogenicity genes in these "islands" originated from other species of bacteria, and in some cases are believed to have been moved into their current position by a process that may have involved insertion sequences. Bacteriophage have also been implicated in the movement of virulence factors between species of bacteria (56), though the prevalence and nature of Bc bacteriophage have not been extensively examined. Due to the particular characteristics of the Bc genome, it has been suggested that Bc may be particularly mutable.

Recently, Mahenthiralingam analyzed the *recA* gene of a number of Bc strains (communication at the sixth annual meeting of the Bc working group, Banff, Canada, April 1999). Sequence analysis

of this widely conserved gene allowed the placement of strains into clusters based on sequence similarity as an indicator of relatedness. Several biological control strains were located in their own cluster, which was distinct from disease causing strain clusters (though the taxonomic distance between clusters is not clear). The biological control strains included AMMD, RAL-3, and two of the three registered strains. The single biological control strain which did not fall into this cluster, M36, was found in a cluster containing several of the epidemic genomovar III strains. Interestingly, M36 was also recently found to contain the Bc Epidemic Strain Marker. On the basis of this latter finding, the registrant had voluntarily discontinued the commercial use of this strain.

Epidemiology and Evolution of Pathogenicity of *B. cepacia*:

How CF patients acquire Bc infection, and the origin and nature of CF pathogenic strains, is critical to the risk assessment of biological control strains. Examining the nature of the CF clinical and environmental populations of Bc, and how they interact, has important implications for determining whether biological control strains can be safely used. For example, it has been argued that even if a biological control strain is not a CF pathogen, it may become one over time. Therefore, it is important to consider issues about the epidemiology and population dynamics, as well as adaption of Bc strains, that may occur with commercial use of biological control strains.

The clinical outcome of Bc infection in CF patients may vary considerably and include transient infection, rapid decline over a period of several weeks, slower decline, or long term chronic infection. Long term infection sometimes culminates in sudden acute decline, which may or may not be associated with replacement of the original colonizing strain with a new strain, often from genomovar III. This variation of symptoms may be argued to reflect lack of disease causality by Bc. However, the determination of Bc as an independent indicator of negative prognosis (30) strongly suggests otherwise. For example, fatalities have occurred in patients with mild CF disease and who were not infected with *Pseudomonas aeruginosa* (14). In addition, the unique presentation of the necrotic fulminating pneumonia associated with some Bc infections also argues for causation due to Bc. The strong correlation between the presence of certain epidemic strains and negative outcome also suggests that Bc is a cause of disease in CF patients.

Another possible reason for the variation in clinical outcome might be explained in part by variation in the capacity of different strains to cause severe disease, as well as differences in individual patient susceptibility.

Bc survives for long periods of time in water and soil, especially the area on or immediately adjacent to roots (rhizosphere), at least in the case of environmental isolates. However, it is susceptible to drying, and was viable for only 45 minutes at low concentration in aerosols (30). As noted above, Bc strain AMMD apparently survives for only a few days on the plant surface. The inability to consistently find Bc in hospital or clinical outpatient environments has suggested that CF patients may acquire Bc from the wider environment (e.g. strains originating in soil). But the common presence of Bc in the environment invites questions as to why Bc infection of CF patients is not even more prevalent.

The uncommon finding of genomovar II or III strains, which make up the considerable majority of clinical isolates, in the soil may argue against the likelihood that soil inhabiting strains cause human disease. However, it is possible that not enough strains have been examined to discover genomovar II or III strains in the soil. If additional genomovar I strains have been found to infect CF patients, as has been suggested (17), the soil may be a reservoir for CF strains.

It has also been suggested that soil inhabiting, possibly nonpathogenic, Bc probably strains may undergo a conversion in the human host or elsewhere to higher levels of pathogenicity (17,23). However, if this is the case, it should probably be reflected in a higher frequency of genomovar I strains in patients. Alternatively, it could be suggested that genomovar I strains might convert to genomovar III strains in the human host. However, DNA hybridization studies that establish genomovar designation involve the entire genome of the tested strains. The genetic difference between genomovars usually represents several percent difference in hybridization, reflecting sequence differences probably equivalent to several hundred of genes (if arranged contiguously, though actual sequence differences are dispersed throughout the genome). Such wholesale conversion seems unlikely in a short period of time. However, with a genome as mutable as that of Bc, this possibility cannot be dismissed.

However, the unique identity of most of the strains infecting CF patients, as well as the inability to determine their origin, indicates that the possibility of a soil reservoir for human pathogenic Bc cannot be ruled out. In addition, one strain colonizing a CF patient could not be distinguished from a soil isolate (17). Apparently, this strain only transiently colonized the patient. Therefore, the pathogenic potential of this strain is unclear.

