A rhizospheric Burkholderia cepacia complex population: genotypic and phenotypic diversity of Burkholderia cenocepacia and Burkholderia ambifaria

Claudia Dalmastri a, Alessia Fiore a, Chiara Alisi b, Annamaria Bevivino a, Silvia Tabacchioni a, Giovanni Giuliano a, Anna Rosa Sprocati b, Lia Segre b, Eshwar Mahenthiralingam c, Luigi Chiarini a,*, Peter Vandamme d

a ENEA (Ente Nazionale per le Nuove Tecnologie, l’Energia e l’Ambiente) C.R. Casaccia, Unità Biotecnologie e Protezione della Salute e degli Ecosistemi, Via Anguillaresse 301, 00060 S. Maria di Galeria, Rome, Italy
b ENEA (Ente Nazionale per le Nuove Tecnologie, l’Energia e l’Ambiente) C.R. Casaccia, Unità Protezione dell’Ambiente, 00060 Rome, Italy
c Cardiff School of Biosciences, Cardiff University, Cardiff CFI 3TL, UK
d Laboratory for Microbiology, University of Gent, B-9000 Gent, Belgium

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Abstract

The Burkholderia cepacia ‘complex’ (Bcc) presently comprises nine species and genomovars. In order to acquire a better comprehension of the species and genomovar distribution and of the genetic diversity among environmental Bcc bacteria, a natural population of 60 bacterial isolates recovered from the rhizosphere of maize and belonging to the Bcc has been characterised to assess the exact taxonomic position, the genetic polymorphism and the metabolic profiles of isolates. The identification of the different species and genomovars was accomplished by a combination of techniques including sodium dodecyl sulfate–polyacrylamide gel electrophoresis of whole-cell proteins and recA-based restriction fragment length polymorphism analyses. The genetic diversity among Bcc isolates was analysed by means of the random amplified polymorphic DNA and amplified fragment length polymorphism techniques; the analysis of molecular variance method was applied to estimate the genetic differences among the various species and genomovars identified within the bacterial population. Metabolic profiles based on carbon source utilisation were obtained by means of the Biolog GN assay and analysed by means of cluster analysis. Forty-four strains were identified as B. ambifaria, 11 as B. cenocepacia recA lineage III-B, four as B. pyrrocinia, and one as B. cepacia genomovar I. Marked genetic differences were observed between B. cenocepacia and B. ambifaria, whereas limited differences were found between B. pyrrocinia and B. ambifaria and between B. pyrrocinia and B. cenocepacia. No significant differences (P < 0.05) were observed between the mean genetic distances of isolates belonging to B. cenocepacia, B. ambifaria, and B. pyrrocinia. Phenotypic analyses revealed that all isolates tested were able to utilise more than 75% of substrates. The highest variability in the number of utilised substrates was found among B. cenocepacia isolates, whereas the lowest was found among B. ambifaria isolates. Cluster analysis of metabolic profiles revealed pronounced differences between B. cenocepacia and B. ambifaria; in contrast, B. pyrrocinia could not be clearly separated either from B. cenocepacia or from B. ambifaria.

Keywords: Burkholderia cepacia complex; Burkholderia ambifaria; Burkholderia cenocepacia; Phenotypic diversity; Genetic polymorphism; Rhizosphere

1. Introduction

Recently, ‘Burkholderia cepacia’ has attracted consider-
and its innate resistance to a wide range of antimicrobial agents [6–8]. Furthermore, it has become increasingly evident that organisms identified as ‘B. cepacia’ appear to be very heterogeneous and constitute a ‘complex’ of at least nine phenotypically similar species or genomovars, i.e. B. cepacia genomovar I, B. multivorans, B. cenocepacia, B. stabilis, B. vietnamensis, B. cepacia genomovar VI, B. ambifaria, B. anthina, and B. pyrrocinia, which can be differentiated on the basis of molecular and biochemical tests [9–16].

