AN ABSTRACT OF THE THESIS OF

Suzanne C. McKenzie Miller for the degree of Master of Science in Soil Science presented on June 1, 2001. Title: Detection of the *Burkholderia cepacia* Complex in Soil Environments.

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Burkholderia cepacia complex (Bcc) bacteria reside in soil, plant rhizospheres, and water, but the prevalence of Bcc in outdoor environments is not clear. In this study, we sampled a variety of soil and rhizosphere environments with which people may have contact: playgrounds, athletic fields, parks, hiking trails, residential yards and gardens. A total of 91 soil samples was obtained from three large U.S. cities (Philadelphia, PA, Cleveland, OH, and Portland, OR). In the first phase of the study, putative Bcc isolates were recovered on *Burkholderia cepacia* selective agar (BCSA) and trypan blue tetracycline medium (TBT). Isolates were sent to the *Burkholderia cepacia* Referral Laboratory and Repository, where they were identified using biochemical tests, growth at 32°C, and polymerase chain reaction (PCR) assays targeting both rRNA and *recA* gene sequences. Bcc isolates were genotyped by using RAPD, PFGE and rep-PCR. A total of

1013 bacterial isolates were examined, and 68 were identified as *B. cepacia* complex. The majority of these were *B. pyrrocinia* or genomovar VII *(B. ambifaria)*; however, a few genomovar III isolates were also recovered. Fourteen (15%) of 91 soil samples yielded Bcc isolates. In the second phase of the study, DNA was extracted from 87 of the 91 soil samples and examined with PCR assays targeting Bcc 16S rRNA gene sequences. By using assays developed by LiPuma et al. (1999), 82% of the soil samples were positive for at least one Bcc genomovar, whereas 94% of samples were positive for at least one Bcc genomovar using the Bauernfeind et al. (1999) assay system. Selected amplicons generated from four soil samples were cloned, and plasmids from multiple transformants (total = 120) were screened by RFLP analysis. Among the clones evaluated from three of four soil samples, 90% or more had the *"Burkholderia"* RFLP pattern. In the remaining soil sample, only 9.5% of the evaluated clones displayed this profile. Sequence analysis of the 463bp 16S rRNA inserts from eight clones with the *"Burkholderia"* RFLP pattern indicated that all were from members of the Bcc. However, the four soil samples from which these clones were generated did not yield isolates identified as Bcc. This study indicates that the use of selective media may not be the best way to estimate the environmental prevalence of Bcc in soils. The natural populations of Bcc in soils with which people commonly have contact may be much higher than previously estimated.

Detection of the *Burkholderia cepacia* Complex in Soil Environments

by

Suzanne C. McKenzie Miller

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Suzanne C. McKenzie Miller, Author

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Dedicated to my father, Stuart W. McKenzie,

for his lifelong example of science in service only to truth.

DETECTION OF THE *BURKHOLDERIA CEPACIA* COMPLEX IN SOIL ENVIRONMENTS

CHAPTER 1. INTRODUCTION TO THE THESIS

The bacterial complex *Burkholderia cepacia* is today at the center of a vigorous scientific debate. Some physicians and clinical microbiologists contend that this naturally antibiotic resistant bacterium should be viewed as a dangerous human pathogen, believing it responsible for fatal infections in susceptible people, namely those with cystic fibrosis (Day, 1998; Av-Gay, 1999; Vidaver et al., 1999; Govan et al., 2000). Many of these scientists have been arguing that the *B. cepacia* complex is not widely found in the natural environment (Butler et al., 1995; Mortensen et al., 1995), and that any deliberate introduction of these bacteria constitutes unacceptable risk to vulnerable human populations.

Environmental microbiologists and agricultural researchers argue that the *B. cepacia* complex (Bcc) is found naturally in a wide range of soil, rhizosphere and stream environments (Hagedorn et al., 1987; King and Parke, 1993; Wise et al., 1995; DiCello et al., 1997), and, consequently, that exposure to introduced soil populations of Bcc poses little risk. At issue is the deliberate release of *B. cepacia* in soil and water as a biocontrol agent against plant pathogens and as a bioremediation organism, particularly in the degradation of the human carcinogen trichloroethylene. Should these activities be curtailed or stopped? Are there consistent differences between medically important, or "clinical" strains, and agriculturally or otherwise useful

"environmental" strains? What is known about the natural, helpful, and harmful roles of *Burkholderia cepacia*?

Historically, the *B. cepacia* complex has been investigated by medical and environmental microbiologists using different methods, including the development of different selective media (Hagedorn et al., 1987; Henry et al.,1997). Until recently, the two fields have had minimal communication, and few, if any, research papers referred to the concerns of the other microbiologists. Articles with titles like "Killing fields: a bacterial pesticide may threaten human life" (Day, 1998) did little to encourage collaboration. Add to this the legitimate complexity of *B. cepacia*'s taxonomy, where various researchers divided Bcc into groups based on random amplified polymorphic DNA (RAPD) fingerprints, restriction fragment length polymorphism (RFLP) patterns, "genomovar" status (discussed later), or the source of isolate, and the result was a near-inability to communicate about *B. cepacia* at all, let alone about the risk posed by Bcc to vulnerable humans.

Happily, recent publications have striven for a more conciliatory tone (e.g., "*Burkholderia cepacia* – Friend AND Foe" (Govan et al., 2000)). Efforts to standardize the taxonomy and become more rigorous in identification protocols have greatly increased the ability of researchers from diverse fields to share their findings (Mahenthiralingam et al., 2000a, c). As a result, current studies have made it clear that in fact there are no phenotypic, genomic, or taxonomic criteria with which to distinguish clinical strains of *B. cepacia* from environmental strains (Govan et al., 2000). Attempts to identify the "pathogenic potential" of different strains are currently underway (Parke, unpublished data). As researchers realize that the source

of the isolate may not be an indicator of its human pathogenic capabilities, more work is being done to incorporate environmental and clinical strains into all experiments with the *B. cepacia* complex (IBCWG, 2001).

One yet unresolved point is the environmental prevalence of the *B. cepacia* complex. The few studies performed by medical researchers did not isolate high numbers of *B. cepacia* from the environment (Butler et al., 1995; Mortensen et al., 1995), leading some to doubt the ubiquity of the bacteria in soil. Others seemed to have no trouble isolating bacteria they called *B. cepacia*, but their identification protocols were based on the original species description for *Pseudomonas cepacia* (Hagedorn et al., 1987). A flood of recent taxonomic changes, coupled with the inherent difficulty identifying this organism, casts doubt on some of these studies (Wigley and Burton, 1999). Debates about the risk posed to susceptible human populations often hinge on this important piece of information (EPA, 1999). Should we assume that Bcc is common, and that the numbers of bacteria added in deliberate applications of Bcc is consequently insignificant, or is Bcc actually infrequently encountered in the natural environment?

This study was a collaboration between a medical doctor's research laboratory, and a soil microbiology laboratory, to determine the environmental prevalence of the *Burkholderia cepacia* complex. Our objective was to determine if Bcc is present in soil environments with which people commonly have contact. We identified the presence of Bcc in soil samples using a variety of both culture-based and non-culture-based methods, developed by the environmental and clinical researchers. In so doing we hoped to contribute to the nascent, but promising, dialogue among microbiologists

from diverse fields about the risks and benefits posed by the *Burkholderia cepacia* complex.

CHAPTER 2. LITERATURE REVIEW

TAXONOMY, GENETICS, AND GENERAL CHARACTERISTICS OF THE *BURKHOLDERIA CEPACIA* COMPLEX

History and taxonomy

W. H. Burkholder originally described *Pseudomonas cepacia* in 1950 as the causal agent of sour skin of onion (Burkholder, 1950). In 1992, several new genera were created to further divide the pseudomonads. Authentic pseudomonads were confined to the species belonging to the same lineage as *P. aeruginosa*, the type species; *P. cepacia* and close relatives were moved to the new genus *Burkholderia*, with *B. cepacia* as the type strain (Yabuuchi et al., 1992). In 1995, the genus *Ralstonia* was created to accommodate two species originally misclassified as *Burkholderia* (Yabuuchi et al., 1995). Considerably more species have been added or moved to the *Burkholderia* genus since then; at present, the genus *Burkholderia* comprises 23 species (Coenye et al., 2000a). Bacteria in the *B. cepacia* complex are in the β subclass of Proteobacteria and the Comamonadaceae family, which also includes the genera *Acidovorax, Comamonas, Hydrogenophaga,* and *Ralstonia* (Achouak et al., 1999).

Further analysis of the Bcc genome and extensive polyphasic taxonomic evaluation resulted in the gradual division of *B. cepacia* into 9 genetic species, or genomovars (Gillis et al., 1995; Vandamme et al., 1997; Coenye et al., 2000b; Vandamme et al., 2000; Coeyne et al., 2001; Vandamme et al., 2001) (see Table 2.1).

The term genomovar was introduced to denote phenotypically similar but genotypically distinct groups of strains. "Genomovar" replaces an unclear variety of terms including genomic species, genomic groups, genospecies, and genomospecies (Ursing et al., 1995).

Table 2.1. Genomovars (genetic species) included in the *Burkholderia cepacia* complex

Species name	Genomovar
B. cepacia	I
B. multivorans	II
----	III
B. stabilis	IV
B. vietnamiensis	V
----	VI
B. ambifaria	VII
----	VIII
B. pyrrocinia	IX

The nine genomovars of *B. cepacia* share a moderate level of DNA-DNA hybridization (30-50%) but a high degree of 16S rDNA sequence similarity (98-99%). As is shown in Table 2.1, a separate species eponym has not been proposed for three of the genomovars, pending the availability of differential phenotypic assays.

Genomovar I, which includes the type strain, is thus the only "*B. cepacia*", though all nine genomovars together are more typically described as the "*B. cepacia* complex." There is some indication that the *B. cepacia* complex may continue to expand; recent analysis of *recA* sequences show several novel Bcc strains that are thought to represent at least three more putative taxonomic groups (Mahenthiralingam et al., 2001). In this paper, as in much of the literature, the terms *B. cepacia*, *B. cepacia* complex, and the abbreviation Bcc are used interchangeably.

General characteristics

Examination of the *B. cepacia* complex has revealed a fascinating, multifaceted organism. *B. cepacia* is a motile, aerobic, non-differentiating, gram-negative straight rod-shaped bacterium. It is typically catalase positive and weakly oxidase positive (Govan et al., 1996), but exceptions are not infrequent (Bauernfeind et al., 1999). Other sources report that *B. cepacia* can be distinguished by its ability to grow on m-hydroxy benzoate or tryptamine as sole carbon sources (EPA, 1999); however, Bcc strains vary widely in their biochemical characteristics. *B. cepacia* strains are also prone to undergoing phenotypic changes in the course of isolation or identification; such auxotrophic changes can cause the strain to no longer react as expected in basic identification tests. The sole use of biochemical tests for identification of the *B. cepacia* complex is therefore notoriously difficult, nonspecific, and time-consuming (Kiska et al., 1996; Henry et al., 1999). A study by Shelley et al. (2000) found that more than 10% of the 1051 clinical isolates identified by referring laboratories were not, in fact, Bcc. Similarly, Segonds et al. (1999) analyzed 51

presumed Bcc isolates with the API 20NE system augmented by an oxidase test. Problems encountered included an absence of *Burkholderia* species other than *B. cepacia* complex in the database, an inability to differentiate *B. cepacia* complex from *Pseudomonas aureofaciens*, and a high number of unidentified strains (11 of 51 isolates). Some moderate success has been reported using substrate utilization profiles, but only to identify *B. cepacia* at the genus level (Yohalem and Lorbeer, 1994). Cellular fatty acid profiles have also been used, although they do not separate Bcc from close relatives like *B. gladioli* (LiPuma, 1998b; Sfalanga et al., 1999). The most reliable method of identifying Bcc uses a polyphasic approach – that is, an integration of phenotypic, genotypic, and phylogenetic information (Vandamme et al., 1996). For instance, the combined use of selective media, biochemical tests (oxidase, lysine decarboyxylase, o-nitrophenyl-β-galactoside, and acid production from lactose), and a PCR-based fingerprinting assay may be necessary for identification of Bcc (LiPuma, 1998b), although DNA-DNA hybridizations are recommended for confident speciation (Vandamme et al., 1996).

Strains in the *B. cepacia* complex are naturally resistant to a wide range of antibiotics. An example is the *B. cepacia* strain RAL-3, proposed as a seed and seedling treatment for conifers, which is resistant to amikacin, amoxicillin K, carbenicillin, cefamandole, defazoline, cefoperazine, defotaxime, ceftrioxine, defurozine, gentamicin, perperacillin, and tobramicin (EPA, 1999). One celebrated paper records the growth of *B. cepacia* using penicillin G as the sole carbon source (Beckman and Lessie, 1979). Strains isolated from the clinical setting are widely

considered to have even greater resistance, due to the long-term use of antibiotic therapies in CF patients (LiPuma, 1998b; Balandreau et al., 2001).

Strains in the *Burkholderia cepacia* complex show considerable adaptability with regard to habitat and substrate. Some can grow at temperatures as high as 50°C (Vaisanen et al., 1998) and as cool as 4°C (Miller, unpublished data). Other strains can form biofilms which demonstrate extreme adhesiveness, making them hard to remove mechanically and which are resistant to the "slimicides" used to clean industrial machinery (Vaisanen et al, 1998). Finally, the catabolic diversity of Bcc is considerable. Reports abound of Bcc surviving on unusual substrates: pharmaceutical gels (Zani et al., 1997), disinfectants (Nelson et al., 1994), even bottled water (Jayasekara et al., 1998). This allows Bcc to colonize locations as diverse as the space shuttle water system (Koenig and Pierson, 1997), and naval toxic waste facilities (Nelson et al., 1986), and to be isolated from tapwater (Zanetti et al., 2000), printing paper machines (Vaisanen et al., 1998), clothes washers (Mortensen et al., 1995), and the cytoplasm of the arbuscular mycorrhizal fungus *Gigaspora margarita* (Perotto and Bonfante, 1997).

Genetics

Both the antibiotic resistance and the catabolic flexibility of Bcc have their footing in an unusually large and complex genome. *B. cepacia* strains have two to four circular chromosomes and any number of plasmids, with overall genome sizes ranging from 5 to 9 Mb, about twice the size of *Escherichia coli* (Cheng and Lessie, 1994; Lessie et al., 1996). The number of chromosomes, as well as the total genome

size, varies between strains, even within the same genomovar. This large size, and the arrangement into multiple chromosomes, allows for some genetic flexibility, as genes may be duplicated on more than one chromosome; this would supply regions of homology which might facilitate rearrangement, recombination, or more rapid mutation of one of the copies (Lessie et al., 1996).

The flexibility of this large genome is also enhanced by the presence of many insertion sequences (Rodley et al., 1995). Insertion sequences (IS) are transposable (moveable) elements, identified on the basis of their abilities to promote genetic rearrangements and activate the expression of neighboring genes. The insertion sequences in *B. cepacia* are thought to be instrumental in the recruitment of foreign genes for catabolic functions; for example, IS elements have been implicated in the recruitment of genes related to the ability of strain AC1100 to degrade 2,4,5-T (Lessie et al., 1996). IS elements thus play an important role in the capacity to adapt to different environments and to colonize a wide range of ecosystems (DiCello et al., 1997; Bertolla and Simonet, 1999). They may also play a role in pathogenicity. Identical insertion sequences have been identified in both Bcc and the human pathogen *B. pseudomallei* (Mack and Titball, 1998), leading to the speculation that pathogenicity islands from other bacteria could potentially be recruited by Bcc. A protein encoded by an insertion sequence in *Mycobacterium tuberculosis* is homologous to proteins encoded by IS elements of *Agrobacterium tumefaciens*, *Streptomyces lividans*, and *Burkholderia cepacia* (Sfalanga et al.,1999).

Lateral (or "horizontal") exchange of genetic material, via transduction and conjugation, has been reported in the *B. cepacia* complex (Cheng and Lessie, 1994).

Nzula et al. (2000) reported the identification of two *B. cepacia* transducing phages, NS1 and NS2. Significantly, the host range of these phages include environmental and clinical isolates from four genomovars of Bcc, and many strains of *Pseudomonas aeruginosa*. This led the authors to speculate that NS1 and NS2 may be able to transfer virulence factors between clinical and environmental isolates. As transduction is affected by host cell density, a relatively large population may be necessary, suggesting that locations like the rhizosphere may be conducive (Droge et al., 1999; Nzula et al., 2000). Conjugation with the IncW plasmid has been observed with Bcc in sterile and nonsterile soils in the pea spermosphere (Sudarshana and Knudsen, 1995). Again, conjugation occurs more frequently in hot-spots of nutrient availability and bacterial clustering, like the rhizosphere or spermosphere (Droge et al., 1999). Transformation, or the uptake and assimilation of naked foreign DNA, may also be possible in the soil environment, as DNA adsorbed to soil particles is still available to transform bacterial cells (Bertolla and Simonet, 1999). Cheng and Lessie (1994) even proposed an "accretion model of genetic evolution" for Bcc, whereby genes for the catabolism of various substrates were added to a primitive chromosome containing housekeeping genes, presumably via horizontal transfer. This is demonstrated in a study by McGowan et al. (1998), which compared small subunit ribosomal DNA from twenty phenotypically distinct strains of 2,4-D-degrading bacteria. The comparison showed phylogenetic incongruencies, indicating that the gene for 2,4-D degradation must have originated from gene transfer between species to a *B. cepacia* recipient.

Between exchange and rearrangement, it's not surprising that many studies have found a great deal of genetic diversity in both clinical and environmental *B.*

cepacia populations. Wise et al., (1995) for instance, noted that multilocus enzyme electrophoresis (MLEE) patterns of lotic (stream-bank) populations of *B. cepacia* showed that *B. cepacia* is clearly not clonal. The panmictic nature of these environmental strains implies frequent reassortment of genes between the strains. A RAPD fingerprinting study of rhizosphere populations of *B. cepacia* revealed a high degree of genetic diversity; among 83 strains analyzed, 68 distinct haplotypes were found (DiCello et al., 1997). Similarly, a RAPD typing of 627 Bcc strains, mainly clinical, yielded 132 RAPD profiles (Mahenthiralingam et al., 1996). PCR and restriction fragment length polymorphism (RFLP) typing of the flagellin gene (*fliC*) in clinical isolates have revealed a large degree of genetic divergence, typical of recombination events (Winstanley et al., 1999).

In short, the combination of a large genome, non-clonality, and demonstrated rearrangement capabilities results in a rare genetic flexibility. It is this flexibility which is assumed to account for the organism's considerable adaptability in substrate utilization and habitat, and which allows it to colonize compromised human tissues.

MEDICAL IMPORTANCE OF *BURKHOLDERIA CEPACIA*

The U.S. Environmental Protection Agency defines an opportunistic pathogen as "a pathogen requiring hosts having one or more deficiencies in their normal ability to resist infection" (EPA, 1999). *B. cepacia*, while not a "frank" or primary pathogen, has in the last 15 years been recognized as an important opportunistic pathogen in some immunocompromised populations. Thus the *B. cepacia* complex has been a

growing concern among medical professionals, particularly those involved in the treatment of people with cystic fibrosis (CF) (Govan and Deretic, 1996).

Cystic fibrosis

Cystic fibrosis is the most frequently inherited fatal disease in Caucasians, occurring in roughly one in every 3900 live United States births; the median survival age of people with CF is 30 years (LiPuma, 1998b). CF is caused by a three-base pair deletion (the AF 508 mutation) which results in improper transport of the chloride ion in both the secretory and absorptive epithelia. This has widespread effects, most seriously in the digestive and respiratory systems. The lungs of a person with cystic fibrosis are filled with a thick viscid mucus, which impairs normal mucociliary clearance mechanisms and presents a unique bacterial habitat. Persistent microbial colonization and chronic bacterial infection are the direct result, and lung disease accounts for most deaths from cystic fibrosis (Welsh and Smith, 1995; Hogardt et al., 2000).

Bacterial lung infections in CF are associated with a number of bacterial species. Early in life, infections with *Staphylococcus aureus* and *Haemophilus influenzae* are typical. Later, infections with *Pseudomonas aeruginosa* are common. *P. aeruginosa* is isolated from more than 80% of CF patients aged 26 or more (Hogardt et al., 2000), and once established is seldom eradicated. Recently, infections with *Stenotrophomonas maltophelia* and *B. cepacia* have become more common. Although healthy people do not harbor populations of *B. cepacia*, current medical literature estimates that 4% of people in the U.S. with CF are colonized with *B.*

cepacia. This compares with 59.9% colonization with *P. aeruginosa*, 37.5% colonization with *S. aureus*, and 15.4% colonization with *H. influenzae* (LiPuma, 1998b). Eight of the nine Bcc genomovars have been isolated from CF sputum samples; *B. pyrrocinia* alone has not been found clinically (LiPuma, 1998b; Coenye, 2000). Most infections with Bcc occur in CF patients, but occasionally other immunocompromised people may also be infected with Bcc – specifically, those with chronic granulamatous disease, and, rarely, patients in intensive care units suffering from other conditions (LiPuma, 1998b).

"Cepacia syndrome"

Many individuals remain stabley colonized with the *B. cepacia* complex for years; a few clear the infection entirely (LiPuma, 1998a). However, up to 33% of those colonized succumb to what is popularly called "cepacia syndrome", a rapid necrotizing pneumonia with fever, bacteremia, elevation of erythrocyte sedimentation rate, and leukocytosis (Govan and Deretic, 1996). Cepacia syndrome is often fatal within weeks or months (Govan et al., 1996).

It is not currently known what factors are involved in the pathogenesis of Bcc infection. Candidate virulence factors include epithelial cell adherence, intracellular survival, evasion of phagocytic killing, production and shedding of lipopolysaccharide, and production of extracellular products like pigments, lipases, hemolysins, exopolysaccharide, proteases, and siderophores.

1. *Cellular adherence.* Some 60% of Bcc strains express peritrichous fimbriae, which increases the ability of Bcc to adhere to pneumocytes in vitro (Nelson

et al., 1994). One clonal lineage expresses a "giant cable pilus" which allows for enhanced binding to cytokinin 13 in respiratory epithelial cells (Sajjan et al., 2000).

2. *Intracellular survival.* Strains of Bcc can invade and survive in A549 human alveolar epithelial carcinoma cells (Burns et al., 1996). It seems likely that Bcc can transverse the epithelium and enter the blood, leading to the bacteremia which accompanies cepacia syndrome. *B. cepacia* can also survive for an extended period of time (120+ hours) in an activated human macrophage. This allows the bacteria to evade antibiotics; macrophage activation also leads to up-regulation of the inflammatory response (Saini et al., 1999).

Alternatively, *B. cepacia* may survive in free-living amoebae like *Acanthamoeba castellanii* and *A. polyphaga* which are commonly found in human nasal passages (Marolda et al., 1999).

3. *Lipopolysaccaride (LPS).* Bcc LPS can be rough or smooth (Nelson et al., 1994) and has been found to induce the inflammatory marker tumor necrosis factor α (TNF-α) to levels achieved by *E. coli* endotoxin. The potent inflammatory response which follows TNF-α production significantly damages lung tissues (Zughaier et al., 1999b).

4. *Production of extracellular products.* A strain of Bcc produces a melanin-like pigment which scavenges superoxide radicals; this helps Bcc resist oxidative killing (Zughaier et al., 1999a). Hemolysin is also produced and may help induce the severe inflammatory response seen with CF patients (Nelson et al., 1994). Exopolysaccharide produced by Bcc is thought to help with adhesion and in resistance to antimicrobial agents (Cerantola et al., 2000), although it is not as crucial to

virulence as the production of alginate is to *P. aeruginosa* (Nelson et al., 1994). Siderophores compete for iron with host iron-binding proteins like transferrin and lactoferrin; those produced by Bcc include pyochelin, ornibactins, cepabactin, and salicylic acid (formerly called azurechelin) (Darling et al., 1998; Sokol et al., 1999). Regulation of siderophore production may be linked to quorum sensing, or autoinduction; *luxR* and *luxI* homologs (called *cepR* and *cepI*) have been identified in Bcc strains (Lewenza et al., 1999).

The lack of knowledge about what virulence factors are essential for pathogenicity by Bcc makes risk assessment more difficult. Typically, many traits are needed to allow bacteria to successfully infect a host; it is not unusual for dozens of virulence factors to contribute to pathogenesis. Thus the transfer of even a few pathogenesis genes (via horizontal genetic exchange) to a completely saprophytic species would not make that species a pathogen. However, it is not known what factors actually do contribute to pathogenicity of Bcc in vulnerable humans. It may be that the soil populations of Bcc are "almost pathogens," and that the addition of just one or two more genes would allow them to be human opportunists. Until the mechanisms of pathogenicity are more clearly delineated, the risk of creating a pathogen via lateral genetic transfer cannot be dismissed (LiPuma, 1998b; Vandamme et al., 2000).

One obviously important virulence factor is the antibiotic resistance demonstrated by most strains of Bcc. Most strains have inducible chromosomal β-lactamase, leading to resistance to penicillin; others have altered dihydroholate reductase, which results in trimethoprim resistance. Outer-membrane permeability

and an antibiotic efflux pump are also thought to contribute to resistance to antimicrobial agents (LiPuma, 1998b). The inherent antibiotic resistance of *B. cepacia* renders chemotheraputic treatment strategies almost totally ineffective (LiPuma, 1998b), so current medical emphasis is placed on prevention of colonization. As patient-to-patient transmission has been clearly documented (Anderson et al., 1991; Segonds et al., 1997), most of the effort has been directed toward programs to separate colonized and non-colonized patients. This segregation has had a profoundly negative impact on the psychosocial well-being of CF patients (LiPuma, 1998b; Govan et al., 1996). The factors that influence the transmission of Bcc between patients include patient behavior, use of contaminated therapeutic devices, CF clinic practices, and, not least, characteristics of the bacterial strain itself (Mahenthiralingam et al., 1997).

Not all strains of *B. cepacia* have the same capacity to cause human disease. Many of the infections within a given CF clinic are caused by the same clone, indicating that it is more transmissible than others. The best known "epidemic" strain (LiPuma suggested the use of the term "hypertransmissable" (LiPuma, 1998a)) is the Edinburgh/Toronto lineage or ET12 clone, although there are many others. Generally, the hypertransmissible strains appear to cluster within a subgroup of genomovar III, but some are also genomovar II (Vandamme et al., 1997; LiPuma, 1998b; Clode et al., 2000). Efforts to find a distinct marker of hypertransmissible strains have thus far yielded three potential candidates. One is the expression of a giant cable pilus, described earlier (Sajjan et al., 2000). Although the cable pilus undoubtedly contributes to enhanced transmissibility, the cable pilus gene, *cblA*, is found in only

some hypertransmissible strains. A second proposed indicator of hypertransmissibility is a conserved 1.4kb open reading frame termed the "*Burkholderia cepacia* epidemic strain marker", or BCESM. The gene product is not known, but the BCESM shows homology with a family of negative transcriptional regulatory genes (Mahenthiralingam et al., 1997). One report found the BCESM present in the seven hypertransmissible strains studied, absent in all nonepidemic strains, and only rarely found in isolates recovered from the natural environment (Mahenthirlalingam et al., 1997). Finally, a hybrid of two insertion sequences, IS*402* and IS*1356*, have been linked to hypertransmissibility (Tyler et al., 1996). Clode et al. (2000) evaluated 117 sputum isolates of Bcc for all three of these proposed hypertransmissibility markers (*cblA*, BCESM, and IS*402*/IS*1356*). In the 41 epidemic strains, all but two were positive for all three markers. Among the 76 nonepidemic strains, only 11 had the BCESM, and none had *cblA* or IS*402*/IS*1356*. Thus, it appears possible to distinguish hypertransmissible strains from other clinical strains. It is important to note, however, that most infections with Bcc are not from hypertransmissible strains, but from unique strain types. These may not be as easily shared among CF patients, but are potentially just as deadly (LiPuma, 1998b). The preponderance of independently acquired, unique strains suggests that the natural environment may be the source of infection for many CF patients.

