



International *Burkholderia cepacia* Working Group

8th Annual Meeting

**March 21-23, 2003,
The Tower Thistle Hotel, London, UK**

Sponsored by :

Canadian Cystic Fibrosis Foundation

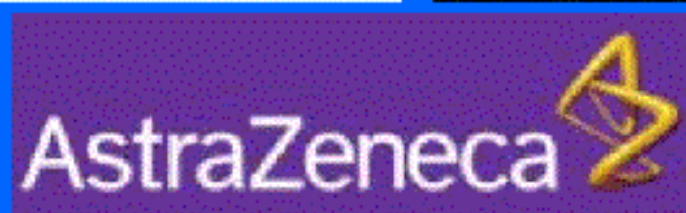
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<http://go.to/cepacia>

- A2 Craig Winstanley: Molecular typing of *Burkholderia cepacia* complex isolates from Brazil
- A27 Alban Ramette: Prevalence and diversity of *Burkholderia cepacia* complex in pristine soils worldwide
- A8 Robert Coutts: Diversity of *Burkholderia* isolates from woodland rhizosphere environments
- A3 Chris Dowson: A unified MLST scheme for the *Burkholderia cepacia* complex
- A4 Christine Segonds: Interest of amplified ribosomal restriction analysis (ARDRA) for the identification of bacteria growing on *Burkholderia cepacia* selective media
- A40 Kirsty Morris: Evaluation of enzyme markers for phenotypic differentiation of the *B. cepacia* complex
10. 00 am End of session and coffee break (until 10.30 am)

Second Presentation Session 10.30am to 1.00pm

**Clinical infections, outcome, transplantation, therapy, models of infection
Chair – Mary Corey (9 abstracts; 2.5 hours)**

- A29 Paul Whitby: Quantitative real-time PCR of *B. cepacia* directly from sputum
- A37 Elizabeth Tullis: Incidence of cepacia syndrome in cystic fibrosis patients infected with *Burkholderia cepacia*
- A39 Andy Jones: Survival of cystic fibrosis patients infected with *Burkholderia cepacia* complex genomovar III, *Burkholderia multivorans* and *Pseudomonas aeruginosa*
- A21 Tony De Soya: Prevalence and clonality of infection with *Burkholderia cepacia* complex genomovars in UK cystic fibrosis patients referred for lung transplantation
- A36 Mary Corey: Survival benefit of lung transplantation in cystic fibrosis patients with *Burkholderia cepacia* genomovar III.
- A26 Cecilia Chapparo: Predictors of outcome in *Burkholderia cepacia* positive patients
- A19 Ross Langley: *Burkholderia cepacia* complex bacteriophage: Their evolving role as potential therapeutic and -transducing agents.
- A24 Uma Sajjan: Short term *in vivo* model that mimics *B. cepacia* lung infection in CF patients
- A20 Steve Bernier & Pam Sokol: Comparative analysis of plant and animal models for the characterization of *Burkholderia cepacia* virulence

Break for Buffet-Lunch.

**Afternoon 2.15pm to 4.30pm Free discussion/collaboration time
(& Jack the Ripper walk)**

4.30pm Afternoon Tea and re-convene for evening session

Third presentation session 5.00pm to 7.00pm

***Burkholderia cepacia* complex virulence and pathogenesis**

Chair – Jane Burns - (8 abstracts; 2 hours)

- A5 Tracey Hunt: Identification of genes required for *in vivo* survival of *Burkholderia cepacia* using signature tagged mutagenesis
- A33 Teresa Urban: Isogenic Mutants of *B. cepacia* BC7 Cable Pili and 22 kDa Adhesin: Effects on Adherence/Invasion of Cultured Squamous Epithelial Cells
- A34 Jason Kim: *Burkholderia cepacia* complex Traverse Polarized Respiratory Epithelium by Disrupting Tight Junctions
- A31 Ute Schwab: The impact of oxygen on the interrelationship between *P. aeruginosa* and *B. multivorans*
- A10 Leo Eberl: The cep quorum-sensing system of *Burkholderia cepacia* H111 provides a regulatory link between surface colonization and pathogenicity
- A35 Catherine Chambers & Pam Sokol: Characterization and specificity of CepR binding to four promoters regulated by CepIR
- A18 Paola Cescutti: News about exopolysaccharides produced by *Burkholderia cepacia* strains.
- A41 John LiPuma; Identification and characterization of a capsular polysaccharide locus in *Burkholderia cepacia* genomovar III

Short break and co-ordination of break out sessions

7.00 - 8.00pm

Group Discussion and breakout sessions - topics:

Clinical epidemiology, management, and therapy- Andrew Jones, leader

Taxonomy, diversity, ecology, evolutionary genomics - Tom Coenye, leader

Genomics, molecular biology, infection - Esh, leader

Finish session at 8.00pm

EVENING MEAL (AT THE HOTEL OR VENUE OF YOUR OWN CHOICE)

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Sunday 23rd March

Full Breakfast from 7.00am

Fourth Presentation session 8.00am to 10.00am

Genomics, molecular biology, virulence, pathogenesis

Chair - David Speert (8 abstracts; 2 hours)

- A38 Barbara Conway: Bacterial cell surface alterations affect the pathophysiology of *Burkholderia cepacia* complex organisms.
- A11 Kostas Konstantinidis: Functional gene content in *Burkholderia* species and in-silico modeling of a DNA-microarray approach to study gene differences in the *B. cepacia* complex
- A1 Tom Coenye: Overrepresentation of immunostimulatory CpG motifs in the *Burkholderia cepacia* genomovar III J2315 genome
- A17 Adam Baldwin: Evaluation of the *Burkholderia cepacia* epidemic strain marker
- A9 Mark Thomas: Organisation, role and regulation of the *tonB* operon in a *B. cepacia* complex genomovar III strain
- A32 Benoit Cournoyer: Analysis of *Burkholderia cepacia* J2315 sigma 70 family of transcriptional factors.
- A6 Miguel Valvano: Identification of a novel tetracycline-responsive regulon in *Burkholderia cepacia*
- A30 Carlos Gonzalez: Plant tissue watersoaking: a type IV secretion system associated trait in *Burkholderia cepacia* strain K56-2

Break for tea/biscuits

Final session 10.30am to 12.00pm - Chair - John LiPuma

Summary of discussion topics:

Clinical epidemiology, therapy & management - Andrew Jones, leader
Taxonomy, diversity, ecology, evolutionary genomics - Tom Coenye, leader
Genomics, molecular biology, infection - Esh, leader

12.00 to 12.30 pm

Planning for future meetings and adjourn

Buffet lunch till 2.00pm

Departure at 2.30pm from Hotel
(also 3 pm coach onward to Wellcome Genome meeting)

8th IBCWG MEETING
21ST MARCH – 23rd MARCH 2003
SUMMARY SCHEDULE

FRIDAY 21st MARCH

BRIDGE SUITE 2

Delegates meet at 6.00pm. Finger buffet is served
Introductory session 7.30pm – 8.30pm

SATURDAY 22nd MARCH

Full Breakfast from 7.00am in the Hotel Restaurant.

BRIDGE SUITE 2

First Session 8.00am to 10.00am.
Break for tea/coffee/biscuits.
Second session 10.30am to 1.00pm.

Finger Buffet-Lunch.

JACK THE RIPPER TOUR with Donald Rumbelo

Meet in the hotel foyer at 2.15pm.
The walk should take approx 2 hrs with a return to the hotel by approx 4.15pm.

BRIDGE SUITE 2

Tea/coffee/biscuits set up from 4.30pm
Third session 5.00pm to 7.00pm.

Syndicate rooms 7.00pm & 8.00pm

Evening session to conclude at approx 8.00pm

SUNDAY 23rd MARCH

Full Breakfast from 7.00am in the Hotel Restaurant..

BRIDGE SUITE 2

First Session 8.00am to 10.00am.
Break for tea/coffee/biscuits.
Second session 10.30am to 12.30pm.

Finger Buffet-Lunch.

To conclude at approx 2.00pm.

FAREWELL AND A SAFE JOURNEY HOME

A1. Overrepresentation of immunostimulatory CpG motifs in the *Burkholderia cepacia* genomovar III J2315 genome

Tom Coenye and Peter Vandamme

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It has been known for almost 20 years that bacterial DNA can trigger an immune response in the vertebrate host and several studies have shown that synthetic oligonucleotides containing unmethylated CpG dinucleotides can induce a similar immune response. Vertebrate DNA shows a significant CpG suppression and approximately 80% of the remaining CpG dinucleotides in vertebrate DNA are methylated at the 5' position of the cytosine. It appears that the vertebrate immune system has evolved to recognise unmethylated CpG motifs and that it can respond with a rapid and coordinated cytokine response, leading to the induction of humoral and cell-mediated immunity. The cellular response to unmethylated CpG-containing DNA is mediated by the Toll-like receptor 9 (TLR9) and TLR9 from different species recognise different CpG motifs. Several studies have indicated that TLR9 mediates the immune response in a concentration-dependent way, i.e. DNA containing multiple CpG motifs has a greater stimulatory capacity. While an immune response normally is protective and self-limiting, the continuous presence of bacteria and their byproducts in the lung of CF patients leads to a vicious cycle where the host response leads to more tissue damage, leading to more infection and further inflammation. In this study we compared the occurrence of CpG dinucleotides and CpG-containing motifs in the *Burkholderia cepacia* genomovar III J2315 genome with the occurrences in other bacterial genomes (including those of other CF pathogens like *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Staphylococcus aureus*). The frequency of CpG dinucleotides in the J2315 genome is not significantly higher than in other CF pathogens, but due to its large genome size, the absolute number of CpG dinucleotides (2692567) is significantly higher than that in other CF pathogens ($P < 0.05$). We also compared the occurrences of 5'-GACGTT-3' (most stimulatory CpG-containing motif for the murine immune system) and 5'-GTCGTT-3' (most stimulatory CpG-containing motif for the human immune system). Both sequences are significantly overrepresented (both in relative as in absolute counts) in the J2315 genome compared to the genomes of other CF pathogens ($P < 0.05$ for all comparisons). Our data indicate that DNA from *B. cepacia* J2315 has the potential to cause a significant proinflammatory response and may play an important part in the pathogenesis of this organism in CF patients. This may be a direct effect or may be the result of an increased sensitivity to the toxic effects of LPS and/or other bacterial products.

A2. Molecular typing of *Burkholderia cepacia* complex isolates from Brazil

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PCR tests were used to assign genomovar status to 39 non-cystic fibrosis (CF) and 11 CF *B. cepacia* complex isolates from patients in hospitals in Brazil. Non-CF isolates were assigned to genomovar IIIA (71.8%), genomovar I (15.4%), *B. vietnamiensis* (7.7%), and *B. multivorans* (5.1%). CF isolates were assigned to genomovar IIIA (18.2%), *B. vietnamiensis* (18.2%), and genomovar I (9.1%). Six CF isolates sharing *recA* PCR-RFLP and RAPD patterns could not be assigned to a genomovar. 16S rDNA

sequence obtained from these isolates indicated a closest relationship to *B. anthina*, but *recA* sequence was equally divergent from several genomovars. PCR screening indicated the presence of *cblA* in only two isolates, whereas the BCESM marker was found in 22 of 28 genomovar IIIA isolates. A type III secretion gene was detected in all but genomovar I isolates. RAPD and PCR-RFLP assays, targeting both *recA* and *fliC*, indicated a large amount of genetic variability amongst the isolates, with many novel patterns being observed. Nine genomovar IIIA isolates from different non-CF patients and clinical sources shared identical RAPD and PCR-RFLP patterns, indicating the presence of a common clone.

A3. A unified MLST scheme for the *Burkholderia cepacia* complex

K. Thickett (1), D. Honeybourne (2), E. Mahenthiralingham (3), and C. G. Dowson (1)

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We have currently developed an MLST scheme for the whole *B. cepacia* complex. Southern blotting of whole chromosomes and restriction digests of total chromosomal DNA from *B. cepacia* complex, (*B. cepacia* genomovars I and III, *B. multivorans*, *B. stabilis* and *B. vietnamiensis*) was used to confirm the presence of loci as single copies. Two genes excluded from the scheme, *mutS* and *trpE*, were present in duplicate, being found on both of the two larger chromosomes of *B. cepacia* complex genomovars I-V. A phylogenetic tree, based upon concatenated MLST sequences, was constructed incorporating 27 isolates from *B. cepacia* complex genomovars I-IX. This tree clearly defined each recognised species or genomovar as discrete clusters supported by significant bootstrap values. Nucleotide sequence divergence within a species or genomovar was in the order of ~1-2% (for the isolates examined) and ~4-8% between species or genomovars. Trees derived from individual MLST loci were frequently non-congruent suggesting that horizontal gene transfer has played a role in the evolutionary origins of the *B. cepacia* complex.

