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BACTERIAL EPIDEMIOLOGY AND TYPING

Flagellin gene PCR-RFLP analysis of a panel of strains from the *Burkholderia cepacia* complex

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***Burkholderia cepacia*, an important opportunist pathogen, is genetically heterogeneous. The *B. cepacia* complex has been subdivided into a number of genospecies or genomovars. A flagellin gene PCR-RFLP method was applied to a representative panel of strains of known genomovar. The technique was able to distinguish strains of *B. multivorans* from other members of the *B. cepacia* complex on the basis of amplicon size (typical of type I rather than type II flagellins) with the exception of one genomovar I strain. There was considerable variation in RFLP patterns amongst the panel of strains; only two pairs of strains were indistinguishable with both *Hae*III and *Msp*I digestion. Where RFLP patterns matched with both enzymes or a single enzyme, matching strains were always in the same genomovar. It was possible to distinguish the UK cystic fibrosis epidemic strain from all other members of the panel, including nine other genomovar III strains. The level of variation suggests that flagellin genotyping is a useful method for discriminating between *B. cepacia* strains.**

Introduction

Burkholderia cepacia is an important opportunist pathogen associated with cystic fibrosis (CF) [1, 2], chronic granulomatous disease (CGD) [3] and septicaemia [4]. There is considerable genetic heterogeneity among CF and other isolates identified as *B. cepacia* and the *B. cepacia* complex has been subdivided into several genomic species or genomovars [5]. Although the majority of CF epidemic isolates, including the UK CF ET12 lineage, belong to the same genomovar (genomovar III), CF isolates belonging to most other genomovars have been isolated [6].

Previous studies reported that the majority of isolates of *B. cepacia* can be classified into two types on the basis of flagellin protein size [7] and demonstrated that the flagellin gene (*fliC*) exhibited considerable heterogeneity in *B. cepacia* [7, 8]. Variation between strains can be observed rapidly by PCR/RFLP analysis of *B. cepacia fliC* amplicons. However, in previous studies, genomovar designations were known for only a few of the strains studied. A panel of representative strains for the *B. cepacia* complex has been proposed [6], including representatives of all major genomic species and genomovars. This study describes the application of the *fliC* PCR/RFLP typing method to the study of genetic variability amongst this panel of strains and assesses the correlation between flagellin genotype and genomovar designation.

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Materials and methods

B. cepacia strains

Most of the bacterial strains used in this study were included in a panel of representative strains reported previously [6] and are listed in Table 1. The strains tested also included PC259, LMG 16232, FC441 and LMG 10929 (*B. vietnamiensis*). Isolates were maintained in the laboratory on nutrient agar.

PCR/RFLP analysis of B. cepacia flagellin genes

Flagellin gene oligonucleotide primers BC4 (CTGGTC GCACAGCAGAACCTGAAC; N-terminal) and BCR12 (ACAG/TGTTCGCGGTTTCCTG; C-terminal) [7] were obtained from Genosys (Cambridge). Cells taken from a nutrient agar plate were suspended in sterile distilled water (20 μl) and boiled for 5 min. This lysed suspension (1 μl) was used directly in a standard

Table 1. Matching *fliC* PCR-RFLP patterns amongst strains of the *B. cepacia* complex

Strain no.	Same *Hae*III digest	Same *Msp*I digest	Same digest with both enzymes
Genomovar I			
ATCC 25416^T	–	ATCC17759, LMG17997	–
ATCC17759	–	ATCC 25416^T LMG 17997	–
CEP509	–	–	–
LMG 17997	–	ATCC 25416^T ATCC17759	–
B. multivorans			
C5393	–	–	–
LMG 13010^T	C1962	CF-A1-1, C1962	C1962
C1576	–	–	–
CF-A1-1	–	C1962, LMG 13010^T	–
JTC	–	–	–
C1962	LMG 13010^T	LMG 13010^T CF-A1-1	LMG 13010^T
ATCC 17616	249-2	249-2	249-2
249-2	ATCC 17616	ATCC 17616	ATCC 17616
Genomovar III*			
J2315 (III-A)	C6433, C5424, BC7, K56-2	–	–
BC7 (III-A)	J2315, C6433, C5424, K56-2	–	–
K56-2 (III-A)	J2315, C6433, C5424, BC7	ATCC 17765, C5424	C5424
C5424 (III-A)	J2315, BC7, C6433, K56-2	K56-2, ATCC 17765	K56-2
C6433 (III-A)	J2315, C5424, BC7, K56-2	–	–
C1394 (III-B)	–	–	–
PC184 (III-B)	–	–	–
CEP511 (III-B)	–	–	–
J415	–	–	–
ATCC 17765 (III-B)	–	K56-2, C5424	–
B. stabilis			
LMG 14294	–	C7322	–
C7322	–	LMG 14294	–
LMG 14086	–	–	–
LMG 18888	–	–	–

