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Burkholderia anthina sp. nov. and *Burkholderia pyrrocinia*, two additional *Burkholderia cepacia* complex bacteria, may confound results of new molecular diagnostic tools

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Abstract

Nineteen *Burkholderia cepacia*-like isolates of human and environmental origin could not be assigned to one of the seven currently established genomovars using recently developed molecular diagnostic tools for *B. cepacia* complex bacteria. Various genotypic and phenotypic characteristics were examined. The results of this polyphasic study allowed classification of the 19 isolates as an eighth *B. cepacia* complex genomovar (*Burkholderia anthina* sp. nov.) and to design tools for its identification in the diagnostic laboratory. In addition, new and published data for *Burkholderia pyrrocinia* indicated that this soil bacterium is also a member of the *B. cepacia* complex. This highlights another potential source for diagnostic problems with *B. cepacia*-like bacteria. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

During the past decade, analysis of the microbiological diversity of *Burkholderia cepacia*-like organisms by using a polyphasic taxonomic approach has clarified problems associated with the identification of these bacteria. It demonstrated the presence of several novel organisms such as *Pandoraea* species, which can be misidentified as *B. cepacia* [1,2]. Even more troublesome, it highlighted the unsurpassed taxonomic complexity of this organism that is rightfully considered as a species complex [2,3]. The *B. cepacia* complex comprises at least seven genomic species (referred to as genomovars): *B. cepacia* (genomovar I), *Burkholderia multivorans* (formerly *B. cepacia* genomovar II), *B. cepacia* genomovar III, *Burkholderia stabilis* (formerly *B. cepacia* genomovar IV), *Burkholderia vietnamiensis* (also known as *B. cepacia* genomovar V), *B. cepacia* genomovar VI, and *Burkholderia ambifaria* (*B. cepacia* genomovar VII) [3–6]. These genomovars share a high degree of 16S rDNA (98–100%) and *recA* (94–95%) sequence similarity, and moderate levels of DNA–DNA hybridisation (30–60%) [2–7]. Isolates from cystic fibrosis patients from different geographical regions and from novel environmental niches are being examined to continue to explore the biodiversity of this organism. The results from these studies rapidly challenge newly developed molecular identification approaches [7–11] by the discovery of novel 'atypical' isolates that do not fit into the current classification system.

The current study describes a group of 19 atypical *B. cepacia*-like organisms that could not be allocated to

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one of the current seven members of this species complex. The name *Burkholderia anthina* sp. nov. is proposed to accommodate this eighth genomovar within the *B. cepacia* complex and several approaches for its identification are described. In addition, analysis of the *Burkholderia pyrrocinia* type strain indicated that this soil bacterium represents a ninth *B. cepacia* complex genomovar.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All *B. anthina* isolates examined are listed in Table 1. Reference strains of other *Burkholderia* species and genomovars were described previously [3–6]. All strains were grown aerobically on trypticase soy agar (BBL) and incubated at 28°C unless otherwise indicated.

2.2. DNA extraction

DNA used for DNA–DNA hybridisation, AFLP fingerprinting, and determination of the DNA base composition was prepared as described by Pitcher et al. [12]. DNA used for PCR amplification of the 16S rRNA and the *recA* gene, and for the *recA*-based PCR tests was prepared by heating one to two colonies (picked from an overnight grown plate) at 95°C for 15 min in 20 μl lysis buffer containing 0.25% (v/v) sodium dodecyl sulphate (SDS) and 0.05 M NaOH. Following cell lysis, 180 μl distilled water was added to the lysis buffer and the DNA solutions were stored at −20°C.

