



**International
Burkholderia cepacia
Working Group**



**International *Burkholderia cepacia* Working Group meeting
April 20 - April 23, 2006
Panoramische Zaal
Lange Kruisstraat 6
Gent, Belgium**

Thursday, April 20, 2006

18.00 h Reception City Hall, Botermarkt 1

Friday, April 21, 2006

Lange Kruisstraat 6

8.00 h - ... Registration

9.00 - 12.30 h Session I - Diversity, diagnosis & epidemiology Chair: Peter Vandamme

9.00 - 9.15 h Welcome & introduction to session Peter Vandamme

9:15 - 10.00 h Multilocus sequence analysis of *B. cepacia* complex Chris Dowson

10.00 - 10.30 h Abstracts A1-A2

10.30 - 10.50 h Break

10.50 - 12.30 h Abstracts A3-A8

12.30 - 13.30 h Lunch

13:30 - 17.00 h Session II - Diversity, diagnosis & epidemiology (ctd.); Models & virulence Chair: Tom Coenye

13.30 - 14.15 h Application of bacteria for biocontrol and plant growth promotion: scientific and administrative hurdles Johan Van Vaerenberg

14.15 - 14.45 h Abstracts A9-A10

14.45 - 15.00 h Break
15.00 - 16.30 h Abstracts A11-A15
16.30 - 17.00 h Proposals for the venue of IBCWG2007

Saturday, April 22, 2006

Lange Kruisstraat 6

9:00 - 13.00 h Session III - Clinical issues & pathogenesis Chair: Chris De Boeck

9.00 - 09.10 h Introduction to session Chris De Boeck
9.10 - 09.50 h Treatment of *B. cepacia* complex infections Andy Jones
9.50 - 10.30 h Management of *B. cepacia* complex infections Stuart Elborn

10.30 - 10.50 h Break

10.50 - 13.00 h Abstracts A16 - A24

13.00 - 14.00 h Lunch & free time

Sunday, April 23, 2006

Lange Kruisstraat 6

9:00 - 12.00 h Session IV - Genome studies Chair: Eshwar Mahenthiralingam

9:00 - 9.40 h Comparative genomics of *Burkholderia* Eshwar Mahenthiralingam

9.40 - 10.40 h Abstracts A25 - A28

10.40 - 11.00 h Break

11.00 - 12.00 h Abstracts A29 - A32
12.00 - 13.00 h Close meeting & Lunch

Peter Vandamme



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The 2006 International *Burkholderia cepacia* Working Group meeting is sponsored by:

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Abstract A1

RELIABILITY OF MULTILOCUS SEQUENCE TYPING OF THE *BURKHOLDERIA CEPACIA* COMPLEX IN CYSTIC FIBROSIS

Waine D.J., Henry D.A., Baldwin A., Honeybourne D., Mahenthalingam E., Dowson C.G.
Warwick University, Coventry, UK

Introduction

Colonization with the *Burkholderia cepacia* complex is an important cause of morbidity and mortality in cystic fibrosis. We investigated the evolutionary clock speed of the 7 genes used in the multilocus sequence typing (MLST) scheme for this bacteria.

Methods

At least 2 isolates, separated by months to years, from each of 19 patients were typed using MLST. In total 39 isolates, providing 115 isolate-years, were analyzed.

Results

14 of the 19 patients had no change in strain type over time (mean 7.37 years, range 1.09 to 14.24). 1 patient had a completely new strain, 3 patients had evidence of recombination involving one of the seven housekeeping genes, and 1 patient had evidence of a recombination event and a possible point mutation.

Conclusions

The genes do have a slow evolutionary clock speed and MLST provides a robust and reliable typing technique for CF isolates. A low rate of point mutation was found, with a higher rate of recombination events, in keeping with previous cross-sectional epidemiological data. The study also demonstrated recombination for the first time in a longitudinal in-vivo study.

Abstract A2

MULTILOCUS SEQUENCE TYPING HAS IDENTIFIED DIVERSE *BURKHOLDERIA CEPACIA* COMPLEX STRAINS FROM WATER ENVIRONMENTS THAT ARE INDISTINGUISHABLE FROM STRAINS CAUSING HUMAN DISEASE

Baldwin A.¹, Mahenthiralingam E.², Vandamme P.³, LiPuma J.J.⁴, Dowson C.G.¹

¹Dept. of Biological Sciences, Warwick University, Coventry; ²Cardiff School of Biosciences, Cardiff University, Cardiff, UK; ³Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; ⁴Dept. of Paediatrics and Communicable Diseases, University of Michigan Medical school, Ann Arbor, USA

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; thus, the environment likely acts as a reservoir for infections and outbreaks in humans.

Analysis of diverse Bcc isolates by multilocus sequence typing (MLST) has identified globally distributed *Burkholderia cepacia* complex (Bcc) strains, responsible for causing human infections that were indistinguishable from strains isolated from the environment. Though it has been widely reported that the Bcc can colonise soil environments its prevalence in water sources is less well known.

MLST analysis was performed on 20 isolates taken from water sources and resulted in the identification of 19 strains or sequence types (STs). These isolates belonged to 7 different Bcc species and 3 novel Bcc groups (BCC2, BCC3 and BCC Group K). Comparison of these environmental STs with a large Bcc MLST database (Baldwin et al., 2005) revealed that 25% of the STs found from water (1 ST from US river water and 3 STs from Belgium Schelde river water) were found to have the same ST (ST-67, 266 and 375) or closely related ST (ST-232) to strains responsible for human infection. The latter (ST-232) was found to be in a clonal complex with highly virulent ET12 strains, differing by just 2 loci. This clearly shows that water sources globally are an under-investigated reservoir for the emergence of Bcc in human infection.

We are grateful for the Wellcome Trust in funding the development and application of the Bcc MLST scheme.

Abstract A3

ASSESSMENT OF MOLECULAR METHODS FOR THE RAPID IDENTIFICATION OF *BURKHOLDERIA CEPACIA* COMPLEX (BCC)-POSITIVE SPUTA

Brown A.R., Govan J.R.W.

The Centre for Infectious Diseases, University of Edinburgh, UK

The rapid and accurate diagnosis of respiratory infections is an essential prerequisite for the early implementation of appropriate antimicrobial therapy and infection control measures. Culture-based methods with subsequent biochemical analysis and species-specific PCR offer reliable identification. However, there is an inherent time delay in such procedures, particularly if bacterial counts within sputa are low or isolates atypical. The current segregation policy for CF patients with Bcc infection does not distinguish between species. Accordingly, this study aims to assess non-culture-based methods (PCR and fluorescent in situ hybridization, FISH) for the rapid identification of Bcc-positive sputa within 2-3 hours. A single in-house PCR assay targeted against the 16S rRNA gene has been developed, based on an alignment of representative 16S rRNA sequences of all species within the Bcc. When applied to a blind study of 100 CF isolates (including Bcc and non-Bcc *Burkholderia*, *Pseudomonads* and *Burkholderia*-like organisms), this PCR assay exhibited 100% specificity for the identification of Bcc organisms. A parallel study of a commercially-available *B. cepacia* FISH assay (SeaPro Theranostics) is underway. Preliminary results suggest that whilst the FISH assay is capable of identifying members of the Bcc that are most frequently encountered in the CF lung (including *B. multivorans* and *B. cenocepacia*), certain species, including *B. stabilis*, are not identified. These findings are supported by the analysis of sequences within publicly-available databases. Attempts are underway to broaden the specificity of the FISH assay to encompass all Bcc-members without increasing the rate of false-positives. Following further optimization, the PCR and FISH assays will be applied to CF sputa, and the results compared with those obtained by conventional (culture-based) methods. By assessing rapid molecular methods for the identification of Bcc within CF sputa, this study aims to improve on existing diagnostic methods and enhance the management of Bcc infections in CF patients.

Abstract A4

Proposal for five new species within the *Burkholderia cepacia* complex.

E. Vanlaere¹, A. Baldwin², E. Mahenthiralingam³, C. G. Dowson², G. W. Payne³ and P. Vandamme¹

¹Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; ²Department of Biological Sciences, Warwick University, Coventry, UK; ³Cardiff School of Biosciences, Cardiff University, Cardiff, UK

The *Burkholderia cepacia* complex (Bcc) is an important group of organisms, containing species of ecological, biotechnological and pathogenic interest. Their taxonomy and identification are complex and are being studied through classical polyphasic taxonomic approaches and through multi-locus sequence typing (MLST). We recently demonstrated that *Burkholderia ubonensis*, a species thus far not encountered in CF specimens, represents a tenth genomovar within the complex.

Among thousands of Bcc isolates, we delineated five clusters of strains, both by polyphasic taxonomy as well as through MLST analysis that cannot be classified into one of the ten established Bcc species, and thus represent five novel Bcc species. Two of these novel species have been isolated from CF specimens only; the others have been isolated from infections in CF and non-CF patients, and from environmental sources. Although all five novel species clearly represent rarely encountered bacteria, we have designed novel *recA* gene based PCR tests for their specific identification.

