

International  
*Burkholderia cepacia*  
Working Group

# International *Burkholderia cepacia* Working Group

Annual Meeting

April 19-22, 2007

 UNIVERSITY OF MICHIGAN

University of Michigan, Ann Arbor, MI, USA

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# Agenda

## International *Burkholderia cepacia* Working Group

### 11<sup>th</sup> Annual Meeting

University of Michigan, Ann Arbor, MI, USA

April 19-22, 2007

#### Thursday, April 19

**17:30-19:30** Registration and Opening Reception  
*Biomedical Science Research Building*

#### Friday, April 20

**9:00-12:30** **Session I** (moderator: Mary Corey)  
*Michigan League*

9:00-9:15 Welcome John LiPuma  
Joanna Goldberg

9:15-10:00 "Natural history and clinical outcomes of  
*B. cepacia* complex infection in CF" Liz Tullis

10:00-10:30 Abstracts A1-A2

10:30-10:45 Break

10:45-12:30 Abstracts A3-A8

**12:30-13:30** **Lunch**

**13:30-17:00** **Session II** (moderator: Joanna Goldberg)  
*Michigan League*

13:30-14:15 "Antibiotic resistance in *Burkholderia*  
*cepacia* complex: is there a magic bullet?" Jane L Burns

14:15-14:45 Abstracts A10-A11

14:45-15:00 Break

15:00-16:30 Abstracts A12-A17

16:30-17:00 Discussion- proposals for venue for IBCWG 2008

## **Saturday, April 21**

**8:30–13:00**      **Session III** (moderator: Carlos Gonzalez)  
*Michigan League*

8:30-9:15      “Ecology of *Burkholderia* species”      Jim Tiedje

9:15-9:45      Abstracts A20-A21

9:45-10:00      Break

10:00-11:30      Abstracts A22-A26

11:30-11:45      Break

11:45-13:00      Abstracts A27-A30

**13:00-14:00**      **Lunch**

**Afternoon**      Discussion / Small Group Meetings  
GeneSpring Demo / Training Session  
Presentation of abstracts A18-A19

## **Sunday, April 22**

**9:00–12:00**      **Session IV** (moderator: Esh Mahenthiralingam)  
*Michigan League*

9:00-9:45      “*Burkholderia cenocepacia* virulence:  
The unbearable lightness of surviving”      Miguel Valvano

9:45-10:30      Abstracts A31-A33

10:30-10:45      Break

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## Abstracts

### International *Burkholderia cepacia* Working Group 11<sup>th</sup> Annual Meeting

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Adam Baldwin, Esh Mahenthiralingam and Chris Dowson
- A6 THE UTILITY OF BOX-PCR TYPING OF *BURKHOLDERIA*: COMPARISON TO MLST**  
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- A7 LUNG TRANSPLANT OUTCOME FOR PATIENTS INFECTED WITH *B. CENOCEPACIA* – TORONTO EXPERIENCE**  
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Bridget Foster, Theodore Spilker, John J. LiPuma

\* presenting author if different from submitting author (underlined)

## **A1. THE CHANGING EPIDEMIOLOGY OF *BURKHOLDERIA* INFECTION IN CYSTIC FIBROSIS**

Jordan Keoleian, Theodore Spilker, Amy Liwienski, Bridget Foster, John J. LiPuma

Department of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, USA

Characterization of the epidemiology and natural history of *Burkholderia* infection in cystic fibrosis (CF) is important in patient management, particularly with respect to infection control, and provides a foundation for the investigation of *Burkholderia* virulence. Several studies have demonstrated that *B. multivorans* and *B. cenocepacia* account for the majority of *B. cepacia* complex (Bcc) infection in CF. Bacterial genotyping studies also have identified several so-called 'epidemic' strains of *B. cenocepacia* that infect multiple CF patients. In the present study, we sought to investigate changes in the incidence of infection with *B. multivorans* and *B. cenocepacia* (including epidemic strains) in US CF patients during a nine year period. We performed species identification and genotyping analyses of *Burkholderia* (Bcc and *B. gladioli*) isolates recovered from 1462 CF patients infected during this interval. In 1997, the proportions of *Burkholderia*-infected CF patients infected with *B. cenocepacia* or *B. multivorans* were 51% and 29%, respectively. Since then, the proportion of new *Burkholderia* infection due to *B. cenocepacia* has decreased, while that due to *B. multivorans* has increased. In 2005, only 15% of new *Burkholderia* infection was due to *B. cenocepacia*, while *B. multivorans* accounted for 43% of new *Burkholderia* infection in this patient population. We defined an epidemic strain as one that was identified as infecting  $\geq 10$  CF patients. Our analysis indicates that the proportion of new *B. cenocepacia* infection due to epidemic strains has decreased significantly during the last several years. In 1997, 61% of new *B. cenocepacia* infection involved an epidemic strain, while in 2005, only 15% of patients newly infected with *B. cenocepacia* were

infected with an epidemic strain. The proportion of *Burkholderia* infection due to *B. gladioli* has increased in this interval; in 2005, *B. gladioli* accounted for 27% of new *Burkholderia* infection. In summary, we have observed that during the past nine years there has been a steady decrease in the proportion of new *Burkholderia* infection due to *B. cenocepacia* (particularly epidemic strains), while the proportion of infection due to genotypically unique *B. cenocepacia* and *B. multivorans* has increased. The reasons for these trends are not clear, but may result from improved infection control measures that limit inter-patient transmission of epidemic *B. cenocepacia*. The greater proportion of *Burkholderia* infection due to genotypically distinct *B. cenocepacia* and *B. multivorans* suggests relatively more acquisition from independent sources in the environment.

## **A2. AN 11-YEAR EPIDEMIOLOGICAL AND ANTIMICROBIAL RESISTANCE SURVEY OF *BURKHOLDERIA CEPACIA* COMPLEX ISOLATED IN THE MAJOR PORTUGUESE CYSTIC FIBROSIS CENTER**

Mónica V. Cunha<sup>1</sup>, Ana Pinto-de-Oliveira<sup>1</sup>, Jorge H. Leitão<sup>1</sup>, Sílvia A. Sousa<sup>1</sup>, Luís Meirinhos-Soares<sup>2</sup>, Celeste Barreto<sup>3</sup>, Susana Correia<sup>3</sup>, J. Melo-Cristino<sup>4</sup>, M. J. Salgado<sup>4</sup> and Isabel Sá-Correia<sup>1</sup>

<sup>1</sup>IBB- Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering (CEBQ), Instituto Superior Técnico (IST), Av. Rovisco Pais, 1049-001 Lisboa, Portugal. <sup>2</sup>Direcção de Comprovação da Qualidade do INFARMED, Parque de Saúde de Lisboa, Avenida do Brasil, nº 53, 1749-004 Lisboa, Portugal. <sup>3</sup>Centro Especializado em Fibrose Quística and <sup>4</sup>Laboratório de Bacteriologia, Hospital de Santa Maria, Av. Prof. Egas Moniz, Lisboa

Epidemiological surveys, carried out in different countries, support the idea of a disproportionate representation of the different *Burkholderia cepacia* complex (Bcc) species in cystic fibrosis (CF) respiratory infections, *B. cenocepacia* and *B. multivorans* being the most common. However, the two epidemiological surveys carried out by the IBB-CEBQ/IST laboratory in the major

Portuguese CF Centre, from January 1995 to March 2006, revealed that the distribution of Bcc species among CF patients attending the Hospital de Santa Maria (HSM), in Lisbon, exhibit an exceptionally high incidence of *B. cepacia*. Specifically, 56% of the CF patients harbouring Bcc were infected with *B. cepacia*, 44% with *B. cenocepacia*, 12.5% with *B. stabilis* and 6.25% with *B. multivorans* (Cunha *et al.*, 2003, Cunha *et al.*, 2007). Remarkably, during 2003-2005, the representation of *B. cepacia* was exceptionally high, this species being isolated from 85% of the patients. A detailed molecular analysis revealed that this was mainly due to two *B. cepacia* clones. These clones were indistinguishable from two strains isolated from intrinsically contaminated non-sterile saline solutions for nasal application, detected during routine market surveillance by Infarmed, the Portuguese Medicines and Health Products Authority. The susceptibility patterns to 13 clinically relevant antimicrobials of 94 sequential isolates belonging to the four Bcc species isolated during the period 1997-2002 from 15 CF patients, in the course of chronic infection, were also compared. Although the number of different strains tested is limited, results of the analysis of such a collection of well characterized Bcc isolates provided an useful indication on the influence of the taxonomic status on the in vitro antimicrobial susceptibility of the Bcc. Results are consistent with the notion that clinical Bcc isolates are resistant to the most clinically relevant antimicrobials and suggest an uneven distribution of resistance rates to the antimicrobials tested among the different Bcc species. The *B. cenocepacia* subgroup A isolates tested were the most resistant while *B. stabilis* and *B. multivorans* isolates were the most susceptible. Evidence was also obtained for mutation to increased resistance to, at least, ceftazidime, imipenem and piperacillin/tazobactam, based on the recovery of phenotypic variants within clonal serial isolates, from a same patient, that exhibit increased resistance to these antimicrobials. Remarkably, the variation of the antimicrobial resistance phenotype was associated with long-term chronic lung infection, more severely compromised lung function, and periods of antibiotic therapy.

Cunha *et al.* (2003) *J. Clin. Microbiol.* 41:4113-4120. Cunha *et al.* (2007) DOI: 10.1128/*J. Clin. Microbiol.*00234-07.

### A3. *BURKHOLDERIA* INFECTION IN CHRONIC GRANULOMATOUS DISEASE: EPIDEMIOLOGICAL INSIGHTS

David E. Greenberg<sup>1</sup>, Frida Stock<sup>2</sup>, Steven M. Holland<sup>1</sup> and John J. LiPuma<sup>3</sup>

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Chronic Granulomatous Disease (CGD) is due to a genetic defect in phagocyte superoxide formation in which patients develop recurrent life-threatening infections with *Staphylococcus aureus*, *Serratia marcescens*, *Burkholderia cepacia* complex (Bcc), *Nocardia* species and *Aspergillus* species. While the epidemiology of *Burkholderia* infection in patients with cystic fibrosis (CF) has been extensively investigated, this has not been well studied in CGD. We investigated *Burkholderia* isolates recovered from CGD patients referred to the NIH Clinical Center during the past 10 years. Isolate speciation was performed with a combination of 16S rDNA and *recA* directed PCRs, *recA* RFLP analysis, and *recA* sequence analysis. Isolate genotyping was done by using rep-PCR. Forty-five isolates, obtained from 18 CGD patients (as well as from environmental sampling from one patient's house), were analyzed in a blinded fashion. The majority (73%) of isolates were obtained between the months of July and October. Among the 45 isolates, we identified 30 distinct *Burkholderia* clones. The distribution of these 30 clones in each of the Bcc species represented is: *B. ambifaria* 1 (2.2%); *B. cenocepacia* 4 (8.9%); *B. cepacia* 3 (6.7%); *B. multivorans* 15 (35.6%); *B. gladioli* 2 (4.4%); *B. vietnamiensis* 1 (2.2%) and indeterminate 4 (8.9%). Among the 10 patients from whom more than a single isolate was available for analysis, six demonstrated the presence of >1 clone among serially

recovered isolates. Two patients maintained the same clone for a period of at least one year. Genotyping correctly predicted that two patients infected with the same *B. cenocepacia* strain were siblings. Environmental sampling of their house yielded *Burkholderia* of an indeterminate species, highlighting the difficulty in identifying the environmental source of clinical isolates. This is the largest series of Bcc isolates analyzed from patients with CGD. While the numbers are small (relative to CF isolates), the data indicate that CGD patients develop infections with a similar spectrum of species as seen in CF, although there may be a trend to more *B. multivorans* and indeterminate species.

