

Characterization and Identification of Two Opportunistic Human Bacterial Pathogens in Rice

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Abstract: *Burkholderia cepacia* (*Bc*) and *Pseudomonas aeruginosa* (*Pa*) are both biocontrol agents in agriculture and opportunistic human pathogens in hospitals. Effective management and utilization practice is needed to understand their characteristics and distribution in rice. During the last decade, the two opportunistic human pathogens were detected in 631 samples of rice seed and 117 samples of rice plant in plain, highland and mountainous rice growing areas of China. *Bc* and *Pa* were primarily differentiated by common bacteriological characteristics and pathogenic tests and then identified into species by Biolog and FAME tests. However, the genotypes of *Bc* still could not be distinguished. It has been noted that the *Bc* and *Pa* mainly existed in rice root with the highest distribution frequency in plain areas (6.1% and 16.1%) and lowest in the mountainous areas (1.0% and 7.8%).

Key words: *Burkholderia cepacia*; *Pseudomonas aeruginosa*; opportunistic human bacterial pathogens; characterization; identification; distribution

Rice plant harbors numerous prokaryotes in which some are pathogenic, some are beneficial with promoting growth of plant or antagonistic against pathogens, and most of them are “neutral” ones with unknown functions. The earliest report on rice bacterial diseases was recorded more than 120 years ago^[1]. Over 20 bacterial species were reported to be associated with the rice diseases^[2-3]. Recently environmental protectionists are paying more attention to control the release of toxic chemical and pesticides in agricultural land and dozens of antagonistic bacteria from rice have been evaluated^[4-5]. However, most of the agriculturists are mainly interested in biological control of diseases and totally ignore the knowledge of medical science and environmental protection. As we known the beneficial bacteria from rice may also include opportunistic human pathogens. For example, *Pseudomonas aeruginosa* (*Pa*) is one of the common opportunistic human pathogen and widely distributed in natural environment (freshwater and soil), which also found in air, animal skins, intestines and respiratory tracts etc. It is difficult to control *Pa* infection in hospitals as it infects respiratory tracts in elder and weak patients and causes sapraemia of immune-low babies^[6-7].

At present *Burkholderia cepacia* (*Bc*) has got the attention of many scientists due to current outbreak of severe lung infections (cepacia syndrome). Many countries have banned the use of biocontrol strains of *Bc*^[8].

The bacteria of *Bc* and *Pa* were regarded as one of the weaker plant pathogens or opportunistic human pathogens which have been utilized as a biocontrol agents in agriculture previously. It has been isolated for their pathogenic effect on crops and antagonistic diversity in rice^[2-4, 9]. However, little is known about the characteristics of *Bc* and *Pa* from rice up to date. The present work was conducted to reveal the biological characteristics and distribution of two types of opportunistic human pathogens from rice environment to efficient management and utilization of the multifunctional bacteria in order to provide reliable evidence for biological safety.

MATERIALS AND METHODS

Standard reference strains

Eight standard reference bacterial strains were provided by Belgian Coordinated Collections of Microorganisms (LMG code) and International Rice Research Institute (IR code). Other bacterial strains were provided by Institute of Biotechnology,

Received: 8 February 2006; Accepted: 4 April 2006

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Sample collection and the bacterial isolation

The representative rice seeds weighted 50-250 g were served as a sample collected from rice field during harvest time and the similar variety or line was selected in each rice growing area. The total of 631 rice seed samples were collected, 322 of which was from plain areas (Jiaxing, Yuhang, Shaoxing and Ningbo), 182 from highland area (Jinhua, Dongyang, Lanxi and Xinchang), 102 from mountainous areas (Lishui, Jinyun and Longquan) of Zhejiang Province, and 25 from plain areas (Nanjing, Fuzhou and Kunming) of other provinces. A total of 117 rice plant samples were collected from rice growing areas of Zhejiang Province at the tillering stage of single rice crop (including 8 rice plant samples from Fuzhou and Kunming). The bacteria were isolated from rice seeds by using direct seed crashing or seed germination method [4], and direct crashing method for isolation from rice plant samples.

Pathogenicity test

The seeds of variety IR8 were treated with 70% ethanol for 1 min and washed with sterilized distilled water until the seeds were cleaned up, and then grew in the boxes filled with uncultivated deep soil with more N-fertilizer. Four-week old seedlings were used for inoculation with injection method. A volume of 0.6 mL bacterial suspension (in concentration of 1×10^8 cfu/mL) was injected into the sheaths at the base of a rice plant with a fine hypodermic needle. The inoculated seedling plants were moved to a moisture chamber with 100% RH for 24 h. Observation for development of symptom was started 3 days after inoculation.

