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Working Group

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Transient sputum culture of *Burkholderia cepacia* in cystic fibrosis patients : epidemiology and clinical implications

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Aims: To identify the incidence of transient sputum culture of *B. cepacia* in a large population of cystic fibrosis patients, to document subsequent microbiology following transient culture and to consider possible clinical implications of the transient sputum culture of *B. cepacia*. Methods: All patients who attended the CF program from 1970 to 2001 at The Hospital for Sick Children or at St Michaels/Wellesley Hospital who had at least one positive sputum culture for *B. cepacia* were eligible for the study. Relevant demographic and microbiology data was abstracted from the patient database. The definition used for transient *B. cepacia* sputum culture, was : at least 3 consecutive sputum samples, negative for *B. cepacia*, obtained over greater than a 12-month period following the last positive sputum culture(s) for *B. cepacia*. In general, sputum culture was performed at least every 3 months using selective media for *B. cepacia*. Results: 458 patients tested positive for *B. cepacia*, almost exclusively the ET12 strain of Genomovar III, over the 31 year period. Of these, 211 (46%) had at least one period of transient sputum culture for *B. cepacia*. Of these 211 patients, 44 (21%) remained culture negative for the duration of their follow-up. For this group the median *B. cepacia* negative follow-up period was 11.5 years (range, 1-26 years) with 41 (93%) having greater than 3 years follow-up. Of the 458 *B. cepacia* patients, 167 (36%) had recurrence of *B. cepacia* culture from sputum following a period of transient culture. Of these, 84 had more than one period of transient culture. The median duration of the culture negative period following the first episode of transient culture was 2.6yrs. However, 25% were negative for >5.7 years and 10% for >8.6 years. There was no bimodality of the duration of negative cultures to suggest a possible distinction between undetected chronic infection and re-infection. The lowest proportion of patients with transient culture occurred between 1980 and 1989 during which time the average incidence of new *B. cepacia* acquisition was highest, ranging from 3.1 to 12.4%. Since the segregation of *B. cepacia* positive and negative patients in 1991, 8 patients have had 16 episodes of transient culture. The incidence of new acquisition of *B. cepacia* during this period was <1% per year. Conclusion: Transient culture of *B. cepacia* in cystic fibrosis patients is a common occurrence. One in 10 patients with positive sputum culture for *B. cepacia* will subsequently become culture negative without recurrence. However, one third of patients have single or multiple episodes of transient positive sputum culture, remaining negative on average for 2-3 years between episodes. It is unclear if the recurrence of positive culture reflects detection of ongoing sub-clinical infection or true re-

infection with B. *cepacia*. Thus it is uncertain at what point, if any, a patient with transient culture can be considered clear of *B. cepacia* and hence no longer a risk for transmission.

Environmental and clinical isolates of *Burkholderia cepacia* complex : do differences exist between them?

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Burkholderia cepacia is present in a high number of habitats where it can also represent one of the dominant microbial groups, as in the rhizosphere of crop plants. The basic question which arises from this observation is: do environmental isolates represent a hazard to human health? In our laboratory, three years ago, we started to deal with this question. Our main objectives are (i) the determination of the distribution of the different genomovars in various natural habitats; (ii) the evaluation of similarities and differences between environmental and clinical isolates in respect of candidate virulence and transmissibility factors, genetic polymorphism, and phytopathogenic and plant growth promoting activity. As far as objective (i) is concerned, we have analysed, so far, *B. cepacia* complex isolates from soil (maize rhizosphere) and lotic habitats. Among the nine genomovars which comprise the *B. cepacia* complex, the only one present in high numbers in both types of habitats is *B. cepacia* genomovar III. In the maize rhizosphere is also highly present *B. ambifaria*, whereas in river water and sediments the other most common genomovar is *B. pyrrocinia*. *B. multivorans* was not recovered neither from the maize rhizosphere nor from river water and sediments. Therefore, wondering if the cultivation based method used in these studies could introduce a bias in the recovery of bacterial isolates belonging to the *B. cepacia* complex, we started a study aimed at evaluating the ability of different isolation media to reliably recover all members of the complex from natural habitats and at determining if direct extraction of DNA from the environment may help outline a more real picture of genomovar distribution in natural habitats. Studies concerning objective (ii) started by comparing a large collection of cystic fibrosis and rhizospheric isolates. First results indicate that some of the candidate determinants related to virulence and transmissibility are not confined solely to clinical isolates but are spread also among environmental isolates belonging to different genomovars of the *B. cepacia* complex. Results of RAPD analysis suggest that clinical and environmental populations of *B. cepacia* have quite different genetic structure. Since *B. cepacia* genomovar III is the most common genomovar in cystic fibrosis and, according to preliminary studies, also in natural habitats, our studies concerning objective (ii) are currently focusing on this genomovar. At present a large panel comprising 80 isolates of environmental and clinical origin and belonging to genomovar IIIA and IIIB has been set up. All isolates are being subjected to genetic fingerprinting (RAPD and PFGE) and characterised in respect of various virulence determinants. As far as candidate virulence determinants are concerned, we are particularly interested in exopolysaccharides, because of their role in biofilm formation. Therefore, we are determining the type of exopolysaccharides produced by a restricted number of clinical and environmental isolates belonging to genomovar IIIA and IIIB.

Towards establishing a multilocus sequence typing (MLST) scheme for *Burkholderia cepacia* complex

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The identification of *B.cepacia* complex is challenging. A polyphasic taxonomic approach is often employed in the taxonomic study of *B. cepacia* complex, including total protein electrophoresis, fatty acid analysis, biochemical tests, PCR amplification of rDNA, random PCR amplification (RAPD), pulse-field gel electrophoresis and ribotype analysis. However, molecular diagnostic probes based upon the polymerase chain reaction (PCR) provide a rapid and frequently highly discriminatory means of identifying microbial infection. Recently *recA* sequence analysis has helped to unravel the phylogenetic relationships within the *B. cepacia* complex and has formed the basis for a rapid PCR based identification of *B. cepacia* complex genomovars (1). Multi-locus sequence typing (MLST) is a powerful, portable and rapid means of undertaking identification, typing and population genetic analysis and enables population based analyses not possible by pulse field gel electrophoresis (PFGE) or other macrorestriction profiling and allows the establishment of a world wide data base for the unambiguous identification of different strains. We have currently developed 6 loci for use in an MLST scheme for *B. cepacia* complex including RecA (*recA*), beta subunit of ATP synthase, (*atpD*), poly-hydroxyalkoanoate polymerase (*phaC*), glutamate synthetase (NADPH) large chain precursor (*gltB*), NADH dehydrogenase I chain E and GTP binding protein (*lepA*). Southern blotting of whole chromosomes and restriction digests of total chromosomal DNA from *Burkholderia* complex, (*Burkholderia cepacia* genomovars I and III, *Burkholderia multivorans*, *Burkholderia stabilis* and *Burkholderia vietnamiensis*) has been used to confirm the presence of loci as single copies. We describe preliminary phylogenetic relationships from three loci using isolates widely distributed across a *recA* phylogenetic tree.