#### **A4. COMPARISON OF SENSITIVITY AND SPECIFICITY OF A NESTED PCR ASSAY FOR *B. CEPACIA* COMPLEX WITH CULTURE**

Sarka Vosahlikova<sup>1</sup>, Helena Reitzova<sup>1</sup>, Ondrej Cinek<sup>1</sup>, Marek Omelka<sup>2</sup>, Pavel Drevinek<sup>3</sup>

<sup>1</sup> 2nd Medical School of Charles University, Prague, The Czech Republic <sup>2</sup> Faculty of Mathematics and Physics, Charles University, Prague, The Czech Republic <sup>3</sup> Cardiff School of Biosciences, Cardiff University, Cardiff, Wales, UK

To increase the sensitivity and specificity of diagnostics of *B. cepacia* complex (BCC) infection, we developed a nested PCR assay detecting the pathogen directly from sputum. The aim of our current study was to evaluate the actual benefit of the PCR assay as a diagnostic tool. Over the period of seven years, we analyzed 3098 sputum samples from 412 patients attending the Prague CF centre. Samples were analyzed simultaneously by culture and by nested PCR targeting the *recA* gene. We attempted to approximate the sensitivity calculations by longitudinal analysis of a patient's sputa provided in regular intervals. Using generalized estimating equations; we modeled the likelihood for future culture positivity as a proxy measure of BCC colonization. Two

most significant predictors were: past culture positivity (approximately 30-fold increase in the odds of being positive in subsequent cultures), and the PCR positivity within the past year (approximately 6-fold increase in the odds for subsequent culture positivity). While the first predictor is self-explanatory as BCC colonization is persistent, an independent association of the PCR result may indicate its potential usefulness in the clinical practice. Microbiological results obtained from both methods matched each other in 95.6% of samples by being negative in 2550 and being positive in 413 cases. 117 samples were negative by culture but positive by PCR, while 18 samples were positive by culture and negative by PCR. In six patients, one or more positive PCR results preceded a subsequent finding of BCC by culture. The range of subsequent culture positivity was 2-22 months. In remaining cases, the PCR findings may reflect either an increased in-vitro sensitivity of the PCR assay, or false positivity. The 18 culture-positive, PCR negative samples obtained from 13 patients represented 4.2% of all 431 culture-positive samples. The positivity was neither preceded, nor followed by other positive cultures in 14 of the 18 samples, we thus may assume false culture positivity due to misidentification of another bacterium. However, in three patients further tests confirmed BCC infection, indicating that the PCR test may be false negative in a small proportion of cases. Our dataset of longitudinally obtained samples demonstrates a usefulness of the PCR test, which can detect BCC infection earlier than standard culture.

#### **A5. INVESTIGATING *BURKHOLDERIA CEPACIA* COMPLEX INDUSTRIAL CONTAMINATION BY MULTILOCUS SEQUENCE TYPING**

Adam Baldwin<sup>1</sup>, Esh Mahenthiralingam<sup>2</sup> and Chris Dowson<sup>1</sup>

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*Burkholderia cepacia* complex (Bcc) are a group of opportunistic pathogens that reside in the environment and are capable of infecting vulnerable individuals. Infection control measures aimed at limiting contact between infected persons have failed to prevent new Bcc infections completely. Analysis of diverse Bcc isolates by multilocus sequence typing (MLST) has identified several globally distributed *Burkholderia cepacia* complex (Bcc) strains, responsible for causing human infections that were indistinguishable from strains isolated from the environment. However, even though the majority of CF infections are caused by strains of *B. cenocepacia* IIIA and *B. multivorans*, recovery of these specific groups from natural environments has been limited. In this study we investigated a largely unexplored niche, industrial process contamination, by MLST to assess its potential health risk. Bcc industrial contamination is one of the major and most problematic worldwide for cosmetics, personal care, hygiene, food, plastics, paints, pulp, petroleum and pharmaceutical industries. Even with Good Manufacturing Practice, microbial fouling still occurs and is an expensive and potentially dangerous problem. A collection of 50 suspected Bcc isolates (recovered as contaminants from a variety of personal hygiene products and processing lines, and manufacturing sites) were analysed by MLST and compared to a large database (>1,000 isolates) of environmental and clinically sourced BCC isolates. The majority of contamination-associated isolates were from novel groups such as the little studied *B. cepacia* complex Group K, suggesting this group may possess greater fitness for contamination-associated environments. Identification of *B. cenocepacia* contaminants which were closely related to two major epidemic CF strains (the ET-12 and PHDC lineages) also suggested that certain contamination-associated strains possess the genetic capacity to cause virulent human infection. In total, our preliminary work found that 38% of the industrial isolates examined were associated with strains isolated from human infection. Although, there was no evidence to suggest a causal link

between the contaminated industrial products and the clinical infections, the presence of clonal strains in both niches, with geographic concordance, suggested regional product contamination could pose a potential infection risk. In summary, MLST proved to be a robust method for examining the contamination-associated isolates, particularly as the majority of them fell outside the currently defined Bcc species and hence could not have been characterised using conventional methods. It also showed industrial contamination globally to be an under-investigated reservoir for the potential emergence of Bcc in human infection.

#### **A6. THE UTILITY OF BOX-PCR TYPING OF *BURKHOLDERIA*: COMPARISON TO MLST**

Theodore Spilker<sup>1</sup>, Adam Baldwin<sup>2</sup>, Eshwar Mahenthiralingam<sup>3</sup>, Christopher Dowson<sup>2</sup>, John J. LiPuma<sup>1</sup>

<sup>1</sup> Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, MI, USA <sup>2</sup> Department of Biological Sciences, University of Warwick, Coventry, UK. <sup>3</sup> Cardiff School of Biosciences, Cardiff University, Cardiff, Wales.

Previous studies have demonstrated the utility of repetitive extragenic palindromic PCR genotyping using the BOX A1R primer (BOX-PCR) for investigating the epidemiology of *B. cepacia* complex (Bcc). However, the relatively poor inter-assay reproducibility of this method was an impediment to its use in large scale epidemiologic studies and in the construction of durable genotyping databases. In the present study, we assessed the reproducibility of a modified BOX-PCR method and compared this method to genotyping results obtained using multilocus sequence typing (MLST). Ten Bcc isolates, representing nine distinct strains from five Bcc species, were analyzed by BOX-PCR in duplicate; each duplicate assay was repeated three times. UPGMA cluster analyses of the resulting DNA profiles showed that the average similarity coefficient between replicate assays (n=6) of the same strain was 94.8% (+/-

1.4%). In an analysis of 230 *B. cenocepacia* isolates we found an excellent correlation between dendrograms based on BOX-PCR profile and concatenated MLST sequence data. A BOX-PCR similarity coefficient of 85% (which, based on previous analyses, is the similarity coefficient cutoff we use to define a strain) correlated to a change of approximately 1.3 nt per 100 nt in the concatenated MLST sequence. We conclude that the modified BOX-PCR method provides an inexpensive, rapid and reliable means to assess Bcc for clonal identity. This method is suitable for large scale epidemiologic studies and could prove most useful in screening large collections of isolates to select strains for more comprehensive analysis by MLST.

#### **A7. LUNG TRANSPLANT OUTCOME FOR PATIENTS INFECTED WITH *B.CENOCEPACIA* – TORONTO EXPERIENCE**

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**Background.** Lung transplantation is the only option to prolong survival for cystic fibrosis patients with end-stage lung disease. Unfortunately, for those infected with *B. cenocepacia* the risk of death is high. The Toronto Lung Transplant Program has continued transplanting these patients despite their higher morbidity and mortality risks. We have gone through different strategies over time trying to improve the outcome for these patients. We reviewed outcome with those strategies. **Study Design.** Retrospective study to evaluate different outcomes with the different strategies in all our cystic fibrosis patients infected with *B. cenocepacia*. We recorded transplant date, type of *B. cenocepacia*, post-transplant complications related to *B. cepacia* and survival. **Results.** Between March 1988 and December 2006 we have transplanted 185 patients, 67 of them infected with *B. cenocepacia*. Between March 1988 and August 1995 patients with CF were

all treated with a combination of cephalosporines and amynoglucosides for 2 weeks post-transplant. After 1995 they were treated for up to 4 weeks with a protocol that included a cephalosporin, amynoglucosides, sepra and chloramphenicol. With the introduction of multiple combination bactericidal tests in 1999 we changed the antibiotic protocol to a patient tailored one based on MCBT. More recently a lavage to airway and chest cavity with bethadine before implantation was introduced for BC positive patients. Log-rank analysis of Kaplan Meier survival estimates show significant difference for curves of BC(+) vs BC(-) ( $P < 0.001$ ), but no difference by treatment strategy within BC(+) ( $p = 0.6$ ).

#### **A8. IMPACT OF *BURKHOLDERIA* INFECTION ON LUNG TRANSPLANT ALLOCATION SCORE AMONG U.S. CYSTIC FIBROSIS PATIENTS**

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CF patients awaiting lung transplantation in the US are assigned a Lung Allocation Score (LAS) that combines urgency (i.e., expected candidate lifetime during the next year without transplant) and post-transplant survival (i.e., expected lifetime of the same patient in the first year after transplant) to determine transplantation priority. Diagnosis, age, BMI, diabetes, NYHA class, FVC, PA systolic, resting O<sub>2</sub>, six minute walking distance, and need for continuous ventilation are used to calculate waitlist survival, while FVC, age, creatinine, PCW pressure, ventilator status, and NYHA class are used to calculate post-transplant survival. Currently, infection with *Burkholderia* is not included in the LAS calculation. We assessed the impact of infection with *B. cepacia* complex (Bcc) and *B. gladioli* on CF waitlist and post-transplant survival and its potential impact on

LAS scores. Clinical and demographic data from the CF Foundation Patient Registry, the Scientific Registry of Transplant Recipients, and the Social Security Death Master File were cross-validated and merged with microbiologic data from the *B. cepacia* Research Laboratory and Repository. Cox multivariate survival models were used to analyze 1371 CF patients listed for transplantation from 1997-2006 and, separately, 719 CF patients transplanted from 1997-2006. *Burkholderia* infection was confirmed in 172 (13%) of waitlisted and 88 (12%) of transplanted patients. A waitlist survival model adjusting for LAS factors and duration of *Burkholderia* infection at listing showed that infection with any *Burkholderia* species had increased waitlist mortality compared to uninfected CF patients; however, none of these associations attained statistical significance. A post-transplant model adjusting for LAS factors and time infected before transplant revealed that infection by *B. gladioli* had significantly increased post-transplant mortality compared to uninfected patients. When patients infected with epidemic *B. cenocepacia* strains (PHDC and Midwest clone) were analyzed separately from the remaining *B. cenocepacia* strains, significantly increased post-transplant mortality was found only in non-epidemic *B. cenocepacia*-infected patients. Transplanted *B. multivorans* patients had similar outcomes to uninfected patients, with significantly better survival than patients infected with *B. gladioli* and non-epidemic *B. cenocepacia* strains. Lung allocation scores for 633 patients that include hazards for *Burkholderia* infection showed a tendency for *B. multivorans* and Midwest clone *B. cenocepacia* patients to rank somewhat higher and *B. gladioli* and non-epidemic *B. cenocepacia* patients to rank somewhat lower than uninfected CF patients. We conclude that transplant benefit varies by type of *Burkholderia* infection. *Burkholderia* infection should be added to the current LAS algorithm and should assist in clinical decisions on listing and transplanting CF patients.

