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ORIGINAL PAPER

Phenotypic and biological properties of two antagonist *Bacillus subtilis* strains

C. X. Zhang · X. Zhao · Y. X. Jing · T. Chida · H. Chen · S. H. Shen

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Abstract *Bacillus subtilis* KB-1111 and KB-1122 were studied to illustrate their phenotypic and biological properties. Comparison of KB-1111 with KB-1122 in morphology was carried out by microscopy and agar plate assays. Biological assay of the test strains showed that they may possess different physiological pathways from those of reference strain ATCC6501. The assessment of antagonism against the indicator fungi showed that both test strains had broad antifungal characteristics against eight phytopathogenic fungi. Of those fungal species, *Magnaporthe grisea* P131, *Sclerotinia sclerotiorum*, and *F. oxysporium* exhibited high sensitivity to the test strains.

Keywords *Bacillus subtilis* · Comparison · Intra-specific diversity · Biocontrol

Introduction

Bacillus subtilis is the best-characterized member of the *Bacillus* genus, and has become a paradigm organism for investigating Gram-positive bacterial physiology (Kunst et al. 1997). Biological control by using antagonistic rhizobacteria instead of chemical pesticides to suppress crop diseases, offers a powerful contribution to environmental conservation. The endospore-forming Gram-positive bacterium *B. subtilis*, a beneficial rhizobacterium, has become a good candidate as a biocontrol agent (Bais et al. 2004; Ryu et al. 2004). *B. subtilis* has a well developed secretory system (Tjalsma et al. 2004), which produces structurally diverse secondary metabolites with a wide spectrum of antibiotic activity, and has become very valuable for medical and agricultural applications (Liu et al. 2006). In recent years, efforts to control plant diseases with antagonistic bacterial agents have been made successfully (Marten et al. 2000; Han et al. 2005), and some commercial strains of *B. subtilis* have been marketed as biocontrol agents for fungal diseases of crops (Emmert and Handelsman 1999).

In this paper, we introduce two antagonist *B. subtilis* strains with wide spectrum antifungal activities toward eight general indicators of phytopathogenic fungi, give a comparative analysis of their phenotypic and biological properties, and show their intra-specific differences. Such comparative data may contribute to future attempts to correlate the differential antagonism with genetic characteristics.

Materials and methods

B. subtilis KB-1111 and KB-1122 were test strains in this study. To analyse the phenotypic and biological properties of the test strains, and evaluate their potential in vitro antagonism, a wild type reference strain *B. subtilis* ATCC 6051 was used in this research. All the *B. subtilis* strains were grown in Luria-Bertani (LB) broth, or plated on LB agar. The indicator phytopathogenic fungi *Magnaporthe grisea* P131, *Botrytis cinerea*, *Alternaria alternata*, *Rhizoctonia solani*, *Fusarium*

C. X. Zhang · X. Zhao · Y. X. Jing · H. Chen · S. H. Shen (✉)
Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Xiangshan Nanxincun 20, Haidian District, Beijing 100093, China
e-mail: shshen@ibcas.ac.cn

C. X. Zhang · X. Zhao
Graduate College of Chinese Academy of Sciences, Beijing 100039, China

T. Chida
Chemicals Agrochemicals Planning and Development Department, Tokyo, Japan

oxysporum, *Sclerotinia sclerotiorum*, *Verticillium dahliae* and *Phytophthora capsici*, were incubated on potato dextrose agar (PDA)at 28°C. For microscopic observation, the *B. subtilis* cultures were diluted to an OD_{600} of 0.5, and observed under a Zeiss Axioskop 40 microscope (Carl Zeiss). Images were captured by using the Axiocam MRc digital camera (Carl Zeiss), exported as TIFF files, and adjusted with Adobe Photoshop software (Adobe, San Jose, CA, USA). Swarming observation was carried out on agar plates as described by Kearns et al. (2004), with some modifications. The biological analysis of *B. subtilis* KB-1111 and KB-1122 were carried out according to the description of Holt et al. (1994). The determination of in vitro antagonism of test strains against *M. grisea* P131 and other fungal species were carried out in a hyphal diffusion inhibition assay (Liu et al. 2006) with some modifications.

Results and discussion

The cells of the strains KB-1111, KB-1122 were rod-shaped with the size, respectively, of 0.7 ~ 1.0 × 2 ~ 3 μm, 0.8 ~ 0.9 × 2.1 ~ 4 μm, which were similar to the reference strain ATCC6051 with the size of 0.7 ~ 0.8 × 2 μm (Fig. 1a). KB-1122 had longer length of cells than those of KB-1111. In addition, KB-1122 performed a stronger swarming motility and forming communities in LB broth. In agar plates the colonial forms of KB-1111 and KB-1122 were significantly different (Fig. 1b). The former strain formed mucous wrinkles on nutrient LB agar, lacked any glisten, and had an irregular growing mode. In contrast, the latter showed a weak glisten, well-regulated growing pattern, and spreading smooth surface.

Physiological and biochemical characteristics of the test strains *B. subtilis* KB-1111 and KB-1122 compared with the reference strain in this study are listed in Table 1. Most of the biochemical reactions including the utilization of carbohydrates and phenotypic characteristics of biochemical reactions in all the three strains showed either completely positive ones or negative ones with a few exceptions. In our experiments, the test strains were analysed by morphological observation, physiological and biochemical characteristics, showing that their similarities to *B. subtilis* species. Both KB-1111 and KB-1122 showed phenotypic and biological differences from reference strain *B. subtilis* ATCC6501: firstly, in the utilization of carbohydrates, the strains KB-1111 and KB-1122 could utilize lactose, but not inositol (Table 1); secondly, they demonstrated different strengths of tolerance to 7% NaCl, casein hydrolysis and utilization of urea agar (Table 1); finally, they had different swarming mobility and colony spreading in LB medium (Fig. 1). All these differences suggest that KB-1111 and KB-1122 may have different physiological pathways from those of the type strain, though they belong to *B. subtilis* species.