Identification of the bacteria

Colonial morphology and bacteriological test

Colonial morphology and bacteriological test (Gram stain, fluorescence under UV on KMB, and LOPAT and so on) were performed according to the methods described by Mew et al [10] and Schaad et al [11]. Fluorescent or nonfluorescent strains of *Pseudomonas* were distinguished by means of gram stain and fluorescence under UV light, and further divided into *Bc* and *Pa* group by comparing colonial morphology and bacteriological characteristics of

standard strains. The representative isolates of each strain were selected for Biolog and FAME tests.

Biolog test

The tested strains were cultured on Trypticase Soy Agar (TSA) for 24 h, and the grown cultures were diluted to 5×10^8 cfu/mL with sterilized saline solution. A volume of 150 μ L bacterial suspension was put into each of 96 holes of the Gram-negative (GN) Microplates containing 95 different carbon sources (Biolog Inc., Investment Blvd. 3447, Suite 3 Hayward 94545, USA) and incubated for 24 h at 30°C. The color reaction in each hole was observed and recorded, and analyzed by Biolog procedures (4.01 edition).

Fatty acid methyl ester (FEME) test

The pure tested cultures were grown on NA for 24 h at 28°C and then transferred on TSA plates containing 3% trypticase for exactly 24 h at 28°C. A loopful of cells was harvested with a sterilized loop (4 mm diameter) and transferred to a test tube capped with a Teflon-lined screw-cap. The extraction and preparation of FAME was performed [12]. FAME profiles were obtained by gas-liquid chromatography using a model 5980a gas chromatograph, and identified by the microbial identification software package MIS 4.15 edition (Microbial ID Inc., Newark DE).

RESULTS

Morphological and colonial characteristics of *Bc* and *Pa*

The four standard reference strains and four representative isolates of *Bc* from rice plants and hospitals showed gram-negative, rod, with polar monotrichous or tuft of flagella. When the cultures of genotype I and II of *Bc* were incubated for 4 days at 28°C the colony was winked olive yellow convex with smooth to irregular margin, 2-4 mm in diameter on TSA, and pale green-yellow, nonfluorescent, convex with smooth to wave margin, 2-3 mm in diameter on KMB. The colony of genotype III was butyrous cream white, translucent, convex with smooth margin, 2-3 mm in diameter on TSA, and white mucous, nonfluorescent, convex, 2-3 mm in diameter on KMB. The bacterium was positive for

oxidase, indole and H₂S. Acid produced from glucose and mannitol. No *Bc* growth was observed at 42°C and no significant symptom at inoculated site of rice plants (Table 1).

The three standard reference strains and two representative isolates of *Pa* from rice seeds and plants showed gram-negative, rod, in pairs or forming short line, with polar monotrichous or tuft of flagella. The colony was grey with irregular margin, convex, smooth, 0.8-2.5 mm in diameter, forming water soluble pigment on TSA, and grey with metal shine, blue or yellowish-green fluorescence under UV light on KMB. The bacterium was positive for oxidase, nitrate reduction, gelatin and urea hydrolysis. Most of the strains grew at 42°C. No growth was observed at 4°C with no significant symptom on inoculated site of rice plants.

Pathogenicity tests of *Bc* and *Pa*

The results of pathogenic tests indicated that all

the *Bc* tested cultures didn't show any disease symptom on rice seedlings. However, 2% strains of *Pa* in the favorable conditions induced the small lesions at the base of the inoculation site of rice seedlings, which were bigger than those of the control.

Identification of *Bc* and *Pa* isolates by Biolog and FEME tests

To re-confirm the identification, the eight standard reference strains were re-identified by Biolog and FAME tests, which were supplied by Belgium and International Rice Research Institute. The present results completely matched the original records. The bacterial isolates could be identified as genus level by pathogenicity test, colonial appearance and bacteriological test. However, the rapid identification at species level had to be recognized by Biolog and FAME tests. The similarities between Biolog and FAME tested strains were 0.634–1.000 and 0.510–0.723, respectively (Table 2), suggesting

Table 1. Differentiation of the opportunistic human bacterial pathogens associated with *Bc* and *Pa* from rice environment in colony appearance.