The apparent nonclonality of Bc, with the exception of occasional epidemic strains that infect many patients, is reminiscent of a panmictic species such as *Neisseria meningitidis* (46), indicating widespread gene transfer across the genomovars, with amplification in the frequency of selected epidemic strains (46,23). Linkage disequilibrium analysis of environmental strains demonstrates very high rates of recombination (62). Based on previous genomovar analysis of other environmental strains, these strains most likely belong to genomovar I. Linkage disequilibrium analysis has not been reported between strains from different genomovars. It should be cautioned that linkage disequilibrium or panmixis can occur at different taxonomic levels within a species. For example, the rhizosphere inhabiting *Rhizobium meliloti* was found to consist of two distinct populations which did not share genetic material at an appreciable rate, while at least one of the groups was internally panmictic (46). It could be very useful to assess the level of allele linkage between genomovars, especially genomovars I and III as well as I and II. Such an analysis could assess the likelihood of gene transfer between a genomovar that clearly contains human pathogens and one that contains mostly environmental strains. In addition, genomovar analysis has not been published for registered biocontrol strains or RAL-3, and should be performed as an indicator of potential human pathogenicity.

Several traits or genetic markers have been described as possible indicators of epidemic potential of Bc strains. A new pilus, called cable pilus after its distinct appearance, with the main

component coded by the *cbIA* gene, was found on a major epidemic strain associated with high mortality in North America and Europe (45,51). Neither the registered strains nor strain RAL-3 have the cable pilus. The cable pilus binds mucin found in CF patients. Specific adherence is usually considered to be a precondition to successful infection (12,48), and pili from many known human pathogenic bacteria are associated with pathogenicity (48). The *cbIA* epidemic strain of Bc has probably been found infecting more CF patients than any other epidemic strain. Cable pilus is not necessary for patient to patient transmission or pathogenesis, as determined by subsequent work which found the cable pilus was not associated with several other epidemic strains (31). In addition, two unique (apparently not epidemic) strains were found to have the *cbIA* gene, so it is possible that possession of cable pili is not sufficient for patient to patient transmission. However, since expression of the gene was not determined, it is possible that these strains do not actually produce cable pili. In addition, about 9 percent of the environmental isolates contained the *cbIA* gene.

Another genetic marker has also been found to be associated with epidemic strains. This 1.4 kilobase segment of DNA, designated *Burkholderia cepacia* Epidemic Strain Marker (BCESM), has been found in at least 80% of the epidemic associated isolates, and in nearly 100 percent of the most common clinically encountered epidemic isolates tested (31).

No phenotype has been identified for this marker, but it has significant homology to several bacterial negative transcriptional regulators, and therefore it might regulate the expression of one or more factors involved in epidemic spread of Bc. It is also possible that BCESM is only correlated with epidemic spread, and is not itself a causative factor. However, if Bc is truly panmictic across genomovars or more specifically, across genomovar III (23), than the apparently high linkage disequilibrium of this marker from non-epidemic Bc strains, especially other CF associated non-epidemic strains, suggests that a causal role is more likely. One of the three registered strains of Bc, strain M36, was found to contain BCESM marker and its use has been eliminated by the Registrant. Strain RAL-3 does not possess BCESM.

It is important to note, however, that most clinical CF strains, at least some of which have caused serious infection, have never been associated with epidemics or patient to patient transmission. Therefore, while the epidemic strains may be of greatest concern, lack of epidemic markers, even if they could be determined to be necessary for patient to patient transmission, is not sufficient to determine safety.

Even if the environmental strains, including those used for biological control, were determined to have extremely low potential to cause disease in patients with cystic fibrosis, it has been argued that such strains may have the potential to evolve rapidly into human pathogens, or to transfer traits that enhance pathogenicity to human pathogenic strains.

The potential for environmental strains to become pathogenic cannot be adequately addressed based upon current knowledge, though the rarity of known CF pathogens among environmental genomovar I strains may argue against such changes occurring. Most of the published work

regarding genomovar analysis has focused on clinical CF strains, as should be expected given their importance. However, given the lack of information about the origin of clinical strains, genomovar analysis of large numbers of environmental strains, especially strains that may be considered of economic value as biopesticides (and bioremediation agents), should be undertaken. Since diagnostic tests are available for *B. multivorans* (genomovar II strains), this could be done most easily with this genomovar at this time.

The likelihood that hypothetically nonpathogenic strains could contribute virulence or pathogenicity factors to CF strains depends on the presence of such factors in the biocontrol strains, the frequency of transfer of such traits and the selective pressure to maintain them, and whether the use of biocontrol strains increases contact with CF clinical strains above levels which already occur in the environment.

