

Full Length Research Paper

## Identification of antifungal antibiotics genes of *Bacillus* species isolated from different microhabitats using polymerase chain reaction

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Although many *Bacillus* species are known to be good antibiotic producers capable of acting as biocontrol agents, the underlying antimicrobial mechanisms are often poorly understood. In this study, 3 *Bacillus* strains out of 30, demonstrating over 55% mycelial inhibition against *Mycosphaerella musicola* as well as significant control in plate assays, were examined for the presence of antibiotic biosynthetic genes. The use of 16S rRNA revealed that the three strains belonged to *Bacillus amyloliquefaciens* (BB30), *Bacillus atrophaeus* (BB15) and *Bacillus subtilis* (BB13). Sequence analysis of purified PCR products revealed homology with corresponding genes from other *Bacillus* sp. in the GenBank database. Primers specific for iturin D, surfactin, bacillomycin D, bacillomycin A, fengycin and zwittermycin A were used to amplify biosynthetic genes from these *Bacillus* strains using polymerase chain reaction (PCR). This study reveals the equivalent capability of different *Bacillus* strains from various microhabitats to produce the above-mentioned antibiotics and highlights the possibility of using some strains as potential biocontrol agents under different microhabitats distant from their original habitat. Furthermore, it will enable researchers to develop rational strategies for the application of the antagonists and their metabolites within an agroecosystem.

**Key words:** *Bacillus* sp., antagonism, lipopeptides, polymerase chain reaction (PCR).

### INTRODUCTION

Many *Bacillus* species are capable of producing a wide variety of secondary metabolites that are diverse in structure and function (Isabel et al., 2011). Sporulating

Gram positive bacteria like *Bacillus* spp. have been used effectively for plant disease control and its role as biocontrol agent for managing the pathogen in crop

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plants are well reported (Hanene et al., 2012). The production of metabolites with antimicrobial activity is one determinant of their ability to control plant diseases (Athukorala et al., 2009). The Gram-positive bacterium *Bacillus* produces a large number of antibiotics, which are classified as ribosomal or non-ribosomal. Non-ribosomally synthesized circular oligopeptides that contain a fatty acid chain exhibit potent antibacterial or antifungal activity, as represented by surfactin, the iturin C group and fengycin (Compaore et al., 2013). A variety of non-ribosomally produced small lipopeptides belonging to the surfactin family: surfactin and lichenysins (Ramya et al., 2014); the iturin family: iturin A, C, D, and E, bacillomycin A, D, F and L and the fengycin family: fengicins and plipastatins (Compaore et al., 2013); as well as aminopolyols such as zwittermycin A (Luo et al., 2011) are also common. Among these antimicrobial compounds, cyclic lipopeptides (LPs) of the surfactin, iturin and fengycin families (Cao et al., 2011) have well-recognized potential uses in biotechnology and biopharmaceutical applications because of their surfactant properties. Different groups of LPs can confer an advantage to the producing *Bacillus* strains in specific ecological niches (Mukherjee and Das, 2005). The biocontrol agent *Bacillus* produces several classes of broad spectrum lipopeptide antibiotics which are effective suppressors of many plant pathogens (Nagorska et al., 2007; Ongena and Jacques, 2008).

There are reports that *Bacillus* spp. especially *Bacillus subtilis*, *Bacillus atrophaeus* and *Bacillus amyloliquefaciens* have been effective against plant and fruit diseases caused by soil-borne, aerial, or post-harvest fungal diseases (Broggini et al., 2005). In most situations, the involvement of antibiotics has been established (Leclere et al., 2005). Detection of antibiotic production in a particular bacterium is important in determining its competence to be a good biocontrol agent for plant diseases (Duncan et al., 2006). Screening candidate strains for particular antibiotic-encoding sequences followed by direct detection of the antibiotic profile of a particular bacterium provides a rapid approach in comparison with the conventional method of selection (de Souza and Raaijmakers, 2003). PCR based detection of antibiotic biosynthetic genes is very appropriate approach in this regard. Since the *Bacillus* strains used in this study were isolated from different microhabitats, this study would be important for the understanding of the niche specificity of the antibiotics produced by *Bacillus* species.

## MATERIALS AND METHODS

### Fungal pathogen

Infected leaves showing yellow Sigatoka disease symptoms were collected and isolated in V8 juice agar medium. The fungus was purified by using the single hyphal tip technique (Rangaswami, 1972)

maintained under 25°C and used for further studies. Cultures started to produce conidia approximately 26 days later. The pure culture of *M. musicola* was maintained on V-8 juice agar slants throughout the period of study.