Reference :
(1) Mahenthiralingam, E., Bischof, J., Byrne, S.K., Radomski, C., Davies, J.E., Av-Gay, Y., Vandamme, P., DNA based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis, Burkholderia multivorans*, *Burkholderia stabilis* and *Burkholderia cepacia* genomovars I and III. Journal of Clinical Microbiology 2000 38:3165-73

Multilocus restriction typing, a novel tool for studying global epidemiology of *Burkholderia cepacia* complex infection in cystic fibrosis

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We evaluated the use of a novel typing method, multilocus restriction typing (MLRT), for investigation of the global epidemiology of *B. cepacia* complex genomovar III. With MLRT variation at several loci is indexed by restriction analysis of PCR-amplified genes. Data obtained by MLRT and pulsed-field gel electrophoresis (PFGE) analysis of a large number of *B. cepacia* genomovar III isolates (including isolates belonging to epidemic lineages and environmental isolates) show a strong correlation. MLRT extends the utility of isolate genotyping by allowing comparisons of isolates collected in larger-scale (both temporal and spatial) studies. The portability of MLRT data will facilitate comparison of data obtained in different laboratories. In addition data obtained with MLRT can be used in studies of bacterial population structure. Our preliminary results show that the majority of *B. cepacia* genomovar III strains examined group into three major clonal complexes, comprising the epidemic ET12, PHDC and Midwest clonal lineages. Our analysis indicates that these clonal complexes are characterized by varying degrees of genetic recombination. The observation that genetic recombination is frequent within some *B. cepacia* genomovar III populations can have important implications for the biotechnological use of *B. cepacia* complex organisms.

Burkholderia cepacia -- 2002 Regulatory updates

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In the United States, there are two major laws that apply to the commercial production of members of the *Burkholderia cepacia* complex (Bcc)for uses such as microbial pesticides, drain cleaners, production of specialty chemicals, and bioremediation of recalcitrant organics : the Federal Insecticide, Fungenticide, and Rodenticide Act (FIFRA), and the Toxic Substances Control Act(TSCA). In 2002, major actions were taken by EPA under both laws : a data call in was issued for Bcc microbial pesticides under FIFRA, and premarket approval for other Bcc environmental applications was proposed under TSCA. The risks, and regulatory requirements, of both actions will be discussed, with a focus on the TSCA premarket approval proposal.

Characterisation of the *Burkholderia cepacia* epidemic strain marker (BCESM) for virulence factors contributing to the spread of the bacterium among patients with cystic fibrosis.

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Over the last 15 years *Burkholderia cepacia* has emerged as a highly virulent cystic fibrosis (CF) pathogen that can lead to an irreversibly fatal decline in health, known as *B. cepacia* syndrome. This syndrome is characterised by acute necrotising pneumonia and occasionally bacteremia, with the majority of infections (~70%) accounted for by genomovar III strains of the *B. cepacia* complex. These chronic pulmonary infections of CF patients airways is a major cause of their morbidity and mortality, therefore it is a priority for the CF community to identify factors

involved in the virulence and spread of *B. cepacia*. Despite advances in our understanding of the taxonomy and epidemiology of *B. cepacia* knowledge of the virulence mechanisms which facilitate infections are limited. The *B. cepacia* epidemic strain marker (BCESM) was identified in strains associated with patient-to-patient spread by Random Amplified Polymorphic DNA (RAPD) fingerprinting. The DNA is now known to be specifically associated with strains for genomovar III, where it is stable within genomovar III-A strains, while less stable in III-B strains. An isogenic pair of CF strains, one encoding BCESM (CEP54B) and the other lacking it (CEP54A) have been derived from the genomovar III-B CF strain CEP54 originating from an epidemic in Cleveland, USA. In both a rat model of chronic lung infection and mouse models of acute infection, strain CEP54B encoding the BCESM has been shown to be better able to persist during infection than the isogenic BCESM negative strain. This suggests that the BCESM DNA encodes factors responsible for persistence in vivo which may contribute to the transmission and virulence of genomovar III strains. We have shown that the BCESM region remains stable during chronic infection of CF patients by examining sequential isolates by pulsed-field gel electrophoresis (PFGE) followed by Southern hybridization analysis for the presence of the marker. These data indicate that loss of the marker observed in genomovar III-B strains is not associated with CF infection and substantiates it potential *in vivo* role. The deletion of the BCESM in the genomovar III-B strain CEP54A was investigated by comparison with the same region in the genomovar III-A strain, J2315, the genome sequencing strain. Genes encoding amidases and a major porin protein have been found to occur downstream of the BCESM region in strain J2315. Upstream of the BCESM in strain J2315 occur a putative insertion sequence, lipid biosynthesis genes and acetyl homo-serine lactone biosynthetic genes. The presence or absence of these open-reading frames in strain CEP54A is being investigated by PCR-mediated mapping. Bioinformatic analysis of genes present at the BCESM locus within the genome sequence and the full extent of the BCESM deletion occuring in the less virulent strain CEP54A will be presented.
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Identification and characterization of a novel insertion element of *Burkholderia cepacia* genomovar III