The ability for pathogenicity traits to be transferred depends on several factors. Some bacteria are naturally transformation competent. Interestingly, most of the very limited number of panmictic strains discussed by Maynard Smith (46) are naturally transformation competent, though one transformation competent strain was not panmictic. As an overall measure of genetic exchange, linkage disequilibrium analysis could give an indication of the likelihood of transfer. Other mechanisms for transferring traits include bacteriophage and conjugal plasmids. Information about both of these mechanisms as well as the level of natural transformation competence is not well known in Bc. Plasmids are common in Bc, and some may have potential to be transferred during conjugation (22,28). At least one Bc plasmid with apparent conjugational ability harbors a streptomycin resistance gene and is capable of transfer to several other bacterial species (22).

In addition, the wide host range plasmid RP4 replicates in Bc, and therefore similar naturally occurring plasmids containing this replicon (replication machinery), are probably capable of transfer between Bc strains (47).

Bacteriophage have been known to transfer virulence factors between bacteria, for example cholera toxin has been found to be carried by a bacteriophage (56). Limited knowledge of Bc bacteriophage limits our ability to determine the frequency of transduction between Bc strains at this time.

IS elements also have been implicated in the movement of genes between bacterial strains. As discussed above, DNA sequences bordering pathogenicity genes originating in other species indicate insertion by IS-like elements or bacteriophage.

In other bacterial pathogens, evidence for transfer of virulence or pathogenicity factors is convincing. For example, pathogenicity islands in enteric species have often been shown to have a GC content that differs significantly from the rest of the chromosome, suggesting an origin in a species with different DNA base composition (18,19). Since closely related species usually have similar base composition, the species that originally contained these pathogenicity genes are

probably not even closely related to the species that contain them now. In general, however, bacteria are more likely to transfer genes to closely related strains due to greater similarity of such factors as promoter structure and gene expression regulatory proteins, restriction and modification systems which eliminate foreign DNA, and compatibility with the genome.

A recent prominent case of transfer of virulence concerns the well published case of enterohemorrhagic *E. coli* O157:H7 (58). In this case, a previously less pathogenic strain of *E. coli* acquired a shiga-like toxin as well as other changes, which created a more virulent pathogen. There are currently not enough data to determine whether transfer of genes between biocontrol strains and clinical strains of Bc could be important for increasing pathogenicity. In the absence of detailed information, based on the common ability of bacteria to share genetic material in various environments, it is reasonable to assume that this ability exists in Bc. There is considerable evidence that a number of human pathogens engage in horizontal gene transfer of virulence genes (34).

In addition to the possible ability to transfer genes between Bc strains, consideration must be given to the importance of traits that might be transferred, the likelihood of transfer, and whether the likelihood of transfer might be changed with the use of a Bc biopesticide.

Many traits are undoubtedly required to allow bacteria to infect humans, even a pathogen that has a limited ability to cause infection in an immunocompetent host. In well characterized pathogens, dozens of traits are known to contribute to pathogenesis (12). The addition of a single or a few pathogenicity genes in a completely saprophytic and noncommensal species would not be expected to make it a pathogen. However, cases of transfer of one or a few virulence factors between strains or species that are already pathogens, and which enhance virulence, are known, as noted above. In some cases single genes have been found to make a substantial difference in pathogenicity.

In the case of Bc, virulence factors are not well defined (see section below), especially in the case of biological control strains. It is therefore not possible to define whether important pathogenicity traits could be transferred from biocontrol to clinical strains.

Of certain importance in controlling Bc infections is antibiotic sensitivity. Bc is already broadly resistant to antibiotics, and transfer of new antibiotic resistance genes from a biocontrol strain to a clinical strain could have serious consequences. In general, clinical strains have been found to be resistant to a wider variety of antibiotics than environmental strains (28,59). However, general antibiotic resistance is probably less important than whether environmental strains carry specific antibiotic resistance for which clinical strains are sensitive.

Antibiotic resistance transfer between environmental or biocontrol bacteria and clinical strains has rarely, if ever, been documented. A case of possible vancomycin resistance transfer between *Bacillus popilliae*, the first biological control bacterium registered for use in the United States, and several human pathogenic enterococci, such as *E. faecium*, has recently been reported (43). The authors suggest that the agricultural use of *B. popilliae* may have been responsible for the

acquisition of vancomycin resistance in enterococci.