Table 1
List of *B. anthina* strains studied

Strain number[a]	Other strain designations	Source
LMG 16670	J2552[b], BPC51	rhizosphere of *Carludoucas palmata* (UK)
LMG 20980	W92B, J2863, CCUG 46047	rhizosphere soil (Nashville, USA)
LMG 20982	C1658	hospital environment (Manchester, UK)
LMG 20983	C1765	sputum, cystic fibrosis patient (Blackpool, UK)
R-131	J2553[b], BPC22	*Sanseviera* leaf (Edinburgh, UK)
R-4183	W92	rhizosphere soil (Nashville, USA)
R-4190	W89B	rhizosphere soil, vine plant (Edinburgh, UK)
R-9942	FC0976	environmental strain
R-11752	J2927	*Petunia* rhizosphere (Dundee, UK)
R-11753	J2928	*Petunia* rhizosphere (Dundee, UK)
R-11755	J2950	*Begonia* rhizosphere (Dundee, UK)
R-11757	J2944	*Begonia* rhizosphere (Dundee, UK)
R-11758	J2951	*Dracaena* rhizosphere (Dundee, UK)
R-11759	J2949	*Begonia* rhizosphere (Dundee, UK)
R-11761	J2941	*Petunia* rhizosphere (Dundee, UK)
R-11763	J2946	*Begonia* rhizosphere (Dundee, UK)
R-11766	J2945	*Begonia* rhizosphere (Dundee, UK)
R-13393	C1660	hospital environment (Manchester, UK)
R-16022	AU1293	sputum, cystic fibrosis patient (USA)

[a]LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie Gent, Universiteit Gent, Ghent, Belgium.
[b]See reference [25].

2.3. 16S rDNA sequencing

The nearly complete sequences of the 16S rRNA gene of strain R-4183 was amplified by PCR using conserved primers (5′-AGAGTTTGATCCTGGCTGAG-3′ and 5′-AAGGAGGTGATCCAGCCGCA-3′). The PCR products were purified using a QIAquick PCR purification kit (Qiagen GmbH) according to the manufacturer's instructions. Sequence analysis was performed using an Applied Biosystems 310 DNA Sequencer and the protocols of the manufacturer (Perkin-Elmer) using the ABI Prism™ dye terminator cycle sequencing ready reaction kit. The sequencing primers are those given by Coenye et al. [1]. Sequence assembly was performed using the programme AutoAssembler™ (Perkin-Elmer). Phylogenetic analysis was performed using the BioNumerics software package (Applied Maths), based on the neighbour-joining method [13]. Approximately 1460 bases were used and unknown bases were excluded from the calculations.

2.4. recA gene sequence and restriction fragment length polymorphism (RFLP) analysis

recA gene sequence and RFLP analyses were performed as described before [7]. The sequences were aligned and compared with *recA* sequences determined from *B. cepacia* complex strains analysed in previous studies [4,6,7] using the BioNumerics software package (Applied Maths).

2.5. 16S rRNA- and recA-based PCR assays

Assays for the identification of members of the *B. cepacia* complex based on the rRNA operon and the *recA* gene were performed as described previously [7,9].

A *B. anthina*-specific *recA*-based PCR test was developed based on *recA* sequences determined during this and previous studies. PCR assays were performed in 25 μl standard reaction mixtures [7] using 20 pmol of primers, BCRG81, 5′-TACGGTCCGGAATCGTCG-3′, and BCRG82, 5′-CGCACCGACGCATAGAAT-3′ (see Section 3 for design criteria). Amplification was carried out using a Techne Touchgene Thermal Cycler (Jencons-PLS, Leighton Buzzard, UK). Thirty amplification cycles were completed, each consisting of 30 s of denaturation at 94°C, 45 s at an annealing temperature of 61°C and 60 s of extension at 72°C. A final elongation of 7 min at 72°C completed the cycle. One third of the PCR mixture was then analysed by gel electrophoresis for the presence of a 473 bp product.

2.6. AFLP (amplified fragment length polymorphism) fingerprinting

The preparation of template DNA (using restriction enzymes *Apa*I and *Taq*I), amplification (using primers A00 and T00 in the preselective PCR and primers B07 [labelled with the fluorescent dye 6-FAM] and T11 in the selective PCR), separation of the fragments using an ABI Prism 377 automated DNA sequencer and numerical analysis were performed as described previously [14]. Data for the reference strains of *B. cepacia* genomovars were generated during previous studies [5,6,14].

