Characterization of *Burkholderia rhizoxinica* and *B. endofungorum* Isolated from Clinical Specimens

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**Abstract**

Eight isolates submitted to CDC from 1989 to 2006 from clinical specimens were initially identified as members of the genus *Burkholderia* based on preliminary cellular fatty acid analysis and/or 16S rRNA gene sequencing. With the recent descriptions of the new species *B. rhizoxinica* and *B. endofungorum*, which are considered endosymbiotic bacteria in *Rhizopus microsporus* fungi, we now identify seven of these clinical isolates as *B. rhizoxinica* and one as *B. endofungorum* based on biochemical testing, 16S rRNA, and DNA-DNA hybridization results. We also further characterize these isolates by assessing toxin production and/or by multiple locus sequence typing.

**Introduction**

Recently, *Burkholderia* spp. have been described that are not only endosymbionts in *Rhizopus microsporus*, a saprotrophic fungi, but are also responsible for the production of the toxins rhizoxin and rhizinon which had been previously believed to have been produced solely by the fungi [1,2,3]. Rhizinon is a important virulence factor for infection of plants by *Rhizopus* and has antimitotic activity [4,5,6]. Rhizinon is a cyclopeptide exhibiting fatal hepatotoxic effects [3,5,7,8]. *Burkholderia rhizoxinica* produces rhizinon and is now associated with the ability of *Rhizopus* to cause rice seedling blight. *Burkholderia endofungorum* was recently shown to produce rhizinon. Both bacteria appear to reside in the cytosol of the fungal cell [6,9].

Clinical isolates H2199 (Ohio - 2002), H3620 (New Mexico - 2005), H500 (California - 1997), G8810 (North Carolina - 1993), G7344 (Oregon - 1992), H2592 (District of Columbia - 2003), H3977 (South Dakota - 2006), and G4101 (New York - 1989) were submitted to CDC from 1989 to 2006 by various public health agencies in the U.S. and placed in our archive. All but one were isolated from blood specimens with the exception being H2592 which was from a wound. These isolates were obtained from five different geographical regions: two were from California; two from the Midwest; and one from the South. Isolates were tested biochemically using standard biochemical testing, 16S rRNA gene sequencing and DNA-DNA hybridization. We now identify these clinically derived strains as either *B. rhizoxinica* or *B. endofungorum*. We also further characterize them by cellular fatty acid analysis (CFA) and/or and multiple locus sequence typing (MLST).

**Materials and Methods**

Biochemical testing was performed on all strains and cellular fatty acid (CFA) analysis was performed on strains H500, G7344, G8810 and G4101 as described by Weyant et al. [10].

We performed DNA-DNA hybridization as previously described [11]. In brief: Cells were harvested and lysed, and the chromosomal DNA was isolated and purified. DNA from the type strains of *B. rhizoxinica* (HKI 454\(^1\)) and *B. endofungorum* (HKI 456\(^1\)) were labeled with \(\text{[}\text{32P}\text{]}\text{dCTP}\) using a commercial nick translation kit (Invitrogen Life Technologies, Carlsbad, CA) and tested for reassociation to unlabeled DNA from the same strains (homologous reaction). Reassociation of *B. rhizoxinica* DNA was tested with DNA from H3977 and H2199 and that of *B. endofungorum* was tested with DNA from G4101. A reciprocal reaction using labeled G4101 DNA was also performed. Relative binding ratios and percent divergence were calculated as described previously [11].

DNA sequencing was performed using methods and primers as previously described. In brief: whole cell suspensions of bacteria were used for PCR. Bacteria were grown by plating one loop (1 ml) of stock cell suspension (heavy suspension of *Burkholderia* spp. in defibrinated rabbit blood, stored at ~70°C until use) on trypticase soy agar with 5% defibrinated sheep blood (SBA) (BB/L Microbiology systems, Cockeysville, MD) and incubating aerobically 1–2 days at 37°C. A single colony was suspended in 200 ml of 10 mM Tris, pH 8.0 in a 1.5 ml Millipore 0.22 µm filter unit (Millipore, Bedford, MA), heated at 95°C for 30 min, and...
Results