*Where known the designation of a strain following subdivision of genomovar III into two groups on the basis of *recA* sequence is indicated [11].

amplification mixture. Amplifications were done in 50-μl volumes containing Taq DNA polymerase (Helena Biosciences, Sunderland, Tyne and Wear) 2.5 units, 1× TaqMaster (Helena Biosciences), 200 nM of each primer (BC4 and BCR12), 1× Taq buffer, 2.5 mM $MgCl_2$ and 100 μM nucleotides (dATP, dCTP, dGTP, dTTP) for 30 cycles consisting of 95°C (1 min), 60°C (1 min) and 72°C (2 min).

Amplified product samples (5 μl) were digested with the restriction endonucleases *Hae*III and *Msp*I using the conditions recommended by the supplier (Helena Biosciences). These digests were then subjected to electrophoresis on MetaPhor agarose 2% w/v gels (Flowgen) alongside pUC19/*Msp*I (Helena Biosciences) or Novagen PCR markers (CN Biosciences, Nottingham).

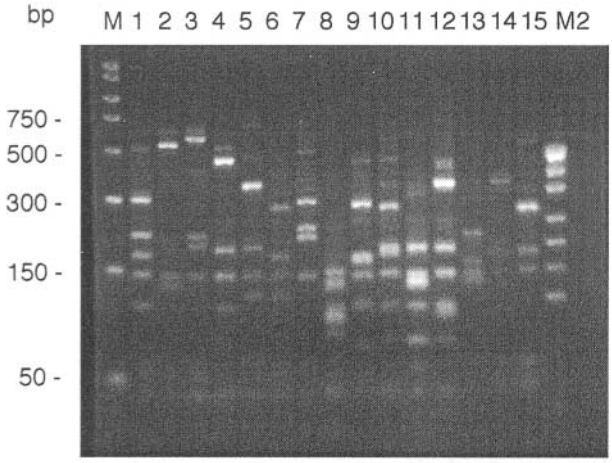

Fig. 1. *fliC* PCR-RFLP patterns generated with the endonuclease *Hae*III. The gel includes all the *Hae*III patterns generated from strains in this study: lane **1**, ATCC 17759; **2**, C6433; **3**, ATCC 17616; **4**, C1962; **5**, JTC; **6**, J415; **7**, ATCC 25416; **8**, CEP509; **9**, LMG 17997; **10**, C5393; **11**, C1576; **12**, CF-A1-1; **13**, PC184; **14**, CEP511; **15**, ATCC17765; **M** and **M2**, PCR markers and pUC19/*Msp*I respectively.

Results

Flagellin gene amplicons were obtained from 26 of the 30 strains tested. All four strains that failed to yield an amplified product were from the same genospecies (*B. vietnamiensis*, formerly genomovar V). Of the remaining 26 strains, nine yielded larger amplified products indicative of type I flagellins (1.4 kb compared with 1.0 kb for type II flagellins). Eight of these strains were from the same genospecies (*B. multivorans*, formerly genomovar II).

PCR/RFLP analysis of *fliC* amplicons indicated extensive variation amongst the panel of strains (Fig.