## **A9. IS SUSCEPTIBILITY TO BCC INFECTION INFLUENCED BY NON-CFTR MODIFIER GENES?**

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The Canadian Cystic Fibrosis Modifier Gene Project aims to find genetic variants outside of the CFTR causal gene that explain the extreme variability in CF disease phenotype. Lung disease is the main cause of early mortality in CF patients, but the course of lung disease and long term survival cannot be predicted by CFTR genotype. We are developing complex lung phenotype profiles, based on longitudinal lung function and infection with typical CF bacteria. Growth, diabetes, intestinal and liver disease phenotypes are also being investigated. Over 2500 patients from across Canada have been enrolled, along with the parents of roughly half of the patients. Candidate genes have been selected for analysis that are known or hypothesized to be involved with infection susceptibility and inflammatory pathways. And a genome wide linkage scan is being conducted in a subset of 200 families with multiple CF siblings, in order to find novel genetic loci that may be involved in the CF disease process. Regional analysis of Canadian data has shown that infection with *Burkholderia cepacia* complex is extremely variable. Modifier genes may explain some of this variation, as well as the variable susceptibility to infection within regions of high Bcc prevalence.

## **A10. DIFFERENTIAL SUSCEPTIBILITY OF THE BURKHOLDERIA CEPACIA COMPLEX TO SYNTHETIC AND NATURAL CATIONIC ANTIMICROBIAL PEPTIDES**

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The *Burkholderia cepacia* complex (Bcc) is a group of closely related Gram-negative bacteria that are opportunistic pathogens in individuals with cystic fibrosis or chronic granulomatous disease. Bcc infections may be life-threatening in part due to their high level intrinsic resistance to various cationic antimicrobial agents, including aminoglycosides and polymyxin. In the last couple of decades, naturally occurring cationic peptides with broad spectrum antimicrobial properties have been discovered from many different organisms, from bacteria to humans. Our goal was to investigate if any of these cationic antimicrobial agents had inhibitory growth effects against species within the Bcc. Antimicrobial resistance was assessed by measuring the minimal inhibitory concentration (MIC) of each of the cationic antimicrobial agents to isolates from the Bcc experimental strain panel (Mahenthiralingam *et al.* 2000). All *B. cepacia* and *B. cenocepacia* isolates were highly resistant to the antimicrobials tested, while a *B. multivorans* laboratory isolate was highly susceptible to CP26, CP29, two synthetic

and natural cationic antimicrobial peptides, with the exception of one *B. multivorans* isolate which will be investigated further. Our data may aid in identification of the specific bacterial factors responsible for cationic antimicrobial resistance in the Bcc and facilitate the design of novel therapeutic agents. Future experiments will further investigate cationic antimicrobial resistance in the Bcc to provide insights into bacterial factors responsible for Bcc resistance.

#### A11. ANTIMICROBIAL RESISTANCE IN THE CF PATHOGENS *PSEUDOMONAS AERUGINOSA* AND *BURKHOLDERIA CEPACIA*: MECHANISMS OF BIOCIDES RESISTANCE

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*Pseudomonas aeruginosa* and the *Burkholderia cepacia* complex represent problematic cystic fibrosis pathogens

**Table 1. Antimicrobial Minimum Inhibitory Concentrations**

Species* (n)	µg ml <sup>-1</sup>									units
	Bac2a	K24	E2	E6	CP26	CP29	Ind <sup>a</sup>	LL37	PPM <sup>a</sup>	PMB <sup>a</sup>
<i>B. cepacia</i> (3)	>128	>128	>128	>128	>128	>128	>128	>128	128	>600
<i>B. multivorans</i> (7)	>128	>128	>128	>128	>128	>128 <sup>b</sup>	>128	>128	≥128	>600
<i>B. cenocepacia</i> (10)	>128	>128	>128	>128	>128	≥128	>128	>128	≥128	>600 <sup>c</sup>
<b>Strain</b>										
<i>B. multivorans</i> 249-2	>128	>128	128	128	8	4	32	32	8	150

\**B. stabilis*, *B. vietnamiensis*, and an additional isolate of *B. cepacia* to be tested

<sup>a</sup>Ind = indolicidin; PPM = polyphemusin; PMB = polymyxin B

<sup>b</sup>except *B. multivorans* CF-A1-1 (MIC of 64 µg/ml)

<sup>c</sup>except *B. cenocepacia* J2315 (MIC of 300 units)

analogues of an insect cecropin, and polyphemusin, a naturally occurring horseshoe crab peptide (Table 1). In conclusion, the members of the Bcc tested thus far are extremely resistant to synthetic

with innate multidrug resistance. The aim of this study was to investigate antimicrobial resistance in these CF pathogens, particularly focusing on mechanisms of biocide resistance. Biocides such as chlorhexidine and cetylpyridinium chloride are widely used as first line antiseptics in cleaning and disinfection of the hospital and home environment. Biocide resistance profiles were determined for panels of representative *P.*

*aeruginosa* and *B. cepacia* complex strains (45 and 100 strains respectively). Strains were chosen based on: (i) their Multilocus Sequence (MLST) type being representative of the genetic diversity within each species and (ii) their clinical background and association with transmissibility. Minimum inhibitory concentrations (MICs) for two biocides, chlorhexidine and cetylpyridinium chloride (CPC) were determined using broth dilution assays. Within the *B. cepacia* complex the biocide resistance patterns varied, however, *B. cenocepacia* strains were significantly more resistant to chlorhexidine than other species (mean MIC = 54.5 µg/ml). However a *B. multivorans* strain, LMG 16660 had higher chlorhexidine and CPC MICs than other *B. multivorans* strains (90 and >200 µg/ml respectively). This strain was involved in an outbreak in a hospital in Glasgow. Further investigation of 19 Glasgow strains involved in this outbreak is taking place. *P. aeruginosa* strains had significantly lower MIC values in chlorhexidine than *B. cenocepacia* strains (mean MIC = 18.64 µg/ml). Antibiotic susceptibility profiles were also determined. Many of the strains tested were resistant to multiple antibiotics but there was no correlation between those strains with high resistance to the two biocides tested and antibiotic resistance. A microarray experiment was carried out to determine global gene expression in *B. cenocepacia* J2315 in response to exposure to 5 µg/ml of chlorhexidine. Significant up-regulation of gene expression in 11 genes was seen in (i) multidrug efflux pump genes; (ii) response regulator proteins; (iii) secretion proteins. The majority of down-regulated genes were involved with flagellar production and chemotaxis. This research was funded by grants from the Big Lottery Fund and the UK Cystic Fibrosis Trust.

## **A12. RESISTANCE OF *BURKHOLDERIA CEPACIA* COMPLEX BIOFILMS TO DISINFECTANTS**

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*Burkholderia cepacia* complex (Bcc) bacteria are opportunistic pathogens that can cause severe infections in cystic fibrosis (CF) patients. Moreover, most Bcc bacteria are intrinsically resistant to many antimicrobial agents, which complicate the effective treatment of infected CF patients even more. Regular cleaning and disinfection of medical devices, in order to prevent the acquisition of these micro-organisms, is often stressed in infection control guidelines (ICG) for CF patients. Unfortunately, problems regarding the efficacy of several disinfectants for removing and/or killing of these micro-organisms have been reported. Biofilms often have an increased resistance towards antimicrobial agents, compared to their free-living planktonic counterparts. As such, the biofilm-forming capability of Bcc bacteria may contribute significantly to this problem. The goal of our study was to determine the effect of several commonly used disinfectants on Bcc biofilms with reference to the effect on planktonic cells. *B. cenocepacia* LMG 18828 was used for all experiments and disinfection procedures were based on the recommendations given in several ICG. The tested disinfectants are NaOCl, chlorhexidine, cetrimide, HAC (a commercial disinfectant that contains chlorhexidine and cetrimide), hydrogen peroxide, acetic acid, ethanol and chloroxylenol. The effects of warm water (70°C) and microwaves were also examined. To allow high-throughput evaluation of multiple treatments, biofilms were grown in 96-well microtiter plates. The effects of the treatments on biofilm biomass were evaluated using a crystal violet staining, while reductions in the number of viable sessile cells were determined using a resazurin-based viability staining. Finally we also considered possible regrowth of treated biofilms by performing a viability staining after an additional incubation of 24h. For comparison we also evaluated the effect of these treatments on planktonic cells using the European Suspension Test (EST). Our results show a good efficiency on planktonic cells as reductions of at least 99.999% were obtained for all treatments. However none of the treatments resulted in a complete removal of biofilm biomass and some treatments did not

result in a complete eradication of all viable sessile cells. Regrowth of biofilms was observed when using NaOCl (0.05% - 5 min), chlorhexidine (0.015%/0.05% - 15 min) and H<sub>2</sub>O<sub>2</sub> (0.5%/1% - 30 min). Although underlying reasons for the increased resistance are at present unclear, the presence of strong catalase activity may partially explain the resistance to H<sub>2</sub>O<sub>2</sub>. In this study we used a combination of three tests to assess the efficacy of multiple disinfection treatments on *B. cenocepacia* LMG18828 biofilms. Although most treatments showed good results when applied to planktonic cells, several appeared to be insufficient for complete biofilm removal. Hence, we recommend an evaluation of disinfection procedures on both planktonic as well as on sessile cells.

### **A13. EXPLORING ESSENTIAL GENES OF *BURKHOLDERIA CENOCEPACIA* FOR NOVEL ANTIMICROBIAL TARGETS**

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*Burkholderia cepacia* complex bacteria are multi-drug resistant due to innate and acquired mechanisms of resistance. As treatment options for *B. cepacia* complex remain limited the need for novel antibiotics is beyond doubt. Given the short period of time in which antibiotic resistance arises against new drugs, this effort must be continuous and developed at a fast pace.