These eight clinical isolates shared common phenotypic characteristics. They were nonmotile, gram-negative, cocccobacilli. They produced oxidase but did not produce catalase, indole, urease, did not reduce nitrite, did not hydrolyze gelatin or esculin, and did not utilize citrate. Nitrates were reduced by only one isolate H3977. No acid production in the slant or butt of triple-sugar iron agar was noted. There was no growth on MacConkey agar, Salmonella Shigella agar or on cetrimide agar. No acid production was observed in King’s oxidation-fermentation base from D-glucose, D-slyose, mannitol, lactose, sucrose, and maltose. Variable reactions were produced with limus milk. We were unable to demonstrate catalase production and motility with either of these clinical strains or with the type strains of B. rhizoxinica (HKI 454) and B. endofungorum (HKI 456) in our laboratory even though these characteristics were reported by Partida-Martinez and coworkers [6]. Partida-Martinez and coworkers noted that growth of these bacteria was poor in pure culture and did not allow for consistent biochemical characteristics [6].

Analysis of the 16S rRNA gene sequences indicates that H2199, H3620, H500, G3810, G7344, H2592, H3977 have ≥99.4% identity to the 16S rRNA gene sequence for the B. rhizoxinica type strain (HKI 454) and G4101 has an identity of 99.4% to the sequence for the B. endofungorum type strain (HKI 456) (Figure 1).

Four strains were selected for cellular fatty acid analysis (CFA). H500, G7344, G3810 and G4101 shared a unique profile which is easily recognized by the presence of two cyclopropane acids (17:0 cyc, 19:0 cyc 11:12), 16:0 and 18:1 w7c as major acids (8–27%), and smaller amounts (1–5%) of six hydroxy acids (3-OH-14:0, 2-OH-16:1, 2-OH-16:0, 3-OH-16:0, 2-OH-18:1, 2-OH-19:0 cyc). The CFA composition of H500, G7344, G3810 and G4101 is consistent with that of the type strains of B. rhizoxinica and B. endofungorum [6], and is most similar to that of the CFA group containing B. cepacia, B. gladioli, B. mallei and B. pseudomallei [10].

The results of DNA relatedness studies are given in Table 1. Isolates H3977 and H2199 exhibited greater than 78% relatedness (RBR) under both the optimum and stringent reassociation criteria and had divergence (D) of less than 5 to the labeled DNA from the B. rhizoxinica type strain. Isolate G4101 exhibited 76% relatedness under optimum conditions, but 69% under the more stringent conditions when matched with labeled DNA from the B. endofungorum type strain, however in the reciprocal reaction using labeled G4101 the relatedness is over 70% for both the optimum and stringent reassociation criteria.

The results of the MLST indicate that strains G3810, H2190, H500, G7344, H2592, and H3620 are members of the B. rhizoxinica clade called the “Pacific group” (97.7% to 99.4% identity with B. rhizoxinica type strain), whereas isolate H3977 is more remotely related to this group (95.9% identity) (Figure 2). Strain H3977 is most likely not a member of the known subspecies and appears to be the only known member of a new group within the complex. Alleles for isolate G4101 cluster closely with B. endofungorum spp. from the “Eurasian branch” of endofungal symbionts (99.3% to 99.8% identity) and still has 95.7% identity with the B. endofungorum type strain from Mozambique.

To verify the production of toxins by the clinical isolates the metabolic profiles of two bacterial strains were investigated. Strain H2199 and G7344 proved to be cultivable under the conditions optimized for rhizoxin formation whereas we were unable to grow G4101 for the toxin study. HPLC and MS analyses clearly showed that H2199 and G7344 produce high amounts of cytotoxic rhizoxin analogues (Figure 3).

Discussion

Recently, the toxin rhizin was shown to be produced by endosymbiont B. endofungorum and not by the host R. rhizopus [3]. It is
unknown if the clinical isolate (G4101) of *B. endofungorum* produces rhizonin, but the production of this toxin could be clinically significant since rhizonin is a known hepatotoxin. Further studies will determine if this strain produces rhizonin. However, in all hitherto examined cases there has been no evidence for the involvement of *Burkholderia* symbionts for the development of zygomycoses [21,22].

