Identification of *Burkholderia* species and genomovars from cystic fibrosis patients by AFLP fingerprinting

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AFLP is a genomic fingerprinting technique based on the selective amplification of restriction fragments from a total double-digest of genomic DNA. The applicability of this method to differentiate between species and genomovars of the genus *Burkholderia* was tested, with particular emphasis on taxa occurring in cystic fibrosis patients. In this study, 78 well-characterized strains and field isolates were investigated by two methods of AFLP fingerprinting. In the manual procedure, a radioactively labelled primer was used, amplified fragments were separated by conventional PAGE and the patterns were revealed by autoradiography. In the automated procedure, a fluorescently labelled primer was used in combination with electrophoresis and on-line data collection by means of an automated DNA sequencer. Overall, there was good agreement between the two AFLP procedures and the data were mostly consistent with results obtained from SDS-PAGE of whole-cell proteins and DNA–DNA hybridization experiments. The automated AFLP procedure has considerable technical advantages compared with the manual AFLP procedure, but a thorough visual analysis of the DNA profiles was required to avoid misidentification of some *Burkholderia cepacia* genomovar III strains.

Keywords: AFLP, *Burkholderia*, identification

INTRODUCTION

The genus *Burkholderia* was established by Yabuuchi *et al.* (1992) for *Pseudomonas* species belonging to rRNA group II (Palleroni *et al.*, 1973). At present, the genus *Burkholderia* contains 16 validly named species: *Burkholderia andropogonis*, *Burkholderia caryophylli*, *Burkholderia cepacia* (the type species), *Burkholderia gladioli*, *Burkholderia glathei*, *Burkholderia glumae*, *Burkholderia graminis*, *Burkholderia mallei*, *Burkholderia multivorans*, *Burkholderia norimbergensis*, *Burkholderia phenazinium*, *Burkholderia plantarii*, *Burkholderia pseudomallei*, *Burkholderia pyrrocinia*, *Burkholderia thailandensis* and *Burkholderia vietnamiensis* (Gillis *et al.*, 1995; Vandamme *et al.*, 1997; Wittke *et al.*, 1997; Viallard *et al.*, 1998; Brett *et al.*, 1998; Coenye *et al.*, 1999). In addition, three phenotypically similar genomic species or genomovars of *B. cepacia* have been described (Vandamme *et al.*, 1997).

Because of their phenotypic similarities, *B. cepacia* genomovars I, III and IV, *B. multivorans* (formerly *B. cepacia* genomovar II) and *B. vietnamiensis* are referred to together as the *B. cepacia* complex (Vandamme *et al.*, 1997).

During the last decade, increasing numbers of infections in cystic fibrosis (CF) patients by strains belonging to the *B. cepacia* complex and *B. gladioli* have been reported (Anonymous, 1992; Govan & Deretic, 1996; Govan *et al.*, 1996). In about 20% of the patients, infection leads to the ‘*B. cepacia* syndrome’, a necrotizing pneumonia associated with a rapid and often fatal clinical deterioration (Isles *et al.*, 1984; Govan & Deretic, 1996). Other *B. cepacia*-colonized CF patients show no or only a slow deterioration of lung function (Govan *et al.*, 1993). Epidemic strains causing multiple deaths in the CF population and strains causing ‘*B. cepacia* syndrome’ have mostly been identified as *B. cepacia* genomovar III (Vandamme *et al.*, 1997; Coenye *et al.*, 1998). Present data indicate that correct identification of

Abbreviation: CF, cystic fibrosis.

Table 1. List of the strains studied

The clusters in which the strains appear in Figs 1 (manual procedure) and 2 (automated procedure) are shown. LMG, BCCM/LMG Culture Collection, Laboratorium voor Microbiologie Gent, Universiteit Gent, Belgium. NK, Not known, ND, not determined; s, separate position.

