

INTERNATIONAL *BURKHOLDERIA CEPACIA* WORKING GROUP

Fifth Annual Meeting **Saturday, April 8, 2000**

***Burkholderia cepacia* Research Laboratory and Repository**

John J. LiPuma, M.D.

The *B. cepacia* Research Laboratory and Repository, established in Spring 1997 and funded by the Cystic Fibrosis Foundation (U.S.), has received nearly 1900 bacterial isolates recovered from 928 persons receiving care in 138 treatment centers in 111 cities in the US. Referring laboratories are asked to provide presumptive identification and methods used for species determination for each isolate submitted. A polyphasic evaluation including phenotypic (selective media, biochemical tests, and commercial test kits) and genotypic (genus- and species-specific PCR assays) analysis is used to confirm species identification. Among the 1871 isolates for which analysis is complete, 1386 had been identified as *B. cepacia* by the referring lab and 485 were either identified as another species or not identified to the species level. Among the 1386 isolates referred as *B. cepacia*, 1244 were confirmed as *B. cepacia* complex (Bcc); the remaining 142 (10%) were NOT Bcc. Among the 485 isolates referred as unidentified or as another species, 174 (36%) were, in fact, identified as Bcc. The specific methods used by referring laboratories in the initial evaluation of submitted isolates were reviewed in detail for 1051 isolates. Among the 108 laboratories submitting these isolates, nine different commercial test systems were reported as primary methods of identification. The positive predictive values of these systems for identification of Bcc ranged from 71% to 98%. Negative predictive values ranged from 50% to 82%. Seventy-six laboratories reported the use of 'secondary' or 'supplemental' tests; among these, 57 different combinations of tests were described. These data indicate that laboratories vary considerably in the methods used to evaluate Bcc and related species. Furthermore, rates of misidentification of Bcc based on phenotypic assays alone remain high.

This work was supported with funding from the Cystic Fibrosis Foundation.

Taxonomy and identification of *B. cepacia*-like organisms

Tom Coenye, Severine Laevens & Peter Vandamme

Our research focusses on the taxonomy and identification of *B. cepacia*-like organisms isolated from different ecological niches. A polyphasic taxonomic study that included DNA-DNA hybridisations, DNA base ratio determinations, 16S rDNA sequence analysis, whole-cell protein and fatty acid analyses, and an extensive biochemical characterisation was performed on 16 *B. cepacia*-like strains isolated from the environment, animals and human clinical samples. The isolates belonged to the genus *Burkholderia* and were phylogenetically closely related to *Burkholderia graminis*, *Burkholderia caribensis* and *Burkholderia phenazinium* and had a G+C content between 61.9 and 62.2 mol%. Seven strains isolated from the rhizosphere were assigned to the new species *Burkholderia caledonica*. Nine strains isolated from the environment, animals and human clinical samples were assigned to the new species *Burkholderia fungorum*. Recently, several unidentified *B. cepacia*-like strains isolated from cystic fibrosis patients were also classified as *B. fungorum*. Currently we are also investigating a large number of *Ralstonia pickettii*-like strains from different ecological niches (including CF patients). Our polyphasic taxonomic study (including 16S rDNA sequencing, SDS-PAGE of whole-cell proteins, DNA-DNA hybridisations and *gyrB* RFLP) seems to indicate that several new species are present in this group of strains.

Taxonomy of *B. cepacia*-like biocontrol strains

Tom Coenye, Eshwar Mahenthiralingam & Peter Vandamme

One of the most studied biocontrol isolates is *B. cepacia* AMMD (LMG 19182). This strain was isolated from healthy-appearing pea plants in Wisconsin (USA) in 1985. Several studies have shown that strain LMG 19182 is very effective in controlling phytopathogenic *Phytophthora* species (responsible for pre- and post-emergence damping-off in peas) and *Aphanomyces euteiches* (responsible for root-rot and the main factor limiting pea production in the midwest of the USA). In an ongoing survey of *B. cepacia*-like isolates by means of whole-cell protein analysis, a large number of isolates from human and environmental specimens exhibited striking similarity towards *B. cepacia* LMG 19182. We performed a polyphasic taxonomic study for the further characterisation of these isolates. The genotypic and phenotypic characteristics of the strains investigated seem to indicate that they will have to be classified as a new member of the *B. cepacia* complex.

Presentations

***B. cepacia*: CF center director perspective - Susanna McColley**

Abstract not available

Five year survival model of CF - Bruce Marshall

Abstract not available

Short and Long Term Mortality Risks of *Burkholderia cepacia* and *Pseudomonas aeruginosa*

Mary Corey, Annie Dupuis, Elizabeth Tullis.

Authors: Pulmonary infection with *Pseudomonas aeruginosa* (Pa) is common in all CF populations, whereas *Burkholderia cepacia* (Bc) infection varies tremendously in different CF populations. Both are associated with declining lung function and mortality. In previous survival analyses, the significance of Pa risk was confounded with that of pulmonary function, so that for a given age and level of FEV₁ no additional risk was attributed to Pa infection. In contrast, Bc increased mortality risk at all ages and FEV₁ levels.

In order to assess the independent and interacting risks posed by infection with Pa and Bc, Cox proportional hazards regression was conducted in a cohort of 1251 patients with CF attending Toronto CF clinics during 1970-1999. Virtually all Bc in this cohort is Genomovar III, which has been characterized by both epidemic and virulent infection in CF. For one set of survival models, FEV₁ was excluded, so that the short term effects of Pa could be quantified along with those of Bc. Overall, Pa was observed in 74% of the patients, Bc in 21%. 402 (32%) patients died over the 30-year period. When Pa and Bc infections were coded as absent before the first positive sputum, and present thereafter, the hazard ratios associated with Pa and Bc were 3.7 and 4.4 in single models, 2.7 and 4.1 in a combined model. However, when an additional category was added to reflect the disappearance of the organism from culture samples, the hazard associated with Bc infection was greatly increased when Pa was "gone" from sputum cultures. The hazard ratios for both Pa and Bc were increased in younger patients, but not in females, although, as expected, female risk was higher than males across all infection categories.

For predicting longer term survival, FEV₁ and its rate of decline, as well as % of ideal weight and its rate of decline, age, sex, and pancreatic function status (a surrogate for CFTR genotype), were included in models with

Pa and Bc. In separate analyses, covariate values were lagged by 6 months, 1, 2 and 5 years, to assess the best combination of short and long term predictors. These models excluded patients too young for pulmonary function testing (less than 6-8 years of age). Bc was significant in 6 month, 1 and 2 year models, although hazard ratios were reduced because they did not capture the effect of rapid death due to “cepacia syndrome”, since covariate lag times preceded the first positive culture of Bc. Bc was not significant in five-year models, while Pa and pancreatic insufficiency were competing predictors. Neither were significant in shorter term models because of confounding with FEV₁. These results support the hypothesis that Pa infection reflects a susceptibility directly related to CFTR genotype. Bc infection is an independent risk factor, and has been clearly shown to be transmissible, and potentially overwhelming. However, the hazard associated with Bc infection falls dramatically over time. Further research is needed to detect host factors or Genomovar III subcategories to explain the extreme variability in outcome for patients infected with this strain of Bc.

Surveying the Scope and Significance of *Burkholderia cepacia* complex as Pathogens during Lung Transplantation in the United States

George B. Mallory, Jr., MD

There have been no new data reported recently in the medical literature on the potential significance of *Burkholderia cepacia* in the context of lung transplantation for cystic fibrosis (CF). At present, few organisms have been sent to the US *B. cepacia* repository lab with the specific identifying information of having been cultured from a transplant patient (LiPuma, personal communication).

There are presently 35 lung transplant centers performing lung transplantation in CF patients in the USA according to the Cystic Fibrosis Foundation. Thirteen (37%) have performed 5 or more lung transplants on CF recipients over the past four years. An uncertain number (likely the majority) of programs specifically exclude recipients with *B. cepacia* from consideration in their programs. In an era during which the *B. cepacia* complex is being subdivided into species and when clinical correlation between species and clinical outcome is sorely needed, further investigation of the role of *B. cepacia* in the highly vulnerable lung transplant populations is needed, even if in a retrospective fashion.

A survey tool has been developed and will be sent to US Lung Transplant Centers performing lung transplantation in CF patients. Copies will be available at the conference. Overall experience with candidates and recipients with *B. cepacia* complex and individual outcomes will be sought. There will be a concomitant effort to inform the transplant program personnel of the existence of the US repository and to encourage its use in the future. If *B. cepacia* complex organisms have previously been sent to Dr. LiPuma, an attempt will be made to identify these particular specimens. It is uncertain how many of the organisms in the repository have been derived from transplant patients and whether there will be adequate resources to investigate these particular organisms further. It is anticipated that the survey instrument may be extended to Canada, Australia, and Europe in the future.