Abstract A5

ISOLATION OF *BURKHOLDERIA. CEPACIA*-COMPLEX MEMBERS: FAILURE OF GROWTH ON A SELECTIVE MEDIUM

Cocchi P, Ravenni N, Taccetti G, Campana S.

Meyer Hospital, Cystic Fibrosis Centre, Dept. of Pediatrics, Florence, Italy

Highly selective media circumvent the problem of isolation of *Burkholderia cepacia* complex bacteria from respiratory samples of CF patients, while identification of Bcc members is still a challenge for clinical laboratories. The wide variability of Bcc bacteria must always carefully be evaluated in order to isolate and identify all isolates potentially belonging to the Bcc.

Our attention was focused on 22 strains, grown on Mac Conkey Agar (MCK), isolated from respiratory samples of 15 patients being followed at the Tuscany CF Centre. These strains had an uncommon phenotype and were not identified by biochemical tests.

We found using the 16S rDNA sequencing a powerful tool for the identification of unusual bacteria isolated from CF respiratory samples.

We amplified a 1000-bp portion of the 16S rRNA gene as described before, then we determined the nucleotide sequence and matched it with a public database (NCBI).

Five out of 22 isolates (22.7%) belonged to the Bcc, as confirmed by *recA*- based PCR and RFLP assays, and were grown on MCK but not on *Burkholderia cepacia* Selective Agar (BCSA). One of these strains belonged to *B. multivorans*, while the genomovar *status* of the other strains remains unknown. These isolates showed a characteristic flavoured odour and wax-like colony morphology.

In conclusion some Bcc members, one *B. multivorans* and 4 strains with undetermined genomovar, failed to grow on a highly selective medium such as BCSA suggesting that the prevalence of Bcc could be underestimated. These findings may have important implications in prevention and management of Bcc pulmonary infections in CF patients.

Abstract A6

USE OF RAMAN SPECTROSCOPY FOR THE IDENTIFICATION OF *BURKHOLDERIA SPP*

Willemse H.F.M.¹, Maquelin K.¹, Scholtes M.J.¹, Vandamme P.A.R.², van Belkum A.³, Puppels G.J.¹

¹Erasmus MC, Center for Optical Diagnostics and Therapy, Rotterdam, The Netherlands ; ²Ghent University, Laboratorium voor Microbiologie, Ghent, Belgium ; ³Erasmus MC, Dept. of Medical Microbiology & Infectious Diseases, Rotterdam, The Netherlands

In CF patients pulmonary infections with organisms from the *Burkholderia cepacia* complex (Bcc) have a considerable impact on clinical outcome and may lead to a fatal pneumonia known as cepacia syndrome. Members of the Bcc are well recognized as nosocomial and outbreak related pathogens and are able to colonize CF patients.

Species identification of Bcc organisms can be obtained with phenotypic methods such as automated commercial systems. These systems perform identification with varying but insufficient accuracy. In general, no differentiation can be made between the different species within the Bcc so further identification with expensive and laborious molecular methods is required.

A powerful and inexpensive tool for the identification of microorganisms is Raman spectroscopy. Vibrational spectra obtained with this technique are highly specific and reflect the overall molecular composition of a sample. They can serve as spectroscopic fingerprints and enable the accurate identification of microorganisms. Furthermore, results can be obtained the same day the initial culture is available.

To evaluate the usefulness of Raman spectroscopy for the diagnosis of *Burkholderia spp*, a panel of 50 well-characterized clinical strains was used. The complete analysis was reproduced in three independent sessions.

Using Raman spectroscopy rapid discrimination between *Burkholderia spp.* and related organisms is 94 % accurate and separation of species within and outside the complex is of similar accuracy. Rates of correct identification of Bcc isolates by automated systems such as the BD Phoenix (Becton Dickinson) and the VITEK2 (bioMerieux) were 73 % and 78 % respectively.

The highest clinical relevance lies in the discrimination of *Burkholderia cenocepacia* and *Burkholderia multivorans*, since these are the most prevalent and probably the most virulent species within the complex. Raman spectroscopy identified 7 out of 9 *B. cenocepacia* strains (77.8 %) and 8 out of 9 *B. multivorans* strains (88.9 %) correctly.

These first results indicate that Raman spectroscopy is an accurate and reproducible method to identify microorganisms frequently found in CF patients and to discriminate clinically relevant species within the *B. cepacia* complex.

Abstract A7

Use of MALDI-TOF mass spectrometry for a rapid identification of *Burkholderia cepacia* complex.

E. Vanlaere¹, K. Sergeant², P. Dawyndt¹, B. Samyn², B. Devreese² and P. Vandamme¹.

¹Laboratory for Microbiology, University of Gent, Gent, Belgium; ²Laboratory of Protein Biochemistry and Protein Engineering, University of Gent, Gent, Belgium

The identification of organisms cultured from respiratory specimens obtained from CF patients is not straightforward and misidentifications occur often. We evaluated the identification of *Burkholderia cepacia* complex (BCC)-like organisms using Matrix Assisted Laser Desorption/Ionisation Time Of Flight mass spectrometry (MALDI-TOF MS) of intact microbial cells. In this technique, laser light vaporizes large biomolecules of intact cells achieving free gas-phase ions that were separated by their mass-to-charge (m/z) ratios. The data are recorded as mass spectra. We investigated 60 BCC strains and 40 non-BCC strains representing 20 species that are commonly misidentified as BCC. Colonies grown for 24 h were suspended in 0.5 ml 0.1 % TFA, 2 µl of which was mixed with 2 µl matrix solvent (49 ACN: 49 Isopropanol: 2 0.1% TFA saturated with alpha-cyano). Subsequently 1 µl of this mixture was spotted onto the sample slide and allowed to air dry prior to analysis.

We obtained bacterial mass spectra in the m/z range 2-18 kDa. The data were analysed by Bionumerics 4.0 software and subjected to a cluster analysis using Pearson's correlation coefficient. Except for *Burkholderia gladioli* and some *Ralstonia* sp., all reference species were easily distinguished from BCC. Most strains belonging to BCC species grouped in species specific clusters, except for *Burkholderia pyrrocinia* and *Burkholderia anthina* that constitute a single cluster. The same was observed for most of the other taxa examined. In the mass spectra some peaks could be used as biomarkers to distinguish between species or even strains. Moreover, this method is quick, easy to perform and rather cheap.

These data suggest that MALDI-TOF MS of intact cells could be a powerful tool for the rapid identification of BCC-like organisms recovered from CF patients.

Abstract A8

MOLECULAR CHARACTERIZATION AND ENVIRONMENTAL DISTRIBUTION OF THE *BURKHOLDERIA CENOCEPACIA* B&B CLONE

Graindorge A.¹, Menard A.¹, Bouvet C.², Miollan R.², Gaillard S.³, de Montclos H.⁴, Laurent F.^{1,4}, Cournoyer B.¹

¹Research group on Bacterial Opportunistic Pathogens and Environment, UMR CNRS 5557 Ecologie Microbienne, Université Lyon 1, Villeurbanne ; ²Unité Départementale de Lutte contre les Infections Nosocomiales (UDLIN) ; ³Service de Réanimation (ICU); ⁴Laboratoire de Microbiologie, Centre Hospitalier Fleyriat, Bourg-en-Bresse, France

A *Burkholderia cepacia* complex (Bcc) nosocomial outbreak occurred between February and July 2004 at the intensive care unit of the Fleyriat Hospital Center (France, near Lyon). Bcc strains were isolated from seven ventilated patients, and were found involved in respiratory tract infections, with one infection which unquestionably led to death of a patient. High Bcc-like bacterial counts were observed in this last instance, with numbers as high as 10⁷ CFU per ml found in the bronchoalveolar lavage fluid. In this talk, we will present a summary of the data obtained so far on the characterization of the Bcc strains collected during the outbreak. We will also present data concerning the environmental origin and dissemination of these strains. The strains were analysed by phenotypic and molecular methods enabling their identification as *B. cenocepacia*. *recA* PCR-RFLP (restriction fragment length polymorphisms) analyses of the strains revealed profiles matching those of the *B. cenocepacia* IIIA subgroup, which is well-known to include the most virulent Bcc strains. PCR screenings of most if not all virulence genes and epidemic markers characterized so far in the Bcc were performed. Interestingly, the *cblA* gene was not detected but all strains harboured the *B. cepacia* epidemic-strain marker known as BCESM. *SpeI*-PFGE (pulsed field gel electrophoresis) analyses were performed and showed all strains from the patients to belong to an unique epidemic clone, which was named B&B (standing for Bourg-en-Bresse). Hospital reservoirs of the B&B clone were analysed. During the outbreak, B&B strains could be recovered from the inspiratory and expiratory branches of respiratory circuits, from water reservoirs of incubator humidifiers, from tap water, and from antiseptic products and their distributors. Soil and water environments including wastewater treatment plants in the area of the hospital are being currently analysed for the presence of the B&B clone and other *B. cenocepacia* strains. A summary of the data obtained so far about the environmental population of *B. cenocepacia* found in the Bourg-en-Bresse area will be presented.

Abstract A9

INTEREST OF INTRODUCING BACTERIAL PARTNERS OF CULTIVATED PLANTS FROM THEIR COUNTRY OF ORIGIN: THE CASE OF MAIZE ASSOCIATED BURKHOLDERIA TROPICA

Guyon S., Flattin M., Balandreau J.