Since the B. cepacia complex (Bcc) comprises multiple genomovars and species, a priority task facing microbiologists is the exact definition of the pathogenicity of the different genomovars and species found in environmental samples. Govan et al. [17] described a clinical B. cepacia genomovar I isolate that was identical to an environmental isolate. More recently, LiPuma et al. [18] have identified from agricultural soil a B. cenocepacia strain that has been frequently isolated from CF patients in the USA, showing that, at least as far as B. cenocepacia is concerned, human pathogenic strains are not necessarily distinct from environmental ones. However, to approach this question in a more comprehensive way including all genomovars and species of the Bcc, preliminary information about the species and genomovar composition of Bcc populations in different natural habitats as well as about their genetic and phenotypic diversity is urgently needed. In fact, so far, very few studies have dealt with the distribution of species belonging to the Bcc in the natural environment [19,20]. According to these, B. cenocepacia and B. ambifaria seem to predominate in environmental samples, in particular in the maize rhizosphere, whereas B. cepacia genomovar I and B. pyrrocinia are less frequently recovered. In contrast, clinical isolates have been extensively analysed with respect to their genomovar status [8,20–23]. Most clinical strains have been assigned to B. cenocepacia and B. multivorans, which are considered the most virulent species, whereas the remaining species and genomovars are very sparsely represented in clinical samples. Thus, an extensive comparison between species composition in natural habitats and clinical samples may help to better define the biological origins of strains infecting CF patients and the risks posed by strains likely to be encountered in the natural environment and those developed for commercial use as biocontrol and bioremediation agents. Furthermore, so far, no data concerning the genetic or phenotypic diversity of environmental populations of the different genomovars and species constituting the Bcc are available, with the only exception of a recent work by Coenye et al. [24] on B. cenocepacia. Several other studies have dealt with the genetic polymorphism and metabolic diversity of natural populations of ‘B. cepacia’ but mainly at the complex level [25–29].

In particular, Dalmastri et al. [26] investigated the genetic polymorphism of different rhizospheric Bcc populations and compared their genetic structure. One of these bacterial populations, named MVPC1, was further investigated in the present study in order to determine (i) the species composition, and (ii) the genetic polymorphism and phenotypic diversity of the different species and genomovars present in this population. The identification of the different species and genomovars was accomplished by a combination of techniques including sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins and recA-based restriction fragment length polymorphism (RFLP) analyses and polymerase chain reaction (PCR) assays. The genetic diversity among Bcc isolates was analysed by means of the random amplified polymorphic DNA (RAPD) technique and by the amplified length polymorphism (AFLP) technique. Both techniques are useful to highlight variability among strains belonging to closely related taxa, such as genomovars and species comprising the Bcc. The RAPD technique has been mostly used to investigate genetic polymorphism in bacterial isolates belonging to the same species or to closely related genomic species, whereas the AFLP technique has also been applied to assess differences among species. The analysis of molecular variance (AMOVA) method [30] was applied to estimate the genetic differences among the various species and genomovars identified within the bacterial population. The phenotypic diversity among Bcc isolates was investigated by assessing the ability of bacterial isolates to utilise 95 selected carbon sources by means of the Biolog GN assay. The metabolic profiles obtained were subjected to cluster analysis to evaluate similarities among bacterial isolates.

2. Materials and methods

2.1. Isolation of bacterial strains

Bacterial isolates were obtained from the rhizosphere of Zea mays (cv Airone, Agra) cultivated in a field located at Pieve d’Olmi (Italy) [26]. Maize has been cultivated for at least 5 years in this field. Four plants were collected after 40 days of plant growth. Roots were excised from plants and loosely adhering soil was removed. Afterwards roots were weighed and cut into small pieces (0.2–0.7 cm). Two grams of each root sample were crushed in a sterile mortar and resuspended in phosphate-buffered saline (Flow Laboratories). Serial dilutions of these suspensions were plated onto the selective medium PCAT [31] and incubated for 48 h at 28°C to estimate colony-forming units (cfu) of micro-organisms belonging to Bcc. As previously reported by Dalmastri et al. [26], Bcc population density amounted to 6.20 ± 0.22 log cfu g⁻¹ root fresh weight. Bacterial isolations were performed with samples diluted to the same level from PCAT plates with 50–100 colonies. Colonies showing the characteristic B. cepacia morphology were purified through serial transfers on the same medium and cryopreserved at −80°C in 30% glycerol.
2.2. Assignment of bacterial isolates to the Bcc

To identify Bcc strains, amplification of 16S rDNA was performed on 2 µl of lysate cell suspension obtained from a single colony from each isolate, according to the procedure described by Di Cello et al. [25]. Restriction analysis of the amplified 16S rDNAs (ARDRA) with the enzyme Alu I made it possible to assign to the Bcc the isolates showing the ARDRA pattern typical of this bacterial species.