### Pathogenicity

Young banana plants (cv. Cavendish) used for inoculation were approximately 1 m high with three to four leaves. Plants were moved into the glass house 30 days prior to inoculation to allow for acclimatization. Plants were watered every third day and exposed to normal sunlight hours. The adaxial surface of the leaves was lightly abraded with a syringe needle to remove a portion of the waxy cuticle. An agar plug overgrown with mycelial culture (MM 13) was then placed onto the epidermis, covered with clear strip of parafilm and marked. Two leaves per plant were inoculated with four replications maintained. Plants were inspected regularly for symptom development and record the typical leaf spot symptoms. The symptoms that appeared on the leaves of banana was assessed after one month at weekly intervals from the date of inoculation by visual observation. *M. musicola* was re-isolated on V-8 medium from the inoculated plants and the infected leaves. This experiment was carried out in PL-480 glasshouse, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India.

### Bacterial isolates and culture conditions

Thirty strains of *Bacillus* species collected from healthy banana rhizosphere soil with different origins were used in this study. New cultures of each bacterium were started by streaking them onto Nutrient agar medium (NA) and incubated at 28°C for 24 h. Pure cultures of each bacterium were maintained in Nutrient agar medium (NA) and stored at -80°C.

### In-vitro efficacy of *Bacillus* sp. against *Mycosphaerella musicola*

The *Bacillus* strains were evaluated against foliar pathogen, *Mycosphaerella musicola* by dual culture technique as described by Dennis and Webster, 1971. The plates were incubated at room temperature (28±2 °C) for five days and the mycelial inhibition of pathogen was measured in millimeter. PDA medium inoculated with the pathogen alone served as the control. Five replications were maintained.

### DNA extraction, PCR analysis and BLAST search

Rhizosphere *Bacillus* spp. was grown in nutrient broth and incubated at 37°C on a rotary shaker at 180 rpm for 16 h. Total DNA (including chromosomal and plasmid DNA) was extracted as described by Robertson et al. (1990) with slight modifications. Purified DNA was quantified by UV spectrophotometry. Products were sequenced at the Bioserve Biotechnologies Pvt. Ltd, Genome Valley, Hyderabad, India. Specific homologies for the sequences were searched for in the GenBank database through the NCBI n-BLAST search and submitted.

### PCR detection of antibiotic biosynthesis genes

Primers and all the reagents were obtained from Bangalore Genei Pvt. Ltd., Bangalore, India. The primers used for molecular

**Table 1.** List of antibiotic primers used in this study.

Primer	Sequence (5' – 3')	Antibiotic	Amplicon size (bp)	References
ITUD-F1 ITUD-R1	CCCCTGTTCTAGATGATCGGAGGAATCTC TGCATCGATTCTGTCCATCTAACCGGCATC	Iturin D	1203	Ramarathnam, 2007
SFP-F SFP-R	ATG AAG ATT TAC GGA ATT TA TTATAA AAG CTC TTC GTA CG	Surfactin	675	Ramarathnam, 2007
BACA-F BACA-R	TGAAACAAAGGCATATGCTC AAAAATGCATCTGCCGTTCC	Bacillomycin A	482	Athukorala et al., 2009
BACD-F1 BACD-R1	TTGAAYGTCAGYGCSCTTT TGCGMAAATAATGGSGTCGT	Bacillomycin D	875	Ramarathnam, 2007
ZWITF2 ZWITR1	TTG GGA GAA TAT ACA GCT CT GAC CTT TTG AAA TGG GCG TA	Zwittermycin	875	Ramarathnam, 2007
FEN D-F FEN D-R	CCTGCAGAAGGAGGAGAAGTGAAG TGCTCATCGTCTTCCGTTTC	Fengycin D	964	Athukorala et al., 2009

analyses are given in Table 1. PCR amplifications were carried out in 20- $\mu$ l reaction mixtures. Samples were quickly transferred in Mastercycler gradient (Eppendorf, Germany) with the following cycle conditions: initial activation at 95°C for 15 min; 40 cycles of 95°C for 1 min, 55°C or 52°C for 1 min, and 72°C extension for 1.5 min; and a final extension at 72°C for 7 min. A total of 5  $\mu$ l of each amplification reaction was analyzed by electrophoresis using a 1.5% agarose gel followed by ethidium bromide staining and ultraviolet visualization.