A4. Interest of amplified ribosomal restriction analysis (ARDRA) for the identification of bacteria growing on *Burkholderia cepacia* selective media

C. Segonds, S. Paute, and G. Chabanon

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The recovery of *Burkholderia cepacia* from the sputum of patients with cystic fibrosis (CF) requires the use of selective media. It has been recently pointed out that bacteria belonging to other genera, mainly *Ralstonia* and *Pandoraea* species, were able to grow on these media. The differentiation of these bacteria from *Burkholderia* species, essential for the management of patients with CF, is difficult by usual manual or automated phenotypic methods. Thus, a molecular tool equally applicable to all bacteria growing on *Burkholderia cepacia* selective media would be of interest. In the present study, we assessed the performance of amplified ribosomal restriction analysis (ARDRA) for the

identification of *Burkholderia*, *Ralstonia* and *Pandoraea* species involved in CF. The bacterial strains tested included type and reference strains representative of the *Burkholderia cepacia* complex, i.e. *Burkholderia cepacia* genomovar I, *Burkholderia multivorans*, *Burkholderia cepacia* genomovar III, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, *Burkholderia cepacia* genomovar VI, *Burkholderia ambifaria*, *Burkholderia anthina*, *Burkholderia pyrrocinia*; *Burkholderia gladioli*; *Ralstonia pickettii*, *Ralstonia mannitolilytica*, *Ralstonia gilardii* and *Ralstonia taiwanensis*; *Pandoraea pnomenusa*, *Pandoraea sputorum*, *Pandoraea pulmonicola*, and *Pandoraea apista*. Besides, 106 clinical isolates recovered from 104 French CF patients and transmitted to the Observatoire cepacia in 2001 were analyzed. ARDRA was performed using the primers fd1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3') and rd1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3') for the amplification of the 16S rDNA gene. The PCR products were digested with the following endonucleases: *AluI*, *CfoI*, *DdeI*, *MspI*, *NciI*, and *XmnI*. Within the type and reference strains, *Burkholderia cepacia* genomovar I and III could not be differentiated, as well as *Burkholderia stabilis* and *Burkholderia pyrrocinia*; *Pandoraea pnomenusa* and *Pandoraea sputorum*; *Pandoraea pulmonicola* and *Pandoraea apista*. All the other species harboured specific ARDRA profiles. Among the 106 clinical isolates, 50 were classified by use of ARDRA as *Burkholderia cepacia* genomovar I or III, 46 as *Burkholderia multivorans*, 2 as *Burkholderia stabilis* or *B. pyrrocinia*, 4 as *Burkholderia vietnamiensis*, 2 as *Burkholderia gladioli*, and 2 as *Ralstonia mannitolilytica*. Two patients simultaneously harboured a *Burkholderia cepacia* genomovar I or III strain and a *Burkholderia multivorans* strain. Thus, the ARDRA method tested in this study, though unable to separate all *Burkholderia cepacia* complex species, and all *Pandoraea* species, proves to be a useful identification tool for clinical microbiology laboratories involved in CF; it requires the constitution of a data bank of profiles, but presents the advantage of being equally applicable to the main organisms growing on *Burkholderia cepacia* selective media, i.e. *Burkholderia*, *Ralstonia* and *Pandoraea*; it provides sufficient information to physicians for the management of colonized patients, and sound epidemiological data, when used on a national scale.

A5. Identification of genes required for *in vivo* survival of *Burkholderia cepacia* using signature tagged mutagenesis

T.A. Hunt (1), C. Kooi (2), P.A. Sokol (2), and M.A. Valvano (1)

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Cystic fibrosis (CF) patients are at particularly high risk for *B. cepacia* infections. These infections are difficult to treat due to the resistance of this microorganism to many clinically useful drugs, as well as the inherent resistance to cationic peptides. Many virulence factors have been postulated to play a role in *B. cepacia* infections, however the mechanisms of pathogenicity have not been well described. We hypothesize that several *B. cepacia* genes are preferentially expressed following interactions with the host, and that potential virulence factors that would otherwise not be identified using classical *in vitro* techniques may be discovered using *in vivo* approaches. Our objective is to identify characteristics of *B. cepacia* which cause pathogenesis and allow the chronic colonization of the lungs of CF patients. We have chosen to use the methodology of signature tagged mutagenesis (STM) in order to identify genes of *B. cepacia* that are important for *in vivo* survival. This technique was chosen since it is a genome-wide identification strategy, and it is an unbiased

approach for gene identification. We have constructed unique transposable elements and utilized these to produce nearly 3000 *B. cepacia* mutants for use in STM. Using the rat agar bead model of lung infection, we analyzed pools of the mutants and following the infections, the mutants were screened using real-time PCR. Mutants which did not survive *in vivo* were re-grouped into smaller pools and used for a secondary round of rat infections. Following the secondary screen, we found 118 *B. cepacia* mutants which contained a mutation in a gene important for survival *in vivo*. A self-cloning technique was utilized to identify the chromosomal genes containing the transposon insertion to identify genes important for *in vivo* survival. The gene homologues found could be grouped into one of six categories: genes involved in regulation, DNA replication and repair genes, cellular metabolism genes, genes which encoded membrane proteins, genes of unknown function, and true virulence or anti-host factors. These studies demonstrate that STM is an efficient method that can be modified for use in *B. cepacia* to uncover genes important for survival *in vivo*. With the recent *B. cepacia* sequencing project by Sanger, the characterization of these genes will be enhanced as we will be able to study not only the gene which was interrupted in each STM mutant, but also obtain information about the surrounding genomic region. In addition, this study will identify genes important for the establishment and persistence of *B. cepacia* in pulmonary infections of CF patients, and may therefore identify potential new therapeutic targets for drug therapy.

A6. Identification of a novel tetracycline-responsive regulon in *Burkholderia cepacia*

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ABSTRACT: *Burkholderia cepacia*, a bacterium found in the soil, is emerging as an important opportunistic pathogen in patients with cystic fibrosis (CF). Due to the compromised mucociliary clearance of the respiratory tract that results from this genetic disease, chronic infections are often established in the lungs of CF patients. Because of the inherent antibiotic resistance of *B. cepacia*, high concentrations of antimicrobials are often required for the treatment of infections. The membrane of *B. cepacia* has been demonstrated to be highly impermeable which is partially responsible for the resistance to most therapeutic agents. We are primarily interested in the stress responses of *B. cepacia* to these agents during treatment, particularly changes in the outer membrane that may lead to altered membrane permeability. Since it has been shown that the efflux pump NorM can mediate resistance to the cationic peptide polymyxinB in the presence of tetracycline, we analyzed several isolates of *B. cepacia* from soil and clinical samples to identify outer membrane proteins responsive to changes in extracellular concentrations of tetracycline. Growth of *B. cepacia* in concentrations of tetracycline 100 g/ml was associated with a drastic reduction in a 23 kDa outer membrane protein subsequently identified by tandem mass spectrometry as a peptidoglycan-associated lipoprotein (pal) homologue. However, since tetracycline is known to chelate magnesium, we wanted to determine if the effects were due to the intracellular action of tetracycline or to the extracellular metal chelating properties of tetracycline. Addition of excess magnesium to the growth medium abrogated the effect seen on the lipoprotein suggesting that the response is due to the latter. In addition, changes in the expression of several other large outer membrane proteins were observed but these remained unaffected by supplementation of magnesium. Preliminary evidence suggests that these proteins may be involved in iron transport and the changes seen in the presence of tetracycline may be due to the chelation of iron, another property of this drug. 2D gel electrophoresis of bacterial cell lysates revealed that the expression of several cytosolic proteins is regulated by growth in high tetracycline concentrations. Thus, the changes observed in the

outer membrane profiles appear to be part of a more global response involving many cellular proteins. These observations reflect that the presence of antibiotics, usually used in high concentration for the treatment of *B. cepacia* infections, elicits stress responses by modifying the extracellular concentration of metal ions. A proteomics approach is currently being used to identify both cytosolic and outer membrane proteins involved in these responses. This will contribute to our understanding of the cellular changes needed for adaptation to low magnesium or low iron concentrations that may occur in the host and in intracellular environments, as well as in the soil.

A7. Genetic polymorphism of *recA* subgroups of *B. cepacia* genomovar III

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At present *B. cepacia* genomovar III comprises four lineages (subgroups) based on *recA* gene sequence, III-A, III-B, III-C, and III-D. The clinical and ecological implications of this subdivision are not yet fully understood. Apparently, lineage III-A comprises the most virulent strains, and has been found exclusively in clinical settings, so far; lineage III-B has been isolated from specimens and seems to be widely spread in natural habitats (plant rhizosphere, soil, river sediments). Very little is known about lineages III-C and III-D, except that, so far, they have been isolated from environmental or clinical sources, respectively. To improve our understanding of the ecology of the lineages of *B. cepacia* genomovar III, around 200 isolates belonging to genomovar III were analysed to investigate the distribution of the different lineages among CF patients and in natural habitats; furthermore, their genetic diversity was investigated by means of the RAPD technique. Environmental isolates were recovered from maize roots harvested at three different sites in Italy, whereas clinical ones were isolated from a single cystic fibrosis center (G. Gaslini, Genoa). Among environmental isolates only subgroup III-B was detected, whereas among clinical isolates subgroups III-A, III-B, and III-D were found. It is noteworthy that among clinical isolates the dominant lineages were III-A and III-D; III-B was recovered from a small percentage of patients. Lineage III-C was absent among both environmental and clinical isolates. RAPD analysis revealed highly different degrees of genetic polymorphism among the various subgroups. In particular, similar low values of mean genetic distance (GD) were found in subgroups III-A and III-D, whereas III-B showed significantly higher GD than the other subgroups. It is noteworthy that both clinical and environmental isolates of III-B had very similar GD values. Furthermore, all clinical and environmental III-B isolates represented unique genetic fingerprints, whereas III-A and III-D isolates were grouped in 3 and 1 clusters or RAPD types, respectively. In other words, transmissible strains seem to be found more often within genomovar III-A and III-D than within genomovar III-B. These results suggest a prevalent acquisition of *B. cepacia* genomovar III-B by CF patients from the environment, whereas *B. cepacia* genomovar III-A and III-D seem to be prevalently acquired by patient-to-patient transmission.

A8. Diversity of *Burkholderia* isolates from woodland rhizosphere environments

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The aim of this project was to determine the genetic diversity among UK *Burkholderia cepacia* isolates from various environmental niches, principally woodland tree rhizospheres and onions. Genus determination was made using PCR-amplification and fatty acid methyl ester profiling. Genetic diversity was investigated by repetitive sequence genetic PCR fingerprinting. Several onion isolates were similar to clinical isolates but others were diverse. Some environmental isolates were possibly synonymous with *B. cepacia* and *B. gladioli* but most from woodland rhizospheres were distinct and clustered together. The 16S rRNA genes of representatives from these clusters were PCR-amplified, sequenced and phylogenetically compared with all known *Burkholderia*, and related species. This revealed that the rhizospheric isolates had closest affinity with *Burkholderia* spp. with known bioremediative and biocontrol capabilities and were unrelated to taxa comprising plant or human pathogenic strains. Our conclusions are that all of the analyses investigated revealed that environmental and onion isolates of *B. cepacia* complex bacteria are genetically diverse but that woodland rhizospheric isolates are related to each other and unrelated to plant or human pathogenic strains. We contend that woodland rhizospheric isolates of *B. cepacia* are potentially good candidates for use in bioremediation and biocontrol, as they appear distinct from plant or human pathogenic strains.