Cultural code	Origin	Identity	Colony appearance on TSA	Colony appearance on KMB
*LMG1222 ^T	Onion	<i>B. cepacia</i> I	Winked olive yellow, convex with smooth to irregular margin	Pale green-yellow colony, non-fluorescence
*IR06854B	Rice plants in the Philippines	<i>B. cepacia</i> I	Winked olive yellow, convex with smooth to irregular margin	Pale green-yellow colony, non-fluorescence
*LMG18606	Rice seeds in the Philippines	<i>B. cepacia</i> II	Winked olive yellow, convex with smooth to irregular margin	Pale green-yellow colony, non-fluorescence
*LMG19587	Hospitals in Europe	<i>B. cepacia</i> III	Butyrous cream white, translucent, convex with smooth margin	Pale green-yellow colony, non-fluorescence
T3-18845	Rice seeds in China	<i>B. cepacia</i> I	Winked olive yellow, convex with smooth to irregular margin	Pale green-yellow colony, non-fluorescence
T1-03749	Rice seeds in China	<i>B. cepacia</i> II	Winked olive yellow, convex with smooth to irregular margin	Pale green-yellow colony, non-fluorescence
T3-18806	Rice plants in China	<i>B. cepacia</i> I	Winked olive yellow, convex with smooth to irregular margin	Pale green-yellow colony, non-fluorescence
H2003005	Hospitals in China	<i>B. cepacia</i> I	Winked olive yellow, convex with smooth to irregular margin	Pale green-yellow colony, non-fluorescence
*LMG 1242 ^T	Soil	<i>P. aeruginosa</i>	Convex, smooth, blue-green to grey with irregular margin	Water soluble pigment, blue or yellow-green fluorescent under UV light
*IR07358	Rice seeds in the Philippines	<i>P. aeruginosa</i>	Convex, smooth, blue-green to grey with irregular margin	Water soluble pigment, blue or yellow-green fluorescent under UV light
*IR07342	Rice plants in the Philippines	<i>P. aeruginosa</i>	Convex, smooth, blue-green to grey with irregular margin	Water soluble pigment, blue or yellow-green fluorescent under UV light
Y99115	Rice seeds in China	<i>P. aeruginosa</i>	Convex, smooth, blue-green to grey with irregular margin	Water soluble pigment, blue or yellow-green fluorescent under UV light
S30297	Rice plants in China	<i>P. aeruginosa</i>	Convex, smooth, blue-green to grey with irregular margin	Water soluble pigment, blue or yellow-green fluorescent under UV light

* means standard reference strain; ^T means type strain.

Table 2. Biolog and FAME identity of opportunistic human bacterial pathogens associated with *Bc* and *Pa* from rice.

Cultural code	Biolog identity	Biolog similarity	FAME identity	FAME similarity
*LMG1222 ^T	<i>B. cepacia</i> I	0.725	<i>B. cepacia</i>	0.702
*LMG18606	<i>B. cepacia</i> II	0.634	<i>B. cepacia</i>	0.606
*LMG19587	<i>B. cepacia</i> III	0.771	<i>B. cepacia</i>	0.635
*IR06854B	<i>B. cepacia</i> I	0.815	<i>B. cepacia</i>	0.638
T3-18845	<i>B. cepacia</i> I	0.920	<i>B. cepacia</i>	0.622
T1-03749	<i>B. cepacia</i> II	0.636	<i>B. cepacia</i>	0.521
T3-18806	<i>B. cepacia</i> I	0.802	<i>B. cepacia</i>	0.704
H2003005	<i>B. cepacia</i> I	0.785	<i>B. cepacia</i>	0.583
*LMG 1242 ^T	<i>P. aeruginosa</i> ^T	1.000	<i>P. aeruginosa</i>	0.635
*IR07358	<i>P. aeruginosa</i>	0.821	<i>P. aeruginosa</i>	0.539
*IR07342	<i>P. aeruginosa</i>	0.880	<i>P. aeruginosa</i>	0.601
*IR10707	<i>P. aeruginosa</i>	0.880	<i>P. aeruginosa</i>	0.510
Y99115	<i>P. aeruginosa</i>	0.836	<i>P. aeruginosa</i>	0.702
S30297	<i>P. aeruginosa</i>	0.811	<i>P. aeruginosa</i>	0.723
CB98001	<i>P. aeruginosa</i>	0.728	<i>P. aeruginosa</i>	0.615
CB98663	<i>P. aeruginosa</i>	0.864	<i>P. aeruginosa</i>	0.603

* means standard reference strain and the result of present identification accorded with the original; ^T means type strain.

that the both identification methods are applicable for the strains of *Bc* and *Pa*. The Biolog and FAME similarities of the four representative isolates of *Bc* and four of *Pa* were 0.636–0.920 and 0.728–0.864, and 0.521–0.700 and 0.603–0.723, respectively. This indicated the reliability of the identification results of *Bc* and *Pa* in rice plants.

Distribution of *Bc* and *Pa* on different parts of rice plants

The highest amount of total bacteria biomass was found in rice roots followed in sheaths and seeds (Table 3). The highest amount of *Bc* and *Pa* populations were in roots and seeds, and the lowest were in sheaths and leaves. This indicated *Bc* and *Pa* mainly survived in rice roots and seeds.