Bacterial genomes contain many essential genes that remain unexplored as targets for antimicrobial drug development. Despite the success in identifying putative essential genes this route has not yet delivered new antibiotics to the marketplace. This may be due in part to the lack of functional understanding for many of these new targets, hence the impossibility of activity-based inhibitor screens. In addition, the characterization of novel essential genes is complicated by the unfeasibility of obtaining

knockout strains, simply because they are non viable. The construction of conditional-growth mutants in essential genes offers an alternative approach. Essential genes in these mutants can be regulated to express their cognate protein products at lower levels. Thus, these mutants are hypersensitive to molecules that inhibit these proteins. Previously, a transposon-delivery system of the rhamnose promoter for the generation of conditional-growth essential gene mutants in *B. cenocepacia* K56-2 was developed. We propose to generate a genomic library of conditional-growth mutants in *B. cenocepacia* by large-scale transposon delivery of the rhamnose system. We hypothesize that by examining the dose-response curve of conditional-growth mutants to a wide panel of small molecules under inducing and sub-inducing conditions, it is possible to identify target-inhibitor pairs of target-inhibitors as a starting point in antibacterial drug development. A small collection of conditional-growth mutants was used to prioritize potential antimicrobial targets. A rhamnose-regulated *mraY* strain was chosen to develop an antibiotic hypersensitivity assay. *MraY* is an essential murein biosynthesis enzyme, which is inhibited by tunicamycin. This strain was cultured with various antibiotics at a range of concentrations to generate dose response curves, either in inducing or sub-inducing concentrations of rhamnose. As expected, hypersensitivity to tunicamycin was observed, as sub-inducing conditions shifted the dose response curve of the *mraY*-specific antibiotic tunicamycin into lower effective concentrations. However, no such effect was seen for unrelated antibiotics. These results suggest that the conditional-growth mutants constitute a valuable detection tool for novel specific antibiotics. A high throughput strategy to generate a *B.cenocepacia* K56-2 conditional-growth mutant library, representative of all essential genes, is under development.

#### **A14. POTENTIAL USE OF BACTERIOPHAGES IN THE TREATMENT OF PATHOGENIC *BURKHOLDERIA CEPACIA* COMPLEX INFECTIONS**

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Respiratory infections caused by members of the *Burkholderia cepacia* complex (Bcc) are of major concern in cystic fibrosis patients due to their transmissibility, poor clinical outcomes and resistance to treatment with chemical antibiotics. The use of lytic bacteriophages in the treatment of bacterial infections, a practice termed phage therapy, has experienced a resurgence in recent years as a potential treatment for antimicrobial-resistant or otherwise intractable bacterial infections. Phages may be a viable alternative to chemical antibiotics in the treatment of Bcc infections in the CF lung. A number of novel bacteriophages able to lyse pathogenic Bcc strains have been isolated from agricultural soil samples collected from sites in the continental United States. Large-volume soil eluates were concentrated by batch chromatography on DEAE cellulose followed by ultrafiltration. This concentration method was used in conjunction with a more traditional, small-volume enrichment method for the isolation of phages. Based on an initial screening of strain virulence in a mouse model, phages able to lyse *B. cenocepacia* strain PC184 and *B. dolosa* strain AU0158 were selected for further study. Fifteen phages capable of lysing one or both of these strains have been isolated from soil samples collected from New York, North Carolina, Michigan and Illinois. Seven of the isolated phages exhibit polyvalency between *B. dolosa* and *B. cenocepacia*. Two novel bacteriophages which lyse strain PC184, the

podophage BcepIL2 and myophage BcepNY5, have been selected for more detailed analysis. Phage BcepNY5 was found to be inactivated by lipopolysaccharide (LPS) purified from strain PC184, while phage BcepIL2 was not. This suggests that LPS is used as the major receptor for phage BcepNY5, but not for BcepIL2. Stable variants of strain PC184 which show reduced sensitivity to phages BcepIL2 and BcepNY5 have been isolated. By superinfecting these insensitive PC184 variants with the wild-type phages, several phage variants have been isolated which are able to largely overcome the bacterial insensitivity to these phages. This indicates that bacterial resistance to phage, often cited as a major barrier to phage therapy, may be overcome relatively easily in this system.

#### **A15. DEVELOPMENT OF A PHAGE THERAPY STRATEGY FOR THE *BURKHOLDERIA CEPACIA* COMPLEX**

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The *Burkholderia cepacia* complex (Bcc) is comprised of nine closely related bacterial species that are problematic pathogens, especially in individuals with cystic fibrosis. These bacteria have become a critical clinical problem because they are highly resistant to most antibiotic agents and therefore incurable. In individuals with cystic fibrosis, Bcc establish either a long-term chronic infection or a rapidly fatal, uncontrollable disease that includes necrotizing pneumonia and bacteremia. In order to eliminate or better control BCC infections, alternative antibacterial strategies are required. One potential strategy to eradicate infection is through the application of bacteriophages, viruses specifically adapted to infecting and killing bacterial cells. Because phages are highly specific for a particular bacterial strain, several different phages may be used together to improve the chance that one phage will attack the bacterium. In order to develop

phage therapy for the Bcc, new phages must be isolated. Furthermore, in order to ensure patient safety and gain approval by governmental regulatory agencies, the phages must be well characterized. Towards this end, we have isolated several new Bcc specific bacteriophages and are characterizing their biological activity. KS9 is a lambda-like member of the Siphoviridae and a prophage of *B. pyrrocinia* LMG 21824 that plaques on *B. cenocepacia* K56-2 and *B. ambifaria* LMG 19467. The KS9 genome is a mosaic somewhat related to phage phiE125 from *B. thailandensis* E125 and phage phi1026b from *B. pseudomallei* 1026b, as well as uncharacterized prophage elements of *B. cenocepacia* PC184. KS10 is a Mu-like phage with homology to other Mu phage including BcepMu (also known as KS4 and DK4). KS10 is harbored by *B. cenocepacia* K56-2 and plaques on *B. ambifaria* LMG 19467. In order to investigate the ability of different phages to inhibit Bcc growth in vivo, we have developed an animal infection model utilizing larvae of the greater wax moth (*Galleria mellonella*). The results obtained from Bcc infection of this wax worm model can be used to ascertain the virulence of different strains and Bcc species, and correlates well with results observed with other Bcc infection models. We are currently using this infection model to determine whether phage therapy against the Bcc is efficacious in vivo.

#### **A16. COMPARATIVE GENOMIC HYBRIDIZATIONS USING OLIGONUCLEOTIDE MICROARRAYS TO ANALYZE CONSERVED GENE CONTENT IN CLOSELY RELATED BURKHOLDERIA STRAINS**

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The scientific community is increasingly finding the current microbial species definition (> 70% DNA-DNA reassociation values) lacking as it often leads species to be composed of a heterogenous set of strains from many environmental niches. The development of approaches for defining species that yield a higher resolution of the genetic differences between closely related organisms is now imperative. One such approach, microarray-based comparative genomic hybridizations (CGH), is gaining popularity due to design flexibility, higher accuracy, and rapid determination of conserved genes between strains. CGH can be used to investigate if species-specific gene sets (signatures) exist when a microarray designed for one species is used to hybridize strains of different, related species. However, the performance of microarrays with strains of varying levels of relatedness is poorly understood, particularly for oligonucleotide microarrays that are more sensitive to mismatches. These issues were investigated using an Agilent 60-mer microarray containing probes corresponding to genomic sequences from 3 *Burkholderia cenocepacia* strains. We performed CGH with *B. cenocepacia* strains and strains of closely related species and compared the results to known genome sequence data. The 8 genomes used in this study form a gradient of relatedness from 79-100% average nucleotide identity (ANI with 95% ANI representing the current limit for species) to a reference genome represented on the array. Under highly stringent hybridization conditions, our results indicate that these microarrays can be robustly used between strains that are as distantly related as ~92% ANI. False error rates have also been quantitated to ensure the accuracy of the results. Using CGH, we were able to estimate the evolutionary distance between the reference strain and a related strain *Burkholderia* sp. H111 and these results were confirmed by sequence data. A set of ~2252 microarray spots were conserved among all the strains and represent many genes that encode proteins involved in housekeeping functions, while spots not conserved represent many phage and hypothetical genes.

## **A17. GENE EXPRESSION CHANGES OF J2315 INDUCED DURING GROWTH IN CF SPUTUM**

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We characterized gene expression occurring in *Burkholderia cenocepacia* strain J2315 in response to growth in CF airway. Three sputum specimens recovered from three adult CF patients (infected with the ET-12 strain) were spiked with J2315 to mimic the lower respiratory tract environment. Bacterial cells were harvested at mid-log phase and their RNA was processed according to standard protocols for microarrays analysis. A direct comparison of the test sample (growth in sputum) versus the control (growth in a basal salt medium supplemented with glucose and casein aminoacids) was performed by hybridisation of fluorescently labelled cDNA on the *B. cenocepacia* 11K custom microarray. In total, six microarrays representing three sputum-specific duplicates and two additional self-hybridization arrays (to correct potential dye bias) were carried out. Out of 8,740 J2315-specific features present on the microarray, signals from 8,533 spots were consistently above the background noise. By applying t-test statistics on the sputum triplicates (each of which was derived from averaging two patient's sputum replicates), 2,007 genes were found to change significantly their expression level ( $p \leq 0.05$ , no multiple test correction). By filtering on minimal of 2-fold change, 183 genes were shown to be over-expressed, while 296 were down-regulated in the presence of sputum. To extend the breadth of potentially interesting altered gene expression hits, an alternative analysis based on six replicates of the sputum

experiment was also performed; out of 3,457 genes with  $p \leq 0.05$  (Benjamini and Hochberg false discovery rate), 414 of them appeared up- and 539 down-regulated. Significantly up-regulated genes included two genes encoding efflux pumps of the RND family, several flagellar genes, genes protecting the cells against reactive oxygen species, metalloproteases and highly expressed oxidoreductases belonging to two discrete operons. Under-expression was observed in genes encoding ribosomal proteins, urease structural proteins, the cable pilus operon and in clustered genes lying within one genomic island. Confirmatory RT-PCR of eleven selected up-regulated genes is in progress. Also, two genes: ferric reductase-like transmembrane component (BCAL 270) and multidrug efflux protein (BCAM 1947), have been selected for mutagenesis to investigate their pathogenic phenotype. In summary, transcriptomic analysis of the growth of *B. cenocepacia* within CF sputum has revealed the importance of novel genetic pathways as well as those associated with antimicrobial and host resistance, motility and iron metabolism. Supported by Wellcome Trust grant No. 075586 and CFFT's *Burkholderia* Array Project

## **A18. SEQUENCING AND ANNOTATION OF TWO BURKHOLDERIA GENOMES AT BROAD INSTITUTE**

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The Microbial Sequencing Center at the Broad Institute is actively involved in sequencing several pathogenic bacteria. As part of this larger initiative, we collaborated with members of the *Burkholderia* research community to sequence two important *Burkholderia* genomes: *B. dolosa* (strain

AU0158) and *B. cenocepacia* (strain PC184). The goal of this project is to use sequence-based information to gain a better understanding of the role these bacteria play in Cystic Fibrosis pathogenesis. Genome annotation is an important step in the transformation of raw sequence data into useful data. Despite great progress in the area of prokaryotic gene finding, no single method or tool can be used for annotation of a wide range of microbial genomes. With these challenges in mind, we have developed an evidence-based annotation system that is robust, scalable and can easily be customized to any genome. We will summarize our annotation methodology and discuss our preliminary findings from the comparative analysis of these two strains of *Burkholderia* genomes.

