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Detection of antimicrobial peptide genes from antagonistic *Bacillus subtilis* (Bs_Ane) isolated from Neil Islands of Andaman, India

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Abstract

Aim: The present study aimed to identify the native potential *Bacillus* strains of Neil Islands, which have biocontrol potential against major bacterial and fungal plant diseases of Andaman Islands.

Methodology: Twenty five *Bacillus* spp. isolated from chilli rhizosphere soils of Neil Island, South Andaman, India were characterized for antimicrobial potential by *in vitro* and glass house studies. Further, the antimicrobial potential was confirmed by PCR amplification of AMP biosynthesis genes.

Results: Among 25 strains, *Bacillus subtilis* (Bs_Ane) strain showed better *in vitro* antagonistic potential (growth inhibition) against three hytopathogens: *Ralstonia solanacearum* (15.2 mm), *Xanthomonas oryzae* pv. *oryzae* (29.3 mm) and *Colletotrichum gleosporioides* (48%). In glass house studies, Bs_Ane strain showed significant bio-control efficacy (88.9%) against chilli bacterial wilt disease. The strain identity of *Bacillus subtilis* was confirmed by 16s rRNA gene analysis and biologi based phenotypic fingerprinting. The antimicrobial potential of strain was further ascertained by the presence of eight antimicrobial peptide (AMP) gene biosynthesis in PCR amplifications, which confirmed the synthesis of five antibiotics (surfactin, bacilysin, subtilin, subtilisin and fengycins) by Bs_Ane strain.

Interpretation: The overall results revealed that *Bacillus subtilis* (Bs_Ane) strain from Neil islands could be used as a potential biocontrol agent either single/ in consortium while formulating broad spectrum plant disease management in Andaman Islands.

Introduction

Members of the genus *Bacillus* are Gram-positive, aerobic, endospore-forming bacteria often considered microbial factories for the production of a vast array of biologically active molecules which includes antimicrobial compounds (Fickers, 2012) and plant growth promoting substances (Collins and Jacobson, 2003). Most of the species of genus *Bacillus* are considered as safe microorganisms as they possess remarkable abilities to synthesize many substances that have been successfully used in agriculture (Stein, 2005). They are found in diverse environments such as soil, clays, rocks, dust, aquatic environments, vegetation, food and in the gastrointestinal tracts of various insects, animals and environments of extremity (Nicholson, 2002). The major antimicrobial compounds of *Bacillus* species include peptide and lipopeptide antibiotics and bacteriocins (Stein, 2005). The production of antimicrobial substances and sporulation capacity confer wide range survival of *Bacillus* strains in different environments.

There are different mechanisms of action including antibiosis, induction of defense responses in the host plant, and competition for nutrient sources and space (Choudhary and Johri, 2009; Solanki et al., 2012) reported for effective bio control efficacy of *Bacillus* species. The secondary metabolites produced by several species and strains of genus *Bacillus* have been found to show antibacterial or antifungal activity against different phytopathogens (Katz and Demain, 1977; Ongena et al., 2008, Yu et al., 2002, Santhiyya et al., 2015). The antagonistic efficacy of *Bacillus* species has also been mainly linked to the presence of AMP biosynthetic genes such as bmyB, fenD, ituC, and srfA which involves the production of cyclic lipopeptides such as bacillomycin, fengycin, iturin and surfactin A having direct antimicrobial activities (Mora et al., 2011).

The Andaman and Nicobar Islands archipelago situated in the Bay of Bengal which is near to Myanmar in the North, Thailand in the East and Indonesia in the South. The hot, humid and tropical climatic conditions prevailing in islands favors many plant diseases which results in severe yield loss of crop plants. The usage of chemical is not recommended due to fragile nature and uniqueness of island environment, but the organic disease management is encouraged. Neil island is one among the agriculturally important area where cultivation practices are being carried out throughout the year. The reports on usage of native microbes for control of plant diseases in Andaman Islands is meagre. In view of the above all, an attempt was made to study the antagonistic activity of *Bacillus* strain, Bs_Ane isolated from chilli rhizosphere soil of Neil Islands of Andaman and Nicobar Islands, against some important and native plant pathogens, and its correlation with the presence of antibiotic synthetic genes in the Bs_Ane strain.

Materials and Methods

Collection of soil samples and isolation of bacteria : The soil samples were collected from healthy rhizosphere of chilli plants from 20 different fields of Neil Islands. *Bacillus* was isolated from chilli rhizosphere soils of Neil Island (South Andaman), India by selective isolation technique as suggested by Travers et al. (1987). Briefly, one gram of soil was incubated for 10 min at 80°C followed by mixing with one ml of sterile water. Then 100 µl of solution was streaked on nutrient agar plates and incubated for 24-48 hrs at 28±2°C. The dominant colonies were selected and sub cultured based on its morphological characters. A total of 25 strains were purified and maintained in 20% glycerol for long term storage.