Several studies have undertaken to determine if there is a difference between clinical and environmental strains of Bcc. Genomovar status is not enough; all genomovars except *B. pyrrocinia* have been found in the CF lung, although the pathogenesis of some colonizations has not been established (LiPuma, 1998b). It is

also true that most infections are caused by genomovar III and II (LiPuma, in press; Henry, 1999). A study of the Bcc flagellin gene (*fliC*), in which 57 isolates were evaluated, found that an RFLP digest of the gene produced many different groups, but that isolates from the natural environment fell into all of the major *fliC*-digest categories (Winstanley et al., 1999). Other methods to distinguish "environmental" from "clinical" have also failed. Attempts include analysis of LPS (Nelson et al., 1984), siderophore production (Darling et al., 1998), outermembrane protein (OMP) profiles (Livesly et al., 1998), method of isolation (Yohalem and Lorbeer, 1997) and isoenzyme profiles (Yohalem and Lorbeer, 1994).

USE OF THE *BURKHOLDERIA CEPACIA* COMPLEX IN BIOCONTROL

Biocontrol

The use of *B. cepacia* in agriculture has great promise. In an age when more plant pathogens are demonstrating pesticide resistance, greater interest in biological control is shown by researchers and commercial interests alike (Govan et al., 1996; EPA, 1999). Biological control is defined as a reduction in the numbers and/or activities of a pathogen using one or more organisms. Often, biological controls are more subtle and operate more slowly than their chemical counterparts, but they can be more stable and longer lasting than other control methods (Graham and Mitchell, 1998). *B. cepacia* has shown itself to be useful as a bioprotectant against fungal, oomycete and even bacterial plant pathogens, as well as being a plant-growth

promotor. It is most often used to control seedling and root diseases, where it replaces chemicals like captan, thiram, benlate, and thiobendazole (EPA, 1999).

Mechanisms of biocontrol

The *B. cepacia* complex uses a wide array of mechanisms to control plant pathogens. These include the production of volatile antifungal compounds, pigments, siderophores, and antibiotics. Examples of antibiotics produced include altericidins, cepacin A and B, and pyrrolnitrin.

Antifungal protection

Burkholderia cepacia strains are capable of considerable antifungal activity, which can protect commercially valuable plants from fungal pathogens. For instance, the *B. cepacia* strain PCII produces four quinolinones which demonstrate antifungal properties (Moon et al., 1996). A study examining *Fusarium* colonization of soil aggregates recorded that aggregate colonization was inhibited by the presence of *B. cepacia* strain MRTii (Toyota et al., 1996). Other researchers have noted that *B. cepacia* restricted the growth and induced morphological abnormalities in phytopathogenic fungi (Upadhyay and Jayaswal, 1992).

Applicability to commercial crops has been demonstrated; greenhouse trials of Bcc strain 2.2N demonstrated the protection of six commercially important plants, including tomato, grape, pepper, wheat, banana, and peanut, from nine pathogenic fungi (Cain et al., 2000). *B. cepacia* has also been shown to help control crown rot in wheat (Huang and Wong, 1998). Strain 5.5B controls *Rhizoctonia* stem rot of

poinsettia when cuttings are rooted in polyfoam rooting cubes (Cartwright et al., 1995). This has become particularly important since the removal of the chemical fungicide benlate from greenhouse use. A soil drench of *B. cepacia* strain D1 protected cotton seedlings from damping off caused by *Rhizoctonia solani* (Zaki et al., 1998). Strain AMMD prevents Aphanomyces root rot and Pythium damping off when applied to pea seeds (King and Parke, 1993). When *B. cepacia* was applied to tomato and pepper seeds in combination with the fungal antagonist *Gliocladium virens*, the seedlings were able to withstand an otherwise deadly mix of *Rhizoctonia solani*, *Pythium ultimum*, *Scleortium rolfsii* and *Fusarium oxysporum* f. sp. *lycopersici* (Mao et al., 1998a). Bcc may be helpful in controlling post-harvest diseases as well, such as green mold of lemons caused by *Penicillium digitatum* (Smilanick and Denis-Arrue, 1992), and blue mold and gray mold on apple and pear (Janisiewicz and Roitman, 1988).

Antibiotics produced by the *B. cepacia* complex have been shown to be effective against bacteria as well as fungi. For instance, the Bcc strain NB-1 produces pyrrolnitrin, which was active against a broad spectrum of filamentous fungi, yeasts, and especially gram-positive bacteria. Pyrrolnitrin was shown to suppress aerial mycelium and spore formation by inhibiting the electron transport system of the pathogen (El-Banna and Winkelmann, 1998). A new *Burkholderia cepacia* strain called PVFi5A has been isolated from the tomato rhizosphere which suppresses the growth of both fungal and 38 bacterial plant disease agents (Sfalanga et al., 1999). Bacterial plant pathogens inhibited by PVFi5A include *Erwinia carotovora* pv. *carotovora, Pseudomonas syringae* pv. *phaseolicola, Pseudomonas syringae* pv.

tomato, Xanthomonas campestris pv. *vesicatoria*, and *Clavibacter michiganensis* subsp. *michiganensis*.

Plant-growth promotion

B. cepacia has also been shown to be a plant growth promoting [rhizo]bacterium, or PGPR. Plant growth promotion may result from indirect action, such as the biocontrol of soilborne diseases that, while they do not kill the plant outright, nonetheless reduce plant growth. Alternatively, PGPR may directly assist the host plant by providing it with nitrogen, phosphorus, or iron, or by making phytohormones (Bowen and Rovira, 1999). Specific strains within the *B. cepacia* complex increased the yield and health of red pepper (Moon et al., 1996), sorghum (Chiarini et al., 1998) and corn (Bevivino et al., 1998). The PGPR activity of Bcc strain MCI 7 on corn was present in greenhouse tests in soils with and without the fungal pathogen *Fusarium moniliforme* (Bevivino et al., 2000). *B. cepacia* also shows potential for use with grasses (Nijhuis et al., 1993), and has been shown to act as a "biofertilizer" for rice cultivated in low-pH, low-fertility soils (Govan et al., 2000). This may be related to the fact that some strains of *B. cepacia*, in genomovar V (*B. vietnamiensis*), can fix dinitrogen (Gillis et al., 1995), although generally the properties of bacteria that allow them to be PGPR may be both varied and complex.

USE OF THE *BURKHOLDERIA CEPACIA* COMPLEX AS A BIOREMEDIATION AGENT

Bioremediation

The use of microorganisms to remediate, or "clean up" soil and water contaminated with toxic compounds, is called bioremediation. *B. cepacia*'s extremely wide range of potential substrates makes it a natural candidate for bioremediation projects. It can use some chlorinated aromatic compounds as energy sources; other compounds it degrades using cometabolic strategies.

Remediation of halogenated compounds

Certain strains of *B. cepacia* are more than a typical saprophyte because they are capable of degrading a particularly problematic class of compounds – the synthetic chlorinated hydrocarbons. Many of these compounds were manufactured as solvents, and were so stable and effective that they were used in a variety of settings: degreasing metals, dry cleaning, manufacture of plastics, and insect and rodent control (Ensley, 1991). Unfortunately, their widespread use and stability have led compounds such as trichloroethylene (TCE) to be among the most frequently detected groundwater contaminants in the United States; the EPA has designated TCE a Priority Pollutant (Arp, 1995). This is problematic because TCE can be both directly toxic and carcinogenic (Ensley, 1991) and is even more potently carcinogenic when partially degraded to vinyl chloride (Newman and Wackett, 1997).

TCE degradation by *B. cepacia*

B. cepacia strain G4, which expresses the enzyme toluene-2-monooxygenase (*t2mo*), degrades TCE significantly more quickly than toluene-oxidizing bacteria without *t2mo* (Leahy et al., 1996). The K_s and V_{max} values determined for TCE degradation by *B. cepacia* strain G4 were 3 μM and 8 nmol/min per mg protein, respectively (Folsom et al., 1990). Although slower in the field, in situ remediation of TCE contamination by strain G4 has been documented (Krumme et al., 1993). The TCE degradation pathway of the *B. cepacia* complex does require induction by toluene. TCE degradation can thus suffer both from competitive inhibition (in which the active site of the enzyme is occupied by the inducing compound, toluene, instead of the target compound, TCE) and catabolite repression, or diauxie, in which an organism fails to make inducible enzymes when a more easily digestible energy source is present (Leahy et al., 1996; Folsom et al., 1990). The difficulties of induction and repression have been practically solved by inserting the toluene-2-monooxygenase gene from *B. cepacia* into a *Pseudomonas fluorescens* strain, where it is expressed constitutively and therefore does not have to be induced. However, the release of a recombinant organism is subject to regulatory approval and application of this technology is consequently not straightforward (Yee et al., 1998).

Other compounds degraded by *B. cepacia*

It is apt that one of the genomovars of the *B. cepacia* complex has the species name "multivorans", as some strains within the complex seem capable of metabolizing a fabulous array of compounds. For example, when inoculated at high cell densities,

three strains of the *B. cepacia* complex isolated from a gas manufacturing plant in Australia degrade high molecular weight polycyclic aromatic hydrocarbons (PAHs) like benzopyrene, dibenzanthracene, and coronene. These toxic, mutagenic and carcinogenic compounds bioaccumulate in food chains and are considered highly recalcitrant. However, the studied Bcc strains thrived in 63 days of incubation with PAHs as the sole carbon and energy source (Juhasz et al., 1996). In another study, *B. cepacia,* in combination with three other bacterial species, has been demonstrated to help degrade a mixture of 13 chlorinated anilines, benzenes, and benzoates in soil slurry. Seventy percent of the chlorine was eliminated after 25 days, decontaminating soils enough to allow plants (wheat and cress) to grow (Brunsback and Reineke, 1995). Bcc strain DB01 is capable of using phthalate as the sole source of carbon and energy; phthalates and phthalate esters are widely used in the manufacture of plastics, textiles, and munitions (Chang and Zylstra, 1998). Other notable compounds metabolized by *B. cepacia* include 2,4-dichlorophenoxy-acetic acid (2,4-D) (Daugherty and Karel, 1994; Jacobsen, 1997) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). In fact, *B. cepacia* degrades 2,4,5-T, the active agent in Agent Orange, 20,000 times faster than any other degradative organism (Govan et al., 1996).

CONCLUSION

In summary, it appears that the same characteristic which makes the *B. cepacia* complex such a good bioremediation and biocontrol agent – namely, the large and dynamic genome which leads to great adaptability in substrate and in habitat – is the very characteristic which makes *B. cepacia* a potential danger to susceptible people,

providing it with antibiotic resistance and free genetic exchange. Such genetic mutability makes unambiguous distinctions between clinical and environmental strains futile, even if they existed; it seems possible that any "good" Bcc strain could become a "bad" one given a conducive environment and appropriate selective pressure. The most appropriate question thus is not, "How can we differentiate helpful Bcc from harmful Bcc?" but "What are the risks associated with adding Bcc to the populations already present in the environment?" Evaluating these risks depends absolutely on a correct estimation of natural environmental populations of Bcc, especially in locations where people may come into contact with them. In this study, we used clinical and environmental, culture-based and non-culture-based methods to determine the prevalence of the *B. cepacia* complex in soil environments with which people have contact.

CHAPTER 3. MATERIALS AND METHODS

SAMPLING

Sample locations were chosen to represent a wide range of soil microbiological habitats within the urban and suburban areas of Philadelphia, PA, Cleveland, OH, and Portland, OR. All soil and rhizosphere samples were taken from places where people commonly contact soil, such as playgrounds, gardens, and golf courses. A clean hand trowel was surface sterilized by soaking in 10% bleach solution (0.5% sodium hypochlorite) for 1 minute. Excess bleach solution was then shaken off and the trowel partially air-dried. At the sampling site, the trowel was inserted in the soil up to the handle to remove any residual chlorine, then removed; the soil sample was taken about 1 cm ahead of the first insertion site. Soil to a depth of about 5 cm was placed in a sterile plastic bag. The plastic bag was then sealed and placed on ice in a cooler chest. All soil samples remained on ice until processing in the lab (per Wollum, 1994).

Soil samples were processed within 72 hours of collection. The soil was mixed inside the plastic baggie by vigorous manual massaging; sticks, stones, worms and other large objects were removed. The mixed soil was then dispensed into several containers. Several grams of soil were placed in a film canister and immediately frozen at –20°C. This would later be used in the direct extraction of DNA and examination of the extracts with the PCR. Soil water content was determined gravimetrically on 20 g of soil dried at 105°C for 48 hours. Finally, approximately 1 gram of soil was placed in a preweighed tube containing 10 ml of sterile 0.1M $MgSO_4$ buffer. The tube was placed in an ultrasonic cleaner (Mettler Electronics Corporation,

model ME 4.6, Anaheim, CA) for two minutes to dislodge bacteria from the soil particles, and then two serial 10-fold dilutions were made, with vigorous vortexing at all steps. Aliquots (100µl) of all three dilutions were plated onto two media selective for Bcc: Trypan-Blue Tetracycline agar (TBT) (Hagedorn et al., 1987), and *Burkholderia cepacia* Selective Agar (BCSA) (Henry et al., 1997). Both media were amended with nystatin at a final concentration of 50 µg/ml, to inhibit fungal growth. Selectivity for *B. cepacia* in TBT agar is provided by a combination of trypan blue and tetracycline, whereas polymyxin, gentamycin, and vancomycin inhibit growth of non-Bcc bacteria on BCSA. Plates were incubated at room temperature (20-22°C) until counted, and then stored in the dark at 4°C prior to colony transfer and isolation of pure cultures.

Sample types collected for each city are summarized in Table 3.1. Soil samples which contained plant roots were often divided into "bulk soil" and "rhizosphere" components. The bulk soil component was handled as above. Rhizosphere samples were treated similarly, but with the following modifications. Plant roots were removed from the soil and shaken to dislodge any loosely adhering soil. The root was then cut into suitable lengths (1 to 4 cm) with sterile instruments, placed in a preweighed sterile tube containing 10 ml of 0.1M $MgSO_4$, and sonicated for 2 minutes. Dilutions were made and plated as above. Afterwards, the root segments were removed from the tube, blotted dry, and weighed. No rhizosphere soil was retained for direct extraction or for soil moisture evaluation; the latter was assumed not to be significantly different from the parallel bulk soil sample.

Special treatment was also necessary for two samples of vegetables purchased at a farm stand. The vegetables (beets and lima bean pods) were placed in sterile plastic bags. After transport to the lab, 15 ml of sterile buffer was added to each of the bags and serial dilutions were made directly out of the bag. The amount of soil adhering to the vegetables was small; no attempt was made to estimate the exact amount of soil present or its moisture content.

Table 3.1. Sample types collected in each city

	Locations	Bulk soil samples	Rhizosphere samples	Other samples	Total samples
Philadelphia	30	27	5	2	34
Cleveland	30	28	11	1	40
Portland	30	30	5	0	35
TOTAL	90	85	21	3	109

ISOLATION OF BACTERIA

The number of bacteria in each major morphology type was noted. Representatives of each recorded morphology type were isolated on the same media from whence they came and grown again at room temperature. Isolated colonies were then grown in non-selective broth culture (Luria-Broth [LB], Kings-B broth [KB], Trypticase Soy broth [TSB] or 25% TSB) with orbital shaking at 150 rpm for at least 24 hours or until turbid. Several media were often tried in an effort to grow the isolate in broth culture. Broth (100 µl) was plated on the same nonselective media. The

resulting bacterial lawn was swabbed up and placed in 1.5-ml cryostorage vials containing 1 ml of LB with 7% DMSO as a cryoprotectant. Notes about growth on selective and nonselective solid and liquid media were maintained for each isolate. Isolates were stored at –80°C until sent to the *Burkholderia cepacia* Referral Laboratory and Repository (BcRLR) (University of Michigan Medical Center, Ann Arbor, MI) for identification. For transport, isolates were grown from frozen stock in 5 ml of nonselective media with orbital shaking at 150 rpm. Sterile transport swabs (BBL CultureSwab Plus, Becton-Dickenson, Sparks MD) were swirled in the broth to inoculate and then mailed by overnight courier.

IDENTIFICATION OF ISOLATES (PERFORMED BY BcRLR)

Isolates were initially screened for the ability to grow on the selective medium BCSA and for the ability to grow at 32°C. Bacteria were plated from the transport swabs onto nonselective (Mueller-Hinton [MH]) agar, and incubated at both room temperature (20-22°C) and 32°C. Colonies were taken from the MH plates and grown also on BCSA, at room temperature and at 32°C. Bacteria taken from the MH plates were stored at -80°C, with 15% glycerol as a cryoprotectant.

All isolates were tested for oxidase production using 1% tetramethyl *p*-phenylenediamine dihydrochloride. Isolates which were positive with the *Burkholderia-Ralstonia* PCR assay (described later) were also tested with the following biochemical tests: reactivity with lysine decarboxylase, reactivity with o-nitrophenyl-β-D-galactoside (ONPG), and oxidation-fermentation of sucrose and

lactose (Remel, Lenexa, KS) as described (McMenamin et al., 2000). A set of isolates for which identification was still ambiguous was investigated with the RapID NF Plus Kit (Remel, Lexena, KS) according to the manufacturer's instructions.

All isolates which grew on MH agar were tested with the *Burkholderia-Ralstonia* PCR assay, using a boil-lysis procedure. A loopful of bacteria was placed in 500-1000 µl of UV-irradiated sterile water in a 1.5-ml centrifuge tube and pelleted by centrifugation at 5,000 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended, heated at 100°C for 20 minutes and then cooled to room temperature. This was pelleted with centrifugation and 5 µl of supernatant was used as a template in a PCR assay; the primer pair targeted the 16S gene of members of the *Burkholderia* and *Ralstonia* genera. The PCR assay was conducted as previously described (LiPuma et al.,1999).

Isolates which were positive in the above assay were subjected to a second assay, using purified DNA as template. DNA was purified using the Easy-DNA kit (Invitrogen, Carlsbad, CA) with modifications described previously (LiPuma et al., 1999). More PCR assays, targeting the 16S and *recA* genes and designed to differentiate between species in the *Burkholderia cepacia* complex, were performed as previously described (LiPuma et al., 1999; Mahenthiralingam et al., 2000a). If isolates were positive on the *Burkholderia-Ralstonia* PCR assay but negative on the other 16S and *recA* assays, they were assayed with a PCR primer pair designed to amplify *B. gladioli* (Whitby et al., 2000). PCR assays included a eubacterial primer as a positive control, and a water blank as a negative control.

Isolates identified as Bcc were assigned to one of the species in the complex using restriction fragment length polymorphism (RFLP) on the PCR-amplified *recA* gene (Mahenthiralingam et al., 2000a). A few isolates were not conclusively speciated after this step; in these cases the *recA* gene was sequenced. Sequence data was evaluated manually using Chomas software (Technelysium Pty Ltd, Gold Coast, Australia) (LiPuma et al., in press). Genotypes of the Bcc isolates were obtained using random amplified polymorphic DNA (RAPD) typing and pulsed field gel electrophoresis (PFGE).

EXTRACTION OF DNA FROM SOIL SAMPLES

Frozen aliquots of all soil samples were used to provide DNA for a series of PCR assays. DNA was extracted using the BIO101 FastDNA SPIN Kit for Soil (Q-Biogene, Carlsbad, CA). Soil samples were extracted according to the manufacturer's instructions with a few modifications. Briefly, 978 µl of 200 mM sodium phosphate buffer, 122 µl of MT buffer, and 300-400 µg of soil (including any small roots present in the sample) were placed in a FastDNA tube, which already contained silica and ceramic beads in a variety of sizes. This was shaken in the FastPrep homogenization unit for 30 seconds at a setting of 5.5, and then centrifuged at 4°C and 14,000 x *g* for 15 minutes. The supernatant was transferred to a clean tube on ice. Protein precipitating solution (250 µl) was added, and the mixture centrifuged at 14,000 x *g* for 5 minutes. The supernatant was again transferred to a clean tube, and 1 ml of silica DNA binding matrix added. The tube was gently mixed by inverting for 2 minutes and then allowed to settle for 3 minutes. The pellet was resuspended by pipetting, and

600-µl aliquots were transferred to the Spinfilter and centrifuged at 14,000 x *g* for 1 minute. When the pellet had been collected on the Spinfilter, it was washed with a salt-ethanol solution, then centrifuged at 14,000 x *g* for 2 minutes to dry. The filters, with the pellets in them, were placed in clean catch tubes and air-dried for 5 minutes, with a Kim-Wipe placed over them to avoid air-borne contamination. DNA-free water (100 µl) was added to the pellet and the mixture very gently stirred with a pipette tip to resuspend the silica. The filters were centrifuged at 14,000 x *g* for 1 minute to elute the DNA to the catch tube.

Samples were extracted in duplicate. Every other extraction run (the FastPrep instrument allows 12 tubes to be run at one time) included a blank, in which 300 µl of DNA-free water was substituted for soil, and a spike. Two types of spikes were prepared; some were made by adding Bcc cells to autoclaved soil, and some by adding Bcc cells to field soil. The spikes and the bacterial strains they contained are listed in Table 3.2.

Table 3.2. Spiked soil samples used in DNA extractions

Spike number	Soil	Bcc strain added as overnight broth (genomovar)
1	Field (non-autoclaved)	AMMD (VII)
2	Autoclaved	FC461 (I), FC 147 (II), cep49 (II), cep 40 (V)
3	Autoclaved	Bcc 232 (VI), FC 147 (II), cep49 (II)
4	Autoclaved	FC 461 (I), cep 40 (V)

DNA was quantified using a DNA fluorometer (Hoefer Scientific Instruments model TKO 100, San Francisco, CA) and Hoechst's dye. A standard of calf thymus DNA (Sigma Chemical Co., St. Louis, MO) at 25 ng/µl was used to calibrate the fluorometer each time it was used. Extracted, purified DNA was standardized to a concentration of 25 ng/µl and stored in water at –20°C until use.

PCR ASSAYS

Two different sets of primer pairs – eight pairs in all – were used to ascertain if Bcc DNA was present in soil samples. All primer pairs targeted different sections of the 16S (*rrn*) gene. Five primer pairs were designed by LiPuma et al. (1999), and three were constructed by Bauernfeind et al. (1999). The PCR scheme was designed in stages. First, the samples were tested with an assay designed to amplify bacterial DNA. Samples which were negative for this or any other step were run again, using more or less DNA; all negative samples were assayed at least twice. If positive, the samples were tested with another more specific assay, to amplify members of the *Burkholderia-Ralstonia* genera. If the sample was positive on the *Burkholderia-Ralstonia* assay, it was run with all six Bcc-specific assays. These were designed to amplify different subgroups of genomovars within the *B. cepacia* complex.

Sensitivity and selectivity of all primer pairs were ascertained using DNA extracted from a set of 35 known strains, representing genomovars I-VII as well as other bacterial species (see Table 3.3). The strains were grown from frozen stock overnight in 5 ml LB (AMMD was grown in 5 ml KB) at 25°C with orbital shaking at 150 rpm. Cells were centrifuged for 15 minutes at 14,000 x *g*, and then resuspended

in 100 µl water. DNA was extracted from the pellet using the Bio101 FastDNA kit according to the manufacturer's instructions, using the Cell Lysis Solution "TC". DNA was quantified and stored as before.

PCR assays were performed using 50 µl reaction mixtures containing 1 x PCR buffer (Promega Corp., Madison, WI), 0.06% bovine serum albumin, 2 mM $MgCl_2$ (Promega), each deoxynucleoside triphosphate at a concentration of 0.20 mM (Promega), the forward and reverse primer each at a concentration of 0.20mM, and 2 U of *Taq* DNA polymerase (Promega). The only exceptions were assays using the primer pair PC-SSF, PC-SSR (described later: see Table 3.4) (LiPuma et al., 1999), which used a cocktail containing 1.5 mM $MgCl_2$. The amount of DNA per assay was 50 ng per reaction vessel in reactions with pure cultures, and varied from 50 ng to 250 ng per reaction vessel in reactions with DNA extracted from soil. Typically, the reaction was first run with 150 ng of DNA and then other amounts were tried if the initial run was negative.

For each primer pair, the Mg^{2+} concentration and the optimal annealing temperature were determined using DNA extracted from pure cultures. Thermalcycler parameters were slightly different than those published (LiPuma et al., 1999; Bauernfeind et al., 1999). These data are listed in Table 3.4. LiPuma primer pairs used the following thermalcycler parameters: denature for 3 minutes at 95°C, then 30 cycles with 1 minute at 94°C, 1 minute at the annealing temperature, and 1 minute at72°C. The final extension step was 4 minutes long. Samples were then held at 25°C until removed from the thermalcycler and placed at 4°C. Reactions using

Table 3.3. Strains used to test sensitivity and selectivity of PCR primer pairs

Strain	Other names	LMG accession number [1]	Origin[2]	Genomovar
cep 31	ATCC 25416^T	LMG 1222^T	Onion, USA	I
cep 80	ATCC 17759	LMG 2161	Soil, Trinidad	I
FC 461		LMG 17997	UTI, Belgium	I
cep 509		LMG 18821	CF, Australia	I
cep 144	ATCC 17616	LMG 17588	Soil, USA	II
FC 445		LMG 13010^T	CF, Belgium	II
FC 769	CP-A1-1	LMG 18825	CF-e, UK	II
cep 781	C1576	LMG 16660	CF-e, UK	II
c5393		LMG 18822	CF, Canada	II
cep 24	PC 184	LMG 18829	CF-e, USA	III
FC 475	BC7	LMG 18826	CF-e, Canada	III
FC 505	K56-2	LMG 18863	CF-e, Canada	III
FC 511		LMG 18830	CF-e, Australia	III
cep 565	J2315	LMG 16656	CF-e, UK	III
c5424		LMG 18827	CF-e, Canada	III
c6433		LMG 18828	CF-e, Canada	III
FC 367		LMG 14294	CF, Belgium	IV
FC 472		LMG 14086	Respirator, UK	IV
FC 779		LMG 18888	Clinical, Belgium	IV
c7322		LMG 18870	CF, Canada	IV
cep 40	PC 259	LMG 18835	CF, USA	V
FC 369		LMG 10929^T	Rice, Vietnam	V
FC 441		LMG 18836	CGD, Canada	V
Bcc 232			CF	VI
Bcc 305			CF	VI
AMMD		LMG 19182^T	Pea rhizo., USA	VII
Bcc 118			CF, USA	VII
Bcc 267			CF, Australia	VII
B. gladioli		LMG 2216		
B. caribensis		LMG 18531^T		
Ralstonia pickettii		LMG 5942^T		
Pseudomonas aeruginosa				
Pseudomonas stutzeri				
Serratia marsescens				
Achromobacter cycloclastes				

[1] Laboratorium Microbiologie Ghent Culture Collection, Universiteit Ghent, Ghent, Belgium.
[2] Abbreviations: CF, cystic fibrosis infection; CF-e, strain that has spread epidemically among patients with CF; CGD, infection of chronic granulomatous disease patient; UTI, urinary tract infection.