Strain	Source	Cluster	
		Automated	Manual
<i>B. cepacia</i> genomovar I			
LMG 16670	Rhizosphere of <i>Carludovica palmata</i> (UK)	1	ND
LMG 16671	Rotting bark, orchid house (UK)	1	ND
LMG 14087	Wound swab (UK, 1988)	1	4
LMG 1222 ^T	<i>Allium cepa</i>	1	4
LMG 6988	Leg wound (Sweden, 1972)	s	4
LMG 2161	Forest soil (Trinidad)	6	ND
LMG 16672	Rhizosphere of banana plant (UK)	6	ND
R-1464*	CF patient (Australia)	6	4
LMG 14095	CF patient (UK, 1974)	6	4
<i>B. cepacia</i> genomovar III			
LMG 16658	CF patient (UK, Newcastle epidemic reference)	8	1
LMG 16656	CF patient (UK, 1989, ET12 reference)	8	1
LMG 16659	CF patient (UK, Manchester epidemic reference)	8	1
LMG 16667*	Non-CF patient, respiratory secretions (UK)	11	6
LMG 13011	CF patient (Belgium, 1988)	11	6
LMG 13012	CF patient (Belgium, 1988)	11	6
LMG 13054	CF patient (Belgium, 1987)	11	6
LMG 14274	CF patient (Belgium, 1993)	11	6
LMG 14942	CF patient (Belgium, 1993)	11	6
LMG 14276	CF patient (Belgium, 1993)	11	6
LMG 16661	CF patient (UK)	11	6
LMG 13053	CF patient (Belgium, 1987)	11	6
LMG 16662	CF patient (UK)	11	1
LMG 13318	CF patient (UK, 1992)	12	5
LMG 13325	CF patient (UK, 1992)	12	5
LMG 13320	CF patient (UK, 1992)	12	5
LMG 13316	CF patient (UK, 1991)	12	5
LMG 13307	CF patient (UK, 1992)	12	5
LMG 12615	CF patient (UK, 1992)	12	5
LMG 12614	CF patient (UK, 1992)	12	5
LMG 13308	CF patient (UK, 1992)	12	5
LMG 14088	CF patient (UK, 1993)	12	1
LMG 13327	CF patient (UK, 1992)	12	5
<i>B. cepacia</i> genomovar IV			
LMG 13017	Blood culture (Belgium, 1988)	5	10
LMG 14086	Respirator (UK, 1970)	5	10
LMG 15950	Water bath, CF ward (Belgium, 1994)	5	10
LMG 14940	CF patient (Belgium, 1994)	5	10
LMG 15949	CF patient (Belgium, 1993)	5	10
LMG 14294	CF patient (Belgium, 1993)	5	10
LMG 7000	Blood (Sweden, 1983)	5	10
LMG 14295	CF patient (Belgium, 1993)	5	10
LMG 14291	CF patient (Belgium, 1993)	5	10

Table 1 (cont.)