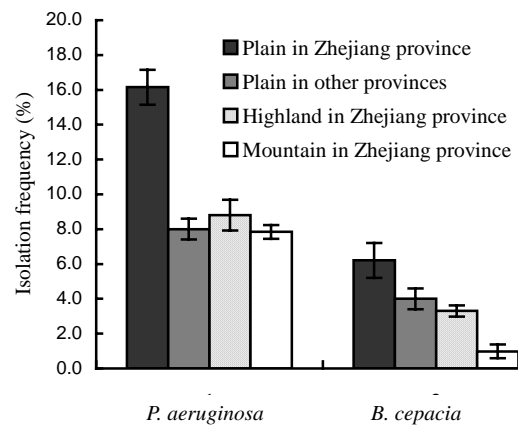
Distribution frequency of *Bc* and *Pa* from different rice growing areas

Based on the isolation frequency of *Bc* and *Pa* from the 631 rice samples in four rice growing areas,

Table 3. Distribution of the opportunistic human bacterial pathogens associated with *Bc* and *Pa* on different parts of rice plants.

Plant part	Total bacteria (10 ⁶ cfu/g)	<i>B. cepacia</i> (%)	<i>P. aeruginosa</i> (%)
Leaf	16.10 c	0.27 c	1.01 c
Sheath	24.30 b	0.23 c	0.72 c
Seed	3.10 d	0.50 b	1.43 b
Root	56.30 a	2.68 a	3.27 a

Means followed by a same letter in a column are not significantly different at the 5% level by DMRT.

**Fig. 1. Isolation frequency of the two opportunistic human bacterial pathogens associated with rice seeds from plain, highland and mountain of rice growing areas.**

the distribution frequency of *Bc* and *Pa* was 16.1% and 6.1%, respectively, which was the highest among the four areas, and the lowest in the mountainous area which was 1.0% and 7.8%, respectively. The distribution frequencies of *Bc* were significantly lower than those of *Pa* in the four rice-growing areas (Fig. 1).

DISCUSSION

During last decade, over ten thousands of bacteria were isolated from 631 rice seed and 177 rice plant samples in plain, highland and mountainous rice growing areas of China. The frequency of diversity and isolation of non-pathogenic bacteria and opportunistic human pathogens were surveyed. Several groups of genera were distinguished by

colony appearance, pathogenic test and classic bacteriological test. In addition, more than 700 representative strains and 80 standard reference strains^[13] were identified by Biolog and FAME tests. Eleven species or types of *Pseudomonas* and 23 other species with 14 genera of non-pathogenic bacteria including some biocontrol agents were also identified. In 1999 we were the first to identify some species of non-pathogenic or antagonistic strains in China^[4], and concluded that rice seed is an important source of biocontrol agents. In 2002 seven bacterial species or types were found in rice seeds (based on Biolog identification), indicating that both beneficial and pathogenic bacteria existed in rice seeds, and confirmed the diversity of procaryotes from rice in China^[13]. The present results further revealed that the opportunistic human pathogens of *Bc* and *Pa* were detected in rice seeds along with the beneficial and pathogenic bacteria. However, the variation of the two opportunistic human pathogens and their impact on mammals need to be further investigated. Previously, nine genotypes of *Bc* strains have been reported in plants, environments, hospitals and human^[8], but only two of them were detected in this study reflecting that the more sensitive and rapid identification methods should be developed.

Yu et al^[14] reported that the highest amount of antagonistic bacteria against rice sheath blight was found in sheath, followed by leaf and seed under three different seeding patterns (direct row seeding, direct broadcast seeding and transplanted seeding). The results were similar to those of total bacteria biomass in this study. However, the distribution frequencies of *Bc* and *Pa* were in the order, i.e. root>seed>leaf >sheath. This might be related with wide distribution of *Bc* and *Pa* bacteria in natural freshwater and soil^[13]. The higher population of *Bc* and *Pa* in rice seeds might be attributed to irrigation during maturity and contamination by soil particles at the harvesting stage. The similar reason could be used to explain the highest *Bc* and *Pa* population in plain areas and the lowest in the mountainous rice growing areas. Usually in plain areas the chance of bacterial spread due to hospital waste is more than those in the other areas. Correspondingly, the distribution frequencies of *Bc* and *Pa* in rice growing areas of other provinces may be lower due to lesser number of samples tested. However, further work should be done to confirm the findings.

ACKNOWLEDGMENTS

Thanks to the postgraduate students for their help during this work. This study was supported by the National Natural Science Foundation of China (Nos. 30370951, 30270891).

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