A9. Organisation, role and regulation of the *tonB* operon in a *B. cepacia* complex genomovar III strain

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Members of the *Burkholderia cepacia* complex are recognised as serious pathogens of patients with cystic fibrosis (CF). The CF lung provides an iron limiting environment, suggesting that iron acquisition mechanisms are important for bacterial colonisation. To characterise the iron acquisition mechanisms of these organisms in more detail, we used a novel transposon to isolate mutants which exhibited unregulated siderophore production. Of 14,000 transposon mutants screened, three were found to overproduce the siderophores ornibactin and pyochelin under iron replete conditions. Cloning and DNA sequence analysis of genomic DNA flanking the transposon showed that the transposon had integrated into the *exbB* gene in each case. This gene was located downstream of a gene encoding a protein homologous to TonB and upstream of a gene encoding a protein with high homology to ExbD. TonB, ExbB and ExbD are required for iron uptake in Gram-negative bacteria by forming a complex in the cytoplasmic membrane and transducing energy derived from the proton motive force into the transport of Fe(III) across the outer membrane. The role of these genes in iron uptake was confirmed by examining the effect of *exbB* disruption on (i) growth in the presence of different iron sources (ii) resistance to hydrogen peroxide and (iii) transport of Fe(III) and haem. Upstream of *tonB* is a gene, *bfd*, encoding a 78 amino acid polypeptide exhibiting high homology to the bacterioferritin-associated ferredoxin of *E. coli*. RT-PCR and primer extension analysis demonstrated that these four genes comprise an operon under control of a single promoter. Consistent with this, polar insertions within *bfd* or the *bfd-tonB* intergenic region gave rise to a TonB-negative phenotype. Using a strain harbouring a chromosomal *exbB-lacZ* transcriptional fusion, the *bfd* promoter was shown to be fully repressed by 5 μ M ferric chloride. The *B. cepacia* complex *fur*

gene, encoding the global regulator of iron regulated genes, was cloned under control of the *tac* promoter and expressed in an *E. coli* Δfur strain harbouring a plasmid borne *bfd-lacZ* fusion. *B. cepacia* complex Fur was shown to efficiently repress the *bfd* promoter in *E. coli* in response to iron. The Fur titration assay (FURTA) allowed the identification of the Fur binding site (Fur box) overlapping the *bfd* promoter.

A10. The cep quorum-sensing system of *Burkholderia cepacia* H111 provides a regulatory link between surface colonization and pathogenicity

B. Huber (1), K. Riedel (1), M. Köthe (1), I. Steinmetz (2), M. Givskov (3), S. Molin (3) and L. Eberl (1)

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Burkholderia cepacia and *Pseudomonas aeruginosa* often co-exist as mixed biofilms in the lungs of patients suffering from cystic fibrosis (CF). The isolation of thirteen random mini-Tn5 insertion mutants of *B. cepacia* H111 that are defective in biofilm formation on a polystyrene surface is reported. A detailed quantitative analysis of the biofilm structures formed by wild type and mutant strains revealed that the isolated mutants are impaired in their abilities to develop a typical three-dimensional biofilm structure. Molecular investigations showed that the genes required for biofilm maturation fall into several classes: (i) genes encoding for surface proteins, (ii) genes involved in the biogenesis and maintenance of an integral outer membrane, and (iii) genes encoding regulatory factors. Three of the regulatory mutants produced greatly reduced amounts of N-octanoylhomoserine lactone (C8-HSL). This compound serves as the major signal molecule of the cep quorum-sensing system, which consists of the AHL synthase CepI and the cognate C8-HSL receptor protein CepR. Furthermore, it is shown that *B. cepacia* H111 effectively kills the nematode *Caenorhabditis elegans*. Depending on the medium used for growth of the bacterium two different killing modes were observed. On high-osmolarity medium the nematodes became paralysed and died within 24 hours. Using filter assays we provide evidence that this killing mode involves the production of an extracellular toxin. On nematode growth medium killing occurs over the course of 2-3 days and involves the accumulation of bacteria in the intestinal lumen of *C. elegans*. It is demonstrated that the cep quorum-sensing system of H111 is required for efficient killing of *C. elegans* under both killing conditions. Work currently under way employs proteomics to identify those quorum sensing regulated proteins that are required for surface colonization and pathogenicity of the organism.

A11. Functional gene content in *Burkholderia* species and in-silico modeling of a DNA-microarray approach to study gene differences in the *B. cepacia* complex.

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Burkholderia species have among the largest bacterial genomes; however, what drives genome expansion and what ecological benefits accrue for these species remain speculative. We have recently completed a comparative analysis of all completely

sequenced genomes and have discovered definite trends between functional gene content and genome size (Konstantinidis and Tiedje, submitted). In particular, we have found that large genomes preferentially accumulate metabolism, transport and regulation genes as opposed to informational ones. A constant or species-specific relationship between the remaining gene functional categories and genome size was observed. Our results clearly explain what is gained in a large genome and correlate with the broader metabolic versatility and ecological success shown by species with larger genomes. We have preliminary evidence, based on the available partial genomic sequences, that *Burkholderia* species closely follow these trends with genome size. To further understand genome evolution in Burkholderiaceae, it is also important to link genomic information e.g. genetic differences and similarities between species to information on the ecology of the species. For instance, is there a gene core conserved for *Burkholderia* at the species and ecotype levels? Can keystone genes, usually but not necessarily ones that have ecological function and are key to ecological success of the species, be identified for *Burkholderia*? DNA microarrays are viewed as a powerful tool for these research questions because of having great potential in detecting genetic differences and similarities between closely related strains. However, their appropriateness for the *B. cepacia* complex (Bcc) case remains unclear. In particular, can a whole genome microarray based on a single genome e.g. that of strain Sanger J2315, be used for DNA-DNA or expression studies with other Bcc strains? We investigated this issue using the existing knowledge on the evolutionary distances (DNA-DNA similarity and 16S rRNA data) between the Bcc species and the available genomic sequences for four *Burkholderia* strains. Our results suggest that such a microarray, will have a significant number of false negatives when used with distantly related Bcc strains because nucleotide sequences have considerably diverged at levels of 30-45% DNA-DNA similarity relatedness (which are common between Bcc species or genomovars). We have obtained comparable results in a similar analysis of three additional bacterial groups namely the Enterics, Mycobacteria and Streptococci. Conclusively, we believe that microarrays based on several reference Bcc strains are needed to give an adequate coverage of the whole Bcc. Our results also indicate that an oligo-array (one specific 50 mer per gene) can perform comparably to a cDNA array for expression or DNA-DNA studies when evolutionary distance in minimum e.g. within species or between highly related species or genomovars.

A12. The pro-inflammatory activity of *Burkholderia cepacia* complex genomovars; the role of lipopolysaccharide chemotypes

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Burkholderia cepacia complex organisms are now known to comprise nine closely related species known as genomovars. Genomovar III is the most prevalent and appears the most virulent. We have demonstrated a clear association between sepsis and early post-pulmonary transplant death with pre-transplant cepacia genomovar III infection (Lancet 2001). We have investigated the biological activity of an extended reference panel of 33 strains using whole cell lysates to induce the septic shock-related cytokines, IL-1, IL-6 and TNF α from differentiated U937 human macrophages. We found varying biological activity within and between genomovars. Genomovar II *B. multivorans* strains were, in general, poor cytokine inducers with the exception of the *B. multivorans* type strain LMG 13010. Genomovar III strains possessed the greatest cytokine induction activity although certain genomovar III strains were poor cytokine inducers using this system. There was variable cytokine induction capacity of the

various ET-12 clones tested; LMG 12614 and LMG 16656 (J2315) were potent cytokine inducers whilst LMG 18863 (K562) and LMG 18826 (BC7) were relatively modest cytokine inducers. One genomovar III strain, LMG 16654, associated with the first UK report of Cepacia syndrome, but not transmissibility, was a potent TNF α inducer. There was no clear pattern of cytokine induction relating to source of strain origin. Of the Genomovar I strains tested, LMG 17997 was the only cytokine inducer of note, and was of non-CF origin (isolated from a urinary tract infection). This strong cytokine induction was not seen for other non-CF strains included in the panel; genomovar III strain LMG 18832 (urinary tract infection), *B. multivorans*, strains LMG 17588 (soil), LMG 18823 (lab), or those from *B. vietnamiensis*, LMG 18835 (soil) and LMG 10929 (rice). Cytokine induction activity was CD-14 dependent and could be inhibited by polymixin B suggesting lipopolysaccharide was the main biologically active component of the lysates. The variability of cytokine induction was not simply related to lipopolysaccharide chemotype. Genomovar I strains were predominantly smooth with LMG 17997, a non-CF strain, a notable exception with a partial rough chemotype. Genomovar II strains were mostly smooth chemotype (the exceptions being rough LPS strains LMG 13010, a potent cytokine inducer, and LMG 14273, a poor cytokine inducer). Genomovar III strains were similarly either rough, partial rough or smooth. There were differences in the LPS chemotype of the various ET-12 clones tested; LMG 12614 and LMG 16656 (J2315) were of rough LPS whilst LMG 18863 (K562) were and LMG 18826 (BC7) of smooth chemotype. Of note LMG 16654 a non ET12 clone with potent cytokine induction activity was smooth chemotype whilst a *B. multivorans* strain of poor cytokine induction, LMG 14273, was rough chemotype. We purified lipopolysaccharide from two strains bearing rough lipopolysaccharide, a genomovar III (LMG12614) and a genomovar II strain (LMG 14273). Using the purified lipopolysaccharide, as a stimulant in the same tissue culture system, the observed divergence in biological activity was preserved. Different structural motifs in the purified lipopolysaccharide were noted on polyacrylamide gels. We conclude that variations in lipopolysaccharide from BCC strains may in part explain the variability in clinical outcomes associated with infections with these organisms.

A13. Design and evaluation of a universal *Burkholderia* species *recA* probe

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The genus *Burkholderia* currently consists of 23 distinct species and 2 genomovars. These include: *B. gladioli*, *B. plantarii*, *B. glumae*, *B. pseudomallei*, *B. mallei*, *B. thailandensis*, *B. caryophylli*, *B. graminis*, *B. caledonica*, *B. fungorum*, *B. caribensis*, *B. glathei*, *B. kururiensis*, *B. sacchari*, *B. andropogonis*, *B. phenazinium* and the *B. cepacia* complex. In turn, the *Burkholderia cepacia* complex consists of *B. cenocepacia* (formerly genomovar III) *B. vietnamiensis* (formerly genomovar V), *B. multivorans* (formerly genomovar II), *B. cepacia* genomovar VI, *B. pyrrocinia* (formerly genomovar IX), *B. stabilis* (formerly genomovar IV), *B. ambifaria* (formerly genomovar VII), *B. cepacia* genomovar I and *B. anthina*. In addition there are a number of well-characterised strains such as *Burkholderia* LB400, which are known to belong to the genus. The *recA* gene has proven to be a very useful means to identify members of the *Burkholderia cepacia* complex. Currently, available PCR primers are unable to amplify *recA* beyond this group. We are interested in understanding the total diversity of all *Burkholderia* species associated with natural and polluted environments. The *recA* gene forms an excellent basis upon which to develop a universal *Burkholderia* species molecular diagnostic. It would also allow comparison between the *recA* and the 16S rRNA genes as bacterial identification tools for this group. Using existing *recA* sequence data from the *B. cepacia* complex, *Burkholderia* strain LB400, *B.*

pseudomallei and other β -proteobacteria, a primer set was designed to amplify an 869bp fragment of the *recA* gene from *Burkholderia* species. The primers were evaluated on 9 representative strains from the *B. cepacia* complex and 16 other *Burkholderia* species reference strains. It was found to amplify all but *B. andropogonis* and *B. phenazinium*. However, the primers also amplified *recA* outside of the *Burkholderia* genus, detecting four species of *Pandoraea*, which have recently been separated from the *Burkholderia*. Nucleotide sequence data will be obtained from this set of PCR fragments and will complement the existing database of *B. cepacia* complex *recA* information. Further *recA*-based primers that can universally identify *Burkholderia* species will be designed and tested as a means to determine their diversity in various habitats.

A14. *Burkholderia dolosa* sp. nov., a formal name for *Burkholderia cepacia* genomovar VI

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Since *Burkholderia cepacia* genomovar III was renamed *Burkholderia cenocepacia*, only *B. cepacia* genomovar VI awaited a formal binomial designation. Here we report several distinguishing biochemical and molecular tests that allow the differentiation of *B. cepacia* genomovar VI from other *B. cepacia* complex organisms and propose the name *Burkholderia dolosa* for this taxon. In contrast to representatives of the other members of the *B. cepacia* complex, *B. dolosa* does not grow on PCAT medium and does not assimilate azelaic acid, salicin or tryptamine. In addition, *B. dolosa* can be differentiated from other *B. cepacia* complex organisms by *recA* RFLP (using restriction enzymes *HaeIII* and *MnlI*), 16S rDNA RFLP (using restriction enzymes *AuI*, *CfoI* and *DdeI*) and a 16S rDNA-based PCR assay.