Strain	Source	Cluster	
		Automated	Manual
<i>B. multivorans</i>			
LMG 16660	CF patient (UK, Glasgow epidemic reference)	9	7
LMG 16668	Hospital flower vase (UK)	9	7
LMG 14273	CF patient (Belgium, 1993)	9	7
LMG 14280	CF patient (Belgium, 1993)	9	7
LMG 14282	CF patient (Belgium, 1993)	9	7
LMG 14281	CF patient (Belgium, 1993)	9	7
LMG 13010 ^T	CF patient (Belgium, 1992)	9	7
LMG 14293	CF patient (Belgium, 1993)	9	7
LMG 17588	Soil (USA)	9	7
LMG 14275	CF patient (Belgium, 1993)	9	7
<i>B. vietnamiensis</i>			
LMG 16232	CF patient (Sweden, 1993)	7	2
LMG 16234*	CF patient (Sweden, 1993)	7	2
LMG 16233*	CF patient (Sweden, 1993)	7	2
LMG 6998	Blood (Sweden, 1978)	7	2
LMG 10929 ^T	Rice rhizosphere (Vietnam)	7	2
LMG 11347*	Rice rhizosphere (Vietnam)	7	2
<i>B. gladioli</i>			
R-2011*	CF patient (USA)	2	9
LMG 2216 ^T	<i>Gladiolus</i> sp. (USA)	2	9
R-2015*	CF patient (UK)	2	9
LMG 6882	<i>Gladiolus</i> sp. (USA)	2	9
R-2014*	CF patient (USA)	2	9
LMG 2121*	NK	2	9
R-2012*	CF patient (USA)	2	9
R-645*	CF patient (Canada)	2	9
LMG 11626	Poisoned bongkrek (Java, Indonesia)	2	9
<i>B. glathei</i>			
LMG 14190 ^T	Soil (Germany)	s	s
<i>P. aeruginosa</i>			
LMG 1242 ^T	NK	4	8
LMG 6395	Blood culture	4	8
LMG 5827	Lung	4	8
<i>B. plantarii</i>			
LMG 10907	Soil (Japan)	3	3
LMG 9035 ^T	<i>Oryza sativa</i> (Japan)	3	3
<i>B. caryophylli</i>			
LMG 2155 ^T	<i>Dianthus caryophyllus</i> (USA)	s	s
<i>B. glumae</i>			
LMG 1277	<i>Oryza sativa</i> (Japan)	10	11
LMG 2196 ^T	<i>Oryza sativa</i> (Japan)	10	11
LMG 6952	<i>Oryza sativa</i> (Japan)	10	11
<i>B. andropogonis</i>			
LMG 2129 ^T	<i>Sorghum bicolor</i> (USA)	s	s
<i>B. pyrrocinia</i>			
LMG 14191 ^T	Soil	s	4

* Strains not included in the taxonomic study of Vandamme *et al.* (1997).

Burkholderia strains from CF patients is important in infection control but is far from straightforward using conventional methods [e.g. selective media, phenotypic characteristics, cellular fatty acid analysis or rRNA gene-derived probes (Leff *et al.*, 1995; Simpson *et al.*, 1994; Kiska *et al.*, 1996)]. The most reliable identification method for *Burkholderia* species is the comparison of one-dimensional protein electrophoretic patterns combined with DNA–DNA hybridizations (Vandamme *et al.*, 1997). However, these techniques are not accessible to most laboratories, they are time-consuming and interpretation of protein-pattern similarity is not straightforward (Vandamme *et al.*, 1997).

AFLP is a genomic fingerprinting technique that is based on the selective amplification of restriction fragments (Vos *et al.*, 1995), and ample evidence has been provided to show that this technique can be applied in bacterial taxonomy for the differentiation of closely related taxa (Janssen *et al.*, 1996, 1997; Huys *et al.*, 1996; Koeleman *et al.*, 1998; Desai *et al.*, 1998). The aims of the present study were: (i) to determine whether it is possible to distinguish *Burkholderia* species and genomovars using AFLP fingerprinting, (ii) to compare two procedures of AFLP fingerprinting, one involving a radioactively labelled primer, conventional PAGE and data collection after autoradiography (referred to below as the 'manual' procedure) and one involving a fluorescently labelled primer and an automated DNA sequencer for electrophoresis and on-line data collection (referred to below as the 'automated' procedure), and (iii) to compare the results of the two AFLP methods with existing taxonomic data.

METHODS

Strains and culture conditions. Strains used in this study are listed in Table 1. All of the strains were routinely grown on Trypticase soy agar (BBL; Becton Dickinson Microbiology Systems) and were incubated in air at 28 or 37 °C. Sixty-eight strains listed in Table 1 were identified previously by using a polyphasic taxonomic approach (Vandamme *et al.*, 1997). Ten additional reference strains and field isolates were included in the present study, following preliminary identification as *B. cepacia* complex or *B. gladioli* by SDS-PAGE of whole-cell proteins (data not shown). A relatively large number (23) of *B. cepacia* genomovar III strains was included to assess the diversity of this heterogeneous group, which seems predominant in CF patients (Vandamme *et al.*, 1997).