2.7. Determination of the DNA base composition

DNA was enzymatically degraded into nucleosides as described by Mesbah et al. [15]. The obtained nucleoside mixture was then separated by high-performance liquid chromatography using a Waters SymmetryShield C8 column thermostated at 37°C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5% acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

2.8. DNA–DNA hybridisation

DNA–DNA hybridisations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki et al. [16], using a HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements. The hybridisation temperature was 50°C.

2.9. Polyacrylamide gel electrophoresis (PAGE) of whole-cell proteins

After an incubation period of 48 h, whole-cell protein extracts were prepared and SDS–PAGE was performed as described before [17]. The densitometric analysis, normalisation and interpolation of the protein profiles, and numerical analysis were performed using the GelCompar software package version 4.2 (Applied Maths). Similarity levels between the patterns were calculated using the Pearson product moment correlation coefficient and are expressed as percentage similarity for convenience. Data for the reference strains of *B. cepacia* genomovars were generated during previous studies [3–6].

2.10. Fatty acid methyl ester analysis

After an incubation period of 24 h at 35°C, a loopful of well-grown cells was harvested and fatty acid methyl esters were prepared, separated and identified using the Microbial Identification System (Microbial ID) as described before [18].

2.11. Biochemical characterisation

Tests were performed as described previously [19]. Briefly, pure cultures were stored at −70°C in Mueller Hinton broth (Difco) with 8% dimethylsulfoxide. Frozen isolates were subcultured to Columbia agar containing 5% sheep blood (PML Microbiologicals) before testing. The primary identification system used was the API Rapid NE system (Biomerieux Vitek Inc.), supplemented with glucose, maltose, lactose, xylose, sucrose and adonitol oxidation-fermentation (OF) sugars, and an adaptation of Moeller lysine and ornithine decarboxylases (Difco). All incubations were done at 35°C except where otherwise mentioned. Growth on trypticase soy agar (Becton Dickinson) at 35°C and 42°C was observed for appearance and pigment production.

3. Results

3.1. recA RFLP analysis

The *recA* RFLP patterns of the *B. anthina* isolates differed from those of the recognised members of the *B. cepacia* complex and from that of the *B. pyrrocinia* type strain. Two different patterns, designated T and AS could be distinguished among the 19 isolates examined (Fig. 1). The *B. pyrrocinia* type strain had a unique *recA* RFLP pattern, designated M, and occupied a distinct position in the dendrogram (Fig. 1).

3.2. PCR assays

In the 16S rRNA-based PCR assays, DNA fragments of the expected size were amplified for all *B. anthina* isolates investigated with the primer pair RHG-F and RHG-R (specific for the genera *Burkholderia*, *Pandoraea* and *Ralstonia*) and with the primer pair PC-SSF and PC-SSR (specific for *B. cepacia* genomovars I and III, *B. stabilis* and *B. ambifaria*). Using primer pairs BC-GII and BC-R, and BC-GV and BC-R (specific for *B. multivorans*, and for *B. multivorans* and *B. vietnamiensis*, respectively), no amplification product was obtained (data not shown).

None of the *B. anthina* isolates yielded PCR products with the *recA*-based PCR assays described for the other *B. cepacia* complex genomovars (data not shown).