Comparison of DNA sequence data between the homologous resistance genes in the species involved suggests that this is unlikely, however. While the sequence from the four enterococci are very highly conserved, the sequence homology with *B. popilliae* is between 68% and 76%. This degree of sequence divergence is unlikely to have occurred in the 50 years of agricultural use of *B. popilliae* in the United States. However, the similarity between the genes does indicate the possibility of transfer between these species at an earlier date.

In addition, resistance to many antibiotics may occur by more than one mechanism. Differences, in the permeability of the bacterial outer membrane, insensitivity of the molecular target(s), inactivation of the antibiotic, or active removal from the cell may all be involved in resistance to a given antibiotic. Possession of several mechanisms may confer a higher level of resistance than a single mechanism alone. Therefore, it is possible that coincidence of an antibiotic phenotype between two strains may not sufficiently describe the potential for those strains to augment each other's antibiotic resistance by gene transfer.

As a first step in determining the potential hazard due to transferred antibiotic resistance, the in vitro resistance of proposed biological control strains should be determined and compared with that of clinical Bc strains.

Another important consideration concerning the possible transfer of pathogenicity traits from biological control to clinical Bc is the likelihood of contact between the strains which might allow gene transfer. Because the reservoir of clinical Bc strains is unknown at present, the possibility that they are found in the soil must be entertained. At least one definition of opportunistic pathogens, as distinguished from primary pathogens, is that opportunistic pathogens are not dependant on their host for survival (11). From this definition we would expect to find the main reservoir for clinical Bc to be other than the human host. Efforts should be made to determine whether genomovar II, III and IV strains can survive in agricultural (nonsterile) soil or rhizosphere. As mentioned above, it would also be useful to determine whether strains from these genomovars can be discovered in the soil or rhizosphere. The prohibition against using currently registered strains in non-agricultural (residential or recreational) environments reduces the possibility of contact between the strains involved and CF patients.

B. vietnamiensis has occasionally been isolated from CF patients as well as the soil environment and could theoretically act as a bridge between biocontrol strains and important clinical strains. However, the limited reports of isolation of *B. vietnamiensis* from the environment are from Asia, and Vietnam in particular, especially from the rice rhizosphere (54). This suggests that this species may prefer a different soil environment than other species in the Bc complex, and therefore may not often come into contact with biocontrol species. However,

the reported CF patients infected with *B. vietnamiensis* are mostly from Scandinavia, and it is unexplained how or where they acquired these infections.

Another important consideration in the possible effect of biocontrol strains on the evolution of clinical pathogenesis is whether these strains would increase the opportunity to transfer new pathogenicity traits to the clinical strains. Consideration should be given to the fact that the biological control strains occur naturally. The prevalence of the particular genotypes used in biological control is generally unknown, though there is no reason to believe that they carry pathogenicity traits that differ from other naturally occurring soil strains. This is especially true when the apparent panmictic nature of environmental strains (62) is considered, which implies frequent reassortment of genes between strains.

Traits that enhance survival in the soil, rhizosphere, or other environments with which CF patients may come into contact may be considered important in risk assessment because they may increase the possibility for the patient to come into contact with the bacteria, or to contact them in higher numbers. Little is known about traits that confer rhizosphere fitness, though antibiotic and siderophore production have been implicated, as well as utilization by rhizosphere bacteria of carbohydrates exuded by plant roots (44) and adherence to root cells (4).

It may also be argued that the sheer volume of applied biocontrol bacteria could affect the potential to transfer traits between strains. However, the acreage covered by agricultural crops is only a fraction of the soil environment likely to be occupied by Bc strains. Furthermore, current biocontrol strains are only used on a small fraction of crop land in the United States.

Bc may be applied at a rate of about 1.5×10^{11} CFU/acre. It is difficult to determine the amount of naturally occurring Bc in agricultural fields because these concentrations vary with conditions such as soil moisture. In addition, Bc cells are not uniformly distributed, being found at concentrations of around 10^5 to 10^8 CFU/g root in the rhizosphere and typically at much lower numbers in bare soil. The amount of one Bc biocontrol strain has been shown to return to background levels in about 5 to 6 weeks (3).

To summarize, in considering the potential for biological control strains of Bc to cause disease in CF patients, epidemic strain markers have been used as indicators of potential pathogenicity. However, many clinical CF strains do not cause epidemics, so absence of these markers is not sufficient to determine lack of CF pathogenicity.

In addition, it has been suggested that the potential for biological control strains of Bc to become serious CF pathogens, even if initially nonpathogenic to CF patients, should be considered before commercial use is allowed. The possibility of nonpathogenic strains of Bc to become CF pathogens is unknown, but the rarity of genomovar I strains that are CF pathogens may argue against such events. In addition, while the ability of various Bc strains to transfer traits is likely, it is unclear that traits important for human CF pathogenicity would be found in biological control strains of Bc. Some pathogenicity traits can function in both plants and animals, as has been

shown for *P. aeruginosa* (see following section), but whether these traits originated in plant or animal pathogens is unknown.