2.3. Assignment of Bcc isolates to species and genomovars of the ‘complex’

Bacterial isolates were identified at the species level by means of SDS-PAGE of whole-cell proteins and recA-based RFLP analyses as described by Mahenthiralingam et al. [32] and Fiore et al. [19]. Numerical analyses of the banding patterns were performed using the BioNumerics software package (Applied Maths). The identity of B. pyrocinia was confirmed by DNA-DNA hybridisation experiments (Vandamme et al., unpublished results).

2.4. RAPD fingerprinting

Amplification reactions of genomic DNAs were performed on 2 µl of lysate cell suspension obtained from a single colony from each isolate as previously described [25]. Four distinct reactions were performed using four different 10-mer primers (AP5, AP12, CD1 and 1253) having different GC contents (Table 1). These primers were chosen on the basis of their ability to put in evidence differences in genetic polymorphism of Bcc populations [25,27], own data).

The amplification patterns were analysed both visually and with the aid of computer analysis (Kodak 1D Image Analysis Software, NY, USA). For each isolate, patterns obtained with each primer were merged together and considered as its genetic fingerprint. Different isolates exhibiting genetic fingerprints differing by no more than two bands were considered genetically related and, thus, belonged to the same RAPD type. Isolates producing genetic fingerprints that did not match others within the bacterial population were designated as unique.

2.5. AFLP fingerprinting

The preparation of template DNA was performed as described by Jiang et al. [33] with some modifications. One microgram of chromosomal DNA was digested with the restriction enzyme TaqI at 65°C for 1 h. Then, the second restriction enzyme EcoRI, the adapters to a final concentration of 0.1 µM for EcoRI and 1 µM for TaqI, and T4 DNA ligase were added; the second digestion and the ligation reactions were performed simultaneously at 37°C for 3 h.

For selective amplification of restriction fragments, EcoRI primer E1sG and TaqI primer T1sG were used (Table 1). The PCR reaction was performed on 0.5 µl of template DNA (about 10 ng) in 20 µl of Taq Pharmacia buffer containing 200 µM of each dNTP, 250 pg µl⁻¹ labelled primer E1sG, 1.5 ng µl⁻¹ primer T1sG and 0.5 U Taq polymerase (Pharmacia). The PCR was performed with a Perkin Elmer 9600 thermal cycler employing the following programme: 2 min at 72°C and then 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 2 min.

Three microliters of each amplified product were dried by using speed vacuum for 10 min, resuspended in 2 µl of loading buffer composed of bromophenol blue and deionised formamide (1:5 v/v), heated for 3 min at 95°C to denature the DNA and quick-chilled on ice. Two microliters of GENESCAN-2500 size standard, double-stranded DNA fragments, sized between 37 and 14079 bp, with one of the strands labelled with the fluorescent dye ROX (PE Applied Biosystems), were added to each sample. Amplified fragments were separated on a 5.33% (w/v) denaturing polyacrylamide gel containing 41.6% (w/v) urea in 1 x TBE buffer, on an Applied Biosystems 373 DNA sequencer. Gels were run at 2000 V for 7 h. During the run, the data were captured using the ABI Prism Sequencing program 2.12 (PE Applied Biosystems). Lane tracking and normalisation was performed using the GENESCAN 672 software 1.2.2 (PE Applied Biosystems).

2.6. Analysis of RAPD type diversity

The evenness index (E) was used to analyse the RAPD type diversity of the different Bcc species. This index describes the distribution of RAPD type abundance [34].

2.7. Analysis of molecular variance of PCR amplification products and calculation of mean genetic distance index

The vector of the presence or absence of RAPD and AFLP markers (1 for the presence or 0 for the absence of each band on gels) for each strain was used to compute the measure of the genetic distance for each pair of strains.