#### **A19. MICROARRAY WORK FLOWCHART AND PROCESS FOR CYSTIC FIBROSIS FOUNDATION THERAPEUTICS *BURKHOLDERIA CEPACIA* PROJECT TO BE DONE AT MOGENE**

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MOgene, LC, St. Louis, MO 63141

Cystic Fibrosis Foundation Therapeutics has contracted MOgene to perform Microarray work for *Burkholderia cepacia* project. I will present the microarray work process which will include sample submission, sample quality requirements and the process employed for doing the arrays at MOgene with data transfer to UNC. Some preliminary work done at MOgene for CFF will also be presented.

#### **A20. GENETICS OF HOST SPECIFICITY IN *BURKHOLDERIA CENOCEPACIA***

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Viswanathan<sup>1</sup>, Joshua J. Buysse<sup>1</sup>, A. Cody  
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*Burkholderia cenocepacia* is an opportunistic pathogen capable of infecting a wide range of hosts, including humans, plants, nematodes, and fungi. The hypothesis underlying this study is that *Burkholderia cepacia* complex (Bcc) strains can infect varied natural host subsets, and there are distinct genes associated with infection of each natural host. Our goal is to identify and characterize genes that contribute to host specificity in Bcc. We have collected human and environmental isolates, including three collections from cystic fibrosis patients in the Midwest from 1987-89, 1991-93, and 2004-06; clinical isolates from other geographic areas; and >500 environmental isolates from the rhizosphere and soil in Michigan in 2003-2004. Analysis by *recA* RFLP and multilocus sequence typing indicates that our clinical collection is highly biased toward the Midwest clone, which we have not detected among the environmental isolates. We have developed an onion bulb scale infection model, a nematode model, and a fungal inhibition assay to examine virulence, complemented by testing for phenotypes related to virulence, such as siderophore production, and genes such as *pehA*, which encodes pectate hydrolase. Greater than 95% of clinical isolates cause minimal maceration in onion, and do not contain the *pehA* gene. In contrast, most environmental isolates are highly virulent in onion model and are *pehA*-positive. Most environmental isolates inhibit the fungus *Rhizoctonia solani*, and contain the *prnD* gene, which is involved in pyrrolnitrin biosynthesis. The ability to inhibit fungi is less common in the clinical isolates, although most do contain the *prnD* gene. The *esmR* epidemic strain marker is present in ~60% of both the clinical and environmental isolates. Interestingly, most Midwest clone isolates from 1987-93, but less than half of the more recent Midwest clone isolates, contain this marker. In the nematode model, we have identified both clinical and environmental

isolates that are virulent, including one clinical isolate that is also highly virulent in onions and inhibits fungal growth. Transposon mutagenesis of this isolate and analysis of mutants in the onion and nematode models to identify mutations that reduce virulence is in progress. To facilitate future analysis of correlations between genes identified as required for virulence and host specificity, we will construct whole genome arrays with 1200-2400 clinical and environmental isolates. We hope to identify genes that are essential for the broad host range exhibited by some *Burkholderia cepacia* complex isolates, as well as genes that are specifically involved in virulence in a single host.

#### **A21. IS ELEMENTS AND DIVERSIFICATION OF BURKHOLDERIA CENOCEPACIA**

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IS (Insertion Sequences) elements are small mobile genetic entities able to insert into genomic DNA sequences of bacteria. These elements can play a role in genetic rearrangements and duplication processes by favoring recombination between chromosomes and/or plasmids. They can also inactivate gene sequences or modify their expression profiles by inserting into a promoter region. Three naturally occurring and active IS elements, IS402, IS407, and IS1416, have been reported in the *Burkholderia cepacia* complex (1). These elements were found broadly distributed in this bacterial complex, and to vary significantly in copy number from one strain to another. High copy numbers of IS407 were reported in the ET12 clone, which can lead to the cepacia syndrome among individuals with cystic fibrosis, of *B. cenocepacia* (2). IS402 was found responsible of the inactivation of

the O antigen lipopolysaccharide biosynthesis cluster in several strains of the ET12 clone (3). This latter IS was also suggested to have a significantly higher copy number in clinical rather than environmental strains of Bcc (2). Similarly, another element, named IS1363, was found to have a distribution mainly restricted to the PHDC and ET12 epidemic clones of *B. cenocepacia* (4). These observations suggest a possible relationship between IS elements and the diversification of *B. cenocepacia* into infra-specific lineages, including a role in the adaptation of *B. cenocepacia* to CF lungs. This possibility led us to initiate an analysis of the distribution and localization of IS elements among strains of *B. cenocepacia* of different origins including strains recovered from a recent nosocomial outbreak (5). This study was divided into three main parts: (A) an exhaustive analysis of IS elements among the *B. cenocepacia* genome sequences including analysis of their insertion sites, (B) an analysis of the distribution of insertion sites and of their colonization by IS elements among collections of clinical and environmental strains, and (C) an analysis of the abundance and variations of IS copy numbers in a soil population of *B. cenocepacia* of an experimental field divided into sections undergoing various treatments. These investigations allowed us to identify a novel group of IS elements, to observe IS-driven large scale genome rearrangements, to identify several IS inactivated genes, and observe great variations in the occupancy of insertion sites among closely related isolates. A summary of these observations and their ecological/functional significance will be presented.

1. Miché et al. 2001. Environmental Microbiology 3: 766-773; 2. Kenna et al. 2006. J. Med. Microbiol. 55:1-10; 3. Ortega et al. 2005. J. Bacteriol. 187:1324-1333; 4. Liu et al. 2003. J. Clin. Microbiol. 41:2471-2476; 5. Graindorge et al. 2006. IBCWG, 11<sup>th</sup> Annual Meeting, Gent, Belgium.

## **A22. CLONAL DIVERSITY AND HOST RANGE OF *BURKHOLDERIA CENOCEPACIA* FROM AGRICULTURAL AND CLINICAL SOURCES**

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Center for Microbial Pathogenesis, Michigan State University

*Burkholderia cenocepacia*, a member of *Burkholderia cepacia complex* genomovar III (Bcc III), is an opportunistic pathogen capable of causing disease in onions and nematodes, as well as pneumonia in cystic fibrosis (CF) patients. To investigate the diversity of bacterial clones comprising natural populations of *B. cenocepacia*, 43 clinical and 24 environmental isolates were characterized by multilocus sequence typing (MLST). The clinical isolates were obtained from CF patients in the United States between 1987 and 1993. Twenty of the environmental isolates were obtained from rhizosphere in Michigan (n= 19) during 2004 and in Mexico (n=1). The remaining environmental isolates were obtained from the soil of onion fields in Michigan during 2004. Among the 67 isolates, MLST resolved 17 distinct allelic profiles, or sequence types (STs) which all fall into the Bcc IIIB lineage. Fifteen STs that fell within the BccIIIB lineage were new and could not be assigned STs by the PubMLST database. Twenty-nine of the clinical strains were indistinguishable from one another and matched the ST-40 profile, also known as the Midwest clone. Among the other IIIB clinical isolates, MLST resolved 8 new variant genotypes. A consensus phylogeny using the minimum evolution algorithm revealed the 8 clinical variant genotypes were distributed within the IIIB lineage. The agricultural strains yielded matches to the ST-36 and ST-37 genotypes in addition to 7 new variant genotypes. Consensus phylogeny revealed the environment variant genotypes belong to 7 closely related STs that cluster with ST-36

and ST-37. This cluster also includes two sequenced strains (AU1054 and HI2424) both of which belong to the Bcc PHDC clone, a human pathogen that has been recovered from CF patients in North America and Europe. Split decomposition produced a phylogenetic network exhibiting several parallel paths indicative of past recombination and recurrent mutation. The Bcc IIIB lineage demonstrated the highest degree of reticulation in comparison to the other Bcc III lineages (A, C, D, and E), a result consistent with the extensive genetic diversity observed among environmental isolates. The results support the hypothesis that in addition to person-to-person transmission, a variety of *B. cenocepacia* clones prevail in the natural environment acting as a reservoir for clinical infections.

## **A23. DYNAMICS AND IDENTITY OF *BURKHOLDERIA* IN DIVERSE FARMING SYSTEMS**

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Farming systems research projects conducted at the Center for Environmental Farming Systems in Goldsboro, NC, are large-scale, long-term replicated studies initiated in 1998-1999. Soil microbiological properties are being examined along with soil chemical and physical properties, crop yield and quality, pest and beneficial organism dynamics, and the economics of production. Bacterial communities in agricultural soils represent a key factor in healthy ecosystem functioning. Several bacterial groups play significant roles in sustainable soil systems and in plant health. In particular, *Burkholderia* species have been studied by various groups because of their association with plant disease-suppressive and plant growth-promoting effects. Members of this genus have been shown to fix nitrogen, induce resistance in plants to pathogens, promote plant growth and act as biocontrol agents. All treatment plots (0.10 to 6.0 ha in size) contained 5 randomly and GPS-mapped sampling points. Samples from each point,

collected 1x to 4x per year from 1999-2006 were composed of 30 cores and a thoroughly mixed subsample was placed on ice for microbial analysis. Other subsamples of each composite sample were analyzed for multiple physical and biological properties by collaborators. Samples were diluted and total culturable bacteria, fluorescent *Pseudomonas* and *Burkholderia* populations were assessed using soil dilution assays. Year and Date of sampling dramatically impacted bacterial populations in each assay. Distinct and consistent trends in each population were observed. For example, bacterial populations were typically relatively high in the early spring, declined during the summer and increased again by September. All soil samples, bacterial cultures and DNA (when extracted) have been archived for future analysis. For example, dominant and representative *Burkholderia* strains from each plot on each sample date were archived as live cultures. BOX-PCR genomic fingerprint analysis of a limited number of strains (72) from the baseline sample, prior to implementation of farming system treatments, revealed a high level of diversity. The 16S rDNA and Recombinase (RecA) genes were sequenced from strains representing the observed diversity. BLAST and phylogenetic analysis demonstrated that soil type was the dominant factor that determined species content, based on the limited number of strains analyzed to date. Identified species included, *B. caribensis*, *B. cenocepacia*, *B. cepacia*, *B. pyrrocinia*, *B. terrae*, *B. tropicalis* and uncultured *Burkholderia*. Strains isolated over a 6-yr period on the 81 ha experimental site should provide a productive resource to evaluate species composition as determined by space, time and farming system.