However, not enough is known about the origins of CF pathogenic strains of Bc to be confident about the pathogenic potential of biological control strains. EPA has restricted the use registered biological control strains of Bc to minimize exposure to CF patients. Without increased exposure, the possibility for these strains to become CF pathogens is minimized. Similarly, since the amount of biological control bacteria added to the environment through commercial use is not expected to increase the natural levels of Bc, the potential for transfer of traits, or change to CF pathogenicity, should not be increased.

Virulence and Pathogenicity Traits of *Burkholderia cepacia*:

Virulence and pathogenicity traits are known for many human pathogens, and pathogenic and nonpathogenic strains of these bacteria can sometimes be distinguished by examining them for the presence or absence of these traits. If pathogenicity and virulence traits necessary for infection of CF patients could be identified, then the occurrence of these traits in biological control strains would contribute greatly to the risk assessment of these strains. In this section, the nature of virulence traits is considered. In so doing, cautions about the use of these traits in risk assessment are discussed. In addition, putative virulence and pathogenicity traits of CF clinical strains are reviewed. Though not a virulence trait, the BCESM, as a possible marker of pathogenicity, has already been used in regulating Bc biological control strains. The presence of the BCESM was used as a basis for the elimination of registered Bc strain M36 from use.

An opportunistic pathogen such as Bc, like primary or frank pathogens, must be able to survive and reproduce in its human host under conditions that are prohibitive to saprophytic bacteria. Primary pathogens have been described as having an absolute requirement for replication in the host to assure its long term survival and must possess mechanisms for host to host transmission (11). By contrast, opportunistic pathogens are able to infect their host only under certain conditions, or only infect a small subset of the total host population. This requires that the opportunistic pathogen primarily lives and reproduces in an environment other than the host, or is usually commensal in the host. In the case of Bc, this concept is consistent with the *de novo* appearance of numerous “unique” infectious clones, as noted above.

While differences exist between these primary and opportunistic pathogens, most of the immune responses of most compromised hosts remain at least partially functional. Therefore, a pathogen such as Bc must still have adaptations to avoid, disarm, or resist the existing defense mechanisms of the CF patient. It must also have traits that cause disease symptoms, which distinguish it from commensal organisms that reside in humans.

However, the absence of particular virulence traits may not be sufficient to predict a lack of pathogenicity to CF patients. Not all traits are necessary for infection, and there is often redundancy or substitution of other pathogenicity traits. For example, some *E. coli* strains are uropathogenic while others infect through the digestive system. These strains have different

pathogenicity and virulence traits involving different adhesion proteins and toxins (48). Examples of differences in pathogenicity factors between strains that invade the same tissue may be fewer, but there are examples of strains that produce the same or similar symptoms possessing different traits. For instance, many *E. coli* diarrheal strains possess different pili or other specific adhesion structures (48). *Listeria monocytogenes*, usually considered to be an opportunistic pathogen of humans, produces several phospholipases. Either one is apparently sufficient for escape from the cell vacuole, while elimination of both eliminates virulence (12). Therefore, the absence of a particular trait may be compensated for by functional redundancy, which is common in human pathogens.

Conversely, the presence of certain traits often associated with human pathogenicity may not be sufficient to substantiate the ability to cause disease in humans. For example, type III protein secretion systems have been found in many gram negative human pathogenic bacteria (13,24). These secretion pathways contain highly integrated component proteins, conserved across many bacterial species, and which consist of about 20 or more genes. The type III pathway has been found to secrete a number of different proteins in these bacteria, in particular virulence and pathogenicity factors. A type III secretion pathway has been described for *Pseudomonas aeruginosa*, an opportunistic human and plant pathogen and the most important pathogen of cystic fibrosis patients.

However, type III secretion genes are also found in plant pathogens, such as *Pseudomonas syringae* and *Xanthomonas campestris*, which have never been shown to cause disease in humans or animals (24). Therefore, for example, the presence of type III secretion genes in a biological control strain of Bc would not be sufficient to determine that it could be a pathogen of CF patients. On the other hand, the presence of these genes in biological control strains, and the absence in CF strains might raise concerns for potential transfer from biological to CF strains (see above).

Some traits that contribute to virulence or pathogenicity in plants may also contribute to virulence or pathogenicity in humans. Rahme and others (42) screened mutagenized *Pseudomonas aeruginosa* for pathogenicity on *Arabidopsis* plants. Mutants with reduced virulence were then screened for infectivity on mice. Mutations in nine genes were found to eliminate or reduce pathogenicity in both mice and plants.