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Table 1: Primers used in RAPD and AFLP analyses

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>GC content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP5</td>
<td>5'-TCGGCGCTGGCG-3'</td>
<td>80</td>
</tr>
<tr>
<td>AP12</td>
<td>5'-CGGCCCCCTGCG-3'</td>
<td>90</td>
</tr>
<tr>
<td>CD1</td>
<td>5'-GTTGTCGCGCGC-3'</td>
<td>70</td>
</tr>
<tr>
<td>L235</td>
<td>5'-GAATCCCTGAC-3'</td>
<td>60</td>
</tr>
<tr>
<td>E1sG</td>
<td>5'-GATGCGTACCAGCTGAGCG-3'</td>
<td>53</td>
</tr>
<tr>
<td>T1sG</td>
<td>5'-GATGCGTACCAGCGAG-3'</td>
<td>59</td>
</tr>
</tbody>
</table>

*The E1sG primer was labelled at its 5' end with 5-carboxyfluorescein, a phospho-ramidite dye (Pharmacia).
*Selective bases at 3' ends are underlined.
The Euclidean metric measurement (E) of Excoffier et al. [30], as defined by Huff et al. [35], was used: \( E = e_{xy}^2 = n(1-2n_{xy}/2n) \), where \( 2n_{xy} \) is the number of markers shared by two individuals and \( n \) is the total number of polymorphic sites.

The genetic structure of bacterial populations was investigated by an analysis of the variance framework. Information on RAPD and AFLP pattern divergence was incorporated into an analysis of variance format, derived from a matrix of squared distances among all pairs of RAPD and AFLP patterns. This AMOVA produces estimates of variance components reflecting the correlation of RAPD and AFLP pattern diversity at different levels of hierarchical subdivision. The significance of the variance components is tested using a permutational approach, eliminating the normality assumption that is conventional for analysis of variance but inappropriate for molecular data.

Internal variability of each population was calculated using the Euclidean distances between all possible combinations of RAPD and AFLP patterns taken in pairs. The mean number of differences or mean genetic distance (GD) between all pairs of strains of a single population was considered as an estimation of its molecular diversity.

The AMOVA and the calculation of the mean GD were performed using Arlequin ver. 1.1 software [36].

2.8. Metabolic fingerprinting

Substrate utilisation profiles of 27 MVPC1 isolates were generated using Biolog GN plates (Biolog, Hayward, CA, USA). Colonies grown on Biolog universal growth agar (1 day old) were harvested and processed according to the manufacturer’s instruction: the cell suspension was adjusted to 40% of transmittance (equal to 0.35 OD\(_{600}\)) and added to the GN plates (150 \( \mu \)l per well). The plates were incubated at 28°C in the dark. Three replica plates were prepared for each strain. Purple colour formation in the GN plates was measured by the Biolog Microstation Reader after 4, 8, 12, 24, 48, 72 and 96 h of incubation.

2.9. Analysis of substrate utilisation profiles

Utilisation of the different Biolog substrates by bacterial isolates was assessed either by the presence or absence of colour development or by measuring the highest OD\(_{590}\) values reached for each substrate during the incubation period. Data were analysed by cluster analysis using a partitioning around medoids procedure [37,38]. An attempt was made to analyse the complete data set, i.e. all 95 substrates were simultaneously included in the clustering process. However, numerically unstable results were obtained as the number of substrates (playing the role of variables) exceeded the number of isolates (cases). The 95 substrates were then randomly divided into four groups after some data management, i.e. substrates which were not utilised by any bacterial isolate were excluded. Each set of substrates comprised 20–22 different carbon sources, and cluster analysis was performed on these four smaller data sets using S-PLUS software (S-PLUS 2000 Professional Edition for Windows, Math Soft).

3. Results

3.1. Identification of Bcc isolates

Sixty bacterial isolates belonging to the Bcc recovered from the rhizosphere of maize [26] have been characterised to assess their species status. Whole-cell protein electrophoresis was first used to confirm the identification of the isolates as Bcc (data not shown). Accurate species level identification was further achieved by recA RFLP analyses. Forty-four isolates were identified as \( B. \) \( \text{ambifaria} \) (representing two distinct RFLP types designated AE and N), 11 as \( B. \) \( \text{cenocapacia} \) (two RFLP types designated AD and I), four as \( B. \) \( \text{pyrrocinia} \) (one RFLP type designated P), and one as \( B. \) \( \text{cepacia} \) genomovar I (RFLP type E) (Table 2). Fig. 1 illustrates the recA RFLP types of the isolates examined. It is worth noting that all \( B. \) \( \text{cenocapacia} \) isolates were assigned to recA lineage III-B, as defined by Mahenthiralingam et al. [32].