Isolation and purification of genomic DNA for AFLP fingerprinting. DNA was isolated and purified as described by Pitcher *et al.* (1989). DNA concentrations were determined by measuring the A_{260} in a Uvikon model 940 spectrophotometer.

Manual AFLP fingerprinting. The preparation of template DNA for PCR was performed essentially as described by Huys *et al.* (1996). One microgram of chromosomal DNA was digested with the restriction enzymes *Apal* and *TaqI* (Pharmacia Biotech). After complete digestion, double-stranded restriction-halvesite-specific adaptors were ligated to the restriction fragments with T4 ligase (Pharmacia Biotech). After precipitation with 7.5 M ammonium acetate and 50 %

(v/v) 2-propanol, templates were resuspended in 100 µl T0-1E (10 mM Tris/HCl, 0.1 mM EDTA, pH 7.6). For selective PCR amplification of restriction fragments, the *Apal* primer B07 (5'-GACTGCGTACAGGCCCG-3') and *TaqI* primer T11 (5'-GATGAGTCCTGACCGAG-3') (selective bases at the 3' ends are underlined) were used. The B07 primer was labelled at its 5' end with T4 kinase (Pharmacia Biotech) by using [γ -³²P]ATP (Amersham) as described by Vos *et al.* (1995). Amplification reactions, electrophoresis, visualization of fragments, densitometric scanning and numerical analysis were performed as described previously (Janssen *et al.*, 1996).

Automated AFLP fingerprinting. The preparation of template DNA for PCR was performed as described above. In a pre-selective PCR, the sequences with adaptors ligated to both ends were exponentially amplified. Four microlitres of template DNA was combined with 0.5 µl 10 µM *TaqI* primer T00 (5'-GACTGCGTACAGGCCCG-3'), 0.5 µl 10 µM *Apal* primer A00 (5'-GATGAGTCCTGACCGA-3') and 15 µl AFLP Amplification Core Mix (PE Applied Biosystems), containing nucleotides, *Taq* polymerase and buffer. The reactions were performed on a Perkin Elmer 9600 thermal cycler using the following thermal profile: 2 min at 72 °C and then 20 cycles of 20 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C (with all ramp times entered as 0.01). Ten microlitres of the pre-selective PCR product was diluted with 190 µl T0-1E. For selective PCR amplification, primer B07 (New England Biolabs) labelled with 6-carboxyfluorescein (6-FAM), a phosphoramidite dye, and primer T11 (New England Biolabs) were used. The diluted pre-selective PCR product (1.5 µl) was combined with 0.5 µl 5 µM 6-FAM-labelled primer B07, 0.5 µl 1 µM primer T11 and 7.5 µl AFLP Amplification Core Mix. The following thermal cycle profile for touchdown PCR was used: 2 min at 72 °C; cycle 1 was 20 s at 94 °C, 30 s at 66 °C and 2 min at 72 °C; in each of the next nine cycles, the annealing temperature was lowered by 1 °C from 65 to 57 °C; cycles 10–30 were 20 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C; followed by 30 min at 60 °C for final extension (with all ramp times entered as 0.01). The selective PCR products were diluted with 20 µl TE (10 mM Tris/HCl, 1 mM EDTA, pH 7.6). Amplified fragments were separated on a 4.25 % denaturing polyacrylamide gel with a length of 36 cm, on an ABI Prism 377 DNA Sequencer. Gels were prepared according to the manufacturer's guidelines. Loading buffer was prepared by mixing 1.25 µl deionized formamide, 0.25 µl blue dextran/50 mM EDTA loading solution and 0.5 µl GENESCAN-500[TAMRA] size standard [double-stranded DNA fragments, sized between 35 and 500 bp, with one of the strands labelled with the fluorescent dye TAMRA (6-carboxytetramethylrhodamine, an NHS-ester dye)] (PE Applied Biosystems). Two microlitres of the loading buffer was mixed with 1 µl diluted selective PCR product, heated for 3 min at 95 °C to denature the DNA and quick-chilled on ice before loading on the gel. Gels were run at 3000 V for 2 h at a constant temperature of 51 °C. During the run, the data were captured using the ABI Prism 377 Data Collection software and the raw data were stored on disk (PowerPC; Apple Macintosh). Lane tracking and normalization was performed using the GENESCAN 2.1 software (PE Applied Biosystems). Each gel track was then imported into the software package GELCOMPAR 4.1 (Applied Maths) using the program ABICON (Applied Maths). Similarity levels between the patterns were calculated with the Pearson product-moment correlation coefficient and are expressed as percentage similarity for convenience.