Phytopathogens of solanaceous crops : Cultures of three plant pathogens viz., *Ralstonia solanacearum* (isolated from chilli), *Xanthomonas oryzae* (isolated from rice) and *Colletotrichum gloeosporioides* (isolated from chilli fruit) causing solanaceous bacterial wilt, rice bacterial blight and chilli fruit rot, respectively in Andaman Islands were obtained from the culture collection center, Plant Pathology Laboratory, CIARI, Port Blair. All the cultures were properly maintained by sub culturing on their respective media under laboratory conditions.

Screening of bacteria for antimicrobial activity against phytopathogens : Antibacterial antagonistic ability of the rhizosphere bacilli against virulent *R. solanacearum* and *Xanthomonas oryzae* strains were evaluated by agar diffusion assay method on KB-medium (Ramesh and Gauri, 2012). Briefly, 24-48 hr old grown *R. solanacearum* and *X. oryzae* strains were mixed in the King's B medium (0.25 µl ml⁻¹ at 10⁸ CFU ml⁻¹) separately. Twenty µl of the exponential growth phase antagonistic bacterial cultures (approximately 3.5 x 10⁸ CFU ml⁻¹) were added in the 7mm wells and plates were incubated at 28°C for 24 h. sterile water was used as negative control and suitable replications were maintained and the experiment was replicated thrice. Inhibition zone around the well (radius in mm) was measured after 48 hrs and the antagonistic activity as zone was calculated.

Antifungal antagonistic effect of strain Bs_Ane was evaluated by dual-culture assay using relative growth of *C. gloeosporioides* (Zhou et al., 2011). A 5-mm agar plug from an agar culture of *C. gloeosporioides* was placed at the center of the PDA plate, and antagonistic strains were streaked at 2 equidistance sites at 2.5 cm from the centre or margin. Control plates were maintained only with *C. gloeosporioides* in the absence of test strain as control. The treatments were replicated thrice and the plates were incubated at 28°C for 7 days. The antagonistic effect of test strain on *C. gloeosporioides* was observed based on the relative growth of fungi in treatment and control.

Identification of potential *Bacillus* strain : The most potential strain Bs_Ane was subjected to Biolog based phenotyping using the Microlog system (Biolog, Inc., Hayward, CA). Bacterial cell suspensions prepared as per manufacturer's instructions were pipetted into each of the 96 wells of the micro plates and incubated at 28-30°C for 16–24 hrs and then read with an automated plate reader (Biolog, Inc.).

For 16S rRNA gene amplification, 1.5-kb RNA gene fragment was amplified by PCR using primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGA TCCAGCCGCA-3') described by Edwards *et al.* (1989). Reaction (50µl) contained 100 ng genomic DNA, 2.5 mM concentrations of each deoxynucleotide triphosphate, 10 pMol concentrations of each forward (pA) and reverse (pH) primer, 25 mM MgCl₂, 10X Taq buffer A (GeNeI) and 3 U of Taq DNA polymerase. Polymerase chain reaction was performed in a C1000TM Thermal Cycler (Bio-Rad, Germany) with an initial denaturation of 92 °C for 2 min and 10 s followed by 35 cycles of 92 °C for 1 min, 48 °C for 30 s and 72 °C for 2 min and 10 s and a final extension step of 72 °C for 6 min and 10 s. The PCR products were resolved using 1% agarose gel. 16S rRNA gene was sequenced and analyzed by Xcelris (Ahmedabad, India). The sequences obtained were concatenated, edited and end trimmed in DNA Baser software and CLC sequence viewer and blast search was performed in NCBI database.

PCR detection of antibiotic biosynthesis genes : The presence of antibiotic genes in *Bacillus* strain Bs_Ane were identified using PCR with different set of primers as described by Chung *et al.*, 2008 (Table 2). PCR amplifications were carried out in 50-µl reaction mixtures. Samples were quickly transferred to C1000TM Thermal Cycler (Bio-Rad, Germany) with the following cycle conditions: initial activation at 95°C for 15min; 40 cycles of 95°C for 1min, 55°C or 52°C for 1min, and 72 °C extension for 1.5min; and a final extension at 72 °C for 7min. A total of 5µl of each amplification reaction was analyzed by electrophoresis using 1.5% agarose gel followed by ethidium bromide staining and ultraviolet visualization.