The full-length *recA* gene sequences from strains LMG 16670, LMG 20980, LMG 20982, LMG 20983, R-4190 and R-11761 (EMBL accession numbers AF456060, AF456059, AF456034, AF456051, AF456058 and AF456061, respectively) were aligned against published *recA* sequences for each *B. cepacia* complex genomovar [4,6,7] and 48 novel *recA* sequences representative of distinct *recA* gene RFLP types from other *B. cepacia* complex isolates (E. Mahenthiralingam, unpublished data). The nucleotide at position 210, G, in the *recA* gene was found to

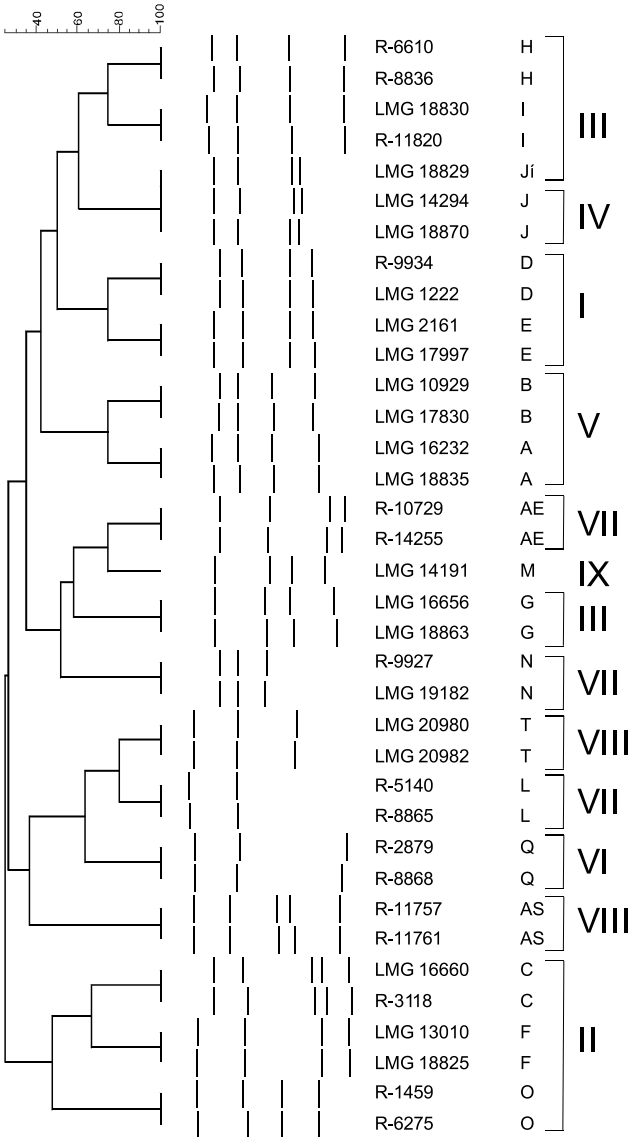

Fig. 1. Numerical analysis and computer-generated reproductions of *Hae*III – *recA* gene restriction profiles of *B. cepacia* complex bacteria. Data for non-*B. anthina* strains were taken from previous studies [5–7]. Roman numerals indicate genomovar numbers. RFLP types D and E represent *B. cepacia* (genomovar I), RFLP types C, F and O represent *B. multivorans*, RFLP types G, H, I and J′ represent *B. cepacia* genomovar III, RFLP type J represents *B. stabilis*, RFLP types A and B represent *B. vietnamiensis*, RFLP type Q represents *B. cepacia* genomovar VI, RFLP types AE, N and L represent *B. ambifaria*, and RFLP types AS and T represent *B. anthina* (types J and J′ are identical but represent different genomovars [7]).

be conserved in all *B. anthina* sequences and absent in other *B. cepacia* complex *recA* genes, except for *B. ambifaria* and two sequences from strains of currently undetermined genomovar. Base position 648, A, was completely specific to *B. anthina recA* genes. Two 18 nucleotide primers, BCRG81 (position 193–210 in the *recA* gene) and BCRG82 (the reverse complement of position 665–648 in the *recA* gene), were designed to detect the latter bases and specifically amplify a 473 bp PCR product from *B. anthina* strains. All *B. anthina* strains tested positive with these primers (data not shown). DNA from strains representative of 51 *recA* gene RFLP types, including all current genomovars of the *B. cepacia* complex and several novel *recA* RFLP types, were negative in the *B. anthina* PCR (data not shown).

