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NOTE

Diversity Analysis of *Burkholderia cepacia* Complex in the Water Bodies of West Lake, Hangzhou, China

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A survey of *Burkholderia cepacia* complex (Bcc) species was conducted in water bodies of West Lake in China. A total of 670 bacterial isolates were recovered on selective media. Out of them, 39.6% (265 isolates) were assigned to the following species: *Burkholderia multivorans*, *Burkholderia cenocepacia recA* lineage IIIA, IIIB, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, and *Burkholderia seminalis* while *B. cenocepacia* is documented as a dominant Bcc species in water of West Lake. In addition, all Bcc isolates tested were PCR negative for the *cblA* and *esmR* transmissibility marker genes except *B. cenocepacia* IIIB A8 which was positive for *esmR* genelater. The present study raises great concerns on the role of West Lake as a "reservoir" for potential Bcc pathogenic strains.

***Keywords*:** *Burkholderia cepacia* complex, West Lake, detection, diversity

Burkholderia cepacia complex (Bcc) is a collection of genetically distinct but phenotypically similar bacteria (Fang *et al*., 2010). It currently comprises *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, *B. pyrrocinia, B. ubonensis*, *B. latens*, *B. diffusa*, *B. arboris*, *B. seminalis*, *B. metallica*, *B. contaminans*, and *B. lata* (Vermis *et al*., 2002; Mahenthiralingam *et al*., 2008; Vanlaere *et al*., 2008, 2009). Bacteria belonging to Bcc, originally identified as plant pathogens (Burkholder, 1950), have emerged as opportunistic human pathogens in 1980s, causing devastating lung infections in patients with cystic fibrosis (CF) and being responsible for various nosocomial infections in immunocompromised patients (Govan and Deretic, 1996; Coenye and Vandamme, 2003).

Bcc has been found to be widely distributed in habitats such as soil, water, plant rizhosphere, animal surfaces, human and hospital environment (Ramette *et al*., 2005; Zhang and Xie, 2007). Nowadays many Bcc strains have been isolated from both clinical and environmental sources, such as *B. cenocepacia* strain recovered from the majority of CF patients in USA might be recovered from agriculture soils (LiPuma *et al*., 2002) and *B. multivorans* IST455 which was isolated from CF patient's sputum shared the same sequence type (ST) with strain recovered from river Schelde (Baldwin *et al*., 2007). Thus, the natural environment including water habitats could be acting as a "reservoir" for infectious Bcc pathogens. An examination of Bcc species in river waters Schelde and Leie in Belgium were conducted by Vermis *et al*. (2003) and several species had been recovered. Subsequently, a lethal disease was reported in fish in China and the pathogen was identified as *B. cepacia* by API2ONE identification system (Jin *et al*., 2005). Therefore, the occurrence of Bcc in water habitats should be investigated more extensively.

West Lake, located in center of Hangzhou, China, is a world famous tourist lake visited by over 10 millions tourists every year and also a vast fresh water rearing pond. There is a famous Chinese dish known as "West Lake Vinegar Fish" was made from the fishes cultivated in the Lake. The risk posed by Bcc in water of West Lake is not only harmful to fish but also an alarming threat to the activities and diet of people in West Lake area. Therefore, it is quite necessary for examination of Bcc distribution in the Lake.

In order to approach these questions, we investigated the distribution of Bcc organisms in water of West Lake for one year. The species status of Bcc organisms was assessed by combination of *recA-Hae*III restriction fragment length polymorphism (RFLP) assays, species-specific PCR tests, *recA* gene sequencing, and multilocus sequence typing (MLST) analysis. In addition, two genetic markers, *cblA* and *esmR*, which are associated with transmissibility, were detected from the isolates recovered from water of West Lake.

Sampling and processing

Monthly sampling was carried out in the first day of each month from May 2006 to April 2007. Water samples were obtained from 10 different fixed sites of the lake, 30 cm below the surface by standard methods as recommended in section 9030 of Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1998), and

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Table 1. Detection of *B. cepacia* complex by culture-based method in water samples collected in West Lake, Hangzhou, China

