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Antimicrobial Peptide Genes Present in Indigenous Isolates of *Bacillus* spp. Exhibiting Antimicrobical Properties

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A B S T R A C T

An attempt was made to detect the presence of antimicrobial peptide genes (AMPs) in ten different indigenous isolates of *Bacillus* spp. proved to be effective against *M. incognita*, *Fusarium oxysporum* f.sp.*lycopersici* and their disease complex. The study revealed the presence of iturin A, iturin C, iturin D, surfactin, bacilomycin D, fengycin D, zwitermycin, bacillomycin bac D, bacilysin bcc AB and fengycin CAE in the above isolates of *Bacillus* spp. Among the ten different AMPs, the iturin A gene was found to present in all the ten isolates of *Bacillus* spp. Of all indigenous isolates B. thuringiensis (TLBRE2), *B. cereus* (CLB2D) were in possession of higher number of eight AMPs. It was followed by *B. tequilensis* (TLB2) with seven genes, *B. licheniformis* and *B. Cereus* (TSB 3, CLB2 and TSB4D) with six genes, *B. weihenstephanensis* (CLB3) and *B. subtilis* (TLBRE1) with four genes and *B. subtilis* (TSB5) with three genes.

Introduction

Bacillus spp. is well known for its antimicrobial properties. The members of *Bacillus* spp., produce a wide array of molecules of diverse function. For example *B. subtilis*, has 4-5 per cent of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial peptides (AMP). The diverse antibiotics produced by *Bacillus* spp. includes iturin, surfactin, fengycin, bacillomycin, zwittermicin, bacilysin, mersacidin, ericin, subtilin etc with broad spectrum action and it is reported to vary from species to species. As evidenced by genome sequencing, bacteria dedicate up to 20 per cent of their genome to the biosynthesis of secondary metabolites underscoring the importance of these small molecules to the fitness of the organism in its native environment.

In this regard the antimicrobial peptides of *Bacillus* spp. specifically play a vital role in protecting plants against diseases and pests. Tamalika Sarangi (2014) collected ten indigenous isolates of *Bacillus* spp.

experimented for their biocontrol potential and proved to be effective against *M. incognita* and *Fusarium oxysporum f.sp.lycopersici*.

Hence the present study is programmed to detect the AMPs genes present in indigenous isolates of *Bacillus* spp. responsible for their broad spectrum action.

Materials and Methods

Isolation of genomic DNA

The genomic DNA of all endophytic isolates were isolated using the standard protocol of Cetyl Trimethyl Ammonium Bromide (CTAB) method proposed by Knapp and Chandlee (1996) with slight modifications made by Melody (1997). The actively grown above bacterial culture of 25 ml broth was taken in a centrifuge tube and centrifuged at 6,000 rpm for 5 min at 4ºC. After the removal of supernatant, the pellet was suspended in 1 ml Tris (TE) buffer added with 0.5 ml of 1-butanol, vortexed well to mix with the cells in order to remove extracellular materials and centrifuged at 5000 rpm for 5 min at 4^{o}C. The supernatant was discarded and the pellet was resuspended in 2 ml of TE buffer and centrifuged at 6,000 rpm at 4^{o}C for 5 min to remove all traces of butanol. The pellet was again re-suspended in 1ml TE buffer added with 100 µl of lysozyme (10 mg ml^{-1} freshly prepared) and incubated at room temperature for 5 min. After incubation, 100 µl of 10 per cent Sodium Dodecyl Sulphate (SDS) and 25 µl of 100 µg ml^{-1} proteinase K were added, mixed well and incubated at 37ºC for an hour. To the above mixture 200 µl of 5 M NaCl was added and mixed well. With this 150 µl of CTAB solution was added, mixed well and incubated at 65^{o}C for 10 min. The mixture was extracted with 1 ml of phenol: chloroform mixture in the ratio of 25:24, mixed well and centrifuged at 6000 rpm for 15 min at 4ºC. The aqueous layer was transferred carefully to a 2 ml microfuge tube and DNA was precipitated by adding 0.6 volume of ice cold iso-propanol, incubated overnight at - 20ºC. The DNA was pelleted by centrifugation at 12,000 rpm for 15 min at 4ºC. The pellet was washed with 70 per cent ethanol and dried under vacuum for 10 min and resuspended in 50 µl of TE buffer. One µl DNAse, free RNAse (10 mg ml^{-1}) was also added by swirling and incubated at 37ºC for 30 min. The isolated DNA was stored at - 20ºC for further use.

