

## Gram-Positive Bacteria Associated with Rice in China and Their Antagonists Against the Pathogens of Sheath Blight and Bakanae Disease in Rice

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**Abstract:** It is necessary to understand the bacterial populations associated with rice so as to provide more information and natural resources for effective management of major diseases in rice. A survey on screening and identification of gram-positive bacteria was conducted during 1998–2004. Seven hundred and fifty-six rice samples were collected from Zhejiang, Jiangsu, Fujian and Yunnan Provinces, China. Over 1000 bacterial isolates were isolated and tested for colony morphology, pathogenicity, and some characteristics of bacteriology including Gram staining, fluorescent pigment on Kings medium B and microscopic observation for endospore. Together with five standard reference strains, 74 representative gram-positive bacterial isolates were confirmed by Biolog and gas chromatographic analysis of fatty acid methyl esters. Five bacterial species of *Bacillus* and other three genera were identified and isolates from *Bacillus subtilis* and *Bacillus megaterium*, exhibited the most effective inhibition against the pathogens of sheath blight and bakanae disease of rice. A few isolates from *Bacillus pumilus* and *Bacillus megaterium* showed weak virulent on rice together with some virulent isolates, risk should be considered when isolates from these species were screened for biocontrol agents.

**Key words:** bacterial identification; bacterial population; antagonistic bacteria; sheath blight; bakanae disease; rice

Rice (*Oryza sativa* L.) is one of the most important crops, which feeds half of the population in the world. It's the principle of the Integrated Pest Management (IPM) to maintain the pest damage under an economical permitted level by means of resistant cultivars and biological control. Some of the bacteria from rice plants are pathogenic<sup>[1]</sup>, but most of them are nonpathogenic ones, which provide abundant resources for the biological control to rice diseases. It is a first key step of biological control with antagonistic bacterial to understand the composition of the bacterial population on the rice plant. Much research work has been done on the gram-negative bacteria, while the research on the gram-positive bacteria has ever been limited to the *Bacillus* spp., for example, *Bacillus subtilis* (B916) has been successfully applied to control rice sheath blight caused by *Rhizoctonia solani*<sup>[2,3]</sup>. However, there has hardly developed a systematic study on the gram-

positive bacterial composition, pathogenicity, and the antagonistic effect against the two major fungal pathogens causing sheath blight and bakanae disease of rice in China.

The aims of this study are attempted to determine the major composition of the gram-positive bacteria on the rice plant and explore their antagonism against *Rhizoctonia solani* and *Fusarium moniliforme*, thus providing information and bio-resources for the further development and use of biological control agents against the rice diseases.

## MATERIALS AND METHODS

### Reference strains

Five standard bacterial reference strains from the Laboratory of Microbiology, University of Gent in Belgium (LMG) and the International Rice Research Institute (IRRI) in the Philippines were used in the experiments. Four bacteria strains (B2, B5, B7, and B8) of biocontrol agents from vegetables and paddy soil<sup>[4]</sup> and strains of *Rhizoctonia solani* and *Fusarium*

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*moniliforme* were provided by the Biotechnology Institute of Zhejiang University.

### Sample collection and isolation of bacteria

The experiments were conducted in single crop rice during 1998 to 2004 in China. The leaf and sheath samples were collected at tillering and booting stage and grain samples (50 to 250 g per sample) from milky to maturity stage in the paddy fields. Six hundred and thirty-one seed samples were collected, of which 606 from Zhejiang and 11 from Jiangsu, 8 from Fujian and 6 from Yunnan Province. During the rice growth stage, 117 samples of rice plants were collected from Zhejiang, 4 from Fujian and 4 from Yunnan. The crashing method for isolation of the bacteria from rice plant was used<sup>[5]</sup>. The isolation of bacteria from rice seeds was followed by Xie et al<sup>[6]</sup>.