3.2. RAPD fingerprinting

Amplification of genomic DNAs of the Bcc isolates with the four primers listed in Table 1 gave rise to a total of 52 bands, with dimensions ranging from 150 to 2300 bp. Each RAPD pattern was represented by eight to 28 bands (mean value, 19 bands). Each isolate showed the same amplification pattern in two independent experiments (data not shown). All isolates belonging to \( B. \) \( \text{cepacia} \) genomovar I, \( B. \) \( \text{cenocapacia} \) and \( B. \) \( \text{pyrrocinia} \) had unique RAPD fingerprints (Table 3). Among the 44 isolates be-
longing to *B. ambifaria*, 35 had unique RAPD fingerprints whereas the remaining nine isolates were clustered in three distinct RAPD types, each comprising three isolates (Table 3).

### 3.3. AFLP fingerprinting

E1sG and T1sG primers, which recognise restriction sites of enzymes EcoRI and TaqI, respectively, were used to selectively amplify restriction fragments from genomic DNA of all bacterial isolates. Amplification gave rise to a total of 168 markers with a length of 170–361 nucleotides. Each AFLP pattern was represented by 15–70 bands (mean value, 35 bands). No identical patterns were found. A subset of 20 isolates comprising representatives of all species showed the same amplification patterns in two independent experiments (data not shown).

### 3.4. Genetic variability among Bcc isolates

RAPD patterns were used to evaluate the genetic diversity of the Bcc isolates by means of the GD and *E* indices, whereas both RAPD and AFLP patterns were used to evaluate the genetic differences among the Bcc species by means of the AMOVA method. GD values, calculated from RAPD data, among isolates belonging to *B. cenocepacia*, *B. ambifaria* and *B. pyrrocinia* were 16.45, 18.31 and 16.90, respectively; no significant differences among the GDs of the three species were found (*P* > 0.05) (Table 3). Based on RAPD types, the *E* values were quite similar for *B. cenocepacia* (*E* = 1), *B. pyrrocinia* (*E* = 1), and *B. ambifaria* (*E* = 0.98) (Table 3).

Analyzing RAPD and AFLP patterns by means of the AMOVA method, we observed that, although most genetic variability in the rhizosphere population was due to differences among isolates within the population, a statistically significant percentage of the molecular variance was attributable to genetic differences among *B. cenocepacia*, *B. ambifaria* and *B. pyrrocinia* (Table 4). Interestingly, both genetic fingerprinting techniques yielded similar values of molecular variance. Analyzing the percentage of variation at a lower hierarchical level, i.e. between species taken in pairs, higher genetic differences were observed between *B. cenocepacia* and *B. ambifaria*, whereas lower genetic differences were observed between *B. pyrrocinia* and *B. ambifaria* and between *B. pyrrocinia* and *B. cenocepacia* (Table 4). It is worth noting that in the two latter cases the AFLP technique showed a higher inter-species discriminatory power than the RAPD technique, whereas no difference in discriminatory power of the two techniques was observed comparing percentages of variation between *B. cenocepacia* and *B. ambifaria*.

### 3.5. Utilisation of 95 carbon sources

A subset of 27 MVPC1 isolates were tested for the ability to utilise a variety of carbon sources. This subset comprised all *B. cepacia* genomovar I, *B. cenocepacia* and *B. pyrrocinia* isolates, and 11 *B. ambifaria* isolates showing unique RAPD fingerprints. Table 5 shows substrate utilisation by the Bcc isolates. The percentage of utilised substrates varied in the range of 76–89%, 81–84%, and 76–84% for *B. cenocepacia*, *B. ambifaria* and *B. pyrrocinia* isolates, respectively. Taken as a whole, the subset of 27 isolates showed an average utilisation of carbon sources of 80 ± 4%.

### Table 3

<table>
<thead>
<tr>
<th>B. cepacia gen. I</th>
<th>B. pyrrocinia</th>
<th>B. cenocepacia</th>
<th>B. ambifaria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of RAPD types</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Number of unique RAPD fingerprints</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Genetic distance</td>
<td>18.31 ± 4.59</td>
<td>16.45 ± 6.32</td>
<td>16.90 ± 4.01</td>
</tr>
<tr>
<td>Evenness</td>
<td>0.98</td>
<td>0.85</td>
<td>0.85</td>
</tr>
</tbody>
</table>

*Values are the mean number ± S.D. of differences between all pairs of isolates belonging to each genomovar.