Determining the Prevalence of *B. cepacia* complex in the Republic of Ireland.

Jonathan Collins. Philip G. Murphy.

Identification of *Burkholderia cepacia* within the Republic of Ireland has been confined to commercial biochemical test systems which have been shown to have an unacceptable degree of error. Neither the prevalence nor the genomovar status of *B. cepacia* have been evaluated in the Republic of Ireland even though we have the highest rate of Cystic Fibrosis in the world. In light of this a National *B. cepacia* Prevalence Project

is being established. We have coordinated with CF units throughout Ireland to supply us with sputum samples from each of these patients as well as isolated *B. cepacia* and other closely related genera. We have also acquired archived isolates on which we will conduct retrospective analyses. Our analyses will utilise genomovar typing based on PCR-RFLP of the *RecA* gene and the 16S rRNA gene. Gas Chromatography- Mass Spectroscopy (GC-MS) will be used to assess the fatty acid composition of the various *B. cepacia* isolates and to compare with a library of spectra from internationally typed strains. Typing will be based primarily on PFGE and RAPD techniques. We are currently evaluating a technique known as Infrequent Restriction Site-PCR (IRS-PCR) as an alternative means of typing *B. cepacia* isolates.

Characterisation of *Burkholderia cepacia* Complex Pathogens in Cystic Fibrosis Patients from Northern Ireland

Kerstin Dunbar, Andrew McDowell, John E. Moore, Eshwar Mahenthiralingam, J. Stuart Elborn

Colonisation of the cystic fibrosis (CF) lung with organisms of the *Burkholderia cepacia* complex (BCC) leads to increased patient morbidity and mortality. Major complications of BCC infection include resistance to anti-pseudomonal antibiotics, the development of “*Burkholderia cepacia* syndrome” and patient to patient transmission. Due to the clinical problems associated with BCC organisms it is imperative transmissible strains posing a risk to the CF patient are identified. As a result, the objective of this study was to examine the genetic relatedness of BCC isolates prepared from the sputum of CF patients in Northern Ireland. A total of 32 isolates were prepared from sputum by culture on MAST *B. cepacia* selective agar. The identity of these organisms as BCC pathogens was confirmed using the API 20 NE phenotypic identification system as well as PCR detection of BCC *recA* and 16S rRNA gene sequences. The genomovar status of these isolates was determined by restriction fragment length polymorphism analysis of the amplified BCC *recA* gene. Speciation results revealed 29 of the isolates (90%) were *B. cepacia* genomovar III (epidemic strain) while the remaining 3 samples (10%) were *B. multivorans* (formerly *B. cepacia* genomovar II). The genomovar isolates were typed by PCR-based random amplification of polymorphic DNA (RAPD) which served to identify the presence of identical or related strains within the Northern Ireland CF population. Amplified DNA fragments were resolved on homogeneous polyacrylamide gels and detected by silver staining. Based on differences in three or more DNA banding patterns, the RAPD profiles for the BCC pathogens fell into a number of distinct groups. With this information, we have been able to assess cross-infection of different BCC strains within our CF community. In addition, clinical data compiled from the patients has enabled us to evaluate the potential virulence of strains represented by the various RAPD groups. From this study we have concluded that RAPD analysis is an extremely valuable diagnostic tool enabling cross-infection to be assessed within a hospital setting as well as the identification of prevalent and clinically relevant BCC strains.

The use of pulsed-field gel electrophoresis for genomic fingerprinting of the *Burkholderia cepacia* complex

Catherine J Doherty and John R W Govan

Ideal criteria for typing or fingerprinting bacterial pathogens include stability, reproducibility and adequate discriminatory power. The use of pulsed-field gel electrophoresis (PFGE) following digestion of whole genomic DNA with the rare cutting enzymes *XbaI* and *SpeI* conforms to these criteria, and is accepted by some researchers as the gold standard for molecular genotyping of the *B. cepacia* complex. This presentation is based on five years experience of PFGE for epidemiological studies of the *B. cepacia* complex. Topics to be covered include techniques for DNA extraction procedures, choice and use of restriction enzymes, electrophoretic pulse times, gel recording and interpretation. Initially, lysis buffer containing proteinase K was used in the DNA extraction protocol for isolates of *B. cepacia*. However, this procedure does not always produce clear, easily

visible banding patterns following digestion with restriction enzyme *XbaI* and subsequent separation of DNA fragments by PFGE. This makes comparison of profiles difficult. An alternative method for DNA extraction was developed using Triton-X-100 as a substitute for proteinase K. This resulted in consistently clear, well-defined profiles showing good resolution. Interestingly, the ET/12 strain of *B. cepacia* is one of the few lineages which resists lysis by Triton-X-100 and requires an additional lysis step using proteinase K. A number of variable parameters are necessary to obtain good separation of DNA fragments by PFGE with pulse times being the most crucial factor. The present PFGE protocol includes digestion of chromosomal DNA from isolates of *B. cepacia* with restriction enzyme *XbaI* and/or *SpeI* using initial and final pulse times of 2.9s and 35.4s respectively, for 20h at 14°C. Discussion will include the use of PFGE for ongoing surveillance of *B. cepacia* and other CF pathogens, including identification of emerging epidemic strains and evidence of clonality between environmental and clinical isolates of *B. cepacia* genomovar I.

Molecular Detection and Speciation of *Burkholderia cepacia* Complex Pathogens in Cystic Fibrosis Sputum.

Andrew McDowell, Kerstin Dunbar, Eshwar Mahenthiralingham, John E. Moore, A. Kevin Webb, S. Lorraine Martin and J. Stuart Elborn

Within our diagnostic laboratory we routinely use PCR-based analysis of the *Burkholderia cepacia* complex (BCC) *recA* gene to speciate genomovars cultured from cystic fibrosis (CF) sputum. Speciation of these isolates has been achieved using *recA* restriction fragment length polymorphism (RFLP) analysis and genomovar specific *recA* primers. We have now applied PCR-RFLP analysis of the *recA* gene to direct speciation of BCC organisms within sputum. Due to the specificity of the primers BCR1 and BCR2, directed to the 5' and 3' ends of the *recA* open reading frame respectively, the *recA* gene of all BCC genomovars can be successfully amplified from sputum DNA preparations. Investigations have shown that BCC speciation results obtained upon direct analysis of sputum agree with those obtained after culture of the organisms on *Burkholderia cepacia* selective agars (BCSA). Detection of BCC pathogens within crude CF sputum represents a considerable advantage to the diagnostic laboratory as it negates the necessity for initial culture of the organism (unless required for further analyses) thereby reducing sample processing time. This method may also prove valuable for speciation of non-culturable genomovars. We have applied *recA* PCR-RFLP analysis to the speciation of BCC pathogens in 100 sputum samples obtained from a UK CF centre. Of all the sputa examined, 13 were found to be positive for the organisms as assessed by culture on BCSA and PCR detection of BCC *recA* and 16S rRNA gene sequences. All the BCC positive sputum samples were found to contain strains of *B. cepacia* genomovar III (epidemic strain). However, the samples differed with respect to the *recA* phylogenetic cluster group to which the *B. cepacia* genomovar III strains belonged. Of the 13 sputa speciated, 9 (approximately 70%) contained strains of genomovar III-A. The cable pilin subunit gene (*cblA*) and *Burkholderia cepacia* epidemic strain marker (BCESM) were detected in 56% (n=5) and 78% (n=7) of these samples respectively. The remaining 4 sputa samples (approximately 30%) were found to contain strains of genomovar III-B. None of these latter samples contained *cblA* or BCESM. This study has served to demonstrate the potential of *recA* speciation for direct analysis of sputum samples within a clinical setting. We are now investigating the application of genomovar specific *recA* primers to speciation of BCC pathogens in crude sputum.

Virulence factor profiling of *Burkholderia cepacia* genomovars.

Kevin M. Markey, Philip G. Murphy, Máire M. Callaghan.