Ecologie Microbienne, UMR5557 CNRS-Université Cl. Bernard Lyon1, Bâtiment G. Mendel, Villeurbanne, France

Maize has been domesticated from Teosintle in Mexico some 7000 years ago. Teosintle and maize cultivated in the ancient way often host large numbers of a nitrogen fixing endophyte, *Burkholderia tropica*. In spite of many attempts, this bacterium has not been found in French soils, nor in maize grown in France, where it can be replaced by the undesirable *B. cenocepacia* species, a threat for Cystic Fibrosis affected people. Seeds of maize are devoid of *Burkholderia*. It is hypothesized that maize has been exported to Europe as seeds without its adapted bacterial partner. The work reported here is a preliminary investigation of the possible interest of an introduction of *Burkholderia tropica* in maize cultivation outside Mexico. Two *B. tropica* strains, BM16 and BM273, isolated from Mexican traditional maize were used to inoculate the modern CV Centena, under security green house conditions. Plant colonization was monitored using traditional techniques (CFU counts on a selective medium) and molecular techniques, using a *B. tropica* specific set of primers. Both techniques confirm the absence of this bacterial species in uninoculated control treatments. Both techniques showed a quick colonization of the plants, first at the root level, then at the shoot level. Colonization is both external and internal. Bacteria after a month can be shown by direct PCR amplification of extracted plant DNA more readily than by CFU counts, suggesting that the bacteria could enter a viable but non culturable state. Inoculation does not increase the total number of *Burkholderia* but, in inoculated plants *B. tropica* represents a very large majority of *Burkholderia* present. Moreover, inoculation increased plant biomass very much. A field experiment showed the same growth stimulation (+28% over control in fresh shoot biomass at day 47). Both effects, viz. plant growth promotion and decrease in hazardous *Burkholderia* species, are two important benefits supporting the proposal to reintroduce its normal bacterial partner into maize.

Abstract A10

DEVELOPMENT AND VALIDATION OF A PROTOTYPE 16S rRNA-BASED TAXONOMIC MICROARRAY FOR MEMBERS OF THE GENUS *BURKHOLDERIA*

Schönmann S., Wimmersberger C., Loy A., Vandamme P., Sobek J., Eberl L.

Dept. of Microbiology, University of Zurich, Switzerland

Members of the genus *Burkholderia* are ubiquitously distributed in nature and have been isolated from soil, water, the rhizospheres of various plants, industrial settings, hospital environments and from infected humans. *Burkholderia* strains have an enormous biotechnological potential and have been used for bioremediation of recalcitrant xenobiotics, plant growth promotion, and biocontrol purposes. However, *Burkholderia* strains have also emerged as opportunistic pathogens of humans, particularly those with cystic fibrosis.

The aim of this project is the development of a 16S rRNA-based taxonomic microarray that allows a rapid and reliable identification of isolates as well as an assessment of the *Burkholderia* diversity in natural samples. To this end, a prototype *Burkholderia* phylochip was designed and manufactured. It is composed of 152 probes that cover the entire known diversity of the genera *Burkholderia* and *Pandorea* and target 16S rRNA sequences at various taxonomic levels, ranging from higher phyla to species. The probes are currently validated with a representative set of reference strains. For amplification of 16S rRNA genes novel primer sets were designed that were shown to specifically amplify genes from all *Burkholderia* reference strains but not from closely related *Ralstonia* species. The hybridization conditions were optimized with respect to an optimal signal-noise ratio and to limit false positive signals (less than 4% of the designed probes).

Ongoing work assesses the applicability, sensitivity, and detection limits of the phylochip for the analysis of both clinical and environmental samples. Microarray data will be compared to results obtained from sequencing of respective clone libraries.

Abstract A11

THE EFFECT OF COLONY MORPHOLOGY ON *BURKHOLDERIA CENOCEPACIA* VIRULENCE

Bernier S.P., ¹ Cardona S.T., ² Bouvier M., ¹ Drevinek P., ³ Mahenthiralingam E., ³ Valvano M.A., ² and Sokol P.A.¹

¹ University of Calgary Health Sciences Centre, Faculty of Medicine, Department of Microbiology and Infectious Diseases, Calgary, Alberta, Canada.

² The University of Western Ontario, Department of Microbiology and Immunology, Infectious Diseases research Group, London, Ontario, Canada.

³ Cardiff School of Biosciences, Cardiff University, Cardiff, Wales.

Bacteria are able to adapt quickly to different environments by triggering responses that may be beneficial for their survival and increase their fitness. One of these adaptive strategies is the modification of their cell surface which is evident by different colony morphotypes. When grown on regular Luria Bertani agar medium, *Burkholderia cenocepacia* strains exhibit two types of colony morphology, rough and shiny. When rough strains of *B. cenocepacia* are grown in liquid culture and on alfalfa seedlings, shiny colony variants appear randomly for most of the strains tested. Ninety-three K56-2 shiny variants obtained via liquid cultures were collected and clustered using high throughput phenotypic characterization of the following phenotypes: biofilm formation, pellicle formation, Congo red binding, protease activity, AHL production, swarming and swimming motility, and virulence in the alfalfa infection model. Most shiny variants were deficient in biofilm formation and virulence in alfalfa. Variation in protease activity, AHL production, and virulence in alfalfa were used as parameters for clustering the shiny variants into fifteen groups. Representative variants from seven of the fifteen groups were selected for further analysis. Transmission electronic microscopy demonstrated that the extracellular matrix surrounding bacterial cells was absent or reduced in shiny variants compared to their rough wild type. Three shiny variants were tested for virulence in the agar bead model and were determined to produce significantly less histopathology than wild type K56-2. Expression of some virulence traits was shown to be altered in these variants likely contributing to the decreased virulence. Some shiny variants also had reduced virulence in the *Caenorhabditis elegans* model. The *B. cenocepacia* K56-2 shiny variants had identical PFGE patterns compared to the rough wild type suggesting that it is unlikely that chromosomal rearrangements are involved in the cell surface modifications. Studies are in progress to determine mechanism(s) leading to this phenotypic variation which frequently leads to a less virulent phenotype in at least one infection model.

Abstract A12

RICE PLANT MODEL TO STUDY *BURKHOLDERIA* PATHOGENESIS

Venturi V., Bertani I., Degrassi G., Devescovi G., Ferluga S., Solis S., Steindler L.

Bacteriology Group, International Centre for Genetic Engineering & Biotechnology, Trieste, Italy; Plant Bacteriology Group, International Centre for Genetic Engineering & Biotechnology, Biosafety Outstation, Ca' Tron di Roncade, Treviso, Italy

The two species *Burkholderia glumae* and *Burkholderia plantarii* are very close phylogenetically to the BCC complex and are rice pathogens. *B. plantarii* is a seed- and soil-borne plant pathogen which causes rice seedling blight in rice nursery boxes whereas *B. glumae* causes panicle blight, grain and seedling rot especially when temperatures and humidity are high. We have initiated studies on these organisms with the aim of understanding molecular pathogenic mechanisms used to infect rice and possibly also relate them to members of the BCC complex. At present we are using the type strain of each species and have identified and created knock-out mutants in the global regulatory systems of quorum sensing and of the stationary phase RpoS sigma factor. Having performed pathogenicity assays (seedling blight with *B. plantarii* and grain rot for *B. glumae*) it was observed that both regulatory mechanisms are involved in causing seedling blight whereas they did not play a major role in causing grain rot. In *B. glumae*, we also purified and identified the corresponding genetic determinants of two secreted endo-polygalacturonases. These enzymes are involved in the de-polymerization of pectin and could play a role in grain rot. The two enzymes are isozymes and only when both are inactivated a decrease of grain rot is observed. At present we are investigating how the production of the two enzymes is regulated. We are now also analyzing pathogenic isolates of both species isolated from rice in different geographic locations around the world.

Abstract A13

CLINICAL AND ENVIRONMENTAL *BURKHOLDERIA CENOCEPACIA* STRAINS: EVALUATION OF PATHOGENICITY BY IN VITRO AND IN VIVO MODELS

Pirone L.^{1,2}, Bragonzi A.³, Auriche C.², Chiarini L.¹, Conese M.³, Ascenzioni F.², Bevivino A.¹

¹ENEA C. R. Casaccia, Dept. of Biotechnologies, Rome ; ²Dept. of Cellular Biology and Development, University of Rome "La Sapienza", Rome; ³Institute for Experimental Treatment of Cystic Fibrosis, HS Raffaele Scientific, Milan, Italy

Corresponding author: Annamaria Bevivino, ENEA Casaccia Research Center, Department of Biotechnologies, Protection of Health and Ecosystems, Plant Genetics and Genomics Section

Via Anguillarese 301, 00060 S. Maria di Galeria, Rome, Italy

Email address: annamaria.bevivino@casaccia.enea.it

Burkholderia cenocepacia is an important opportunistic human pathogen, particularly in persons with cystic fibrosis (CF) and has also been isolated from natural environments. Challenging questions to clinical and environmental microbiologists are whether exist differences in the pathogenicity among *B. cenocepacia* strains of different origin. In the present study, we determined the invasion and infection capacity of clinical and environmental *B. cenocepacia* strains, by means of in vitro cellular CF models and a murine model for chronic infection. First, we investigated the distribution of *B. cenocepacia* genomic island genes as well as phenotypic traits and genetic markers related to virulence and transmissibility. We observed that some of the candidate determinants are not confined solely to clinical isolates but are also found among environmental isolates. Results obtained from the in vitro infection assays revealed that clinical *B. cenocepacia* strains were able to invade both non-CF and CF epithelial cells of pulmonary origin, with a higher level of internalization in the latter ones. Similarly, the environmental *B. cenocepacia* strains showed a higher level of internalization in CF cells, although to a lesser extent than clinical strains. As in vivo model, we chose an established and well characterized mouse agar bead model for chronic infection. We found differences in the percentage of mortality and infected mice between clinical and environmental strains. Although the environmental strains cannot be classified as the most virulent strains, they were able to chronically infect the murine lung.