#### **A24. IDENTIFICATION AND PATHOGENICITY OF *BURKHOLDERIA CEPACIA* COMPLEX STRAINS ISOLATED FROM THE RHIZOSPHERE OF ONION AND ORGANIC SOILS**

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Genotypic identification and pathogenicity characterization were performed on *Burkholderia cepacia* complex (Bcc) strains isolated from the rhizosphere of onion and organic soils. A total of 3,798 putative Bcc isolates were recovered on PCAT and TB-T semi-selective media during the 2004 growing season from six commercial onion fields located in two counties in Michigan. Putative Bcc isolates were identified as Bcc by hybridization to a 16S rDNA probe followed by duplex PCR using primers targeted to the 16S rDNA and *recA* sequences. A total of 1,290 rhizosphere (980) and soil (310) strains were assigned to the species *B. cepacia* (genomovar I – 160), *B. cenocepacia* (genomovar III – 480), *B. ambifaria* (genomovar VII – 623), and *B. pyrrocinia* (genomovar IX – 27), by means of *recA* (RFLP) analysis. Bcc strains were characterized for pathogenicity in an onion bulb scale assay. The onion pathogenicity assay revealed symptoms of watersoaking, maceration and/or necrosis. The extent of onion bulb scale maceration was rated on a scale of 0-3, with 0 = 0% maceration, 1 = 1-33% macerated area, 2 = 34-66% macerated area, and 3 = 67-100% macerated area. The majority of strains identified as *B. cepacia* (85%), *B. cenocepacia* (90%), and *B. ambifaria* (76%) exhibited an onion maceration rating of 2 to 3, indicating that all of these species have the potential to cause sour skin rot disease of onion. In addition, *B. cepacia*, *B. cenocepacia*, and *B. ambifaria* were isolated from naturally diseased onions exhibiting soft rot symptoms typical of sour skin disease, first described by W. H. Burkholder in 1950. This is the first report of *B. cenocepacia* and *B. ambifaria* causing sour skin disease in onion in the USA. A phylogenetic analysis of *recA* sequences from representative Bcc type and panel strains along with strains collected in this study revealed that the *B. cenocepacia* strains associated with onion grouped within the III-

B lineage and are most closely related to strain AU1054, a strain isolated from a CF patient. This study revealed that the onion rhizosphere is a natural habitat and a potential environmental source of *B. cenocepacia*. Further genetic variation studies are being conducted to compare *B. cenocepacia* strains associated with onion and those of clinical origin.

## **A25. CHARACTERISATION OF AN ENZYME WITH LYASE ACTIVITY FOR CEPACIAN EPS**

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The work carried out in our laboratory in the last years was focussed on the structural characterisation of EPS produced by bacteria belonging to the *Burkholderia cepacia* Complex (BCC). These microbes express very often a mucoid phenotype, associated with the production of abundant amounts of exopolysaccharides (EPS). Four different types of EPS were found to be produced by this group of bacteria, and one of them (to so called cepacian) was synthesised by about 95% of the strains examined in our laboratory. This EPS has unique structural features: i) a highly branched negatively charged heptasaccharide repeating unit; ii) a completely substituted glucuronic acid residue; iii) a rhamnose residue in the D absolute configuration; iv) a hydrophobic character due to heavy substitution with acetyl groups. This EPS was not found to be produced by other bacterial species. The unusual characteristics of the primary structure of cepacian prompted the investigation of its macromolecular solution properties, by means of HP-SEC, 2D NOESY NMR, AFM and viscosity. The data showed that the polymer chain presents a rather rigid

conformation and is capable of forming chains association. These macromolecular characteristics nicely explain the cepacian ability to form biofilms. Enzyme degrading EPS are useful tools for the structural determination and modification, and since no enzymes are commercially available for cepacian, we started to search for them. An enzyme with lyase activity was isolated from a *Bacillus* species living in water tanks. The characterisation of the enzyme determined its optimum pH and temperature, and its molecular weight. The saccharidic products of its action were investigated by means of MALDI-MS and NMR. It was shown to be active only on cepacian and not on other bacterial EPS. Although the lyase cleaved a disaccharide side chain leaving a polysaccharide of high molecular weight, the solution viscosity of the latter diminished. Therefore, the determination of O-acetyl groups position, impossible to carry out on the intact cepacian because of its high viscosity, is currently under investigation on the lyase products. This information will explain why the macromolecular properties of cepacian differ passing from the native to the de-O-acetylated sample.

## **A26. CHARACTERIZATION OF A CLUSTER OF GENES INVOLVED IN CORE LIPOPOLYSACCHARIDE BIOSYNTHESIS AND O ANTIGEN LIGATION IN BURKHOLDERIA CENOCEPACIA**

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*Burkholderia cenocepacia*, is an emerging opportunistic pathogen associated with infections in patients with cystic fibrosis. *B. cenocepacia* are inherently multi-drug resistant bacteria found ubiquitously in the environment, which display formidable resistance to all classes of antimicrobial peptides. The genetic and biochemical basis for this resistance are currently unknown. We

hypothesize that the intrinsic antibiotic resistance of *B. cenocepacia*, and in particular the extreme resistance to antimicrobial peptides, is associated with unique properties of the bacterial cell envelope in these organisms. The lipopolysaccharide (LPS) is a major component of the bacterial outer membrane and consists of the lipid A or endotoxin, core oligosaccharide and O antigen. The core oligosaccharide has an inner domain (inner core) usually made of heptoses and *keto-deoxy*-octulosonic acid (Kdo) and is structurally well conserved. We recently constructed a heptoseless LPS *B. cenocepacia* mutant, which demonstrates that the heptose residues of the LPS inner core are not essential for viability of this bacterium but are important for resistance to antimicrobial peptides. Glycosyltransferase genes for the inner core LPS synthesis were identified in the proximity of the O antigen gene cluster. We also identified a putative core biosynthesis cluster in *B. cenocepacia* K56-2 that encodes putative glycosyltransferases and the WaaL O antigen ligase. Taking advantage of a single crossover mutagenesis method, we inactivated several genes in this cluster including *waaL* and other putative glycosyltransferase genes. These mutants provided a panel of isogenic strains with various core oligosaccharide truncations that were used to elucidate the core oligosaccharide structure in strain K56-2. Data indicate that the K56-2 core oligosaccharide consists of a backbone composed of 3 heptoses and Kdo, with Ko and Ara4N linked to the Kdo, and glucose and rhamnose residues linked to heptose I and heptose II, respectively. We have also determined that K56-2 O antigen is attached to heptose II and that a glucose residue is critical for ligation, suggesting that this residue is required for the WaaL protein recognition of the core acceptor. These results allowed to perform a functional assignment of core oligosaccharide glycosyltransferases. Furthermore, we have examined the sensitivity of core LPS mutants to the cationic peptide polymyxin B, and show here that the level of core truncation is directly proportional with an increase in sensitivity to this peptide, suggesting that a complete core

oligosaccharide is required for antimicrobial peptide resistance, possibly reflecting the intactness of the outer membrane.

## **A27. EXOPOLYSACCHARIDE BIOSYNTHESIS AND REGULATION IN *BURKHOLDERIA CEPACIA* COMPLEX ISOLATES**

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Bacteria of the *Burkholderia cepacia* complex (Bcc) have emerged as opportunistic pathogens in patients with cystic fibrosis. Around 80% of the Bcc isolates recovered from CF patients produce large amounts of exopolysaccharide (EPS), suggesting a possible role for this EPS in Bcc pathogenesis. Cepacian is the main EPS produced by Bcc isolates and is composed of branched acetylated heptasaccharide repeat-units with D-glucose, D-rhamnose, D-mannose, D-galactose and D-glucuronic acid in the ratio 1:1:1:3:1, respectively. The pathway leading to the nucleotide sugar precursors biosynthesis was proposed and the biosynthetic cluster of genes was identified (1). This gene cluster is present in all Bcc strains from which the genome sequence is available and it comprises 12 *bce* genes. Database searches for homologous proteins and secondary structure analysis for the deduced amino acid sequences revealed genes predicted to encode enzymes required for the formation of nucleotide sugars, glycosyltransferases for the assembly of the repeat-unit and other proteins involved in polymerization, export and regulation of EPS production. The functional analysis of BceA demonstrated a bifunctional protein with phosphomannose isomerase and GDP-mannose pyrophosphorylase activities. The lack of functional *bceA* gene does not affect EPS production yield but the viscosity of aqueous solutions prepared with this mutant EPS was significantly reduced when

compared with the wild-type biopolymer (2). BceC codes for a UDP-glucose dehydrogenase and is involved in the synthesis of UDP-glucuronic acid. BceB was shown to contain glycosyltransferase activity and mediate incorporation of glucose-1-phosphate into membrane lipids (3). Two other genes analysed were *bceD* and *bceF*, which we demonstrated to encode a phosphotyrosine phosphatase and tyrosine autokinase, respectively (4). The disruption of *bceF* resulted in the abolishment of cepacian accumulation in the culture medium, but 75% of the parental strain's EPS production yield was still registered for the *bceD* mutant. The EPS produced by the *bceD* mutant led to less viscous solutions, suggesting a lower molecular mass for this mutant biopolymer. The size of the biofilm produced in vitro by *bceD* and *bceF* mutants was smaller than the size of the biofilm formed by the parental *B. cenocepacia* strain, indicating that BceD and BceF play a role in the establishment of biofilms of maximal size.

<sup>1</sup>Moreira LM *et al. Biochem Biophys Res Commun*, 312: 323-333, 2003. <sup>2</sup>Sousa SA *et al. Biochem Biophys Res Commun*, 353: 200-206, 2007. <sup>3</sup>Videira PA *et al. J Bacteriol*, 187: 5013-5018, 2005. <sup>4</sup>Ferreira AS *et al. Appl Environ Microbiol*, 73: 524-534, 2007

## **A28. PROTEOMIC INVESTIGATION OF EXOPOLYSACCHARIDE PRODUCTION IN *BURKHOLDERIA CENOCEPACIA***

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*Burkholderia cenocepacia* is known to be capable of exopolysaccharide (EPS) production. EPS production in *Pseudomonas aeruginosa*, the other major cystic fibrosis pathogen, is very well characterized as the mucoid phenotype and results from the production of the polysaccharide alginate. Alginate production, a significant characteristic in *P. aeruginosa* infection in

cystic fibrosis, typically results from conversion of an originally non-mucoid strain and is correlated with loss of pulmonary function. The EPS produced in *B. cenocepacia* is chemically distinct from alginate and indeed *B. cenocepacia* complex bacteria are known to be able to produce at least four different polysaccharides. We are investigating 2 pairs of isogenic strains of *B. cenocepacia* with different colony morphology due to EPS production.

Firstly, a matte (C1394) and shiny (C1394mp2) pair were investigated, the shiny variant being obtained by passage through a pulmonary model of infection. Previously it has been shown that C1394mp2, unlike C1394, produced the polysaccharide cepacian and persisted in the pulmonary infection model. Here this pair were characterized by 2D-PAGE which revealed that proteomic differences in the shiny strain at the protein level included the loss of alkyl hydroperoxide reductase subunit C (AhpC) and increased production of flagellin proteins (Chung and Speert 2007). The second pair we are currently investigating is non-mucoid C8963 and mucoid C9343. These are sequential isolates from a CF patient and have been determined to be isogenic. C9343 produces a frank mucoid phenotype on yeast extract media, which results from the production of the polysaccharides PS-I and cepacian as well as dextran. This isolate has previously been shown to be deficient in production of quorum sensing signaling molecules and biofilm formation and also to have reduced interaction with neutrophils and macrophages as well as reduced clearance in a mouse model of infection. We are currently investigating the effect of conversion to the mucoid phenotype at the proteome level by 2D-PAGE. At least 5 spot differences have so far been identified and are being identified by mass spectrometry at the University of Victoria proteomics facility.