The virulence mechanisms of *B. cepacia* strains are poorly understood. In particular, the characteristics of certain strains responsible for 'cepacia syndrome' in CF patients remain obscure. The production of virulence

factors such as hemolysins, lipases, proteases, LPS and siderophores in *B. cepacia* strains has been documented. However, no one factor has been found to be singularly associated with ‘*cepacia syndrome*’. A comparison of virulence factors produced by the various genomovars may demonstrate a differing profile of factors evident in the highly transmissible pathogenic strains versus non-pathogenic strains. Furthermore, the presence of a synergistic effect between factors may be significant in the ability of some strains to cause a rapid fatal decline in lung function. The aim of this study is to screen *B. cepacia* strains representative of various genomovars for a range of biochemical and molecular virulence and transmissibility factors. Strains are currently being examined for the production of easily identifiable extracellular components such as hemolysins, phospholipase C, LPS, lipase and siderophores. Strains are also being screened for the BCESM marker to determine the virulence factor profiles of epidemic strains. Preliminary evidence indicates that few or no strains produce phospholipase C yet lipases are produced, and seemingly to a greater extent by genomovar II strains rather than genomovars I, III, IV and V. Further work is required to establish a profile of virulence factor distribution amongst the *B. cepacia* genomovars and to investigate their role in the pathogenesis of ‘*cepacia syndrome*’.

Induction of antibiotic resistance in *B. cepacia*

Bindu M. Nair, Adam Griffith, Amy Lee, Jane L. Burns

Treatment of *Burkholderia cepacia* infections in CF is often limited by intrinsic or acquired antibiotic resistance. In general, bacterial resistance mechanisms include drug modification, alteration or overproduction of the target, limited cell wall permeability and drug efflux, all of which have been demonstrated in *B. cepacia*. We have identified an inducible efflux operon from *B. cepacia* (*ceoR/lpE/ceoAB/opcM*) that confers resistance to chloramphenicol (Cam), ciprofloxacin (Cip) and trimethoprim (Tmp) and shows significant homology to the *Pseudomonas aeruginosa* MexEF/OprN efflux system. Experiments conducted with radiolabeled chloramphenicol in the presence and absence of an energy uncoupler demonstrates the energy dependence of the *ceo* efflux system. Sequence information identifies CeoR as a potential LysR type transcriptional regulator that is oriented divergently from the putative structural genes *lpE/ceoAB/opcM*. Promoter probe experiments conducted with a *ceoR-lacZ* fusion construct show a concentration-dependent effect of salicylate (SA, which is a *B. cepacia* siderophore) on LacZ activity. This correlates with a converse effect of iron concentration. Susceptibility testing results have also shown that antibiotic resistance can be induced with the addition of exogenous SA or by growth under iron-limited conditions, which induce endogenous SA production. Ongoing efforts are directed towards understanding transcriptional activity of *ceoR* and *lpE/ceoAB/opcM*, in the presence and absence of Cam, Cip and Tmp as well as varying levels of SA.

Invasion and Intracellular Survival of *Burkholderia cepacia*

Christian D. Mohr, Mladen Tomich, Christine A. Herfst

Burkholderia cepacia is an important pulmonary pathogen in immunocompromised patients and patients with cystic fibrosis (CF). In CF patients, *B. cepacia* colonization of the lung can lead to necrotizing pneumonia and septicemia, often resulting in a rapid and ultimately fatal clinical decline. The mechanisms by which *B. cepacia* is able to invade deeper tissues of the lung and ultimately become blood-borne are poorly understood. We have recently shown that *B. cepacia* strain J2315, a clinical isolate responsible for epidemic outbreaks and mortality in CF patients, can invade and survive intracellularly in cultured macrophages and respiratory epithelial cells. In order to identify and characterize the genetic determinants required for invasion, we are currently screening random transposon-generated mutants of *B. cepacia* strain J2315 for their ability to invade cultured A549 respiratory epithelial cells. We have also identified and are characterizing the role of type III secretion genes in *B. cepacia* pathogenesis. Our goal is to further define the pathogenic mechanisms responsible for the emergence of *B. cepacia* as an important opportunistic pathogen in CF patients.

Localization of *Burkholderia cepacia* in well-differentiated human tracheobronchial epithelial cultures

Ute Schwab, Peter Gilligan, Richard Boucher, Scott Randell, and James Yankaskas

Burkholderia cepacia has emerged as a serious respiratory pathogen in cystic fibrosis (CF) patients. However, the pathophysiology of *B. cepacia* infection in CF is still poorly understood. Prior studies describe cellular invasion and intracellular growth of *B. cepacia* in A549 pulmonary cells as pathogenic mechanisms. We used well-differentiated human air-liquid interface (ALI) cell cultures from a non-CF patient to study the location of *B. cepacia* in mucus and cells. Mature cultures, 2-4 weeks following confluence, were infected with a *B. cepacia* isolate from the ET12 lineage when the airway cells were fully differentiated into ciliated and columnar cells producing mucus. Bacteria were grown in ALI culture medium for 4 hours (log-phase) or grown on agar plates overnight (stationary-phase) and then suspended in culture medium. Approximately 5×10^8 cfu of log- or stationary-phase bacteria were transferred in 50 μ l of ALI medium to well-differentiated 12 mm diameter cultures and incubated at 37°C in 5% CO₂ for 1, 2 or 5 days. Cultures were fixed with osmium tetroxide-perfluorocarbon to preserve the mucous layer and assessed using light microscopy (LM), transmission (TEM) and scanning electron microscopy (SEM). Our initial studies, inoculating 5×10^5 cfu of log-phase bacteria and incubating for 1 day, did not reveal bacteria in the mucous or cell layers, or epithelial damage by LM or TEM. However, after 1-day incubation with the higher inoculum (5×10^8 cfu) of log-phase *B. cepacia*, planktonic bacteria were seen in the mucous layer, but the majority of bacteria seemed to be aggregated when analyzed by LM and TEM. Bacterial aggregates apparently trapped in mucus could also be seen by SEM. We observed by TEM intra- and intercellular single and aggregated bacteria. After a 5-day incubation period, LM and TEM of cultures infected with log-phase bacteria showed significant necrosis of the columnar and ciliated cells. However, no damage was seen in the basal cell layer. Cell cultures infected with stationary-phase bacteria contained mainly planktonic bacteria in the mucous layer and bacterial aggregates were not observed even when the cultures were incubated with bacteria for 2 days. Our data show that *B. cepacia* at high inoculation density formed aggregates in the mucous layer and also appeared as single or multiple intra- and intercellular bacteria. Future experiments will determine whether *B. cepacia* uses cellular penetration and/or transepithelial invasion of airway epithelial cells and what role the aggregate formation plays in the pathogenesis of *B. cepacia* airway infection.

The Toxic Substances Control Act: Uses and Oversight of Products Containing *Burkholderia cepacia*

P. Sayre, V. Hart, and R. Brinkhuis

There are a number of commercial uses of *Burkholderia cepacia* which are potentially subject to oversight under the U.S. EPA's Toxic Substance Control Act (TSCA). A search of in-house databases, the open literature, the patent literature, and other sources indicated that *B. cepacia* could be used in a number of products, including those intended for the following: bioremediation, waste water treatment, biomining/bioleaching, desulfurization of oil and coal, oil recovery, biomass conversion, drain cleaners, and specialty chemical production. This search strategy was gradually narrowed. The next examination revealed specific references to bacteria used in a number of these areas, with highest frequency of use noted in bioremediation (75 references) and specialty chemicals (seven references). Narrowing the search further to just applications involving pseudomonads further focused in on these two application areas, with eight applications for bioremediation and six applications for specialty chemical production located. Although bioremediation applications were expected given the diversity metabolic capabilities of pseudomonads in general, the number of uses for specialty chemical production were not anticipated. Specialty chemical application areas included use of pseudomonads for production of enzymes

such as lipases, and production of bioplastics. *B. cepacia* isolates represented a large portion of the pseudomonad applications located, with four applications in bioremediation, and six in the specialty chemical area. Possible paths forward for addressing the use of *B. cepacia* in such commercial products under TSCA include voluntary approaches (to gather information from industry, inform industry through mechanisms such as fact sheets, and reduce usage in commercial products) to regulatory options under Sections 4, 5, 6, and 8 of TSCA. Other approaches are also possible.

Susceptibility of *Burkholderia cepacia* to ultraviolet light.