### Pathogenicity test

Susceptible variety IR8 was used for inoculation. To eliminate potential infection of other pathogens, the seeds were treated with 70% ethanol for 1 min and washed with sterile distilled water for four times and soaked and germinated with the distilled D-water. The rice plants were raised in the garden soil, in which rice was never grown before. A volume of 0.6 mL bacterial suspension (in concentration of  $1 \times 10^8$  cfu/mL) was injected into the rice plant with a fine hypodermic needle. The procedures followed those established by Xie et al<sup>[7]</sup>.

### Identification of the bacteria

#### *Gram staining and colony morphological test*

Colony morphological test and some bacteriological characteristics including Gram staining, growth on KMB for fluorescence and microscopic examination of stained endospore were based on Schaad's<sup>[8]</sup>. According to the results of Gram staining and fluorescent pigment on KMB the gram-negative bacteria should be excluded. The gram-positive bacteria were differentiated by their colony morphology and production of endospore and further divided into several groups after compared with the standard reference strains. Some representative bacteria strains will be selected for the Biolog and

FAME test. Some of the bacterial isolates with higher Biolog and FAME similarity were also saved as reference strains.

#### *Biolog identification*

After the primary identification, 74 bacteria isolates selected were further identified by Biolog. A 96-well MicroPlate designed to test the ability of inoculated bacteria to utilize (oxidize) a panel of 95 different carbon sources and nutrients in the plate were pre-selected. The Gram-negative (GN) MicroPlate characterizes and differentiates GN aerobic bacteria. Gram-positive (GP) MicroPlate is used for GP aerobic bacteria. The Biolog, MicroLog release 4.01 software was used. This special software allows identification of the bacteria from their metabolic patterns in Biolog MicroPlates based on the similarity.

#### *Gas chromatographic analysis of fatty acid methyl esters (FAME) identification*

Pure cultures were grown on NA for 24 h at 28°C. Grown cultures were transferred onto trypticase soy agar (TSA) plates containing 3% trypticase soy broth (TSB) and 1.5% Bacto-Agar (Difco), for 24 h at 28°C. A loopful of cells was harvested with sterile loop (4 mm diameter) and transferred to a test tube covered with a Teflon-lined screw cap. FAME profiles were obtained by gas-liquid chromatography using a model 5980A Gas Chromatograph (Hewlett-Packard Co., Avodale, Pa) equipped with a 5% phenylmethyl silicone capillary column (25 m by 0.2 mm; Hewlett-Packard), an automated sampler, a flame ionization detection system, and an integrator. The whole process was controlled by the computer program under 25–28°C. FAME fingerprints were identified by using a microbial identification system software package (MIS version 4.15 obtained from Microbial ID, Inc., Newark, Delaware) and a calibration mixture of known standards<sup>[9]</sup>.

### Inhibition to pathogenic fungi

The procedures for inhibition to the pathogenic fungi were followed the Mew et al<sup>[5]</sup>.

## RESULTS

### Isolation of the gram-positive bacteria associated with rice

One thousand and fifteen gram-positive bacterial strains were isolated from the rice samples. Seventy-four typical ones were selected for further analyses. It showed that 46 of them from the rice seeds, 9 from the rice leaves and sheaths of Zhejiang, 8 from the rice seeds of Jiangsu, 6 from the rice leaves and seeds of Fujian, and 5 from the rice leaves and seeds of Yunnan (Table 1). These isolates produced no fluorescence or diffused pigments. The pathogenicity test showed that most of the bacterial strains were nonpathogenic to rice. Eleven of the bacterial strains from the rice seeds of Zhejiang Province were weakly

virulent, which had a tendency to enhance the virulence in the favorable environmental conditions. Risk should be considered when making use of these bacteria strains as the biological agents since, about 2–3% of them may cause small lesions on rice sheath.