Table 3.4. Primer pairs used in 16S rDNA PCR assays

Reference	Primer pair	Target[1]	Annealing temperature
LiPuma et al., 1999	UFPL, URPL	Kingdom Bacteria	55°C
	RHG-F, RHG-R	Members of *Burkholderia* and *Ralstonia* genera	55°C
	BC-GII, BC-R	Genomovar II	54°C
	BC-GV, BC-R	Genomovar V and some Genomovar II	55°C
	PC-SSF, PC- SSR	Genomovars I, most III, IV and VII	53°C
Bauernfeind et al., 1999	Eub16-1, CeMuVi-16-2	Genomovars I-VII	53°C
	Eub 16-1, Ce-16-2	Genomovars I, III, IV and VII	56°C
	Eub 16-1, MuVi-16-2	Genomovars II, V and VII	53°C

[1]In the interests of clarity, genomovar designations have been used instead of species names. Genomovar II = *B. multivorans*: Genomovar IV = *B. stabilis*: Genomovar V = *B. vietnamiensis*: Genomovar VII = *B. ambifaria*.

Bauernfeind primer pairs had the following parameters: 5 minutes of denaturing at 95°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at the annealing temperature, and 45 seconds at 72°C. The final extension step was 7 minutes long; samples were then held at 25°C. All PCR assays included a positive DNA control (50 µl DNA from a pure culture of Bcc which should amplify with the primer pair used),

negative DNA control (50 µl DNA from a pure culture of Bcc or a close relative which should not amplify with the primer pair used), and a water blank (includes all ingredients except DNA) (Pepper and Pillai, 1994).

PCR products were separated from genomic DNA by gel electrophoresis on 1% agarose gels and visualized with ethidium bromide. A band on the gel was considered a positive reaction, even if faint. No bands were seen which were not at the same position on the gel as the positive control. One quarter of all reactions were repeated to assess reproducibility.

LIMIT OF DETECTION OF PCR ASSAYS

To ascertain the limit of detection of the above PCR assays, Bcc was added to autoclaved soil. The soil used was of the Jory series, a clay loam which is 39.5% clay, 39.8% silt, and 20.7% sand. Dry soil was autoclaved twice, 24 hours apart, to sterilize it. Dry, sterile soil (1 g) was placed in a sterile tube, and 300 µl of a broth culture of *Pseudomonas aeruginosa* containing approximately 4 x 10^8 CFU was added to represent "background" bacterial populations. Serial dilutions of broth cultures of two Bcc strains (*B. vietnamiensis* FC 441 (LMG 18836); *B. ambifaria* AMMD (LMG 19182)) were added in amounts ranging from 10 CFU g^{-1} soil to 10^8 CFU g^{-1} soil. The soil/broth was gently mixed – it was muddy – and allowed to stand 20 minutes. All dilutions of each broth culture were also plated on TSA to determine the actual CFU/ml. Wet soil (300 mg) from each dilution was placed into an extraction tube and extracted as above. DNA was quantified and PCR assays were run as described

earlier. Controls included a unit with soil, *P. aeruginosa*, and 100 µl sterile water; a unit with soil and 400 µl sterile water; and two units with sterile water only.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) SCREENING AND SEQUENCING

To verify that Bcc DNA was being amplified in the foregoing PCR assays, selected amplicons were cloned and screened with RFLP. Amplicons were generated from four soil samples using the primer pair Eub 16-1: CeMuVi-16-2, which amplifies genomovars I-VII. Amplicons were purified with an ethanol precipitation, and then ligated into pGEM-T vector and transformed into *E. coli* JM109 cells. The latter steps were part of the pGEM-T Easy Vector System (Promega Corp., Madison, WI) and performed according to the manufacturer's instructions, with the suggested modifications for greater numbers of clones. Clones were screened to determine whether inserts were present by using α-complementation with X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside). Clones with inserts were then screened with PCR to see if the insert was the correct size, again using the primer pair Eub-16-1: CeMuVi-16-2 and 2 µl of LB broth culture of the clone as template. Clones which did not have an insert, or which contained an insert of the incorrect size, were not considered further.

A total of 120 clones which did have the correctly sized insert were screened using a digest with the restriction enzyme *Sau*961 (Promega). The amplicons from the PCR assay, above, were used as template. Each reaction vessel included 7.5 µl water, 2 µl of 10x buffer (Promega), 10 µl (approximately 120 ng) DNA, and 2.5 U of

*Sau*96I. The reagents were mixed by pipetting and centrifuged briefly to collect the contents at the bottom of the tube, then incubated in a water bath at 37°C for 16 hours. The reaction was stopped by adding 4 µl of 6x gel loading dye (0.0625 g bromophenol blue, 0.0625 g xylene cyanol, and 3.75 g Ficoll in 25 ml water) to each tube and again centrifuging briefly. DNA fragments were separated by gel electrophoresis in a 3% agarose gel (Metaphor, BioWhittaker Molecular Applications, Rockland, ME) at 4°C and visualized with ethidium bromide. The results were compared to a computer digest of published Bcc sequences (Wisconsin Package Version 10.1, Genetics Computer Group (GCG), Madison, WI) and to positive controls digested with the clones.

Two clones which had the "*Burkholderia*" pattern were sequenced from each of the 4 soil samples (8 total). Three clones representing non-Bcc patterns were also sequenced. The clones were grown overnight in 3 ml LB and vector DNA prepared from it using the Eppendorf Perfectprep Plasmid Mini Kit (Hamburg, Germany) according to the manufacturer's instructions. Nucleotide sequence data were obtained using T7 and SP6 primers. The sequencing was performed using *Taq* dye terminator chemistry and an ABI cycle sequencer (Central Services Laboratory, Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR). The resulting sequences were used to search for similarities among known sequences using the basic local alignment tool (BLAST) at the National Center for Biotechnology Information (NCBI, Bethesda, MD).

CHAPTER 4: RESULTS

ISOLATION OF BACTERIA ON SELECTIVE MEDIA

Putative Bcc colonies were recovered from both BCSA and TBT. Overall, the mean recovery on BCSA was 6.0 $\log_{10}$ CFU g^{-1} soil dry weight; on TBT it was 5.4 $\log_{10}$ g^{-1} soil dry weight.[3] The mean population recovered was not significantly different between the cities and between the two selective media (ANOVA, p-value = 0.24) with the exception of Philadelphia TBT counts, which were lower (ANOVA, p-value = 1.93×10^{-13}). These results are represented in Figure 4.1. Colony counts from Philadelphia samples on TBT were lower due to rampant fungal growth on the plates. In the Portland and Cleveland samples, TBT was amended with nystatin (50 μg/ml) to control fungal growth.

Some soil samples yielded more colonies when plated on TBT; others yielded more on BCSA. A histogram of the number of BCSA colonies (log CFU g^{-1} dry soil on BCSA) minus the number of TBT colonies (log CFU g^{-1} dry soil on TBT) shows that most soil samples had about the same counts on both media (Figure 4.2). A few samples had higher counts on TBT (the negative numbers on the histogram); more samples had higher counts on BCSA. Thus, although BCSA tended to have higher numbers, and soil samples tended to have slightly higher counts on BCSA, TBT was a more effective medium for some samples.

There were a few soil samples from which no colonies were recovered on either media; these "zero" plates were somewhat more common with TBT (7.3% of

[3] Henceforth, "log" will mean "$\log_{10}$", as opposed to the natural log (ln).

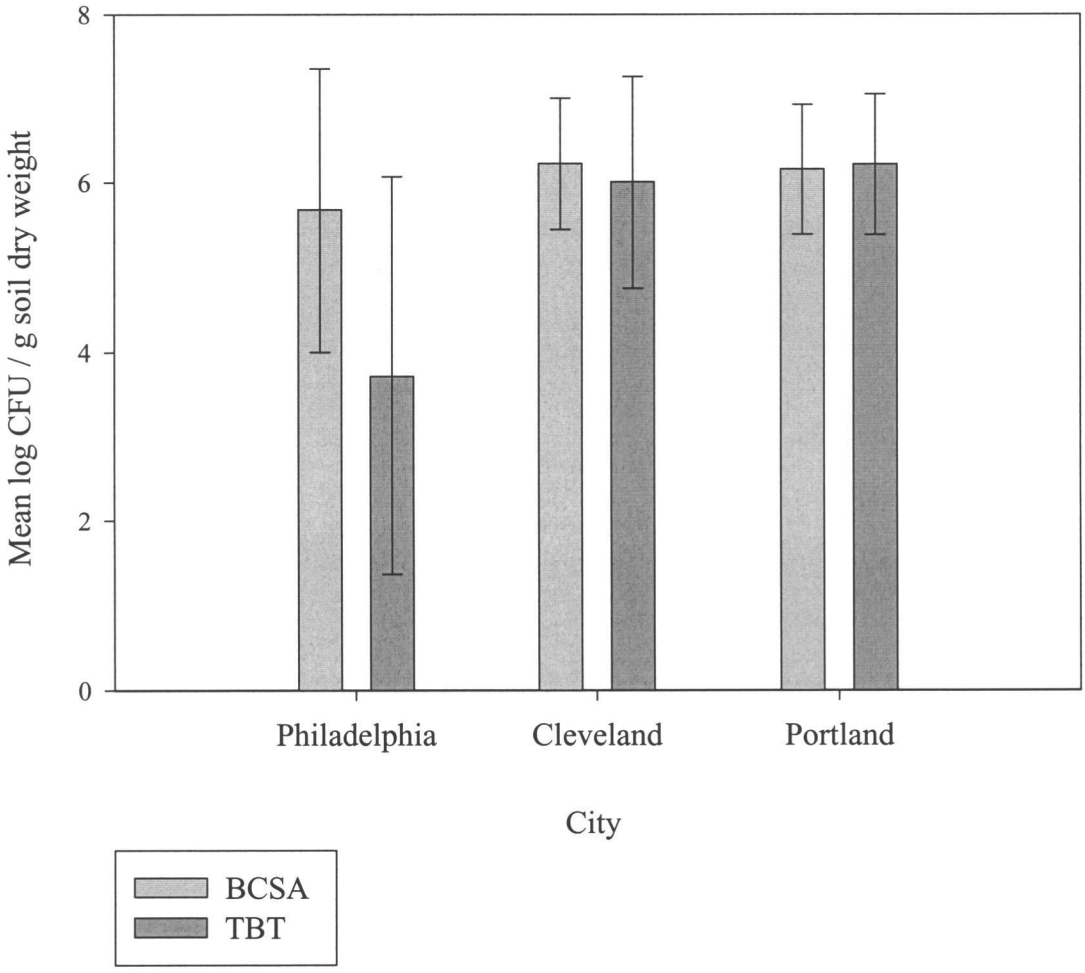


Figure 4.1. Mean log CFU gram^{-1} soil dry weight, on BCSA and TBT. Error bars represent the standard deviation of the mean.

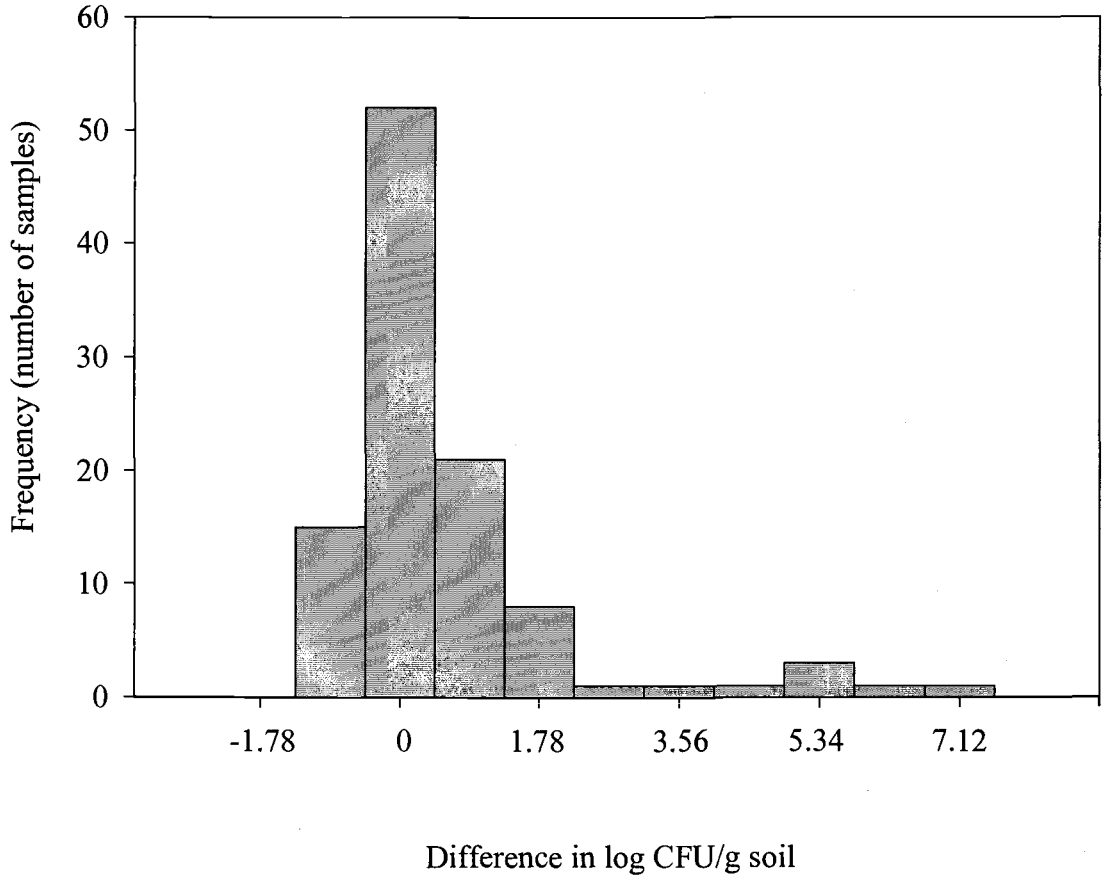


Figure 4.2. Histogram of the difference between log CFU/g soil on BCSA and log CFU/g soil on TBT.

total) than with BCSA (0.9% of total). All of the zeros were from the Philadelphia samples, suggesting that this may be another side effect of the fungal contamination problem experienced with the Philadelphia samples.

A total of 1260 bacterial colonies were chosen from TBT and BCSA and streaked for purity on the same media from which they came; this plate was called the "isolation plate." Some colonies (9%) did not grow on the isolation plates. This phenomenon was noted slightly more frequently on TBT than on BCSA (9.2% vs 8.9%, respectively). It is possible that the bacteria on the original plate were not actually metabolizing the media, but were instead growing on attendant nutrients from the plated soil slurry. Alternatively, the bacteria could be using metabolites or be protected from selective agents due to the growth of nearby colonies. When isolated, the bacteria were no longer fed or protected, and thus unable to grow.

The color and morphology of the isolates were noted at each step; up to 10 different colony types were observed on each medium. The most common colony type isolated from the BCSA plates was medium-large glossy orange; 22% of the colonies originally counted had this morphology. The most common morphology isolated from TBT was small light blue, with 25.5% of the colonies originally counted displaying this morphology.

In both media, there were differences between the cities and between soil and rhizosphere samples. These are displayed graphically in Figures 4.3 and 4.4. Of particular interest is the different colony distribution, and relative lack of colony diversity (richness), evident in the Cleveland samples on both media. This is particularly interesting in the light of the relative lack of Bcc recovered from the

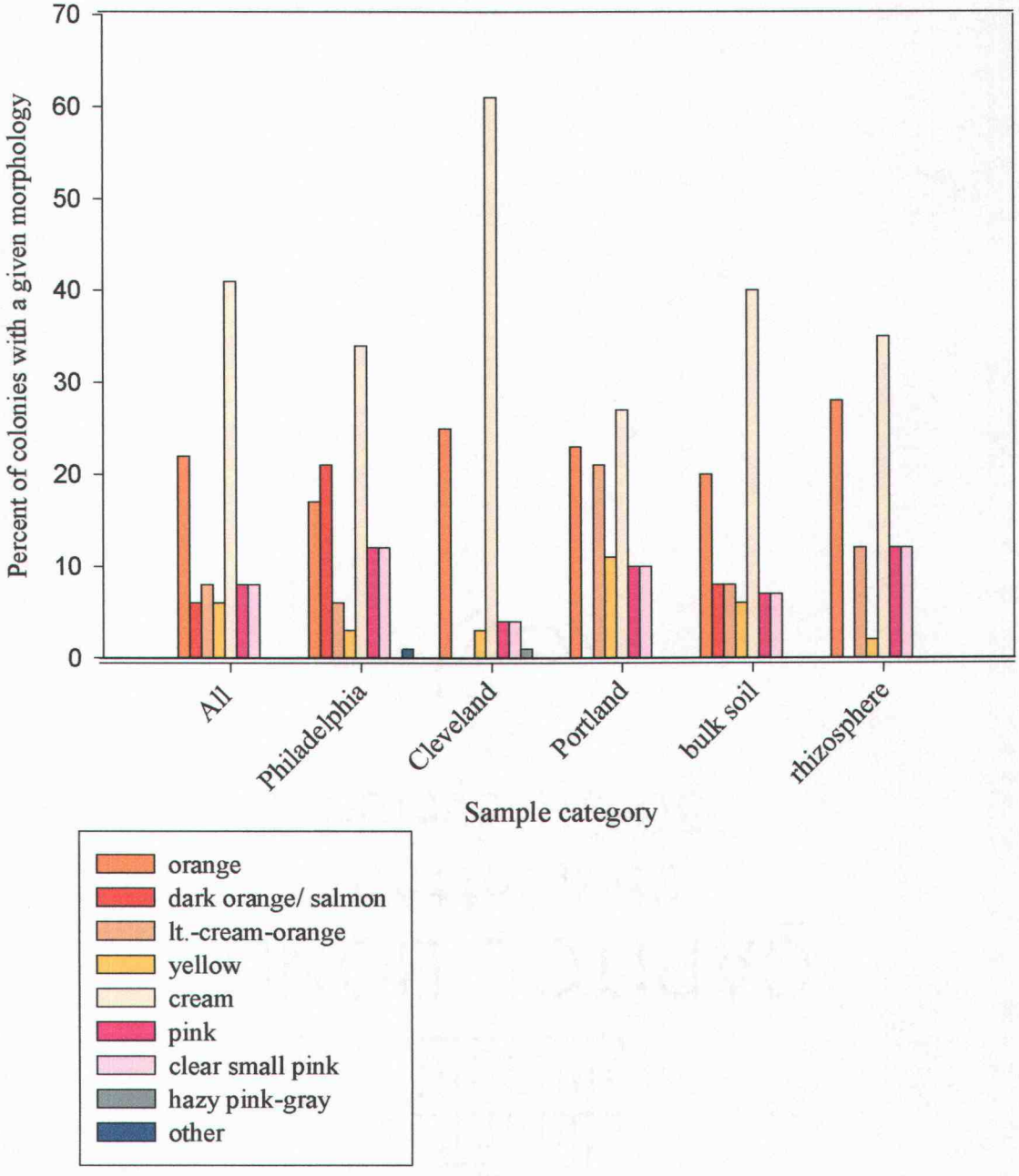


Figure 4.3. Major colony morphologies on initial BCSA plates.

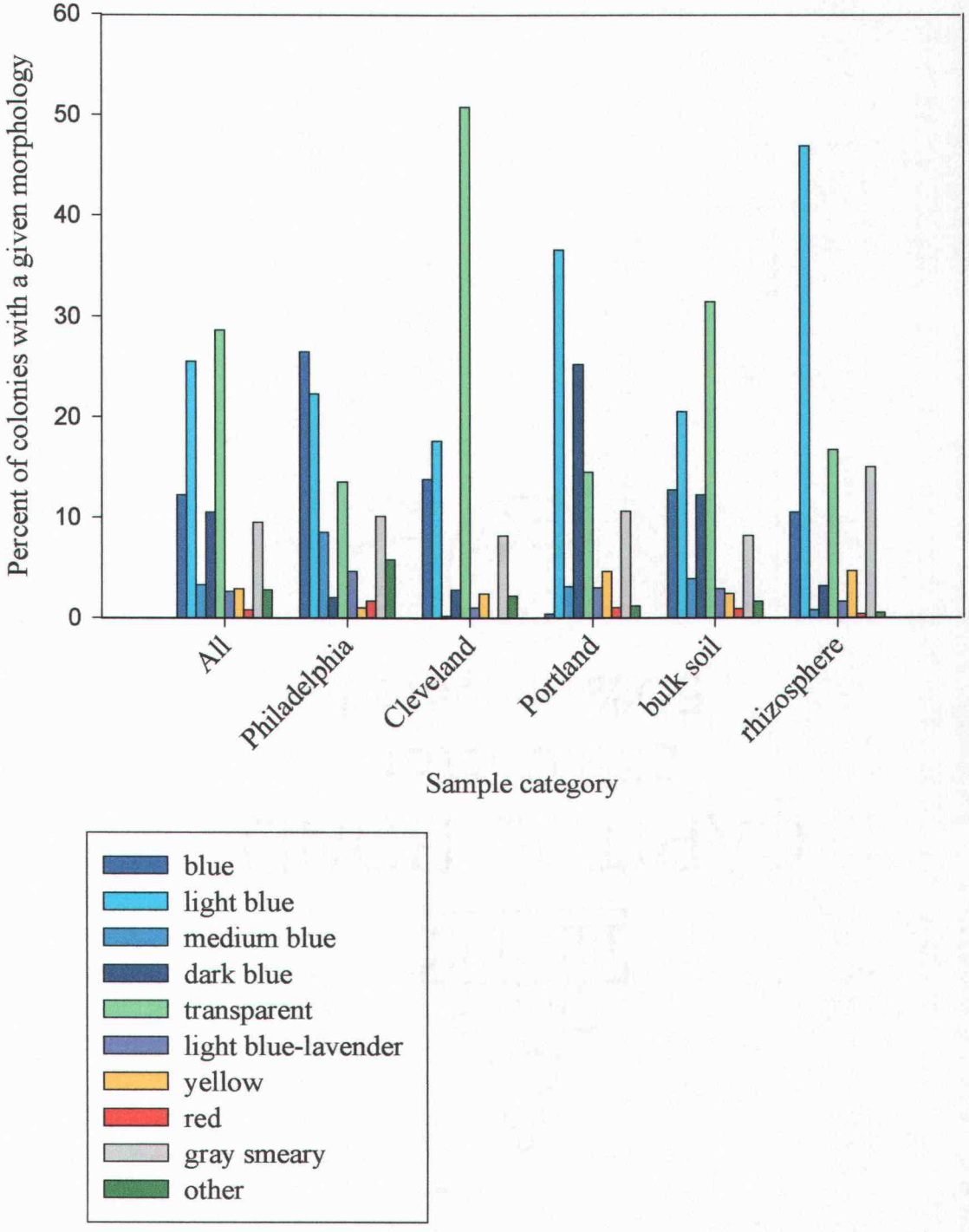


Figure 4.4. Major colony mophologies on initial TBT plates.

Cleveland samples (discussed later); it appears that the character of the bacterial community recovered from the Cleveland samples was different from that recovered from the other two cities. However, analyses of variance did not show differences between cities with regard to the number of different colony types ultimately selected ($\alpha = 0.05$).

Another group of isolates grew on the isolation plates but did not grow in nonselective broth media. This was much more commonly encountered with isolates from TBT than with isolates from BCSA (12.3% vs. 3.2%, respectively). All but three isolates from BCSA grew in 5 ml of LB broth. Isolates from TBT were grown most successfully in KB; 36% of isolates were cultured in this broth medium. Other broth media used included LB (32%), 25% TSB (6%), and full-strength TSB (3%). The difficulty in culturing isolates from TBT in broth, and the eventual success with 25% TSB for otherwise unculturable colonies, may be because TBT is not a rich medium, with only 2 g glucose, 1 g L-asparagine, and 0.5 g $NaHCO_3$ per liter. Bacteria growing on TBT may not be able to adjust to an abrupt switch to a rich medium like full strength TSB. However, BCSA is both rich (10 g sucrose, 10 g D-lactose, 10 g trypticase peptone, and 1.5 g yeast extract per liter) and high in salt (5 g NaCl per liter). It is not surprising that isolates from BCSA would grow well in a rich and salty broth like LB.

Thus, although a total of 1260 isolates were originally selected, 114 did not survive isolation and another 88 were not culturable in any of the broth media used. Another 23 died during storage at –80°C. The total number of isolates raised and mailed to the *Burkholderia cepacia* Referral Laboratory and Repository for

identification and genotyping was 1035. This represents 82% of the original total number of colonies selected.

RHIZOSPHERE SAMPLES

Rhizosphere samples had higher numbers of CFU g^{-1} soil dry weight than did bulk soil samples. The mean difference in CFU between rhizosphere and bulk soil samples was 0.80 log CFU g^{-1} soil on BCSA, and 1.06 log CFU g^{-1} soil on TBT. These differences were statistically significant (one tailed t-test assuming unequal variance: $p = 0.0004$ for BCSA, $p = 0.018$ for TBT).

Overall, the rhizosphere samples did not yield more different types of colonies than bulk soil samples. On BCSA, the mean total number of collected morphologies per sample was identical in the two sample types (5 morphologies per sample). On TBT, a slight difference was observed: the mean total number of collected morphologies per sample was 3.6 with bulk soil, and 3.0 with rhizosphere samples. However, this bulk soil / rhizosphere difference was not significant (one tailed p-value = 0.063).

IDENTIFICATION OF ISOLATES

A total of 1035 isolates was received by the BcRLR and cultured on MH agar at room temperature and at 32°C. Some isolates (251, or 24.3%) did not grow on MH agar at 32°C. None of these 251 were identified as Bcc. Of the 784 which did grow on MH at 32°C, only 449 (57.3%) grew on BCSA at 32°C. Again, of the 335 which did not grow at the elevated temperature, none were identified as Bcc.

Among the 1035 isolates incubated at room temperature, 22 isolates (2.1%) did not grow. Of the remaining 1013, 792 grew on BCSA, and 221 did not grow on BCSA, again at room temperature. None of the 221 that did not grow in BCSA was confirmed as Bcc.

The 1013 which grew on MH at room temperature – the least stringent culture conditions – were also subjected to boil-lysis screening with *Burkholderia-Ralstonia* PCR. A total of 93 isolates were positive with this assay, whereas 920 were negative. A subset of 25 of the negative isolates was chosen, all of which grew on BCSA, and the PCR assay repeated with purified DNA. Again, all 25 were negative with the *Burkholderia-Ralstonia* assay.

The *Burkholderia-Ralstonia* PCR assay was repeated with the 93 isolates which were positive with the initial assay, this time using purified DNA. Here, 78 (83.9%) were positive and 15 were negative. PCR assays (16S and *recA*) performed on the 15 which were negative confirmed that they were not in fact Bcc. The 78 were also evaluated using species-specific 16S PCR assays and *recA* PCR assays, as well as RFLP with the *recA* amplicon. This resulted in 68 isolates being identified as members of the Bcc complex. The other 10 were identified using the RapID NF system: the results are summarized in Table 4.1. It is especially interesting to note that two of the isolates appear in fact to be Bcc after all, even after giving a negative result on the battery of PCR assays used.