Evaluation of Bs_Ane for chilli bacterial wilt in pot trials : The

biocontrol efficacy of Bs_Ane strain was tested against chilli bacterial wilt pathogen *Ralstonia solanacearum* under glass house condition. The virulent pathogen strain CRs- Sg1 was collected from culture collection center, Plant Pathology Laboratory, CIARI, Port Blair. Thirty-day-old chilli plants (*Capsicum annuum*) were transferred to 15 cm diameter plastic pots containing potting mixture of 2:1:1 (Red soil: FYM: Sand) and maintained under glass house conditions. After acclimatization for one week period, freshly prepared 50 ml of Bs_Ane bacterial suspension (10⁸cfu ml⁻¹) were poured in the soil at the base of each plant. One week after inoculation of bio control, a 20 ml suspension of *R. solanacearum* strain (5×10⁷cfu ml⁻¹) was poured in to the soil to give a final bacterial concentration of 10⁶cfu g⁻¹ d.wt. Treated plants were incubated at 28-32 °C and at 80-90% RH under glasshouse conditions for wilt development up to 30 days. Three replications were maintained per treatment with nine chilli plants in three pots. Wilt incidence was recorded at weekly interval up to one month, and wilting percentage was calculated by the following formula as given by Guo *et al.* (2004). The disease incidence and bio-control efficiency were calculated.

Results and Discussion

A total of 25 *Bacillus* spp. were obtained from 20 soil samples collected from rhizosphere soils of chilli from Neil Islands, India (Data not shown). All the strains were screened for the antagonistic activity against three phytopathogens viz., *Ralstonia solanacearum*, *Xanthomonas oryzae* pv. *oryzae* and *Colletotrichum gloeosporioides* *in vitro*. Notably, strain Bs_Ane showed highest apparent antagonistic activity against all the three pathogens tested (Table 1). It showed inhibition zone of 15.2 mm and 29.3 mm against *R. solanacearum* and *X. oryzae*, respectively, in agar diffusion method whereas in case of chili fruit rot pathogen *C. gloeosporioides*, strain Bs_Ane showed 48% of growth inhibition in dual culture assay. Earlier, the antagonistic potential against multiple plant pathogens was described by Chung *et al.* (2008) in *Bacillus subtilis* strain ME488, where this strain could suppress the growth of 39 out of 42 plant pathogens tested using *in-vitro* antagonistic assay. Also, Sayed *et al.* (2014) reported that the *Bacillus* strains isolated from extreme arid soils of Saudi Arabia could inhibit the multiple

Table 1 : Antagonistic efficacy of *Bacillus subtilis* strain Bs_Ane

Strain name	Antagonistic efficacy (<i>in vitro</i>)				Antagonistic efficacy against <i>Ralstonia solanacearum</i> in pot trials		
	<i>Ralstonia solanacearum</i>		<i>Xanthomonas oryzae</i>		<i>Colletotrichum gloeosporioides</i>		
	Mean diameter of inhibition zone (mm) ^x	Degree of antagonism ^y	Mean diameter of inhibition zone (mm) ^x	Degree of antagonism ^y	Mycelial growth of pathogen (mm) ^x	Reduction overcontrol (%)	Wilt incidence ^z (%)
							Biocontrol efficacy (%)
Bs_Ane	15.0	+++	29.0	++++	15	48	11.1
							88.9

^x - Values are mean of three replications; ^y -Weak '+' (width of inhibited growth area 1–5 mm), medium '++' (6–10 mm), strong '+++ (11–20 mm), very strong '++++' (over 20 mm) (Arsenijevic *et al.*, 1998).

Table 2 : Primers used for PCR detection of antibiotic biosynthesis in *Bacillus subtilis* strain (Bs_Ane) strain

Antibiotic	Genes	Primers*	Sequences	PCR product size
Surfactin	srfA	SRFA-F1	52-AGAGCACATTGAGCGTTACAAA	626bp
		SRFA-R1	52-CAGCATCTCGTTCAACTTTCAC	
	sfp	SFP-F1	52-ATGAAGATTACGGAATTTA	675bp
		SFP-R1	52-TTATAAAAGCTCTTCGTACG	
Bacilysin	bacD	BACD-F1	52-AAAAACAGTATTGGTYATCGCTGA	749bp
		BACD-R1	52-CCATGATGCCTTCKATRGTGAT	
Subtilin	spaC,spas	SPACS-F1	52-CCGGACAGGAGTATTTAAGGA	460bp
		SPACS-R1	52-CAGTTACAAGTTAGTGTTTGAAGGA	
	spaB	SPAB-ERIB-F1	52-GCACAGATGGAAAATCTGAAG	688bp
		SPAB-ERIB-R1	52-GAAAATTGCTCCCAAATGA	
Subtilosin	alba	ALBA-F1	52-TTGTTTATAGACAGATGTTTCCA	625bp
		ALBA-R1	52-GGCTCTCTTTTCGCATGAGT	
	albF	ALBF-F1	52-TCAACAGCTGGATGAACGAAC	888bp
		ALBF-R1	52-AGGCGGTAYGTTTGCTGWATCT	
Fengycin	fenB	FENB-F1	52-CCTGGAGAAAGAATATACCGTACCY	670bp
		FENB-R1	52-GCTGGTTCAGTTKGATCACAT