Overall, these data demonstrate the pathogenic potential of environmental isolates and indicate that *B. cenocepacia* virulence could not be related to the *recA* lineage status. These studies suggest that potential reservoirs outside of clinics might play a role in the acquisition of infections and reinforce the idea that clinical strains are not specialized pathogens but, rather, environmental strains able to infect CF patients with the traits acquired to survive in natural ecosystems.

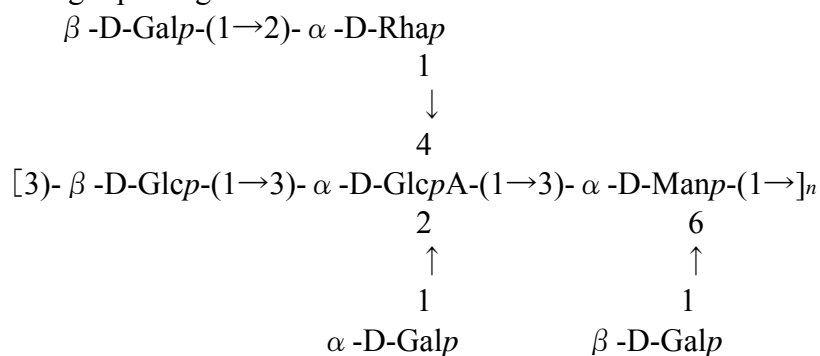
This work was supported by a grant from Italian Cystic Fibrosis Research Foundation (*ffc*), Verona, Italy (FFC #11-2004).

Abstract A14

THE AGGREGATION PROPERTIES OF CEPACIAN AND ITS PRESENCE IN BIOLOGICAL SAMPLES

Cescutti P., Herasimenka Y., Rizzo R.
Department of Biochemistry, Biophysics and Macromolecular Chemistry
University of Trieste, Trieste, Italy

Following the research line on the investigation of the structure-function relationships of exopolysaccharides (EPS) produced by strains of the *Burkholderia cepacia* complex (BCC), we have investigated the conformational and macromolecular properties of cepacian, the EPS produced by the majority of environmental and clinical isolates. The polysaccharide has the following repeating unit:



The rhamnose in the D absolute configuration and the completely substituted glucuronic acid residue are two unusual characteristics that introduce constraints in the conformational properties.

Although it was described that EPS production was not the major requirement for BCC biofilms, the presence of the polysaccharide was found to contribute in terms of matrix thickness. Therefore, the assessment of EPS ability to aggregate and to form viscous solutions is an important item to understand biofilm formation. Our results showed that cepacian formed highly viscous solutions in water, while addition of dimethylsulfoxide (DMSO) caused a drastic decrease in viscosity. DMSO is a chaotropic solvent that disrupts polysaccharide aggregation by weakening intermolecular hydrogen bonds. Hence, the decrease of intrinsic viscosity as a function of DMSO addition is a result of polymer disaggregation. At the same time Atomic Force Microscopy (AFM) experiments were recorded on samples deposited both from water and from DMSO solutions. The microimages of the polymeric strands showed larger diameters when obtained from water with respect to DMSO. Molecular modelling investigations revealed an intense network of inter-residues hydrogen bonding, which are responsible, together with the 1-3 glycosidic linkages of the backbone, of the formation of a rigid and rather elongated chain, a character compatible with the formation of aggregates.

We recently started to investigate the presence of cepacian in CF patients sputum samples. Although the high viscosity of the samples, the abundant glycosylation of the mucin and the difficulty in finding suitable negative controls somewhat impeded the obtainment of definitive results, the data collected strongly pointed at the presence of cepacian in the biological samples.

Abstract A15

BURKHOLDERIA CENOCEPACIA UTILIZES FERRITIN AS AN IRON SOURCE

Whitby P.W., VanWagoner T.M., Morton D.J., Seale T.W., Sokol P.A., Stull T.L.

University of Oklahoma Health Sciences Center, Dept. of Pediatrics, Oklahoma City, USA

Burkholderia cenocepacia is a member of the *Burkholderia cepacia* complex, a group of genetically similar species that inhabit a number of environmental niches including the lungs of patients with cystic fibrosis. To colonize the lung this bacterium requires a source of iron to satisfy the nutritional requirements for this important metal. Because of the high potential for damage in lung tissue resulting from oxygen-iron interactions, this metal is sequestered by a number of mechanisms that render it potentially unavailable to invading microorganisms. Such mechanisms include the intracellular and extracellular presence of the iron binding protein ferritin. Ferritin has a highly stable macromolecular structure and may contain up to 4,500 iron atoms per molecule. To date, there has been no known report of a pathogenic bacterial species that directly utilizes iron sequestered by this macromolecule. To examine the ability of ferritin to support growth of *B. cenocepacia* J2315, iron deficient media was supplemented with varied concentrations of ferritin and the growth kinetics characterized over a 40 hr period. Our results indicated that *B. cenocepacia* J2315 utilizes iron bound by ferritin. Further studies examining the mechanisms of iron uptake from ferritin indicated that iron utilization results from a proteolytic degradation of this otherwise stable macromolecular structure. Since it is known that the ferritin concentration is significantly higher in the cystic fibrosis lung than in healthy lungs, this novel iron acquisition mechanism may contribute to the pathogenesis of *Burkholderia cepacia* infections in people with cystic fibrosis.

Abstract A16

BACTERAEMIA WITH BURKHOLDERIA CEPACIA IN CF

Malfroot A.¹, De Wachter E.¹, De Schutter I.¹, Wybo I.², Pierard D.², Lauwers S.²

¹CF Centre, ²Dept. of Microbiology AZ-VUB- Brussels, Belgium

The last decade several Gram-negative non-fermenting bacilli (GN-NFB) other than *Pseudomonas aeruginosa* are newly described in CF, some as highly transmissible pathogens causing rapid respiratory deterioration. *B. cepacia* complex is best documented. CF patients are chronically infected with Gram-positive or negative organisms, receive multiple antibiotics, however bloodstream infections are uncommon in immunocompetent CF patients. Bloodstream infections with Gram-negative non-fermenting bacilli may be increasing as reported also in haematology and intensive care units, in immunocompromised patients.

Methods

A total of 129 pediatric and adult patients are treated in our centre, 26.2% are colonized by *P. aeruginosa*, *B. cepacia* is present in only 4 patients. If required for frequent intravenous antibiotic courses, patients receive a central venous access device or Porta-catheter PAC (BARD Company). Strict sterile manipulation is instructed and mandatory. Sputum samples are grown on conventional media and *B. cepacia* selective medium (Mast Diagnostics, UK). Blood cultures are performed in patients with persisting fever or chills despite correct antibiotic treatment.

Results

All blood cultures remained negative, except in 2 patients: a 29 yrs old male and a 27 yrs old female both on a lung transplantation waiting list. Both showed *B. cepacia* genomovar III in their sputum cultures, and had a BARD PAC. *B. cepacia* was cultured repeatedly from their blood samples, taken at a peripheral vein as well as in the PAC system. Moreover *B. cepacia* was isolated again in one patient after removal of the PAC and replacement of another PAC at the contra lateral site of the chest. The male patient died before transplantation, the female CF died 2 weeks after successful transplantation of intractable *B. cepacia* septicaemia. *P. aeruginosa* was never recovered in any of the CF blood samples, even in patients with longstanding intravascular PAC and end-stage respiratory failure.

Discussion

GN-NFB septicaemia may be increasing in CF due to better identification techniques, older ages, aggressive treatment and the use of long-term catheters. Use of PAC in CF is probably most prolonged compared to patients with malignant disease, whose duration of survival is often short, or in whom it can be removed after complete cure. In CF, GN-NFB septicaemia may increase mortality. Moreover, it may compromise transplantation. It is unclear why never *P. aeruginosa* or *S. aureus* but only GN-NFB blood infections were seen in these immunocompetent CF patients.