Molecular detection of antibiotic genes of *Bacillus* spp

Iturin A

The forward primer ITUD1F (5'GATGCGATCTCCTTGGATGT3') and reverse primer ITUD1R (5'ATCGTCATG TGCTGCTTGAG3') were used for amplification of iturin A gene (647 bp) (Ramarathnam, 2007). The mixture of 20 µl contained approximately 50 ηg of total DNA, 5 mM each dNTPs, 20 pmol of each forward and reverse primer and 0.5 U of *Taq* DNA polymerase. The PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the conditions of initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 10 min.

Iturin C

The forward primer ITUCF1 (5′CCCCCTCGGTCAAGTGAATA3') and reverse primer ITUCR1 (5′TTGGTTAAG CCCTGATGCTC3') were used for amplification of iturin C gene (594 bp) (Chung *et al.,* 2008). The 20 µl mixture contained approximately 50 ηg of total DNA,

5 mM each dNTPs, 20 pmol of each forward and reverse primer and 0.5 U of *Taq* DNA polymerase. The PCR amplification was performed as above in a thermocycler (Eppendorf Master cycler, German) using the conditions of initial denaturation at 95^0C for 15 min, 40 cycles consisting of 95°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1.5 min (primer extension) and final extension at 72°C for 7 min.

Iturin D

The forward primer ITUD-F1 (5'TTGAAYGTCAGYGCSCCTTT3') and reverse primer ITUD-R1 (5'TGCGMAAATAATGGSGTCGT3') were used for amplification of iturin D gene (482 bp) (Chung *et al.,* 2008). The 20 µl mixture contained approximately 50 ηg of total DNA, 5 mM each dNTPs, 20 pmol of each forward and reverse primer and 0.5 U of *Taq* DNA polymerase. The PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the conditions of initial denaturation at 95°C for 15 min, 40 cycles consisting of 94°C for 1 min (denaturation), 57°C for 1 min (annealing), 72°C for 1.5 min (primer extension) and final extension at 72°C for 10 min.

Surfactin

The forward primer SUR3F (5'ACAGTATGGAGGCATGGTC3') and reverse primer SUR3R (5'TTCCGCC ACTTTTTCAGTTT3') were used for amplification of surfactin gene (441 bp) (Ramarathnam, 2007). The 40 µl PCR reaction mixture contained DNA template 50 ηg, 1X Taq buffer, 0.2 mM of each of dNTP mixture, 1 µM of each primers, 1.5 mM $MgCl_2$ and 2U of *Taq* DNA polymerase. The PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the conditions of initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 57°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 10 min.

Bacillomycin D

The forward primer BACC1F (5'GAAGGACACGGCAGAGAGTC3') and reverse primer BACC1 R (5'CGCTGATGACTGTTCATGCT 3') were used for amplification of bacillomycin D gene (875 bp) (Athukorala *et al.,* 2009). The 20 µl mixture contained approximately 50 ηg of total DNA, 5 mM each dNTPs, 20 pmol of each forward primer and reverse primer and 0.5 U of *Taq* DNA polymerase. The PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the conditions of initial denaturation at 94°C for 3 min, 40 cycles consisting of 94 °C for 1 min (denaturation), 59°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 10 min.

Fengycin D

The forward primer FEND1F (5'TTTGGC AGCAGGAGAAGTTT3') and reverse primer FEND1 R (5'GCTGTCCGTT CTGCTTTTTC3') were used for amplification of fengycin gene (964 bp) (Athukorala *et al.,* 2009). The 20 µl mixture contained approximately 50 ηg of total DNA, 5 mM each dNTPs, 20 pmol of each forward primer and reverse primer and 0.5 U of *Taq* DNA polymerase. The PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the conditions of initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 10 min.

Zwittermycin

The forward primer ZWT1F (5'TTTGGC AGCAGGAGAAGTTT3') and reverse primer ZWT1 R (5'GCTGTCCGTTC TGCTTTTTC3') were used for amplification of Zwittermycin gene (779 bp) (Athukorala *et al.,* 2009). The 20 µl mixture contained approximately 50 ηg of total DNA, 5 mM each dNTPs, 20 pmol of each forward and reverse primer and 0.5 U of *Taq* DNA polymerase.The PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the conditions of initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 57°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 10 min.

