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Burkholderia cepacia Genomovar III Is a Common Plant-Associated Bacterium

JACQUES BALANDREAU,[1]* VERONIQUE VIALLARD,[1] BENOIT COURNOYER,[1] TOM COENYE,[2] SEVERINE LAEVENS,[2] AND PETER VANDAMME[2]†

Ecologie Microbienne, UMR 5557 CNRS-Université Claude Bernard Lyon I, Villeurbanne, France,[1] and Laboratorium voor Microbiologie, Universiteit Gent, B-9000 Ghent, Belgium[2]

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A polyphasic taxonomic study involving DNA-DNA hybridization, whole-cell protein electrophoresis, and 16S ribosomal DNA sequence analysis revealed that a group of *Burkholderia cepacia*-like organisms isolated from the rhizosphere or tissues of maize, wheat, and lupine belong to *B. cepacia* genomovar III, a genomic species associated with "cepacia syndrome" in cystic fibrosis patients. The present study also revealed considerable protein electrophoretic heterogeneity within this species and demonstrated that the *B. cepacia* complex consists of two independent phylogenetic lineages.

In a survey of nonnative plant rhizosphere bacteria conducted in La Côte Saint André (France) with maize and in Kapunda (South Australia, Australia) with wheat, high levels of two groups of *Burkholderia* strains were found. The first group was characterized by using a polyphasic approach and formed a new taxon, *Burkholderia graminis* (15). Strains of the second group (designated phenon B) were found to be closely related to the *Burkholderia cepacia* complex; large numbers of these strains were present on roots, and more recently, new isolates were also obtained from inside the tissues of wheat and lupine in Kapunda (Table 1). Here, characterization of this taxonomic group was revisited by including reference strains of the *B. cepacia* complex in DNA-DNA hybridization, whole-cell protein electrophoretic, and 16S ribosomal DNA (rDNA) sequence analyses.

Total DNA-DNA hybridization analyses were performed by using two methods, one involving tritiated reference DNAs (Table 2) and one involving photobiotin-labeled probes (Table 3). In a preliminary study, the two methods showed good correlation. For instance, the levels of hybridization of strain AUS 27 DNA with DNA of strain LMG 12614 were 65% when tritiated DNA was used and 63% when photobiotin-labeled DNA was used.

In the first experiments we used tritiated reference DNAs of eight isolates, including two rhizosphere isolates (AUS 27 and C3B1M), one recent cystic fibrosis isolate (1–36), and reference strains of *B. cepacia* genomovar I (ATCC 25416^T), *Burkholderia vietnamiensis* (LMG 10929^T), *Burkholderia multivorans* (1–45), *Burkholderia pyrrocinia* (ATCC 15958^T), and *B. graminis* (ATCC 700544^T). DNAs from 15 of our rhizosphere isolates, reference strains belonging to *B. cepacia* genomovar I (ATCC 25416^T and LMG 6964) and genomovar III (LMG 12614, LMG 16661, and LMG 6988), and *B. pyrrocinia* (ATCC 15958^T), and three recent cystic fibrosis isolates (strains 751, 1–36, and 1–47) were hybridized with these radioactively labeled DNAs. When hybridized with labeled DNA of strain AUS 27, all rhizosphere isolates except m35b showed levels of DNA-DNA hybridization greater than 65% and differences in melting temperatures (ΔT_m values) less than 5°C, indicating that they belong to the same genomic species (12). When they were hybridized with labeled DNA of strain C3B1M, slightly lower values (as low as 61%) were obtained, indicating a certain degree of genomic heterogeneity in this species. Strain m35b showed significant but low levels of hybridization (40 to 48%) with all reference strains and thus does not belong to any of the genomovars examined. The possibility that this strain could belong to *Burkholderia stabilis* was not eliminated and will be tested further. *B. cepacia* genomovar III reference strains exhibited levels of hybridization of 58 to 76% with labeled DNA of strain AUS 27, indicating that the rhizosphere isolates belong to *B. cepacia* genomovar III. Reference strains of the other *B. cepacia* genomovars and of *B. pyrrocinia* exhibited levels of DNA-DNA hybridization between 39 and 60%, values which are in complete agreement with values reported previously (13). The levels of hybridization with DNA of the *B. graminis* type strain were much lower (13 to 15%). The three recent cystic fibrosis isolates (strains 1–36, 751, and 1–47) showed levels of hybridization between 63 and 76% with AUS 27 DNA with ΔT_m values less than 5°C. These data show unambiguously that these three isolates also belong to the same genomic species as AUS 27 (i.e., *B. cepacia* genomovar III).

A second group of DNA-DNA hybridization experiments (Table 3) was performed in order to substantiate the relationships among *B. cepacia* genomovar III strains. In addition, a representative endophytic isolate was included. Values between 63 and 82% were obtained, which confirmed that all of these isolates belong to a single genomic species.