A15. The CepRI quorum sensing system contributes to virulence in *Burkholderia cepacia* respiratory infections

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The *cepIR* genes encode an *N*-acyl homoserine lactone (*N*-AHL) dependent quorum sensing system. CepI codes for an *N*-AHL synthase that directs the synthesis of the cell signaling molecules *N*-octanoyl homoserine lactone and *N*-hexanoyl homoserine lactone. CepR is a transcriptional regulator that binds *N*-AHLs and activates or represses target genes. Although present in most genomovars of the *Burkholderia cepacia* complex, these genes have been primarily characterized in genomovar III strains. The CepIR system positively regulates expression of protease and *cepI*. It negatively regulates genes involved in the biosynthesis of the siderophore ornibactin. This system has also been shown to be required for swarming motility and to play a role in biofilm formation. The objectives of this study were to determine the importance of *cepIR* in virulence in two animal models. Sprague-Dawley rats were infected with agar beads containing K56-2, K56-

I2 (*cepI::Tp⁵*), or K56-R2 (*cepR::Tn5-OT182*). At 10 days post infection, lungs were removed and subjected to quantitative bacteriology and quantitative histopathology analyses. There was no difference between the number of bacteria recovered from the lungs of animals infected with the mutants or the parent strain; however, the extent of lung histopathological changes was significantly lower in lungs infected with K56-I2 or K56-R2 compared to the parent strain. Intranasal infections were also performed with K56 and K56-I2 in *Cftr*^{-/-} mice. The experiment was terminated after five days when one of four mice infected with K56-2 died and three were ill. Four of five mice infected with K56-I2 remained healthy. There were significantly more bacteria recovered from the lungs of mice infected with the parent strain than K56-I2. K56-2 was also invasive as evident by the presence of bacteria in the spleen, whereas K56-I2 was not recovered from the spleen of any of the mice. Lung homogenates from mice infected with K56-2 had significantly higher levels of the inflammatory mediators murine macrophage inflammatory protein-2, IL-1 beta, and IL-6 than those from K56-I2 infected animals. The results obtained in these two infection models indicate that a functional CepIR quorum sensing system contributes to the severity of *B. cepacia* infections.

A16. An updated version of the *Burkholderia cepacia* complex experimental strain panel

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To aid in identification, epidemiological tracking and virulence studies, a set of strains representative of the first five known genomovars of the *B. cepacia* complex was assembled. A review of the literature reveals that this panel has been used extensively in various areas of research (for a complete list of publications using this panel, see <http://go.to/cepacia>, follow link to *B. cepacia* experimental strain panel). Since its assembly, however, several additional species within the *B. cepacia* complex have been described and therefore we propose to update the previously described panel. Strains were selected such that they represent the currently known diversity in isolation sources and geography, and the intra-species diversity. The strains included in the expanded panel were cultured as described previously. All isolates were identified to the species level using a polyphasic approach. To avoid the inclusion of members of the same clone all strains were typed using previously described typing methods. Molecular typing showed that all strains had clearly different fingerprint patterns. Four *B. cepacia* genomovar VI strains are included in the panel. Strains CEP021 and AU0645 were isolated from CF patients in the USA, while E12 was isolated from a CF patient in the UK. Strain STM1441 was isolated from the rhizosphere of *Alysicarpus glumaceus* in Senegal. Three *B. ambifaria* strains are included in the panel. DNA-based fingerprinting showed that all possessed a unique genetic fingerprint. The type strain of *B. ambifaria*, AMMD^T, was isolated from the rhizosphere of peas (*Pisum sativum* L.) in the USA. It is one of the most studied biocontrol isolates. *B. ambifaria* ATCC 53266 is another strain with possible biocontrol applications; it was isolated from corn roots in the USA. Strain CEP0996 was isolated from the sputum of a CF patient in Australia. Four *B. anthina* strains are included. The type strain W92^T was isolated from the rhizosphere of a houseplant in Nashville (USA) in 1997, while strain C1765 was isolated from the respiratory tract of a CF patient in the UK. J2552 was isolated from the rhizosphere of *Carludauca palmata* in the tropical aquatic house of a botanic garden in the UK. Isolate AU1293 was isolated from the respiratory tract of a CF patient in

the US; this patient has been chronically infected with this organism for over two years. Four *B. pyrrocinia* isolates are included in the panel. The type strain ATCC 15958^T was isolated from soil. *B. pyrrocinia* ATCC 39277 was isolated from cornfield soil in the USA. Isolate BC011 was isolated from a blackwater stream in the USA. Isolate C1469 was isolated from a CF patient attending a treatment center in the UK. All strains were deposited in and can be obtained from the BCCM/LMG Bacteria Collection, Ghent, Belgium. On the website (<http://go.to/cepacia>) all available information on the updated *B. cepacia* complex experimental strain panel will be compiled.

A17. Evaluation of the *Burkholderia cepacia* epidemic strain marker.

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Burkholderia cepacia complex are a group of bacterium that have emerged as a highly virulent cystic fibrosis (CF) pathogen that can lead to an irreversibly fatal decline in health. The majority of infections (~70%) are accounted for by genomovar III strains of the *B. cepacia* complex. These chronic pulmonary infections are a major cause of CF patient morbidity and mortality, therefore identification of factors involved in the virulence and spread of *B. cepacia* is required. Despite advances in our understanding of the taxonomy and epidemiology of *B. cepacia* complex, knowledge is limited of the virulence mechanisms which facilitate infections. The *B. cepacia* Epidemic Strain Marker (BCESM) was initially identified in strains associated with spread among CF patients; it is now known to be specifically associated with strains for genomovar III and located on the second largest chromosome. Although associated with certain virulent strains of *B. cepacia* genomovar III, the extent of the BCESM locus and whether it encodes genes involved in virulence is not known. The BCESM was investigated by using sequence data emerging from the *B. cepacia* genomovar III strain J2315 genome project coupled with a comparative Southern hybridisation approach. Bioinformatic analysis demonstrated that the BCESM is on a low G+C genomic island in *B. cepacia* (60.8% compared with an average of 66.9% G+C content), encoding putative genes for an amidase, a major porin, an insertion sequence, lipid and acetyl homo-serine lactone (AHL) biosynthetic loci. The presence or absence of these open-reading frames and flanking loci was determined within a collection of 281 different *B. cepacia* complex strains by hybridisation to genomic DNA arrays. The extent of BCESM region determined to be approximately ~25Kb and with the genes encoding AHL synthase and a Porin at respective ends of the island. This region was present in 56% of the clinical genomovar III strains examined as an intact genomic island. Site-directed mutagenesis has been performed on the amidase, AHL-synthase and porin encoding genes. The knockout mutants will be characterised for virulence in a rat agar bead model of CF infection and a plant model. This work was funded by the UK Cystic Fibrosis Trust.

A18. News about exopolysaccharides produced by *Burkholderia cepacia* strains.

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The exopolysaccharides produced by clinical strains of *B. cepacia* isolated at the Regional Centre for CF in Trieste (BTS) were investigated. Most of the strains belonged to genomovars I and III. They produced an exopolysaccharide common to the majority of the *B. cepacia* strains studied and reported in the literature (1-3) and for which the name Cepacian was proposed. Two strains, BTS2 and BTS13, produced a different polymer composed of galactose and 3-deoxy-D manno-2-octulosonic acid (Kdo), and named Galactan-Kdo (4). The structure of the latter polysaccharide produced by the strain BTS13 was completely determined. Interestingly enough, the strain BTS13, when grown on a medium rich in mannitol (5), produced also a polysaccharide composed only of fructose, named Levan. It was previously reported that the Levan polymer was produced by other bacterial strains belonging to the genus *Streptococcus* and *Acetobacter*. Considering the possible dangerousness of the environmental *B. cepacia* strains, a study of the structure of exopolysaccharides produced by clinical and environmental strains of *B. cepacia* belonging mostly to genomovar III was undertaken. Twelve strains were examined and all except one produced the exopolysaccharide Cepacian. The strains produced Cepacian either alone or in combination with other polymers; the latter containing the same neutral sugars of Cepacian (rhamnose, glucose, galactose and mannose) but in different ratios. Only one of the clinical strains examined produced the Galactan-Kdo polysaccharide, thus indicating that this polymer is not a peculiarity of the *B. cepacia* strains isolated from patients referring to the CF Centre in Trieste. Our study showed that about 90% of the *B. cepacia* strains examined produced the polysaccharide Cepacian, and the remaining 10% synthesised the Galactan-Kdo polymer. The molecular weight and the degree of acetyl substitution of the Cepacian depend on the growth medium used. Moreover, there seem to be no correlation between genomovar and type of exopolysaccharide produced. At the same time, the investigation of the macromolecular and solution properties of Cepacian is in course (6). In particular, the aggregation properties of the polymer will be studied in the presence of chaotropic solvents, like dimethylsulfoxide. The overall aim is to better understand the aggregation characteristics of the Cepacian polymer chain. This information is extremely relevant to define the structure-function relationship and to correlate them to the "in vivo" situation.

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A19. *Burkholderia cepacia* complex bacteriophage: Their evolving role as potential therapeutic and transducing agents.

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The *Burkholderia cepacia* complex (Bcc) comprises at least nine genomovars. Although, each genomovar has been cultured from clinical material, the majority of isolates from cystic fibrosis (CF) patients are *B. cenocepacia* and *B. multivorans* (formerly genomovars III and II, respectively) (1). In the last decade, most Bcc research has focused on the taxonomy and epidemiology of the complex. Since the role of bacteriophages has been relatively neglected, and could be important in terms of therapy and genomic plasticity, we investigated the prevalence of lysogeny in Bcc and sought lytic phage from the natural environment. Our data indicated that 50% of Bcc strains are lysogenised and that all the bacteriophage, isolated so far, have a host range which extends across the genomovars. In some cases, this host range also extends to *Pseudomonas aeruginosa*, *Burkholderia gladioli* and *B. pseudomallei*. The implications of inter-species gene transfer and the acquisition of foreign virulence genes could have an impact on the modus operandi of different Bcc strains (2-4). Bcc infections are notoriously difficult to treat. Although antibiotic resistance is variable and perhaps overated, use of current antibiotic agents does not eradicate the bacteria (5). A novel approach to the treatment of Bcc infections is offered by the isolation of lytic bacteriophage from soil and plant rhizospheres. Classic bacteriophage therapy involved the use of whole phage particles, but phage resistance is common and particles are removed by the host immune system (6-7). The aim of our research is to isolate genes expressing phage lytic enzymes, including lysins and holins to provide an alternative strategy in treating Bcc infections (8).

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A20. Comparative analysis of plant and animal models for the characterization of *Burkholderia cepacia* virulence

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Alternative host models have been developed for some important human pathogens. For example, a multihost pathogenesis system has been used for identifying virulence associated-genes in *Pseudomonas aeruginosa*. Alternative host models used include: *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Galleria mellonella*. The utilisation of plants and animals as infection models has allowed the identification of new virulence genes and common virulence factors in both plant and animal infections. Although appropriate animal models are currently used for studying *Burkholderia cepacia* complex (Bcc) chronic respiratory infections including agar bead models in rats and mice, the CFTR^{-/-} knockout mouse, and the leukopenic mouse model, alternative infection models for preliminary analysis of Bcc virulence have not yet been described, with the exception of the intracellular models in amoebae and macrophages. A simple alfalfa model was developed as an alternative infection model for Bcc virulence studies. Symptoms of disease were observed in wounded alfalfa seedlings within 7 days following inoculation of 10¹-10⁵ colony-forming units of most Bcc strains. Strains from seven genomovars of the Bcc were tested for virulence in the alfalfa model, and the degree of virulence generally correlated among strains belonging to the same genomovar. Strains of *B. multivorans* and some strains of *B. stabilis* did not cause symptoms of disease in alfalfa seedlings. Representative strains from five different genomovars were also tested for virulence using the rat agar bead model. Most of the strains tested were able to establish chronic lung infections with the exception of the *B. stabilis* strains. Most of the strains that were virulent in the alfalfa infection model were also virulent in the lung infection model. Therefore, this alfalfa infection model may be a useful tool for assessing virulence of strains of the Bcc.