Sampling time	Season in Hangzhou	Water temperature (°C)	No. of bacterial isolates[a]	No. of Bcc isolates[b]	Species composition
Jun., 2006		24.6	22	8	*B. stabilis*
Jul., 2006	Summer	28.2	107	68	*B. stabilis*
Aug., 2006		30.4	115	85	*B. cenocepacia* IIIA
Sep., 2006		26.3	58	12	*B. cenocepacia* IIIB (6) *B. vietnamiensis* (5) *B. seminalis* (1)
	Autumn				
Oct., 2006		21.9	156	48	*B. multivorans*
Nov., 2006		14.5	45	2	*B. cenocepacia* IIIA
Dec., 2006		8.0	21	0	
Jan., 2007	Winter	5.4	40	21	*B. cenocepacia* IIIA
Feb., 2007		9.8	25	0	
Mar., 2007		12.0	21	0	
Apr., 2007	Spring	16.4	19	0	
May, 2007		17.8	41	21	*B. cenocepacia* IIIA

[a] Isolates were recovered on PCAT medium.
[b] Bcc isolates were identified by primers BCR1/BCR2.

mixed together. The samples were maintained at a temperature of 4°C and processed within 2 h of sampling.

Three liters of water samples were filtered through 0.45 μm pore-size Millipore membranes (45 mm diameter) with a vacuum pump (Autoscience, China). The membranes (4-8 membranes for each 3 L sample) were washed with sterile water to obtain a final volume of 2 ml, which was divided into two aliquots, one was used for molecular analysis, and another was used for cultured analysis.

Analysis of Bcc species composition by molecular methods

An aliquot of 1 ml each concentrated sample was centrifuged at 4,000 rpm for 15 min. The resulting pellet was resuspended in 0.5 ml of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). DNAs of each sample were extracted using method described by Fera *et al.* (2004). Then they were subjected to nested PCRs: the first PCR run was performed with 2 μl of DNA extract, with the primers of 5'-ACAGTGTCTGC ATTCGTG-3' and 5'-CTCTTCTTCGTCCATCGCCTC-3' and the annealing temperature of 58°C, the second run was carried out with 1 μl of first-run PCR product, the species-specific primers of Bcc and their annealing temperature was referred to the report of Mahenthiralingam *et al*. (2000).

Eight of the 12 water-extracted DNAs were positive with about 1,110-bp amplicons obtained for first-run of PCR. The second-run of nested PCR with species-specific primers revealed the presence of the following Bcc species: *B. multivorans*, *B. cenocepacia* IIIA and IIIB, *B. stabilis*, *B. vietnamiensis* (data not shown).

Isolation and identification of *B. cepacia* complex

An aliquot of 1 ml water was inoculated on ten plates of selective medium PCAT (Zhang and Xie, 2007). Individual bacterial colonies formed after 2-3 days of incubation at 28°C were recovered from culture plates. DNAs of each bacterial isolate were extracted according to the method of Vermis *et al*. (2003) and were assigned to *B. cepacia* complex by means of PCR amplification of *recA* gene using specific primer pairs for Bcc, BCR1 and BCR2, by the procedure described by Zhang and Xie (2007).

A total of 670 putative Bcc isolates were recovered from 8 sample collections. Screening PCR amplification of the *recA* gene with specific primers for Bcc, BCR1 and BCR2 revealed that 265 of them belong to Bcc, from which an amplicon of about 1041-bp was obtained (Table 1).

Species status of Bcc isolates

The species composition of Bcc isolates were analyzed by a combination of *recA*-restriction fragment length polymorphism (RFLP) assays, specific-species PCR tests, *recA* gene analysis and MLST analysis.

RFLP analysis was performed according to the method proposed by Mahenthiralingam *et al*. (2000). After amplification with primers BCR1 and BCR2, 8 μl of the PCR product was combined with 1 U of *Hae*III (Fermentas, USA) in a total volume of 15 μl and incubated at 37°C for 3 h. RFLP products were analyzed by agarose gel electrophoresis (2%) using 0.5× Tris-EDTA buffer. Banding patterns of RFLPs were manually analyzed and compared to those shown by Bcc reference strains (Mahenthiralingam *et al*., 2000). Digestion with *Hae*III of *recA* gene resulted in seven different restriction patterns (F, G, I, H, J, B, A) among 265 Bcc isolates. Each pattern was assigned an alphabetical code as described previously (Mahenthiralingam *et al*., 2000; Petrucca *et al*., 2003; Dalmastri *et al*., 2005; Pirone *et al*., 2005). The result revealed that 265 Bcc isolates could be attributed to *B. multivorans*, *B. cenocepacia* IIIA, *B. cenocepacia* IIIB, *B. stabilis*, and *B. vietnamiensis* based on the RFLP patterns of *recA* gene.