3.3. PAGE of whole-cell proteins

Duplicate protein extracts were prepared to check the reproducibility of the growth conditions and the preparation of the extracts. The correlation level between duplicate protein patterns was more than 93% (data not shown). The overall whole-cell protein profiles of *B. anthina* isolates resembled those of *B. cepacia* genomovars I and III and *B. ambifaria* isolates, but were clearly different from those of other *B. cepacia* complex species and other *Burkholderia* reference species present in the database (data not shown). Numerical analysis of the profiles did not allow differentiation of *B. anthina*, *B. cepacia* genomovars I and III, and *B. ambifaria* isolates (data not shown).

3.4. AFLP fingerprinting

The reproducibility was checked by preparing PCR products in duplicate and was always higher than 92% (data not shown). Following numerical analysis, all of the *B. anthina* isolates examined formed a single cluster delineated above a similarity level of 40%, which was clearly different from the clusters comprising other *Burkholderia* species or *B. cepacia* complex genomovars (data not shown).

3.5. Phylogenetic analysis

The 16S rRNA gene sequence of strain R-4183 (EMBL accession number AJ420880) was compared with the available 16S rRNA gene sequences of representatives of the *beta-Proteobacteria*. Strain R-4183 clustered among members of the *B. cepacia* complex, with similarity levels ranging from 98.7% to 99.6%. Similarity levels towards other *Burkholderia* species were below 97.7%. Similarity levels towards representatives of other taxa belonging to the *beta-Proteobacteria* were below 95.1%.

3.6. DNA–DNA hybridisation and G+C content analysis

The DNA–DNA hybridisation results between strains LMG 16670, R-4183, and LMG 20980 revealed binding values above 71% (Table 2). The DNA–DNA hybridisation values between the latter strains and representatives of (i) *B. cepacia* genomovar I (53–61%), (ii) *B. multivorans* (34–45%), (iii) *B. cepacia* genomovar III (51%), (iv) *B. stabilis* (53–63%), (v) *B. vietnamiensis* (42–59%), (vi) *B. cepacia* genomovar VI (40–55%), (vii) *B. ambifaria* (61%), and (viii) *B. pyrrocinia* (31%) were intermediate or low (Table 2).

Table 2
DNA–DNA binding values and mol% G+C of all strains examined

	DNA binding values with *B. anthina* strain:		
	LMG 16670	R-4183	LMG 20980^T
B. anthina			
LMG 16670	100		
R-4183	85	100	
LMG 20980		71	100
B. cepacia genomovar I			
LMG 1222^T	55	61	
LMG 14087		53	59
B. multivorans			
LMG 13010^T	45	34	
B. cepacia genomovar III			
LMG 16659	51		
B. stabilis			
LMG 14294^T	53	63	
B. vietnamiensis			
LMG 10929^T	42	59	
B. cepacia genomovar VI			
LMG 18941	40	55	
B. ambifaria			
LMG 19182 ^{T}	61		
B. pyrrocinia			
LMG 14191^T	31		

The G+C contents for *B. anthina* strains LMG 16670, R-4183, and LMG 20980 were 66.6, 66.4, and 66.5 mol%, respectively.