The 68 isolates which were positive were identified at the species level, as indicated in Table 4.2. *B. pyrrocinia* was by far the most common species, representing 73.5% of the total isolates, although genomovar III and *B. ambifaria* were

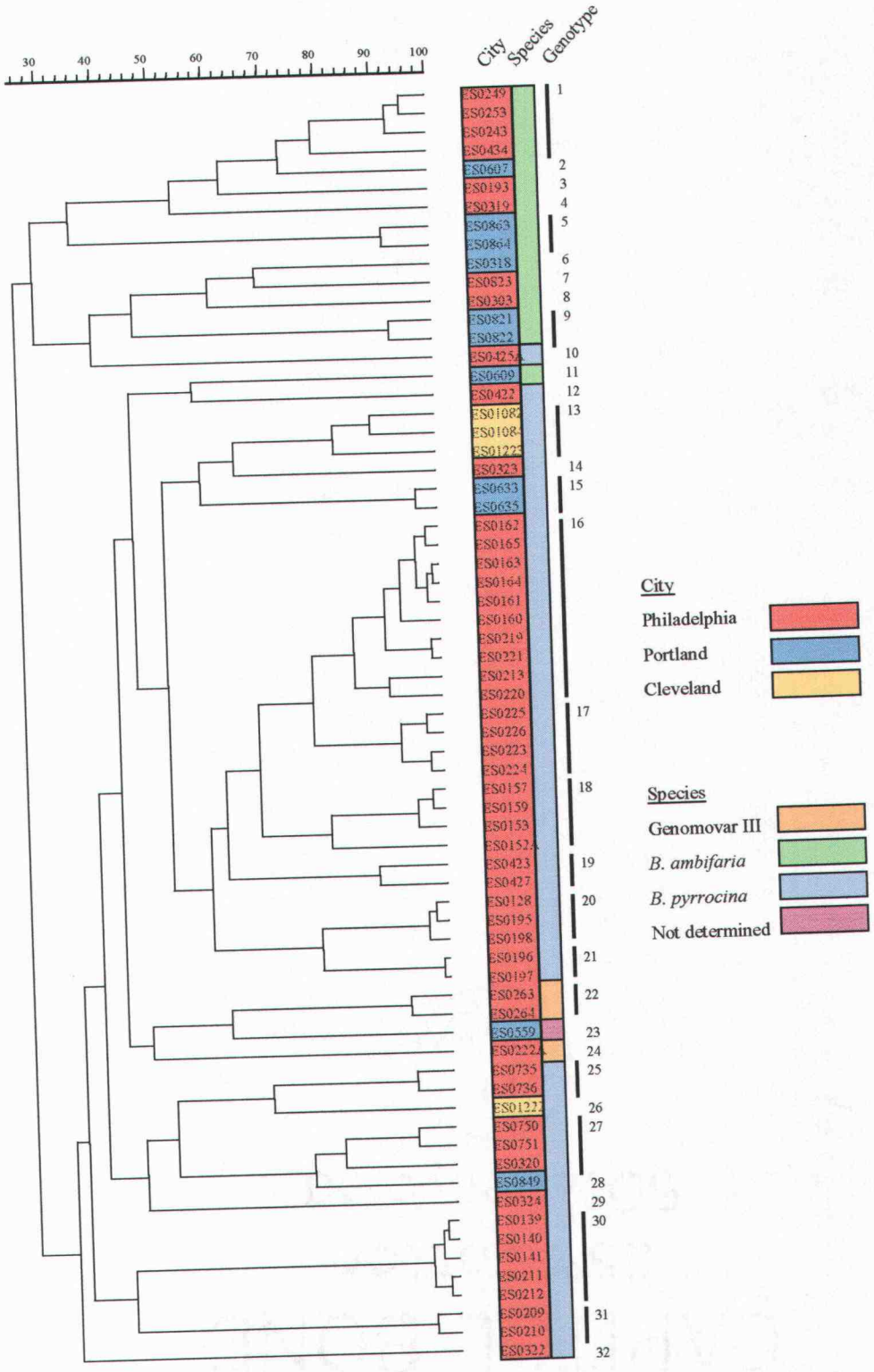
also identified. Genotyping reveals that there were 32 clones among the 68 Bcc isolates, displayed graphically in Figure 4.5. The 68 Bcc isolates represent 6.5% of the isolates screened, and 5.4% of the isolates originally selected from the two media.

Table 4.1. Identification of isolates which were positive with *Burkholderia-Ralstonia* PCR assays but were not positive on Bcc-specific PCR assays

Isolate number	Identification according to the RapID NF Plus Kit
154	Questionable identification as *Flavomonas odoratum*
639	Inadequate identification as *Pseudomonas pseudoalcaligenes*
836	Inadequate identification as *Pseudomonas pseudoalcaligenes*
839	Adequate identification as *Brevendumonas vesicularis*
842	Adequate identification as *Brevendumonas vesicularis*
928	Satisfactory identification as *Burkholderia cepacia*
1148	Implicit identification as *Alcaligenes xylosoxidans*
1160	Satisfactory identification as *Morax lacunata*
1205	Implicit identification as *Vibrio hollisae*
1363	Adequate identification as *Burkholderia cepacia*

Figure 4.5. Product moment-UPGMA cluster analysis of BOX-PCR profiles of Bcc isolates recovered from urban soil samples. 32 genotypes (strains) are identified among 68 isolates from three cities. (LiPuma et al., unpublished data)

Figure 4.5


30
40
50
60
70
80
90
100
City
Species
Genotype
ES0249
ES0253
ES0243
ES0434
ES0607
ES0193
ES0319
ES0863
ES0864
ES0318
ES0823
ES0303
ES0821
ES0822
ES0425A
ES0609
ES0422
ES0108
ES0323
ES0633
ES0635
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ES0161
ES0160
ES0219
ES0221
ES0213
ES0220
ES0225
ES0226
ES0223
ES0224
ES0157
ES0159
ES0153
ES0152A
ES0423
ES0427
ES0128
ES0195
ES0198
ES0196
ES0197
ES0263
ES0264
ES0559
ES0222A
ES0735
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6
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8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
City
Philadelphia
Portland
Cleveland
Species
Genomovar III
B. ambifaria
B. pyrrocina
Not determined

RELATIONSHIP OF BCC ISOLATES TO SAMPLING LOCATION AND MORPHOLOGY

Table 4.2 summarizes the data pertaining to those isolates which were determined to be Bcc. Overall, Bcc was isolated from 15% of sample sites. Philadelphia samples yielded by far the most Bcc: 79% of the Bcc isolates were from Philadelphia. Nineteen percent of the total isolates came from a single sampling site, a gopher hole in a cemetery. Neither Portland nor Cleveland samples had nearly as many Bcc isolates; in fact, samples from Cleveland yielded only 4 Bcc isolates, all of which came from a single soil sample. It is interesting to note that more than twice as many Bcc isolates came from TBT (45; 66% of total) as from BCSA (23; 34% of total), although in 7 cases (78% of samples) Bcc was recovered on both BCSA and TBT. The soil samples out of which Bcc was cultured were not significantly different from the others with regard to water content.

There was no clear rhizosphere enrichment effect. Bcc was isolated from 3 of 20, or 15%, of rhizosphere samples. Similarly, bulk soil yielded Bcc in 14.7% of the samples (13 of 88). In only one sample was Bcc cultured from a rhizosphere sample when it was not cultured from the parallel soil sample; this was true for location 120, the purchased rosebud impatiens from a garden store. In another sample (51, fallow flowerbed), Bcc was isolated from the bulk soil fraction but not the rhizosphere. Bcc was isolated from the rhizospheres of clover, grass, and a rosebud impatiens. It was not isolated from 4 other grass rhizosphere samples, 6 turf samples, or the rhizospheres of tomato (in soil and in potting mix), lettuce, harvested corn, dandelion, or wild geranium.

Table 4.2. Samples from which Bcc was isolated

Location #	City	Location	# isolates from TBT	# isolates from BCSA	Species/ genomovars (# isolates)
38	Philadelphia	Centerfield of baseball diamond	4	1	*B. pyrrocinia* (5)
40	Philadelphia	Forest floor next to hiking trail	6	3	*B. pyrrocinia* (9)
41	Philadelphia	Muck from edge of creek in park	1	0	*B. pyrrocinia* (1)
42	Philadelphia	Under apple tree in residential yard	4	4	*B. pyrrocinia* (7) G'var III (1)
43	Philadelphia	Next to paved play area in park	4	6	*B. pyrrocinia* (10)
49	Philadelphia	Base of a statue at art museum	3	1	*B. ambifaria* (5)
51	Philadelphia	Fallow flowerbed in arboretum	2	0	G'var III (2)
62	Philadelphia	Soil clinging to beets purchased at a farm stand	1	0	*B. ambifaria* (1)
67	Philadelphia	Small animal burrow in cemetery	8	4	*B. pyrrocinia* (10) *B. ambifaria* (2)
95	Cleveland	Bank of small stream in park	2	2	*B. pyrrocinia* (4)
105	Portland	Turf in goalie box of soccer field	3	0	*B. ambifaria* (3)
110	Portland	Near mud puddle in park	4	0	*B. ambifaria* (4)
120	Portland	Soil from pot of rosebud impatiens purchased at local garden center	0	1	*B. pyrrocinia* (1)
124	Portland	Public flower gardens	3	0	*B. pyrrocinia* (3)
TOTAL			45	23	= 68 Bcc isolates

Tables 4.3 and 4.4 display the morphological data pertaining to the Bcc isolates. Although there is no one clear "cepacia morphology," there does seem to be a correlation with certain morphology types. Gray-pink colonies on BCSA, for instance, seem to be a typical Bcc morphology, as do pale blue and medium blue colonies on TBT. Knowing this may help in initial colony selection. For instance, many of the colonies isolated from BCSA were orange (22%). As only 1 orange isolate was identified as Bcc, it makes sense not to select orange colonies in favor of other, more promising morphologies, such as gray-pink.

The resolving power of morphological data is not great, however. There were 14 Bcc isolates from Philadelphia that looked pale or silvery blue, but this was only 34% of the total 41 pale or silvery blue isolates selected from TBT. Similarly, there were 22 medium blue colonies selected from the Philadelphia samples, and only 6 of those, or 27%, were identified as Bcc. Still, it is interesting to note that there were very few gray-pink colonies noted from the Cleveland (3) or Portland (1) samples; that is, there seems to be a connection between the frequency of Bcc morphologies noted and the number of isolates actually identified as Bcc from a given city.

EXTRACTION OF DNA FROM SOIL SAMPLES

DNA was extracted from 87 soil samples. The mean amount of DNA extracted was 3.6 μg DNA g^{-1} soil dry weight, as evaluated with a fluorometer; the median was 17 ng g^{-1} soil dry weight. The amount of DNA extracted varied considerably from sample to sample, as is shown in histogram form in Figure 4.6. All of the soil samples yielded at least a small amount of DNA, however. The minimum

Table 4.3. Morphologies on BCSA: number of Bcc isolates with a given morphology, as described on isolation plate

	Cream	Orange	Gray-pink	Gray-green	Pink	Very dark gray	Brown	Yellow	TOTAL
Philadelphia	1	1	13	1	1	1			18
Cleveland	1							1	2
Portland							1		1
Totals	2	1	13	1	1	1	1	1	21

Table 4.4. Morphologies on TBT: number of Bcc isolates with a given morphology, as described on isolation plate

	Pale or silvery blue	Medium blue	Lavender or pink	Dark blue	Slate blue	TOTAL
Philadelphia	14	6	3	8	1	32
Cleveland			2			2
Portland	2	8	2			12
Totals	16	14	7	8	1	46

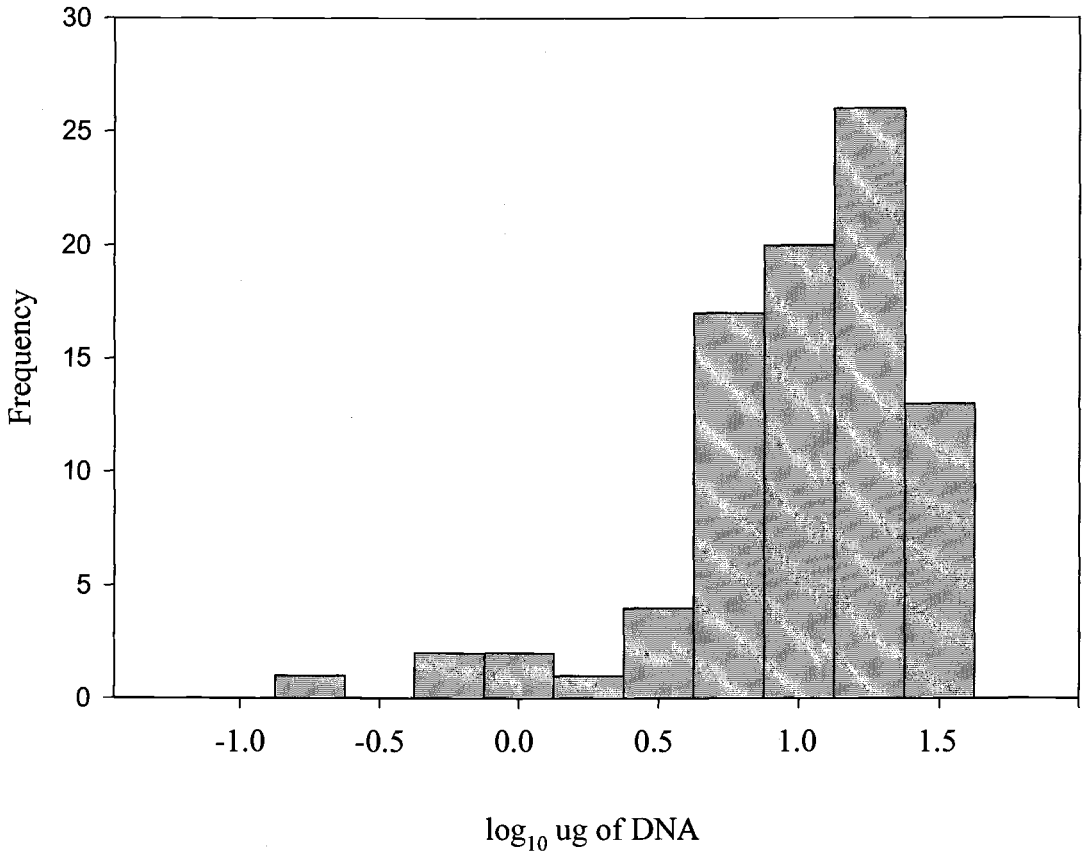


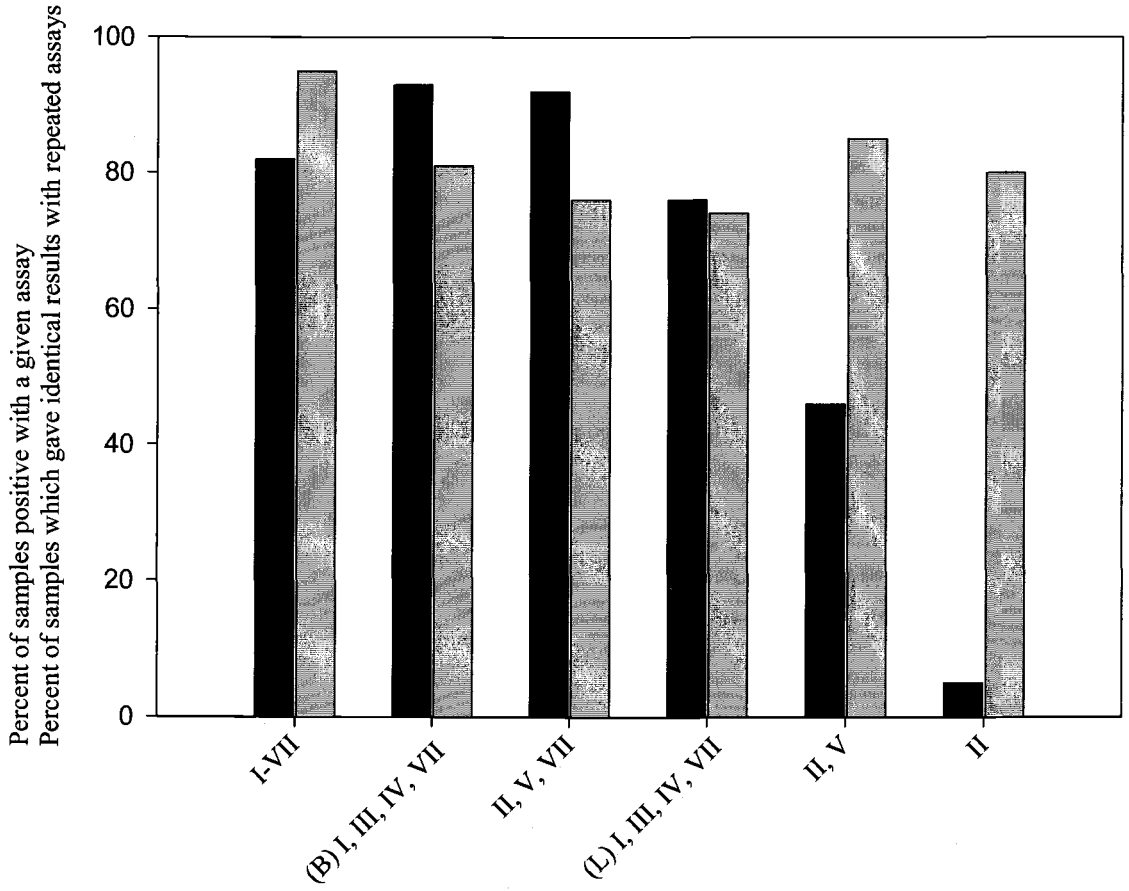
Figure 4.6. Histogram of the quantity of DNA extracted from soil samples.

was 0.12 ng DNA g^{-1} soil dry weight, whereas the maximum was 40.3 μg DNA g^{-1} soil dry weight. Some very low values are not surprising given the inhospitality of some sampled environments (playground sand, for instance). Assuming a bacterial population of 10^9 bacteria g^{-1} soil, and 5 to 8 fg of DNA per cell, the total amount of DNA extracted should have been about 8 to 13 μg DNA g^{-1} soil dry weight. Thus the extraction efficiency of the procedure, using the mean amount of DNA extracted, can be estimated to be between 28% and 45%. The extracted DNA was occasionally faintly discolored with humic acids or other colored materials.

No DNA was detected in extraction blanks when they were evaluated with the fluorometer. Spikes made by adding bacteria to nonautoclaved soil contained considerably more DNA than spikes constructed using twice-autoclaved soil; the mean amount of extracted DNA was 4.5 μg DNA g^{-1} soil for nonautoclaved and 0.21μg DNA g^{-1} soil for autoclaved soil. DNA was successfully extracted from all spikes.

16S rDNA PCR ASSAYS

The results of the PCR assays are consolidated in Figure 4.7. All 87 soil samples from which DNA had been extracted were evaluated with a bacterial primer pair, and all but one sample was positive for bacterial DNA. This sample was from the dusty, dry sand in front of home plate on a baseball diamond. Three more samples were negative at the "genus" level, using the *Burkholderia-Ralstonia* primer pair; they were from wet sand along a creek, sand from a playground, and bark mulch underneath a swingset. The remaining 83 soil samples were all evaluated with the six Bcc-specific PCR assays. Generally, there were more positive results using the



Percent positive
Percent reproducibility

Primer pair	Target genomovars
Eub-16-1, CeMuVi-16-2	I-VII
Eub-16-1, Ce-16-2	I, III, IV, VII
Eub-16-1, MuVi-16-2	II, V, VII
PC-SSF, PC-SSR	I, III, IV, VII
BC-GV, BC-R	II, V
BC-GII, BC-R	II

Figure 4.7. Results of 16S rDNA PCR assays on DNA extracted from soil samples. (B) and (L) refer to primers designed by Bauernfeind et al. (1999) and LiPuma et al. (1999), respectively.

Bauernfeind primer pairs. Overall, 94% of the 87 soil samples from which DNA had been extracted were positive in at least one of the Bauernfeind Bcc-specific PCR assays, whereas 82% were positive in at least one of the LiPuma Bcc-specific assays. Many of the assays were initially negative and were repeated with more or less template DNA. The optimum amount of template solution did vary between samples, as has been observed previously (Kuske et al., 1998).

One-quarter of all the PCR assays were repeated to assess reproducibility. Reproducibility here means providing the same result in repeated assays (positive-positive and negative-negative). The overall reproducibility was 80.6%. Bauernfeind assays were 83.9% reproducible, and LiPuma assays were 78% reproducible. The lowest score for an individual primer pair was PC-SSF and PC-SSR, which gave the same result only 74% of the time. Fully 71.7% of all the changes were associated with faint bands, suggesting that in some cases the amount of template DNA, or the amount of a potentially inhibiting co-extracted substance, was near a threshold concentration for detection.

LIMIT OF DETECTION

The DNA extracted from the limit of detection experiment was evaluated using the same two sets of primer pairs. Low amounts (0.17 - 1.17ng DNA g^{-1} soil, dry weight) of DNA were extracted from the prepared soils, as was the case for other autoclaved, spiked soil samples. In all assays, the limit of detection was 10^5 CFU g^{-1} soil dry weight. The amount of "background" bacteria present at this level was 4×10^8 CFU g^{-1} soil, or approximately a $1/10^4$ ratio. This is slightly less than the 10^9 CFU

figure suggested by Cullen and Hirsch (1998) as an appropriate estimate of the bacterial population in a 'typical' gram of soil. Background populations are important, as the effect of diluting the target DNA into a larger pool of sample DNA is to lower achievable detection sensitivity (Kuske et al., 1998).

There were some slight differences based on the primer pair used and the strain used. The limit of detection could be improved to 50 CFU g^{-1} soil dry weight by running two sequential cycles of PCR, and using the product of the first assay as template for the second. Similar results were reported by Bell et al. (1999) with sequential PCR. No amplification on any of the blanks was observed in this procedure. However, it was considered too vulnerable to PCR error to use with the soil samples (Speksnijder et al., 2001).

CLONING AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ASSAYS

Clones (120) were generated from four soil samples; each clone contained a vector with a 463 bp insert, which was the amplicon from PCR assays designed to capture genomovars I – VII. A PCR screen of these clones revealed that most (97.6%) had the correctly sized insert. A digest with *Sau*96I showed that 82.7% of the correctly sized inserts had the *Burkholderia* pattern, reproduced in Figure 4.8. The RFLP assay results are collected in Table 4.5. In sum, in three of the four soil samples, more than 90% of the evaluated clones had the *Burkholderia* pattern. In soil sample 102, only 9.5% of the clones had the *Burkholderia* pattern. None of these soil samples yielded isolates which were identified as Bcc.

Table 4.5. Results from RFLP and sequencing of 463 bp segment of 16S rDNA

Sample number	Location	Bcc cultured on selective media from sample?	Percent of clones with *Burkholderia* RFLP pattern	Bcc sequences?
57	Vegetable garden	No	96.4%	Yes
68	Edge of jogging/cycling path	No	100%	Yes
70	Golf course	No	90.0%	Yes
102	Flowerbed in botanical garden	No	9.5%	Yes

Of the 96 clones with the *Burkholderia* RFLP pattern, eight were selected for sequencing. Two were selected from each soil sample. All of the sequences were identified as Bcc using the BLAST program. Although species designations are difficult given the rapidly changing taxonomy and using a 463 bp segment, the best matches were genomovar I, genomovar II, and genomovar III. Three clones displaying non-*Burkholderia* patterns were also sequenced. These were identified as a chimeric sequence, an unidentified soil clone, and *Zoogloea ramigeria*.

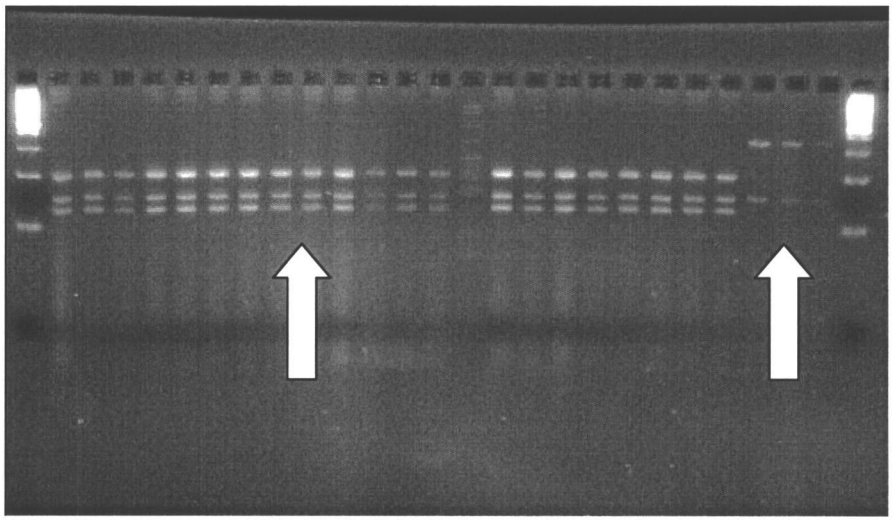


Figure 4.8. RFLP assay with *Sau* 96I digest of 463 bp fragment of *rrn* gene. Left arrow points to *Burkholderia* pattern; right arrow points to one of the non-*Burkholderia* patterns. Far left and far right lanes are molecular ladders, with 100, 200, and 300 bp bands.

CHAPTER 5. DISCUSSION

The *Burkholderia cepacia* complex has emerged in recent years as an important human opportunistic pathogen, particularly for people with cystic fibrosis. It also holds great promise as an agent of biocontrol of many plant pathogens, and as a bioremediation organism for the degradation of a wide range of recalcitrant compounds. Although several attempts have been made to distinguish between helpful and harmful strains of Bcc, clear demarcations between environmentally useful and clinically dangerous strains have not been found. Moreover, a small but steady number of Bcc infections in CF patients each year occur from strains that have not been previously encountered in the clinical setting. These strains are presumed to come from the natural environment, further blurring the lines between natural, beneficial, and potentially hazardous strains of *B. cepacia.*

The understandable hesitancy of many medical researchers to advocate the use of Bcc as an agent of biocontrol or bioremediation is supported by conflicting reports on the prevalence of Bcc in the natural soil environment. If, as some have proposed, Bcc is rarely encountered in soils, then deliberately adding any strain of Bcc to soil may well constitute an unacceptable risk to vulnerable people. If, however, Bcc is commonly found in soils, the risk posed by agricultural and engineering use may be negligible. Essential to the process of determining the occurrence of Bcc in soils was the use of the protocols mutually acceptable to the medical and environmental microbiology communities, including rigorous identification procedures which reflect the most current taxonomy. This study examined the prevalence of Bcc in urban and

suburban soil environments, using a combination of culture-based and non-culture based methods.

ISOLATION OF BACTERIA ON SELECTIVE MEDIA

Although isolating bacteria from soil can be time consuming and difficult, it is preferred over non-culturing techniques to answer certain questions. With the isolate in hand, a wide range of tests are possible, and it is possible to obtain important information such as genomovar status and presence or absence of hypertransmissibility factors. Isolation of members of the *Burkholderia cepacia* complex was attempted on two different selective media, BCSA and TBT. Both have reported considerable selectivity for Bcc; 93.6% of the clinical isolates that were cultured on BCSA were Bcc (Henry et al., 1997) whereas 72% of the colonies from environmental samples that were cultured on TBT were identified as *Pseudomonas cepacia* (Hagedorn et al., 1987). Our results showed substantially lower selectivity. Only 8.8% of the isolates (nonrandomly selected) from TBT were identified as Bcc, as were only 2.9% of isolates from BCSA. Overall, 5.4% of the 1260 isolates originally selected were identified as Bcc.

The discrepancy between our results and previous studies with these media could stem from several issues. First, BCSA was developed for use in the clinical setting, and is consequently a rich medium, amended with polymyxin B, vancomycin, and gentamycin. It is possible that the richness of BCSA placed too much metabolic stress on nutrient deprived soil populations of Bcc; a leaner medium might be better for capturing bacteria from the natural environment.

A second possibility is that the antibiotics used are either too selective or not selective enough. It is entirely possible that, in the huge variety of soil-living bacteria, there are other, non-Bcc members that are able to overcome the formidable antibiotic selectivity of BCSA. These other bacteria may have overwhelmed any Bcc that were present on the plates. Alternatively, it is possible that environmental strains of Bcc do not have, or do not express, the genes for antibiotic resistance that characterize clinical strains, and consequently weren't able to grow on BCSA at all. Substantially lower antibiotic resistance by environmental strains was reported by Butler et al. (1985). However, others report the isolation from soil of Bcc with considerable antibiotic resistance. Strain PVFi5A, isolated from the rhizosphere of tomato and described by Sfalanga et al. (1999), showed resistance to erythromycin, carbenicillin, gentamycin, kanamycin, neomycin, polymyxin B, rifampin, spectinomycin, streptomycin, tetracycline, ampicillin, and penicillin G.