To evaluate the potential of antifungal activity of the antagonist *B. subtilis* KB-1111 and KB-1122, their in vitro antagonism were tested with eight indicator fungi pathogens. Strains KB-1111 and KB-1122 displayed a broad antifungal spectrum (Table 2), suggesting that they are of the significance as biocontrol agents in agricultural production. Among all the eight group experiments, KB-1122 had better inhibition against plant pathogenic fungi than KB-1111, particularly, against *F. oxysporium*, *M. grisea* P131 and *S. sclerotiorum*, in which, the latter two are harmful and cause dramatic yield losses to many crops and horticultural plants in production practice. To our knowledge, *M. grisea* is the most worldwide

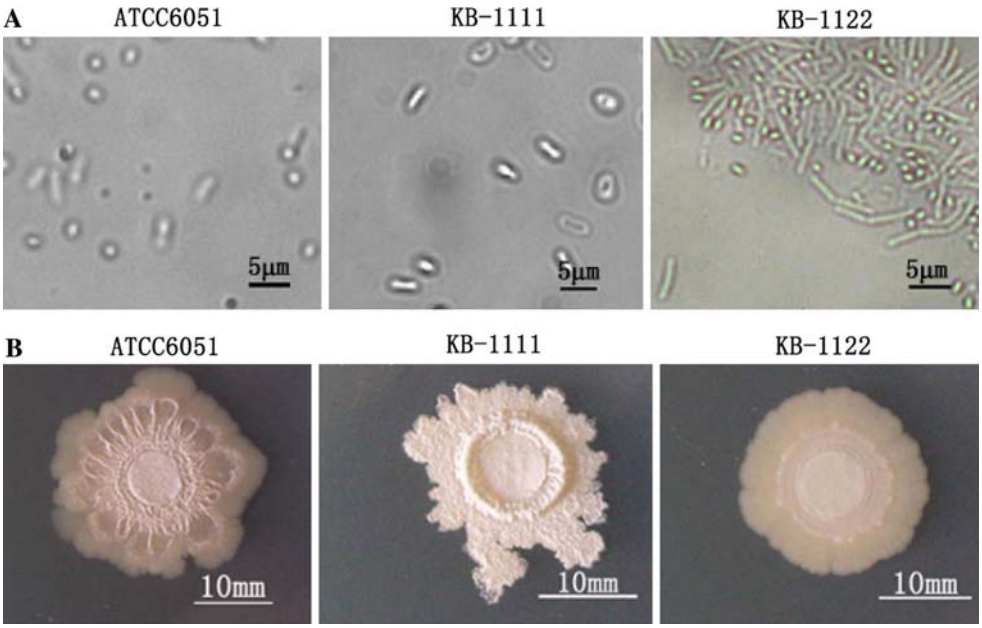

Fig. 1 Morphological characters of *Bacillus subtilis* cells and colonies. (**a**) Microscopic images; (**b**) Colonies spreading on LB agar

Table 1 Physiological characteristics of the strains *B. subtilis* KB-1111 and KB-1122

Characteristics	ATCC6051	KB-1111	KB-1122
Gram-stain	+	+	+
Growth in 7% NaCl	+	+	−
Gelatin hydrolysis	+	+	+
Casein hydrolysis	−	+	−
Starch hydrolysis	+	+	+
Oxidase activity	+	+	+
Nitrate Reductive	+	+	+
M-R reaction	−	−	−
V-P reaction	+	+	+
Catalase activity	+	+	+
Lysozyme reaction	+	+	+
Deamination	−	−	−
Indole production	−	−	−
Urease Agar	−	+	−
Sodium Azide reaction	−	−	−
H_2S production	−	−	−
Utilization of carbonhydrates			
Tyrosine catabolism	−	−	−
Citric acid catabolism	+	+	+
D-glucose	+	+	+
Sucrose	+	+	+
Mannitol	+	+	+
Galactose	+	+	+
Fructose	+	+	+
Starch	+	+	+
Maltose	+	+	+
Trehalose	+	+	+
Sorbitol	−	−	−
Inositol	+	−	−
Lactose	−	+	+

Table 2 Inhibition of indicator pathogens by *B. subtilis* KB-1111 and KB-1122

Indicator pathogens	Inhibition profile		
	ATCC6051	KB-1111	KB-1122
Magnaporthe grisea P131	+	++	+++
Sclerotinia sclerotiorum	+	+	++
Botrytis cinerea	−	+	+
Fusarium oxysporium	−	+	++
Verticillium dahliae	−	+	+
Phytophthora capsici	−	+	+
Rhizoctonia solani	−	+	+
Alternaria alternate	−	+	+

Note: (−): No inhibition or the inhibition zone was not totally clear

(+): The inhibition zone is less than 10 mm

(++): The inhibition zone is from 10 mm to 20 mm

(+++): The inhibition zone is above 20 mm

destructive pathogen of rice and the principal model organism for elucidating the molecular basis of fungal disease for many years (Dean et al. 2005; Soundararajan et al. 2004). In this study, KB-1111 and KB-1122 characterized as *B. subtilis* possessed a wide spectrum of antifungal activities against the most frequently occurring pathogenic fungi that cause diseases in crop and horticultural plants, showing possible application in biological control in future.

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