Moreover, species-specific PCR tests were carried out to find the putative species status of each Bcc isolate according to the procedure reported previously (Mahenthiralingam *et al*., 2000; Vandamme *et al*., 2002; Pirone *et al*., 2005). In agreement with the result of RFLP assay of *recA* gene, the species-specific PCR analysis allowed us to assign all 265 Bcc isolates to respective species.

To definitively assess the species status of the Bcc isolates, the complete *recA* gene sequence of 18 Bcc isolates representative of different RFLP type were determined and aligned to other known Bcc sequences deposited in GenBank. All sequences obtained were more than 96% similar to those of

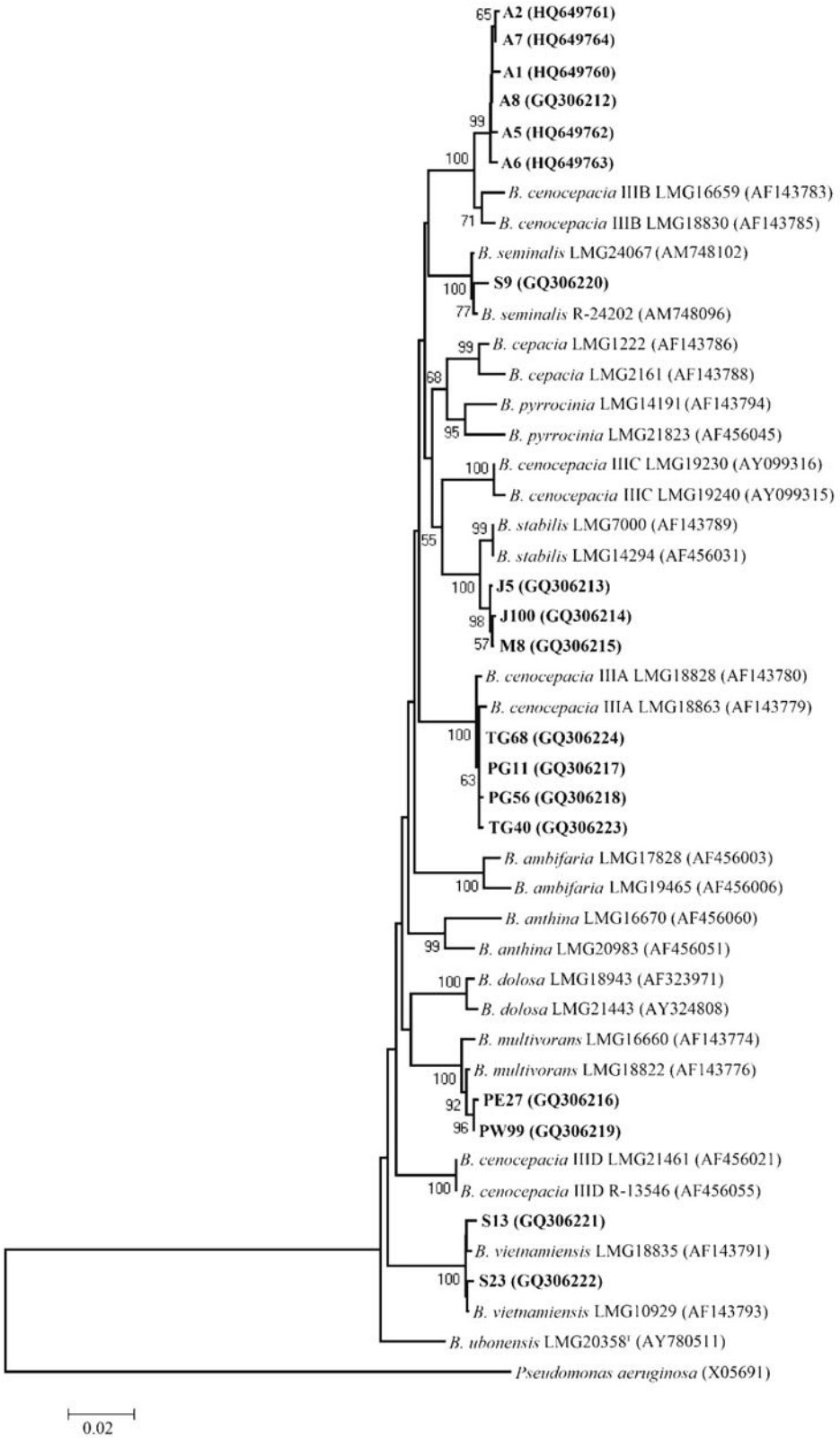

Fig. 1. Phylogenetic tree derived from the *recA* gene sequence analysis on reference strains of each *B. cepacia* complex species and representative isolates of different *B. cepacia* complex species from the West Lake in China. The tree was generated by the neighbor-joining method based on the two-parameter Kimura correction of evolutionary distances. Bootstrap analyses (1,000 replicates) for node values from 50% are indicated. *Pseudomonas aeruginosa* was used as the outgroup. Bar=1% sequence dissimilarity.