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The *Burkholderia cepacia* complex (Bcc) consists of several closely related yet distinct bacterial species (or genomovars); among these, genomovar III (GIII) accounts for the majority of Bcc

isolated from sputum of cystic fibrosis (CF) patients. In a study to identify factors contributing to the virulence of GIII, a 200 bp DNA fragment with high homology to a putative transposase of *Pseudomonas* spp. was identified by using a subtractive hybridization strategy. By using this fragment as a probe, several clones containing the putative transposase and flanking sequences were recovered from a genomic library of GIII strain PC8. DNA sequence analysis of these clones identified a 1.4 kb segment containing 14 bp perfect terminal inverted repeats (TIR, nt 33 to 46, nt 1369 to1382). Additional 12 bp inverted repeats (IR1, nt 42 to 55, nt 66 to 77) and 11 bp inverted repeats (IR2, nt 1051 to 1061, nt 1072 to 1082) were also identified. Potential –35 promoter regions (TGACCA), inbeded in the 14 bp TIRs, and a putative ribosomal binding site downstream of IR1 were followed by a 1025 bp open reading frame (orf).
The deduced amino acid sequence of the orf showed 61 % and 58% identity to the IS1328 protein of *Yersinia enterocolitica* and TnpA of *Enterobacter aerogenes*, respectively. The deduced protein had a high isoelectric point (10.01) characteristic of transposases. Southern analysis of 11 GIII strains using an internal segment of the orf as probe indicated that they contained varying copy numbers of the putative insertion element (IS). Dot blot and PCR assays of nearly 900 Bcc isolates (representing all genomovars) recovered from CF and environmental sources indicate that this IS is restricted to GIII strains that infect multiple persons with CF. In summary, we have identified a 1.4 kb fragment that has all the characteristics of an insertion element. The disproportionate distribution of this novel IS among Bcc species suggests a potential role in the virulence of GIII strains – particularly among so-called epidemic strains. The function and the role of this IS in contributing to virulence are being more completely characterized in ongoing studies.

Biocide and antibiotic suceptibility 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 frequently cause fatal respiratory infection in patients with cystic fibrosis. *B. cepacia* bacteria are intrinsically resistant to many antimicrobial compounds and may even survive within and contaminate disinfectant solutions. These organisms have been responsible for the contamination of both a salt solution preserved with benzylkonium chloride (Geftic *et al*., 1979) and a povidone-iodine antiseptic solution (Anderson *et al*. 1991). However, limited knowledge is available as regards their molecular basis of resistance. The resistance of *B. cepacia* to the antimicrobial compounds chlorhexidine diacetate (CHX) and triclosan was investigated. The objectives of this study were to (1) isolate and (2) characterise both chlorhexidine and triclosan-sensitive mutants of *B. cepacia* using a random transposon mutagenesis system. *B. cepacia* genomovar III strain K56-2, a clinical isolate recovered from a cystic fibrosis patient was used. Strain K56-2 showed a high degree of resistance to both compounds; the MICs of chlorhexidine and triclosan were 200 μg/ml and >1000 μg/ml, respectively. The suicide vector pOT182, carrying a self-cloning transposon system, was mobilized into strain K56-2 and transposon mutants isolated on selective media containing tetracycline. Individual mutants (N = 2000) were then replica plated onto plates containing either chlorhexidine or triclosan. Four chlorhexidine-sensitive and two triclosan-sensitive mutants were isolated after this screen. All six mutants showed a substantial increase in sensitivity to the biocides tested (up to a 50% increase in sensitivity). Also a number of the mutants showed cross suseptibility to various antibiotics e.g Trimethoprim, Chloramphenicol, Ciprofloxacin, Novobiocin and Nalidixic acid. Two of the chlorhexidine-sensitive mutants and one of the triclosan-sensitive mutants were genetically characterized and found to carry transposon insertions within genes homologous to: (i) *lytB*, encoding a protein associated with polymyxin resistance in *Burkholderia pseudomallei*, (ii) *hslU*, encoding a poorly defined heat-shock protein in *Lactobacillus leichmannii* and (iii) *tmpA* encoding a probable transmembrane protein in *Ralstonia*. The genetic basis of the remaining biocide sensitive mutants will be determined. 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.

Is there a role for the *Burkholderia cepacia* mucoid phenotype in CF lung infection?

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Bacterial phenotype influences resistance to host defenses in some bacterial species, but the role of bacterial phenotype in virulence of *Burkholderia cepacia* has not been determined. A distinguishing feature of *Pseudomonas aeruginosa* pulmonary isolates from cystic fibrosis (CF) patients is the mucoid phenotype, resulting from overproduction of the exopolysaccharide alginate. The conversion of *P. aeruginosa* to a mucoid phenotype has been associated with the establishment of chronic respiratory infection in CF. In addition, alginate overproduction has been linked to inhibition of phagocytosis, protection from reactive oxygen intermediates, and altered biofilm architecture, resulting in increased antibiotic resistance. We are interested in determining if overproduction of exopolysaccharide in *B. cepacia* confers similar advantages on the bacteria. We have obtained two genomovar III, RAPD group 1 *B. cepacia* isolates from a CF patient, one of which gave rise to mucoid colonies when grown on Luria broth agar. The non-mucoid isolate was capable of biofilm formation and quorum sensing signal production, whereas the mucoid isolate was not. Only the mucoid isolate produced exopolysaccharide when grown on mannitol. This isolate was also more resistant to antibiotics. We are using complementation and transposon mutagenesis strategies to identify genes necessary for production of the mucoid phenotype in *B. cepacia*. Further, we will characterize the effect of the mucoid phenotype on microcolony formation, phagocytosis by macrophages, and persistence in a murine model of infection. These studies may provide information about the role of the mucoid phenotype in *B. cepacia* respiratory infection in CF.