*Evenness was calculated as follows: *E* = *H*/*H*<sub>max</sub>, where *H* = Σ(−*p*<sub>i</sub>log<sub>2</sub>*p*<sub>i</sub>) ( *p*<sub>i</sub> is the proportion of the *i*th RAPD type), *H*<sub>max</sub> = log<sub>2</sub>(*S*) and *S* is the total number of RAPD types and unique RAPD fingerprints.
MVPC1 isolates utilised 85 out of 95 carbon sources; a common set of 76 substrates (90% of utilised carbon sources) were metabolised by each Bcc species, except B. cepacia genovar I.

3.6. Phenotypic variability among Bcc isolates

Cluster analysis of metabolic profiles based on the presence or absence of colour development grouped bacterial isolates independently of their species assignment (data not shown). Therefore, metabolic profiles based on the highest OD590 values reached for each substrate during the incubation period were used for the cluster analysis. Cluster analysis of the complete data set comprising all 95 carbon sources and of the four smaller data sets each comprising 20–22 carbon sources yielded very similar results. In Fig. 2 are shown the results of the cluster analysis of one of the small data sets. Taken altogether the results of the cluster analysis of the four small data sets show that most B. cenocepacia and B. ambifaria isolates clustered in separate groups. In particular, most B. ambifaria isolates clustered in a single group, whereas most B. cenocepacia isolates were split into two or three groups. The four B. pyrrocinia isolates did not cluster separately from B. cenocepacia and B. ambifaria and were spread among three or four groups.

4. Discussion

Sixty bacterial isolates belonging to the Bcc recovered from the rhizosphere of maize and previously investigated by Dalmastri et al. [26] have been analysed to assess their species status and the genotypic and phenotypic diversity of the species present in this population. This is the first extensive study in which a single natural Bcc population has been subjected to both genetic and metabolic fingerprinting to assess intraspecific diversity. Forty-four isolates were identified as B. ambifaria, 11 as B. cenocepacia recA lineage III-B, four as B. pyrrocinia, and one as B. cepacia genovar I. Our results indicate, along with previous findings of Fiore et al. [19] and Bevivino et al. [20], that, among Bcc species, these are the most easily encountered in the rhizosphere of maize. These data may help to better define the relationships between environmental and clinical strains belonging to the Bcc. In fact, a preliminary step in the evaluation of the potential pathogenicity of environmental isolates of the Bcc is to

Table 4
Percentage of variation between and within species and genovars of the Bcc isolated from the rhizosphere of maize calculated by means of AMOVA

<table>
<thead>
<tr>
<th>Variance component</th>
<th>RAPD</th>
<th>AFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cenocepacia v. B. ambifaria v. B. pyrrocinia</td>
<td>7.01</td>
<td>8.28</td>
</tr>
<tr>
<td>B. cenocepacia v. B. ambifaria</td>
<td>9.02</td>
<td>9.02</td>
</tr>
<tr>
<td>B. pyrrocinia v. B. ambifaria</td>
<td>2.42</td>
<td>7.50</td>
</tr>
<tr>
<td>B. pyrrocinia v. B. cenocepacia</td>
<td>−5.83</td>
<td>2.41</td>
</tr>
<tr>
<td>Within species</td>
<td>92.99</td>
<td>91.72</td>
</tr>
</tbody>
</table>

In all cases, the probability (P) of having a more extreme variance component than the observed value is P < 0.001.