Abstract A17

THE EPIDEMIOLOGY OF *BURKHOLDERIA* SPECIES INFECTION AT THE MANCHESTER ADULT CYSTIC FIBROSIS CENTRE

France M.¹, Dodd M.E.¹, Govan J.R.², Doherty C.J.², Webb A.K.¹, Jones A.M.¹

¹Manchester Adult Cystic Fibrosis Centre; ²Medical Microbiology, University of Edinburgh, United Kingdom

Introduction

We have recorded all cases of *Burkholderia* species infection at our centre since 1983. We have had a policy of microbiological surveillance for cross-infection with *Burkholderia* species at our CF centre since 1991 and all isolates are sent to a reference microbiology laboratory for strain typing. We introduced partial segregation in 1991 and full segregation in 1993 for CF patients with *Burkholderia* species infection. All isolates available in the repository were genomovar typed in 2001, including retrospective typing of previously stored isolates. All subsequent new isolates since 2001 have been genomovar typed. We have a policy of sending sputum for culture, including on selective cepacia media, at each clinic visit and for all inpatients stays.

Methods

We reviewed the results of strain and genomovar typing of all available *Burkholderia* isolates at our centre from 1983-2005.

Results

The incidence/prevalence of infection with of *Burkholderia* species between 1983-1990 was below 5% / 9% each year. There was a rise in incidence/prevalence of *Burkholderia* species between 1991 to 1994 with a peak of 15.6% / 31.2% in 1992. Following full segregation the incidence has fallen to below 3% for all but one year and the prevalence has gradually reduced to 9.3% in 2005. In the past 11 years since segregation there have been 6 cases of infection with epidemic *B. cenocepacia* strains and 13 with unique strains of *Burkholderia* species. In the past 6 years, there has been just 1 case of new infection with an epidemic *B. cenocepacia* strain and 9 cases of new infections with unique strains of *Burkholderia* species within the centre.

Conclusions

Segregation has controlled spread of epidemic *B. cenocepacia* strains. Unique strains of *Burkholderia* species now account for the majority of new infections at the Manchester Adult Cystic Fibrosis Centre.

Abstract A18

COMPARATIVE *IN VITRO* ACTIVITY OF TEMOCILLIN, MEROPENEM, CEFTAZIDIME AND PIPERACILLIN/TAZOBACTAM AGAINST PANEL STRAINS AND CLINICAL ISOLATES OF *BURKHOLDERIA CEPACIA* COMPLEX FROM 9 DIFFERENT GENOMOVARS

Carryn S.¹, Tulkens P.M.¹, and Vandamme P.²

¹Unité de pharmacologie cellulaire et moléculaire, Université catholique de Louvain, Brussels, Belgium; ²Laboratorium voor Microbiologie, Faculteit Wetenschappen, Universiteit Gent, Gent, Belgium

Purpose

B. cepacia complex (Bcc) infection in cystic fibrosis (CF) patient is associated with increased morbidity and mortality. Temocillin (TMO), a semisynthetic 6- α -methoxy β -lactam, has already been successfully used in a pilot study for the treatment of pulmonary infections in CF patients infected with Bcc. We determined the susceptibility of well characterized panel strains and clinical isolates of Bcc to TMO in comparison with 3 other β -lactams used in CF patients: meropenem (MER), ceftazidime (CTZ), and piperacillin/tazobactam (PTZ).

Methods

The MICs were measured by microdilution in Mueller-Hinton broth using the CLSI standard method. *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were included as control strains. CLSI breakpoints for Enterobacteriaceae were used for MER, CTZ, and PTZ and that of Fuchs *et al.* (1985 Eur J Clin Microbiol 4:30-33) for TMO.

Results

The table below shows the MIC50 and MIC90 obtained on 100 Bcc strains representing 9 genomovars (n= 30 and 35 for *B. multivorans* and *B. cenocepacia*, respectively, and 5 for genomovars I, and IV to IX).

β -lactam	MIC50 μ g/ml	MIC90 μ g/ml	Breakpoint μ g/ml	% susceptibility %
TMO	8	32	16	81
MER	4	16	4	66
CTZ	4	> 128	8	70
PTZ	16	> 128	16	51

The susceptibility pattern was similar among the different genomovars. Interestingly, 7 strains were susceptible only to TMO, while 6/35 *B. multivorans* and 7/30 *B. cenocepacia* were resistant to all the antimicrobials tested.

Conclusion

TMO was active against more strains than any of the 3 other comparators. Combined with the results of the clinical pilot studies, our data suggest a potential therapeutic use for TMO in CF patients infected with *B. cepacia* complex.

Abstract A19

RESISTANCE OF *BURKHOLDERIA CEPACIA* COMPLEX BIOFILMS TO DISINFECTANTS

Peeters E., Bruneel S., Nelis H.J., Coenye T.

Laboratorium voor Farmaceutische Microbiologie, Universiteit Gent, Gent, Belgium

Burkholderia cepacia complex (Bcc) bacteria are opportunistic pathogens that are intrinsically resistant to many antimicrobial agents. Apart from the availability of a limited number of antibiotics for the treatment of Bcc infected CF patients, problems regarding the efficacy of disinfectants for removing and/or killing of these microorganisms have also been reported. It is well known that biofilm cells are often more resistant to antimicrobial agents than planktonic cells. Hence the biofilm-forming capability of Bcc bacteria may thus 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. In a first phase we developed and optimised various assays to quantify biofilm biomass in microtiter plates. Subsequently we used these assays to compare the efficacy of various antimicrobial treatments.

Using three different staining techniques we can now rapidly assess the effect of any given antimicrobial agent on the total biofilm biomass (using crystal violet staining), on the total number of viable cells in the biofilm (using fluorescein diacetate staining) and on the amount of extracellular matrix formed (using DMMB staining), in 96-well microtiter plates. This set-up allows high-throughput initial screening of antimicrobial agents. The European Suspension Test (EST) was chosen to determine the effect of disinfectants on planktonic cells, whereas the microtiter plate methods described above were used to evaluate the effect of these agents on cells in biofilms. Our initial results show that several commonly used disinfectants (including sodium hypochlorite) show significantly reduced activity towards Bcc biofilms, compared to planktonic cells. This treatment failure can have important consequences for infection control in CF. Further investigations considering the possibility of regrowth of Bcc biofilms after an antimicrobial treatment are ongoing.

Abstract A20

COMPARISON OF ANTIBIOTIC SUSCEPTIBILITY OF *BURKHOLDERIA CEPACIA* COMPLEX (BCC) STRAINS WHEN GROWN PLANKTONICALLY OR AS A BIOFILM *IN VITRO*

Caraher E.M.^{1,2}, Reynolds G.¹, Murphy P.^{3,4}, McClean S.^{1,2}, Callaghan M.^{1,2}

¹Dept. of Applied Science, ITT-Dublin, Tallaght, Dublin 24, Ireland; ²National Institute of Cellular Biotechnology; ³The Adelaide, Meath Incorporating the National Children's Hospital (AMNCH), Tallaght, Dublin 24, Ireland; ⁴Dept. of Clinical Microbiology, Trinity College, Dublin 2, Ireland

The *Burkholderia cepacia* complex (Bcc) is a group of highly problematic pathogens in the Cystic Fibrosis (CF) community that are associated with an increase in morbidity and mortality in these patients. Bcc is innately resistant to antibiotics thus antibiotic therapies used in an attempt to eradicate the infection are usually ineffective. The objectives of this study were to determine the antibiotic susceptibility of three species of Bcc and to compare whether susceptibility patterns vary depending on the mode of growth of bacterial culture, i.e. comparing planktonic cultures to biofilm cultures. Using a modified method to determine biofilm susceptibility to antibiotics, we assessed the susceptibility of six strains representative of three different species of Bcc, *B. multivorans*, *B. cenocepacia* and *B. dolosa* and compared the results to conventionally determined MICs. The biofilm inhibitory concentrations (BIC) were considerably higher than the corresponding MICs when the strains were incubated with either meropenem or piperacillin-tazobactam but were comparable for amikacin, tobramycin, azithromycin, ceftazidime and ciprofloxacin. Treatment with colistin was ineffective at killing Bcc regardless of mode of growth.

B. dolosa was less susceptible to most of the antibiotics tested compared to the other two species. The data presented here suggest a re-evaluation of the appropriate uses of conventional susceptibility testing and a consideration that biofilm susceptibility testing may be more clinically appropriate for determining antibiotic therapy for CF Bcc infections.

Abstract A21

INTRACELLULAR SURVIVAL OF *BURKHOLDERIA CENOCEPACIA* IN MACROPHAGES

Valvano M.A., Lamothe J., Maloney K.E.