Exopolysaccharides produced by CF strains of *Burkholderia cepacia*

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In previous investigations several CF clinical strains of *B. cepacia* were typed and cultivated to obtain suitable amount of exopolysaccharides (EPS). The strains investigated belonged to the genomovars I, IIIA. The analysis of the primary structure of the polymers produced showed that two different EPSs were synthesised by different bacterial strains. The first was identical to that described by our as well as other groups (Cerantola S., Lemassu-Jacquier A., and Montrozier H. (1999) *Eur. J. Biochem*. 260, 373-383; Cescutti P., Bosco M., Picotti F., Impallomeni G., Leitpo J.H., Richau J. and Sß-Correia I. (2000) *Biochem. Biophys. Res. Comm*. 273, 1088-1094; Linker A., Evans L.R. and Impallomeni G. (2001) *Carbohydr. Res*. 335, 45-54) and the second one was a totally different polysaccharide exhibiting a galactan backbone which included a Kdo residue. The latter polysaccharide is identical to the exopolysaccharide produced by *B. pseudomallei* (Nimitz M., Wray V., Domke T., Brenneke B., Haussler S. and Steinmetz I. (1997). *Eur. J. Biochem*. 250, 608-616), which is known as intracellular pathogen. The above study definitively confirmed that clinical strains of *B. cepacia* do produce different EPS whose diagnostic relevance should be thoroughly investigated. Besides the investigation on EPSs' primary structure, our group in Trieste is currently interested in the macromolecular and solution properties of these polymers. In particular, the EPS first described as produced by *B. cepacia* (Cescutti P., Bosco M., Picotti F., Impallomeni G., Leitpo J.H., Richau J. and Sa-Correia I. (2000) *Biochem. Biophys. Res. Comm*. 273, 1088-1094) was thoroughly characterised. The polymer exhibited a very elongated structure conferring high viscosity to aqueous solutions. In addition to this, aggregation of polysaccharidic chains is induced either by low pH and low temperature or by positive ions. Molecular weight measurements showed that the aggregation were rather specific exhibiting a chain stoichiometry equal to 2 and 4. The aggregate formation involved interaction at the carboxylic group level of the glucuronic acid residues present in the polymer repeating unit. About one third of these groups were hidden in the core of the aggregated structure as detected by potentiometric titration measurements. In analogy with alginate produced by *P. aeruginosa*, the biophysical characterisation of the exopolysaccharide produced by B. cepacia will certainly contribute to understand the micro-environment surrounding the bacterial cells. Such an investigation will contribute to clarify the conditions of the infection maintenance.

Lack of correlation of serotype and genomovar in *Burkholderia cepacia* complex

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The O antigen is the immunodominant component of lipopolysaccharide (LPS) and is responsible for serotype specificity. At least nine distinct serotypes of *Burkholderia cepacia* strains have been recognized (1, 2) and the structure of each of these has been determined (reviewed in 3). With the more recent appreciation that several distinct species (initially classified as genomovars) comprise the *B. cepacia* complex, it was of interest to determine whether a relationship exists between serotype and genomovar. We obtained the 9 serotype strains from Dr. Henri Monteil, as well as an additional serotype O5 strain, PC222 (4), and for each, determined species (genomovar) using species-specific 16S rRNA and *recA*-directed PCR (5, 6). If PCR results were equivocal, the genomovar was determined by *recA* sequence analysis. The results are shown in Table 1. Of particular note, the two serotype O5 strains belong to different genomovars and strains of various serotypes belong to genomovar III. During our sequence analysis of the O antigen locus of PC222, we noted the presence of a *wbpM* homolog. In *Pseudomonas aeruginosa*, *wbpM* is important for O antigen synthesis and is highly conserved among various serogroup strains (7, 8). We PCR amplified an internal portion of the *wbpM* gene from all of the *B. cepacia* serotype strains. Each of the PCR products from *B. cepacia* was smaller than that from *P. aeruginosa* and sequence analysis confirmed this size difference. There is a high degree of sequence similarity between the various *B. cepacia* isolates, however this similarity does not follow the pattern of genomovar typing. Thus, neither serotype nor sequence of *wbpM* correlates with genomovar typing of the *B. cepacia* complex strains. This suggests that either the O antigen locus can be transferred between various genomovars or that various genomovars can emerge from strains of the same serotype. Experiments to distinguish between these hypotheses, as well as to delineate the evolution of O antigen, are currently ongoing.

Table 1. Serotype and genomovar of *B. cepacia* complex strains.

Serotype	Strain	Genomovar (species ID method)
O1	CIP 8235	IV (rDNA and *recA* PCR)
O2	CIP 8236	III (rDNA and *recA* PCR)
O3	CIP 8237	III (rDNA and *recA* PCR)
O4	CIP 8238	III (rDNA and *recA* PCR)
O5	CIP 8239	Novel (*recA* sequence)
O6	CIP 8240	Novel (*recA* sequence)
O7	ATCC 17759	I (*recA* sequence)
O8	CDC 99	Novel (*recA* sequence)
O9	CDC 86	III (*recA* sequence)
O5	PC222	IV (rDNA and *recA* PCR)

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Burkholderia cepacia genomovar III persistence in the mouse in relation to colony morphology

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B. cepacia is recognized as a serious respiratory pathogen in patients with cystic fibrosis (CF), however the pathogenesis of this bacterium is poorly understood. Our laboratory has developed a pulmonary infection model using neutropenic BALB/c mice to study *B. cepacia* infection and evaluate putative virulence determinants in *B. cepacia* strains. We have previously reported differential virulence among strains within the *B. cepacia* complex, particularly between *B. multivorans* (genomovar II) and *B. cepacia* genomovar III strains. In our model, genomovar III strains were consistently cleared from the murine lung by Day 4, however we were able to isolate a persistent variant of genomovar III strain C1394 after sequential intranasal mouse passage. Comparative studies showed that strain C1394 and its persistent derivative C1394mp2 shared the same genetic profile but differed by colonial morphology. Colonies of C1394 maintained a matte, dry appearance whereas C1394mp2 colonies were shinier, but did not display the mucoid phenotype typical of *Pseudomonas aeruginosa* strains in patients with CF. These morphological differences were more pronounced when strains were grown on media supplemented with congo red dye or on yeast mannitol (YEM) media. Both strains were found to produce exopolysaccharide (EPS) in markedly different amounts on YEM agar on which the shiny phenotype correlated with greater EPS production. Moreover electron microscopy revealed increased presence of pili in the shiny persistent derivative C1394mp2. In summary we have (through *in vivo* selection) isolated a persistent derivative of a non-persistent genomovar III strain, and have discovered morphological differences that may be associated with differential persistence in the murine lung. These studies may describe a role for colonial morphology in *B. cepacia* virulence. We are currently using 2-D protein gels to identify the determinants that are involved in the shiny phenotype and persistence in the murine host.