Whole-cell protein extracts were prepared from 48-h cultures of all of the *B. cepacia* genomovar III strains and several additional endophytic isolates. Data for the reference strains were obtained from previous studies (3a, 13, 14). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses were performed as described previously (13). Protein profiles were analyzed by using the GelCompar software package (version 4.2; Applied Maths, Kortrijk, Belgium). Levels of similarity between the pat-

* Corresponding author. Mailing address: Ecologie Microbienne, UMR 5557 CNRS-Université Claude Bernard Lyon I, Bât. 741, 43 Boulevard du 11 Novembre, 69622 Villeurbanne (cedex), France. Phone: 04 72 44 82 00. Fax: 04 72 43 12 23. E-mail: balandreau@univ-lyon1.fr.

† Present address: Laboratorium voor Farmaceutische Microbiologie, Universiteit Gent, B-9000 Ghent, Belgium.

TABLE 1. Strains used in this study

Strain	Other designation[a]	Reference or source	Ecology
Rhizosphere strains			
AUS 13	LMG 19240	15	Continuous wheat plot (Kapunda, Australia)
AUS 26	LMG 19247	15	Continuous wheat plot (Kapunda, Australia)
AUS 27	LMG 19238	15	Continuous wheat plot (Kapunda, Australia)
AUS 30	LMG 19245	15	Continuous wheat plot (Kapunda, Australia)
AUS 12	LMG 19243	15	Wheat pasture rotation (Kapunda, Australia)
AUS 29	LMG 19246	15	Wheat pasture rotation (Kapunda, Australia)
AUS 31	LMG 19244	15	Wheat pasture rotation (Kapunda, Australia)
AUS 32	LMG 19248	15	Wheat pasture rotation (Kapunda, Australia)
AUS 34	LMG 19242	15	Wheat pasture rotation (Kapunda, Australia)
AUS 36	LMG 19239	15	Wheat pasture rotation (Kapunda, Australia)
AUS 37	LMG 19241	15	Wheat pasture rotation (Kapunda, Australia)
C3B1M	R-13371	15	Maize rhizosphere (Côte Saint André, France)
m32	R-13369	15	Maize rhizosphere (Côte Saint André, France)
m35b	R-13370	15	Maize rhizosphere (Côte Saint André, France)
Endophytes			
WS11.7	LMG 19232	K. Ophel-Keller	Wheat shoot endophyte (Kapunda, Australia)
WS9.1	LMG 19231	K. Ophel-Keller	Wheat shoot endophyte (Kapunda, Australia)
WR2.5	LMG 19237	K. Ophel-Keller	Wheat root endophyte (Kapunda, Australia)
WR2.6	LMG 19230	K. Ophel-Keller	Wheat root endophyte (Kapunda, Australia)
LS2.4	LMG 19233	K. Ophel-Keller	Lupine shoot endophyte (Kapunda, Australia)
LS12.9	LMG 19234	K. Ophel-Keller	Lupine shoot endophyte (Kapunda, Australia)
LR1.4	LMG 19235	K. Ophel Keller	Lupine root endophyte (Kapunda, Australia)
LR14.9	LMG 19236	K. Ophel-Keller	Lupine root endophyte (Kapunda, Australia)
Clinical isolates			
1–36	R-9061	C. Segonds	Cystic fibrosis patient (Clermont-Ferrand, France)
751	R-11750	C. Segonds	Non-cystic fibrosis hemoculture, (Bordeaux, France)
Reference strains			
B. cepacia (I)[b]	ATCC 25416[Tc]	1	Onion sour skin
B. cepacia (I)	LMG 6964	Haywood, 1965	Tomato
B. multivorans (II)	1–45	11	Cystic fibrosis patient (France)
B. cepacia (III)	LMG 13053	3	Cystic fibrosis sputum (Belgium)
B. cepacia (III)	LMG 16661	13	Cystic fibrosis sputum (United Kingdom)
B. cepacia (III)	LMG 12614	13	Cystic fibrosis sputum (United Kingdom)
B. cepacia (III)	LMG 12615	13	Cystic fibrosis sputum (United Kingdom)
B. cepacia (III)	LMG 16659	13	Cystic fibrosis sputum (United Kingdom)
B. cepacia (III)	LMG 6988		Leg wound, (Sweden, 1972)
B. vietnamiensis (V)	LMG 10929[T]	5	Rice rhizosphere
B. pyrrocinia	ATCC 15958[T]	7	Soil
B. graminis	ATCC 700544[T]	15	Maize rhizosphere

[a] ATCC, American Type Culture Collection, Manassas, Va.; LMG, Culture Collection, Laboratory of Microbiology, State University of Ghent, Ghent, Belgium.
[b] The roman numerals in parentheses indicate genomovars.
[c] T = type strain.