#### Statistical analysis

The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines Gomez and Gomez, 1984. The percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at significant level ( $P < 0.05$ ) and means were compared by Duncan's Multiple Range Test (DMRT).

## RESULTS

### Pathogenicity

Inoculation of *M. musicola* was done on banana plant, cv. Grand Naine at 2<sup>nd</sup> - 3<sup>rd</sup> leaf stage and maintained under controlled environmental conditions. Symptoms were seen on the leaves from three months after inoculation with *M. musicola* isolate. Differences in the plants were very distinct between inoculated and uninoculated banana leaves. A successful infection showed typical symptom after 12 weeks on inoculated leaves. The inoculation resulted in spot with a dark brown sunken

centre, often surrounded by a yellow halo, spot developed a grey, dried out centre and a peripheral black ring, whereas the symptoms did not develop on uninoculated leaves. In order to prove the Koch postulates, the pathogen was reisolated on V-8 juice agar medium from the diseased leaves. Again it was inoculated to banana leaves, which resulted in the production of the above symptoms (Figure 1).

### Selection of *Bacillus* strains

Thirty isolates from Sigatoka infected banana soils that were initially screened for *in-vitro* inhibition of *M. musicola* on PDA plates were selected. Ten isolates inhibited pathogens, with inhibition by three of these isolates resulting in zones of inhibition that were greater in size (Table 2). These three isolates were identified from sequence of the 16S rDNA gene as *B. amyloliquefaciens* (BB30), *B. atrophaeus* (BB15) and *B. subtilis* (BB13) with accession numbers viz., KF921503, KF921504 and KF921505.

### Identification of antibiotics produced by *Bacillus* strains

PCR was used to screen for genes involved in the biosynthesis of eight antibiotics reported to be produced by three strains of *Bacillus* sp. In most cases, primer pairs were specific for genes involved in biosynthesis of an individual antibiotic. A set of PCR experiments was



Over all view

Inoculation with pin prick plus  
spore suspension spray

Symptom expression

**Figure 1.** Pathogenicity test of *Mycosphaerella musicola* on banana (cv. Grand Naine).

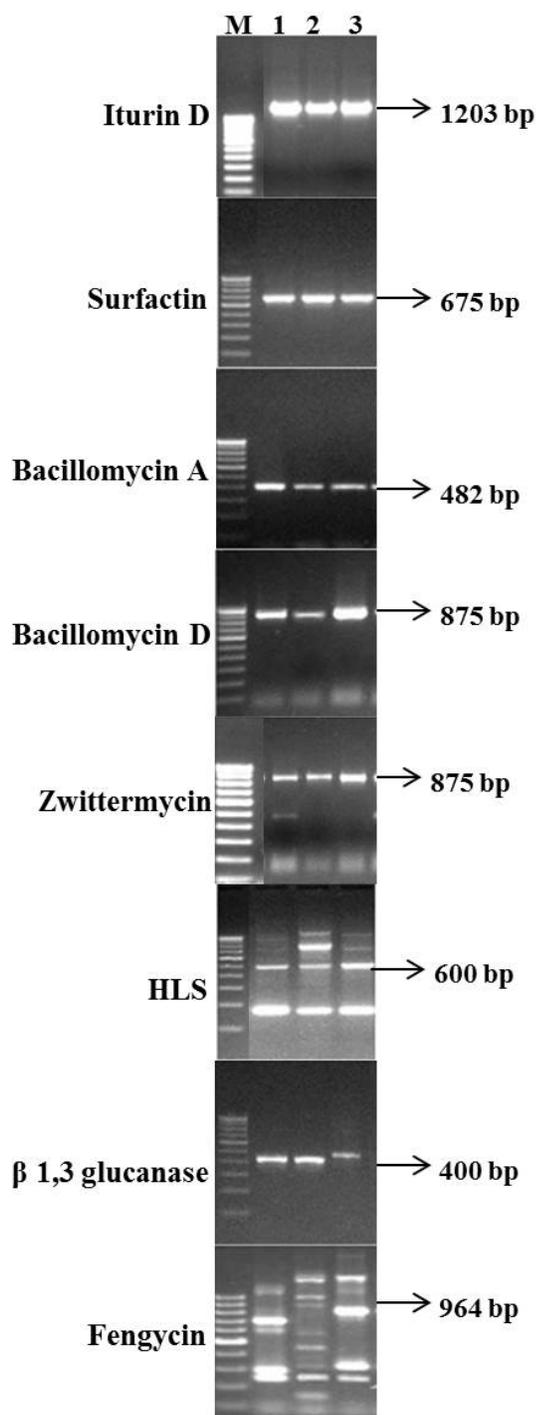
**Table 2.** *In-vitro* antagonism of *Bacillus* strains against *M. musicola*.