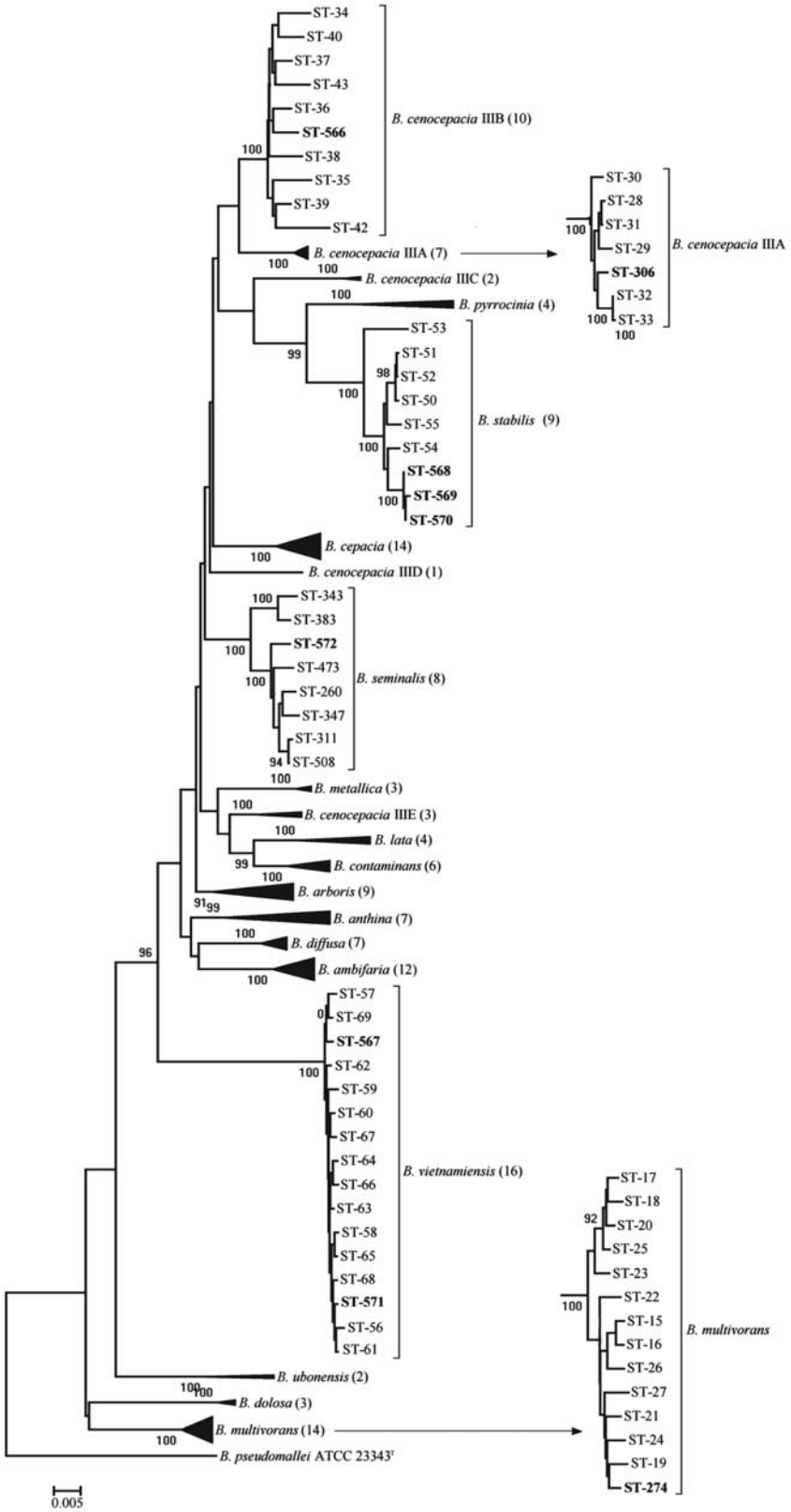

Fig. 2. Phylogenetic tree of concatenated nucleotide sequences from seven loci (*atpD*, *gltD*, *gyrB*, *recA*, *lepA*, *phaC*, and *trpB*) of Bcc bacteria and representative isolates of different *B. cepacia* complex species from the West Lake in China. The tree was generated by the neighbor-joining method based on the two-parameter Kimura correction of evolutionary distances. Bootstrap analyses (1,000 replicates) for node values from 50% are indicated. *B. pseudomallei* ATCC 23343 was used as the outgroup. Bar=1% sequence dissimilarity.

other members of the Bcc. Phylogenetic trees were generated using the genetic distance-based neighbor-joining algorithms within MEGA version 4.0 (http://www.megasoftware.net/). In agreement with the result of *recA* RFLP and species-specific PCR, *recA* gene sequence analysis allowed us to assign the 17 Bcc isolates except S9 to respective species. The phylogenetic analysis revealed that isolate S9 and strains of *B. seminalis* clustered within a group and well separated from other species of Bcc (Fig. 1). It is therefore considered that the isolate should be identified as *B. seminalis*.

Multilocus sequence typing (MLST) analysis was performed subsequently to verify the attained results. Fifteen isolates representative of different RFLP type in different species were analyzed by MLST scheme, all were successfully sequenced at all seven loci (*atpD*, *gltB*, *gyrB*, *recA*, *lepA*, *phaC*, and *trpB*), resulting in a total of nine STs. They are ST274, ST306, ST566, ST568, ST569, ST570, ST567, ST571, and ST572. Indeed, only two already known STs were recovered: ST-274, which was assigned to three CF *B. multivorans* strains BCC0264 (Australia), SBL03-454 and SBL04-409 (New Zealand), as reported by Baldwin *et al*. (2008), and ST-306, which was assigned to two Canada CF *B. cenocepacia* IIIA isolates D1541 and D1065 (Waine *et al*., 2007). Our results ensured that all the isolates belonged to *B. multivorans* and *B. cenocepacia* IIIA shared the same STs with CF strains. The taxonomic position of the isolates was evidenced by the tree (Fig. 2). The result is in agreement with the identification performed by *recA* gene analysis above.