Kevin G. Kerr,

The application of molecular typing techniques has done much to elucidate the epidemiology of infection associated with *Burkholderia cepacia*. However, the routes of transmission between patients are not well characterized. There is evidence, albeit limited, to suggest that the airborne route may be of importance in the transmission of this bacterium and, given the renewed interest in ultraviolet germicidal irradiation as an infection control measure following the emergence of multi-drug resistant tuberculosis, this technology may have a role in the prevention of spread of respiratory tract pathogens in cystic fibrosis. Details of a proposed pilot study which will investigate the effectiveness of UVGI in a CF center will be presented. As a preliminary, an in-vitro study of the UVGI susceptibility of *B. cepacia* was conducted using strains from each of the currently recognised genomovars of this bacterium. Strains of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* were also examined. Experiments were conducted in a polycarbonate chamber with 4 UV lamps (total output 7W) with integral downward reflectors that emitted UVGI at a wavelength of 253.7nm. Plates with lawns of each strain were exposed to UV intensities of 11.32-12.41W/m² for varying time periods. The duration of the sample UV exposure period was controlled by a shutter located directly below the lamps and by using this, it was possible to vary the UV dose (J/m²)

The UV susceptibility constant (k [m²J⁻¹]) was calculated using the formula:

$$k = \frac{-\ln(N_t/N_0)}{H_{eff}}$$

[where N_t = mean cfu at time, t ; N_0 = mean cfu at $t = 0$ s; H_{eff} = effective UV dose (J/m²)]

All the bacteria investigated were killed by a dose of 118 Jm⁻², with doses required to give a one log reduction in bacterial numbers ranging from 28.33-57.5 Jm⁻². With the exception of one genomovar I strain, which was the most susceptible isolate tested ($k = 0.2062$), *B. cepacia* was less susceptible to UVGI than *P. aeruginosa*, but more susceptible than *S. maltophilia*. However, no strain of *B. cepacia* survived more than 8s exposure to UVGI. These preliminary results are encouraging, but we now intend to investigate the activity of UVGI against aerosols of *B. cepacia*. We have also shown that *B. cepacia* can parasitize the free-living amoeba *Acanthamoeba polyphaga* [Landers P, Kerr KG, *et al*; Eur J Clin Microbiol Infect Dis - in press] and we will, accordingly, investigate the UVGI susceptibility of intra-amoebal bacteria

Clinical and environmental isolates of *Burkholderia cepacia* exhibit differential cytotoxicity towards macrophages

Anatoli Melnikov, Olga Zaborina, B.S. Prabhakar, A.M. Chakrabarty and William Hendrickson

Burkholderia cepacia is an emerging opportunistic pathogen that causes fatal infections in patients suffering from cystic fibrosis (CF) and chronic granulomatous disease. Various environmental isolates of *B. cepacia* are, however, capable of degrading environmental pollutants such as trichloroethylene, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), etc, and are also highly effective in controlling plant diseases caused by nematodes and fungi. Such strains have therefore been proposed for environmental release to cleanup toxic dump sites or as

biopesticides. Various efforts to distinguish between clinical and environmental isolates of *B. cepacia* with regard to their virulence characteristics have produced ambiguous results, suggesting that newer methods are needed to test for the presence or absence of pathogenic potential in *B. cepacia* strains proposed for environmental release. We now report that two strains of *B. cepacia*, isolated from the lungs of CF patients, secrete cytotoxic factors that allow macrophage cell death through activation of ATP-inducible macrophage surface-associated P2Z (P2X₇) receptors. Two environmental strains had reduced activity in this regard. We further demonstrate that while all the strains secrete enzymes that have nucleoside diphosphate kinase (Ndk), adenylate kinase (Ak) and 5'-nucleotidase activity, the level of secretion of the 5'-nucleotidase (and ATPase) appears to be lower in the environmental strains than the clinical strains. The secretion of these enzymes is specifically activated in presence of eukaryotic proteins such as 2-macroglobulin. Since the P2Z receptors promote macrophage cell death only in presence of mM concentrations of ATP, and since the secreted ATP-utilizing enzymes, particularly 5'-nucleotidase, generate various phosphorylated or nonphosphorylated adenine nucleotides that may even be better agonists than ATP in activating the P2Z receptors or may act through activation of additional purinergic receptors, such enzymes may play an important role in allowing *B. cepacia* to evade host defense.

Genetic characterization of the plant watersoaking phenotype expressed by plant and clinical *Burkholderia cepacia*.

E.G. Medrano and C.F. Gonzalez.

Burkholderia cepacia has historically been regarded as a plant pathogen causing onion soft rot. However, *B. cepacia* is now of wider interest in agriculture, biotechnology, and medicine. We have observed that plant isolates that have been cured of an endo-polygalacturonase-encoding plasmid or that have a 35-kb deletion of the plasmid do not macerate plant tissue but still cause a watersoaking (wts) reaction in the tissue. We have previously reported this same wts phenotype in 14/15 genomovar III isolates obtained from the IBCWG collection and tested on plant tissue. Using transposon mutagenesis we have generated a series of wts-negative mutants in both a plant and clinical isolate. A BLAST search using the translated sequence of a clinical wts - negative mutant has revealed homology to a porin, suggesting the potential involvement of this protein in the wts phenotype. Furthermore, a Chou-Fasman protein structural analysis of the sequenced gene predicts a pattern of hydrophobic and hydrophilic residues consistent with known porin architecture. Since wts activity has been proposed to result from host cell electrolyte leakage induced by a pathogen effector molecule it is possible that a porin mutation could affect the secretion of a bacterial virulence factor involved in wts. Sequence analysis of several other mutants is being conducted in an effort to elucidate and compare wts symptoms produced by *B. cepacia* of plant and clinical origin.

Sunday, April 9, 2000

Multiple Combination Bactericidal Antibiotic Testing for Acute Exacerbations of Cystic Fibrosis Associated with *Burkholderia cepacia* Infection.

Shawn Aaron, Elizabeth Tullis, David Haase, Pearce Wilcox, Noni MacDonald

Background: Traditional single antibiotic susceptibility testing methods have limited relevance in cases of CF-associated *B. cepacia* infection since many *B. cepacia* organisms are pan-resistant to individual antibiotics. We

therefore developed an *in-vitro* method called multiple combination bactericidal antibiotic testing (MCBT), for testing and evaluating combinations of single, double, and triple antibiotics for bactericidal activity against isolates of *B. cepacia*. We studied 119 consecutively-referred *B. cepacia* isolates with MCBT testing and found: 1) That the majority were resistant to all single antibiotics; 2) That triple antibiotic combinations were more likely to be bactericidal than double combinations, and; 3) That antagonism (growth of the organism when an additional antibiotic was added to a previously bactericidal combination) was common.

Objective: This randomized, double-blind, controlled clinical trial is designed to prospectively assess whether the use of combination antibiotic therapy, directed by results from MCBT testing, improves bacteriologic and clinical outcomes in patients with acute pulmonary exacerbations of cystic fibrosis who are known to be colonized with *Burkholderia cepacia* organisms.

Timetable: The trial will recruit and randomize patients from four Canadian CF centers over an 18 month period. Follow-up of patients will continue for 12 months after the last patient has been randomized into the study.

Study Design: Randomized, double-blind, controlled clinical trial.

Patient Population: One-hundred and twenty CF patients known to be colonized with *B. cepacia* will submit sputum at 3 month intervals for MCBT testing. The study will randomize the first 82 of these 120 patients who develop an exacerbation of pulmonary disease over the 18 month randomization period.

Intervention: Patients randomized to the control group will receive a 14 day course of any two intravenous antibiotics \pm one inhaled antibiotic (tobramycin/TOBI) chosen by their CF physician based on results from conventional sputum culture and sensitivity testing. Patients randomized to MCBT- directed therapy will have their antibiotic therapy chosen based on the result of the MCBT sensitivity test. MCBT-directed therapy will consist of two intravenous antibiotics \pm inhaled tobramycin/TOBI.

Outcomes: The primary outcome measure will be the time, measured in months, from randomization until the patient's next pulmonary exacerbation. Other outcomes will be measured at the end of the 14 day treatment period and will include: 1) changes in sputum bacterial densities; 2) changes in the forced expiratory volume in one second and the forced vital capacity (FEV₁ and FVC); 3) changes in oxygenation; 4) changes in dyspnea index scores; 5) proportion of 'treatment failures' within 14 days of entry into the trial; 6) hospital length of stay; and 7) adverse effects.

Analysis: The final analysis will be performed on an 'intention to treat' basis. Kaplan-Meier survival curves will be used to describe the probability of remaining exacerbation-free in the two treatment arms as a function of time from completion of the 14-day study treatment. The curves will be compared using the log-rank test statistic. Subsequently, Cox proportional hazards modeling will be performed to adjust for other variables which could potentially affect the time to next exacerbation.

Conclusion: This clinical trial offers a unique opportunity to determine whether MCBT-directed antibiotic therapy results in superior clinical and bacteriologic outcomes when compared to usual antibiotic therapy for *B. cepacia*-associated CF pulmonary exacerbations.