## **A29. BURKHOLDERIA DOLOSA EXPRESSES THE POLYSACCHARIDE POLY-N-ACETYL-GLUCOSAMINE (PNAG)**

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Joanna B. Goldberg<sup>1</sup> and Gregory Priebe<sup>2,3</sup>

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*Burkholderia dolosa*, previously described as genomovar VI of the *Burkholderia cepacia* complex (Bcc), can cause chronic lung infections in cystic fibrosis (CF) patients and has been associated with patient-to-patient transmission and worse clinical outcomes. The polysaccharide poly-N-acetylglucosamine (PNAG) has been shown to be expressed on *Staphylococcus aureus* isolated from the lungs of CF patients. Antibodies generated to the deacetylated form, dPNAG, are opsonic and protective against *S. aureus*. Using monoclonal and polyclonal antibodies, we detected dPNAG on *B. dolosa* by immunofluorescence in a murine model of *B. dolosa* lung infection and in the explanted lung of a CF patient colonized with *B. dolosa*. Recently, the complete genome sequence of *B. dolosa* strain AU0158 has been made available by the Broad Institute at MIT. Using the *Escherichia coli* K12 PNAG biosynthetic loci (*pga*) for homology searches with the BLAST family of programs, we have recognized a locus potentially responsible for PNAG production in *B. dolosa*. This ~4 kb region consists of four genes that we have tentatively named *pgaA*, *pgaB*, *pgaC*, and *pgaD*, based on their similarity to the *E. coli* K12 *pga* genes. These genes are proposed to encode an outer membrane protein, a carbohydrate esterase, a glycosyl transferase, and an inner membrane protein, respectively. The four predicted protein sequences are 56%, 70%, 78% and 0% similar to their *E. coli* K12 counterparts.

We constructed a *pgaA* insertional mutant in *B. dolosa* strain AU0158 by allelic exchange. The wild-type and mutant were compared for colonization after intranasal inoculation of FVB/NJ mice. One day after infection with  $5 \times 10^8$  CFU, mice infected with the wild-type and mutant had similar levels of bacteria in the lungs and spleen. However, after 8 days, the levels of bacteria in the lung were significantly lower ( $p < 0.001$ ) in mice infected with the *pgaA* mutant, while the levels in the spleen were not significantly different. These findings indicate that dPNAG is expressed on *B. dolosa* and that this expression may be important for bacterial persistence in the lung but not for dissemination to and survival in the spleen.

## **A30. COLONY MORPHOLOGY CORRELATES WITH BIOFILM FORMATION AND VIRULENCE IN BURKHOLDERIA CENOCEPACIA**

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Bacteria often modify their cell surface to adapt to environmental changes. In some cases, cell surface modifications can be detected by changes in colony morphology. *Burkholderia cenocepacia* strains typically have a rough colony morphology; however, shiny colony variants (shv) can spontaneously appear when grown *in vitro*. The appearance of shv was demonstrated in six *B. cenocepacia* strains with a typically rough phenotype, although the frequency varied between strains. With the exception of strain BC7, shv colonies were more frequently isolated from cultures obtained from infected alfalfa seedlings than from cultures grown in liquid medium. To determine whether K56-2 shv were defective in forming biofilms on abiotic surfaces, 93 independent shv obtained via liquid cultures were evaluated for their ability to form biofilms on polystyrene pegs. All shv had at least a 50% reduction in biomass on

polystyrene pegs when compared to the rough K56-2. The ability to form floating pellicles was also analyzed and pellicles formed by shv were either absent or not as rigid or stable as found in the rough strain. Of the 93 shv selected by growth in liquid medium, 80% were avirulent in the alfalfa seedling infection model. K56-2 shv were characterized with respect to protease activity, *N*-acylhomoserine lactone production, swarming, and swimming motility and clustered into 15 representative groups. With the exception of biofilm formation and alfalfa virulence, other phenotypic properties varied between independently isolated shv, although these phenotypes were generally stable upon repeated passage. Selected shv were analyzed for virulence in cell and animal models. K56-2-S15, K56-2-S76 and K56-2-S86 were tested for virulence in the chronic agar bead model of respiratory infection and were determined to produce significantly less lung histopathology than the rough wild type K56-2 although they were able to establish a chronic infection. K56-2-S76 and K56-2-S86 were examined for their ability to invade and replicate in the CF bronchial epithelial cell line IB3-1. These shv had similar invasion frequencies; however, they did not replicate, whereas K56-2 generally increased 1-2 logs in 24 hr. Transmission electron microscopy on K56-2-S76 and K56-2-S86 revealed an extracellular-like matrix surrounding bacterial cells was absent or reduced in both strains compared to their rough wild type. In summary, shv generally produced less biofilms and were less virulent in infection models suggesting that there is a correlation between colony morphology, biofilm formation and virulence in *B. cenocepacia*.

### **A31. STUDIES ON THE FIRST CLINICAL ISOLATES OF *BURKHOLDERIA GLUMAE***

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*Burkholderia glumae* is pathogenic to rice (*Oryza sativa*) producing symptoms that include seedling blight, seedling rot and grain rot; all these are often considered to be manifestations of a single disease which is usually called just 'panicle blight'. Panicle blight is a recurring problem in rice-producing areas in the United States, Japan and Korea and its incidence is increasing in recent years. *B. glumae* is very closely related to species belonging to the *Burkholderia cepacia* complex (BCC) which are important opportunistic human pathogens for specific groups like cystic fibrosis (CF) and chronic granulomatous disease (CDG) patients. Recently two strains of *B. glumae* have been isolated in the USA from infants with CGD. We report that both of clinical strains have retained the capacity to cause severe disease symptoms in rice. As previously reported in the rice isolate *B. glumae* BGR1 and in members of the BCC, also in the clinical isolates of *B. glumae*, the TofI/R acyl homoserine lactone (AHL) quorum sensing played a pivotal role in virulence. In addition we determined that AHL quorum sensing in *B. glumae* clinical isolate AU6208 regulates the LipA secreted lipase as well as regulating toxoflavin, the phytotoxin produced by *B. glumae*. Importantly, *B. glumae* AU6208 *lipA* mutants were no longer pathogenic to rice indicating that the lipase is an important virulence factor. The rice model could therefore prove useful in studying *Burkholderia* pathogenesis. Finally it was also observed that spontaneous mutations in the *tofR* regulator are responsible for phenotypic conversion in this species.

### **A32. MUTATION OF A NOVEL SENSOR KINASE-RESPONSE REGULATOR HYBRID INCREASES *BURKHOLDERIA CENOCEPACIA* VIRULENCE**

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*Burkholderia cenocepacia* is a multi-drug resistant Gram-negative bacillus that is found ubiquitously in the environment. *B. cenocepacia* has emerged as an important opportunistic pathogen, establishing chronic lung infections in the immunocompromised patients with chronic granulomatous disease and most commonly, with cystic fibrosis (CF). Very little is known about how this respiratory pathogen can cause disease and how it can survive in the airways of CF patients. Rapid and specific adaptations by *B. cenocepacia* to the CF airway environment likely play an important role in the development of persistence. Sensing and adaptation to new environmental conditions by bacterial pathogens is commonly governed by two-component regulatory systems leading to modification of gene expression patterns required for bacterial survival. Recently, a global virulence regulator PA4856 (RetS) has been identified in *P. aeruginosa*. Using BLASTP analysis, we identified a RetS homolog in *B. cenocepacia* and hypothesized that this regulator might be part of a signal transduction network regulating the expression of virulence factors of *B. cenocepacia* during the infection. By genetic analyses employing mutagenesis and complementation studies in *B. cenocepacia* K56-2, we report the identification of *BCAM0379*, encoding a putative sensor kinase. Biofilm and adherence assays revealed that inactivation of *BCAM0379* leads to a hyperadherent phenotype to both abiotic surfaces and lung epithelial cells. Trypan blue exclusion assays demonstrated that *B. cenocepacia* K56-2  $\Delta BCAM0379$  is more cytotoxic toward macrophages than the wild-

type strain. Finally, preliminary in vivo studies using the *Caenorhabditis elegans* “fast killing” assay also suggest that mutation of *BCAM0379* increases the virulence of *B. cenocepacia* K56-2. Our results suggest that *BCAM0379* encodes a component of a regulatory cascade controlling the expression of virulence factors potentially involved in bacterial persistence and infection in the host.

### **A33. RpoN-DEPENDENT COLONY VARIANTS OF *BURKHOLDERIA CENOCEPACIA* WITH DEFECTS IN BIOFILM FORMATION AND INTRACELLULAR SURVIVAL**

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*Burkholderia cenocepacia*, a multi-drug resistant Gram-negative bacillus, is an opportunistic pathogen frequently associated with infections in patients with cystic fibrosis. Our working model from the ET12 clonal lineage, strain K56-2, can undergo phenotypic variation generating two colonial variants: (i) a rugose variant forming corrugated and dry colonies that take up Congo red (herein designated as K56-2R), and (ii) a smooth variant characterized by colonies with a glossy and wet appearance that are unable to retain Congo red (herein designated as a K56-2W). Using transmission electron microscopy we demonstrated the presence of an extracellular fibrous matrix surrounding K56-2R cells that is absent in K56-2W variant. Considering that extracellular material plays an important role in biofilm formation we compared the biofilm forming capacity of the two variants. Our results show that K56-2R colonies produced at least twice more biofilm mass than the K56-2W variants. We also compared the ability of the two variants to survive in murine macrophages RAW264.7. The two K56-2 colonial variants behave differently in RAW264.7 cells. In macrophages infected with K56-2W, the

acidification of the bacteria-containing vacuoles proceeds normally, suggesting that these bacteria cannot delay the maturation of the phagosome. In contrast, K56-2R containing vacuoles delayed acidification, as we have found with the clonal ET12 strain J2315, suggesting these bacterial cells can survive inside macrophages. We identified two sigma 54-specific transcriptional regulators (*BCAL2913* and *BCAM1342*) that control biofilm formation and demonstrated that RpoN controls the associated colony morphology phenotypes. We generated *rpoN* mutants in the K56-2R and K56-2W genetic backgrounds and characterized the mutants for biofilm formation and survival in macrophages RAW264.7. The K56-2R *rpoN* mutant exhibited decreased biofilm formation and survival in RAW264.7 macrophages, while changes were observed with the K56-2W *rpoN* counterpart. Our results suggest that RpoN controls, at least in part, phenotypic colony variations that are implicated in biofilm formation and bacterial survival in macrophages.

#### **A34. GENOMIC ISLAND DNA RELEASED FROM CYSTIC FIBROSIS ISOLATES OF *PSEUDOMONAS AERUGINOSA* AND *BURKHOLDERIA SPECIES***

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Pathogenicity islands, which are members of the family of genomic islands, are known to harbor genes involved in bacterial virulence. They are part of horizontally transferred gene clusters present in many pathogenic bacteria and may encode both virulence factors and secretion systems such as components of the type III secretion apparatus. They are usually characterized by determining genome sequences from clinical isolates and comparing the sequences from laboratory

strains that are likely to be avirulent. We have recently reported that a cystic fibrosis (CF) isolate of *Pseudomonas aeruginosa* releases in the growth medium a segment of DNA that is rich in CpG sequences and harbors many genes normally involved in pathogenicity. This specific DNA fragment is believed to be looped out of its chromosomal location and released extracellularly when bacteria are grown in a rich medium containing eukaryotic proteins. This particular CpG-rich DNA has also anti-tumor activity (Mahfouz et al, Plasmid, **57**, 4 -17, 2007). The ability of several CF pathogenic strains of *P. aeruginosa* and *Burkholderia cenocepacia* and other *Burkholderia* species to release DNA segments that differ in size and other characteristics such as GC content will be described with regard to their pathogenic potential.