Table 5
Substrate utilisation* by Bcc isolates

<table>
<thead>
<tr>
<th>Carbon sources on Biolog GN plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Bcc isolates</td>
</tr>
<tr>
<td>Tween 40, Tween 80, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-fructose, L-fucose, D-galactose, α-D-glucose, D-mannitol, β-mannose, D-psicose, D-sorbitol, sucrose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, p-hydroxyphenyl acetic acid, bromosuccinic acid, histidine, cis-aconitic acid, succinic acid, citric acid, α-ketobutyric acid, formic acid, D-galactonic acid lactone, α-ketovaleric acid, D-alanine, L-phenylalanine, phenylethylamine, D-galacturonate, D,L-lactic acid, L-alanine, L-proline, D-gluconic acid, malonic acid, L-alanylglycine, L-proglyutamic acid, 2-aminoethanol, D-glucosaminic acid, propionic acid, L-asparagine, D-glucoronic acid, quinic acid, L-aspartic acid, L-serine, α-hydroxybutyryl acid, β-hydroxybutyric acid, D-saccharic acid, L-glutamic acid, D,L-ε-glycerocephosphate, sebacic acid, D,L-carnitine, D-glucose-6-phosphate, succinic acid, γ-aminobutyric acid, 2,3-butanediol, D,L-glycerolphosphate</td>
</tr>
<tr>
<td>Some Bcc isolates</td>
</tr>
<tr>
<td>glucogen (1, 11, 10, 4), m-inositol (1, 9, 11, 4), α-D-lactose (1, 7, 7, 2), lactulose (0, 8, 11, 0), maltose (0, 2, 1, 0), β-methyl-α-glucoside (0, 5, 10, 4), raffinose (1, 10, 11, 4), xyitol (0, 10, 11, 3), α-hydroxybutyric acid (0, 0, 0, 1), itaconic acid (0, 8, 10, 3), α-ketoglutaric acid (0, 10, 11, 3), glucuronamide (0, 11, 10, 3), alaniamide (1, 10, 11, 4), glycy1-L-glutamic acid (1, 7, 7, 0), L-serine (1, 11, 9, 4), L-threonine (1, 11, 9, 4), uracil acid (0, 10, 11, 4), mison (0, 6, 6, 0, 0), uridine (1, 1, 1, 1, 0), thymidine (1, 1, 1, 1, 1), putrescine (1, 10, 8, 2), α-D-glucose-1-phosphate (1, 11, 10, 4), α-threholase (0, 11, 11, 1)</td>
</tr>
<tr>
<td>No Bcc isolates</td>
</tr>
<tr>
<td>erythritol, α-melibiose, α-cyclodextrin, dextrin, gentobiose, L-rhamnose, N-acetyl-D-galactosamine, turanose, glycy1-L-aspartic acid, D-cellobiose</td>
</tr>
</tbody>
</table>

*Utilisation was defined as colour development determined spectrophotometrically by the Biolog Microstation Reader (see Section 2).

*Figures in parentheses refer to the number of positive isolates belonging to B. cepacia genovar I, B. cenocepacia, B. ambifaria and B. pyrrocinia, respectively.
elucidate how the distribution of species and genomovars in CF compares with that among Bcc residing in the natural environment. The present study reinforces the view that the distribution of species in environmental samples differs widely from that in clinical samples. In fact, as revealed by recent studies [21–23], most clinical isolates belong to B. multivorans and B. cenocepacia, the species seemingly best adapted to the CF lung and including the major epidemic strains of the Bcc, whereas significantly fewer isolates belong to other species. Hence, the wide diffusion of B. cenocepacia in the environment, in particular in the rhizosphere of crop plants ([13,19], this study), raises the question of natural habitats as potential reservoirs of pathogenic strains. The recent isolation by LiPuma et al. [18] of an epidemic strain of B. cenocepacia in agricultural soil makes it still more urgent to define more specifically the preferred natural habitats of this species and, in general, of each Bcc species. It is noteworthy that all B. cenocepacia isolates analysed in the present study belong to the recA lineage III-B. It is becoming evident that the four phylogenetic lineages constituting B. cenocepacia (recA lineages III-A, III-B, III-C and III-D) [16] have different ecological distributions. In fact, so far bacteria belonging to recA lineages III-A and III-D have been recovered only from clinical samples, whereas bacteria belonging to recA lineage III-C have been found only in environmental samples; in contrast, bacteria belonging to recA lineage III-B have been found in both environmental and clinical samples ([16,32], Tabacchioni et al., manuscript in preparation). As far as B. ambifaria is concerned, it is worth noting that a considerable number of Bcc strains with significant plant growth-promoting ability have been identified as B. ambifaria [15,39–41], a species highly represented in natural habitats. On the other hand, in the most extensive study so far conducted on the species status of Bcc strains of clinical origin, B. ambifaria represents only 0.7% of all isolates [23], whereas in two studies carried out recently in Italy no isolates of B. ambifaria were found among clinical isolates [20,22]. The uneven distribution of B. ambifaria in clinical and natural environments suggests that it may play a minor role as an opportunistic pathogen in CF-related infections. In general, the results of the present study, in comparison with data concerning clinical isolates [22,23], suggest that there may be differences in the potential pathogenicity of environmental isolates belonging to different species and genomovars and reinforce the observation of LiPuma et al. [23] that the disproportionate representation of the different species belonging to the Bcc among CF patients implies critical differences in their pathobiology.