Dept. of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada

Strains of the *Burkholderia cepacia* complex (Bcc) are opportunistic bacteria that can cause life-threatening infections in patients with cystic fibrosis and chronic granulomatous disease. Previous work in our laboratory has shown that Bcc isolates can persist in membrane-bound vacuoles within amoeba and macrophages without bacterial replication, but the detailed mechanism of bacterial persistence is unknown. In this study, we have investigated the survival of the *B. cenocepacia* strain J2315 within RAW264.7 murine macrophages. Strain J2315 is a prototypic isolate of the widespread and transmissible ET12 clone. We have shown that unlike heat-inactivated bacteria, live *B. cenocepacia* J2315 survived for up to 6 h post-infection in vacuoles that accumulated LAMP1 and LAMP2 but did not fuse with lysosomes. Using fluorescent fluid phase probes, we also demonstrated that *B. cenocepacia*-containing vacuoles (BcCVs) continued to interact with early endosomes, and maintained a luminal pH of 6.4 ± 0.12 . In contrast, vacuoles containing heat-inactivated bacteria had an average pH of 4.8 ± 0.03 and rapidly merged with lysosomes. Additional experiments using concanamycin A, a specific inhibitor of the vacuolar H⁺-ATPase, revealed that vacuoles containing live bacteria did not exclude the H⁺-ATPase. This mode of bacterial survival did not require type III secretion, as no differences were found between wild type and a type III secretion mutant strain.

In a previous study, we employed signature-tagged mutagenesis and an agar bead model of chronic pulmonary infection in rats to identify *B. cenocepacia* genes that are required for bacterial survival *in vivo*. One of the most significantly attenuated mutants had an insertion in the *mgtC* gene, which encodes a membrane protein of unknown function. We now show that *mgtC* is also needed for growth of *B. cenocepacia* in magnesium-depleted medium and bacterial survival within murine macrophages. Using fluorescence microscopy we show that *B. cenocepacia mgtC* mutants, unlike the parental isolate, co-localize with the fluorescent lysosomal probe LysoTracker Red. By 2 h post-infection, *mgtC* mutants expressing monomeric red fluorescent protein cannot retain the protein within the bacterial cytoplasm.

Collectively, our results suggest that Bcc may delay maturation to prevent rapid fusion with lysosomes, persisting longer in early phagosomes, and thereby escaping and/or at least temporarily avoiding the microbicidal activities of the host cell. Furthermore, the *mgtC* mutant fails to induce delayed phagolysosomal fusion, resulting in BcCVs rapidly targeted to lysosomal compartments, where intracellular bacteria are destroyed, suggesting the MgtC protein plays a critical role in promoting the survival of *B. cenocepacia*.

Abstract A22

RESPONSE OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS TO THE CYSTIC FIBROSIS PATHOGENS *BURKHOLDERIA MULTIVORANS* AND *BURKHOLDERIA CENOCEPACIA*

MacDonald K.L., Speert D.P.

University of British Columbia, Dept. of Microbiology and Immunology, Vancouver, Canada

Burkholderia cepacia complex (BCC) bacteria are responsible for pulmonary infections which can evolve into fatal overwhelming septicemia in chronic granulomatous disease or cystic fibrosis (CF) patients. BCC is a putative intracellular pathogen, as inferred from its survival in murine or human cultured epithelial and macrophage cell lines and its persistence in amoebae and murine models.

Dendritic cells (DCs), one of the resident lung phagocytes, are crucial cells linking innate and adaptive immunity; as such they may serve as systemic vectors for this putative intracellular pathogen. We hypothesize that BCC modulates the normal functions of primary human monocyte-derived DCs, to subvert antibacterial mechanisms and persist intracellularly. Based on their clinical importance in CF and murine model experimental data, *B. multivorans* and *B. cenocepacia* were chosen for these experiments. Peripheral blood mononuclear cells from healthy adults were isolated by Ficoll-Paque density gradient centrifugation. After lymphocyte depletion, monocytes were cultured in granulocyte-macrophage colony stimulating factor and interleukin-4 and differentiated into DCs. Non-opsonic host-pathogen interactions were characterized using a microscopy based association assay to measure bacterial binding and uptake into DCs. Cytochalasin D was used to inhibit actin-mediated uptake, enabling differentiation between binding and phagocytosis. After two hours, both *B. multivorans* and *B. cenocepacia* were bound and ingested by DCs, and avoided inducing host cell cytotoxicity, as assessed by lactate dehydrogenase release. Effects of the bacteria on maturation of DCs from phagocytic cells to antigen presenting cells were determined using flow cytometry. It appeared that *B. cenocepacia*, but not *B. multivorans*, impaired DC maturation; DCs co-incubated for 24 hours at a low multiplicity of infection with *B. cenocepacia*, but not *B. multivorans*, had reduced expression of maturation and co-stimulatory markers when compared with standard BCC lipopolysaccharide-matured DCs. We are now investigating induction of apoptosis or necrosis in DCs by *B. cenocepacia* and production of cytokines and superoxide by DCs exposed to BCC. Future experiments using a modified antibiotic protection assay and electron and confocal microscopy will explore possible intracellular persistence and alteration of antigen processing. In conclusion, we believe that *B. cenocepacia* may be pathogenic in certain susceptible hosts by interfering with normal DC maturation.

Abstract A23

INTRACELLULAR TRAFFICKING AND REPLICATION OF BURKHOLDERIA CENOCEPACIA IN HUMAN CYSTIC FIBROSIS AIRWAY EPITHELIAL CELLS

Sajjan U., Yang J., Hershenson M., LiPuma J.

University of Michigan, Dept. of Pediatrics and Communicable Diseases, Ann Arbor, USA

We investigated the trafficking of *Burkholderia cenocepacia*, an opportunistic respiratory pathogen of persons with cystic fibrosis (CF), in immortalized CF airway epithelial cells *in vitro*. Our results indicate that bacteria enter cells in a process involving actin rearrangement. Whereas both live and heat-killed bacteria reside transiently in early endosomes, only live bacteria escape from late endosomes to co-localize in vesicles positive for lysosomal membrane marker Lamp1, endoplasmic reticulum (ER) membrane marker calnexin, and autophagosome marker monodansylcadaverin (MDC). Twenty four hours after infection, microcolonies of live bacteria were observed in the perinuclear area co-localizing with calnexin. In contrast, after ingestion, dead bacteria co-localized with late endosome marker Rab7, and lysosome markers Lamp1 and cathepsin-D, but not with calnexin or MDC. Six to eight hours after ingestion of dead bacteria, degraded bacterial particles were observed in the cytoplasm and in vesicles positive for cathepsin D. These results indicate that live *B. cenocepacia* gain entry into human CF airway cells by endocytosis, escape from late endosomes to enter autophagosomes that fail to fuse with mature lysosomes, and undergo replication in the ER. This survival and replication strategy may contribute to the capacity of *B. cenocepacia* to persist in the lungs of infected CF patients.

Abstract A24

INTERACTIONS OF DIFFERENT *BURKHOLDERIA CEPACIA* COMPLEX SPECIES WITH TIGHT JUNCTIONS OF LUNG EPITHELIAL CELLS *IN VITRO*

Duff C.¹, Mullen T.¹, Murphy P.², Callaghan M.¹, McClean S.¹

¹ITT Dublin, Dept. of Science, and National Institute for Cellular Biotechnology, Dublin 24, Ireland; ²Adelaide, Meath and incorporating the National Children's Hospital, Tallaght, Dublin, Ireland

The aim of this study was to investigate how *Burkholderia cepacia* complex (Bcc) strains cross the epithelial barrier of the lung and cause septicaemia in a subgroup of cystic fibrosis patients. We have examined the invasion of six Bcc species in different lung epithelial cell models: A549, 16HBE-14o- and Calu-3 cells, in order to investigate the role that Bcc-lung cell interactions play on virulence. The latter two cell lines form well-differentiated monolayers when grown on semi-permeable supports, allowing us to study the effects that Bcc strains have on epithelial integrity and tight junction proteins. Invasion of *B. dolosa* strains was comparable to those observed for *B. cenocepacia* and *B. multivorans* which may explain its virulence in certain patients. Invasion of Calu-3 and 16HBE14o- cells by *B. multivorans* strains was reduced when the cells were grown as tight polarised monolayers compared with those grown on plastic, suggesting basolateral receptors are required for the process. In contrast, four *B. cenocepacia* strains showed comparable invasion of both cell lines, irrespective of culture model.

All species of Bcc examined reduced the transepithelial resistance of polarised Calu-3 cell monolayers. However, while strains of *B. cepacia*, *B. multivorans* and *B. stabilis* strains readily translocated across the epithelial monolayer, *B. cenocepacia* translocated less readily. Both *B. multivorans* and *B. cenocepacia* reduced expression of ZO-1 in Calu-3 cells, but not E-cadherin or claudin-1 as determined by both Western blotting and immunofluorescence.

Overall, the findings that Bcc strains from several species, which differ greatly in their virulence, have the potential to disrupt tight junctions and to translocate across the epithelium, demonstrate that the potential to cause septicaemia is not limited to the two most commonly found species.