Totally, five species *B. multivorans*, *B. cenocepacia* IIIA and IIIB, *B. stabilis*, *B. vietnamiensis*, and *B. seminalis* were recovered from the water bodies of West Lake in China. *B. cenocepacia* is the most predominant Bcc species, which occupied 50.8% of the total Bcc isolates and 48.5% was *B. cenocepacia* IIIA .What's more, *B. stabilis*, *B. multivorans*, *B. vietnamiensis*, and *B. seminalis* dominated 28.8%, 18.2%, 1.89%, and 0.38%, respectively.

Detection of putative transmissibility markers

The potential pathogenicity of the Bcc strains isolated in this study was evaluated with the presence, by PCR, of *cblA* and of BCESM gene (Mahenthiralingam *et al*., 1997; Clode *et al*., 2000). Among all the Bcc isolates, no *cblA* gene was detected among all the Bcc isolates. One isolate A8 which belonged to *B. cenocepacia* IIIB was found to be positive for the presence of BCESM gene. The *esmR* marker was found to be related to strain transmissibility (Mahenthiralingam *et al*., 1997) but not an absolute marker for transmissible strains (McDowell *et al*., 2004), however, the presence of *esmR* DNA in isolate A8 recovered from water of West Lake reinforces the hypothesis that the water habitat may serve as a "reservoir" of pathogenic strains belonging to this species. This is the first time that *esmR* marker gene was detected in water samples of West Lake.

Our results clearly indicate that totally species composition and abundance of Bcc in water of West Lake in China vary dramatically. Five species of Bcc have been detected at present study by both uncultured-based and culture-independent method while *B. cenocepacia* was the most dominant species, followed by *B. stabilis*, *B. multivorans*, *B. vietnamiensis*, and *B. seminalis*. To our knowledge, this is the first report of *B. stabilis* and *B. seminalis* in water habitat. In addition, the recovery of STs which have only been recovered in pathogenic strains and the existence of *esmR* marker gene in A8 indicate the risk posed by Bcc organisms in water of West Lake to immunocompromised patients in China. Therefore, more studies are required to evaluate the pathogenicity of these Bcc isolates to human beings.

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