Growth of *Burkholderia cepacia* in airway surface liquid

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Mucus is poorly cleared from the lungs of cystic fibrosis (CF) patients and thus forms stationary mucus plaques and plugs. *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., J. Clin. Invest. 2002;109:317-325). Accordingly, bacteria trapped in mucus appear to grow in an oxygen-starved environment. We have shown that *Pseudomonas aeruginosa* is able to grow in airway surface liquid (ASL) under anaerobic conditions. However, this bacterium is known to respire anaerobically using nitrate as alternative electron acceptor, which has been found in both sputum and ASL in sufficient concentrations to support growth. Although *Burkholderia cepacia* has been localized in the exudate of bronchial lumen in CF patients, there are no data on growth of *B. cepacia* in oxygen-reduced environments. To test for growth of *B. cepacia* in ASL we collected samples of native ASL from the apical cell surface of CF well-differentiated human airway epithelial cultures 3 to 4 weeks old, which were maintained in antibiotic-free media. For the bacterial growth studies, we incubated 30 µl of ASL with low inocula (~10^2 cfu/0.5 µl PBS) of *B. cepacia* BC-7 (genomovar III, cable pili positive) and *B. multivorans* J-1 (genomovar II), which were grown overnight on sheep blood agar and then suspended/diluted in PBS. Their growth was monitored for 3 days under aerobic and anaerobic conditions (Coy anaerobic chamber) at 37°C in round bottom 96 well plates. The number of viable bacteria was determined at 1, 2 and 3 days of incubation by serial dilutions plated onto sheep blood agar and cultured under aerobic conditions for bacterial enumeration. We show that *B. cepacia* BC-7 and J-1 grow aerobically and anaerobically in ASL from CF patients suggesting that *B. cepacia* has the capacity to survive under oxygen reduced conditions in CF airways.

Identification and characterization of locus required for cable pilus biogenesis in *Burkholderia cepacia*

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Burkholderia cepacia is an important pulmonary pathogen in immunocompromised patients and patients with cystic fibrosis (CF). In CF patients, *B. cepacia* colonization of the lung can lead to necrotizing pneumonia and septicemia, often resulting in a rapid and ultimately fatal clinical decline. Virulence and high transmissibility of strains of *B. cepacia* have been correlated with expression of unique peritrichous adherence organelles, designated cable pili. Cable pili have been shown to bind human mucins and respiratory epithelial cells, and likely promote *B. cepacia* colonization of the CF lung. We have undertaken a genetic analysis of *B. cepacia* cable pili and have identified in a CF clinical isolate of *B. cepacia* a locus encoding four genes involved in cable pilus biogenesis. All four genes, designated *cblB*, *cblA*, *cblC*, and *cblD*, are predicted to encode proteins with significant homology to structural and assembly components of the CS1 family of pili, involved in colonization of the human intestine by enterotoxigenic *Escherichia coli*. In order to examine the role of the *cbl* genes in *B. cepacia* cable pilus biogenesis, we insertionally inactivated the *cblA* gene, encoding the major pilin subunit, and *cblD*, encoding the putative minor pilin subunit and initiator of cable pilus assembly. Both the *cblA* and the *cblD* mutants are blocked in cable pilus morphogenesis and fail to export CblA, the major pilin subunit. Transcriptional fusion analysis of the *cbl* locus revealed a growth phase-dependent regulation of *cbl* gene expression, with maximal activity during stationary phase. Studies are currently underway to characterize the cable pilus assembly pathway and to further define the regulation of *cbl* gene expression.

Identification and analysis of the Cable pili gene cluster in *Burkholderia cepacia*

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One of the highly transmissible and virulent strains of the *B. cepacia* complex belongs to genomovar IIIa and expresses morphologically unique cable pili. The *cblA* gene encoding the major pilin subunit was earlier cloned and sequenced and its protein product shown to be highly similar to the structural protein of colonization factor antigens of enterotoxigenic *E. coli*. We have now identified and analyzed 4 other genes on the cable pilin operon: *cblB* is at the 5' end of *cblA*, while *cblC*, *cblD* and *cblE* are at the 3' end. The orientation of the first four genes is identical to that of the corresponding genes in *E.coli* CFA/I, and the family of CS operons. Products of *cblB*, *cblC* and *cblD* show 28 to 33% identity and 50-55% similarity to the corresponding gene products of CS and CFA/I operons. By analogy to the CFA/I and CS operons, and computer based structural analysis we predict that CblB is one of the FGL family of periplasmic chaperones and plays an important role in pilus assembly, CblC functions as an outermembrane usher protein and as a platform for pilus assembly, and CblD is a minor pilin and plays a role in initiation of pilus polymerization. Both CblA and CblD proteins have a beta-zipper domain at the C-terminus, which is recognized by FGL-like periplasmic chaperones. Unlike CFA/I and CS operons, which have only 4 genes, there is another open reading frame (*cblE*) at the 3' end of *cblD* that encodes a large protein with high similarity to sensory proteins of two component regulatory systems. Computer based 3-D structural analysis indicates the presence of a sensory domain at the N-terminus and a response domain at the C-terminus of the protein. CblE also has a putative histidine kinase domain which is characteristic of sensory proteins of two component regulatory systems. A functional role of CblE as a transcription regulator of cable operon genes is yet to be demonstrated. In summary, the cable operon is organized like CFA/I and CS operons of *E. coli* with the exception that there is an additional gene at the 3' end (*cblE*) encoding a putative sensory protein.

Development of genomic oligonucleotide microarrays for *Burkholderia* sp. LB400

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Burkholderia sp. LB400, a PCB-degrading bacterium, is the first among *Burkholderia* species to have its genome sequenced. In this study, we optimized the microarray method and designed oligonucleotide probes for the LB400 genome. An oligonucleotide-based microarray approach was selected because of its flexibility in design and reliability of probe quality. On the level of microarray production, post-processing and target labeling, several parameters were optimized to gain a higher consistency and sensitivity. For this methodological study, an oligo-array was constructed using a limited set of genes of two PCB-degrading bacteria, *Burkholderia* sp. LB400 and *Rhodococcus* sp. RHA1. Out of 7 different printing substrate types, Superaldehyde slides (Telechem) were selected. 70-mers were selected over 45-mers, and C6-amino-modification of the probes over unmodified probes. When comparing 4 different spotting concentrations, 60 mM was observed to give the optimal result for the selected slide. For the development of a genome scale microarray, C6-amino-modified 70-mers were designed using the ArrayDesigner 2.0 software package (Premier Biosoft International). The Tm for the probes was optimized at 80 +/- 10 °C. To improve the specificity of the probes, BLAST was run against the LB400 genome to screen out cross-homology regions prior to the design. The successfully designed probes covered 83.4 % (7,264 probes) of the whole genome (8,708 ORFs).