Recent research (J. Burns, Sixth Meeting of the International *Burkholderia cepacia* Working Group, Banff, Canada, April 9-11,1999) has demonstrated that salicylate may be involved in the regulation of antibiotic resistance in Bc. Salicylate has also been implicated in the induction of resistance of plants to pathogens, called systemic acquired resistance (SAR). Several biological control bacteria produce salicylate. In the case of *P. aeruginosa*, (as well as other bacteria), the production of salicylate in the rhizosphere has been associated with protection of plants from disease (8). Therefore, it may be possible for some plant pathogens to possess plant virulence traits that contribute to human pathogenicity. However, microbes such as Bc, selected for control of plant disease, typically are not plant pathogens.

Several virulence or pathogenicity traits have been proposed for Bc strains infecting CF patients. Due in part to the unavailability of adequate animal models, it is currently unknown how important any of these traits are to pathogenicity in CF patients.

Bc lipopolysaccharide (LPS) has been found to induce inflammatory markers to a high degree. Tumor necrosis factor alpha (TNF- α) is induced to a 10 fold higher level by LPS from clinical and environmental strains of Bc than by LPS from *P. aeruginosa*. Levels of TNF- α induction are comparable to those induced by *E. coli* endotoxin (16,66). Testing of the registered biological control strains of Bc or RAL-3 for inflammatory response has not been reported.

Bc produces catalase, which has been associated with resistance to killing by phagocytes, in chronic granulomatous disease patients. Bc is also resistant to non-oxidative killing by neutrophils (49). A Bc epidemic strain was recently found to produce a melanin-like pigment which scavenges superoxide radicals, which is part of the oxidative killing mechanism of human monocytes. The melanin was secreted into the growth medium, and may therefore have a role in Bc resistance to oxidative killing (65).

Recently, a hemolysin has been described in Bc that shares properties, such as neutrophil nucleosome degradation at low concentrations, with known lipopeptide toxins (16). Along with neutrophil degranulation at higher concentrations, this may help to explain the severe inflammatory response associated with Bc in CF patients (16,66). The gene coding for this hemolysin has been reported to occur in the biological control strain Bc AMMD (17).

Other factors that have been suggested as possible contributors to human pathogenesis include lipases, proteases, and siderophores (16). However, as discussed above for secretory III pathway genes, these traits may be found in saprophytes or plant pathogens and must be clearly demonstrated to contribute to pathogenicity before they can reliably be used to screen biological control strains for potential human pathogenicity. The presence of putative virulence traits has not been examined in the registered biological control strains or RAL-3.

In conclusion, it has been difficult to determine if traits identified in Bc strains actually contribute to pathogenicity. The presence or absence of a given pathogenicity trait may not be sufficient to confidently identify a strain as a pathogen. Possible redundancy or alternate routes to pathogenicity make absence of a trait less predictive of non-pathogenicity. Possession of a trait generally associated with pathogenicity may not be predictive since many of these traits can have alternative functions not associated with pathogenicity.

No current single test system is adequate to identify Bc pathogenicity traits for CF, suggesting that several lines of evidence may be necessary to have confidence that a trait really is important for pathogenicity. For example, presence in a high percentage of known CF pathogenic strains, homology with known human pathogenicity proteins or other compounds that have been well characterized in other pathogens, and appropriate reaction in test systems, such as human

immune system cell lines, might need to be considered together to make a reasonable weight of the evidence case for or against pathogenicity.

One critical requirement for drawing correlations between possible pathogenicity factors and strain virulence is that the clinical history of the strains used to make correlations is well known. Only strains that have substantial data demonstrating that they cause disease should be used for this purpose. Some strains appear to colonize patients only transiently, and it is not clear that they are capable of causing disease. Possession or absence of a particular trait in a poorly characterized strain may confuse the assessment of the trait's importance in the disease process. Non-epidemic causing strains may be used if the data supporting their ability to cause disease is sufficient.

Since none of the traits currently under investigation have been confirmed to be important for pathogenicity or virulence to CF patients, they have not been used in risk assessment for RAL-3 or currently registered biological control strains. How and whether to use these or other putative virulence or pathogenicity traits in risk assessment should be considered further.

Animal Testing and Human Host Susceptibility Factors:

As discussed above, Tier I acute toxicity and pathogenicity tests are performed to examine the ability of the biological control strain to cause adverse effects in test animals. These tests typically use immunocompetent rodents which are necropsied and examined for signs of toxicity, infection, and clearance.