PFGE standardization project - Philip Murphy

Abstract not available

A promising animal model of *B. cepacia* lung infection in CF

U. Sajjan, G. Thanassoulis, M. Wu, G. Downey, G. Kent and J. Forstner

A suitable model of chronic lung infection by *B. cepacia* is required for advancing our knowledge of the pathogenesis of CF lung disease. We have developed a model in liquid-fed, long-surviving UNC *cftr* knockout (-/-) mice. This was achieved by intranasal instillation of *B. cepacia* (Genomovar III, RAPD type 2, BCESM +ve, *cblA* /adhesin +ve) into groups of UNC *cftr* (-/-) (n=13) and *cftr* (+/+) (n=12) mice once every 3 days for a total of four doses. Mice were sacrificed nine days later, and the lungs examined for histopathology and evidence of chronic colonization by *B. cepacia*, using quantitative viability counts and immunofluorescence with an anti-*B. cepacia* antibody. Inflammatory cells in the bronchoalveolar lavage (BAL) fluid were determined by flow cytometry.

All *cftr* (-/-) mice had moderate to severe infiltration of both mono- and polymorphonuclear inflammatory cells in almost all parts of the lung parenchyma. In severely affected interstitial foci, complete consolidation of lungs was observed. The infiltrate was composed predominantly of macrophages, but neutrophils were also abundant. Airways were characterized by moderate to severe peribronchiolar lymphoplasmacytic cuffing and Clara cell hypertrophy/hyperplasia in the distal small airways. A PAS positive (mucus-like) secretion was noted from unusual 'domed' Clara cells in many areas. The major cell type in the BAL fluid was macrophages, with neutrophils accounting for 19.7% of the total cells. *B. cepacia* cultured from lung ranged from 1×10^4 to 5×10^5 (mean of 1.5×10^5) cfu/g lung tissue. By immunolocalization, *B. cepacia* microcolonies were observed all over the lungs, being most densely clustered in heavily infiltrated peribronchial and perivascular areas, and in areas of consolidated pneumonia and pathologically thickened alveolar septa. The antibody did not react with lung tissue of animals instilled with PBS (control). The lungs of *cftr* (+/+) mice were histologically much more normal in appearance. There were some patchy areas of mononuclear inflammatory cell infiltration in the parenchyma, indicative of mild to moderate interstitial pneumonitis. There were also randomly distributed small areas of peribronchiolar and perivascular lymphocyte and plasma cell infiltration. BAL fluid contained fewer neutrophils (7.8% vs 19.7%). Only three out of eight animals had culturable *B. cepacia* in their lungs, with the bacterial load ranging from 2.9×10^2 to 1×10^3 cfu/g lung tissue, which is much less than *cftr* (-/-) mice. Consistent with this observation, far fewer *B. cepacia* microcolonies were detected by immunofluorescence in the lungs of *cftr* (+/+) mice, but the bacteria detected were in close association with areas of inflammatory cell infiltration. In summary, repeated nasal instillation of *B. cepacia* followed by 9 days of no treatment, gave histological and microbiological evidence of lung infection in UNC mice. Homozygous *cftr* (-/-) mice were more susceptible to *B. cepacia* than *cftr* (+/+) mice. It is expected that this animal model will be very useful for studies of chronic lung infection in human CF.

Differential Pathogenicity of Genomovars of *Burkholderia cepacia* in a Murine Intranasal Infection Model.

David P. Speert, Keith Halsey and Karen Chu.

Burkholderia cepacia is an important pathogen in patients with cystic fibrosis, and there is a striking difference in outcome among patients infected with strains from the different genomovars/species within the complex. We developed an animal model of pulmonary infection to try to identify the bacterial determinants responsible for apparent differential virulence among strains within the *B. cepacia* complex. Mature BALB/c mice were rendered mildly neutropenic by administering cyclophosphamide (150 mg/kg IP every 5 days); more frequent administration of cyclophosphamide resulted in unacceptable morbidity. One day after the first dose of cyclophosphamide, mice were challenged intranasally with 1.6×10^4 CFU of overnight grown bacteria from one of the *B. cepacia* genomovars/species. Animals were killed after 16 days or earlier if there was evidence of unacceptable morbidity (excessive weight loss, ruffling of fur, listlessness, etc.). A total of 17 bacterial strains were evaluated in immunosuppressed and control animals. Groups of three animals were evaluated at each time point (30 minutes post-inoculation and 16 days or earlier). Substantial differences were observed among the genomovars/species as follows:

- **Genomovar II** (*B. multivorans*). Six strains tested – 4/6 strains persisted at stable numbers in the lungs (10^5 CFU gram lung tissue) at day 16, but the animals looked relatively unaffected. Most control animals cleared all bacteria from the lungs but minimal numbers persisted for 2/6 strains.
- **Genomovar III**. Six strains tested – 4/6 strains caused unacceptable morbidity, and the animals were killed at day 5. In all cases there were no viable bacteria in any organ (lung or spleen). Of the animals killed at day 16, 3/3 which received one of the strains were culture-negative, but bacteria persisted at 2×10^4 in 1/3 of the animals which had received the other strain; all these animals appeared healthy.
- **Genomovar IV** (*B. stabilis*). One strain tested – All animals which received the one strain were healthy at day 16 and were culture-negative.
- **Genomovar V** (*B. vietnamiensis*). One strain tested – One animal was killed at day 8 and two at day 10 because of morbidity. Substantial growth over the initial inoculum was seen in the lungs of all cyclophosphamide-treated animals and persistence was seen in the lungs of the normal controls.
- **Genomovar VI**. One strain tested – All mice were killed at day 14 and the results were virtually the same as for *B. vietnamiensis*; there was bacterial growth in the lungs of the cyclophosphamide-treated mice and persistence in the lungs of the controls.

In conclusion, we describe a new animal model for evaluation of putative virulence determinants in *B. cepacia* strains from different genomovars/species. Using this model, we intend to evaluate the bacterial features which permit differential persistence or intoxication among the strains. Results of ongoing histopathological studies will be presented. Supported with funds from the Canadian Cystic Fibrosis Foundation

N-acyl homoserine lactone formation by *Burkholderia multivorans* and *B. vietnamiensis* isolates

F. Yao, H. Zhou, and T.G. Lessie.

The *Chromobacterium violaceum* autoinducer-deficient mutant CV026 is a sensitive reporter strain for detection of N-acyl homoserine lactones (AHLs) produced by *B. vietnamiensis*, but not a good indicator of AHL production by other members of the "*B. cepacia* complex." We have exploited strain CV026 to isolate variants of *B. multivorans* 17616 which produce significant amounts of AHLs not detected in cultures of their wild type parent. AHLs from culture filtrates of *B. multivorans* and *B. vietnamiensis* were adsorbed on mixed resin containing vinyl pyrrolidone and divinyl benzene, eluted with methanol, and resolved by thin-layer chromatography. Representative *B. vietnamiensis* isolates produced two AHLs which comigrated with hexanoyl and octanoyl homoserine lactones. The AHL overproducing variant of *B. multivorans* 17616 produced similar AHLs as well as a third species of intermediate mobility. The results suggest that other members of the "*B. cepacia* complex" may have the potential to give rise to variants forming increased amounts of AHL. We have used PCR primers homologous to the *cepI* and *cepR* genes of the genomovar III strain K56-2 described by Lewenza et al (J. Bacteriol. 181: 748-756) to screen for genes specifying autoinducer synthase and transcriptional activator in representative *B. vietnamiensis* and *B. multivorans* isolates. The results suggest that the organization of these genes in *B. vietnamiensis* is similar to that in strain K56-2. We are examining the wild type and AHL-overproducing variants of strains 17616 in an effort to understand the mechanisms governing AHL formation.

N-acyl homoserine lactone-Mediated Interspecies Communication between *Burkholderia cepacia* and *Pseudomonas aeruginosa*.

S. Lewenza and P.A. Sokol

B. cepacia and *P. aeruginosa* are opportunistic pathogens that commonly cause pulmonary infections in cystic fibrosis patients. Both organisms possess quorum sensing regulatory systems to control the production of