Genomic characterization of *Burkholderia* sp. strain LB400 and comparative analysis with other *Burkholderia* genomes

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Very little is known about ecological features that differentiate clinical from environmental strains in the *Burkholderia cepacia* complex. It is also speculative as to what determines the great metabolic diversity of the *Burkholderia* species. Genomic approach is believed to provide new insight into these questions. The *Burkholderia* sp. strain LB400 is a soil isolate with exceptional characteristics in the degradation of polychlorinated biphenyls. Its genome sequence has been partially determined (8 X coverage), and annotated by the Joint Genome Institute (http://www.jgi.doe.gov/). At present, 8708 candidate genes (ORFs) have been predicted from 351 contigs (>10 reads and > 1kb size totaling 9.6 Mb). Phylogenetic analysis (16S rRNA data) places the strain LB400 outside the *Burkholderia cepacia* cluster with *B. phenazinium*, *B. graminis* and *B. caribiensis*. We investigated the genome of the strain LB400 as representative of the innocuous soil isolates and a useful out-group, in attempts to characterize the genome of this organism and to define the backbone gene set in the *Burkholderia* species. Pulse field gel electrophoresis has revealed that these ORFs are distributed in at least three mega-replicons, a common feature among the *Burkholderia cepacia* strains. The genome sequence data revealed that the genome has many but relatively short length (<20-30 kb) duplications. 1653 ORFs have at least one BLAST hit within the genome with > 50% identify at amino acid level over more than the 80% of their length. Transposase and/or insertion like elements as well as unknown or hypothetical function proteins are over-represented among the replicated ORFs. Moreover, transcription-related, carbohydrate transport and lipid metabolism genes are enriched in the LB400 genome relatively to other sequenced bacterial genomes. These findings are in compliance with the hypothesis that *Burkholderia* have a large and plastic genome and potential for metabolic diversification. To find a common gene set for the *Burkholderia* genus, the LB400 genome was compared with those for *B. cepacia* strain J2315 and *B. pseudomallei* strain K96243, which are currently being sequenced by the Sanger Center (both at a comparable stage of sequence coverage with strain LB400; available at http//www.sanger.ac.uk/). 4009 ORFs in the LB400 genome appeared to have a homolog with the latter species, suggesting a genus-specific backbone gene set. We are currently working on the annotation of these ORFs as well as the annotation of what is uniquely found in the LB400 genome.

Redox proteins as virulence factors of *Burkholderia cepacia*

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We previously demonstrated that several clinical and environmental isolates of *Burkholderia cepacia* secreted ATP-utilizing enzymes to the outside medium; the secretion of these enzymes by a cystic fibrosis (CF) lung isolate strain 38 was shown to be greatly enhanced in presence of alpha 2-macroglobulin. Fractionation of the growth medium of a CF isolate strain 71 demonstrated the presence of two additional proteins, homologues of *Pseudomonas aeruginosa* azurin and cytochrome c551 which are normally involved in electron transfer during denitrification. A Q-Sepharose column flow through (QSFT) fraction of the growth medium of *B. cepacia* strain 71 enriched with the azurin and cytochrome c551 homologues triggered apoptosis in macrophages and mast cells, leading to their cell death. Preincubation of the QSFT fraction with anti-azurin and anti-cytochrome c551 antibodies greatly reduced the cell death. Isolation of RNA from azurin-cytochrome c551-treated macrophages and DNA chip analysis of the expression of proapoptotic and anti-apoptotic genes did not demonstrate significant activation of such genes. However, azurin-cytochrome c551 treatment led to a higher level of p53 tumor suppressor protein in the macrophages. The putative role of p53, a known inducer of apoptosis, in the apoptotic death of macrophages mediated by azurin and cytochrome c551 will be discussed.

Cloning of a *Burkholderia cepacia* metalloprotease gene

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Many bacteria produce extracellular zinc metalloproteases that may contribute to their virulence. *Burkholderia cepacia* strains produce a 36 kDa zinc metalloprotease, PSCP. Previous studies

have shown that PSCP is capable of cleaving human IgA, IgM, IgG, transferrin, lactoferrin and collagen and has been implicated in lung disease. Immunization with synthetic peptides based on a conserved metalloprotease epitope reduces the severity of experimental *B. cepacia* respiratory infections. A 2.6 kb fragment that contained a putative zinc metalloprotease homolog was cloned from *B. cepacia* genomovar III strain Pc715j and determined to code for PSCP. The predicted amino acid sequence of this gene suggested a pre-proenzyme stucture with a molecular weight of 59.8 kDa. The sequence has a 13 amino acid region identical to the previously determined mature N-terminal sequence of PSCP. Processing at this predicted site would yield a mature protease of 36.8 kDa. PSCP was shown to have a conserved zinc metalloprotease active site motif. A PSCP mutant was constructed by inserting a trimethoprim cassette into the PSCP gene. Interestingly, the mutant retained its proteolytic activity on skim milk agar; however, cell-free supernatants demonstrated decreased proteolytic activity using hide blue azure as a substrate. A previously identified 40 kDa protease was determined not to be a precursor of PSCP, suggesting that *B. cepacia* produces at least two zinc metalloproteases.

Genetic characterization of plant tissue watersoaking in *Burkholderia cepacia*

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Burkholderia cepacia was originally identified as the causal agent of onion soft rot. This versatile bacterium is now recognized as a significant opportunistic human pathogen of patients with cystic fibrosis. Taxonomic studies have determined that representatives of *B. cepacia* can be divided into seven genomic species or genomovars that are collectively referred to as the *B. cepacia* complex. Our laboratory has previously reported that plant tissue watersoaking (ptw), a plant disease associated phenotype, was expressed by genomovars I and III. The ptw phenotype results from electrolyte leakage of plant host cells. Plant tissue watersoaking activity is a pathogenic attribute of *B. cepacia* plant pathogens that is necessary for plant disease. The fact that isolates of genomovar I and III cause watersoaking of plant tissue suggests the potential for a common factor that affects plant cell integrity. Plasposon mutagenesis was used to generate a bank of 5000 *B. cepacia* mutants of the genomovar III strain K56-2. From this bank, we have identified over 50 ptw-negative mutants. The DNA flanking the transposon insert of several ptw-negative mutants was used to probe a genomic library of strain K56-2. From over 80 kb of genomic DNA sequence data, we identified a cluster of eleven genes in a 45 kb region that showed similarity to components of a transport system. The *ptw* cluster appears to encode for a type IV secretion system in *B. cepacia* strain K56-2. Insertions in genes *ptw*C, *ptw*D and *ptw*I were obtained using *in vitro* mutagenesis. The disrupted genes were introduced into K56-2 using

a suicide vector to facilitate gene replacement. Mutant strains confirmed as having a disruption in gene *ptw*C or *ptw*D were negative for the ptw phenotype as well as mobilization of a transfer deficient plasmid. Collectively, our results indicate that the ptw effector moiety is transported from the cell via a type IV secretion system. Type IV secretion systems have been demonstrated to transport pathogenicity factors in the plant pathogen *Agrobacterium tumefaciens*; and virulence factors in the human pathogens *Helicobacter pylori* and *Bordetella pertussis*.