In the case of Bc biological control strains, no toxicity or pathogenicity was observed in these tests, and clearance was accomplished. However, given the compromised nature of CF patients due to recessive cystic fibrosis transmembrane conductance regulator (CFTR) genes, these animal studies are not considered to be conclusive for the subpopulation with CF.

Adequate animal models that accurately replicate the phenotype of human disease are difficult to develop, and especially difficult for opportunistic human pathogens that may be even less likely to infect species other than humans.

Animal models often do not reproduce the human phenotype with high fidelity, which is necessary for use in risk assessment testing. Even when a genetic defect is known and a defective gene has been cloned, as is the case with cystic fibrosis, it is possible that several different defective alleles exist which confer phenotypes of differing severity. More than 720 different CFTR mutations have been identified which give rise to CF (41).

In addition, other individual differences in genetic background (for example, possible differences in alleles for other immune system functions), as well as medical history, make it likely that susceptibility is a complex combination of factors that will vary considerably between patients (33). While the variety of clinical outcomes for CF patients has prompted a focus on differences in the pathogenicity of the Bc strains, it is also recognized that patient differences may greatly

affect outcomes. For example, coinfection of CF patients with Bc and *P. aeruginosa* has been speculated to cause different clinical outcomes in Bc infections (16). Recent data revealed very different disease progression from infection of different patients with isolates of the Bc ET12 epidemic lineage (Corey, M. et al, Sixth IBCWG Meeting, April 9-11, 1999, Banff, Canada). This suggests that host differences might play a role in disease severity. ET12 has been associated with a high level of patient to patient spread and rapid decline, but recent data from Toronto includes patients infected with ET12 lineage isolates who experienced long term colonization.

Genetic and anatomical differences between host species, such as humans and rodents, can be expected to be even greater than those between CF patients. A CFTR knockout mouse, where the normal gene has been replaced with a defective human CFTR gene, has been developed (7). Versions of this mouse apparently do not replicate the human lung CF phenotype well enough to be used in biopesticide testing. In addition, the CF mice developed in Europe are very fragile and not generally available. There is currently no adequate animal model for testing the human pathogenicity of Bc strains.

Testing of the adequacy of potential animal models requires well characterized clinical strains. Known non-pathogenic strains are also valuable, but non-clinical strains are not known to be definitely non-pathogenic. Bc strain collections are now available, for example at the University of British Columbia. Many of the CF clinical strains in these collections are known to be pathogenic to CF patients, but many are also not well characterized regarding virulence. The same caution should be taken in choosing strains for animal model validation, as was discussed above concerning virulence genes. In the case of clinical strains at least, it is desirable to use strains of known pathogenicity.

In addition to animal models, *in vitro* models are being developed. For example, researchers at the University of Toronto have been working with a lung epithelial cell line to examine the effects of Bc on epithelial tissue and possible invasiveness (Uma Sajjan, Sixth Meeting of the IBCWG, Banff, Canada April 9-11, 1999). While such cell lines have obvious limitations, they may contribute significantly to our understanding of Bc pathogenicity. Differences in cell death have been seen between known pathogens, such as those of the ET-12 lineage, compared to strains that are less virulent.

CF patients mount a humoral response to Bc infection, developing IgA, IgG, and IgM antibodies to Bc LPS and outer membrane proteins (2,36). Clinical outcome is, however, independent of the magnitude of the antibody response (36). Govan (16) points out that normal opsonization processes are disrupted in the CF lung due to the presence of host (e.g. elastase) and bacterial proteases.

While defective CFTR are the ultimate cause of CF pathology, the specific reasons that this defective protein allows infection by Bc is currently unknown, even for better characterized pathogens like *P. aeruginosa* (41). Recent work has identified normal CFTR as a receptor for *P.*

aeruginosa, by which it is normally internalized into lung epithelial cells, contributing to clearance from normal human lungs (41). Epithelial internalization in CF patients is absent or reduced not only where CFTR is absent, but also in the most common defective allele, found in 70% of CF patients. In contrast to *P. aeruginosa*, Bc does not bind to CFTR, and apparently is not cleared by it. However, both species have been found to bind mucin in CF lungs (41).

In conclusion, no adequate animal model has yet been developed for the purpose of testing the pathogenicity of Bc strains to CF patients. Prospects for developing such a model are probably not promising at this time. The CFTR mouse would have been expected to replicate the human CFTR lung phenotype better than less specific approaches, such as immune system suppression by ceratimide or cyclophosphamide. It cannot be expected that an adequate CF animal model for risk assessment will be developed in the near future, leaving us with the other approaches discussed above for determining the pathogenicity of proposed biological control strains of CF.