### Identification of the gram-positive bacteria associated with rice

#### *Reliability of the method for identification of the gram-positive bacteria*

Three standard gram-positive bacterial strains and two gram-negative bacterial strains from LMG and IRRI were re-identified to ensure the reliability of the identification methods used. The results of the present identification were consistent with those of the original (Table 2). The genera of the bacterial strains

**Table 1. Isolation and characterization of gram-positive bacteria associated with rice.**

| Original No.                               | Origin            | Isolation position | No. of strains tested | Gram staining | Pathogenicity test |
|--|-------------------|--------------------|-----------------------|---------------|--------------------|
| B97010                                     | LMG               | Rice seed          | 1                     | +             | Avirulent          |
| B97012                                     | LMG               | Rice seed          | 1                     | +             | Avirulent          |
| I11342                                     | IRRI              | Rice seed          | 1                     | +             | Avirulent          |
| IR01806                                    | IRRI              | Rice seed          | 1                     | —             | Virulent           |
| IR10707                                    | IRRI              | Rice seed          | 1                     | —             | Avirulent          |
| CB96201, 96205, 98200, 98206, 98207        | Zhejiang Province | Rice seed          | 5                     | +             | Weak virulent      |
| CB96233, 96236, 98255, 98261, 98266, 98269 | Zhejiang Province | Rice seed          | 6                     | +             | Weak virulent      |
| S98011-S98012                              | Jiangsu Province  | Rice seed          | 2                     | +             | Avirulent          |
| S99031-S99032                              | Jiangsu Province  | Rice seed          | 2                     | +             | Avirulent          |
| S00041                                     | Jiangsu Province  | Rice seed          | 1                     | +             | Avirulent          |
| S01044-S01046                              | Jiangsu Province  | Rice seed          | 3                     | +             | Avirulent          |
| S98211-S98216                              | Zhejiang Province | Rice seed          | 6                     | +             | Avirulent          |
| S98221-S98225                              | Zhejiang Province | Rice seed          | 5                     | +             | Avirulent          |
| S00211-S00212                              | Zhejiang Province | Rice seed          | 2                     | +             | Avirulent          |
| S00241-S00244                              | Zhejiang Province | Rice seed          | 4                     | +             | Avirulent          |
| S01251-S01254                              | Zhejiang Province | Rice seed          | 4                     | +             | Avirulent          |
| S03261-S03271                              | Zhejiang Province | Rice seed          | 11                    | +             | Avirulent          |
| S04291-S04293                              | Zhejiang Province | Rice seed          | 3                     | +             | Avirulent          |
| S23780, T26511, S40005, Y40084             | Zhejiang Province | Rice leaf, sheath  | 4                     | +             | Avirulent          |
| T16436, T16439, Y28336, S33655, S40011     | Zhejiang Province | Rice leaf, sheath  | 5                     | +             | Avirulent          |
| T00311-T00314                              | Fujian Province   | Rice plant, seed   | 4                     | +             | Avirulent          |
| T01321-T01322                              | Fujian Province   | Rice plant, seed   | 2                     | +             | Avirulent          |
| T99431-T99433                              | Yunnan Province   | Rice plant, seed   | 3                     | +             | Avirulent          |
| T00451-T00452                              | Yunnan Province   | Rice plant, seed   | 2                     | +             | Avirulent          |

could be determined based on their colony morphology, pathogenicity and bacteriological characteristics. However, on the species level they had to be distinguished by Biolog and FAME. The five standard reference strains showed their similarity above 0.64 and 0.70 by Biolog and FAME, respectively. It indicates that the two methods are reliable for identification of the gram-positive bacteria.