Fig. 1 : PCR detection of antibiotic biosynthesis genes in Bs_Ane strain. Lane M is a 100bp ladder. Lane 1 is srfA, Lane 2 is sfp, Lane 3 is bacD, Lane 4 is spaC/spaS, Lane 5 is spaB, Lane 6 is alba, Lane 7 is albF, Lane 8 is fenB

fungal pathogens *in-vitro* (*Fusarium oxysporum*, *Sclerotinia sclerotiorum*)

The strain Bs_Ane was further subjected for identification using 16S rRNA gene sequence analysis and BIOLOG based phenotyping. BLAST analysis of 16S rRNA gene sequence further confirmed the identity of the strain, Bs_Ane as *Bacillus subtilis*, since sequence homology was greater than 99% to that

of *Bacillus subtilis* of GenBank. The 16S rRNA gene sequence of Bs_Ane was assigned GenBank Accession number (KP864636). Similarly BIOLOG identification based on the utilization of 71 carbon sources and 23 chemical sensitivity assays also confirmed the identity of the strain as *Bacillus subtilis* (0.75 similarity co-efficient).

In glass house studies, the wilt incidence in chilli was recorded 10 days after inoculation of Bs_Ane and up to 30 days. In the experiment as compared to control (100%) the bacterial strain Bs_Ane (11.1%) showed significant reduction in disease incidence. The biocontrol efficiency of Bs_Ane in greenhouse was 88.9% when compared to control. Similarly the biocontrol efficacy of *Bacillus* strains against bacterial wilt disease was recorded in different crops under glass house conditions. Zhong *et al.*, 2011 reported that the bio-organic fertiliser (BOF) fortified with a mixed inoculation of *Bacillus* strains QL-5 and QL-18 effectively decreased *R. solanacearum* disease incidence in both greenhouse and field conditions. Similarly Yuan *et al.* (2013) reported that bacillus enriched biofertilizers could suppress tobacco bacterial wilt up to 85.1% using under glass house conditions. Also, Kurabachew *et al.* (2013) reported that *Bacillus cereus* strain BC1AW could significantly reduce the incidence of bacterial wilt in tomato genotypes King Kong 2 (moderately resistant) and L390 (susceptible) in the pot experiments by 46.8% and in by 33.6% respectively.

Antimicrobial peptide genes (AMPs) has been reported for biocontrol activity of *Bacillus subtilis* against wide range of plant pathogens (Joshi and Mc Spadden 2006; Romero *et al.*, 2007, Gonzalez-Sanchez *et al.*, 2010). The presence of AMP biosynthetic genes were ascertained by PCR (Fig. 1) in the Bs_Ane strain and the result revealed the amplification of eight antimicrobial peptide synthetic gene primers viz., surfactin- srfA gene (626 bp) and sfp gene (675 bp), Bacilysin D- bacD gene

(749 bp), subtilin- spaC/S gene (460 bp) and spaB gene (688 bp), subtilosin- albA gene (625 bp) and albF gene (888 bp), and fengycin- fenB gene (670 bp) (Table 2). The results of detection of antibiotics biosynthesis genes showed that presence of five antibiotics viz., surfactin, bacilysin, subtilin, subtilosin and fengycins produced by Bs_Ane. Hence, the production of antibiotics might be one of the important mechanisms for the antagonistic efficiency of *B. subtilis* (Bs_Ane) against bacterial and fungal phytopathogens *in vitro*. Similarly, the co-production of antibiotics by single *Bacillus* strains has been recorded earlier. Kim *et al.*, (2010) demonstrated the production of iturin, fengycin, and surfactin by *B. subtilis* CMB32, similar to that observed in strain P7 (Yao *et al.*, 2003). Also, *B. subtilis* strain ME488 with iturin, bacilysin and mersacidin antibiotics played a vital role in suppression of *Fusarium* wilt of cucumber and *Phytophthora* blight of pepper (Chung *et al.*, 2008).

In the present study, *Bacillus subtilis* strain Bs_Ane collected from chilli rhizosphere of Neil Islands, India showed good *in vitro* antagonistic activity against fungal and bacterial plant pathogens tested *in vitro* and also showed positive for production of five AMP genes. So this could be effectively used as broad spectrum potential biocontrol agent for the management of plant diseases, either as a single or in consortia with other potential strains in different formulations after confirming the field efficacy by further studies.

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