A21. Prevalence and clonality of infection with *Burkholderia cepacia* complex genomovars in UK cystic fibrosis patients referred for lung transplantation

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The Freeman Hospital Transplant unit serves Cystic Fibrosis units in Northern England, Scotland and both Northern and Southern Ireland. We have assessed the distribution and clonality of genomovar infections in patients referred to this centre. We performed a phenotypic and molecular analysis of all suspected BCC organisms. A retrospective review of the pulmonary transplant database at the Freeman Hospital, from 1989 (program start) to the present date, was performed. Initial phenotypic analyses were performed using the API 20 NE diagnostic system. All BCC isolates (greater than 150 isolates) were assessed blinded using *B. cepacia* complex specific primers BCR1 and BCR2, the recA PCR-RFLP speciation results were also confirmed with genomovar specific primers. In specimens found to be genomovar III, amplification of cable pilus sequences and the *Burkholderia cepacia* epidemic strain marker was performed as previously described. We used Pulsed-Field Gel-Electrophoresis to assess clonality of infection and also to assess any possible change in strains pre and post transplant. 32 patients referred from 9 centres were initially identified as having *B. cepacia* in their sputum using phenotypic tests. Thirteen of these patients were transplanted at

this centre; the post-transplant outcome results of 11 of these have previously been reported.[De Soyza, Lancet 2001] The remaining patients were assessed for transplantation at this centre and are either awaiting transplantation or have died. We were able to confirm that 28 of the 32 patients identified did have *B. cepacia* complex infections using PCR based techniques. One isolate was found to be *Brevundimonas vesicularis* on re-testing whilst the remaining 3 isolates await species designation. The most prevalent genomovar was genomovar III with 16 patients affected (50%) of these 14 were genomovar III-A (44%) and two were III-B (6%). *B. multivorans*, genomovar II, was the second most prevalent organism with 10 patients infected (31%). The remainder were infected with genomovar V, *B. vietnamiensis*, (n=2, 6%). Genomovar III-A strains were identified and of greater than 20 isolates tested all were cable pilus positive. As controls isolates from eight *B. multivorans* and two *B. vietnamiensis* infected patients were tested and all were cable pilus negative. All of the above genomovar III strains that were cblA positive were also BCESM positive. PFGE revealed that the ET-12 genomovar III-A clone was responsible for infection in 13 of 14 genomovar III-A infections. The remaining genomovar III strains were all unique. One patient infected with ET-12, died of Cepacia Syndrome on the active waiting list for transplant. All of the deaths following transplantation where stored isolates were recoverable were associated with the ET-12 clone. Since our initial findings, we have adapted our management of the GIII infected patients and two ET-12 infected patients have been successfully transplanted (survival greater than 9 months). Where pre and post transplant strains were available PFGE confirmed clonality of the strain within a patient. All *B. multivorans* infections were attributable to unique strains as were the *B. vietnamiensis* infections. Our experience has been different to that of the N. Carolina study where poor transplant outcomes were associated with a variety of genomovar III strains (all *cbl* negative). We conclude that genomovar III-A ET-12 infections are the predominant infection in patients referred to our UK centre and this strain has been associated with poor transplant outcomes using standard management.

A22. Isolation and characterisation of chlorhexidine and triclosan-sensitive mutants of *Burkholderia cepacia* K56-2

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The *Burkholderia cepacia* complex consists of gram-negative opportunistic pathogens which cause severe and frequently fatal respiratory infections in patients with cystic fibrosis. *B. cepacia* bacteria are intrinsically resistant to many antimicrobial compounds and have been reported to survive within and contaminate disinfectant solutions. However, specific mechanisms of resistance and their genetic basis remain poorly understood. In this study the resistance of *B. cepacia* to the widely used antimicrobial compounds chlorhexidine diacetate and triclosan was investigated. The main objectives were to (1) isolate and (2) characterise triclosan and chlorhexidine-sensitive mutants of this organism using a random transposon mutagenesis system. *B. cepacia* K56-2, a clinical isolate recovered from a cystic fibrosis patient was used. This strain is highly resistant to both triclosan and chlorhexidine with an MIC value of 200 µg/ml and >1000 µg/ml respectively. The suicide vector pOT182, carrying a self-cloning transposon system was mobilized into strain K56-2 via *E. coli* SM10. Transposon mutants were isolated on selective media containing tetracycline and 2000 individual mutants were screened for biocide sensitivity by replica plating onto medium containing either triclosan or chlorhexidine. From this screen two triclosan and four chlorhexidine-sensitive mutants were isolated. All mutants showed a substantial increase in sensitivity to the biocides tested. In addition cross-sensitivity to other biocides and altered

antibiotic susceptibility was also observed when compared to the wild-type strain. All six mutants have been genetically characterised and a number of different genes involved in biocide resistance identified. Although, two of the four chlorhexidine-sensitive mutants had transposon insertions at different locations within the same gene, as a result only one of the mutants were retained for further analysis. Triclosan-sensitive mutants were found to carry transposon insertions within genes homologous to (1) *tmpA*; a probable membrane protein and (2) *lcfL*; a long chain fatty acid CoA ligase protein. Chlorhexidine-sensitive mutants were found to carry transposon insertions within genes homologous to (1) *LytB*; encoding a protein involved in polymyxin resistance, (2) *hslU*; encoding a poorly defined heat shock protein and (3) *glfA*; a glycosyltransferase. Site directed mutagenesis of the *tmpA* gene has been achieved and confirmed its role in resistance to triclosan. Cosmids encoding all the biocide-resistance associated genes have been identified from a *B. cepacia* genomovar III K56-2 library and will be used to complement the transposon mutants. The identification of genes involved in disinfectant resistance and elucidation of the mechanism of biocide action may lead to the development of more effective and even novel antimicrobial compounds.

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A23. Feasibility of signature-tagged transposon mutagenesis for investigation of the environmental fitness of the *Burkholderia cepacia* complex

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The *Burkholderia cepacia* complex (BCC) represents an abundant group of environmental bacteria. Members are commonly found in soil and river sediments, but have also adapted highly polluted sites. The BCC exhibits great metabolic diversity, and some members possess the potential for biotechnological exploitation as biocontrol and bioremediation agents. Biocontrol strains such as *Burkholderia ambifaria* AMMD are dominant rhizosphere bacteria and antagonise soil-borne plant pathogens. They confer protection to crops such as peas and tomato, thus promoting crop growth and increasing harvest yields. The bioremediation strain *Burkholderia vietnamiensis* G4 (ATCC 53617) can degrade organic pollutants such as toluene, phenol, and trichloroethylene through degradative enzymes encoded on the large self-transmissible plasmid pTOM. A fundamental question in environmental genomics is what genes are expressed in a given habitat, which allow an organism to prosper and be considered fit? Biocontrol and bioremediation are both multi-factorial biological processes, and only a limited number of associated genes have been identified. We propose to use the powerful functional genomic approach of signature-tagged transposon mutagenesis STM, to identify genes essential for biocontrol and bioremediation in the BCC. STM technology has been used extensively to identify bacterial pathogenicity genes, but has not yet been used to study interactions in the natural environment. STM enables a large number of mutants to be screened simultaneously in a single model, due to the generation of tagged mutants which can be monitored. The tags consist of a 40 bp unique oligonucleotide sequence, flanked by two 20 bp invariable arms that allow PCR amplification and labelling of the tag sequence. Individual tags are incorporated into a modified mini-Tn5 transposon, which is used to mutagenise the bacterial chromosome. The uniquely tagged mutants generated are organised into 96-well plates where each well contains a mutant with the tag corresponding to a designated well. These mutant banks are then subjected to a selective pressure in the form of an infection model/microcosm, before the mutants are retrieved. A comparison of hybridisation patterns of the input and output

labelled tags against a complete set of the 96 tag sequences, will reveal mutants present in the input but not output pool. Mutants failing to survive the selective pressure (normally lost in such screens) can be identified, revealing the identity of genes essential to the process being examined. In this study, separate mating experiments were carried out between *B. vietnamiensis* G4 and 96 uniquely tagged *E. coli* S17.1::pUT-miniTn5Km2. Mutant DNA was digested with *EcoRI* and probed for the transposon kanamycin gene; a differently sized single band was detected for each mutant indicating random transposon insertion had occurred. Sixty signature-tagged mutant banks have been created, which will be screened for "unfit" mutants in rhizosphere colonisation and phenol degradation microcosms (currently under development). Preliminary screening results suggest that there is an unusually high percentage of phenol mutants (0.92%) compared to auxotrophic mutants (0.62%). This suggests that there is a hot spot for transposon mutagenesis in the phenol pathway, which could be associated with the plasmid location.

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A24. Short term *in vivo* model that mimics *B. cepacia* lung infection in CF patients

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In CF patients, respiratory infection with *B. cepacia* complex (Bcc), especially genomovar III strains has been recognized as an important risk factor. About 20% of infected patients succumb to the acute fatal cepacia syndrome either within a year or after being chronically infected for several years. Previously, we demonstrated that repeated exposure to *B. cepacia* (genomovar III, ET12 strain) caused chronic lung infection only in CFTR (-/-) mice (a long term model). In the present study, we have attempted to create a short term model of lung infection, in preparation for possible use in testing novel therapeutic approaches and/or virulence of other members of Bcc. Liquid fed CFTR (-/-) or littermate control (CFTR (+/+)) mice, age 10-12 weeks (20-24 per group) were infected intranasally with the isolate BC7 (1×10^8 cfu) and sacrificed after 4 hr, 1, 3, or 5 days (4-6 mice at each time point). Lungs were aseptically removed, fixed in 10% formalin and processed for histological evaluation, or homogenized and tested for bacterial persistence and cytokine levels. Lungs from mice instilled with PBS alone were used to determine the basal levels of cytokines. At 4 hr, CFTR (-/-) mice showed excessive pro-inflammatory cytokine TNF- α , and chemokine KC, (5 and 16 fold respectively) compared to CFTR (+/+) mice, and 28 and 1600 fold, respectively, over basal levels. By 24 hr both groups of mice showed almost equivalent increased levels of TNF- α and KC (9 and 300 fold respectively, over basal levels), and equivalent numbers of bacteria in their lungs. However CFTR (+/+) mice exhibited a greater inflammatory response than the CFTR (-/-) mice. By day 3, CFTR (-/-) mice showed more bacteria than CFTR (+/+) mice (1×10^5 vs 1×10^3 cfu/lung). At the same time, both groups of mice showed severe inflammation, but almost normal levels of TNF- α and KC. By day 5, CFTR (-/-) mice showed an increase in the number of bacteria (1×10^6 cfu/lung) indicating bacterial proliferation, severe lung inflammation and slightly higher levels of both TNF- α and KC. In contrast, CFTR (+/+) mice had cleared all bacteria, had normal cytokine levels and only mild inflammation. Immunostaining of lung sections (at day 3) for bacteria, neutrophils, and macrophages, revealed bacteria in inflammatory foci, and co-localized with neutrophils and macrophages. In CFTR (-/-) mice, most of the phagocytosed bacteria were intact, whereas in CFTR (+/+) mice, bacteria within macrophages and neutrophils were mostly fragmented. In summary, after a single intranasal dose of isolate BC7 (1×10^8 cfu), *B. cepacia* persisted better and caused severe inflammation only in CFTR (-/-), but not in CFTR (+/+) mice. These pathological changes were distinguishable between

day 3 and 5. Because it requires only 3-5 days, this model is useful to test virulence of other members of Bcc and/or the effects of novel therapeutic agents.

A25. Genomovar status and DNA fingerprinting of *Burkholderia cepacia* complex isolates from Italian cystic fibrosis patients.

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Introduction: *B. cepacia* complex has emerged as a major pathogen in patients with cystic fibrosis (CF). These organisms are resistant to a broad range of antibiotics and exist as nine distinct genomovars which vary in their degree of transmissibility and virulence.

Aims: To investigate the national prevalence and genomovar status of *B. cepacia* complex in patients from 16 (61.1%) Italian CF centers.

Methods: 178 isolates presumed to be *B. cepacia* complex were obtained from 178 CF patients attending 16 centers. The genomovar status of these isolates was determined by means of a polyphasic approach including whole cell protein analysis, DNA-DNA hybridisation experiments, and recA sequence and RFLP analysis. Pulsed-field gel electrophoresis (PFGE) using the restriction enzymes *Xba*I and *Spe*I was performed to analyse the clonality of the isolates.

Results: 167 out of 178 strains belonged to the *B. cepacia* complex, 1 was *Pseudomonas* spp., 7 *B. gladioli*, 1 *Inquilinus limosus*, 1 *Alcaligenes xylosoxidans*, and 1 *P. huttienensis*. Genomovar analysis showed that *B. cenocepacia* (genomovar III) was the most prevalent *B. cepacia* complex species (70.6%), followed by *B. cepacia* (genomovar I - 9.0%), *B. stabilis* (genomovar IV - 7.2%), *B. multivorans* (genomovar II ; 5.4%) and *B. pyrrocinia* (genomovar IX ; 4.8%). *B. vietnamiensis* (genomovar V ; 0.6%), genomovar VI (0%), *B. ambifaria* (genomovar VII ; 0%) and *B. anthina* (genomovar VIII ; 0%) were not or only rarely found.

Genomic fingerprinting has currently been performed on 135 *B. cepacia* complex isolates cultured from patients attending 11 Italian CF clinics. PFGE revealed 57 unique strains. The remaining isolates were distributed amongst 19 clusters. The number of isolates within these clusters ranged from two to eight. Clusters of eight patients sharing the same *B. cenocepacia* clone were identified in two separate CF centres. Two clusters (one with 5 patients from a first center and one with 3 patients from a second center) were identified as *B. pyrrocinia*.

Discussion:

-167/178 (94%) isolates were correctly identified as *B. cepacia* complex demonstrating a good performance of the microbiology laboratories.