TBT was developed for use with environmental strains of *B. cepacia* (Hagedorn, et al., 1987) and, in fact, more than twice as many Bcc isolates were recovered from TBT than from BCSA. This could be because of the smaller number of antibiotics used (tetracycline only), or perhaps because of the relative meagerness of the medium. However, 8.8%, even non-randomly selected, is still a far smaller percentage of colonies that are Bcc than the published values of 72% (Hagedorn et al., 1987). It may be that the soil strains of Bcc we happened to encounter did not have resistance even to tetracycline. In that respect, it would have been interesting to have included a selective medium in which the basis of selectivity was not resistance to antibiotics or other substances. One such alternative might have been *Pseudomonas*

cepacia agar (PCAT), which uses as a basis of selectivity the ability to metabolize unusual substances, such as azelaic acid and tryptamine as the sole sources of carbon and nitrogen, respectively (Burbage and Sasser, 1982). Other studies have reported considerable success isolating Bcc using PCAT (Bevivino et al., 1998; Balandreau et al., 2001), which has been reported to provide a degree of selectivity for Bcc of more than 70% (DiCello et al., 1997). However, Hagedorn et al. (1987) reported significantly greater success in isolating Bcc from some soils with TBT rather than PCAT.

A significant problem in evaluating previous reports of media designed to select Bcc from the environment is that the taxonomy of Bcc, and indeed of the entire genus *Burkholderia*, has changed rapidly. As discussed earlier, "*B. cepacia*" has gone from being considered a single *Pseudomonas* species to a being a *Burkholderia* complex of no less than nine species (genomovars) in a few years' time. Although a few researchers have kept current of the increasing taxonomic complexity, most have not. Add to this the notorious difficulty in identifying Bcc with widely available biochemical test schemes (Segonds et al., 1999; van Pelt et al., 1999) and it becomes extremely difficult to know how much confidence to place in an identification of "*B. cepacia*". Hagedorn et al. (1987) openly acknowledged this latter difficulty in noting that "it is highly likely that some of the *P. cepacia* isolates [from that study] are actually closely related *Pseudomonas* species."

Perhaps the best example of this difficulty is a 1999 study by Wigley and Burton, in which 21 environmental isolates were identified as *B. cepacia*. Bcc isolates were identified by growth on five selective media, gram stain, and use of the API

20NE system. The 21 Bcc isolates represented a recovery rate of 20% from samples tested. Subsequent rigorous testing of the isolates, including amplified 16S rRNA gene restriction fragment length polymorphism analysis (ARDRA), *recA*-specific PCR, and protein profile analysis, demonstrated that only 1 of the 21 isolates were actually members of the *B. cepacia* complex. The other twenty were tentatively identified as a variety of bacterial species, including members of the *Ralstonia, Serratia, Chromobacterium, Pseudomonas* and *Enterobacter* genera (Mahenthiralingam et al., 2000). It is thus not unlikely that previous reports of success isolating Bcc on various selective media included isolates which were not, by current taxonomic definition, Bcc. This may help explain the difference between our results and those from previous studies; in any case, it emphasizes the importance of conducting a study of environmental populations of Bcc using the most current identification protocols available.

Some earlier studies have also reported isolating Bcc infrequently. Mortensen et al. (1995) sampled in homes, salad bars and food markets. Of the 916 samples collected, only 25, or 2.7%, were positive for Bcc. Butler et al. (1995) collected 55 soil, rhizosphere, vegetation and water samples from a botanical complex and cultured Bcc from 12 samples (21.8%). These researchers cultured bacteria on PC and MacConkey agar (BBL Microbiological, Cockeysville, MD) and Mast cepacia agar (Mast Diagnostics, Ltd., Bootle, U.K.). Together with our results, this suggests that Bcc is in fact not easy to recover on selective media. It may be that non-culture-based methods are necessary for an accurate assessment of the prevalence of Bcc in the environment.

Less easily explained is the difference between the cities – specifically, the reason why the preponderance of isolates were found in Philadelphia. Two things were different for the Philadelphia samples; first of all, the time of year in which the samples were collected. Philadelphia samples were collected in September of 1999, and Cleveland and Portland were sampled in May and June of 2000. The weather was warm and wet in all three cases, however, and soil moisture was not significantly different between the three cities (data not shown).

A second difference is that there were no additions of antifungal agents to the media for the Philadelphia plates, whereas nystatin was added to the media (50 μg/ml) for both Cleveland and Portland. We did not initially add nystatin to the plates because we had not seen significant fungal growth in the small pilot study performed previously (Miller, unpublished data). However, the lack of nystatin in the Philadelphia media resulted in nearly uncountable TBT plates and severe fungus contamination of BCSA plates. The addition of nystatin did not seem to have an adverse affect on total colony numbers, as was demonstrated in an experiment performed prior to sampling in Cleveland and Portland (data not shown). Moreover, Hagedorn et al. (1987) used nystatin at a concentration of 50 μg/ml and reported good success isolating *B. cepacia*. Finally, the PCR results do not reflect a similar difference between the cities, lending further support to the idea that change in the media may be responsible for the difference. It's thus not known why the samples from Philadelphia yielded so many more Bcc isolates than did samples from Cleveland or Portland.

It is interesting to note that, although more Bcc was isolated from TBT than from BCSA, all isolates which were ultimately identified as Bcc were able to grow on BCSA. It may be that soil strains of Bcc were more capable of growing on BCSA after first growing on TBT. Temperature may also be a useful screening tool, as all of the Bcc isolates were capable of growth at 32°C. As is typical with this complex, some Bcc isolates were PCR-negative but positive according to biochemical tests. This again underscores the difficulty in identifying Bcc and the importance of using a polyphasic approach.

By far the most common species/genomovar isolated from the soil samples was *B. pyrrocinia* (considered to be genomovar IX). *B. ambifaria* (genomovar VII) was also isolated relatively frequently, and a few genomovar III isolates were recovered. The genomovar III isolates did not cluster with clinically epidemic strains. *B. pyrrocinia* and *B. ambifaria* are known to be present in soil environments (Coeyne, 2000; Vandamme, 2001), and recovering them in higher numbers was not surprising. More interesting was the fact that no genomovar I was isolated, despite the general supposition that genomovar I is common in the environment (Govan et al., 1996). Also interesting was the lack of *B. vietnamiensis* (genomovar V). The plant-growth-promoting behavior of some *B. vietnamiensis* strains made it a potentially likely rhizosphere colonizer, but no *B. vietnamiensis* strains were isolated in this study in rhizosphere or bulk soil samples.

Reference strains of genomovars I – VII were successfully grown on TBT and BCSA, ruling out a categorical inability of any genomovar to be cultured on these media. It may be that some genomovars, or some strains, make the soil - media

transition better than others, due to assumption of the viable but not culturable (VBNC) state, loss of antibiotic resistance, or other factors.

It was not surprising to see the large number of genotypes (32 clones among 68 isolates), given the predilection of Bcc for genetic variability and nonclonal populations (Wise et al., 1994). However, not all clones came from the same sample; two isolates, taken from samples approximately 1 mile apart, were clonal.

RHIZOSPHERE ENRICHMENT

We had sampled the rhizosphere in 20 of 107 samples (18.7%). Bcc is a known rhizosphere colonizer; populations of up to 10^5 CFU g^{-1} root have been identified on the roots of peas (King and Parke, 1993). Maize (corn) also sustains large populations of Bcc, where it can comprise 4 to 35% of the total culturable rhizobacteria (Hebbar et al., 1992; Nacamulli et al., 1997). Balendreau et al. (2001) isolated Bcc from the rhizosphere of maize, wheat, and lupine. Other plant hosts known to support Bcc in the rhizosphere include tomatoes (Sfalanga et al., 1999) and perennial ryegrass (Nijhuis et al., 1993). It thus was theorized that populations of Bcc would be enriched by the presence of a plant root, and that otherwise low – and possibly undetectable – populations of Bcc would be detectable in rhizosphere samples. Indeed, this was the basis for a planned experiment using the rhizosphere of peas to enrich for Bcc in soil, and thus bring low populations up to detectable levels (see Appendix B).

Rhizobacteria which could grow on the selective media we used were more abundant than bacteria in the bulk soil (by about 1 log unit g^{-1} soil). There were no

more or less morphological types of colonies present on the rhizosphere plates, however, indicating that diversity of colonies was not noticeably affected by the presence of the root. Also, Bcc was not isolated from the rhizosphere any more frequently than from the bulk soil samples. The best explanation here lies with the nonrandom selection of isolates. One colony type of each morphology was selected, so if rhizosphere samples had more Bcc (with, presumably, similar morphologies), we would still have chosen only one colony. Other explanations include the host plants; it may be that the plants we chose were not good hosts for Bcc, or that the Bcc populations on those plants were not able to overcome the selectivity of the two media. Methodology may also differ. Some other studies blended or ground the roots of plants and plated the root slurry (Bevivino et al., 1998; Balandreau et al., 2001), instead of plating only the adhering soil, as we did. Blending and plating root slurry would have recovered endophytic populations in addition to populations external to the root, and endophytic populations of Bcc can be substantial (Hallman et al., 1999).

EXTRACTION OF DNA FROM SOIL SAMPLES AND EVALUATION WITH THE PCR

The second half of this study was to use non-culture-based methods to look for the presence of *B. cepacia* complex in soil samples. It is well known that 90 to 99% of the bacteria in soil are not culturable using conventional methods (Cullen and Hirsch, 1998), and it seemed possible that some Bcc strains might be included in the unculturable majority. We thus directly extracted DNA from soils and evaluated the extracts for the presence of Bcc DNA using two independently developed sets of 16S PCR assays which targeted the 16S ribosomal gene.

Other studies have established beadbeating as a fast and easy method of extracting bacterial DNA from soil without coextracting undue amounts of plant or fungal DNA (Borneman et al., 1996), and the use of spin columns to purify crude DNA extracts has been similarly confirmed (Frostegard et al., 1999). This combination coextracts a minimum of humic acids, which have a similar molecular weight and net charge as DNA, and thus are readily copurified (Holdben, 1994) but which can inhibit *Taq* DNA polymerase (Cullen and Hirsch, 1998). The amount of DNA extracted from the soil samples in our study spanned five orders of magnitude (from 0.12 ng to 40.3 μg DNA g^{-1} soil). This is not surprising given the inhospitality of some sampled environments, like playground dust and streambank sand. Because many of the "soil" environments sampled in this study were not actually soil, the estimated extraction efficiency of 28 to 45% – based on the mean amount of DNA extracted per g soil – may not be a good index of the performance of this procedure. However, even this falls within the range of published values and indicates that a reasonable proportion of DNA was extracted. It should further be noted that DNA was extracted from soil that had been frozen at –20°C. Stenberg et al. (1998) concluded that storage of soil samples at –20°C for up to 13 months did not affect the microflora of annually frozen soils in any obvious way. Although soil microflora in Swedish soils may well be different from those in the United States, it seemed clear that freezing was preferable to refrigeration.

PCR assays with the 16S gene have been used in other studies of soil bacterial populations (Pepper and Pillai, 1994; Dojka, 1998; Bell et al., 1999). There are advantages to using ribosomal genes as a PCR target; one is the high copy number of

rrn genes (6, on three chromosomes, in most strains of Bcc) (Lessie et al., 1996). There are many published sequences of this gene, facilitating comparison with sequences generated in the course of this study. Finally, the primers give one product of one size with pure cultures, unlike some other primer pairs which target the *recA* gene (Mahenthiralingam et al., 2000). Disadvantages of the 16S gene are, principally, its highly conserved nature. There may well be other members of the β-proteobacteria which are similar to Bcc and which were not tested in the creation of the primer pairs. A highly conserved gene is also an unlikely source of easily gained differentiablity between genomovars, which by definition are 98 to 99% homologous in their 16S rDNA sequences (Coeyne, 2000). Although other researchers have used partial 16S sequences to identify bacteria at the species level (Borneman et al., 1996), the genomovar identifications made in this study as a result of sequencing a 463-bp segment (30%) of the 16S gene in Bcc are tentative due to the high degree of inter-genomovar homology.

The performance of the 16S rDNA PCR assays was very consistent. Reproducibility of results ranged from good (74%) to excellent (95%), depending on the primer pair. The results from the two PCR schemes generally supported one another, although there were differences between the primer pairs. For instance, both the Bauernfeind and LiPuma groups developed a primer pair which, in trials with known strains, captured genomovars I and III, *B. stabilis* (IV) and *B. ambifaria* (VII). In assays with soil extracts, however, these primer pairs did not perform identically; 76% of samples were positive with the LiPuma version, whereas 93% were positive with the Bauernfeind primer pair. These differences could result from varying

sensitivities of the primer pairs to the target DNA, or to various inhibiting contaminants which may have been co-extracted with the DNA. Hogardt et al. (2000) found that some otherwise indistinguishable Bcc strains had a "lack of sensitivity" to PCR assays which were not readily explained. Two of the isolates in this study also gave conflicting biochemical and PCR assay results. Thus, the difference could lie with the target Bcc DNA, the primer pair or a hypothetical contaminant.

Most soil samples were positive for one of the Bcc-specific PCR assays; 82% were positive using one of the LiPuma assays, and 94% positive in at least one of the Bauernfeind assays. This is much higher than the results of isolation from selected media; only 14 samples out of 107, or 15%, yielded isolates which were identified as Bcc. Several possibilities for the discrepancy immediately suggest themselves. One possibility is the limit of detection of plating on selective media, as opposed to the limit of detection of the PCR assays. It is difficult to know what the limit of detection of our culturing effort actually was, given the non-random nature of colony selection; the PCR assays had a limit of detection of 10^5 CFU g^{-1} soil. Moreover, if numbers of the desired bacterium are low, they may be impossible to detect via plate culture, as they will be swamped by more numerous and faster growing organisms. Different detection limits are seen in clinical studies where a patient may be "culture-negative" but "PCR-positive" for Bcc (Whitby et al., 1998). It could also be that the bacteria are culturable but not able to grow with the selective agents in these two media, as previously discussed.

Alternatively, it could be that the bacteria are viable but not culturable. Bacteria in the viable but not culturable (VBNC) state are thought to be common in a

substrate-limited habitat like soil (McDougald et al., 1998). VBNC may be a stress-response performed by non-differentiating bacteria; in lieu of, for instance, spore formation, a bacterium capable of VBNC undergoes a series of distinct physiological changes in response to environmental stress. These responses include a thickening of the cell wall, condensation of the DNA, reduction of the amount of RNA, metabolic slowdown, and development of nonculturablility. Interestingly, it has been suggested that PCR assays to detect VBNC cells are made more difficult, due to the condensation of genomic DNA, and that the limit of detection of VBNC cells is therefore higher than for normal cells (McDougald et al., 1998). As it has been shown that bacteria in the VBNC state do not lose pathogenicity or virulence (McDougald et al., 1998), the ability to ascertain the presence of Bcc in this state could be very helpful in delineating the risk posed by environmental populations to susceptible people.

A third possibility is that the bacteria were in fact not present in the soil, and that the PCR assays were amplifying Bcc DNA remaining from previous populations which had survived degradation by being adsorbed to soil colloids. Extracellular DNA in soils can be bound to sand, clay, and humic materials (Bertolla and Simonet, 1999). Clay minerals in particular are highly reactive; one g of pure montmorillonite is able to adsorb up to 30 mg of DNA – the equivalent of 10^{13} *E. coli* genomes. DNA, typically negatively charged, binds to the positively charged edges of clay minerals. It also binds to clay surfaces, helped by bridges formed by divalent cations like calcium and magnesium (Paget and Simonet, 1994). In these locations the DNA is protected from nucleolytic degradation and can persist for untold lengths of time: some have

reported adding labeled DNA to soil and then detecting it with PCR assays for up to 130 days (Paget et al., 1992; Bertolla and Simonet, 1999). Frostegard et al. (1999) reported that DNA added to high-clay soils adsorbed so strongly to the clay that it was necessary to add RNA to the soils in order to recover more than a few percent of the DNA added.

Our experience with spiked, autoclaved soils supports the hypothesis that at least a portion of the extracted DNA was from nonviable cells. Autoclaved soils were used in this study in both the limit of detection experiment, and in the construction of spikes for extraction. A substantial amount (ca. 10^9 CFU g^{-1} soil) of bacteria were added in all cases, but the amount of DNA extracted from these previously autoclaved soils was much lower than the mean for unautoclaved soil samples. The difference may reflect the lack of extracellular DNA in the autoclaved soils, which may have been disrupted in the sterilizing process. The much lower amount of DNA extracted from spiked, autoclaved soils suggests that at least part of the DNA extracted from soil samples may not have come from live cells, but from DNA adsorbed to various soil particles (Cullen and Hirsch, 1998). This in turn suggests that Bcc may in fact not be present at all in some soil samples that amplified in the PCR assays, but that DNA from previous Bcc populations was still in the soil.

A final explanation for the discrepancy between culture-based and non-culture-based estimations of Bcc prevalence is that the PCR assays are not sufficiently selective, and are amplifying non-Bcc DNA. This is clearly the case at least part of the time, as is shown in the cloning and sequencing data; in one soil sample, 90% of the tested clones had an RFLP pattern that corresponded to *Zoogloea ramigeria*, and

which was confirmed by sequencing. The presence of some non-Bcc patterns in the RFLP digest was fully expected, as the PCR and cloning techniques we used have been shown to introduce errors (Speksnijder et al., 2001). Nor is it particularly surprising, given the highly conserved nature of the 16S gene, that other bacterial DNA is able to give a positive signal in these PCR assays. It is important to note, however, that all four soil samples contained sequences which were definitely identified as part of the *B. cepacia* complex. It is thus possible that the non-Bcc DNA helped amplify the Bcc signal, but that Bcc DNA was also present in many of the samples.

The 16S rDNA PCR assays are far from a perfect tool for determining the environmental prevalence of Bcc. Their selectivity is less than ideal, leading one to question the overwhelmingly positive results obtained using them. However, the screening and sequencing portion of the study demonstrated that *Burkholderia cepacia* complex DNA was being amplified in all four soil samples, and indeed accounted for the majority of the DNA amplified in three of the four samples. Finally, no Bcc was isolated from any of these four samples in which Bcc DNA was conclusively present. Although the 16S PCR assays may overestimate the prevalence of Bcc in the environment, culturing on the currently available selective media clearly underestimates Bcc populations. These results underscore the need for better techniques of identifying bacterial soil populations. More significantly, they indicate that the natural environmental prevalence of the *B. cepacia* complex is higher, and possibly much higher, than previous studies have indicated.

SUGGESTIONS FOR FURTHER RESEARCH

The data from this study are not adequate to definitively answer the question of what populations of Bcc are present in soil environments. The limit of detection experiment was performed by adding bacteria to autoclaved, sterile soil with a high clay content (39.5%). Only low amounts of DNA were extracted from the tested soil, although 10^9 bacteria per g soil were added. This suggests that the bacteria, or, after lysing, bacterial DNA, were strongly adsorbed to the soil colloids, as was discussed earlier (Paget et al., 1992; Frostegard et al., 1999). Holben reports that the DNA recovered from two soils with similar organic content but different clay content (8.1 and 48% respectively) differed; the yield of DNA from the high clay soil was only about 15 to 25% of that from the low clay soil. Interestingly, both soils had similar bacterial counts (Holben, 1994). It thus seems possible that the use of autoclaved soil with a high clay content may have resulted in a higher estimation of the limit of detection than was actually the case with soil samples. In other words, the high limit of detection may be an artifact of the method used to estimate it and the actual populations of Bcc in the sampled soils is yet unknown.

Other questions also remain. For instance, *B. multivorans* (genomovar II) is a clinically important strain, responsible for cases of cepacia syndrome in the U.S. (LiPuma, in press) and elsewhere. Although there are reports of a hypertransmissible or "epidemic" *B. multivorans* strain (Segonds, 1999), new and unique *B. multivorans* strain types are acquired each year by CF patients. The source of *B. multivorans* is not known. This study showed only 5% of soils were positive with *B. multivorans*–specific primers. If this is accurate, it reaffirms that not all genomovars are equally

common in soils, and raises the possibility of another source of infection with *B. multivorans* besides contact with the soil environment.

Similarly, the 45% of soils that were positive with the *B. vietnamiensis*-specific primer pair lead to questions about the culturability of *B. vietnamiensis*. If *B. vietnamiensis* DNA was present in nearly half of sampled soils, why were none isolated? Could some of the plant-associated strains like *B. vietnamiensis* occupy internal plant tissue, where they would have been captured by the DNA extraction from fine plant roots included in the soil samples? The overwhelming preponderance of *B. pyrrocinia* among the isolates raises still other questions. Are some genomovars, like *B. vietnamiensis*, simply more difficult to culture than others, like *B. pyrrocinia*? Are some more resistant to DNA extraction and evaluation by the PCR, perhaps as a part of the VBNC state? Clearly, more research on the ecology of this bacterial complex – and of the best methodologies to study it – are necessary to further our understanding of *B. cepacia*'s presence and population in soils.

CHAPTER 6: SUMMARY

Advancements in the taxonomy of the *B. cepacia* complex, coupled with the inherent difficulty in identifying this microorganism, have made it difficult to interpret the wealth of literature on its prevalence in the soil environment. Although many studies have been conducted on Bcc, many questions remain. Is Bcc common in soils? Is it easily isolated? Is it present in soil environments where people may contact it?

This study examined the prevalence of the *B. cepacia* complex in urban and suburban soil environments where people may contact it. We sampled sites such as gardens, baseball fields, golf courses, and playgrounds in three large United States cities. Soil samples were plated on two different selective media and isolates evaluated with a battery of the most current identification protocols. DNA was also extracted from the soil samples and examined using two separate PCR assay systems specific for Bcc.

We found that only a few (5.4%) of the isolates selected were Bcc. RepPCR revealed the presence of 32 genotypes in the 68 isolates. More isolates were selected from the TBT medium than from BCSA, although all of the isolates identified as Bcc grew on BCSA as well. Incubation temperature was another useful tool in screening out non-Bcc isolates, as all Bcc isolates were able to grow at 32°C.

The majority of the soil samples we evaluated were positive for the *B. cepacia* complex as determined by the 16S rDNA PCR assays. The limit of detection of these assays, determined with a high-clay autoclaved soil, was 10^5 CFU/g soil, suggesting

that large populations of Bcc may be present in the soil. Selected amplicons were screened with RFLP and sequenced; all four of the soil samples examined contained Bcc DNA. In three of the four soil samples examined, more than 90% of the clones had a "*Burkholderia*" RFLP pattern. One soil sample contained clones with a different pattern, identified as *Zoogloea ramigeria*.

Although there are many interesting aspects to this study, perhaps the most significant is the difference between the culture-based and nonculture-based methods. Bcc was isolated from only 14% of soil samples on the two selective media. In contrast, 76% and 93% of the soil samples were positive for Bcc DNA according to the two PCR assay systems. The selectivity of the PCR assays is not perfect, and it is possible that some "Bcc-positive" soil samples do not in fact contain Bcc DNA. However, all four soil samples that were further evaluated by sequencing of PCR products contained Bcc DNA, and none of them yielded Bcc isolates on selective media. Of the many possible explanations for this, one of the simplest is that many Bcc isolates are not culturable on the media we used. It follows that use of selective media may not be the best way to estimate the environmental prevalence of Bcc in soils, and, further, that populations of Bcc in soils may be much higher than previously estimated.

The story of bovine spongiform encephalopathy shows that the gap between agricultural or environmental research and clinical microbiology can have grave repercussions. In that sense, a deep concern in the medical community over the deliberate use of *B. cepacia* as a biocontrol or bioremediation agent is well founded and appropriate. Indeed, Bcc is not the only organism with a dual "Jekyll and Hyde"

identity; other bacteria, such as *Pantoea agglomerans, Enterobacter cloacae, Serratia marcescens*, and *Stenotrophomonas maltophilia* all are human opportunistic pathogens capable of causing disease in vulnerable people (EPA, 1999). It is essential that microbiologists from diverse fields join in a cooperative effort to determine the best use of *B. cepacia*'s marvelous potential, while minimizing risks to susceptible humans.

Risk assessment cannot proceed with incorrect or misleading data. Studies that failed to isolate Bcc on selective media may have overlooked nonculturable Bcc. Conversely, studies that isolated large numbers of putative Bcc colonies may not have performed adequately rigorous identification protocols to confirm the identity of Bcc. Our cooperative study between medical and environmental researchers clearly shows that not all Bcc is culturable on two of the most widely used environmental and clinical selective media. Our results also suggest that Bcc is commonly encountered in urban soil environments where people may contact it. Better molecular techniques for determining the presence of Bcc in soil are necessary to adequately determine the populations of Bcc in soils. Developing these techniques, and further deciding the fate of *B. cepacia* application, will take sustained multidisciplinary effort and involve everyone who is interested in *B. cepacia* as a human pathogen, remediation agent, biopesticide, and uniquely intriguing microorganism.