Analysis of mutant derivatives of *Burkholderia multivorans* and *Burkholderia ambifaria* altered with respect to formation of *N*-acyl homoserine lactones.

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B. ambifaria BcF forms higher levels of AHL *in vitro* than most members of the *B. cepacia* complex, whereas *B. multivorans* forms essentially no AHLs, despite the fact that it possesses genes related to quorum sensing. We isolated mutant derivatives of *B. multivorans* ATCC 17616 which produce high levels of AHL. We expected that they might have alterations in the genes encoding AHL synthase (BmuI) and/or AHL-binding transcriptional activator (BmuR). As in the case of the *cepI* and *cepR* genes of the genomovar III isolate strain K56-2, the *bmuI*and *bmuR* genes of *B. multivorans* 17616 were divergently oriented and separated by ca 700 nt. There was a *lux*-box-like sequence and σ^{70}-like promoter immediately upstream of the *bmuI* gene. No promoter was detected in the region immediately upstream of the *bmuR* gene. There was a 396-nt open reading frame which terminated 134-nt upstream of *bmuR*. This ORF was preceded by a σ^{70}-like promoter. We speculate that this putative promoter might direct transcription of both the ORF and *bmuR* gene. Comparison of the *bmuIR* loci of AHL-overproducing derivatives of strain 17616 and their wild type parent failed to reveal differences in nucleotide sequence that might explain the increased formation of AHLs. Presumably the alterations in the mutant strains lie outside of the *bmuIR* locus in other genes governing quorum sensing. We speculate that these might be global regulatory genes whose activity depends upon whether or not the bacteria are host-associated. We also have isolated AHL-deficient mutants of the maize rhizosphere isolate *B. ambifaria* BcF which exhibit reduced antifungal activity against *Saccharomyces cerevisiae*, *Pythium ultimum*, *Rhizoctonia solani*, and *Fusarium oxysporum* f. sp. *lycoperisici*. In certain of the mutants genes related to quorum sensing were absent, suggesting that the *bafIR* locus is a hot spot for deletion. In both *B. ambifaria* and *B. multivorans*, which each contain three chromosomes, genes related to quorum sensing were located on the mid-size chromosome.

New molecular tools for the gene transfer and expression, and rapid detection of essential genes in *Burkholderia cepacia* genomovars

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The extraordinary ability of *B. cepacia* to resist the antimicrobial activity of the majority of clinically useful antibiotics as well as the effects of cationic peptides requires the identification and characterization of novel targets. We postulate that a global genomic analysis leading to the identification of genes that are essential for bacterial survival, especially those genes whose functions are not apparent from homology searches, will uncover potentially novel targets for developing new antimicrobial agents. Unfortunately, genetic studies in *B. cepacia* complex isolates are hampered, in part, due to limited availability of cloning vectors and also by the inherent resistance of these isolates to most of the antibiotics commonly used for genetic selection. Also, in our experience some of the promoters usually common for expression studies in *Escherichia coli* are inefficient for sustaining gene expression in *B. cepacia*. In this work we have used the backbone of the vector pME6000, a derivative of the pBBR1 plasmid (originally isolated from *Bordetella bronchiseptica*), to construct a set of expression vectors for use in *B. cepacia*, which contain either the constitutive promoter of the S7 ribosomal protein gene from *B. fungorum* LB400 or the arabinose-inducible BAD promoter from *Escherichia coli*. Promoter sequences were placed immediately upstream of multiple cloning sites in combination with the minimal sequence of pME6000 required for plasmid maintenance and mobilization functions. The functionality of both vectors was assessed by cloning the eGFP gene and determining the level of protein expression and fluorescence emission in various clinical and environmental isolates of the *B. cepacia* complex. We show that *B. cepacia* cells carrying these constructs display strong fluorescence that allows us to follow the bacterial path within *Acanthamoeba castelanii* and alveolar macrophages by fluorescence microscopy.

We have also constructed a tool for the rapid and facile identification of essential genes at the genomic level. It is based on the use of a broad-spectrum plasposon vector, which has been engineered to detect conditionally expressed essential genes. Using this transposon, which contains a highly regulatable BAD outward promoter, we have detect to date approximately 100 mutants in *B. cepacia* C5424 (Genomovar III - ET12 lineage) that require arabinose for growth. We are currently characterizing the site of transposon insertion by DNA sequencing and preparing a list of candidate essential genes, which will be grouped into one of several categories (cell surface assembly, cellular biosynthetic pathways including TCA cycle and cellular respiration, cellular and DNA repair processes, transcription, DNA replication, and genes of unknown function).

Subtractive hybridization of *B. cepacia* genomovar III

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Species belonging to the *Burkholderia cepacia* complex (Bcc) are of great importance to the scientific community, not only from a medical aspect but also due to their enormous ecological potential as bio-remediators and crop yield enhancing agents. Currently there is little information regarding genetic differences between members of this complex, making the determination of potential pathogenic isolates difficult. To determine genes that are specific for pathogenic strains we performed subtractive hybridization between a known pathogenic Bcc GIII isolate from CF sputum and a previously characterized Bcc GI isolated from the environment. Briefly, the Bcc GI genomic DNA was sheared, biotinylated and hybridized with BcC GIII DNA that had been Sau3AI digested. Hybridized fragments were immobilized using streptavidin coated magnetic beads while non-hybridized fragments were removed and used in a further three rounds of subtraction. The final round was followed by linkage of the remaining Bcc GIII fragments to Sau3AI linkers and PCR. The resulting products were cloned into a TA cloning vector, transformed in to *E. coli* and individual colonies sequenced. To date, we have sequenced approximately 40 individual colonies and performed BLAST searches utilizing the NCBI and Sanger databases to determine significant sequence homology. Interestingly, of the sequenced clones that possessed homology with other known genes/proteins, the majority represented fragments with homology to OMPs and capsular polysaccharide biosynthesis gene products such as the high molecular weight adhesin of *H. influenzae* and WcbC and Wzt2 of *B. thailandensis* and *B. pseudomallei* (resp). These latter fragments did not have identity with the current *B. cepacia* genome sequence. Several sequenced fragments shared homology with regulatory elements in *R. solanacearum* and *P. aeruginosa*. Approximately one third had no significant homology with any gene/protein in the NCBI database. None of the sequenced fragments shared homology with genes associated with central metabolism. Currently we are focusing on sequencing further clones from the subtractive hybridization prior to examining the expression of the associated gene during models of infection.