-The genomovar distribution is characterized by a predominance of *B. cenocepacia* (genomovar III) and relatively few *B. multivorans* isolates.

- Most clusters of patients were colonized by *B. cenocepacia* clones representing different RecA RFLP types.

- This is the first report of transmission of *B. pyrrocinia* among CF patients.

A26. Predictors of outcome in *Burkholderia cepacia* positive patients.

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Cystic fibrosis patients infected with *B. cepacia* have a higher and unpredictable mortality after transplant.

Objective: Our primary objective was to compare the pre-transplant characteristics of CF transplant recipients who survived with those who died within 90 days after transplantation.

Methods: We analyzed our patients *B. cepacia* (+) who have been transplanted from 1988 to 2001 to determine if there were any predictors of outcome. Twelve early deaths among 41 recipients were identified.

Results: Univariate analysis using Cox regression to model survival after lung Tx showed that neither age, sex, creatinine, FIO₂, PaCO₂, diabetes, donor age nor sex had an effect.

A model with donor and recipient CMV serostatus preTx showed that the hazard ratio and P-value for a positive donor were 2.4 and 0.021, respectively, and for a positive recipient the HR was 0.6 with p=0.159. Remarkably, only in one patient was CMV considered a factor in his death. Most of these patients have died of sepsis by *B. cepacia* where CMV infection was not a factor.

Conclusion: a CMV(+) donor increases the risk of dying of sepsis with *B. cepacia* early after lung transplantation. The mechanisms of this remains to be established. Further studies on the effect of CMV on the immunological status of the recipient and/or increased virulence of the *B. cepacia* are needed. Transplanting organs from CMV(+) donors to *B. cepacia* (+) patients should be considered a very high risk procedure.

A27. Prevalence and diversity of *Burkholderia cepacia* complex in pristine soils worldwide

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While most of the studies reported so far deal with clinical isolates or samples coming from areas affected by human activity (e.g., cropped soils, city surroundings), nothing is known about the actual prevalence and diversity of the nine genomovars representing the *Burkholderia cepacia* complex (Bcc) in pristine soils worldwide. Existing data suggest however that those strains could show some patterns of local diversification. For instance, they share unusual biological characteristics with e.g., large genome size, genomic plasticity, recombinations occurring in nature, and they are isolated from different ecological habitats ranging from soils and rivers to human lungs. To test the hypothesis that natural Bcc populations are locally adapted (endemicity), we propose to compare the genetic diversity of Bcc isolated from a worldwide collection of pristine soils with their origin of isolation. Soil samples under investigation were collected in previous studies following a hierarchical sampling scheme scaling from 5 m over 200 m transects, to multiple sites in the same region, to sites in five continental regions. Available methods for isolating Bcc isolates are based on cultivation on semi-selective media and further molecular screenings by PCR

amplification of 16S rRNA and *recA* genes. Based on this methodology, a high throughput molecular method is implemented to screen hundreds of isolates recovered from semi-selective media at low levels of soil dilutions. When a significant collection of candidate Bcc isolates is obtained, further characterization will be performed at the genomovar and genotype levels using genomovar-specific and rep-PCR and/or RAPD primers, respectively. We expect to see a positive correlation between genetic and geographic distances, which would indicate a significant diversification of Bcc in nature. Our preliminary data indicate that Bcc, if present in pristine soils, represent low population levels and semi-selective media containing antibiotics could bias the recovery of a large and diverse population.

A28. Characterization of a locus required for cable pilus biogenesis in *Burkholderia cepacia*: role of cable pili in mediating cell-cell interactions

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Cable pili are unique peritrichous adherence organelles expressed by certain strains of the *Burkholderia cepacia* complex. Cable pili have been shown to mediate binding to both cellular and acellular receptors, and likely promote *B. cepacia* colonization of the respiratory tract in compromised hosts. We have undertaken a genetic analysis of cable pili and have identified a locus encoding structural, accessory and regulatory components of the cable pilus biosynthetic pathway. Mutagenesis of the *cblA* gene, encoding the major structural subunit of cable pili, resulted in a block of cable pilus morphogenesis and expression of the CblA major pilin. Microscopic, biochemical and flow cytometric analyses of *B. cepacia* cultures revealed that cable pilus expression facilitated the formation of diffuse cell networks, whereas lack of cable pilus expression enhanced the formation of compact cell aggregates. Diffuse cell-cell interactions mediated by cable pili may maximize the exposure of pilus adhesin(s) to receptors in the host, thus facilitating colonization. Furthermore, the cable pilus-independent formation of compact cell aggregates may also promote colonization by facilitating co-operative binding to host receptors. Future studies will further characterize both cable pilus-dependent and -independent *B. cepacia* cell-cell interactions.

A29. Quantitative real-time PCR of *B. cepacia* directly from sputum.

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Assessing the role of an individual gene product in a human host is difficult. Often, the closest we can come is to extrapolate from studies performed in an animal model. However, this is not always sufficient to provide answers of expression, regulation and function in the human host. For example, the current animal models of *B. cepacia* infection do not truly mimic the disease progress in humans, such as long term colonization followed by a rapid decline. Over the last several years it has become possible to analyze regulation of individual genes by quantitative real-time PCR (Q-PCR). The requirements for this procedure are a known

gene sequence and a quality RNA sample. With the release of the *Bcc* J2315 sequence we have access to a host of previously undescribed genes. To allow us to utilize this data we have performed preliminary experiments with *B. cepacia* genomovar III grown *in vitro*. More recently we have developed an RNA extraction protocol to purify *Bcc* RNA directly from sputum. Sputum is immediately mixed upon expectoration with a reagent cocktail designed to partially liquefy the sputum, kill both microorganisms and human cells and stabilize the RNA profile of the samples. The samples are then frozen or immediately processed using the Qiagen RNeasy kit to purify the total RNA. Following a DNase treatment, aliquots of the RNA are used to synthesize cDNA for use in the Q-PCR reaction. To ensure that this protocol accurately reproduced the RNA profile of the sputum organisms, we performed the following experiment. Sputum samples were obtained courtesy of Dr Tullis at the Toronto CF center. This center has as its predominant clone an ET12 lineage. Upon expectoration, a sample of sputum was mixed with our reagent cocktail and split into 3 approximately equal aliquots. The semi-viscous material in each aliquot was further lysed by the addition of DTT until the solution liquefied. Following DNase-treatment and cDNA synthesis, each of the three samples was used in triplicate in a Q-PCR reaction with the primer pairs targeting the following genes: 16S rRNA, *cepI*, and *cepR*. These genes were chosen since they are well documented and sequences are available. It was immediately apparent that the levels of 16S rRNA transcripts varied, however the ratio of the *cepI* to *cepR* was fixed between each sample. Using this ratio as a guide, we demonstrated a variance of approximately 8% between aliquots of the original sputum sample. We have repeated this procedure with other sputum samples and shown similar results. Thus, we have established an accurate, reproducible procedure to examine RNA profiles/gene expression during *Bcc* infection in the human lung.

A30. Plant tissue watersoaking: a type IV secretion system associated trait in *Burkholderia cepacia* strain K56-2

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Burkholderia cepacia strain K56-2, a genomovar III representative of the *B. cepacia* complex, is part of the epidemic and clinically problematic ET12 lineage. The strain produced plant tissue watersoaking (ptw) on onion tissue, which is a plant disease associated trait. Using plasmid mutagenesis, mutants in the ptw phenotype were generated. The translated sequence of a disrupted (*ptwD*) gene from a ptw-negative mutant showed homology to TraD of the F-plasmid transfer cluster. Analysis of the region proximal to the transfer gene homologue resulted in the identification of a cluster of 11 genes (*ptwD*, *I*, *E*, *B*, *C*, *W*, *U*, *NF*, *H* and *G*) on a 40 kb locus, located on a resident 92 kb plasmid, with translated products that showed homology to a conjugal type IV secretion system. The ptw phenotype was not observed in K56-2 derivatives with a disruption in *ptwD* or *ptwC*, but was observed in a derivative with a disruption in *ptwI*. Plasmid pMMB207 $\alpha\beta$ was mobilized by strain K56-2 into an isogenic strain and *Pseudomonas syringae*. Plasmid mobilization was not detected when derivative strains with mutations in *ptwD*, *I* or *C* were used as donors. Analysis of K56-2 cultural supernatants revealed that the putative ptw effector(s) was a secreted, heat-stable protein(s) that caused plasmolysis of plant protoplasts. The cytotoxic activity was not observed in derivatives with a disruption in *ptwD* or *ptwC*; however, the derivative of *ptwI* still retained activity. Our results indicate that mobilization of plasmid DNA and transport of the ptw effector(s) to the extracellular milieu is mediated via a plasmid-encoded type IV secretion system.

A31. The impact of oxygen on the interrelationship between *P. aeruginosa* and *B. multivorans*

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It is generally believed that *Burkholderia cepacia* and *Pseudomonas aeruginosa* co-exist in cystic fibrosis (CF) airways. Since in vivo oxygen measurements of the mucopurulent material obstructing the lobar bronchi of chronically infected CF patients showed a rapid decline in pO₂ with increasing depth within the mucopurulent masses (Worlitzsch et al. JCI 109:317, 2002) bacteria trapped in mucus appear to grow in an oxygen-starved environment. In the present study, we evaluated the interspecies relationship between *B. cepacia* and *P. aeruginosa* during growth in airway surface liquid (ASL) under aerobic/anaerobic conditions. To evaluate mixed cultures for aerobic/anaerobic growth in ASL harvested from well-differentiated human airway epithelial cultures (CF and non-CF), equal numbers of *P. aeruginosa* Pae33 (CF isolate) and *B. multivorans* J-1 (CF isolate, genomovar II) were co-incubated, and after 72 hours, serial dilutions were prepared and plated in parallel onto trypticase soy agar and *B. cepacia* selective agar for bacterial enumeration. When mixed cultures of *B. multivorans* and *P. aeruginosa* bacteria were incubated under aerobic conditions, total killing of *B. multivorans* J-1 occurred in the presence of Pae33 at 72 hours of incubation. However, when we incubated mixed cultures of *P. aeruginosa* and *B. multivorans* in ASL under anaerobic conditions we observed a different outcome. Growth of *B. multivorans* J-1 in the presence of *P. aeruginosa* Pae33 was inhibited under anaerobic conditions. These observations were made independently of testing mixed cultures in CF or non-CF ASL. Our data indicate that oxygen availability affects the interaction between *P. aeruginosa* and *B. cepacia*. While *P. aeruginosa* killed *B. multivorans* under aerobic conditions, *B. multivorans* was still able to survive in the presence of *P. aeruginosa* under anaerobic conditions. The survival of *B. cepacia* in the presence of *P. aeruginosa* under conditions of oxygen depletion in our airway epithelial cultures may reflect events occurring in vivo; i.e. co-existence of *B. cepacia* and *P. aeruginosa* in the endobronchial lumen of CF patients as demonstrated by the co-isolation of both bacteria from CF sputum cultures. Further studies are required to elucidate the mechanism(s), which allows *B. multivorans* to compete with *P. aeruginosa* during anaerobic growth in ASL. This work was supported by research grant to Ute Schwab from the North American Cystic Fibrosis Foundation.

A32. Analysis of *Burkholderia cepacia* J2315 sigma 70 family of transcriptional factors.

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ABSTRACT: Transcriptional sigma 70 factors play a key role in gene expression by binding reversibly to the eubacterial DNA-dependent RNA polymerase (RNAP) to form the holoenzyme that will initiate mRNA synthesis. The sigma 70 factor is responsible of the promoter-specific attachment of the complex. Through evolution, selective pressures have favored the emergence of several paralogous lineages of sigma 70, and these factors became a central component of bacterial adaptability to changing environments. The sigma 70 factors have been divided into four

groups according to sequence similarities and promoter-recognition specificities: (1) the essential sigma factors, named the primary sigma, and involved in exponential growth, (2) the stationary phase and alternative actinomycetal sigma factors; (3) the factors involved in sporangium development, flagellin synthesis and heat shock response, and (4) the ones involved in extra-cytoplasmic activities (named ECF). Several of these factors are involved in bacterial virulence. For example, AlgU of *Pseudomonas aeruginosa* is an ECF factor involved in the conversion to mucoidy, and HrpL of *P. syringae* (phytopathogen) is involved in type III secretion. The sigma 70 family is likely to play a key role in *B. cepacia* colonization of the respiratory tract of cystic fibrosis patients. The genome of *B. cepacia* J2315 was recently completed by the Sanger Institute. In this talk, I will present an analysis of the sigma 70 gene family of this genome. Blast searches were performed to recover the gene sequences, and their protein sequences were deduced and classified into the major sigma 70 groups. Phylogenetic analyses were performed to investigate their relationships with well-established bacterial factors, to infer their possible function, and identify duplication events. Our conclusions about the evolution of this gene family in *B. cepacia* and the eubacteria will be presented.