3.7. Cellular fatty acid analysis

The average whole-cell fatty acid profile was determined for nine *B. ambifaria* and 10 *B. anthina* isolates. Following components were present in *B. ambifaria* and *B. anthina*, respectively (in percent): 14:0 (3.6 ± 0.3, 5.0 ± 0.8), 16:1 ω7c (11.0 ± 3.5, 17.1 ± 1.1), 16:0 (26.3 ± 2.0, 28.4 ± 0.9), 17:0 cyclo (11.3 ± 4.9, 4.6 ± 2.5), 16:1 2OH (trace amounts in both species), 16:0 2OH (trace amounts in both species), 16:0 3OH (6.6 ± 0.7, 5.8 ± 0.3), 18:1 ω7c (25.6 ± 6.3, 27.6 ± 5.2), 18:0 (1.1 ± 0.5, 1.0 ± 0.2), 19:0 cyclo ω8c (4.8 ± 2.8, 1.3 ± 0.4), 18:1 2OH (1.4 ± 0.4, 1.0 ± 0.4), 14:0 3OH (5.5 ± 1.1, 5.9 ± 0.5). In addition, *B. ambifaria* also contains 1.1 ± 0.2% 12:0. In these profiles, summed feature 2 (comprising 14:0 3OH, 16:1 iso I, an unidentified fatty acid with equivalent chain length value of 10.928 Da, or 12:0 ALDE, or any combination of these fatty acids), and summed feature 3 (comprising 16:1 ω7c or 15 iso 2OH or both) are listed as 14:0 3OH and 16:1 ω7c, respectively, as these fatty acids have been reported in *Burkholderia* species [20].

Overall, all *B. cepacia* complex isolates had the same fatty acid components in their whole-cell profiles. However, *B. anthina* isolates had strikingly high levels of the unsaturated fatty acids 16:1 ω7c and 18:1 ω7c, and low levels of the cyclic fatty acids 17:0 cyclo and 19:0 cyclo ω8c, suggesting that their physiological condition after 24 h cultivation at 35°C is younger compared to other *B. cepacia* complex bacteria. This allows a clear differentiation between *B. anthina* and *B. cepacia* genomovars I and III, which are otherwise very similar. The same is true, but to a lesser extent, for *B. ambifaria*.

3.8. Biochemical characterisation

Biochemical characteristics were determined for 16 *B. anthina* isolates (isolates R-131, R-4183 and R-16022 were not included). Growth was observed at 30 and at 37°C, but not at 5°C. Growth at 42°C was strain dependent (when present, growth was poor). No pigmented strains were detected, nor was a melanin-like pigment produced on tyrosine agar. Growth on BCSA and MacConkey agar and Simmons citrate agar was observed, and there was no haemolysis on sheep blood agar.

Oxidase activity (slow reaction), and oxidation of glucose, lactose, maltose, and xylose were present in all isolates investigated. Ornithine decarboxylase and liquefaction of gelatin were absent. Hydrolysis of aesculin, nitrate reduction, β-galactosidase and lysine decarboxylase activity, and oxidation of sucrose and adonitol are strain dependent.

The API identification scores (as '*B. cepacia*') were 1067577 (three isolates), excellent identification; 1067777 (nine isolates) very good identification; 1047577 (one isolate), very good identification; 1467777 (one isolate) very good identification; and 0047577 (two isolates) low discrimination.

Two characteristics were particularly helpful to differentiate *B. anthina* from most other *B. cepacia* complex isolates. On BCSA agar, the isolates turned the medium alkaline (pink colour production), which is most unusual for organisms capable of utilising sucrose. In addition, the colony colour and morphology would be best described as 'creamy' in colour and texture whereas most other *B. cepacia* complex isolates were typically grey, and moist or dry (depending on genomovar).

4. Discussion

In an on-going multicenter effort to improve the diagnosis of *B. cepacia* complex infections by studying the taxonomic relationships of large collections of isolates obtained from various ecological niches, we discovered 19 isolates with whole-cell protein profiles resembling those of *B. cepacia* genomovars I and III, and *B. ambifaria* isolates. These isolates could however not be assigned unequivocally to one of these established genomovars within the complex and reacted negative in the *recA*-based PCR assays for the identification of *B. cepacia* genomovars I and III, and *B. ambifaria*. Subsequent analysis by *recA* RFLP revealed two distinct restriction profiles (Fig. 1) that were different from those established in previous studies [6,7]. Analysis of the same isolates by means of AFLP