### Gram-positive bacterial groups associated with rice

The Biolog and FAME identification showed that most of the gram-positive bacteria isolated from rice plants in Zhejiang Province belonged to *Bacillus subtilis* and *Curtobacterium flaccumfaciens* pv. *poinsettiae* while from rice grains, majority of them were *Bacillus subtilis*, *Bacillus cereus* and *Bacillus megaterium*, *Brevibacillus brevis*, *Bacillus licheniformis* and *Microbacterium liquefaciens*. The

gram-positive bacteria isolated from rice plants and grains in Fujian were *Bacillus cereus* and *Bacillus megaterium*, and *Bacillus subtilis* and *Bacillus pumilus* in Yunnan. The gram-positive bacteria from Zhejiang Province seems more diverse in the present study. It may relate with large number of samples and different geographic locations for sample collection.

### Inhibition effects of the gram-positive bacteria associated with rice against the two fungal pathogens of rice

Some of the representative bacteria identified by Biolog and FAME were screened for antagonistic effect against *Rhizoctonia solani* and *Fusarium moniliforme*. The results showed that among the four genera identified, the strains of *Microbacterium liquefaciens* had the weakest antagonistic effect while the strains of *Bacillus* spp. and *Brevibacillus brevis* showed the strongest effect against the two pathogens. Some strains of *Curtobacterium flaccumfaciens* also

Table 2. Biolog and FAME identity of gram-positive bacteria associated with rice.

| Original No.                               | Species or type  | Biolog similarity | FAME similarity |
|--|--|-------------------|-----------------|
| B97010                                     | <i>Brevibacillus brevis</i>                            | 0.68              | 0.71            |
| B97012                                     | <i>Bacillus megaterium</i>                             | 0.66              | 0.70            |
| I11342                                     | <i>Bacillus subtilis</i>                               | 0.64              | 0.75            |
| IR01806                                    | <i>Acidovorax avenae</i> subsp. <i>avenae</i>          | 0.73              | 0.84            |
| IR10707                                    | <i>Pseudomonas aeruginosa</i>                          | 0.88              | 0.50            |
| CB96201, 96205, 98200, 98206, 98207        | <i>Brevibacillus brevis</i>                            | 0.60–0.70         | 0.61–0.70       |
| CB96233, 96236, 98255, 98261, 98266, 98269 | <i>Bacillus megaterium</i>                             | 0.61–0.71         | 0.59–0.73       |
| S98011-S98012                              | <i>Brevibacillus brevis</i>                            | 0.53–0.68         | 0.53–0.66       |
| S99031-S99032                              | <i>Bacillus megaterium</i>                             | 0.50–0.66         | 0.48–0.63       |
| S00041                                     | <i>Bacillus pumilus</i>                                | 0.57–0.65         | 0.56–0.65       |
| S01044-S01046                              | <i>Bacillus subtilis</i>                               | 0.65–0.71         | 0.67–0.75       |
| S98211-S98216                              | <i>Brevibacillus brevis</i>                            | 0.53–0.66         | 0.53–0.66       |
| S98221-S98225                              | <i>Bacillus cereus</i>                                 | 0.51–0.67         | 0.52–0.69       |
| S00211-S00212                              | <i>Bacillus licheniformis</i>                          | 0.51–0.63         | 0.50–0.65       |
| S00241-S00244                              | <i>Bacillus megaterium</i>                             | 0.50–0.70         | 0.60–0.79       |
| S01251-S01254                              | <i>Bacillus pumilus</i>                                | 0.57–0.67         | 0.57–0.70       |
| S03261-S03271                              | <i>Bacillus subtilis</i>                               | 0.65–0.71         | 0.49–0.89       |
| S04291-S04293                              | <i>Microbacterium liquefaciens</i>                     | 0.51–0.70         | 0.48–0.69       |
| S23780, T26511, S40005, Y40084             | <i>Bacillus subtilis</i>                               | 0.70–0.75         | 0.69–0.73       |
| T16436, T16439, Y28336, S33655, S40011     | <i>Curtobacterium flaccumfaciens</i> pv. <i>poin B</i> | 0.43–0.56         | 0.42–0.52       |
| T00311-T00314                              | <i>Bacillus cereus</i>                                 | 0.51–0.72         | 0.52–0.71       |
| T01321-T01322                              | <i>Bacillus megaterium</i>                             | 0.50–0.72         | 0.58–0.78       |
| T99431-T99433                              | <i>Bacillus pumilus</i>                                | 0.57–0.74         | 0.58–0.72       |
| T00451-T00452                              | <i>Bacillus subtilis</i>                               | 0.68–0.71         | 0.49–0.79       |