Abstract A25

A WHOLE-GENOME DNA MICROARRAY FOR *B. CENOCEPACIA*

Leiske D., Karimpour-Fard A., Gill R.T.*

Dept. of Chemical and Biological Engineering, UCB424/ECCH120, University of Colorado, Boulder, CO, USA

Corresponding author: Ryan T. Gill, Department of Chemical and Biological Engineering, UCB424/ECCH120, University of Colorado, Boulder, CO 80309

Email: rtg@colorado.edu

The recent sequencing of the *Burkholderia cenocepacia* genome will enable a broad range of new studies that will improve understanding of the *B. cenocepacia* biology. A key component of such studies will be the development of a commercially available, full-genome DNA microarray for *B. cenocepacia*. The objective of this project, performed in collaboration with the Cystic Fibrosis Foundation and Agilent, Inc., was to design, synthesize, validate, and make-available such a microarray. We have previously discussed the 1st generation *B. cenocepacia* microarray synthesized using the in situ, ink-jet technology of Agilent. These efforts included the evaluation of gene expression in cells cultured in rich versus minimal media along with cells undergoing a heat shock response. We also evaluated microarray performance through comparative hybridizations with *B. cenocepacia* genomic DNA vs *Pseudomonas aeruginosa* PAO1 genomic DNA. These results collectively demonstrated that the microarray performed well by generating reproducible and biologically sensible results. We moved on to design and evaluate the 2nd generation, 11000 feature array, which contains probes for all of the genes annotated within the J2315 genome sequenced by the Sanger Institute as well as probes against unique genes thus far annotated within the *B. cenocepacia* HI2424 or AU1054 genomes. The new array design was initially tested comparing transcription of J2315 before and after heat shock. The results showed that known heat shock response genes exhibited an increase in transcription, thus validating the new design. These heat shock studies have been repeated, using a standardized protocol, by β -testers to compare results between labs. Results confirmed approximately 50% agreement in genes with increased transcription following heat shock, which is a positive endorsement for the array considering the difficulty in achieving lab-to-lab repeatability in microarray experiments. Since then, several labs have already begun to use the microarray for transcriptional profiling and genomic comparisons. Following these tests it was determined that all labelling and hybridization for Cystic Fibrosis Foundation-funded work will be done using a central facility. By decreasing the variability in protocol we hope to enable the creation of a large database of microarray data available to *B. cenocepacia* researchers.

Supported by the Cystic Fibrosis Foundation and Agilent, Inc.

Abstract A26

CHARACTERIZATION OF A STRESS RESPONSE OPERON IN BURKHOLDERIA CENOCEPACIA K56-2

Flannagan R.S.¹, Kooi C.², Sokol P.A.², Valvano M.A.¹

¹University of Western Ontario, London, Ontario, Canada; ²University of Calgary, Calgary Alberta, Canada

Burkholderia cenocepacia, a Gram-negative bacillus found ubiquitously in the environment, has emerged as an opportunistic pathogen that is frequently associated with infections in patients with cystic fibrosis. Within the host environment *B. cenocepacia* encounters a variety of stresses such as oxidative stress, serum complement and cationic peptides. In order to survive, *B. cenocepacia* must adapt to these stresses which is often mediated by the activity of two-component regulatory systems (TCS) and effector proteins. Through analysis of the sequenced genome of *B. cenocepacia* J2315 we identified a six gene operon that encodes a TCS and an HtrA protease. In other Gram-negative bacteria HtrA plays a key role in the bacterial stress response and is regulated by the CpxR/CpxA TCS. Inactivation of the entire operon in *B. cenocepacia* K56-2 using the plasmid pGPΩTp, which results in polar mutations, gave rise to *B. cenocepacia* RSF12. To determine if the operon inactivated in RSF12 was involved in the *B. cenocepacia* stress response, the mutant was cultured under a variety of conditions known to adversely affect HtrA- or Cpx-deficient bacteria. Phenotypic characterization of this mutant revealed that inactivation of the identified operon significantly impaired the growth of RSF12 in the presence of excess Na⁺ or K⁺ ions or upon exposure to ethanol. Interestingly, several known HtrA or Cpx stresses did not adversely affect RSF12 indicating the stress response of *B. cenocepacia* may be unique. Further genetic analyses employing mutagenesis and complementation studies revealed that the observed growth defect in the presence of excess Na⁺ and K⁺ was in fact due to inactivation of the HtrA protease. However, inactivation of the TCS was responsible for the ethanol induced growth impairment. The importance of the HtrA protease in infection was established *in vivo* using the rat agar bead model of lung infection. These animal studies demonstrated that HtrA is essential for survival *in vivo*. A likely scenario is that *B. cenocepacia* encounters a variety of stresses *in vivo* whereby the HtrA protein plays an important role, possibly processing misfolded proteins. Effort in our laboratory is currently focused on determining the physiological role of the HtrA protease.

Abstract A27

CHARACTERIZATION OF THE *BVIIR* QUORUM SENSING SYSTEM IN *BURKHOLDERIA VIETNAMIENSIS*

Malott R.J., Sokol P.A.

University of Calgary Health Sciences Center, Dept. of Microbiology and Infectious Diseases, Calgary, Canada

The *Burkholderia cepacia* complex (Bcc) utilizes *N*-acyl-homoserine lactone (AHL) based quorum sensing systems for regulation of diverse physiological processes. The *cepIR* system is widely distributed among the Bcc. CepI catalyzes the synthesis of the signaling molecules *N*-hexanoyl-homoserine lactone (HHL) and *N*-octanoyl-homoserine lactone (OHL). *B. vietnamiensis* strains also possess the *bviIR* system. BviI is responsible for the synthesis of *N*-decanoyl-homoserine (DHL), *N*-dodecanoyl-homoserine lactone (doDHL), HHL and OHL. Despite possessing both quorum sensing systems, *B. vietnamiensis* AHL production has been shown to be strain dependent. The objectives of this study were to determine if there is a regulatory relationship between the *cepIR* and *bviIR* systems and to examine differences in AHL production and *bviIR* expression of clinical and environmental strains. Using *bviI* and *bviR::luxCDABE* transcriptional fusions in the environmental strain, G4 and a G4 *bviR* mutant, we determined that BviR is required for the expression of *bviI*. Similar transcriptional analysis of the *bviR*, *cepI* and *cepR* promoters revealed that BviR is not involved in the regulation of these genes. AHL bioassays of the G4 *bviI* and *bviR* mutants were both void of detectable DHL, confirming the role of BviR in the positive regulation of *bviI*. Considerable amounts of HHL and OHL remained in the AHL production profiles of the mutants due to the functional *cepIR* system, confirming that the *bviIR* system is not required for the expression of *cepIR*. AHL bioassays were also used to determine the AHL production profiles of *B. vietnamiensis* strains. Only one of four clinical strains and three of four environmental strains produced DHL. PCR analysis revealed that all but the one non-DHL producing environmental strain possessed the *bviIR* genes. Reverse-transcriptase PCR analysis of *bviI* and *bviR* in the clinical non-DHL producing strains indicated that *bviR* was expressed whereas *bviI* was not. Heterologous expression of G4 *bviR* restored DHL production in these strains, indicating that the absence of DHL was due to a lack of induction of *bviI* by BviR. Efforts are ongoing to understand the role of the *B. vietnamiensis* quorum sensing systems in clinical and environmental strains.

Abstract A28

THE ORNIBACTIN BIOSYNTHESIS AND TRANSPORT GENES OF *BURKHOLDERIA CENOCEPACIA* ARE REGULATED BY AN ECF σ FACTOR WHICH IS PART OF THE FUR REGULON

Agnoli K., Lowe C.A., Farmer K.L., Husnain S.I., Thomas M.S.

University of Sheffield Medical School, Division of Genomic Medicine, Sheffield, England

Siderophores are iron-scavenging molecules important for bacterial colonisation of iron-limited environments, such as the human host. Members of the *Burkholderia cenocepacia* complex (BCC) produce the siderophore ornibactin. Production of this siderophore has been shown to increase the virulence of *Burkholderia cenocepacia* in a rat agar bead infection model. Mutagenesis using a novel transposon resulted in the identification of 9 mutants that were unable to produce ornibactin. These mutants carried transposon insertions within one of three linked genes which comprise part of a 15 gene operon responsible for both production and utilisation of this siderophore. Those genes which had not been previously characterised were ascribed putative functions using the BLAST search and alignment programs. The transposons were found to lie within 2 putative non-ribosomal peptide synthetase genes, *orbI* and *orbJ*, and another gene, *orbS*. The *orbS* gene exhibited strong homology to an ECF sigma factor-encoding gene of *Pseudomonas aeruginosa*, *pvdS*. PvdS specifies transcription of genes encoding the biosynthesis and transport of pyoverdine under conditions of iron limitation. The potential role of OrbS as an ECF sigma factor specifying transcription of the ornibactin operon was investigated. Three putative OrbS-dependent promoters were identified by homology to the PvdS-dependent promoter consensus sequence. Reverse-transcriptase PCR and ribonuclease protection assay were carried out to show that these putative promoters were responsible for transcription of the entire operon. Expression of these putative promoters, and that of *orbS*, were analysed in the presence and absence of OrbS under iron-limiting and iron-replete conditions. It was found that OrbS is necessary for the iron-regulated expression of the 3 putative OrbS-dependent promoters, but not for its own expression. Expression from the *orbS* promoter was shown to be iron-regulated in both *B.cenocepacia* and *Escherichia coli*, indicating that its regulator was present in both species. The Fur-titration assay and electromobility shift assay showed that regulation of the *orbS* promoter is mediated by the global regulator of iron-uptake, Fur, and has allowed the identification of the binding site for this protein. Work is in progress to determine the effect of disruption of ornibactin operon function on intracellular survival of *B. cenocepacia*.