A33. Isogenic Mutants of *B. cepacia* BC7 Cable Pili and 22 kDa Adhesin: Effects on Adherence/Invasion of Cultured Squamous Epithelial Cells

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Burkholderia cepacia complex (Bcc) infections in cystic fibrosis (CF) patients can be highly variable in their clinical presentation. Approximately 20% of CF patients infected with Bcc develop the cepacia syndrome, characterized by bacteremia and necrotizing pneumonia. Adherence of bacteria to epithelial cells is considered to be one of the first steps in the infection process. Previous studies have shown that the presence of cable pili and the associated 22 kDa adhesin enhance the ability of Bcc strains of the genomovar IIIa, ET12 lineage to bind to and invade epithelial cell cultures via the cytokeratin 13 receptor, which is expressed abundantly in CF airway epithelia. To determine the specific contribution of the cable pili and the adhesin in adherence and pathogenesis, isogenic knockouts were constructed. Specifically, the genes encoding *cblA* (major pilin subunit), *cblS* (hypothesized regulator), and *adhA* (the gene encoding the 22 kDa adhesin) were individually insertionally inactivated in the BC7 genome with a trimethoprim resistance cassette. PCR analysis of genomic DNA confirmed the interruption of these genes. Immunoblot analysis using an antibody to CblA confirmed its absence in the *cblA*-mutant. The *cblS*-mutant was also found to be deficient in CblA production, suggesting a role for *cblS* in regulation of the cable pilus operon. Both the *cblA*- and *cblS*-mutants showed approximately 50% binding to cytokeratin 13 relative to the parent strain, as revealed by bacterial overlay assays. The *adhA*-mutant expressed CblA by immunoblot analysis, but did not exhibit binding to cytokeratin 13. Preliminary results of an *in vitro* assay, using tracheobronchial epithelial cells differentiated into squamous cell cultures, indicate that all of the mutants show a significant decrease in adherence/invasion. This difference was most pronounced in the *adhA*-mutant. Results thus far indicate that although the cable pilus is not necessary for the expression and function of the 22 kDa adhesin, it enhances the 22 kDa adhesin-mediated binding of *B. cepacia* to cytokeratin 13, as well as adherence/invasion of squamous epithelial cell cultures.

A34. *Burkholderia cepacia* complex Traverse Polarized Respiratory Epithelium by Disrupting Tight Junctions

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Unlike the other respiratory pathogens with which persons with CF are infected, *Burkholderia cepacia* complex (Bcc) can cause necrotizing pneumonia and septicemia. However, the mechanisms by which Bcc traverse respiratory epithelium are unknown. Previous studies have demonstrated that some Bcc can survive and replicate within A549 cells, type II pneumocytes that do not polarize. More recent work suggests that isolates of three different genomovars were able to traverse well-differentiated primary bronchial epithelial, via both inter- and intra-cellular routes. We hypothesize that tight junction disruption by Bcc is integral to transmigration of polarized epithelia. We chose 16HBE14o-, a transformed bronchial epithelial cell line that polarizes when grown on extra-cellular matrix proteins in an air liquid interface (on semi-permeable cell culture inserts), or in liquid covered culture (on coverslips). These cells show organized localization of occludin and ZO-1 at the tight junctions. Polarized 16HBE14o-cell layers were infected at a multiplicity of infection of ~100:1 with one of three Bcc genomovar III strains: PC8, PC184, and AU0355, representing the PHDC, Midwest, and ET12 epidemic lineages. Polarized epithelia were also mock infected with PBS or infected with non-pathogenic *E. coli*, JM109, as negative controls. By confocal microscopy and immunostaining, Bcc infected monolayers showed dislocation of occludin and ZO-1 from the tight junctions. Conversely, control cell showed no disruption of tight junction organization. Polarized cell layers were also exposed to spent bacterial culture supernatant, or to live organisms suspended above cell layers in cell culture inserts through which no organisms could pass, and neither occludin nor ZO-1 was disrupted under these conditions. When 16HBE14o- cells were grown in an air-liquid interface on cell culture inserts (3 µm pore size), all three Bcc strains were detected in the basolateral chamber by 8 hours post-infection, while JM109 never crossed the epithelial layer- even after 24 hours of infection. Transepithelial electrical resistance (TER), a biophysical marker of polarization, was measured at different time points during infection. The average initial TER of the uninfected epithelial cell layers was 550 ohms x cm² (300-850). After infection by each of the three Bcc strains, the TER dropped to ~30% of the initial value by 12 hours (p<0.01), and to less than 10% of initial TER by 18 hours post-infection (p<0.01). The TER of controls remained >80% of initial value for all time points. We also measured the flux of FITC-labeled bovine serum albumin (FITC-BSA) from the apical side to the basolateral side of the monolayer to determine changes in epithelial permeability. In Bcc infected cell layers, FITC-BSA translocated to the basolateral side two to five times more than in control wells (p<0.01). In those epithelial layers infected with PC184 and AU0355, the amount of FITC-BSA translocation doubled that of PC8 infected cell layers (p<0.01). Bcc infected epithelial layers showed no increase in trypan blue uptake or in LDH release when compared to control cell layers. Likewise, no difference was seen between control and experimental cells in TUNEL assays. These results indicate that the changes observed following Bcc infection (decreased TER and increased FITC-BSA translocation) were not the result of cytotoxicity or apoptosis. Finally, TEM images showed contracted epithelial cells with organisms mainly between cells; very few intra-cellular organisms were observed. Taken together, these findings suggest that Bcc traverse polarized respiratory epithelia by dislocating occludin and ZO-1 at tight junctions without direct cytotoxicity.

A35. Characterization and specificity of CepR binding to four promoters regulated by CepIR.

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Quorum sensing has been shown to regulate the expression of virulence factors in many gram-negative bacteria. Quorum sensing is a cell signaling system involving an *N*-acyl homoserine lactone (AHL) synthase and a transcriptional regulator that binds the AHL and activates or represses the expression of certain genes. Two such regulatory mechanisms have been characterized within the *Burkholderia cepacia* complex. CepIR appears to be ubiquitous within the *B. cepacia* complex, while BviIR has been identified only in the species *B. vietnamiensis* (genomovar V). CepIR has been shown to regulate protease expression (*zmpA*), biosynthesis of the siderophore ornibactin (*pvdA*), the synthesis of *N*-octanoyl-HSL and *N*-hexanoyl-HSL (*cepI*), *cepR* expression, and swarming motility. Evidence also suggests that biofilm formation, which has been demonstrated to occur in the CF lung, is a phenomena dependent on a multitude of quorum-sensing regulated genes. Understanding how this regulatory mechanism controls gene expression will be an important factor in characterizing its contribution to the virulence of *B. cepacia*. Four genes previously cloned and characterized in our lab have been shown to be regulated by CepIR. The genes *cepI* and *zmpA* are activated in the presence of OHL, while the expression of *pvdA* and *cepR* are repressed. The promoter regions of each of these genes has been subcloned and the PCR amplified products subjected to gel shift analysis. Each of the promoters demonstrated a mobility shift in the presence of purified CepR at concentrations of 0.2 pmole DNA to 5 pmole CepR. Binding was shown to be specific by competing 32-P labeled *cepI* promoter DNA with unlabelled DNA. A CepR binding box was predicted for each of the promoters. Interestingly, homology of the predicted CepR box between the two negatively or the two positively regulated promoters was significantly greater than when negatively and positively regulated genes were compared to one another, suggesting there may be differences in the consensus sequence for the two types of regulation.

A36. Survival benefit of lung transplantation in cystic fibrosis patients with *Burkholderia cepacia* genomar III.

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Cystic fibrosis (CF) is worldwide the principal indication for bilateral lung transplantation. CF patients infected with *B. cepacia* have had a poor prognosis after this procedure compared to those not infected. We used survival regression models to analyze the effect of lung transplantation on the risk of dying in a cohort of 271 Toronto CF patients with positive sputum cultures for *B. cepacia* from January 1, 1987 to Dec 31, 2001. Virtually all were genomovar III. Cox proportional hazard models were computed with sex and age on entry as fixed covariates, and time-dependent covariates for FEV₁ and lung transplantation. A second set of models was computed for a contemporaneous cohort of 399 patients whose lungs were colonized with *Pseudomonas aeruginosa* only. There were 42 transplants and 133 deaths in the *B. cepacia* cohort, 28 transplants and 35 deaths in the *P. aeruginosa* cohort. Results: Single variable models showed that the hazard ratio was 4.2 for transplanted vs untransplanted

patients, indicating a significantly worse outcome ($p < 0.001$). However, when the effects of sex and FEV₁ were accounted for in a multivariable model the hazard ratio for lung transplant was 0.62 ($p = 0.059$) indicating a marginally significant benefit. In a more complex model the FEV₁ * transplant interaction term was significant ($p = 0.035$) indicating that the effect of transplant on mortality hazard was greater at lower levels of FEV₁. Calculation of the varying risks from the model gave a hazard ratio of 1 at FEV₁ = 27% predicted, 0.5 and 0.3 at FEV₁ = 20% and 15%, respectively. The comparison of models for *B. cepacia* and *P. aeruginosa* gave overall hazard ratios for lung transplant (adjusted for FEV₁ and sex) of 0.57 ($p = 0.026$) in the *B. cepacia* cohort, and 0.18 ($p = 0.029$) in the *P. aeruginosa* cohort. Conclusions: Lung transplantation provides significant survival benefit to CF patients with *B. cepacia* genomovar III, reducing the mortality risk to an average 57% of that for non-transplanted patients at the same level of FEV₁. This compares to a hazard ratio of 18% for transplanted vs non-transplanted patients who have *P. aeruginosa* only. The magnitude of this difference is consistent with previous reports, but this analysis shows that lung transplantation is a positive option for CF patients with *B. cepacia* genomovar III. These findings motivate continued efforts to refine pre- and post-transplant protocols to optimize the outcome for these patients.

A37. Incidence of “cepacia syndrome” in cystic fibrosis patients infected with *Burkholderia cepacia*.

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Aim: To identify the incidence of “cepacia syndrome” (CS) in a large population of cystic fibrosis patients infected with *Burkholderia cepacia*. **Methods:** All patients who attended the CF program from 1970 to 2001 at The Hospital for Sick Children or at St Michael's/Wellesley Hospital who had at least one positive sputum culture for *B. cepacia* were eligible for the study. Relevant clinical, demographic and microbiology data was abstracted from the patient database. To identify individuals with possible CS a model was generated and applied to the available data of all eligible patients. The model identified any patient who died within 12 months of acquisition of *B. cepacia*, any who had an FEV₁ > 35% predicted within 6 months of death, or who had either a white blood count (WBC) > 25x10⁹/L, an ESR > 50mm/hr or fever > 38.5°C recorded from the admission during which they died. All patients who had insufficient data available to apply this model and who had died were also identified. Once identified all available clinical, radiological and microbiological data was reviewed from case notes. CS was confirmed if patients had either a positive blood culture for *B. cepacia*, or a new multifocal pulmonary infiltrate on CXR plus at least 2 of the following clinical features: 1. fever (more than one recorded temp > 38.5°C), 2. WBC > 25x10⁹/L, 3. ESR > 50mm/hr. **Results:** 457 patients tested positive for *B. cepacia*, almost exclusively the ET12, RAPD 2 strain of Genomovar III, over the 32 year study period. 33 patients with a single positive culture and no recurrence over followup of more than 3 years (range 3-26 years) are excluded from the following results. Of the 424 with recurrent or acute infection, 126 (30%) are still alive, 50 (11%) met the criteria for CS and a further 25 (6%) were assessed as having probable CS but not fulfilling the definition due to incomplete data, 181 (43%) died without meeting the definition of CS, and 42 (10%) had insufficient data to draw any conclusions. Of the 298 patients who died, CS was felt to have been the cause of death in 24% of cases. This proportion was the same if we considered only the 210 patients for whom complete data was available to confirm CS or rule it out. Only 21 (28%) of the 75 definite or probable cases of

CS occurred within one year of first positive culture, 17 (23%) 1-3 years later, 22 (29%) 4-10 years later, and 15 (20%) 10-22 years later. **Conclusion:** CS is a significant cause of mortality in CF patients infected with *B. cepacia* genomovar III ET12 strain. Although CS is generally feared as a possible outcome for newly infected patients, this study shows that the risk of CS continues for many years after initial infection. This evidence strongly supports the hypothesis of a triggering event or host-pathogen interaction resulting in the classic overwhelming decline and death of patients.