inhibited the pathogens but its risk should be considered as a biological agent since it may cause plant disease. The antagonistic bacterial strains of *Bacillus subtilis* and *Bacillus megaterium* from different parts of the rice plant and different geographic areas displayed the strongest inhibition effect against the two pathogens in species level. They showed stronger inhibition effect on the *R. solani* than on the *F. moniliforme*. Furthermore, the three strains of *Bacillus subtilis* (B5, B7 and B8) and one strain (B2) of *Bacillus pumilus* from the soil also showed antagonistic effect against the two pathogens.

## DISCUSSION

In the previous study more research work on bacteria associated with rice usually focused on the identification of the pathogens and the screening for the antagonists and less attention on opportunistic pathogenic bacteria and symbiotic ones. There was a description on diversity of the nonpathogenic bacteria associated with rice seeds of single crop in Zhejiang<sup>[6]</sup>; however, no systematic study on gram-positive bacterial associated with rice was conducted. In present study we isolated 1015 gram-positive bacterial strains from the rice plants and seeds of Zhejiang, Jiangsu, Fujian and Yunnan in the past seven years. The Biolog and FAME may be more accurate and faster for identification; however, high cost makes it impractical to identify all the bacteria strains isolated. The best practical way is to divide them into several groups first based on their colony morphology, pathogenicity, and traditional bacteriological characteristics. In this way 74 gram-positive bacterial strains were selected and further confirmed by Biolog and FAME.

The gram-positive bacterial groups associated with rice from some provinces of China are mainly *Bacillus* species, which is different from those in tropic environment<sup>[10]</sup>. It may probably relate with different ecological niches of rice. *Bacillus* spp. is one of the major groups as biocontrol agents. Some strains of *Bacillus* spp. in present study demonstrated quite good inhibition effects against the two pathogens. However, the antagonistic effect greatly varied with the strains or species. The two gram-positive bacteria

isolated from paddy soil were as same as those from the rice plants. They may have mechanism to produce some antagonistic substances against the pathogens. The five bacterial strains isolated from the rice plants of Zhejiang were identified as *Curtobacterium flaccumfaciens* pv. *poin B*, which is a plant pathogenic bacterium in some foreign countries. It is necessary to have further confirmation as their similarity of Biolog and FAME was relatively lower. Furthermore, the result also revealed that some strains of the *Bacillus pumilus* and *Bacillus megaterium* were weakly virulent to rice plants, which are widely distributed in China. As a biocontrol agent its bio-security should be taken into consideration.

It is not clear for the biological characteristics of *Microbacterium liquefaciens* and *Curtobacterium flaccumfaciens* isolated from rice plants. However, their importance should not be neglected since the strains of *Curtobacterium flaccumfaciens* may cause plant bacterial disease while whether the strains of *Microbacterium liquefaciens* are potential biocontrol agents or opportunistic pathogenic bacteria needs to be further studied. When selecting biocontrol agents it should be noted that few nonpathogenic bacterial strains of some species might enhance avirulence as they mix with some pathogenic ones under favorable conditions. We have conducted some research work on the gram-positive bacteria associated with rice but it's just a beginning in this field. The research work on the bacterial groups and their functions of the beneficial gram-positive bacteria should be reinforced. Up to now only 1-5% of the microorganism were described<sup>[12]</sup>, and most of them have not yet been isolated and identified due to the problems of culture and identification. At present study we also found some of the strains could not be identified correctly with the methods used. The more accurate and faster identification methods should be developed for the gram-positive bacteria.

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