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APPENDICES

Appendix A. SAMPLING AND ISOLATE INFORMATION

Table A.1. Sample descriptions and locations

Sample number	Habitat description	Location	City	Date sampled
37	Sampled the strip of grass between street and sidewalk. Crabgrass growing: depth to 5".	Tracy street, on RH side, just past 3rd maple tree from corner	Philadelphia	9/2/99
38	Sampled in center field of baseball field.	Swenson park, at the corner of Conwell and President	Philadelphia	9/2/99
39	Sampled the "model" compost pile.	Pennypack Park Environmental Center, off Verree Road: entrance to center is the first right after Bloomfield as you're heading SW on Verree.	Philadelphia	9/2/99
40	Sampled the forest floor a few feet from the hiking trail through Pennypack Park.	Pennypack Park Environmental Center.	Philadelphia	9/2/99
41	Sampled from the edge of Pennypack Creek.	Pennypack Park Environmental Center.	Philadelphia	9/2/99
42	Sampled from the side yard, under the apple tree, of a private residence.	Corner of Rising Sun and Ripley: side yard of white house	Philadelphia	9/2/99
43	Sampled next to the paved play area, along a fence. Skateboarders nearby liked to bump along over the grass.	Fox Court, at the corner of Cottman and Whittaker	Philadelphia	9/2/99
44	Sampled from baseball diamond immediately to the right of the Cottman St. entrance. Sample taken from in front of home plate.	Burholme Park, at Cottman entrance just past Central Ave.	Philadelphia	9/2/99
45	Sampled loose dirt near the base of the slide, which had evidence of people playing in it. The slide itself and the first part of the "landing" area were on a rubber mat.	Burholme Park, further up the hill (toward Jeanes Hospital)	Philadelphia	9/2/99
46	Sampled wet dirty sand from the water's edge. The sand is coarse but plants are growing in it.	Tookany Creek; access from a small parking area on the SW side immediately after turning onto Tookany Creek Parkway from Central Ave	Philadelphia	9/2/99
47	Sampled from a mud puddle on playing field. Puddle is along the road, near the sidelines.	Playing field along Tookany Creek Parkway, NE side (opposite creek)	Philadelphia	9/2/99

Table A.1 (Continued)

48	Sampled by the inlet to a concrete-surrounded pond.	Pastorius Park, at Millman and Hartwell, in the Chestnut Hill neighborhood	Philadelphia	9/3/99
49	Sampled at the base of a statue/art piece outside on the grounds.	Woodmere Art Museum, intersection of Germantown and Bells Mill Road.	Philadelphia	9/3/99
49A	Sampled at the base of a statue/art piece outside on the grounds.	Woodmere Art Museum, intersection of Germantown and Bells Mill Road.	Philadelphia	9/3/99
50	Sampled in cultivated flower bed labeled "Creeping Lily Turf." (See map of arboretum for more exact location.)	Morris Arboretum (entrance at Northwestern Ave.).	Philadelphia	9/3/99
51	Grass rhizo and surrounding soil in a fallow flowerbed.	Morris Arboretum.	Philadelphia	9/3/99
51A	Grass rhizo and surrounding soil in a fallow flowerbed.	Morris Arboretum.	Philadelphia	9/3/99
52	Grass and other unidentified plants along the bank of a stream.	Morris Arboretum.	Philadelphia	9/3/99
53	Inside the "Fernery", a greenhouse/conservatory filled with ferns.	Morris Arboretum.	Philadelphia	9/3/99
54	Sampled from the enormous rootball of a fallen tree along the banks of the Wissahickon.	Morris Arboretum.	Philadelphia	9/3/99
55A	Sampled corn rhizosphere and bulk soil from the corn patch. The corn stalks had been cut down about 16hrs.	Community vegetable garden space, along Northwestern Ave., very close to Morris Arboretum.	Philadelphia	9/3/99
56	Inoculated pea bed. Peas had been inoculated with a commercial preparation of rhizobacteria before being planted in potting soil mix indoors, then transplanted out.	Community vegetable garden space.	Philadelphia	9/3/99
57	Non-inoculated pea bed. Here the peas were planted directly into the ground and were not treated with any bacteria.	Community vegetable garden space.	Philadelphia	9/3/99
58	Volunteer tomato plant growing in the pathway. Sampled the rhizosphere and surrounding soil.	Community vegetable garden space.	Philadelphia	9/3/99
58A	Volunteer tomato plant growing in the pathway. Sampled the rhizosphere and surrounding soil.	Community vegetable garden space.	Philadelphia	9/3/99

Table A.1 (Continued)

59	Sampled an indoor horse arena, where young girls were learning how to ride.	Fairmount Park, just SW of the intersection of Northwestern Ave and Germantown Pike.	Philadelphia	9/3/99
60	Sampled in the "butterfly house", a screened-in enclosure with butterflies and moths.	Schuykill Center for Environmental Ed. Entrance on Hagy's Mill Road.	Philadelphia	9/3/99
61A	Sampled the "pollywog pond", along the edge of the dock. Sample included some grassy plants growing in the mud there.	Schuykill Center for Environmental Ed.	Philadelphia	9/3/99
62A	Placed beets in baggie. Beets were said to have been harvested no earlier than 8/30/99.	Farmstand at Ridge and Port Royal. Farm is in Collegeville, PA.	Philadelphia	9/3/99
63A	Placed limas (which were in pods) in baggie. Limas were said to have been harvested no earlier than 8/30/99.	Farmstand at Ridge and Port Royal . Farm is in Collegeville, PA.	Philadelphia	9/3/99
64	Sampled bark chips underneath climbing rope. Bottom chips were moist.	Chestnut Hill playground, on Germantown Ave. next to the Chestnut Hill Hotel.	Philadelphia	9/4/99
65	Sampled turf just off the fairway. Golf course does not look intensively managed .	Walnut Lane Golf Course, at the intersection of Walnut and Magdelena, across Walnut from the golf clubhouse	Philadelphia	9/4/99
66	Sampled from the base of a large bougainvillea plant in a pot, inside the conservatory.	Robertson's Florists and Conservatory, at Highland and Germantown	Philadelphia	9/3/99
67	Sampled from near the main office .Sample taken from animal burrow --- recently disturbed soil, surrounded by turf.	West Laurel Hill Cemetery	Philadelphia	9/3/99
68	Sampled grass and weeds growing on shallow soil with lots of gravel, along the canal-side of the towpath.	Towpath of Ohio Canal. Parking lot and entrance on Rockside Road, east of Brecksville Road.	Cleveland	5/12/00
68A	Sampled grass and weeds growing on shallow soil with lots of gravel. Along the canal-side of the towpath.	Towpath of Ohio Canal. Parking lot and entrance on Rockside Road, east of Brecksville Road.	Cleveland	5/12/00
69	Topsoil/mulch sample from garden center.	American Turf Garden Center, 845 Broadway.	Cleveland	5/12/00
70	Turf from golf fairway, by lake.	Shawnee Golf Course, off Egbert Road.	Cleveland	5/12/00
70A	Turf from golf fairway, by lake.	Shawnee Golf Course, off Egbert Road.	Cleveland	5/12/00
71	Turf from golf rough, by trees.	Shawnee Golf Course, off Egbert Road.	Cleveland	5/12/00

Table A.1 (Continued)

71A	Turf from golf rough, by trees.	Shawnee Golf Course, off Egbert Road.	Cleveland	5/12/00
72	Turf from oldest fairway on the course: very thick thatch.	Shawnee Golf Course, off Egbert Road.	Cleveland	5/12/00
72A	Turf from oldest fairway on the course: very thick thatch.	Shawnee Golf Course, off Egbert Road.	Cleveland	5/12/00
73	Sampled residential compost pile (didn't look recently used).	Chis and Sue Coblentz, 23525 Drake Road, Oakwood Village, OH 44146.	Cleveland	5/12/00
74	Sampled residential backyard, near deck: leaf litter present.	Jerry and Karen Ruff, 113 May Ave., Northfield, OH 44067.	Cleveland	5/12/00
75A	Sampled grass growing in sand, near the base of a slide at a public playground.	Lincoln Cowles, playground, on the west side of the intersection of Lincoln and Cowles (just north of the intersection of Northfield and Broadway).	Cleveland	5/12/00
76	Purchased 3 tomato seedlings grown in Cleveland station and sold at local garden retail stores.	Pettiti Garden Centers, Broadway.	Cleveland	5/12/00
76A	Purchased 3 tomato seedlings grown in Cleveland station and sold at local garden retail stores.	Pettiti Garden Centers, Broadway.	Cleveland	5/12/00
77	Sampled an unkempt street-yard under deciduous trees.	Riedham, 100 feet south of intersection with Lomard on the east side of the street.	Cleveland	5/12/00
77A	Dandelion rhizosphere from this sample.	Riedham, 100 feet south of intersection with Lomard on the east side of the street.	Cleveland	5/12/00
77B	Ecomycorrhizal "rhizosphere" from this sample.	Riedham, 100 feet south of intersection with Lomard on the east side of the street.	Cleveland	5/12/00
78	Sampled flowerbed which had been dug up fall of '99 and planted this spring: covered with medium-thin layer of leaf mulch.	Cleveland Botanical Gardens, University Circle Area. Flowerbed on south side of Rose garden.	Cleveland	5/12/00
79	Sampled grass next to trash can next to play area. (Play area "paved" with rubber mats.)	Rockefeller park playground, near University circle/MLK Blvd.	Cleveland	5/12/00
80	Sampled woodsy residential backyard: took grass from near swingset.	Tim and Rebecca Yoder, 3805 Bridgeview Drive, S. Euclid, OH.	Cleveland	5/12/00
81	Sampled soil and bark mulch under public swingset.	Quarry Park North play area, at the corner of Monticello and Belvoir in S. Euclid.	Cleveland	5/12/00
82	Sampled as-yet unplanted residential flowerbed in backyard.	Rohn Thomas and Terri Kent, 4173 Harwood Road, S. Euclid.	Cleveland	5/12/00

Table A.1 (Continued)

83	Sampled lawn in backyard. Standing water was present near the sampling site.	Gizella Tapolyai, 21880 Louis Road, Bedford Heights, OH 44146.	Cleveland	5/12/00
84	Sampled from residential vegetable garden, between newly started rows of veggies in raised bed.	Leo and Dorothy Miller, 22470 Sandalwood, Bedford Heights.	Cleveland	5/12/00
85	Sampled from pea patch in residential veggie garden.	Esther Steckle, 151 Willard Drive, Bedofrd, OH.	Cleveland	5/12/00
86	Sampled from residential raised veggie bed, near the asparagus.	Joe and Marjorie Kotva, 763 Wellmon, Bedford, OH.	Cleveland	5/12/00
87	Sampled from flowerbed in front of residence.	Brad and Lisa Mercurio, 708 Wellmon, Bedford, OH.	Cleveland	5/12/00
88	Sampled from residential garden, in the area where last year's tomatoes were planted.	Jim and LaVonne Miller, 659 McKinley, Bedford, OH.	Cleveland	5/12/00
89	Sampled residential veggie garden which consisted of small but obviously heavily composted raised beds.	Melvin and Parsilla Honsaker, 16106 Maplewood, Maple Heights, OH.	Cleveland	5/12/00
90	Sampled from pony ring - grass.	Rocky River Reservation (RRR) riding stables (public - run by city parks).	Cleveland	5/13/00
91	Sampled from golf course fairway, behind the 3rd hole.	Mastick Woods Golf Course.	Cleveland	5/13/00
91A	Sampled from golf course fairway, behind the 3rd hole.	Mastick Woods Golf Course.	Cleveland	5/13/00
92	Sampled from golf course rough, along side the 9th hole.	Big Met Golf Course.	Cleveland	5/13/00
92A	Sampled from golf course rough, along side the 9th hole.	Big Met Golf Course.	Cleveland	5/13/00
93	Sampled grass growing in baseball diamond of public park.	Baseball diamond at South Mastick picnic area, near entrance (off of Valley Parkway in RRR).	Cleveland	5/13/00
93A	Sampled grass growing in baseball diamond of public park.	Baseball diamond at South Mastick picnic area, near entrance (off of Valley Parkway in RRR).	Cleveland	5/13/00
94	Sampled forest floor from trail side.	Wildflower Trail, RRR. Sampled near stone wall after first stairway.	Cleveland	5/13/00
95	Sampled stream inlet of lake in public park.	North Quarry/Cooks House picnic area, RRR.	Cleveland	5/13/00
96	Sampled farm museum veggie and flower garden.	Historic Stearns Farm Garden, Parma.	Cleveland	5/13/00
97	Sampled soil under a picnic table.	Fernhill Picnic Area, Big Creek Park, near Brookpark road.	Cleveland	5/13/00

Table A.1 (Continued)

98	Sampled sawdust mulch under public swingsets.	Main City Community Park, Gresham.	Portland	6/14/00
99	Sampled mud from the side of Johnson Creek, accessed from the island.	Johnson Creek side, from island in Main City Community Park (go over footbridge).	Portland	6/14/00
100	Molehill in historic cemetery.	Gresham Pioneer Cemetery, downtown Gresham. Site is a few yards from the Spring-water Trail, a popular paved multi-use trail.	Portland	6/14/00
101	Sampled recently mowed grassy area near main paved footpath, under a row of walnut trees.	Powell Butte Nature Park. Access from 162nd and Powell, Gresham.	Portland	6/14/00
102	Sampled flowerbed in woods Heavily amended with OM.	Leach Botanical Garden. 6704 SE 122nd.	Portland	6/14/00
103	Botanical garden compost pile.	Leach Botanical Garden. 6704 SE 122nd.	Portland	6/14/00
104	Sampled from the parking-lot side of creek, off a footpath from the back of the parking area. Soil is very compacted.	Leach Botanical Garden. 6704 SE 122nd.	Portland	6/14/00
105	Sampled worn turf (bare ground in some places: some rather soggy) in goalie area of public soccer field.	Ed Benedict Community Parke, Powell Blvd and SE 104th.	Portland	6/14/00
106	Sampled lawn on the south side of the southernmost reservoir.	Mt. Tabor Park.	Portland	6/14/00
106A	Sampled lawn on the south side of the southernmost reservoir.	Mt. Tabor Park.	Portland	6/14/00
107	Sampled horseshoe pit -- sandy, but some grass is growing in it. Fairly damp.	Creston Park, Powell and SE 42nd.	Portland	6/14/00
108	Sampled the right-hand-most garden spot (belongs to Judy); took sample from area around sweet-pea roots.	Kenilworth Community Gardens, SE 34th and Gladstone.	Portland	6/14/00
109	Sampled the compost-mulch pile in the parking lot.	Kenilworth Community Gardens, SE 34th and Gladstone.	Portland	6/14/00
110	Sampled wet place in turf, opposite duck pond.	Laurelhurst Park, near SE Laurelhurst and SE Ankeny.	Portland	6/14/00
110A	Sampled wet place in turf, opposite duck pond.	Laurelhurst Park, near SE Laurelhurst and SE Ankeny.	Portland	6/14/00
111	Sampled vertical cut in the hillslope, which had been made to accommodate a spur trail to the Creek Trail. Sampled about 2.5 feet below the surface soil.	Hoyt Arboretum, spur trail to Creek trail from parking area off of Fisher.	Portland	6/14/00

Table A.1 (Continued)

112	Sampled creekside/trailside, obtaining wild geranium plant.	Hoyt Arboretum, Creek trail. Sampled area approx. 100 yards NW of parking area.	Portland	6/14/00
112A	Sampled creekside/trailside, obtaining wild geranium plant.	Hoyt Arboretum, Creek trail. Sampled area approx. 100 yards NW of parking area.	Portland	6/14/00
113	Sampled lawn near picnic area.	Council Crest Municipal Park.	Portland	6/14/00
114	Sampled hard-packed trailside soil.	Marquam Trail, downhill from SW Sherwood Drive (just before intersection with Nottingham Drive.)	Portland	6/14/00
115	Sampled sawdust mulch underneath children's play area.	Children's Museum, Barbur Blvd and Hooker. Also opposite Metro Family YMCA Historic Neighborhood House.	Portland	6/14/00
116	Sampled from raised beds with YMCA veggie garden.	Hooker and Barbur Blvd (across Hooker from Children's Museum).	Portland	6/14/00
117	Sampled from U-pick strawberry field.	Thompson Farms, SE 242nd and Bohna Park Road.	Portland	6/14/00
118	Sampled near peas in residential veggie garden raised bed.	McKenzie, 11090 SE 240th Place, Gresham, OR.	Portland	6/15/00
119	Lettuce root-ball from residential veggie garden raised bed.	McKenzie, 11090 SE 240th Place, Gresham, OR.	Portland	6/15/00
119A	Lettuce root-ball from residential veggie garden raised bed.	McKenzie, 11090 SE 240th Place, Gresham, OR.	Portland	6/15/00
120	Purchased rosebud impatients raised in Orient.	Deep Creek Garden Center, SE 242nd, near Stark.	Portland	6/15/00
120A	Purchased rosebud impatients raised in Orient.	Deep Creek Garden Center, SE 242nd, near Stark.	Portland	6/15/00
121	Sampled front flower bed of residence.	Rod Stafford, 2524 NE 42nd Ave, Portland.	Portland	6/15/00
122	Sampled back yard of residence, near rhubarb plant.	Cathy Harder, 2555 NE 28th Ave, Portland.	Portland	6/15/00
123	Sampled primrose garden of private botanical garden.	Berry Botanical Garden. 11505 SW Summerville Ave.	Portland	6/15/00
124	Sampled middle rock garden in private botanical garden.	Berry Botanical Garden. 11505 SW Summerville Ave.	Portland	6/15/00
125	Sampled 1 foot off of trail; forest floor.	Old Main Trail in Tryon Creek State Park, off of Terwilliger in Lake Oswego.	Portland	6/15/00
126	Sampled bulk soil in private veggie garden.	Wayne Poteet: 10995 SE 240th Place, Gresham.	Portland	6/15/00
127	Sampled old horse pasture (in pasture at least 30 years).	Ron Caspell: 11121 SE 240th Place, Gresham.	Portland	6/15/00

Table A.2. Correspondence between isolate number and source sample

Isolate number	Source sample	Isolate number	Source sample	Isolate number	Source sample
151	37	186	42	221	48
152	37	187	42	222	48
153	37	188	42	223	48
154	37	189	42	224	48
155	38	190	42	225	48
156	38	191	42	226	48
157	38	192	42	227	48
158	38	193	42	228	48
159	38	194	42	229	49
160	38	195	43	230	49
161	39	196	43	231	49
162	39	197	43	232	49
163	39	198	43	233	49
164	39	199	43	234	49
165	39	200	43	235	49
166	39	201	45	236	49
167	39	202	45	237	49
168	39	203	46	238	38
169	39	204	46	239	38
170	39	205	46	240	38
171	39	206	46	241	38
172	40	207	46	242	38
173	40	208	46	243	38
174	40	209	46	244	38
175	41	210	46	245	39
176	41	211	47	246	39
177	41	212	47	246B	39
178	41	213	47	247	39
179	41	214	47	248	39
180	41	215	47	249	39
181	41	216	47	250	39
182	41	217	47	251	39
183	41	218	47	252	39
184	41	219	47	253	40
185	42	220	47	254	40

Table A.2 (Continued)

Isolate number	Source sample	Isolate number	Source sample	Isolate number	Source sample
255	40	293	49	331	54
256	40	294	49	332	54
257	40	295	49	333	54
258	40	296	49	334	54
259	41	297	49	335	55A
260	41	298	49	336	55A
261	41	299	49	337	55A
262	41	300	49	338	55A
263	41	301	49A	339	55A
264	41	302	49A	340	55A
265	41	303	49A	341	55A
266	41	304	49A	342	55A
267	41	305	49A	343	56
268	41	306	49A	344	56
269	42	307	49A	345	56
270	42	308	49A	346	56
271	42	309	49A	347	57
272	42	310	49A	348	57
273	43	311	50	349	57
274	43	312	50	350	57
275	43	313	50	351	57
276	43	314	50	352	57
277	47	315	50	353	58
278	47	316	50	354	58
279	47	317	50	355	58
280	47	318	50	356	58
281	47	319	51	357	58
282	47	320	51	358	58
283	48	321	51	359	58
284	48	322	51	360	58
285	48	323	51A	361	58
286	48	324	51A	362	58
287	48	325	52	363	58A
288	48	326	52	364	58A
289	48	327	52	365	58A
290	48	328	52	366	58A
291	48	329	54	367	58A
292	48	330	54	368	58A

Table A.2 (Continued)

Isolate number	Source sample	Isolate number	Source sample	Isolate number	Source sample
369	62A	407	50	445	54
370	62A	408	50	446	54
371	62A	409	50	447	54
372	62A	410	50	448	54
373	62A	411	50	449	56
374	62A	412	50	450	56
375	62A	413	51	451	56
376	62A	414	51	452	56
377	63A	415	51	453	56
378	63A	416	51	454	56
379	63A	417	51	455	56
380	63A	418	51	456	56
381	63A	419	51	457	57
382	64	420	51	458	57
383	64	421	51	459	57
384	64	422	51	460	57
385	64	423	52	461	57
386	64	424	52	462	57
387	64	425	52	463	57
388	64	426	52	464	57
389	65	427	52	465	58
390	65	428	52	466	58
391	67	429	52	467	58
392	67	430	52	468	58
393	67	431	52	469	58
394	67	432	52	470	58
395	67	433	52	471	58
396	67	434	52	472	58
397	67	435	53	473	58
398	67	436	53	474	58
399	67	437	53	475	59
400	67	438	53	476	59
401	49	439	53	477	59
402	49	440	53	478	59
403	50	441	53	479	60
404	50	442	53	480	60
405	50	443	54	481	60
406	50	444	54	482	60

Table A.2 (Continued)

Isolate number	Source sample	Isolate number	Source sample	Isolate number	Source sample
483	60	521	67	559	62A
484	60	522	67	560	62A
485	60	523	67	561	62A
486	60	524	67	562	62A
487	64	525	49A	563	62A
488	64	526	49A	564	62A
489	64	527	49A	565	63A
490	64	528	49A	566	63A
491	64	529	49A	567	63A
492	64	530	49A	568	63A
493	64	531	49A	569	63A
494	64	532	49A	570	63A
495	64	533	49A	580	68
496	64	534	49A	581	68
497	64	535	51A	582	68
498	64	536	51A	583	68
499	65	537	51A	584	68
500	65	538	51A	585	68
501	65	539	51A	586	70
502	65	540	51A	587	70
503	65	541	55A	588	70
504	65	542	55A	589	70
505	66	543	55A	590	70
506	66	544	55A	591	70
507	66	545	55A	592	69
508	66	546	55A	593	69
509	66	547	58A	594	69
510	66	548	58A	595	69
511	66	549	58A	596	69
512	66	550	58A	597	69
513	66	551	58A	598	69
514	66	552	58A	599	69
515	67	553	61A	600	71
516	67	554	61A	601	71
517	67	555	61A	602	71
518	67	556	61A	603	71
519	67	557	62A	604	71
520	67	558	62A	605	71

Table A.2 (Continued)

Isolate number	Source sample	Isolate number	Source sample	Isolate number	Source sample
606	71	644	77	682	82
607	71	645	77	683	82
608	71	646	77	684	82
609	71	647	77	685	82
610	72	648	77	686	82
611	72	649	77	687	82
612	72	650	77	688	82
613	72	651	77	689	82
614	72	652	78	690	83
615	72	653	78	691	83
616	72	654	78	692	83
617	72	655	78	693	83
618	72	656	78	694	83
619	72	657	78	695	83
620	72	658	79	696	84
621	72	659	79	697	84
622	73	660	79	698	84
623	73	661	79	699	84
624	73	662	79	700	84
625	73	663	79	701	84
626	73	664	79	702	84
627	73	665	79	703	84
628	73	666	79	704	85
629	73	667	79	705	85
630	74	668	80	706	85
631	74	669	80	707	85
632	74	670	80	708	85
633	74	671	80	709	85
634	74	672	80	710	85
635	74	673	80	711	85
636	74	674	81	712	86
637	74	675	81	713	86
638	76	676	81	714	86
639	76	677	81	715	86
640	76	678	81	716	86
641	76	679	81	717	86
642	76	680	81	718	86
643	76	681	81	719	86

Table A.2 (Continued)

Isolate number	Source sample	Isolate number	Source sample	Isolate number	Source sample
720	87	758	92	796	97
721	87	759	92	797	68A
722	87	760	92	798	68A
723	87	761	92	799	68A
724	87	762	92	800	68A
725	87	763	92	801	68A
726	88	764	93	802	68A
727	88	765	93	803	68A
728	88	766	93	804	70A
729	88	767	93	805	70A
730	88	768	93	806	70A
731	88	769	93	807	70A
732	89	770	94	808	70A
733	89	771	94	809	70A
734	89	772	94	810	70A
735	89	773	94	811	70A
736	89	774	94	812	71A
737	89	775	94	813	71A
738	89	776	95	814	71A
739	89	777	95	815	71A
740	89	778	95	816	71A
741	89	779	95	817	71A
742	90	780	95	818	71A
743	90	781	95	819	71A
744	90	782	95	820	72A
745	90	783	95	821	72A
746	90	784	96	822	72A
747	90	785	96	823	72A
748	90	786	96	824	72A
749	90	787	96	825	72A
750	91	788	96	826	75A
751	91	789	96	827	75A
752	91	790	97	828	75A
753	91	791	97	829	75A
754	91	792	97	830	75A
755	91	793	97	831	75A
756	91	794	97	832	75A
757	91	795	97	833	75A

Table A.2 (Continued)

Isolate number	Source sample	Isolate number	Source sample	Isolate number	Source sample
834	76A	872	93A	910	77
835	76A	873	93A	911	77
836	76A	874	68	912	78
837	76A	875	68	913	78
838	76A	876	68	914	78
839	77A	877	68	915	78
840	77A	878	68	916	78
841	77A	879	68	917	78
842	77A	880	69	918	79
843	77A	881	69	919	79
844	77B	882	69	920	79
845	77B	883	69	921	79
846	77B	884	70	922	80
847	77B	885	70	923	80
848	77B	886	70	924	80
849	77B	887	70	925	80
850	91A	888	71	926	81
851	91A	889	71	927	81
852	91A	890	71	928	81
853	91A	891	71	929	81
854	91A	892	71	930	81
855	91A	893	71	931	81
856	91A	894	72	932	82
857	91A	895	72	933	82
858	92A	896	73	934	82
859	92A	897	73	935	82
860	92A	898	73	936	82
861	92A	899	73	937	82
862	92A	900	74	938	83
863	92A	901	74	939	83
864	92A	902	74	940	86
865	92A	903	74	941	86
866	93A	904	76	942	84
867	93A	905	76	943	84
868	93A	906	76	944	84
869	93A	907	76	945	84
870	93A	908	77	946	85
871	93A	909	77	947	85

Table A.2 (Continued)

Isolate number	Source sample	Isolate number	Source sample	Isolate number	Source sample
948	85	986	68A	1024	99
949	85	987	68A	1025	99
950	86	988	68A	1026	99
951	86	989	68A	1027	99
952	86	990	70A	1028	99
953	86	991	70A	1029	99
954	87	992	71A	1030	100
955	87	993	71A	1031	100
956	87	994	71A	1032	100
957	87	995	71A	1033	100
958	88	996	72A	1034	100
959	88	997	72A	1035	100
960	89	998	75A	1036	100
961	89	999	75A	1037	100
962	89	1000	75A	1038	101
963	89	1001	75A	1039	101
964	90	1002	76A	1040	101
965	90	1003	76A	1041	101
966	91	1004	76A	1042	101
967	91	1005	76A	1043	102
968	91	1006	77A	1044	102
969	91	1007	77A	1045	102
970	92	1008	77B	1046	102
971	92	1009	77B	1047	102
972	94	1010	77B	1048	103
973	94	1011	77B	1049	103
974	94	1012	91A	1050	103
975	94	1013	91A	1051	103
976	96	1014	91A	1052	103
977	96	1015	91A	1053	104
978	96	1016	92A	1054	104
979	96	1017	92A	1055	104
980	95	1018	92A	1056	104
981	95	1019	92A	1057	104
982	95	1020	93A	1058	105
983	95	1021	93A	1059	105
984	97	1022	98	1060	105
985	97	1023	98	1061	105

Table A.2 (Continued)

Isolate number	Source sample	Isolate number	Source sample	Isolate number	Source sample
1062	105	1100	112	1138	119
1063	106	1101	112	1139	120
1064	106	1102	113	1140	120
1065	106	1103	113	1141	120
1066	106	1104	113	1142	121
1067	106	1105	113	1143	121
1068	106	1106	113	1144	121
1069	106	1107	113	1145	121
1070	107	1108	114	1146	121
1071	107	1109	114	1147	121
1072	107	1110	114	1148	122
1073	107	1111	114	1149	122
1074	107	1112	114	1150	122
1075	107	1113	115	1151	122
1076	108	1114	115	1152	122
1077	108	1115	115	1153	122
1078	108	1116	116	1154	122
1079	108	1117	116	1155	123
1080	108	1118	116	1156	123
1081	109	1119	116	1157	123
1082	109	1120	116	1158	123
1083	109	1121	116	1159	124
1084	109	1122	117	1160	124
1085	109	1123	117	1161	124
1086	110	1124	117	1162	124
1087	110	1125	117	1163	124
1088	110	1126	118	1164	124
1089	110	1127	118	1165	124
1090	110	1128	118	1166	125
1091	110	1129	118	1167	125
1092	111	1130	118	1168	125
1093	111	1131	118	1169	125
1094	111	1132	119	1170	125
1095	111	1133	119	1171	126
1096	112	1134	119	1172	126
1097	112	1135	119	1173	126
1098	112	1136	119	1174	126
1099	112	1137	119	1175	127

Table A.2 (Continued)

Isolate number	Source sample	Isolate number	Source sample	Isolate number	Source sample
1176	127	1214	98	1252	105
1177	127	1215	98	1253	105
1178	127	1216	98	1254	105
1179	127	1217	98	1255	105
1180	127	1218	99	1256	105
1181	127	1219	99	1257	105
1182	106A	1220	99	1258	105
1183	106A	1221	99	1259	106
1184	106A	1222	100	1260	106
1185	106A	1223	100	1261	106
1186	106A	1224	100	1262	106
1187	106A	1225	100	1263	106
1188	110A	1226	100	1264	107
1189	110A	1227	100	1265	107
1190	110A	1228	100	1266	107
1191	110A	1229	101	1267	108
1192	110A	1230	101	1268	108
1193	110A	1231	101	1269	108
1194	112A	1232	101	1270	108
1195	112A	1233	101	1271	108
1196	112A	1234	102	1272	108
1197	112A	1235	102	1273	108
1198	112A	1236	102	1274	109
1199	112A	1237	102	1275	109
1200	112A	1238	102	1276	109
1201	119A	1239	102	1277	109
1202	119A	1240	102	1278	109
1203	119A	1241	102	1279	109
1204	119A	1242	103	1280	109
1205	119A	1243	103	1281	110
1206	119A	1244	103	1282	110
1207	120A	1245	103	1283	110
1208	120A	1246	104	1284	110
1209	120A	1247	104	1285	110
1210	120A	1248	104	1286	110
1211	120A	1249	104	1287	110
1212	98	1250	105	1288	111
1213	98	1251	105	1289	111

Table A.2 (Continued)

Isolate number	Source sample	Isolate number	Source sample	Isolate number	Source sample
1290	111	1328	118	1366	124
1291	111	1329	118	1367	124
1292	112	1330	118	1368	125
1293	112	1331	118	1369	125
1294	112	1332	119	1370	125
1295	112	1333	119	1371	125
1296	112	1334	119	1372	125
1297	112	1335	119	1373	126
1298	113	1336	119	1374	126
1299	113	1337	119	1375	126
1300	113	1338	119	1376	126
1301	113	1339	120	1377	126
1302	114	1340	120	1378	126
1303	114	1341	120	1379	126
1304	114	1342	121	1380	127
1305	114	1343	121	1381	127
1306	114	1344	121	1382	127
1307	114	1345	121	1383	127
1308	114	1346	121	1384	106A
1309	114	1347	121	1385	106A
1310	115	1348	122	1386	106A
1311	115	1349	122	1387	106A
1312	115	1350	122	1388	106A
1313	115	1351	122	1389	110A
1314	116	1352	122	1390	110A
1315	116	1353	122	1391	110A
1316	116	1354	123	1392	110A
1317	116	1355	123	1393	110A
1318	116	1356	123	1394	110A
1319	116	1357	123	1395	110A
1320	117	1358	123	1396	110A
1321	117	1359	123	1397	110A
1322	117	1360	123	1398	112A
1323	117	1361	124	1399	112A
1324	117	1362	124	1400	112A
1325	117	1363	124	1401	112A
1326	118	1364	124	1402	112A
1327	118	1365	124	1403	112A

Table A.2 (Continued)

Isolate number	Source sample
1404	112A
1405	112A
1406	112A
1407	119A
1408	119A
1409	119A
1410	119A
1411	119A
1412	119A
1413	119A
1414	120A
1415	120A
1416	120A
1417	120A

Table A.3. Site types from which soil samples were collected

	Golf course or other turf	Vegetable garden	Flower-bed	Play-ground	Athletic field	Hiking trail	Stream -bank, pond-side	Other
Philadelphia	3	4	3	3	3	2	4	8
Cleveland	6	6	3	3	1	2	1	8
Portland	3	7	4	2	1	6	1	6
TOTAL	12	17	10	8	5	10	6	10

Appendix B. RHIZOSPHERE ASSAYS

INTRODUCTION

The rhizosphere, or the soil immediately surrounding a plant root, is a unique microbiological environment. Supplied with nutrients in the form of root exudates, the rhizosphere often supports a higher population of bacteria than does the relatively nutrient-poor environment of the bulk soil (Alexander, 1977; Bowen and Rovira, 1999). However, not all bacteria grow equally well in the rhizosphere (Grayston et al., 1998). The ability to thrive in the rhizosphere is termed rhizosphere competence. In this appendix, root colonization or demonstration of rhizosphere competence is defined per Parke (1991) as "the proliferation of microorganisms in, on, and around the growing root...[including] dispersal of microbes from a source of inoculum to the actively growing root, and multiplication or growth in the rhizosphere." It should be emphasized that root colonization is an active process, and not simply a temporary association of bacteria and roots in soil.