The presence of an acyl transferase AT gene (*rhiE*) from the rhizoxin biosynthesis gene cluster suggests that the clinical isolates of *B. rhizoxinica* might have the capacity to produce cytotoxic polyketides [23]. By metabolic profiling of the bacterial cultures, we unequivocally showed that strains H2199 and G7344 produce significant amounts of rhizonin derivatives. The production of rhizoxin could influence the course of human infection because the toxin has anti-mitotic activity in mammalian cells and has potential as an antitumor drug. Previous work has demonstrated that derivatives of rhizoxin vary in anti-mitotic activity [20]. Further studies will indicate whether these isolates produce the toxin or derivatives of the toxin.

We report for the first time that strains of *B. rhizoxinica* and *B. endofungorum* have been associated with human clinical specimens. Various *Burkholderia* spp. are known pathogens with *B. pseudomallei* causing melioidosis and *B. mallei* causing glanders [13,24]. There are also cases of opportunistic infection by less pathogenic members of the *Burkholderiaceae* such as in the case of *B. cepacia* infections, especially among cystic fibrosis patients, and infection by *B. thailandensis* [25,26]. Since members of the *B. rhizoxinica* complex are known to form tight associations with their host, fungal involvement is possible, however there was no known detection of fungal infections in these
Table 1. DNA/DNA hybridization.

<table>
<thead>
<tr>
<th>Labeled B. rhizoxinica HKI 454&lt;sup&gt;T&lt;/sup&gt; DNA</th>
<th>RBR @ 65°C</th>
<th>D</th>
<th>RBR @ 80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. rhizoxinica HKI 454&lt;sup&gt;T&lt;/sup&gt;</td>
<td>100</td>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>H3977</td>
<td>83</td>
<td>0.0</td>
<td>84</td>
</tr>
<tr>
<td>H2199</td>
<td>85</td>
<td>2.5</td>
<td>79</td>
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<tr>
<th>Labeled B. endofungorum HKI 456&lt;sup&gt;T&lt;/sup&gt; DNA</th>
<th>RBR @ 65°C</th>
<th>D</th>
<th>RBR @ 80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. endofungorum HKI 456&lt;sup&gt;T&lt;/sup&gt;</td>
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<td>0.0</td>
<td>100</td>
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<tr>
<td>G4101</td>
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<td>3.5</td>
<td>69</td>
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<table>
<thead>
<tr>
<th>Labeled G4101 DNA</th>
<th>RBR @ 65°C</th>
<th>D</th>
<th>RBR @ 80°C</th>
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<tr>
<td>G4101</td>
<td>100</td>
<td>0.0</td>
<td>100</td>
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<tr>
<td>B. endofungorum HKI 456&lt;sup&gt;T&lt;/sup&gt;</td>
<td>82</td>
<td>0.1</td>
<td>72</td>
</tr>
</tbody>
</table>

RBR, relative binding ratio; D, percent divergence.
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Figure 2. Phylogenetic network of *Burkholderia* spp from clinical isolates together with fungal symbionts (B1–B8) based on MLST data. B1: *Burkholderia rhizoxinica* type strain. B5: *B. endofungorum* type strain. BPS: *B. pseudomallei* (outgroup).
doi:10.1371/journal.pone.0015731.g002
cases. Unfortunately the clinical records for these isolates is incomplete and further enquiries did not prove productive. The presence of the bacteria by contamination of the specimens cannot be excluded. Seven of the isolates were derived from blood and one from wound tissue. Since Rhizopus are ubiquitous and some are opportunistic human pathogens, it is possible that the patients were colonized by the fungi and that culturing was attempted only to detect bacterial growth, thus missing the presence of Rhizopus \[27\]. When \( B. \text{rhizoxinica} \) or \( B. \text{endofungorum} \) are detected in clinical specimens, clinicians may wish to check for the presence of fungal involvement.

Supporting Information

Supporting Information S1  List of genes sequenced with GenBank accession numbers.

References