Preparation of high-molecular-mass DNA and DNA-DNA hybridization experiments. Preparation of high-molecular-mass DNA for DNA-DNA hybridization experiments and the determination of the degree of DNA-DNA binding by the initial renaturation rate method were performed as described previously (Vandamme *et al.*, 1992; De Ley *et al.*, 1970). The total DNA concentration was $65 \mu\text{g ml}^{-1}$ and the optimal renaturation temperature in $2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl , $0.015 \text{ M sodium citrate}$, $\text{pH } 7.0$) was 80.7°C .

RESULTS

Reproducibility of AFLP patterns

The reproducibility of the manual AFLP procedure was determined by using the pattern of strain *B. plantarii* LMG 16020 as a reference during each electrophoresis run. Differences observed could mostly be attributed to differences in electrophoresis times

Fig. 1. Dendrogram derived from the unweighted pair group method with averages linkage of correlation coefficients between the AFLP patterns obtained with the manual method.

Fig. 2. Dendrogram derived from the unweighted pair group method with averages linkage of correlation coefficients between the AFLP patterns obtained with the automated procedure.

and variations in background intensity. This is in agreement with previously published results (Huys *et al.*, 1996; Janssen *et al.*, 1997). It was noted that, when patterns that were obtained a few months apart from each other were compared, correlation levels were lower than the correlation level between patterns that were obtained within a short period of time (data not shown). However, between-gel reproducibility was always greater than 92%. The within-gel reproducibility was always greater than 94.2%.

The reproducibility of the automated AFLP procedure was determined by multiple analyses of several strains (LMG 16660, LMG 16668, LMG 14275, LMG 14276 and LMG 6395). For each of these strains, the

Fig. 3. AFLP patterns of a selection of the strains investigated. (a) Computer-processed printout of patterns obtained with the manual procedure. (b) Schematic representation of patterns obtained with the automated procedure. The high-intensity bands responsible for the close grouping of *B. vietnamiensis* and certain *B. cepacia* genomovar III strains using the automated AFLP procedure are indicated.

between-gel correlation between the patterns was greater than 93 %. The within-gel correlation between patterns was greater than 95 %.

Numerical analysis of AFLP patterns

For most of the taxa and strains examined, the two methods gave comparable results (Figs 1 and 2). Fig. 3 shows a number of patterns obtained with the two methods.

In the two analyses, strains of *B. cepacia* genomovar IV (clusters M10 and A5) [cluster numbers starting with M refer to the clusters obtained with the manual method (Fig. 1); cluster numbers starting with A refer to the clusters obtained with the automated method (Fig. 2)], *B. multivorans* (clusters M7 and A9), *B. vietnamiensis* (clusters M2 and A7), *B. gladioli* (clusters M9 and A2), *B. plantarii* (clusters M3 and A3), *B. glumae* (clusters M11 and A10) and *Pseudomonas aeruginosa* (clusters M8 and A4) formed well-delineated clusters. The type strains of *B. glathei* and *B. andropogonis* occupied separate positions in both

dendrograms. The type strain of *B. caryophylli* was closely linked to cluster A4 (*P. aeruginosa*) but occupied a separate position in the dendrogram obtained with the manual AFLP procedure.