virulence factors. The components of a quorum sensing system include an autoinducer synthase that generates *N*-acyl HSL signaling molecules and a transcriptional regulator that binds the autoinducer and subsequently activates or represses the expression of target genes. In *P. aeruginosa*, the LasR-OdDHL [*N*-(3-oxododecanoyl) HSL] and RhIR-BHL (*N*-butyryl HSL) systems control the production of proteases, elastase, exotoxin A, rhamnolipids, lipase and pyocyanin. In *B. cepacia*, the CepR-OHL (*N*-octanoyl HSL) controls the production of protease and the siderophore ornibactin. Two approaches were employed to investigate the possibility that *P. aeruginosa* and *B. cepacia* could use heterologous *N*-acyl HSLs to regulate the expression of potential virulence factors. In the first approach, we examined the ability of *P. aeruginosa* and *B. cepacia* to produce *N*-acyl HSLs that cross-feed the autoinducer synthase mutants of the heterologous species and restore protease or elastase production. *B. cepacia* K56-I2 is a *cepI* reporter strain that does not produce protease unless autoinducers are exogenously supplied that bind CepR leading to the restoration of protease expression. Protease production was restored to the *cepI* reporter K56-I2 (a) when concentrated ethyl acetate extracts from stationary phase *P. aeruginosa* PAO cultures were supplied or (b) in cross-feeding assays with *P. aeruginosa* expressing *lasI* or *rhlI* on high copy plasmids. This suggests that *B. cepacia* can utilize both *P. aeruginosa* HSL molecules (OdDHL and BHL) to control protease production. *P. aeruginosa* PDO100 and PAO214 are *rhlI* and *lasI* reporter strains respectively, that do not produce elastase unless appropriate autoinducers are exogenously supplied. Elastase production was not restored in the *rhlI* reporter PDO100 or the *lasI* reporter PAO214 when concentrated ethyl acetate extracts from *B. cepacia* K56-2 cultures were supplied or in cross-feeding assays with *B. cepacia* expressing *cepI* in high copy. In the second approach, we examined the ability of genes from one species to complement the respective quorum sensing mutations in the heterologous species. The introduction of *rhlI*, but not *lasI*, to the *B. cepacia* *cepI* mutant K56-I2 was able to fully restore protease production. The introduction of *cepI* into the *lasI* mutant PAO214, but not the *rhlI* mutant PDO100, restored parental levels of elastase activity. Cross-feeding and cross-complementation assays suggest that *B. cepacia* CepR is flexible in binding autoinducers synthesized by RhII and LasI and *P. aeruginosa* LasR is capable of binding autoinducers synthesized by CepI. It is possible that a mixed bacterial population of *B. cepacia* and *P. aeruginosa* can coordinately regulate some of their virulence factors and influence the progression of lung disease due to infection with these organisms.

RecA based identification of *B. cepacia* complex species - Esh Mahenthiralingam

Abstract not available

Additional Abstracts

Biochemical and Molecular Approaches for determining Genomovar Status of the *Burkholderia cepacia* complex

D. A. Henry, E. Mahenthiralingam, P. Vandamme, T. Coenye, P. W. Whitby, and D. P. Speert

Recent taxonomic advances have demonstrated that the CF pathogen *Burkholderia cepacia* is actually a family of at least five closely related genomic species (or genomovars) now called the *B. cepacia* complex. Genomovars II, IV and V were formally named *Burkholderia multivorans*, *Burkholderia stabilis* and *Burkholderia vietnamiensis*, respectively (with the epithet ‘*cepacia*’ being reserved for genomovar I); genomovar III has not been formally named yet, pending the availability of differential diagnostic tests. Recent studies indicate that genomovar III may be more pathogenic in CF patients than other genomovars, while genomovars I and V are less commonly found in CF patients. Commercial bacterial identification systems are

not able to separate or confirm the identification of *B. cepacia* complex isolates from closely related species (such as *Burkholderia gladioli*) without the addition of supplementary tests. Therefore it is important to find a simple way to determine genomovar status. In this study over 130 *B. cepacia* complex isolates were identified to genomovar level using whole cell protein electrophoresis and amplified fragment length polymorphic DNA analysis. These and an additional 300 isolates were then examined by PCR using genomovar specific molecular probes based on the Rec A gene for the *B. cepacia* complex, and on the 23S rRNA gene for *B. gladioli*. From this information, comparisons to the more classical and routine biochemical tests found in clinical laboratories were made. It was demonstrated that *B. multivorans* and *B. stabilis* could reliably be separated from the other members of the *B. cepacia* complex by tests such as sucrose and ONPG utilization, and growth at 42°C. As well, a biochemical identification scheme for the identification of *B. gladioli* and *Ralstonia pickettii* and their separation from the *B. cepacia* complex is included. This poster was presented at the 1999 International CF Conference; work has been continuing on this project in an effort to increase the sensitivity of the RecA primers, and to confirm identity of previously unidentified isolates.

The Relationship of Outcome to *Burkholderia cepacia*, Lung Function and Inflammatory Markers in Stable Patients with Cystic Fibrosis.

D Downey, M Dempster, L Martin, M Keogan, B Starcher, J Edgar, D Bilton, JS Elborn

Decreased survival in CF patients has been related to FEV₁, BMI and infection with *B.cepacia*. In this study we have assessed the relationship of lung function and of sputum and blood inflammatory markers to survival. We used data from 39 stable CF patients (32 M, 7 F), 10 infected with *B.cepacia* who were participating in a study of nebulised transgenic human alpha-1-antitrypsin (tg-hAAT). Age, height, BMI, alcohol consumption, heart rate, oxygen saturation, FEV1, FVC, VC, white blood cells (WBC), neutrophil count and C-Reactive Protein (CRP) were measured. Plasma and sputum elastase / AAT complexes, urinary desmosine concentration and sputum elastase, IL 8, soluble TNF receptors (P55) and myeloperoxidase (MPO) were measured. All were measured prior to study drug administration. Five deaths were reported at the end of one year (4 in patients affected with *B.cepacia*), (p=0.011). Mann-Whitney U test was performed to compare the above parameters in survivors and non-survivors. FEV1 was significantly lower in those who died, 1.16 (0.25)L, [mean (SEM)] compared to survivors, 2.01 (0.15)L, p=0.03 as was FVC 1.98 (0.26) L vs 3.11 (0.17)L, p=0.01. There was significantly higher sputum elastase 3.64 (1.58) u/ml vs 1.54 (0.59) u/ml p=0.03, plasma elastase/AAT complexes 274.1 (98.95) ng/ml vs 141.9 (30.02) ng/ml p=0.05, sputum MPO 162.6 (62.18) mcg/ml vs 54.39 (6.93) mcg/ml p=0.03 and urinary desmosines 107.9 (19.90) pM/mg creatinine vs 51.11 (3.31) pM/mg creatinine p=0.001, in those patients who died compared to survivors. There was no significant difference between survivors and those patients who died for, heart rate, oxygen saturation, VC, WBC, neutrophil count, CRP, sputum elastase/ AAT complexes, sputum IL8 and soluble TNF-receptors. This suggests that active inflammation and tissue destruction are associated with a poor outcome in CF. Supported by PPL Therapeutics and the CF Trust (UK)

Prevalence and population diversity of *Burkholderia cepacia* complex species in the environment: previously published results must be carefully interpreted and data double checked

Eshwar Mahenthiralingam, Neil Burton, Severine Laevens and Peter Vandamme

From past scientific literature microbiologists have basically come to associate *Burkholderia cepacia* as a bacterial species which is “ubiquitous” in the natural environment. Last year, intriguing data was published on collection of 21 “*B. cepacia*” isolates recovered from the natural environment and 21 cystic fibrosis patients last year (Wigley, P, and N.F. Burton. 1999. Genotypic and Phenotypic Relationships in *Burkholderia cepacia* isolated from cystic fibrosis patients and the environment. Journal of Applied Microbiology. 86: 460-468.). Because of the difficulties associated with identification of *B. cepacia* and the complex taxonomy of this group of bacteria, 20 of the 21 environmental isolates were re-examined by amplified 16S rRNA gene restriction fragment length polymorphism analysis (ARDRA), FAME and whole-cell protein profile analysis, in order to confirm their identity. Six ARDRA types were found among the 20 isolates and not one of them matched the 3 patterns normally associated with the *B. cepacia* complex. FAME analysis was performed on one environmental strain representative of each ARDRA type. FAME assigned species groups were as follows (FAME score): isolate PW 4, *Ralstonia solanacearum* (0.825); isolate PW 5, *Serratia fonticola* (0.195); isolate PW 9, *Chromobacterium violaceum*. (0.541); isolate PW 10, *Kluyvera ascorbata* (0.498); isolate PW 14, *Pseudomonas putida* (0.650); and isolate PW, 19 *Enterobacter intermedius* (0.286). Whole-cell protein profile analysis and lack of amplification with *recA* specific PCR confirmed that not one of the latter isolates belonged to the *B. cepacia* complex. While FAME identification can only be relied upon to provide an approximation of genus identification, polyphasic analysis by ARDRA, *recA* and protein profile analysis confirmed that 20 of the 21 environmental strains from this study were not members of the *B. cepacia* complex. The ubiquity of the *B. cepacia* complex populations in the natural environment and their diversity remains uncertain. Systematic studies using state-of-the-art identification approaches are urgently required to substantiate previously published observations.

Molecular Detection of CF Pathogens.

Paul W. Whitby, John J. LiPuma, and Terrence L. Stull.