#### **A35. *BURKHOLDERIA MULTIVORANS* IN CHRONIC GRANULOMATOUS DISEASE: VIRULENCE, CELL ENTRY AND CYTOKINE PRODUCTION**

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Chronic granulomatous disease (CGD) is a primary immunodeficiency that affects the phagocyte NADPH oxidase. The impaired phagocyte killing can lead to recurrent, life-threatening infections by a very specific set of bacteria and fungi, including *S. aureus*, *Serratia marcescens*, *Burkholderia cepacia* complex (BCC), *Nocardia* and *Aspergillus* species. Within the BCC, *B. cenocepacia* and *B. multivorans* are frequently isolated. Strains of BCC have been previously shown to enter and survive intracellularly in eukaryotic cells, such as macrophages, respiratory epithelial cell lines, and amoeba. In addition, whole BCC, lysates or purified components have been reported to induce various cytokines

such as IL-6, IL-8 and TNF $\alpha$ . In BCC isolates obtained from patients with cystic fibrosis the ability to enter eukaryotic cells and the BCC-induced proinflammatory activity are highly strain dependent. We characterized and compared several CGD clinical isolates and one environmental isolate of *Burkholderia multivorans* for cell association, entry, and cytokine production after 2 hours infection using healthy donor human elutriated monocytes. The % cell association varied by strain. Clinical isolate CGD1 exhibited high level cell association by Giemsa staining (69.6 $\pm$ 14.3% infected cells), whereas the mucoid clinical isolate CGD2 and the environmental isolate exhibited much less cell association (16.4 $\pm$ 9.2 and 8.8 $\pm$ 5.4%, respectively). Differences in cell entry were confirmed by confocal microscopy using selected strains expressing GFP. Cytokines elaborated in cell culture supernatants differed among the isolates, with the major cytokines detected being TNF $\alpha$  and IL8. CGD1 induced high levels of TNF $\alpha$  (10449 $\pm$ 1992pg/ml), the environmental strain induced intermediate TNF $\alpha$  levels (6698 $\pm$ 2249pg/ml) while CGD2 induced lower TNF $\alpha$  levels (2447 $\pm$ 658pg/ml). Cytokine production required serum in the media and was dependent on the p38 MAPK signaling pathway. Pre-incubation of cells with the p38 signaling inhibitor (SB203580) led to >90% reduction in cytokine response. Virulence of the strains was compared by intraperitoneal challenge of CGD mice. Isolates CGD1 and CGD2 but not the environmental strain caused mortality in CGD mice. Components of the bacteria, such as LPS and exopolysaccharide, may be responsible for the differentially induced responses observed among the strains during the early events of cell infection. These differences may lead to different clinical outcomes.

### **A36. VIRULENCE CHARACTERISTICS OF DIVERSE *B. CENOCEPACIA* ISOLATES AND THE IDENTIFICATION OF VIRULENCE FACTORS USING THE *C. ELEGANS* MODEL FOR PATHOGENICITY.**

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The *C. elegans* model for pathogenic host-bacteria interactions has been used extensively to study virulence many pathogens including *Pseudomonas aeruginosa*, an important opportunistic pathogen for cystic fibrosis patients. *P. aeruginosa* kills *C. elegans* by producing toxins on PGS media (fast killing) or by infecting nematodes on NGM media (slow killing). *Burkholderia cenocepacia* is a ubiquitous organism that is also an important opportunistic pathogen for CF patients. We used the nematode model to determine virulence characteristics of clinical, agricultural and environmental isolates and as an animal host to study virulence in select strains of *B. cenocepacia*. The virulence characteristics of some *B. cenocepacia* are similar to *P. aeruginosa* (e.g. Midwest clone PC184), however some strains cause rapid mortality on NGM (e.g. AU1054, HI2424, and MI onion field isolate 6RT-130) and PGS, while other strains kill slowly on PGS or do not kill at all. *Burkholderia cenocepacia* AU1054 is a Mid-Atlantic CF-patient isolate, that is pathogenic to onions, virulent to worms and has anti-fungal properties. Because of this broad host range, we focused on this strain to understand its mechanism of *C. elegans* killing and to identify its virulence genes by transposon mutagenesis. We have used GFP-labeled *Burkholderia cenocepacia* AU1054 in our experiments. *Burkholderia cenocepacia* AU1054 appears to kill *C. elegans* by infections and by producing toxins. To identify the virulence genes, we used transposon mutagenesis and screened for mutants that did not kill *C. elegans* at 72 h on

NGM plates. We have four mutants that appear to be avirulent to worms. Work is in progress to identify the gene(s) that have been interrupted by the transposon in these mutants, to determine if these genes encode general or specific virulence factors and to characterize the virulence of AU1054 to *C. elegans*. In summary, *C. elegans* has proved useful for assessing virulence characteristics of diverse *B. cenocepacia* strains and to identify virulence factors present in select strains.

### **A37. GALLERIA MELLONELLA: A VALUABLE INVERTEBRATE MODEL FOR ASSESSING VIRULENCE WITHIN THE BURKHOLDERIA CEPACIA COMPLEX?**

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The bacterial species that make up the *Burkholderia cepacia* complex (BCC) are metabolically versatile and can often degrade a wide range of compounds. This has led to considerable interest in members of the BCC as potential bioremediation agents. However, the potential of isolates from across the BCC to act as opportunistic pathogens in human hosts has resulted in a paucity of research in this area. Whilst *B. cenocepacia* and *B. multivorans* are responsible for the majority of infections in cystic fibrosis patients, strains from all of the other species within the complex have been found to cause infection. There are several models available for the study of pathogenicity within the BCC. We decided to use the invertebrate model *Galleria mellonella* (Greater Wax Moth) to investigate the pathogenicity of isolates from across the BCC. *G. mellonella* is a pest of beehives and has been used to investigate virulence in a range of pathogens. The larvae are easy to obtain commercially and the experiments can be run at a fraction of the

cost of the equivalent experimentation in mammals. Unlike some other invertebrate models, *Galleria mellonella* will survive at 37°C allowing experiments to be carried out at the physiological temperature of the human host, as well as environmentally relevant temperatures. The degree of similarity between mammalian and insect innate immune systems, and the importance of innate immunity in protection from microbial infection, mean that studying insect responses to infection is potentially useful. *Galleria mellonella* has been extensively characterised as a model for pathogenicity testing of *Pseudomonas aeruginosa*, producing results that correlate well with data obtained from mammalian studies. We have screened a large selection of isolates from a genetically (MLST) characterised strain panel representing diversity across the BCC. Isolates were screened at a range of inoculate concentrations and incubation temperatures. Differences in pathogenicity were found between species and also between isolates of the same species, indicating that the genomic plasticity of BCC and strain-specific genetic material is important in pathogenicity. Incubation temperature was also found to affect virulence. We propose that *Galleria mellonella* provides a useful, cost-effective and ethically acceptable model for pathogenicity within the BCC with the potential for identifying key virulence.

### **A38. ADHESIN A OF BURKHOLDERIA CENOCEPACIA IS ENCODED ON A PATHOGENICITY ISLAND**

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Members of the *Burkholderia cepacia* complex (Bcc) are capable of causing lung infections in cystic fibrosis and other immuno-compromised patients. Sometimes this infection leads to “cepacia syndrome”, which is characterized by necrotizing pneumonia and sepsis. It is not known what triggers this syndrome, as it does not always occur during all Bcc infections. ET12, a

particularly virulent epidemic lineage of *Burkholderia cenocepacia*, is defined by its expression of cable pili. Strain BC7 of this lineage expresses a protein called Adhesin A (AdhA) that is associated with the cable pili. AdhA has previously been shown to be required for BC7 adherence to and transmigration across human lung squamous epithelial tissue. Since “cepacia syndrome” involves migration across this layer into the bloodstream, understanding the synthesis and regulation of AdhA may be critical to uncovering a way to inhibit this deadly process. Genomic analysis of *B. cenocepacia* J2315 (Sanger), an ET12 strain encoding but not expressing AdhA, revealed that the *adhA* gene was 100% identical to that of BC7. Sequence homology searches of AdhA using the BLAST tool revealed that this protein is highly conserved among numerous bacterial species. We also noted that there were three genes immediately downstream of *adhA*. These genes, which were recognized by Whitby et al. (J. Med. Microbiol. 2006, 55:11-21), may form an operon and appear to encode proteins homologous to all three components of a Type I Secretion System. We hypothesize that these proteins are part of the machinery required to secrete AdhA. Partial sequencing of these three genes (*adhB-D*) in BC7 show 100% identity to the J2315 sequence. To determine whether the genes encoding the putative secretion apparatus are conserved, analyses of the open reading frames (ORFs) using BLASTP and published bacterial genomes were performed. Results show that these ORF's are adjacent to one another in several other species. Most interestingly, in both *Burkholderia multivorans* ATCC17616 and *Ralstonia solanacearum* GMI1000, these genes are arranged in the same order, downstream of an AdhA homolog. Sequence comparisons to a non-ET12 *B. cenocepacia* strain, PC184 (Broad), revealed that the entire ~11,000 base pair region encompassing *adhA-D* was absent from the corresponding region and was not found anywhere else in the PC184 genome. Furthermore, AT-rich sequences were noted in this region in both J2315 and PC184 genomes, suggesting that these genes are part of a pathogenicity island.

### **A39. BORDETELLA INFECTION IN CYSTIC FIBROSIS PATIENTS**

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The spectrum of bacteria recovered from respiratory tract specimens from CF patients appears to be expanding. Several species found in CF cultures are relatively uncommon human pathogens and/or opportunistic species with fastidious growth requirements. Many are phylogenetically closely related and species misidentification using routine phenotypic testing is common. We identified *Bordetella* from cultures of sputum, bronchoalveolar lavage, or pharyngeal, throat, or epiglottis swabs from 27 cystic fibrosis patients in the US. All isolates were initially identified as *Achromobacter* spp. based on phenotypic analysis and a PCR-based assay intended to be specific for *A. xylosoxidans*. However, cluster analysis of DNA profiles of these isolates generated by BOX-PCR genotyping showed that these were in a distinct group that was distantly related to other *A. xylosoxidans* strains (similarity coefficient of 23.8 +/- 10.8%) and included several *Bordetella* type strains. Characterization of these isolates by 16S rDNA sequencing identified 18 as *B. bronchiseptica* / *parapertusis*, three as *B. hinzii*, two as *B. petrii* and four as indeterminate *Bordetella* species. Two patients were chronically infected with *Bordetella* species. *B. petrii* was identified in serial cultures collected during a 15 month interval in one patient. In another patient, the same strain of an indeterminate *Bordetella* species was identified in serial respiratory tract cultures obtained during a 5 year period. These findings suggest that the prevalence of *Bordetella* infection in CF patients may be underrepresented due to misidentification of these species as closely related species such as *Achromobacter* and *Ralstonia*. The clinical significance of *Bordetella* species in CF remains to be determined.