Different results were obtained for *B. cepacia* genomovar I strains. In the dendrogram derived from the manual procedure, all *B. cepacia* genomovar I strains examined grouped in cluster M4, together with the *B. pyrrocinia* type strain. In contrast, after numerical analysis of the patterns obtained with the automated technique, *B. cepacia* genomovar I strains LMG 16670, LMG 16671, LMG 14087 and LMG 1222^T formed cluster A1, while *B. cepacia* genomovar I strain LMG 6988 occupied a somewhat separate position and was linked to cluster A1 at a low similarity level (<20 %). *B. cepacia* genomovar I strains LMG 2161, LMG 16672, R-1464 and LMG 14095 formed cluster A6. The type strain of *B. pyrrocinia* occupied a separate position.

Differences were also noted for *B. cepacia* genomovar III strains. *B. cepacia* genomovar III strains did not

Table 2. DNA–DNA binding values of all strains examined

Strain	DNA–DNA binding value (%)																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
<i>B. cepacia</i> gv. I																								
1. LMG 1222 ^T	100																							
2. LMG 14087	91	100																						
3. LMG 6988	58		100																					
<i>B. multivorans</i>																								
4. LMG 13010 ^T	24			100																				
5. LMG 14273					100																			
6. LMG 14275	42			100	82	100																		
7. LMG 14293					88	89	100																	
<i>B. cepacia</i> gv. IV																								
8. LMG 14294		35				54	31	100																
9. LMG 14940	41						24	100	100															
10. LMG 14086								95†	100†	100														
<i>B. cepacia</i> gv. III*																								
11. LMG 12614 (1)	33										100													
12. LMG 12615 (1)	42			36					54		89	100												
13. LMG 13320 (1)						32		43				92	100											
14. LMG 13327 (1)													93	100										
15. LMG 13011 (2)	33			19									54	58	100									
16. LMG 14274 (2)													76		99	100								
17. LMG 13053 (2)		45		42		40						72	69		89		100							
18. LMG 16661 (2)								47				72					93	100						
19. LMG 16659 (2)												74†					100†		100					
20. LMG 16656																			66†	100				
<i>B. vietnamiensis</i>																								
21. LMG 10929 ^T	36			40					26						35		42		42†		100			
22. LMG 6998	16					30		23			18	23									86	100		
<i>B. gladioli</i>																								
23. LMG 2216 ^T	11			4			24	1	12			13				23							100	
<i>B. pyrrocinia</i>																								
24. LMG 14191 ^T	59	59				31							55		63				54		56			100

* Numbers in parentheses after strain numbers indicate genomic sub-group.

† DNA–DNA binding values obtained during this study; other values are from Vandamme *et al.* (1997).

gv., Genomovar.

form a single cluster in either analysis. With the manual method, most strains grouped together (clusters M5 and M6); however, five strains formed a separate group (cluster M1). Two main clusters of *B. cepacia* genomovar III strains were found after numerical analysis of patterns obtained with the automated method (clusters A11 and A12). Three strains formed a separate group (cluster A8), clustering close to *B. vietnamiensis*.

DNA–DNA hybridizations

The results of the DNA–DNA hybridization experiments performed are shown in Table 2.

DISCUSSION

In the study presented here, we included well-characterized *Burkholderia* reference strains from the environment and human clinical samples to assess whether it is possible to distinguish *Burkholderia* species and genomovars using AFLP fingerprinting. For this investigation, two methods of AFLP fingerprinting were compared, one using a radioactively labelled primer and conventional PAGE and one using a fluorescently labelled primer and separation and

detection of the fragments with an automated DNA sequencer. Furthermore, the data obtained with the two AFLP techniques were compared with existing taxonomic data and discrepant results were evaluated by DNA–DNA hybridization experiments.