strongly suggested that they represented a single genomic species [14]. The DNA–DNA hybridisation values among three of these isolates generated the typical high (>70%) hybridisation levels found within *B. cepacia* complex genomovars and the characteristic intermediate to low hybridisation levels (30–60%) towards representatives of the other *B. cepacia* complex genomovars [2] (Table 2). Comparative sequence analysis of the 16S rDNA gene of strain R-4183 confirmed its assignment to the *B. cepacia* complex. The DNA base ratio of about 66 mol% was also within the range of the *B. cepacia* complex. These data unambiguously indicated that the 19 isolates represented a novel *B. cepacia* complex genomovar for which we propose the name *B. anthina*. The specific epithet is derived from the Greek adjective *anthinos* (hence, N.L. adj. *anthinus*), meaning 'from the garden' or 'from flowers' (referring to the origin of the majority of the isolates in the present collection). The type strain is LMG 20980 (=CCUG 46047) which was isolated in 1997 from the rhizosphere of a house-plant in Nashville (USA).

The identification of *B. anthina*, and particularly its differentiation from *B. cepacia* genomovars I and III, using previously established PCR tests proved not possible. However, as demonstrated in the present study, *recA* RFLP analysis and the newly developed *recA*-based PCR assay provide a simple means to identify this organism. Using recently developed rRNA-based PCR assays [9], *B. anthina* strains could be distinguished from *B. multivorans*, *B. vietnamiensis* and *B. cepacia* genomovar VI, but not from the other members of the *B. cepacia* complex. Biochemically, these isolates were difficult to distinguish from *B. cepacia* genomovars I and III. Characteristics useful to separate *B. anthina* from *B. cepacia* genomovar I and III isolates are primarily the alkaline reaction (pink colour change) on BCSA agar, and the distinctive creamy colony morphology [21]. Other methods that facilitate identification of these isolates to the genomovar level include AFLP fingerprinting, DNA–DNA hybridisation (Table 2) and *recA* gene sequence analysis, but these techniques are primarily research tools. Analyses of the cellular fatty acid components demonstrated that the whole-cell fatty acid profiles of *B. anthina* isolates were similar to those of *B. ambifaria* isolates. However, this approach was particularly useful to separate *B. anthina* from *B. cepacia* genomovars I and III.

Most of the present *B. anthina* strains have been isolated from the rhizosphere soil of garden flowers and houseplants, and from sputum samples of cystic fibrosis patients. The presence of these bacteria in soil of various flowers and green plants, suggests that this can serve as a reservoir for infections. However, during the past years, several thousands of cystic fibrosis-related isolates have been stored and examined in various national reference or referral centres (see http://go.to/cepacia), and thus far only few human *B. anthina* isolates have been collected. One of the isolates (R-16022) was recently described by LiPuma et al. [22] as a strain with 'indeterminate' genomovar status. In the latter study, only one out of 606 *B. cepacia* complex infected cystic fibrosis patients carried *B. anthina*. At present, this patient has been chronically colonised for a period of two years (J.J. LiPuma, unpublished information).

An evaluation of published DNA–DNA hybridisation [3,23] and 16S rDNA sequence [23] data for *B. pyrrocinia*, a soil bacterium described in the 1960s [24], revealed hybridisation and similarity levels as reported between other *B. cepacia* complex bacteria. This was confirmed in the present study by the analysis of the *recA* gene sequence which again revealed values similar to those observed between *B. cepacia* complex bacteria. This was also substantiated by the results of the *recA* RFLP technique (Fig. 1), as the primers used for the initial amplification of the *recA* gene were chosen such that they were specific for *B. cepacia* complex bacteria [7]. Altogether, these data indicate that *B. pyrrocinia* should be considered a ninth genomovar within the *B. cepacia* complex and highlights additional sources of identification problems. Our unpublished observations (J.J. LiPuma, E. Mahenthiralingam, and P. Vandamme) indicated that *B. pyrrocinia* indeed accounts for a considerable percentage of the putative *B. cepacia* complex that cannot be identified to the genomovar level. A full taxonomic characterisation of these cystic fibrosis-related and environmental isolates will be published elsewhere.

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