1). Of the 26 strains yielding amplicons, only six matched the RFLP patterns of another strain for both *Hae*III and *Msp*I. Nine strains matched at least one other strain's *Hae*III pattern whilst differing in the *Msp*I pattern and 13 strains matched at least one other strain's *Msp*I pattern whilst differing in the *Hae*III pattern (Table 1). RFLP patterns were compared with those obtained in previous studies [7, 8]. Only three of the strains used in this study matched with an RFLP group reported in previous studies. Strain ATCC 17759 was included in a previous study (under the designation NCIB 9085) and is in flagellin gene RFLP group IX [7]. Strain J2315, representative of the UK CF epidemic strain, was also included in previous studies (under the designation E241) and is in RFLP group I [7, 8]. The only genuine match between different isolates was between strain C6433 and the RFLP group 'Senegal', identified from strains isolated in a paediatric ward in Senegal [8].

Discussion

PCR amplification of the *fliC* gene with the primers used in this study led to discrimination between genospecies failing to yield a product (*B. vietnamiensis*), genospecies that yielded a type I flagellin gene product (*B. multivorans* and one genomovar I strain) and the remainder, that yielded type II flagellin gene products. In previous studies the one known strain of *B. multivorans* (genomovar II) also gave an amplicon indicative of a type I flagellin (1.4 kb) [7, 8]. One strain reported as genomovar I also yielded an amplicon typical of type I flagellins. As only one of the four genomovar I isolates used in this study yielded a type I flagellin gene product, it is apparent that strains from this genomovar can produce either type I or type II flagellin. The failure to obtain *fliC* amplicons from *B. vietnamiensis* is probably due to the inability of one or both of the primers to bind to the *fliC* genes in this genospecies, and suggests that *B. vietnamiensis fliC* genes are less related to the *fliC* genes of other members of the *B. cepacia* complex than such genes are to each other. It may be possible to rectify this problem with different primers. However, the variable nature of the *fliC* gene makes it difficult to design primers that are effective for all members of the *B. cepacia* complex.

Earlier studies reported that *fliC* PCR-RFLP analysis may be a useful epidemiological tool for identification of CF epidemic strains and for discrimination between these and other strains [7, 8]. The present study has demonstrated that *fliC* genotyping can be used to distinguish strain J2315 (genomovar III, ET12 lineage) from other strains of the genomovar III genospecies, including three containing the BCESM marker [9] and *cblA* [10], both indicative of epidemic strains. Although strain J2315 *fliC* amplicons could be distinguished from those of other genomovar III strains with the endonuclease *Msp*I, *Hae*III digestion yielded patterns that were indistinguishable from four other genomovar III strains. These five strains belong to the same subdivision of genomovar III (III-A) based on *recA* sequences [11] and four have been attributed to the same RAPD and PFGE strain types [6]. Two of these genomovar III strains (K56-2 and C5424) were indistinguishable from each other when digested with either restriction endonuclease. Interestingly, these two strains are both Canadian CF epidemic isolates [6]. The only other strains indistinguishable with either of the two endonucleases were members of the *B. multivorans* genospecies. Strain 249-2 is a derivative of ATCC 17616 [12] which has part of its genome deleted. Therefore, it is not surprising that these two strains are indistinguishable by flagellin genotyping. The apparently unrelated strains C1962 (a UK clinical isolate) and LMG 13010 (a CF isolate from Belgium) were indistinguishable with either enzyme. A number of other strains from the panel shared identical *fliC* RFLP patterns with *Msp*I. In each case, strains sharing RFLP patterns were located in the same genospecies. In this respect, typing by *fliC* RFLPs is consistent with genomovar designation.

The most notable observation in this study is the remarkable degree of variation in *fliC* genes of the *B. cepacia* complex. There were very few matches between the *fliC* PCR-RFLP patterns of strains used in this study and those of strains analysed previously. Although flagellin genes do often exhibit variation [13], the level of *fliC* variation in the *B. cepacia* complex is higher than in many other bacterial pathogens, such as the important CF pathogen *Pseudomonas aeruginosa* [14, 15]. In a study of *fliC* PCR-RFLP analysis of *P. aeruginosa* isolates, although six restriction endonucleases were used, strain discrimination was much lower than in *B. cepacia* [14]. This suggests that flagellin genotyping is a highly discriminatory method in *B. cepacia* and can be used as an epidemiological tool for the identification of the same strain from different sources.

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