terns were computed by using the Pearson product moment correlation coefficient and were expressed as percentages of similarity for convenience. Considerable heterogeneity was apparent, and the strains grouped into two main protein electrophoretic clusters comprising the endophytic isolates (cluster 1) and all of the Australian rhizosphere isolates except isolate AUS 27 (cluster 2), three small clusters comprising two isolates each (clusters 3 to 5), and several isolates with distinct positions in the dendrogram (Fig. 1). Cluster 3 comprises two reference strains (LMG 12614 and LMG 12615) and represents cluster xii described previously (13). Cluster 4 also comprises two reference strains (LMG 13053 and LMG 16661) and corresponds to cluster xi described previously (13). Finally, cluster 5 comprises two French rhizosphere isolates (C3B1M and m32). In spite of this protein electrophoretic heterogeneity, DNA-DNA hybridization data (Tables 2 and 3) demonstrated that all of the isolates shown in Fig. 1 represent a single genomic species; most of the isolates belong to clusters 2 through 5, and one strain, strain W11.7, is a cluster 1 reference isolate. The protein electrophoretic homogeneity of the other cluster 1 isolates indicates that they are members of the genomic species as well.

To investigate the phyletic relatedness of the genomovar III isolates, almost complete 16S rDNA sequences of the following two endophytic isolates and two genomovar III strains were obtained: LMG 12615, LMG 12614, LS2.4 and WS11.7. These sequences and selected GenBank 16S rDNA sequences of 19 representative strains of the *B. cepacia* complex were aligned. A total of 1351 16S rDNA sites were then selected, and sites involving indels (insertions or deletions) were excluded from further analysis. Evolutionary distances (representing the percentages of transversion type differences between sequence pairs) were computed by the method of Jukes and Cantor (9). A phylogenetic tree was inferred by using the neighbor-joining method (10), and bootstrapping was performed (9). This phylo-

TABLE 2. Levels of total DNA hybridization with radioactively labeled DNAs of eight reference strains

Source of unlabeled DNA	% Hybridization with labeled DNA of:							
	AUS 27	C3B1M	1–36	*B. cepacia* ATCC 25416^T	*B. vietnamiensis* LMG 10929^T	*B. multivorans* 1–45	*B. pyrrocinia* ATCC 15958^T	*B. graminis* ATCC 700544^T
AUS 27	100	69 (3.2)[a]	60 (4.3)	51	43 (10)	50	60	15
AUS 37	100	71		50				
AUS 14	100			36	40	39	55	
AUS 26	99							
AUS 32	98			48				
AUS 34	92	66		45				
AUS 13	92	61		46				
AUS 30	91	64		44			53	15
AUS 31	90 (2.8)			41		39		
AUS 12	90							
AUS 29	90							
AUS 36	89							
C3B1M	70 (3.8)	100	63 (3.1)	57	44 (5.6)	40	56	13
m32	75	98						
PHQB17	65	76						15
751	72 (4.2)			43				
LMG 12614	65 (4.2)					42		
LMG 6988	58 (4.9)					35		
1–36	63 (4.6)		100	46	38 (7.3)	44	50	
LMG 16661	76		75 (0.8)	45	41 (5.8)	48		
1–47	66 (4.8)		68 (4)	48	44 (9)	40	49	
ATCC 25416^T	56 (6.5)	52 (5.9)	52 (6.5)	100	39 (8)	41	53	
LMG 6964	56 (6.4)	54 (7.8)	55 (6.8)	66	43	44	55	
ATCC 15958^T	62 (9.2)	55 (9.6)	62 (6.2)	51	43 (6.7)	41	100	
m35b	47	48	44	40	48	43	46	

[a] The values in parentheses are ΔT_m values (in degrees Centigrade).

genetic analysis divided the 16S rDNA sequences of the *B. cepacia* complex into two major clusters: (i) a lineage containing the *B. vietnamiensis* (genomovar V), *B. multivorans* (genomovar II), and LMG 18941 (genomovar VI) DNA sequences, and (ii) a group containing the *B. stabilis* (genomovar IV), *B. pyrrocinia, B. cepacia* genomovar I (ATCC 25416^T and ATCC 17759), *B. cepacia* genomovar III (LMG 12614, LMG 12615, WS11.7, LS2.4, and C3B1M), and unclassified strain m35b sequences. This division was strongly supported by 98% of the bootstrap replicates and should be obtained with any other phylogenetic markers that match classical bacterial evolutionary patterns.

Public health implications. Our 14 isolates represent environmental niches ranging from the rhizosphere to the inner tissues of wheat, lupine, and maize and were obtained in France and in South Australia. These plants are cultivated all over the world, and it is likely that our isolates represent very common bacteria.