Many bacterial traits – most of them unknown – contribute to the rhizosphere competence of a bacterial strain. Potentially helpful bacterial traits include the production of surface polysaccharides, fimbriae and flagella, osmotolerance, ability to use root exudates, growth rate, and resistance to predation (Weller, 1988; Jjemba and Alexander, 1994). The loss of any of these traits may result in the loss of the ability of the strain to colonize roots.

Because not all bacteria are able to colonize roots, the presence of certain plant species enriches for those bacterial strains which are rhizocompetent for that plant

(Grayston et al., 1998; Bowen and Rovira, 1999). This is the basis for the "rhizosphere enrichment" assays (represented by experiments 2-6) that follow. Many strains of the *B. cepacia* complex have been shown to colonize the roots of plants. Host plants supporting rhizopopulations of Bcc include peas (Parke, 1990), maize (Nacamulli et al., 1997), tomatoes (Sfalanga et al., 1999), wheat, lupine (Balandreau et al., 2001) and perennial ryegrass (Nijhuis et al., 1993). The goal of the rhizosphere enrichment assays was to use peas to enrich for Bcc present in soil. We hoped that by enriching for Bcc we would increase our chances of detecting soil populations of Bcc via culture on selective media.

Another set of assays (represented by experiments 1 and 2) were designed to evaluate the rhizosphere competence of many clinical isolates of Bcc. It is common for only some strains of a particular bacterial species to demonstrate rhizosphere competence (Kloepper, 1993). Given the dynamic genome of Bcc, it seemed possible that medically important strains may not have developed, or may have lost, the traits essential for survival and proliferation on a plant root. We hoped to develop an assay which would allow for discrimination between clinical and environmental isolates, hypothesizing that clinically derived strains of Bcc would not be rhizosphere competent, while environmental strains would be rhizosphere competent.

EXPERIMENT 1: RHIZOSPHERE COMPETENCE ASSAY WITH CUCUMBERS

Objectives

The objectives of this assay were to see if this method of estimating the amount of bacteria applied per seed was accurate and effective. We also wanted to see what kind of variability in colonization ability was present among strains of Bcc, when applied to cucumber seeds in this soil medium. We hoped to learn if AMMDR1, a known rhizosphere colonizer of peas, would serve as a positive control for assays with cucumber plants. Ultimately, we hoped to learn if other strains in the *B. cepacia* complex were capable of colonizing the rhizosphere.

Materials and methods

Compost-amended soil (Fertil-Mix, Shamrock Landscape Supply, Corvallis, OR), stored double-bagged at 4°C, was passed through a 4.46 mm soil sieve. Thirty-five 3 oz Dixie Cups (Fort James Corp., Norwalk, CT) were filled with the soil medium and leveled off, and 6 ml of sterile deionized water was added to each cup. The cups were then placed in a plastic box with a lid and kept in the growth chamber at 27°C overnight. Meanwhile, 24-hour broth cultures of 5 bacterial strains from frozen stock were grown in LB (KB for AMMDR1) at 27°C with shaking. These strains were AMMDRI, a rifampicin-resistant mutant of AMMD (genomovar VII); cep 138 (LMG 16659, genomovar III); cep 238 (LMG 16654,genomovar III); cep 509 (LMG 18821, genomovar I), and cep 511 (LMG 18830, genomovar III). Tenfold dilutions of each culture were made in 0.1M $MgSO_4$ and the bacterial populations estimated by

measuring the optical density of the diluted broth at A=540 nm with a spectrophotometer (Hitachi U-1000, Tokyo, Japan). Adequate 0.1M $MgSO_4$ was added to each culture to bring it to an estimated bacterial density of 6×10^8 CFU ml^{-1}. Ten cucumber seeds (Marketmore 86, Territorial Seeds, Cottage Grove, OR) per treatment were placed in non-sterile small plastic weigh boats. This and all subsequent steps were performed in a biosafety hood. Three ml of each bacterial suspension was pipetted onto the appropriate seeds. After 15 minutes, sterile tweezers were used to place 5 seeds into sterile 10 ml 0.1 M $MgSO_4$ dilution blanks (1 seed/blank) for the determination of bacterial inoculum. The other 5 seeds were planted into prepared soil cups (1 seed per cup). Two other treatments were a control (cucumber seeds alone) and broth only (seeds soaked in diluted broth media with no bacteria). These seeds were treated identically to the others in all other respects.

Cups were placed into randomized positions in the plastic boxes (20 cups / box), and the boxes placed in the growth chamber. There were 5 replicate cups per treatment. Ten-fold dilutions were made of the blanks with seeds, and dilutions 10^{-1} – 10^{-5} were plated onto BCSA (Henry et al., 1997). Aliquots from cep509 were also plated onto TBT (Hagedorn et al., 1987). Plates were incubated at room temperature for 3 days when colonies were counted.

Seedlings were harvested 10 days later. Plants were removed from soil and the length of the longest root measured. A 1-cm section of root (from 1 cm to 2 cm below the site of emergence from the seed) was removed using a razor blade and placed into a pre-weighed sterile dilution blank containing 10 ml 0.1M $MgSO_4$. New gloves, razor blades, paper rulers, and paper towels were used with each plant. All tools were

flame sterilized between plants and the work surface below the paper towels was cleaned with 10% bleach solution (0.5% sodium hypochlorite). The blanks were reweighed and sonicated (Mettler Electronics Corporation, model ME 4.6, Anaheim, CA) for 2 minutes. Serial dilutions were made and plated on BCSA. Appropriate negative controls were also plated onto BCSA. The root segment was then removed, blotted dry, and weighed, so that the amount of soil adhering to the root segment could be calculated. The plates were incubated at room temperature and counted after three days.

Results

Approximately 5 x 10^5 CFU of each bacterial strain were applied to each cucumber seed (see Figure B.1). No bacteria were detected on untreated seeds.

There was considerable variability in the amount of growth of the cucumber plants, as reflected in the weights of root segments and the total root lengths (see Table B.1). However, the lengths and weights are normally distributed. We also noted that some of the cups appeared drier than others, suggesting that conditions in the growth chamber may have been non-uniform. Three of the seeds (8.6%) had not germinated; there were no signs of disease. In one cup, the seed had germinated but the root was only a fraction of a centimeter long. None of the non-germinated seeds were in a cup that appeared particularly dry.

The strain AMMDR1 is a known rhizosphere colonizer of peas (Parke, 1990) and in this experiment was used as a positive control. The populations of bacteria

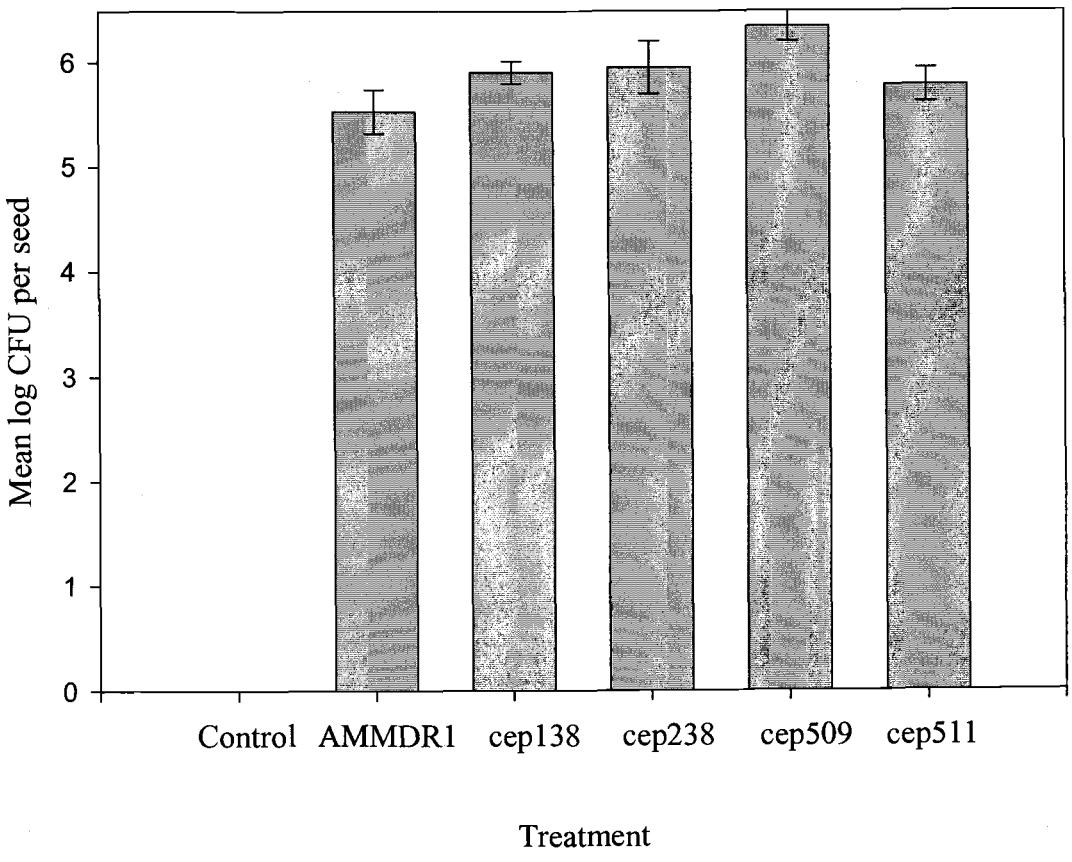

Figure B.1. Mean log CFU applied to cucumber seeds at the start of experiment 1. Error bars represent the standard deviation of the mean.

Table B.1. Root weight, amount of rhizosphere soil, and total root length in rhizosphere competence assay with cucumber plants

	Root weight (mg)	Rhizosphere soil (g)	Total root length (cm)
Mean	79.8	0.106	5.81
Median	69.0	0.103	5.85
Standard deviation	53.2	0.0497	2.412

recovered from the soil adhering to 1 cm of cucumber root was determined for all tested strains by plating on the selective medium BCSA. None of the tested strains were significantly different from AMMDR1 (α= 0.05); this is represented graphically in Figure B.2. Note in particular the high amount of variability in some treatments. Clearly, more experimental units per treatment are necessary to confidently observe differences between the treatments. The controls were also not significantly different from AMMDR1. It is not known how many, if any, of the colonies growing on BCSA were Bcc.

EXPERIMENT 2: RHIZOSPHERE COMPETENCE ASSAY WITH PEAS

Objectives

The objectives of this assay were to see if significant differences were obtained between treated and untreated pea seeds by using substantially more experimental units. We also hoped to learn if AMMDR1 would act as a positive control in

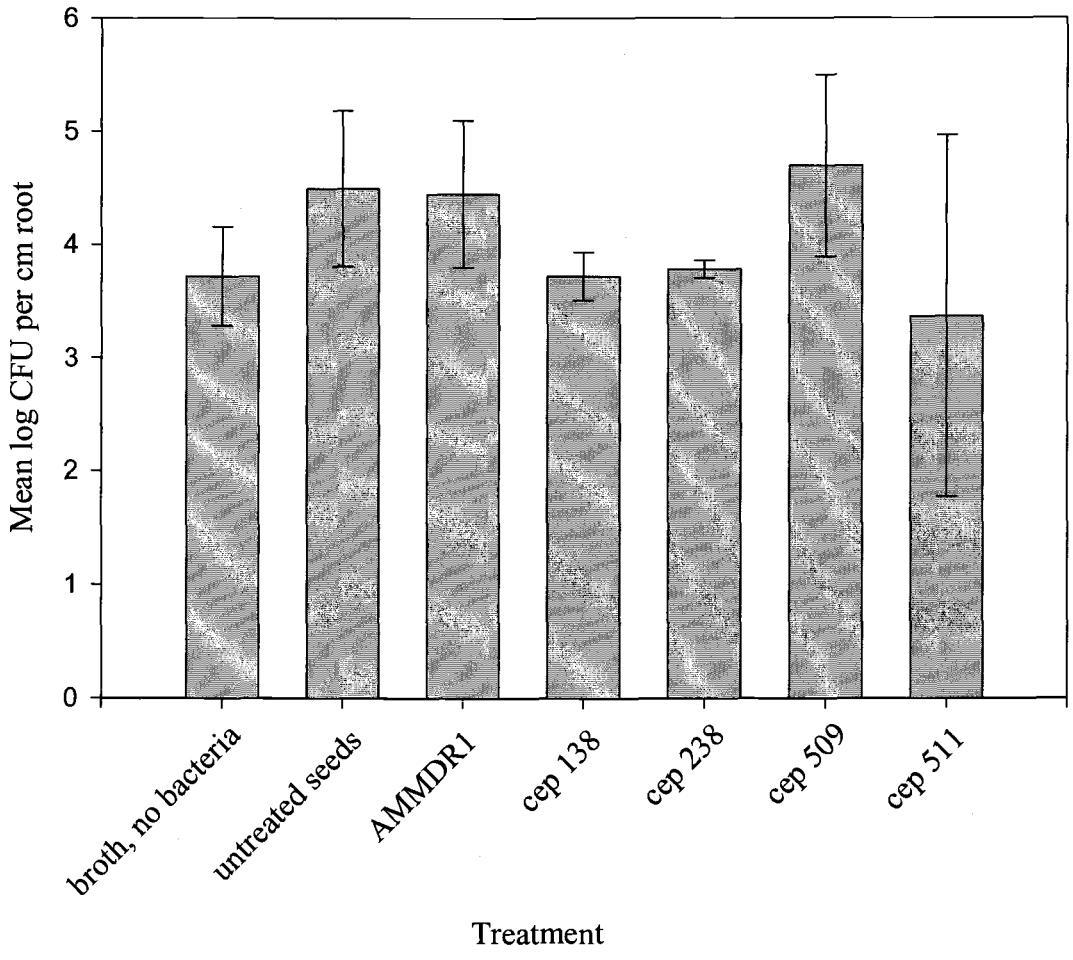

Figure B.2. Mean log CFU recovered per cm cucumber root on BCSA. Error bars represent the standard deviation of the mean.

rhizosphere competence assays in this soil medium when pea seeds were planted. Finally, we wanted to learn if most of the bacteria which were recovered from the treated units were AMMDR1, based on rifampicin resistance.

Materials and methods

Fifty-seven 3 oz Dixie cups were filled with sieved soil medium, watered and kept in the growth chamber overnight as described above. The temperature of the growth chamber was set at 22°C. AMMDR1 was grown from frozen stock in 5 ml KB broth amended with 100µl/ml rifampicin. The following treatments were included: 40 replicates of AMMDR1-coated seeds, 6 replicates with seeds treated with rifampicin and broth but no bacteria, 6 replicates with untreated seeds, and 5 replicates with no seed at all. The titre of cells was determined by spectrophotometry as described above; the target concentration of cells was 6×10^8 CFU/ml, or 6×10^6 CFU/seed assuming 10µl absorbed per seed. Pea seeds (77 Early Perfection) were coated with the bacterial suspension as described above and either planted or placed in a dilution blank. In this experiment, 1 ml dilution blanks were used instead of 10ml blanks. The soil cups were placed in random order in the plastic boxes with 20 cups per box, and one cup of sterile deionized water was placed in the center to maintain humidity. One-hundred-fold dilutions were made from the original blank; 10µl aliquots were plated onto BCSA (10^{-2}, 10^{-4} and 10^{-6} dilution), incubated at room temperature, and counted three days later. Nine soil cups, chosen at random, were also plated on BCSA amended with rifampicin (100 µl/ml) to see what proportion of the colonies on BCSA were the rifampicin-resistant AMMDR1.

The assay was harvested after 15 days of growth. Harvest was performed as described above, except that the amount of rhizosphere soil was not determined. For the 5 replicates with no plant, a small amount of soil was added to the dilution blank, weighed, and plated.

Results

Approximately 1 x 10^7 CFU AMMDR1 were applied per pea seed (6.99 log CFU/seed; standard deviation was 0.27 log CFU/seed). Three of the plants (5%) had not germinated: one unit which had germinated was much muddier than the others. Again, there were no signs of disease. The average root length after 15 days of growth was 28.6 cm (+/- 5.02 cm).

The populations from units which had been plated onto both BCSA and BCSA-rifampicin were not significantly different (one-tailed p-value = 0.45), suggesting that most of the bacteria on the treated plant roots were rifampicin-resistant AMMD. One experimental unit which had not been treated with AMMDR1 was also plated on both media. While there were 4.32 log CFU/cm root on BCSA, no colonies grew on the rifampicin-amended media, supporting the hypothesis that native soil bacteria which may be able to grow on BCSA are not rifampicin-resistant.

Figure B.3 is a graphical comparison between the three treatments. More bacteria were recovered from the AMMDR1 treatment than from the other two (one tailed p-values: 0.0001 and 0.005, from t-tests). There were two control treatments: one in which the seed was not treated at all, and one in which the seed was coated in rifampicin-amended broth, but no bacteria. There were slightly more CFU/cm root on

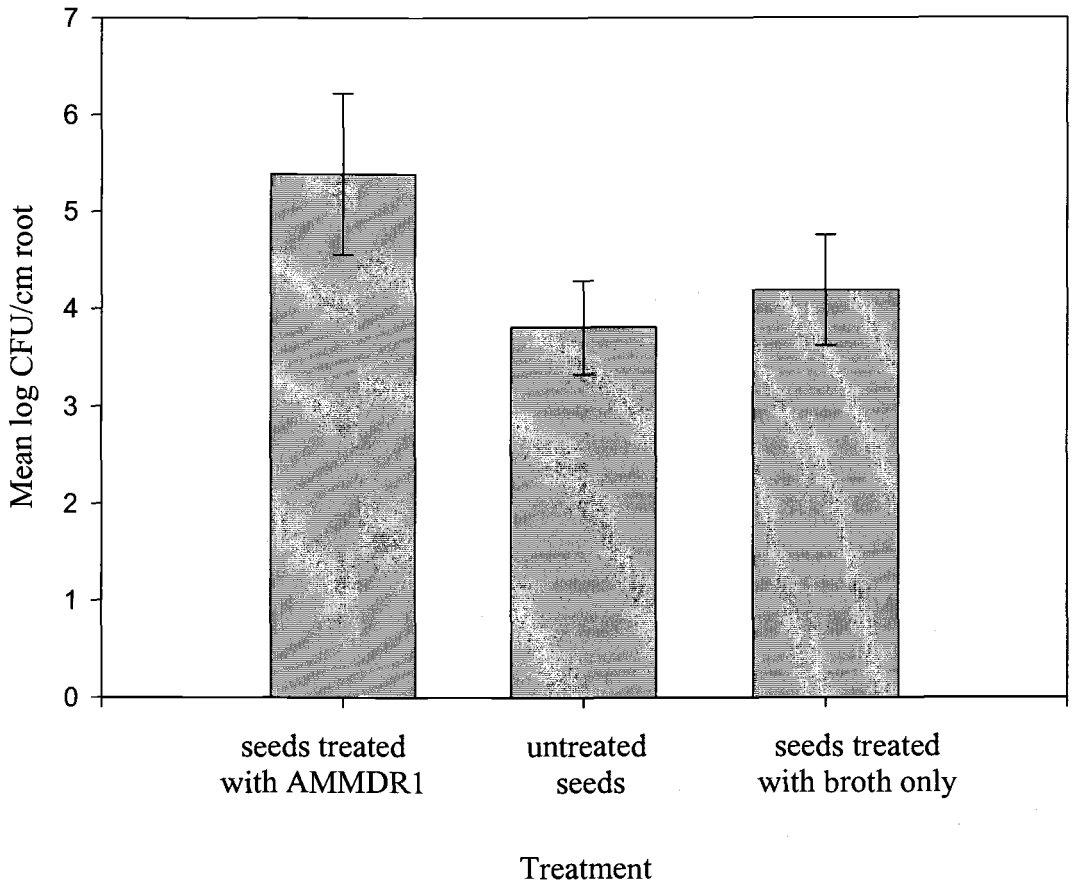

Figure B.3. Mean log CFU recovered per cm pea root on BCSA. Error bars represent the standard deviation of the mean.

units with the rifampicin + broth treatment, but it was not significantly more than the untreated roots (α=0.05; one-tailed p-value = 0.15). The units with no seed planted in them also contained bacteria which grew on BCSA.

EXPERIMENT 3: SEED SURFACE-STERILIZATION ASSAY WITH PEAS

Objectives

The objectives of this assay were to see if planting pea seeds enriched for Bcc. We also wanted to learn if surface-sterilizing the seed altered the recovery of bacteria.

Materials and methods

Forty 3oz Dixie cups were filled with sieved soil medium, watered and allowed to rest in the growth chamber (22°C) overnight as described above. Meanwhile, the Bcc strain G4 (*B. vietnamiensis*) was started from frozen stock in 5 ml of LB. On the day of the experiment the optical density of the bacterial broth was measured and used to create a bacterial suspension of approximately 6 x 10^8 CFU ml^{-1} (estimated to result in 6 x 10^6 CFU $seed^{-1}$). Pea seeds were surface-sterilized by the following procedure. Seeds were bundled together loosely in cheesecloth. They were soaked for 5 minutes in 95% ethanol, 15 minutes in 20% bleach solution (0.5% sodium hypochlorite), and rinsed 3 times (for 10, 15 and 20 minutes, respectively) in copious amounts of sterile deionized water. Five treatments were planned, as is shown in Table B.2, below.

Table B.2. Treatments in experiment 3, seed surface-sterilization assay with peas

Number of replicates	Treatment
10	No treatment; unplanted soil
10	Untreated pea seeds
10	Pea seeds surface disinfested
5	Pea seeds treated with G4
5	Pea seeds surface disinfested and then treated with G4

Several representative seeds from all seed treatments were placed in dilution blanks and plated on either BCSA or LB, to determine inoculum levels. Before seeds were planted into the soil cups, a flame-sterilized spatula was used to place a small amount of soil from 30 soil cups into a pre-weighed 1-ml sterile dilution blank of 0.1$MgSO_4$. This was reweighed to determine the amount of soil added and then plated (at 10^{-2} dilution) onto BCSA. Plates were incubated at room temperature and colonies counted after 3 days.

The assay was harvested after 11 days. The pea plants were removed from soil and all root segments from 1 cm to 2 cm below the site of emergence were excised and placed in a pre-weighed 1 ml sterile dilution blank. Since there were several secondary roots present, the 1 cm – 2 cm excised portion included several root fragments. In the event that no seed had been planted or the seed failed to germinate,

a small amount of soil was placed in the dilution blank instead. The tubes were vortexed briefly to mix the samples and reweighed. Tubes were sonicated for 2 minutes, serial dilutions made, and plated onto BCSA in 10 µl aliquots (final dilutions were 10^{-2}, 10^{-4} and 10^{-6}). The plates were incubated at room temperature until counted.

Results

Inadequate soil was collected to determine putative Bcc populations at the start of the experiment. The average amount of soil placed in the dilution blank was 61.7 mg. Of the 30 soil cups so sampled, only one had countable numbers of colonies (over 25 CFU). An average of 300 CFU/seed was cultured on nonselective media (LB) from the non-surface-sterilized seeds. However, no bacteria were cultured on BCSA from these seeds. No bacteria were cultured on either media from surface-sterilized seeds. The mean number of CFU on seeds treated with G4 was 2.75×10^5 for non-surface-sterilized seeds and 2.45×10^5 for surface-sterilized seeds.

The pea seed did not germinate in four of the experimental units which had included seeds (13%). Three of these four units were in treatments which involved surface sterilizing the pea seeds. Ten of the experimental units had noticeable fungal growth on the soil surface (in one instance, on the surface of the Dixie cup also). All of these units contained seeds (none of the soil-only units were affected). Finally, fungal growth was a problem on the BCSA plates. Plates from nine of the experimental units were uncountable due to rampant fungal growth. Again, all of these units were from treatments which included planting a seed. The fungus

morphology types included low green growth, low white growth, and tall "fuzzy" white growth. In half of the plates, all three fungus morphologies were observed.