Comparison of the two AFLP methods and comparison with existing taxonomic data

Overall, there was good agreement between the two AFLP methods (Figs 1 and 2); however, the automated procedure resulted in the delineation of additional subgroups within some of the taxa examined. Generally, there was good agreement between data obtained by the manual AFLP method and data obtained by SDS-PAGE of whole-cell proteins, but the manual AFLP procedure subdivided *B. cepacia* genomovar III strains into several sub-groups. This is in agreement with the previous finding that *B. cepacia* genomovar III is heterogeneous, as revealed by DNA–DNA hybridization experiments (Vandamme *et al.*, 1997; Table 2). The major differences between the results obtained with the manual AFLP procedure and SDS-PAGE concern strains belonging to *B. cepacia* genomovars I and IV and *B. pyrrocinia*.

B. cepacia* genomovars I and IV and *B. pyrrocinia

Strains LMG 7000, LMG 13017, LMG 14086 and LMG 15950 were identified as *B. cepacia* genomovar I by Vandamme *et al.* (1997) by means of SDS-PAGE of whole-cell proteins. However, with both AFLP methods these four strains clustered with *B. cepacia* genomovar IV reference strains (clusters M10 and A5). The four strains had similar AFLP profiles (data not shown) and DNA–DNA hybridizations of strain LMG 14086 towards *B. cepacia* genomovar IV reference strains LMG 14940 and LMG 14294 revealed binding values of 100 and 95 %, respectively. These results confirm that the four strains belong to *B. cepacia* genomovar IV and that AFLP is superior to SDS-PAGE of whole-cell proteins for the differentiation of *B. cepacia* genomovar I and IV strains.

Another difference between the results obtained with the two AFLP fingerprinting techniques and SDS-PAGE of whole-cell proteins was noticed for *B. cepacia* genomovar I and *B. pyrrocinia* strains. When using the manual AFLP procedure and SDS-PAGE of whole-cell proteins, the five *B. cepacia* genomovar I strains investigated grouped together with the type strain of *B. pyrrocinia* (cluster M4, Fig. 1; Vandamme *et al.*, 1997). When using the automated AFLP procedure, the *B. cepacia* genomovar I strains investigated formed two sub-groups (clusters A1 and A6), while *B. cepacia* genomovar I strain LMG 6988 and *B. pyrrocinia* strain LMG 14191^T occupied separate positions. DNA–DNA hybridizations performed between *B. cepacia* genomovar I strains revealed a high DNA–DNA binding value (91 %) between two cluster A1 strains (Table 2). The intermediate binding values found between LMG 1222^T and LMG 14191^T (59 %) and between LMG 1222^T and LMG 6988 (58 %) support the separate positions of these strains. This suggests that the heterogeneity revealed by using the automated AFLP procedure reflects considerable heterogeneity in terms of DNA–DNA binding levels.

***B. cepacia* genomovar III**

Previously, Vandamme *et al.* (1997) noted the marked genotypic, chemotaxonomic and phenotypic heterogeneity within *B. cepacia* genomovar III strains. Extensive DNA–DNA hybridizations showed that at least two genomic sub-groups (sub-groups 1 and 2) can be found within *B. cepacia* genomovar III (Vandamme *et al.*, 1997; this study). DNA–DNA binding values within these sub-groups are higher than 89 %. DNA–DNA binding values between representatives of the two genomic sub-groups are between 54 and 74 % (Table 2). The AFLP data are in accordance with these DNA–DNA binding levels. Both AFLP methods resulted in the same clustering for the majority of the *B. cepacia* genomovar III strains investigated, except LMG 16662, LMG 14088, LMG 16656, LMG 16658 and LMG 16659 (see below). These genomic sub-groups could not be recognized by SDS-PAGE of whole-cell proteins (data not shown) and there was no

correlation between the genomic sub-grouping and the separation of *B. cepacia* genomovar III strains into three biovars as proposed by Vandamme *et al.* (1997).