TABLE 3. DNA-DNA hybridization of environmental strains with genomovar III representatives

Source of unlabeled DNA	% Hybridization with labeled DNA of:				
	LMG 12614	LMG 13053	LMG 16659	WS11.7	AUS 27
LMG 12614	100				
LMG 13053	79	100			
LMG 16659	67	91	100		
WS11.7	68	82	71	100	
AUS 27	63	82	71	99	100

[a] For hybridization we used photobiotin-labeled probes at 50°C. Each value is the average of the values from at least two hybridization experiments.

We demonstrate here that a significant proportion of these maize-, wheat-, and lupine- associated bacteria are actually members of *B. cepacia* genomovar III. This *B. cepacia* genomovar is particularly relevant for cystic fibrosis as most strains associated with the "cepacia syndrome" belong to it. This syndrome is characterized by a dramatic necrotizing pneumonia that results in rapid death of the patient (6, 8). Recent deadly outbreaks which occurred in many parts of the world have been attributed to strains of genomovar III, suggesting that high transmissibility could be a characteristic of *B. cepacia* genomovar III in the *B. cepacia* complex (13). It has been suggested that the environment is a source of new isolates, but so far no clear evidence of this has been obtained. Attempts to recover *B. cepacia* isolates similar to clinical isolates from soils and other environmental sites have failed, most likely because of the selective agents used (antibiotics). The environmental strains studied here were isolated by using the PCAT medium (2), whose selective power is based solely on the metabolism of unusual sources of carbon and nitrogen (viz., azelaic acid and tryptamine). Thus, our Australian and French isolates represent the first collection of genuinely environmental *B. cepacia* genomovar III strains. This collection opens the way for comparisons of closely related strains of environmental and clinical origin, which could provide some clue about the properties acquired by hospital-adapted strains and their pathogenicity characteristics or genes. It could also help refine strategies for avoiding acquisition of new *Burkholderia* strains in cystic fibrosis treatment centers. Most of the soil isolates have been deposited in the BCCM/LMG Bacteria Collection (University of Ghent, Ghent, Belgium) under the accession numbers shown in Table 1.

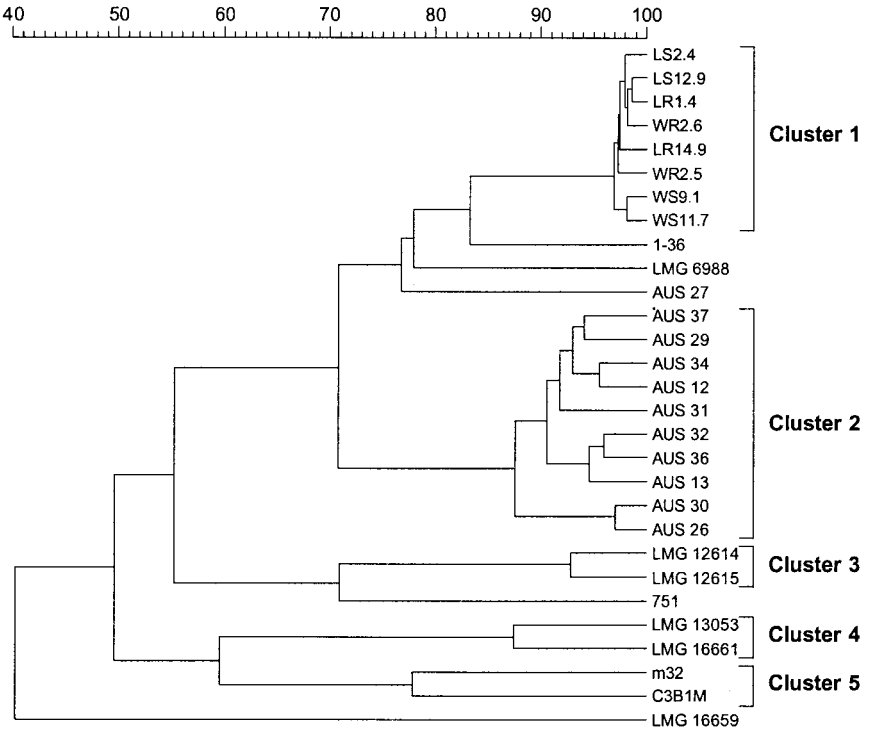

FIG. 1. Dendrogram derived from unweighed pair group average linkage of correlation coefficients (expressed for convenience as percentages of similarity) for the whole-cell protein patterns of the *B. cepacia* genomovar III strains studied.

Nucleotide sequence accession numbers. The nucleotide sequences of strains LMG 12615, LMG 12614, LS2.4, and WS11.7 have been deposited in the GenBank database under accession numbers AF311969, AF311970, AF311971, and AF311972, respectively.

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