Monitoring the Use of *Burkholderia cepacia* for Adverse Effects After Registration:

Current regulations require deposit of registered biological control strains in a nationally recognized culture collection, allowing further testing of these strains as necessary. In addition, FIFRA section 6(a)2 requires reporting of adverse effects to the Agency by the registrant. Adverse effects may also be reported by other parties.

As with other biological control organisms, identification of possible adverse effects caused by registered strains of Bc can only be accomplished if the registered strains are distinguishable from other Bc strains.

While the ability to determine pathogenicity of biocontrol strains remains elusive, the ability to distinguish strains or clones of most bacteria is widely accepted. Genetic markers may be empirically determined without knowledge of a corresponding phenotype (if any). Currently accepted methods to distinguish strains include random fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), or amplified fragment length polymorphisms (AFLPs) (26,55,60). All of these methods use the polymerase chain reaction (PCR), which greatly amplifies specific small sections of DNA to make them visible after separation by electrophoresis (separation of the electrically charged DNA by an applied electric field).

RAPDs are probably most commonly used because the technique relies on short stretches of DNA, called primers, which are randomly chosen. Primers are short unique sequences of DNA that match, and bind to, complementary sequences in the chromosome. Pairs of primers are tested on various strains until ones are found which give different patterns of amplification, depending on differences in the DNA sequence length lying between the primers in different strains.

Current guidelines do not require particular methods to identify and distinguish the registered strain from others. There are also no specific guidelines regarding the number of strains tested to validate the identification methods. This is due in part to the rapid evolution of methods in recent

years. Biopesticide manufacturers also desire to have reliable strain markers to be able to ensure that use of their strain in an unauthorized manner is not occurring. Due to the strain deposit requirements, RAPD primers could be made after a reported incident. However, this could be time consuming. In practice most current bacterial biopesticides have corresponding RAPD markers, though the number of strains against which they have been tested is not fixed. Currently registered Bc strains, as well as strain RAL-3, have RAPD markers.

One possible method for distinguishing strains would be to intentionally mark the registered strain by insertion into the chromosome of specific DNA marker sequences, thereby tagging the strain. Markers could be designed to make it virtually impossible to occur in other strains. These markers would not need to carry an expressible phenotype, thereby avoiding some of the concerns regarding the genetic engineering of these strains.

However, tagging has several possible shortcomings. In particular, if a single marker is used, regardless of its chromosomal position, it could be transferred to other strains. Therefore, either more than one marker, inserted at distant sites in the chromosome should be used, or a single marker should be used in conjunction with other natural markers.

Clearly, if it is considered desirable to mark registered strains, this should be done prior to registration rather than after, when they would not be useful for identification of strains suspected of causing an adverse incident. Currently, however, addition of foreign DNA to strains is not favored.

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APPENDIX

Examples of Biological Control Microorganisms Related to Opportunistic Human Pathogens ^a		
<u>Organism</u>	<u>Predisposing Medical Condition</u>	<u>Biocontrol Use</u>
Bacteria		
<i>Burkholderia cepacia</i> ¹	cystic fibrosis, chronic granulomatous disease	control of fungal, primarily soil borne, plant diseases
<i>Enterobacter (Erwinia/Pantoea) agglomerans</i>	nosocomial, pseudo epidemics	fire blight disease of pome fruits
<i>Enterobacter cloacae</i>	neonates, nosocomial, intensive care patients, elderly	seed treatment for control of fungal diseases
<i>Pseudomonas aeruginosa</i>	burns, cystic fibrosis, sepsis, bacteremia	control of plant parasitic nematodes and fungi, induction of plant resistance (plant diseases)
<i>Serratia marcescens</i>	nosocomial	induced plant resistance (plant pathogens), insect biocontrol
<i>Stenotrophomonas maltophilia</i>	colonizer of cystic fibrosis lungs, catheters, nosocomial pneumonia	control of soil borne fungal diseases
Fungi		
<i>Aspergillus flavus</i> ²	AIDS, cancer chemotherapy, systemic steroid use, cystic fibrosis (ABPA)	reduction of aflatoxin contamination of cotton seed in Arizona
<i>Cryptococcus laurentii</i>	rare nosocomial fungemia, indwelling catheters, neutropenia	post-harvest use on fruit to reduce decay
<i>Fusarium oxysporum</i> and <i>Fusarium solani</i>	immunocompromised (AIDS, organ transplant, cancer chemotherapy)	control of plant diseases, bioherbicides

^a Except where indicated, all examples are taken from research literature rather than EPA registration applications.

¹Three strains registered

²Experimental Use Permit Issued