Abstract A29

TRANSCRIPTOMIC PROFILE OF *BURKHOLDERIA CENOCEPACIA* CULTIVATED IN CYSTIC FIBROSIS SPUTUM

Drevinek P.¹, Gill R.T.², Jones A.³, Mahenthiralingam E.¹

¹Cardiff School of Biosciences, Cardiff University, Cardiff, UK; ²Dept. of Chemical and Biological Engineering, University of Colorado, Boulder, CO, USA; ³Manchester Adult Cystic Fibrosis Unit, Wythenshawe Hospital, Manchester, UK

Studies of the global gene expression in *B. cenocepacia* have been made possible by the development of an 11K (10,807 features) two-channel microarray comprising probes for annotated genes (7,251 features) and intergenic regions (1,489 spots) of the genome of J2315. The array also contains probes for selected genes from *B. cenocepacia* strains AU1054 and HI2424 (1,070 and 387 features, respectively).

Using this array, we examined the changes in gene expression of strain J2315 activated by growth the presence of cystic fibrosis (CF) sputum. J2315 cells (harvested at mid-log growth phase in Luria-Bertani broth) were inoculated into either: (i) minimal growth medium (the control) or, (ii) minimal medium supplemented with 10% (vol/vol) sputum (the test sample). Total RNA was extracted from cultures reaching an OD_{600 nm} of 0.6, converted to cDNA, fluorescently labelled and hybridized to the microarray. The hybridised arrays were scanned with the Agilent scanner and subsequently analyzed by using GeneSpring software. The experiment was performed as two biological replicates which showed 89% similarity in expression data obtained. Almost 93% of J2315-specific features were eligible for statistical analysis (i.e. one sample t-test) in which 289 features were found with the p value < 0.01. Of these genes which showed significant alteration in expression, 50 demonstrated greater than a two-fold increase in expression, while 58 genes were reduced in expression by more than two-fold after growth in the presence of sputum.

Based on our experience with technical performance of the microarray experiment, we highlight two potentially more difficult areas of the protocol. Firstly, multiple aliquots of biological material are usually necessary for RNA extraction, since the input amount of total RNA must be higher than 10 µg. Secondly, the conditions of post-hybridization washing step should follow the manufacturer's recommendations as strictly as possible to avoid producing strong background artefacts. These technical difficulties have now been overcome and our preliminary experience using these microarrays to map gene expression in CF sputum appear to be reproducible and very interesting.

Abstract A30

THE MOLECULAR BASIS OF RESISTANCE TO CATIONIC ANTIMICROBIAL PEPTIDES (cAMPS) IN *BURKHOLDERIA CENOCEPACIA*

Bartholdson J.^{1,2}, Brown A.R.¹, Slack G.¹, Govan J.R.W.¹, Campopiano D.J.²

¹The Centre for Infectious Diseases, University of Edinburgh, UK; ²School of Chemistry, University of Edinburgh, UK

The lipopolysaccharide (LPS) of *Burkholderia cepacia* complex (Bcc) has unique structural and functional properties that influence both the inflammatory potential of the organism and its interaction with antimicrobial agents that act upon the lipid bilayer. One such distinctive feature of Bcc LPS is the presence of 4-amino-4-deoxyarabinose (Ara-4N) moieties that are attached to the phosphate residues of the lipid A backbone. In other bacterial species, the addition of positively-charged Ara-4N moieties to lipid A is associated with increased resistance to polymyxin and other cationic antimicrobial peptides (cAMPs), presumably through the neutralization of the anionic charge of the cell surface. We have been working on a family of cAMPs (β -defensins), with a particular focus on examining the relationship between defensin structure and antimicrobial activity. Whilst β -defensins generally exhibit a broad spectrum of antimicrobial activity, it has recently been reported that Bcc are highly resistant to human β -defensin 3 (HBD3). The molecular mechanism responsible for this resistance remains to be fully elucidated. This study aims to determine whether the Ara-4N substitution of Bcc lipid A is responsible for resistance to β -defensins and other cAMPs. Within the *B. cenocepacia* J2315 genome, we have identified a homologue of the *Salmonella typhimurium* *pmrHFJKLM* gene cluster that is responsible for Ara-4N substitution. We present the results of reverse-transcriptase PCR analysis of the J2315 *pmr* locus to define the boundaries of the locus and identify transcriptional units within it. In parallel, genes from within the J2315 *pmr* locus and other relevant genes located elsewhere within the genome that exhibit homology to known members of the Ara-4N pathway have been cloned, with a view to knocking out individual genes. Mutant strains will be studied to determine changes in the chemical composition of their LPS and their resistance phenotype to polymyxin, β -defensins and other cAMPs.

Abstract A31

THE LIPOPOLYSACCHARIDE INNER CORE OLIGOSACCHARIDE AND ITS CONTRIBUTION TO THE ANTIMICROBIAL PEPTIDE RESISTANCE AND IN VIVO SURVIVAL OF BURKHOLDERIA CENOCEPACIA

Loutet S.A., Ortega X.P., Flannagan R.S., Valvano M.A.

University of Western Ontario, Dept. of Microbiology and Immunology, London, Canada

Burkholderia cenocepacia is an important opportunistic pathogen of patients with cystic fibrosis. The bacterium is inherently resistant to a wide range of antimicrobial agents including high concentrations of antimicrobial peptides. We hypothesized that the lipopolysaccharide (LPS) of *B. cenocepacia* is important for both virulence and resistance to antimicrobial peptides. We identified *hldA* and *hldD* genes in the *B. cenocepacia* strain K56-2. These two genes encode enzymes involved in the modification of heptose sugars prior to their incorporation into the LPS core oligosaccharide. We confirmed the function of these two enzymes through cross-complementation of known *E. coli* mutants. Next, we constructed a mutant, SAL1, which was defective in the expression of both *hldA* and *hldD* and was more sensitive to novobiocin and SDS than the parental strain. The LPS produced by SAL1 consisted of a short lipid A-core oligosaccharide with no detectable O antigen. These results indicated that SAL1 had the deep-rough LPS phenotype. By complementation studies we confirmed that the functions encoded by both genes were required by *B. cenocepacia* for the synthesis of a complete LPS core oligosaccharide.

Additionally, SAL1 was sensitive to the antimicrobial peptides polymyxin B, melittin, and human neutrophil peptide 1. Using the rat agar bead model of lung infection, the SAL1 mutant showed a survival defect *in vivo* as it could not be recovered from the lungs of infected rats 14 days post infection. Together, these data show that the *B. cenocepacia* LPS inner core oligosaccharide is needed for *in vitro* resistance to three structurally unrelated antimicrobial peptides and for *in vivo* survival in a rat model of chronic lung infection. However, the loss of heptose sugars in the inner core oligosaccharide may have an indirect effect on antimicrobial peptide resistance. For instance, loss of other LPS components or destabilization of outer membrane proteins may contribute to the phenotype described for SAL1. We are currently investigating possible links between the LPS core oligosaccharide and antimicrobial peptide resistance in *B. cenocepacia*.

Abstract A32

COMPARATIVE PROTEOMICS OF REPRESENTATIVE MEMBERS OF THE BURKHOLDERIA CEPACIA COMPLEX - EXPLORING THE MOLECULAR BASIS OF PATHOGENIC AND BENEFICIAL TRAITS

Riedel K., Carranza P., Hartmann I., Eberl L.

Dept. of Microbiology, University of Zürich, Zürich, Switzerland

Members of the *Burkholderia cepacia* complex (Bcc) have attracted considerable attention as plant and human pathogens but also because of their biocontrol, plant growth promoting and bioremediation potential. The increasing amount of genomic sequence information of environmental and clinical *Burkholderia* isolates provides meanwhile a solid basis for comparative genome analyses as well as extensive functional analyses of the diverse nature of Bcc strains on the post-transcriptional level.

To identify proteins, which are potential determinants of either pathogenic or beneficial traits the protein profiles of two closely related clinical isolates, *Burkholderia cenocepacia* H111 and J2315, were compared with the one of the environmental strain *Burkholderia* sp. 383 by two dimensional gel electrophoresis (2-DE). Our analysis resulted in a high number (25 – 30% of all detectable spots) of potential strain-specific protein candidates although a higher similarity of the protein pattern of the two clinical isolates was observed. The identification of the most abundant differentially expressed spots by peptide mass fingerprinting and tandem mass spectrometry showed that homologues of one third of the proteins detected as unique spots were present in more than one strain. The majority of the observed differences in the 2-DE originate apparently from small variations in the amino acid composition leading to a horizontal shift of the respective proteins in the gel. A search for genes coding for the remaining differentially expressed proteins in the genomes of the two sequenced strains revealed that (i) two genes coding for proteins with unknown function were exclusively present in J2315 and (ii) one gene that is part of an operon involved in tellurite resistance was exclusively present in the environmental strain 383. Currently, the distribution of these potential marker genes within other clinical and environmental Bcc isolates is investigated by PCR and southern blot analysis.

Our study demonstrated that despite of its known methodical limitations comparative 2-DE is well suited for the identification of strain-specific marker proteins, which could serve as future diagnostic tools or targets for the development of novel anti-infective compounds.