Isolates	Mycelial growth (mm)	% inhibition over control
BB13	52.0 <sup>b</sup>	42.22 <sup>b</sup> (40.52)
BB15	51.0 <sup>b</sup>	43.33 <sup>b</sup> (41.16)
BB30	40.0 <sup>a</sup>	55.55 <sup>a</sup> (48.18)
Control	90.0 <sup>d</sup>	0.00 <sup>d</sup> (0.57)

\*Values are means of five replications. Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at P = 0.05. Values in parentheses are arcsine transformed values.

performed with different primer pairs targeted against additional genes involved in biosynthesis of each of the eight antibiotics. Amplicons of the expected size were

obtained with both primer pairs designed to detect genes involved in biosynthesis of iturin D, surfactin, bacillomycin A, and bacillomycin D (Figure 2). An amplicon of the



**Figure 2.** PCR amplification of *Bacillus* antibiotic gene. Lane M, 100 bp ladder; Lane 1, *Bacillus amyloliquefaciens* (BB30); Lane 2, *Bacillus atrophaeus* (BB15), Lane 3, *Bacillus subtilis* (BB13).

expected size was detected randomly in PCR reactions with either of the primer pairs targeted against the

zwittermycin, homo serine lactone,  $\beta$  - 1,3 glucanase and fengycin genes involved in biosynthesis.

The DNA sequences obtained from these amplicons confirmed the identity of these genes. Analysis of sequence from PCR product from reactions with the ITUD-F1/R1 and SFP-F1/R1, primer pair showed 100% identity with a region of the iturin and surfactin biosynthesis gene cluster while sequence from reactions with the BACD-F1/R1 and BACA-F1/R1 primer pair showed 98% identity with a region of bacillomycin D and A synthetase. Analysis of 1203 and 675 bp PCR product from reactions with the ITUD-F1/R1 and SFP-F1/R1 primer pair showed 100% identity with the ituD and surfactin biosynthesis gene cluster. Analysis of 482 bp and 875 bp PCR product using the BACD-F1/R1 and BACA-F1/R1 primer pair showed 98% identity with bacD and bacA, a gene involved in bacillomycin biosynthesis. Finally, analysis of 875, 600, 400 and 964 bp of PCR products from reactions with the ZWIT-F1/R1 and FEND-F1/R1 primer pairs, respectively, showed 95% identity to zwit and 90% identity to fenD, a gene involved in zwittermycin, homoserine lactone, glucanase and fengycinD biosynthesis.

## DISCUSSION

We have isolated three strains of *Bacillus* sp. with potential for use in commercial agriculture in Tamil Nadu for suppression of foliar disease of banana. In addition to suppression of important foliar diseases, other characteristics of commercial interest associated with isolate BB30, BB15 and BB13 were production of endospores for formulation, stimulation of plant growth development and persistence in association with plant roots. Isolates of *Bacillus* sp. have been successfully commercialized and marketed. The Gustafson product Kodiak is widely used for suppression of cotton diseases in the US (Brannen and Kenney, 1997).

Isolates of *Bacillus* sp. have been studied as components of integrated disease management studies (Jacobsen et al., 2004). Integration of *Bacillus*-based treatments into strategies utilizing resistant varieties has resulted in enhanced disease suppression when these resistant varieties do not provide high levels of disease resistance (Jacobsen et al., 2004).

Isolates BB30, BB15 and BB13 may be useful in like strategies for suppression of yellow Sigatoka disease of banana where desirable varieties of these crops expressing high levels of resistance to all phases of these diseases are not available. Isolates BB30, BB15 and BB13 may also prove useful in strategies combining multiple biocontrol agents. Multiple mechanisms of disease suppression expressed by combinations of microbial antagonists are thought to enhance biocontrol performance (Athukorala et al., 2009).

## Conflict of interest

The authors did not declare any conflict of interests.

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