Identification of the species in the *Burkholderia cepacia* complex by routine clinical microbiology methods is difficult. Phenotypic methods to identify *B. multivorans*, *B. stabilis*, and *B. vietnamiensis* have been reported; however, attempts to identify genomovars I and III have been unsuccessful. The identification of these isolates is further complicated by several other species, also isolated from CF sputum, that share similar phenotypic traits. Such species include *Burkholderia gladioli*, *Stenotrophomonas maltophilia*, *Ralstonia pickettii* and *Alcaligenes xylosoxidans*. To improve the current capability to identify these organisms we have developed species-specific primer pairs that target 16S and 23S rRNA gene sequences and the 16S-23S gene spacer region. Use of these primer pairs in optimized PCRs containing genomic template has allowed detection and identification of the following: *B. cepacia* complex (sens. 68% spec. 87%), *B. vietnamiensis* (sens. 87%, spec. 92%), *B. multivorans* (sens. 100% spec. 99%), genomovars I, III, & IV as a group (sens.100% spec. 99%), genomovars I and III (sens. 100% spec. 100%) genomovars I and IV (sens. 100% spec.100%), *B. gladioli* (sens. 100% spec.100%), and *S. maltophilia* (sens.100% spec. 99%). We have also demonstrated the utility of species-specific PCR as a tool to detect and identify pathogens directly from CF sputum. PCR assays to identify *R. pickettii* and *A. xylosoxidans* by are currently being assessed. rRNA targeted species-specific PCR has the potential to directly identify important pathogens of CF patients.

This work was supported by the Cystic Fibrosis Foundation.

Enhanced adherence of cable-piliated *B. cepacia* to respiratory epithelia of CF transgenic mice and human CF lung explants

Umadevi Sajjan, YiJun Wu, Geraldine Kent and Janet Forstner

Since the initial stage of infections generally involves bacterial binding to host tissues, we have investigated differences in the binding of representative isolates (15 in total) of genomovars I through V to fixed nasal sections of UNC *cfr* (-/-) knockout and *cfr* (+/+) mice, and to human lung explants, biopsy and autopsy tissue of CF and non-CF patients. Binding was highest for the isolates of genomovar III, subgroup RAPD type 2, ET strain, but only if the isolates expressed the cable pili phenotype and its associated 22 kDa adhesin (designated *cblA*+/*Adh*+). Antibodies to the 22 kDa adhesin virtually abolished binding. Significant binding occurred only to *cfr* (-/-) nasal sections or to CF lung sections, and was negligible to *cfr* (+/+) or human non-CF, histologically normal lung sections. Unlike normal epithelia, the hyperplastic epithelia of CF bronchioles were enriched in cytokeratin 13, a 55 kDa protein that we have shown previously acts as a receptor *in vitro* for cable-piliated, adhesin positive *B. cepacia*. Our findings may help to explain the high transmissibility among CF patients of Cbl-positive, genomovar III strains of *B. cepacia*.

Components of a Type II-and a Type IV-Secretory Systems are Required for Survival of *B. cepacia* Within Amoebae and Macrophages

C. Fehlner-Gardiner, T. Hopkins, C. L. Marolda, and M.A. Valvano.

Burkholderia cepacia comprises groups of genomovars (genetically diverse strains with very similar phenotypes) that have emerged as important opportunistic pulmonary pathogens in patients with cystic fibrosis (CF). We have recently shown that several strains of *B. cepacia* can escape intracellular killing by free living amoebae (Marolda *et al.*, Microbiology, 145, 1509, 1999). We have also demonstrated that *B. cepacia* can survive in phagocytic vacuoles of PU5-1.8 murine macrophages for a period of at least five days without significant bacterial replication, and in the presence of macrophage activation (Saini *et al* Microbiology, 145, 3465, 1999). Bacterial entry into macrophages stimulated production of TNF- α , and primed them to release toxic oxygen radicals. Furthermore, infected macrophages primed with IFN- γ produced less nitric oxide than IFN- γ -primed uninfected cells. We propose that the ability of *B. cepacia* to resist intracellular killing by phagocytic cells may play a role in the pathogenicity of cystic fibrosis lung infection. Using a suicide deliver system, we have generated transposon mutants unable to survive within amoebae and macrophages. The background strain is a Genomovar V clinical isolate (*B. vietnamiensis* CEP040) that represents an invasive, non-epidemic strain. The transposon element we used contains an origin of replication that is not functional in *B. cepacia*, thus allowing the self-cloning of the transposon DNA together with flanking chromosomal sequences. DNA sequence analysis revealed insertions in *gspN* (a gene component of a type II secretory system homologous to a similar system in *B. pseudomallei*) and *virB4* (an homologue of a core gene present in type IV secretory systems). The *gspN* mutant was defective in the secretion of hemolysin and phospholipase C activities, probably due to the defect in type II secretion. We are currently characterizing the presence of these two secretory systems in other clinical strains of *B. cepacia* and efforts are being placed to identify secreted substrates with the ultimate goal of determine their role in intracellular survival of *B. cepacia* within professional phagocytes.

***Burkholderia cepacia* (BC) in Lung Transplant Recipients with Cystic Fibrosis (CF)**

J.C. Routh, J.J. LiPuma, R.M. Aris, P.H. Gilligan.

BC has been associated with increased morbidity and mortality in CF patients, particularly those undergoing lung transplantation. This has led many CF centers to declare BC an absolute contraindication to transplant. The recent identification of five distinct species (or genomovars) within the BC complex raises the question of whether all genomovars are equally virulent or if in fact the poor outcomes associated with this organism can be attributed solely to certain genomovars. In addition, a BC virulence factor, the cable pilus, has been associated with enhanced transmissibility and possibly with enhanced virulence. Our goal was to determine the prevalence of the various genomovars and of *cblA*, the gene encoding the cable pilus, in post-transplant and waiting list populations at this CF center and to correlate those results with post-operative mortality. We determined the genomovar and *cblA* gene status of BC isolates from 17 of 19 post-transplant and 6 of 7 waiting list patients by polymerase chain reaction using previously published primers. Among transplant recipients, 12 of 17 were colonized by genomovar I, III, or IV; these patients had a 66% one-year mortality. The 5 transplant recipients carrying genomovars II or V had a 25% one-year mortality. 4 of 6 patients on the transplant waiting list had genomovar II or V. We found all patients at UNC to be negative for *cblA*. Although the numbers are too small to establish statistical significance, these data suggest that patients carrying the BC genomovar I/III/IV group are more likely to have a substantially higher one-year post-transplant mortality rate than patients carrying genomovars II or V.

Isolation of soilborne Genomovar III *Burkholderia cepacia* and lytic phages with inter-genomovar host range.

Carlos F. Gonzalez, G. Louise Mark, Eshwar Mahenthiralingam, and John LiPuma.

Burkholderia cepacia and *B. gladioli* are commonly found in highly organic soils. The objective of this study was to determine the range of *B. cepacia* complex species that could be isolated from organic soils planted to onions. In addition, we sought to determine if bacteriophages could be isolated from the same soils. Sixteen soil samples were taken from four different fields that have been continuously cropped to onions for many years. The samples were taken at a depth of 7.5 cm along an X transect and mixed to form a composite sample for each of the four fields. Each of these four samples was processed and plated to selective medium (TBT). Individual colonies were isolated and presumptively identified as *B. cepacia* complex or *B. gladioli* based on fatty acid methyl ester profiles. A total of fifty-nine *B. cepacia* or *B. gladioli* isolates were recovered from the samples. Based on morphology, ten representative *B. cepacia* isolates were further analyzed to determine species within the *B. cepacia* complex by using rRNA and *recA* gene-targeted PCR. Isolates were also genotyped by using PFGE and RAPD typing, and the presence or absence of the BCESM marker was determined by PCR. Four of the ten isolates were identified as belonging to genomovar III; genotyping demonstrated two strain types, one of which was BCESM positive. Two phages with *B. cepacia* genomovar III hosts were isolated from soil samples. Phage BC-1 is specific for *B. cepacia* BCC1, whereas phage BC-56 is specific for strain K56-2; both phage appear to have a narrow host range at this time. Two independent lytic phages plated on different *B. cepacia* genomovar I hosts and were also shown to plate on genomovar III isolate PC184. This study demonstrates that *B. cepacia* genomovar III are present in the natural environment and that bacteriophages with inter-genomovar host range also exist in the environment. Since phages are known to be vehicles for horizontal gene transfer, *B. cepacia* phages with inter-genomovar host ranges could potentially provide a mechanism for the transfer of virulence genes.