A38. Bacterial cell surface alterations affect the pathophysiology of *Burkholderia cepacia* complex organisms.

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While we are beginning to learn more about the pathophysiology of *Burkholderia cepacia* complex (BCC) infection in cystic fibrosis (CF), a great deal remains to be discovered about the role of proposed bacterial virulence factors. BCC organisms have been shown to synthesize potential virulence factors such as pili, extracellular proteases and lipases, and exopolysaccharide. In addition, these organisms are able to form biofilms and possess acyl-homoserine lactone quorum-sensing systems that regulate expression of some of these factors. We were interested in determining the impact of bacterial cell surface changes on the pathogenicity of these organisms. We are studying two sequential BCC genomovar III patient isolates with identical Random Amplified Polymorphic DNA profiles but dramatically contrasting phenotypes. On both rich and basal media, strain C8963 produced small, shiny, distinct colonies while strain C9343 produced large, wet, mucoid colonies whose edges coalesced with nearby colonies. Growth of C9343 on basal agar containing mannitol resulted in the production of copious amounts of EPS while C8963 produced very little. While C8963 synthesized the quorum sensing molecule octanoyl-homoserine lactone, C9343 was unable to produce this molecule despite the presence of the autoinducer synthase gene *cepl*. In addition, the mucoid C9343 strain was unable to form a biofilm while C8963 formed a biofilm on a variety of surfaces. An attempt to generate a mucoid phenotype in strain C8963 by complementation with DNA from mucoid C9343 was unsuccessful. Our next step will be to generate random mutations in each strain to isolate organisms with altered colony morphologies. This will enable us to begin to understand the role of EPS in BCC virulence.

A39. Survival of cystic fibrosis patients infected with *Burkholderia cepacia* complex genomovar III, *Burkholderia multivorans* and *Pseudomonas aeruginosa*

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Introduction: *Burkholderia cepacia* comprises a number of distinct genomic species, known as genomovars. The majority of cystic fibrosis (CF) clinical isolates belong to genomovars II (*B. multivorans*) and III. The different severity of infection attributable to separate genomovars is unknown. We investigated whether survival differs between patients infected with *B. multivorans*, *B. cepacia* genomovar III and *Pseudomonas aeruginosa*.

Methods: Patients at the Manchester Adult CF Centre who developed chronic infection (1982-2001) with *B. multivorans* and genomovar III were included: each was matched with another CF patient with chronic *P. aeruginosa* infection using the %predicted FEV1 when the *Burkholderia* species was first isolated. The two *Burkholderia* species groups were each compared to their respective control groups for median survival time using the log rank test. *Cepacia syndrome* deaths and numbers progressing from initial to chronic infection were assessed for patients infected with *Burkholderia* species.

Results: Forty-eight patients had an initial infection with *B. multivorans* (n=15) or genomovar III (n=33); 8/15 and 31/33 respectively developed chronic infection (p<0.001). For the *B. multivorans* group and their matched controls there were 4/8 (1x "*cepacia syndrome*") and 3/8 deaths with a median survival of 112 and 114 months respectively (p=0.63). For the genomovar III group and their controls there were 21/31 (2x "*cepacia syndrome*") and 12/31 deaths with a median survival time of 94 and 130 months respectively (p=0.05).

Conclusions: CF patients with *B. cepacia* genomovar III infection have a shortened survival in comparison with those with *P. aeruginosa* infection; however there is no difference in survival between patients infected with *B. multivorans* and *P. aeruginosa*.

A40. Evaluation of enzyme markers for phenotypic differentiation of the *B. cepacia* complex

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In an effort to improve the speed and accuracy of the confirmation of organisms within the *Burkholderia cepacia* complex (BCC), we looked at the application of enzymatic profiling using fluorogenic and chromogenic substrates. The coumarinic compounds 4-methylumbelliferone (MU) and 7-amino-4-methylcoumarin (7AMC) have, like other fluorophores, structural features which predispose towards fluorescence. A collection of 185 strains including organisms from the BCC (genomovars I-VII), *Pseudomonas aeruginosa*, and closely related organisms, were investigated for their abilities to hydrolyze fifty different MU and 7AMC-linked substrates, seven benzoyl-L-amino acids and twelve nitrophenol-linked substrates. These substrates detected a range of hydrolytic enzymes including glycosidases, aminopeptidases, carboxypeptidases and esterases. These organisms were also screened for endopeptidase activity, including elastase, chymotrypsin and other endopeptidases, using fifteen distinct substrates to investigate the presence of pathogenic markers, particularly within the BCC. Thirty BCC organisms representative of the five most commonly isolated genomovars (I-V) were subsequently screened for carbohydrate oxidation (50 tests) and assimilation (100 tests) to see if further differentiation could be achieved between the genomovars. Results. It was determined that *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Burkholderia gladioli*, *Pandoraea sp.* and *Ralstonia picketti* could be differentiated from one another using a combination of the following enzyme substrate tests: hydrolysis of L-histidyl-AMC, L-ornithinyl AMC, L-pyroglyutamyl-AMC, MU- β -xylopyranoside, L-valyl-AMC, L-threonyl-AMC, benzoyl-L-histidine, MU-palmitate, benzoyl-L-alanine, benzoyl DL-leucine, benzoyl-DL-methionine, benzoyl-DL-phenylalanine and MU-phosphate. Seven tests were identified which gave positive results for at least a proportion of all species tested except *Pandoraea sp.*, these tests included hydrolysis of L-histidyl-AMC, L-ornithinyl-AMC, L-threonyl-AMC, MU-palmitate, MU-elaidate, MU-oleate and MU-stearate. A combination of these tests could identify *Pandoraea sp* if all reactions were negative. It was also determined that a combination of the following tests could potentially identify genomovars as I-V: oxidation of ribose, arbutine, salicine, esculine, cellobiose, melibiose, xylitol, β -gentiobiose, D-raffinose, L-fucose, hydrolysis of MU- β -ribofuranoside, MU- β -xylopyranoside, glycyl-L-prolyl-AMC, L-ornithinyl-AMC benzoyl-L-glutamic acid, p-nitrophenyl phenyl-phosphorylcholine, 4-nitrophenyl- α -D-maltoside and 4-nitrophenyl- β -D-maltoside. p-Nitrophenyl p'guanidinobenzoate

proved to be an excellent substrate for the identification of *R. picketti*, discriminating this species from all members of the BCC, as well as *P. aeruginosa* and *Pandoraea sp.* The following endopeptidase substrates also show potential for discrimination between members of the BCC, H-glutamic acid-glycyl-arginyl-pNA, Suc-phenylalanyl-leucyl-phenylalanyl-pNA, H-alanyl-alanyl-phenylalanyl-pNA, H-cys(bzl)-pNA, Suc-alanyl-alanyl-alanyl-pNA, Suc-alanyl-leucyl-prolyl-phenylalanyl-pNA and H-y-glutamic acid-alanyl-glycine-pNA. These compounds are substrates for the following endopeptidases respectively: urokinase, cathepsin G and rat mast cell proteases, chymotrypsin and tripeptidyl peptidase, cystyl aminopeptidase, human and rat leukocyte and porcine pancreatic elastases, peptidyl prolyl cis-trans isomerases and bifunctional glutathionylspermidine synthetase. We conclude that these tests could lead to a purely phenotypic method of BCC identification and that further work in the form of a blind study with a larger collection of strains is required to validate the reliability of the identification scheme.

A41. Identification and characterization of a capsular polysaccharide locus in *Burkholderia cepacia* genomovar III

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In previous work, we employed subtractive hybridization to identify a novel insertion element, designated IS1363, that is specific for epidemic *Burkholderia cepacia* genomovar III strains of the ET12 and PHDC lineages. To characterize regions flanking this element, we constructed a genomic library of PHDC isolate PC8. DNA sequence analysis of an IS1363-containing clone from this library revealed that IS1363 is flanked by ORFs with homology to *dedA*, *yggB* and *wcbA*, three genes that are included in a capsular polysaccharide (CP) biosynthesis cluster previously described in *B. mallei* and *B. pseudomallei*. Further screening of the PHDC library with *wcbA* sequences identified a clone with contiguous DNA encoding a *wcbB* homolog. Additional library screening and sequencing ultimately identified an approximately 27 kb cluster containing 19 ORFs, 13 of which had high homology to the CP biosynthesis genes of *B. pseudomallei*, *B. mallei* or other bacterial pathogens. The presence of this cluster among other genomovar III strains was investigated by PCR analysis using primers specific to six genes within the cluster. The results indicated that this cluster was conserved among all six PHDC isolates screened, but was not present in two ET12 isolates nor in two non-epidemic genomovar III isolates. A search of the J2315 genome database, however, revealed the presence of several of these genes in an organization different from that found in PHDC. This analysis also revealed DNA sequence variation within genes 'conserved' in both PC8 and J2315. Alcian blue staining and Western blot analysis using adsorbed polyclonal rabbit antisera indicated that polysaccharide was produced by PHDC harboring the CP gene cluster. Insertional mutagenesis of several genes in this cluster had various effects on CP production. Whereas, mutation of the *wcbO* homolog had no apparent effect on CP production, mutation of the *wcbD* homolog or a glycosyltransferase gene homolog altered CP expression based on Western blot analysis. Insertional mutation of the *wcbC* homolog appeared to have the greatest effect in diminishing CP production. Analysis of these various mutants for virulence in a mouse model of pulmonary infection is currently underway.

A42. Molecular comparison of isolates of *Burkholderia multivorans* from patients with cystic fibrosis in the United Kingdom: no evidence of patient to patient spread.

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Conflicting reports have appeared in the literature concerning the transmissibility of *Burkholderia multivorans* amongst cystic fibrosis (CF) patients. To address this issue, 55 isolates of *B. multivorans*, received between 2001 and early 2003, from 47 patients from 28 hospitals across the United Kingdom, have been compared by pulsed-field gel electrophoresis (PFGE) of *Xba*I restricted genomic DNA. Identification of isolates was made using *recA* targeted PCR; the identification was also confirmed at the end of the study. The results showed a high degree of genetic diversity between isolates from different patients, and that each patient appeared to harbour their own strain. The only isolates that clustered at greater than 80% similarity were those from the same patient; all the remaining isolates, which were each from different patients, gave unique fingerprints. Flagellin (*fliC*) PCR-RFLP typing was also carried out on these isolates, with 14 different types being identified. PCR-RFLP at the *recA* and *gyrB* loci was carried out on some of the isolates, but little variation was seen, and typing at all three loci (*recA*, *gyrB* and *fliC*) gave no more discrimination than typing at the *fliC* locus alone. Whilst flagellin typing was a useful rapid technique for typing *B. multivorans*, it was not as discriminatory as PFGE. Our results suggest that CF patients acquired *B. multivorans* from the environment, and not from other patients.

A43. Evolution of O antigen genetic loci in *Burkholderia cepacia* complex

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The Gram-negative bacteria of the *Burkholderia cepacia* complex (Bcc), commonly found in soil and water, are opportunistic pathogens that can infect the lungs of cystic fibrosis (CF) patients and can be transmitted among these patients, causing epidemics in the CF community. Lipopolysaccharide (LPS) is an important virulence factor of many Gram-negative bacteria. The O antigen component of LPS is immunodominant and is responsible for serotype specificity. At least sixteen distinct serotypes of Bcc strains have been recognized and the structure of each of these has been determined. The genes required to make and assemble the sugar components of O antigens are generally arranged in a single genetic locus that is hypothesized to be horizontally transferred between bacteria. To understand the evolution of O antigen genetic loci in Bcc, we first isolated a plasmid containing a genomic DNA fragment from Bcc serotype O5 strain PC222, which is genomovar IV (*B. stabilis*). This plasmid induced the expression of Bcc serotype O5 O antigen when in *Escherichia coli*. Sequence analysis of this genetic locus revealed several genes that are involved in O antigen biosynthesis and expression. Among these are genes that resemble O antigen biosynthesis genes in *B. mallei* and *B. pseudomallei*, including *wbiG*, *wbiH*, and *wbiI*. The *wbiI* gene is a part of a larger bifunctional dehydratase/reductase family of genes involved in polysaccharide biosynthesis in many different bacteria. This gene is found in all Bcc strains tested by Southern blot and PCR. Furthermore, there are DNA sequence polymorphisms in the PCR-amplified *wbiI* gene fragment that loosely correspond to the serotype of the strain. Determination of the genomovars by 16SrDNA and *recA* gene sequences of each of these Bcc strains reveals that there is no obvious correlation between serotype (i.e. *wbiI* gene sequence) and genomovar. Experiments to further delineate the evolution of O antigen in Bcc are currently ongoing.