Beside the assessment of the taxonomic status of environmental isolates of Bcc, the investigation of their genetic polymorphism may be useful to characterise the potential environmental reservoirs of clinical isolates. So far, the genetic diversity of natural populations of ‘B. cepacia’ has been investigated only at the complex level [25,27,42]; as a consequence, nothing is known about the genetic polymorphism of the Bcc species present in natural populations. In this study, all species and genomovars proved to be highly diverse; in fact, the E index based on RAPD types ranged from 0.98 to 1, indicating that almost all isolates were genetically unrelated. Furthermore, similar high mean GD values were found for B. cenocepacia, B. ambifaria and B. pyrrocinia, suggesting a high degree of genetic polymorphism within Bcc species found in natural habitats. These results are in agreement with the generally accepted high genetic variability among Bcc strains; however, it has been shown that B. stabilis strains show a remarkably restricted genetic variability [10]. Therefore, findings that are valid for some Bcc species should not be extrapolated to other Bcc species. Thus, more data concerning environmental and clinical isolates of all species are needed to obtain a clearer picture of the degree of genetic polymorphism within all species and genomovars of the Bcc.

AFLP, originally developed by Vos et al. [43] as a universal DNA fingerprinting method, has been shown to have a high potential in characterisation and taxonomy studies, as firstly shown by Janssen et al. [44]. The technique has been successfully employed to study genetic diversity among isolates of different origin belonging to various bacterial species, such as Vibrio cholerae [33], Pseudomonas fluorescens and Pseudomonas putida [45], and, furthermore, as reported by Clerc et al. [46], it allows a clear delineation between intraspecific and interspecific
genetic distances. This technique can be applied also in bacterial taxonomy for the differentiation of closely related taxa and it has been used to identify Burkholderia species and genomovars among CF isolates [47]. Therefore, RAPD and AFLP techniques were used to estimate differences in the genetic structure of the different Bcc species applying the AMOVA method to the patterns generated by these two techniques. Both techniques gave essentially similar results, although the AFLP method proved to have a slightly higher discriminatory power. Pronounced differences in the population genetic structure were observed between B. cenocepacia and B. ambifaria, whereas very low or no differences were observed between B. pyrocinia and B. ambifaria or B. pyrocinia and B. cenocepacia.

An improved picture of microbial diversity is provided by an integration of genetic and functional diversity data [48]. Hence, the substrate utilisation capacities (Biolog) of Bcc isolates were measured to estimate the metabolic diversity of the MVPC1 population. Cluster analysis of metabolic profiles based simply on the presence or absence of colour development failed to clearly separate the Bcc species present in the MVPC1 population, indicating that these species are phenotypically similar, as already shown by Vandamme et al. [9]. Therefore, a more refined method based on the maximum colour reached after a particular incubation period was used to assess substrate utilisation differences between these species [49]. Cluster analysis of metabolic profiles based on such values yielded results in good agreement with the AMOVA data. In fact, pronounced differences were observed between B. cenocepacia and B. ambifaria; in contrast, B. pyrocinia could not be clearly separated either from B. cenocepacia or from B. ambifaria. Cluster analysis also revealed a higher degree of metabolic diversity among B. cenocepacia isolates than among B. ambifaria isolates, which is in contrast with the very similar RAPD-based E and GD values of these two species. In particular, B. ambifaria shows a high degree of metabolic homogeneity compared to the high number of unique RAPD fingerprints. This suggests that there is hidden genetic diversity behind the low phenotypic diversity or, conversely, that different genotypes may show very similar metabolic profiles.

As mentioned above, the different distribution of B. ambifaria and B. cenocepacia in clinical and natural habitats suggests a quite different ecological behaviour of these two species; our genetic and, in particular, phenotypic diversity data reinforce this view. In particular, it may be speculated that the suggested higher metabolic diversity of B. cenocepacia may be associated with the greater potential of this species to colonise habitats as diverse as soil and lungs. In perspective, a more effective integration of phenotypic, genotypic and pathogenicity data concerning all the Bcc species should provide valuable information on the degree of functional differentiation of these species.

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References