Identification of a quorum sensing system in *Burkholderia vietnamiensis*

Barb Conway, E.P. Greenberg

Quorum sensing is a method of gene regulation based on population density that is used by a number of bacteria that have both symbiotic or pathogenic and free-living lifestyles. Autoinducer synthesized during growth binds to a transcriptional activator to control specific genes once a critical concentration of the autoinducer is reached. In *Pseudomonas aeruginosa*, the *las* and *rhl* quorum sensing systems function as part of a cascade of regulation controlling the expression of certain virulence factors. A number of extracellular factors produced by *Burkholderia cepacia* have also been suggested to respond to the addition of exogenous autoinducer from *P. aeruginosa*. We investigated the role of quorum sensing in an environmental *Burkholderia vietnamiensis* isolate. At least four acyl-homoserine lactone (AHL) autoinducer molecules are produced by *B. vietnamiensis* (*cepacia*) G4. The most abundant was N-decanoyl-L-homoserine lactone. *B. vietnamiensis* produced AHLs with six-, eight-, and twelve-carbon acyl groups in lesser amounts. The genes responsible for AHL synthesis, *vieI* and *vieR*, have been cloned and sequenced. They encode polypeptides with sequence similarity to the LuxI family of autoinducer synthases and the LuxR family of transcriptional regulators. Disruption of *vieR*, a LuxR homolog, resulted in production of very little decanoyl-HL and less octanoyl-HL than wild-type, suggesting a role for *VieR* in regulation of autoinducer synthesis. Disruption of *vieI*, a LuxI homolog, resulted in production of a small amount of octanoyl-HL, demonstrating the role of *VieI* in AHL synthesis and suggesting the presence of a second autoinducer synthase in this organism.

Detection, recovery and identification of *Burkholderia cepacia* from the natural environment

Suzanne M. Miller, Jennifer L. Parke, and John J. LiPuma.

The *B. cepacia* complex occurs in the soil, plant rhizosphere, and in water, but the potential for acquisition of human pathogenic strains from these sources is not clear. In this on-going study, we are sampling from a variety of soil and rhizosphere environments with which people may have contact: playgrounds, athletic fields, parks, hiking trails, and residential yards and gardens. In the first phase of the study, we established optimal methods for detection and recovery of *B. cepacia* in environmental samples. Recovery of putative *B. cepacia* isolates was improved when both *Burkholderia cepacia* selective agar (BCSA) and trypan blue tetracycline medium (TBT) were used. Some putative *B. cepacia* isolates failed to grow at 37 °C but grew at room temperature (20 – 22°C). All isolates recovered from selective media were examined by a polymerase chain reaction (PCR) assay targeting *Burkholderia/Ralstonia*-specific rRNA gene sequences. Any isolate identified as *Burkholderia/Ralstonia* was further examined with a panel of biochemical tests (oxidase, ONPG, lysine decarboxylase, oxidation of sucrose and lactose) and PCR assays specific for species of the *B. cepacia* complex. PCR assays for the presence of *B. cepacia* are also performed using DNA directly extracted from soil. Optimal detection of the *B. cepacia* complex in environmental samples is achieved with the use of both culture-based and non-culture-based methods. In the second phase of the study, we will use the same protocol to recover and characterize *B. cepacia* in environmental samples from two cities (Philadelphia, PA and Cleveland, OH) with a high prevalence of *B. cepacia* colonization in cystic fibrosis patients. Our objective is to determine if there is any commonality between isolates recovered from environmental samples and isolates recovered from cystic fibrosis patients. To date, a total of 36 environmental samples from the Corvallis, OR area and 34 environmental samples from the Philadelphia area have been processed; from these a total of 122 bacterial isolates have been evaluated. *B. cepacia*-complex isolates have been recovered from a lawn in a public park, mulch from a U-Pick blueberry farm, commercial topsoil, rhizospheres of corn and pea plants grown in residential vegetable gardens, potting soil in a commercial greenhouse, and riverbank soil from a recreational swimming area.

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***Burkholderia cepacia* Complex Infection in New Zealand Cystic Fibrosis patients**

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Burkholderia cepacia complex is an infrequent isolate from New Zealand cystic fibrosis patients. In Auckland an adult and paediatric clinic provide medical care to 85 cystic fibrosis patients. Despite infrequent colonisation among cystic fibrosis patients, there is considerable anxiety about possible cross infection and infection control measures have been strengthened in recent years. The aim of this study therefore was to confirm the identification of strains of *Burkholderia* and genotype them to determine whether cross infection might be occurring within our population. Isolates of *B. cepacia* complex and *B. gladioli* obtained from 11 adults and children attending these clinics between April 1995 and June 1999 were collected and sent for further identification and analysis at the University of British Columbia. During May/June 1999 four new patients had *B. cepacia* complex identified from respiratory culture. Confirmation of identity was undertaken using standard biochemical tests i.e. the API Rapid NFT system. The strains were also analysed by RAPD, BCESM and by genomovar specific probes as developed and described by the Vancouver Group, and *B. gladioli* specific probe as described by Whitby et.al.. Patient records were reviewed to provide information on possible clinical correlates. 22 isolates from 15 cystic fibrosis patients were analysed. 14 patients were infected with organisms unique to each person. Non identical twins shared the one organism. Six subjects, from whom several samples were obtained, remained infected with their own isolate for periods varying from 1 month to 3 years. Nosocomial acquisition of *B. gladioli* amongst cystic fibrosis patients at Green Lane Hospital was previous reported (Wilsher et al 1997). This organism was re-identified at reference laboratories in England and Belgium as *B. cepacia* identical to the "UK" epidemic strain. This strain is also carried by a patient attending the Starship clinic but to date there has been no cross-infection with other patients. We believe that our infection control measures are preventing cross-infection.

Distribution and characterization of *Burkholderia cepacia* complex species recovered from cystic fibrosis sputum.

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The *B. cepacia* complex is comprised of at least seven distinct yet closely related species. Preliminary data indicate that all species have been recovered from cystic fibrosis (CF) sputum. However, a systematic survey of epidemiologically-defined CF isolates has not yet been performed. To determine the relative frequencies with which *B. cepacia* complex species colonize CF patients we analyzed isolates recovered from 642 persons receiving care in 111 cities in the US. Only one isolate (the first received by this laboratory) from each person was included in this analysis. All isolates were confirmed as *B. cepacia* complex by growth on selective media, biochemical reactivity, and rRNA gene-targeted PCR assay. Genomovar assignment was made on the basis of 16S rRNA and *recA*-targeted PCR and *recA* RFLP analysis. Among the 642 persons, approximately 50% were colonized with *B. cepacia* genomovar III, 35% with *B. multivorans*, and 5% with *B. vietnamiensis*. The remaining 10% were colonized with either genomovar I, *B. stabilis*, genomovar VI, or *recA* 'group C' (potentially a novel species in the *B. cepacia* complex). The *B. cepacia* epidemic strain marker (BCESM) was detected by PCR in 22% of isolates; all BCESM-positive isolates were genomovar III (in total, 43% of genomovar III isolates were BCESM-positive). All isolates were also analyzed for presence of cable pili gene (*cbIA*) sequences by dot blot hybridization and PCR. *cbIA* sequences were detected in the ET12 clone only. These data demonstrate that although genomovar III and *B. multivorans* together account for the majority (85%) of colonized persons, all defined *B. cepacia* complex species are capable of colonizing persons with CF. The presence of either BCESM or *cbIA* is not sufficient to define strains capable of colonizing the CF respiratory tract. The stability of colonization and the clinical outcomes associated with colonization by the various species of the *B. cepacia* complex are currently being assessed.

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Correlation Between In-vivo and In-vitro Models of Invasion by *Burkholderia cepacia*

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Burkholderia cepacia, well described as a respiratory pathogen in cystic fibrosis (CF), has been classified into six genomovars (G), the epidemiology of 5 of which is well-characterized. CF clinical isolates have been found to represent all genomovars, but are predominantly GII and III, with GIII strains primarily associated with increased morbidity and mortality. We hypothesize that the distinct genomovars will differentially invade respiratory epithelium. **METHODS:** GI through GV strains were selected from the *B. cepacia* complex experimental panel and used to infect 1) A549 respiratory epithelial cells in a modified antibiotic protection assay, 2) C57BL/6 mice in an agar bead model of lung infection with culture of lung and spleen at 24 hours after infection. **RESULTS:** A549 invasion in-vitro did not vary significantly by genomovar. Strains from all 5 genomovars tested demonstrated invasion into respiratory epithelial cells. However, in-vivo, the GII, GIII, and GV strains could be recovered from both lung and spleen, the GIV strain could be isolated from lungs, but not spleen, while the GI strain was not recovered from either organ. **CONCLUSION:** GI and GIV strains were invasive in the A549 in-vitro assay, but were unable to cause splenic infection in C57BL/6 mice. The in-vivo assays are consistent with clinical observations of virulence based on genomovar class, but the A549 tissue culture model does not appear to correlate with either the in-vivo or clinical data.