Abstracts without formal presentation

Strain variation during chronic *Burkholderia cepacia* complex infection in cystic fibrosis

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Certain *Burkholderia* species, particularly those within the *B. cepacia* complex, can cause severe pulmonary infection in persons with cystic fibrosis. Although infection is generally persistent after initial acquisition, recent observations indicate that in some patients a change in infecting species – from one *B. cepacia* complex species to another – may occur during the course of chronic infection. In order to ascertain the frequency with which this occurs, and to determine if changes from one strain to another (ie, within the same species) might also occur, we analyzed 1095 *Burkholderia* isolates serially cultured from 379 CF patients receiving care in 112 CF treatment centers in the U. S. Overall, a change in infecting species or strain was found in 24 (6.9%) of 347 patients infected with *B. cepacia* complex species and in three (9%) of 32 patients chronically infected with *B. gladioli*. We identified several patients who were likely co-infected, at least transiently, with more than one *B. cepacia* complex strain. These findings indicate that patient cohorting on the basis of *Burkholderia* infection status may place patients at unnecessary risk of acquiring a potentially more virulent strain. Ongoing isolate analysis is required to monitor for changes in infecting strain that may impact on patient management options.

The IBCWG website : an update

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The website from the IBCWG is located on the allserv – ser ver of the University of Ghent, Belgium. The exact location of the website on this server may be variable and therefore the redirect URL http://go.to/cepacia should be used.

Since the last meeting of the IBCWG in Orlando (October 2001) a new tool was added to the website which registers traffic (Nedstat Basic). An overview of traffic to the site is shown at the Nedstat website, which can be accessed from the IBCWG homepage or directly from http://v1.nedstatbasic.net/s?tab=1&link=5&id=1321371. We have been monitoring traffic since November 19th and between then and March 18th the website had 1038 visitors (mean per day 8.7, range 0-42) from 42 countries (from all continents except Africa). The majority of visitors (37.7%) were from the US, followed by the UK (17.4%), Canada (10.4%), Italy (8.8%) and Belgium (3.4%). Most visitors use Internet Explorer 5.x (59.5%), 6.x (16.2%) or Netscape 4.x (16.2%). Most visitors use a Windows operating system (Windows98 37.8%, WindowsME 18.9%, Windows2000 10.8%, Windows95 8.1% and WindowsXP 5.4%), 13.5% of all visitors used a Mac operating system. So far few problems have been reported in accessing the site (except during occassional maintenance), but people using older version of Netscape (5.x or lower) or Internet Explorer (4.x or lower) may encounter problems, especially if they are working from within a WindowsNT environment. Updates to the site (including updates to the IBCWG publication list) are published regularly (appr. every two weeks) and members of the group are encouraged to submit useful documents and/or links for publication on the website.

Biodiversity and molecular epidemiology of *Ralstonia* species in CF

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The frequency of respiratory tract infections by *Ralstonia* species in persons with cystic fibrosis (CF) and the role of these species in CF pulmonary disease are not well-documented. This may be, in part, due to difficulty in accurate identification of *Ralstonia* species. By using polyphasic analyses we identified 42 *Ralstonia* sp. isolates from sputum culture from 38 CF patients. The majority were identified as *Ralstonia mannitolilytica* or *Ralstonia pickettii*, but *Ralstonia gilardii* and *Ralstonia taiwanensis* were also found. Several of the isolates and similar isolates recovered from other clinical samples and the environment could not be identified to the species level and they were classified as *Ralstonia* sp. Thirteen of these isolates (recovered from CF patients, other clinical samples, soil and water) formed a homogeneous group by SDS-PAGE of whole-cell proteins and most likely form a single novel species. Epidemic spread of *Ralstonia* sp. among CF patients was not seen, but by using genotyping of serial isolates recovered from the same patient we demonstrated chronic colonization.

To facilitate identification of *R. mannitolilytica* and *R. pickettii*, and to avoid their misidentification as other closely related species (in particular *Burkholderia cepacia* complex), we developed 16S rDNA-based PCR assays that allow the sensitive and specific identification of these species. We also developed a 16S rDNA-based PCR assay for the identification of *Ralstonia* sp. to the genus level.

Molecular identification and phylogenetic diversity of the *Burkholderia cepacia* complex based on the recA gene

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B. cepacia is a very problematic opportunistic pathogen in patients with cystic fibrosis (CF) and many other vulnerable individuals. Isolates of this species were shown to belong to five closely related genetic species (genomovars): *Burkholderia vietnamiensis*> (formerly genomovar V), *Burkholderia multivorans* (formerly genomovar II), *Burkholderia stabilis* (formerly genomovar IV), genomovar I and genomovar III. This group of closely related bacteria were collectively designated as the *B. cepacia* complex. Since *B. cepacia* may spread from patient-to-patient and is associated with poor prognosis, rapid identification of strain genomovar is important for the clinical management and treatment of infection. Identification of the different members of the *B. cepacia* complex is not straightforward. Molecular identification approaches based on the *B. cepacia* complex recA gene were developed and proved useful for identification of all five current genomovars by polymerase chain reaction. However, since these assays were first developed, further taxonomic diversity within the *B. cepacia* complex has been identified. *B. cepacia* genomovar VI and *Burkholderia ambifaria* (genomovar VII) have now been formally described. In addition, a new genomovar, VIII, is in the process of being described and will be designated as *Burkholderia anthina*. Isolates previously designated as *Burkholderia pyrrocinnia* have also been found to belong to the *B. cepacia* complex. Nucleotide sequence variation within the recA gene enables differentiation among all these new taxonomic groups and has also identified 4 potentially novel taxonomic clusters within the *B. cepacia* complex. A total of 142 recA gene sequences have been determined from strains representing the taxonomic diversity within the complex. PCR assays specific for genomovar VIII isolates have been developed. Assays based on recA gene restriction fragment length polymorphisms and nucleotide sequence will also be described. Identification of *B. cepacia* complex isolates is vital to further research concerning the epidemiology and pathogenesis of this organism in patients with CF.
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