For five genomovar III strains, the two AFLP procedures revealed different results. Strains LMG 14088 and LMG 16662 grouped together in cluster M1 using the manual AFLP procedure, while LMG 14088 clustered with A12 and LMG 16662 with A11 using the automated AFLP procedure. Using the manual AFLP procedure, *B. cepacia* genomovar III strains LMG 16656 [the Edinburgh–Toronto (ET12) epidemic reference strain], LMG 16658 (the Newcastle epidemic reference strain), LMG 16659 (the Manchester epidemic reference strain), LMG 14088 and LMG 16662 formed cluster M1, while the former three strains constituted a separate cluster A8 using the automated AFLP procedure. Using the automated AFLP procedure, cluster A8 grouped closely with cluster A7 (*B. vietnamiensis*). This similarity of *B. cepacia* genomovar III strains to *B. vietnamiensis* cannot be explained in terms of DNA–DNA binding level, as LMG 16659 exhibited only 42 % DNA–DNA binding with *B. vietnamiensis* LMG 10929^T, while values of 74 % and 100 % were measured towards other genomovar III strains (Table 2). The most probable reason for this apparent high similarity level is the presence of two high-intensity bands (one of approximately 110 bp and one of approximately 265 bp) in both *B. vietnamiensis* and cluster A8 strains (Fig. 3). When these bands were omitted from the numerical analysis, cluster A8 and *B. vietnamiensis* strains formed clearly separated clusters at a similarity level < 30 % (data not shown). The different positions of these epidemic strains in AFLP fingerprinting compared with other *B. cepacia* genomovar III strains is not confirmed by SDS-PAGE of whole-cell proteins (Vandamme *et al.*, 1997) or DNA–DNA hybridizations, as LMG 16659 exhibited a high binding value towards LMG 13053 (100 %) (clusters M6 and A11) and LMG 12615 (74 %) (clusters M5 and A12) and only a moderately high binding value towards LMG 16656 (66 %).

Other taxa

All other taxa included could be clearly separated (Figs 1 and 2). *B. multivorans*, *B. vietnamiensis* and *B. gladioli* all appeared as homogeneous taxa, confirming previous conclusions based on SDS-PAGE of whole-cell proteins and DNA–DNA hybridizations (Christenson *et al.*, 1989; Zhao *et al.*, 1995; Vandamme *et al.*, 1997). Clusters M9 and A2 also contained LMG 11626, the type strain of *Burkholderia covei*; the results of both AFLP methods thus confirm the reported synonymy between *B. gladioli* and *B. covei* (Coenye *et al.*, 1999).

When using the manual procedure, the type strain of *B. caryophylli* occupied a separate position, while this strain grouped closely with cluster A4 (*P. aeruginosa*) when using the automated procedure. The most probable reason for this apparent similarity is the

presence of two high-intensity bands (one of approximately 45 bp and one of approximately 85 bp) in both *P. aeruginosa* and *B. caryophylli* (data not shown).

P. aeruginosa is another predominant pathogen in the respiratory tract of CF patients (Govan & Deretic, 1996) and atypical (non-pigmented) isolates are occasionally misidentified as *B. cepacia*. Since the G+C content of the *P. aeruginosa* genome (67.2 mol%; Palleroni, 1984) is similar to that of members of the *B. cepacia* complex (67–69 mol%; Vandamme *et al.*, 1997), *P. aeruginosa* strains can be identified with AFLP using the same restriction enzymes and primers as those used for *Burkholderia* species, as illustrated in Figs 1 and 2.

Conclusions

Despite considerable technical differences between the manual and automated AFLP fingerprinting procedures, the two approaches yielded comparable clusters following numerical analysis of the patterns. The results obtained with the two methods are mostly in agreement with previously established taxonomic data and DNA–DNA hybridization experiments show that the AFLP procedure is a valuable or even superior alternative to SDS-PAGE of whole-cell proteins. The manual AFLP procedure has the disadvantage of being technically demanding, but all strains investigated could be identified unambiguously. The automated AFLP procedure is technically less demanding and faster than the manual procedure, but several important clinical strains might have been misidentified as *B. vietnamiensis* if the results of the cluster analysis had not been verified by a visual examination of the patterns of all the strains constituting the different clusters.

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