None of the five treatments were significantly different from one another (ANOVA, single factor: p-value = 0.167), although the mean CFU per gram soil and root was highest for the treatment with surface sterilized seeds and G4 added. The data is shown in Figure B.4.

EXPERIMENT 4: ENRICHMENT ASSAY WITH PEA SEEDS

Objectives

The objective of this assay was to see if planting a pea seed enriches for bacterial biotypes able to grow on BCSA (putative Bcc). Bulk soil before peas were planted was compared to rhizosphere soil and bulk soil after peas were planted.

Materials and methods

Soil medium was sieved with a clean #3.5 sieve (5.6 mm opening), and placed in 20 3 oz Dixie cups. Sterile deionized water (6 ml) was added and the cups placed in a plastic box in the growth chamber for 24 hours. The next day, soil samples were taken from 10 of the cups at random and placed in 1 ml dilution blanks. These were vortexed 5 seconds and then sonicated 2 minutes; serial dilutions were made in sterile 0.1M $MgSO_4$ buffer and dilutions 10^{-2}, 10^{-4} and 10^{-6} plated on BCSA. Untreated pea seeds were planted in 10 of the twenty cups. Three seeds were placed in dilution blanks and plated on BCSA and nonselective media (KB). The cups were placed in

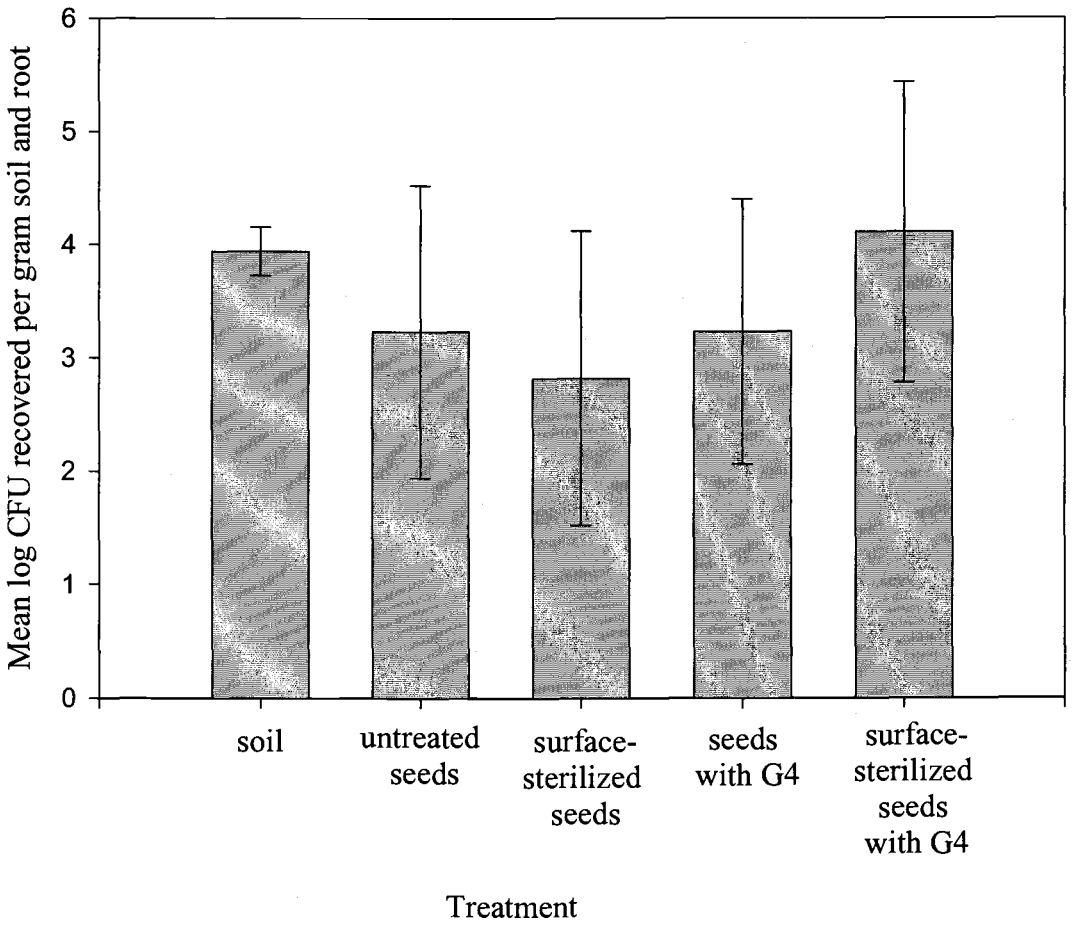

Figure B.4. Mean log CFU recovered per gram pea root and soil on BCSA. Error bars represent the standard deviation of the mean.

random order in the plastic box, with a cup of sterile water to maintain humidity, and the box replaced in the growth chamber.

After nine days, the peas were harvested. In the no-plant treatment, a sterile spatula was used to place a small amount of soil in a dilution blank. In the treatment with plants, the pea seedling was removed, the longest root measured, and the 1 cm – 2 cm segments excised as described above. Again, all paper towels, paper rulers, gloves and razor blades were changed between each experimental unit. Blanks were vortexed, sonicated 2 minutes, and then the 10^{-2}, 10^{-4} and 10^{-6} dilutions plated onto BCSA. Plates were incubated at room temperature for three days, then counted.

Results

The mean number of log CFU/g soil (or per gram soil-and-root) was greater in rhizosphere soil of peas than in units without a pea plant. This difference is significant (α=0.05; two-tailed p-value = 0.031). The 95% confidence interval for the difference between the two treatments is between 0.55 and 0.03 log CFU/g soil (and root). However, there was no significant difference between the number of log CFU/g soil in the rhizosphere of the pea plant and the number of log CFU/g soil prior to the experiment (two-tailed p-value = 0.4795).

EXPERIMENT 5: CHECK OF RIDOMIL IN ENRICHMENT ASSAY WITH PEAS

Objectives

Plant disease caused by fungal or oomycete species had been a problem in earlier experiments. An overarching goal for this experiment was to see if using the antifungal soil drench Ridomil would control disease without affecting the rest of the experiment. Other objectives of this experiment were to answer the following questions. Does planting a pea seed have a significant effect on the number of putative Bcc CFU recovered per gram soil, after taking into account "nutrient loading" and variable growth of seeds? (Nutrient loading is when soil bacteria grow on selective media using nutrients carried along with the soil sample, rather than nutrients in the selective media; it is more common in cases where the soil sample is not very diluted). Does the rate of growth of the pea plant, or the use of Ridomil, have a significant effect on the number of CFU recovered? Finally, is the effect of the plant changed in the presence or absence of Ridomil – that is, is there an interaction between Ridomil and plant presence?

Materials and methods

Sixty 3 oz Dixie cups were filled with sieved soil medium as described above. Thirty cups were watered with 6 ml of sterile deionized water. Thirty cups were watered with 6 ml of solution of the fungicide Ridomil (Novartis, Greensboro, NC) (active ingredient; (R)-2-[2,6-dimethylphenyl-methoxyacetylamino]-propionic acid

methyl ester) and water. The Ridomil was applied at the recommended rate of 0.5 pints per acre, or 4.6×10^{-4} ml/cup. The conversion is shown below.

Rate of application = 0.5 pints Ridolmil /acre

1 pint = 473.176 ml

1 acre = 4046.873 m^2

Area of a Dixie cup = 78.5 cm^2

$$\frac{78.5\ cm^2}{1\ cup} \times \frac{1\ m^2}{10{,}000 cm^2} \times \frac{1\ acre}{4{,}046.87 m^2} \times \frac{0.5\ pints}{1\ acre} \times \frac{473.176\ ml}{1\ pint}$$

$$= 4.58 \times 10^{-4}\ ml\ /cup$$

The cups of soil were placed in plastic boxes with a cup of water for humidity and left in the growth chamber for 24 hours.

Soil samples were collected from 10 randomly chosen cups, and placed in 1 ml dilution blanks. Dilutions of 10^{-2} and 10^{-3} were plated onto BCSA, incubated and counted as usual. Pea seeds were planted in half of the experimental units, making four treatments (plant +/- and Ridomil +/-).The soil cups were placed in random order in the plastic boxes and put in the growth chamber.

Peas were harvested after seven days. In no-plant units, a small amount of soil was added to a pre-weighed 1 ml dilution blank containing sterile 0.1M $MgSO_4$. In units with a pea plant, plants were removed from the cups, the longest root measured, and the portion from 1 cm to 2 cm excised and placed in a dilution blank. All tubes were then reweighed. After vortexing and sonicating, serial dilutions were performed and 10^{-2} and 10^{-3} dilutions were plated onto BCSA. All root segments were removed

from the blank, patted dry, and weighed. In half of the units with plants, the root segments were carefully measured to ascertain the total excised root length.

Results

The number of CFU per gram of soil is higher in the rhizosphere than in bulk soil collected at harvest (one-sided p-value from t-test – 0.0019), after accounting for Ridomil, nutrient loading and root length. The estimate of the plant effect on CFU numbers is 117.89 times; that is, units with a pea plant had an estimated 117.89 times more CFU per gram soil than units without. The 95% confidence interval for this estimate is (5.1, 2735).

The model used to make these estimates is

Mean $\log_{10}$CFU | Ridomil, root length, plant =

$$\underset{(0.098)}{4.14} - \underset{(0.042)}{0.08}\,(\text{root length}) - \underset{(0.118)}{0.22}\,(\text{Ridomil}) + \underset{(0.679)}{2.07}\,(\text{plant}).$$

Marginally convincing evidence exists that longer pea roots are associated with smaller populations of Bcc in the sampled area of the rhizosphere (two-sided p-value = 0.0509). A one-centimeter increase in root length is associated with an estimated 17.5% fewer CFU per gram soil. The data are consistent with there being no effect of Ridomil application (two-sided p-value = 0.0709). Nutrient loading, factored into the model as the amount of soil actually added to the initial dilution blank, was not significant ((p>>>0.10, from an extra sum of squares F-test). Three-way and two-way interactions, including the interaction between plant and Ridomil, were also not significant (p>>>0.10, from extra sum of squares F-test).

There were two outlying influential observations. Analyses performed without these observations did not change the conclusions.

The mean log CFU per gram root and soil was not significantly different between bulk soil at the start of the experiment and that collected in the rhizosphere at harvest with both the Ridomil and non-Ridomil units pooled together (one-sided p-value from t-test = 0.29). This is shown graphically in Figure B.5.

The mean root weight after 7 days of growth was 0.058g (+/- 0.012g). The mean amount of soil, or roots and soil, collected was 0.43g (+/- 0.18 g), so roots comprised an average of 9.5 to 23% of the weight in rhizosphere samples.

The mean excised root length was 12.68 cm (+/- 2.59 cm). The mean tap root length was 16.07 cm +/- 2.17 cm.

EXPERIMENT 6: ENRICHMENT ASSAY WITH AIR-DRIED SOILS AND PAPER TOWELS

Objectives

The objectives of this experiment were to develop a protocol for future experiments with pea seed enrichment of theorized "latent" populations of Bcc in air-dried soils. In particular, we hoped to see if we could successfully construct a gnotobiotic system, and if using Ridomil as a seed treatment could control oomyete growth.

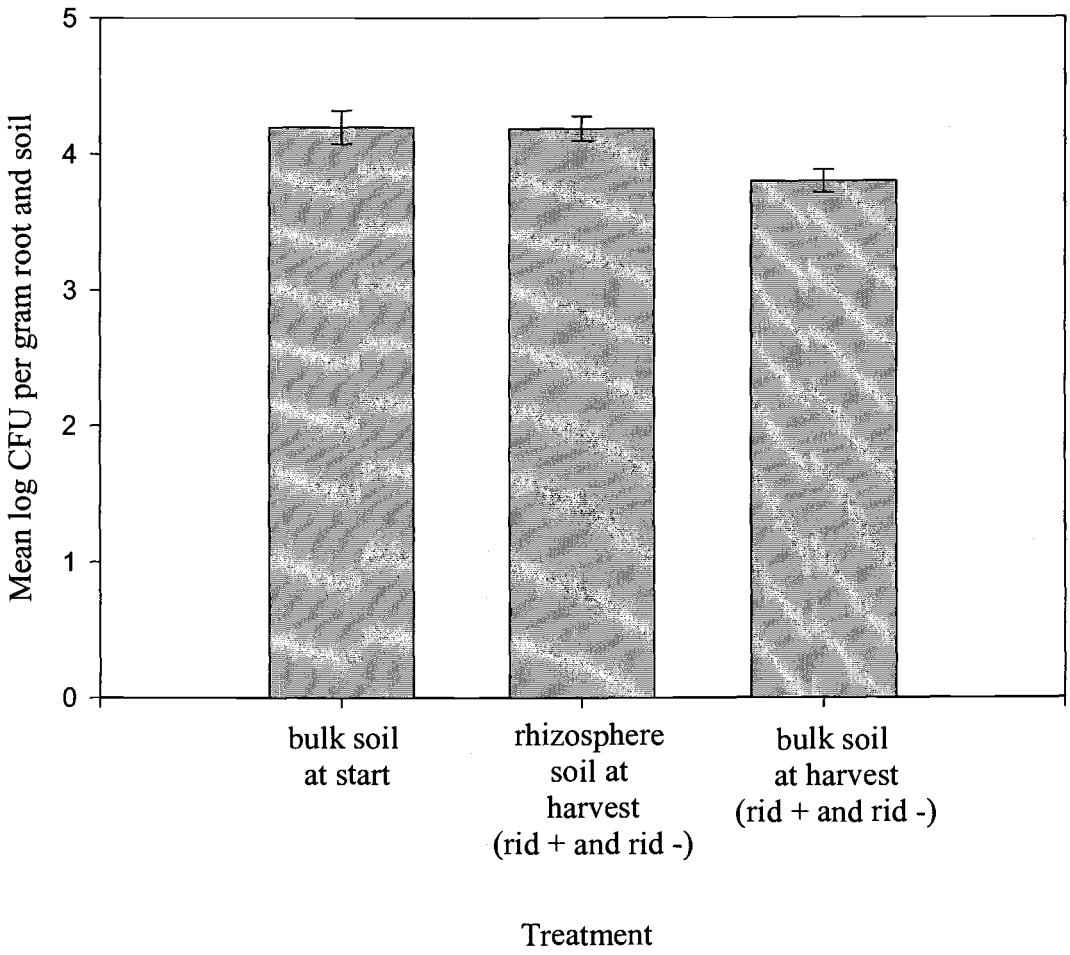

Figure B.5. Mean log CFU recovered per gram pea root and soil on BCSA. Error bars represent the standard deviation of the mean.

Materials and methods

The treatments for experiment 6 are listed in Table B.3, below.

Table B.3. Treatments in enrichment assay with air-dried soil

Replicates (10 pea seeds per replicate)	Treatments
5	Seeds surface disinfested Soil present (seeds treated with Ridomil)
5	Seeds surface disinfested No soil
5	Seeds not surface disinfested Soil present (seeds treated with Ridomil)
5	Seeds not surface disinfested No soil

Pea seeds were surface disinfested as described earlier. After removal from the last water bath, the pea seeds were blotted dry on sterile paper towels.

Pea seeds were treated with Ridomil according to the label instructions. Briefly, a 1:100 solution was made in sterile water. Sixty pea seeds were placed on a sterile petri plate in a fume hood, and 4.8 ml of Ridomil solution added and stirred around the seeds. After one minute the seeds were removed to another petri plate to dry. Surface sterilized seeds and non-surface sterilized seeds were treated with Ridomil in separate batches.

Experimental units were prepared as follows. A sterile paper towel with a penciled line 1 inch from the top was placed on a bleached-and-dried lab surface. The paper towel was wet by sprinkling sterile water over it. One-tablespoon of air dried soil was sprinkled over the wet paper towel with a sterile spoon. Sterile tweezers were used to place 10 pea seeds at equal intervals along the ruled line on the paper towel. Using a clean pair of gloves, the towel was rolled up and placed in a washed, bleached and dried conetainer in a rack. Plastic wrap was placed around the top and held in place with a rubber band. The lab surface was cleaned with 10% bleach and dried between each replicate.

Paper cups with sterile deionized water were placed underneath the conetainers, to keep the paper towels wet. The conetainers were incubated on the bench top for seven days before harvest.

At harvest, the paper towel was unrolled on a bleached and dried lab surface. A sterile blade was used to cut roots just below the pea seed. If necessary, the roots were cut into shorter (1-4 cm) lengths so that they would fit in the 10 ml dilution blank (with 0.1M $MgSO_4$). Blanks were sonicated for two minutes and 10-fold dilutions made. The suspension was plated onto BCSA. The roots were then removed from the test tube and the total length measured. Plates were incubated at room temperature and counted after three days.

Results

There was no growth on selective media from the roots of any surface-sterilized soil-less replicates. This confirms our ability to construct a gnotobiotic system.

Replicates containing soil did not grow as well as replicates without soil, as measured by cm root harvested per conetainer. The mean root length in the units differed between the four treatments (p-value from ANOVA = 1.44×10^{-5}). The difference between the two soil treatments was not significantly different, nor was the difference between the two non-soil treatments (two-sided p-value from t-test: 0.21 and 0.83, respectively). The variable growth between the treatments is shown graphically in Figure B.6. Germination accounted for some of the difference: only 60% of the seeds germinated in the treatment with soil, and only 33% germinated in the treatment with soil and surface sterilization. All soil-less units had 100% germination. Some of the seeds which did not germinate had fuzzy pythium-like growth; other seeds were simply soft and rotten. Many of the seeds which had germinated in the soil units had short, stunted roots with obvious decay.

Only 3 of the 10 units with soil added yielded colonies on selective media. This was not enough to compare treatments.

DISCUSSION

The differing rhizosphere competence of bacterial strains was the basis for the development of two types of assays. The first type, rhizosphere competence assays, were designed to distinguish clinical from environmental strains of the *B. cepacia*

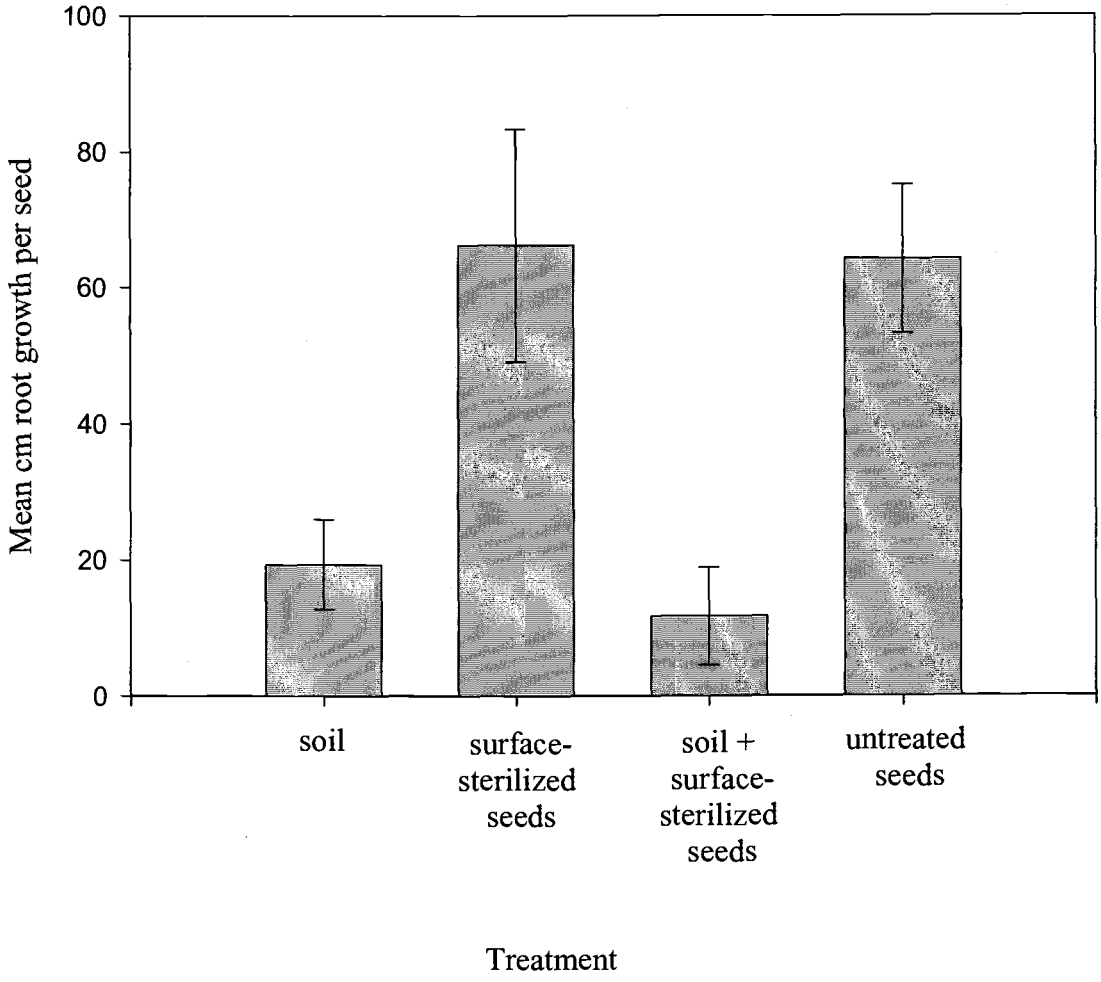

Figure B.6. Mean cm root growth per pea seed. Error bars represent the standard deviation of the mean.

complex. The second type, rhizosphere enrichment assays, sought to enrich for low numbers of (presumed rhizocompetent) Bcc populations in bulk soil, and bring them up to detectable levels on selective media. While neither assay type achieved these goals, valuable data were gathered and some helpful procedural details noted.

Variability

Others have noted high variability in rhizosphere populations of bacteria (Loper, 1984). This was certainly the case in our experiments. High variability was almost certainly exacerbated by the low numbers of experimental units per treatment. Using more experimental units appears essential in order to tease out differences in root colonization ability in experiment 1, for example, or in experiment 3. Experiment 2 did show significant differences between treatments; in that assay, 40 experimental units were treated with bacteria, as opposed to the more typical 5 or 10 units per treatment in the other assays.

Similarly, a reasonably large (ca. 0.5 g) aliquot of soil, or root-and-soil, must be collected for dilution plating. Samples which were too small resulted in populations below the limit of detection in assays 3 and 6.

Identification of Bcc

All colonies growing on selective media were deemed "putative Bcc" in these assays. However, other parts of this study determined that only a fraction of the bacteria from soil which can grow on BCSA and TBT are in fact Bcc (see Chapter 4,

Results). The lack of ability to confidently identify Bcc calls into question the results from all of these assays.

The exception is experiment 2, in which selected samples were plated onto both BCSA and BCSA amended with rifampicin. There was no difference between the plate counts on these two media, which suggests that nearly all the bacteria recovered from AMMDR1-treated seed was in fact AMMDR1. Others have successfully used antibiotic resistance as a marker in order to track bacteria in the rhizosphere (Jjemba and Alexander, 1999). This may prove a useful tool in further rhizosphere competence assays, if antibiotic resistance markers could be found for all tested strains. Unfortunately, it is not helpful in the rhizosphere enrichment assays. It thus seems prudent to postpone any further enrichment assays until a more accurate method of assessing Bcc populations is developed. Meanwhile, the interpretation of any of the six assays described here must be done very carefully, as populations of Bcc may have little relationship to the CFU recovered on the selective media used.

Plant disease and use of the fungicide Ridomil

Some of these assays had considerable problems with fungal or oomycete plant diseases, while others did not. Experiment 3, in which G4 was added to pea seeds, and experiment 6, in which seeds were "planted" in a paper towel system, were two of the hardest hit assays; indeed, disease is a major confounding variable in both, making it very difficult to have confidence in the results. It is not obvious why some of the assays should have been affected and not others. The soil medium was treated

identically in assays 3 and 4, yet only assay 3 experienced such overwhelming damping-off.

Ridomil was used as both a soil drench (experiment 5) and as a seed treatment (experiment 6). In experiment 5, there was very little damping-off, and few signs of decay or disease observed. Moreover, the presence of Ridomil did not seem to affect the number of CFU recovered from the root segments. However, the use of Ridomil as a seed treatment in the paper towel assays was ineffective. Thirty-three percent of seeds in one treatment, and 60% of seeds in another treatment, failed to germinate. Many were covered with a fluffy fungus or were soft and rotten. Of the seeds that germinated, many roots showed signs of decay and disease. If the paper towel assay system is adopted, another antifungal agent or other changes will need to be incorporated. For instance, it might be possible to harvest only the first three plant roots from each conetainer. This would allow for only 30% germination and might facilitate comparison between treatments even with significant disease present.

Cucumbers and peas as host plants

Cucumber was chosen as a host plant for rhizosphere colonization assays due to its ability to grow at clinically important temperatures (37°C). Cucumbers also grow well in the tested assay system. However, care should be taken not to let assays with cucumbers run too long, as the root system of cucumber is comprised of many tiny roots which break upon harvest. Also, AMMDR1, a known rhizosphere colonizer of peas (Parke, 1990), may not be a good positive control for assays with cucumbers (though more experimental units would help confirm this – see above).

Peas are the preferred host plant for these assays. The seeds are large and easy to coat with bacteria. Pea plants have a strong taproot, facilitating harvest and measurement. Assays may be conducted at room temperature in the laboratory, instead of requiring a growth chamber. Finally, strains of Bcc have been shown to colonize pea roots (Parke, 1990).

The root length data of pea plants from the various assays is consolidated below. Based on these data, it seems best to harvest the assay after 7 to 9 days of growth.

Table B.4. Composite data on root length of peas grown in 3 oz paper cups

Experiment	Days of growth	Units with pea seed	Cm root (st.dev.)
2	15	52	28.6 (5.02)
4	9	10	16.1 (2.17)
5	7	30	16.1 (2.43)

Enrichment of Bcc in the rhizosphere

It is difficult to see a clear enrichment effect from these assays. In experiments 4 and 5, the mean log CFU from rhizosphere soil was higher than from bulk soil taken from seedless units which had been in the growth chamber with the pea plants. However, the mean log CFU from rhizosphere soil was not higher than from bulk soil at the start of the experiment. (The fungal problems of experiments 3 and 6 make it difficult to evaluate them.) It may well be that Bcc makes up a greater proportion of

bacteria on the BCSA plates after the rhizosphere enrichment, but without more data this is only speculation. Again, identification of Bcc is paramount in the evaluation of these assays.

Other methodological details

The method of estimating bacterial density based on optical density and applying the bacteria to seeds worked well with different bacterial strains (experiments 1, 2, and 3).

Excising the segment of roots from 1 cm to 2 cm results in more than 1 cm of root being placed in the dilution blank. The mean total excised root length from assay 5 was 12.68 cm (st.dev. 2.59 cm). Assays which express results per cm root should ascertain the actual length of the root segments included in the dilution. Similarly, if the results are to be compared to bulk soil, the total weight of root and soil or, better, rhizosphere soil alone (without the root) should be determined (experiment 2).

Antifungal agents like nystatin, cycloheximide and crystal violet should be incorporated into selective media to minimize fungal growth (experiment 3).

SUMMARY

The rhizosphere competence of members of the *B. cepacia* complex is a tempting potential tool to use both for enrichment of Bcc in soil samples, and for discrimination between Bcc strains. These assays represent some preliminary work toward that end, but significant procedural challenges remain. Adequate control of fungal diseases is a problem, especially when Ridomil may not be used as a soil

drench, as is the case in the paper towel system. High variability between treatments dictates the necessity of using a much greater number of experimental units. Most importantly, the ability to confidently identify Bcc, and distinguish introduced Bcc from "indigenous" (soil-borne) Bcc, is essential for the success of these experiments.