Bacilysin bacD

The forward primer BACDF1 (5′AAAAAC AGTATTGGTYATCGCTGA3') and reverse primer BACDR1 (5′CCATGATGCC TTCKATRCTGAT3') were used for amplification of Bacilysin bacD gene (749bp) (Chung *et al.,* 2008). The 20 µl mixture contained approximately 50 ηg of total DNA, 5 mM each dNTPs, 20 pmol of each forward primer and reverse primer and 0.5 U of *Taq* DNA polymerase. The PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the conditions of initial denaturation at 95°C for 15 min, 40 cycles consisting of 95°C for 1 min (denaturation), 52°C for 1 min (annealing), 72°C for 1.5 min (primer extension) and final extension at 72°C for 7 min.

Bacilysin bacAB

The forward primer BACAB-F1 (5′CTTCTCCAAGGGGTGAACAG3') and reverse primer BACAB-R1 (5′TGTAGG TTTCACCGGCTTTC3') were used for amplification of Bacilysin bacAB gene (815bp) (Chung *et al.,* 2008). The 20 µl mixture contained approximately 50 ηg of total DNA, 5 mM each dNTPs, 20 pmol of each forward and reverse primer and 0.5 U of *Taq* DNA polymerase. The PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the conditions of initial denaturation at 95°C for 15 min, 40 cycles consisting of 95°C for 1 min (denaturation), 50°C for 1 min (annealing), 72°C for 1.5 min (primer extension) and final extension at 72°C for 7min.

Fengycin CAE

The forward primer FENCEA F1(5′CCCATCCGACYGTAGAAG3') and reverse primer FENCEA R1 (5′GTGTAAGC RGCAAGYAGCAC3') were used for amplification of Fengycin CAE gene (820 bp) (Chung *et al.,* 2008). The 20 µl mixture contained approximately 50 ηg of total DNA, 5 mM each dNTPs, 20 pmol of each forward and reverse primer and 0.5 U of *Taq* DNA polymerase. The PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the conditions of initial denaturation at 95°C for 15 min, 40 cycles consisting of 95°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1.5 min (primer extension) and final extension at 72°C for 7 min

Results and Discussion

The isolated ten endophytic bacteria as *Bacillus* spp. were subjected to PCR for the detection of antibiotic biosynthetic genes and the result of the study is furnished in table 1.

Iturin A

The specific primers meant for iturin A gene bind with the genomic DNA and amplified to form an amplicon size of 647 bp. Therefore this positive reaction of PCR revealed that all

the ten species / isolates *viz*. *B. weihenstephanensis*, *B. cereus*, *B. subtilis*, *B. licheniformis*, *B. thuringiensis* and *B. tequilensis* possessing the iturin A gene.

Iturin C

In contrast, only five isolates of *Bacillus* spp. *viz*. *B. weihenstephanensis* (CLB3), *B. subtilis* (TLBRE1), *B. thuringiensis* (TLBRE2) and *B. cereus* (CLB2 and CLB2D) were found to have iturin C gene since the test undertaken for the detection of antibiotic biosynthetic gene showed positive reaction of binding of specific primers with the genomic DNA and amplifying to form an amplicon size of 594 bp which is characteristic for the detection of iturin C gene.

Iturin D

In the test for the detection of iturin D gene it is observed that six native isolates *viz. B. subtilis* (TSB5), *B. weihenstephanensis* (CLB3), *B. tequilensis* (TLB2), *B. thuringiensis* (TLBRE2) and *B. cereus* (TSB4D and CLB2) were found in possession of iturin D gene as the test is positive by binding of specific primers with the genomic DNA and amplifying to form an amplicon size of 482 bp which is characteristic of the iturin D gene.

Surfactin

With regard to the detection of antibiotic biosynthetic gene, the surfactin gene was found to present only in seven isolates *viz*. *B. weihenstephanensis* (CLB3), *B. thuringiensis* (TLBRE2), *B. subtilis* (TLBRE1), *B. cereus* (CLB2, TSB4D and CLB2D) and *B. tequilensis* (TLB2) through positive reaction showing the binding of specific primers meant for surfactin with genomic DNA and amplifying to form an amplicon size of 441 bp.

Bacillomycin D

Among ten isolates of *Bacillus* spp. subjected for the detection of Bacillomycin D gene except two native species / isolates of *B. weihenstephanensis* (TSB4) and *B. cereus* (CLB2) others showed the possession of the above antibiotic biosynthetic gene. It was confirmed through the positive reaction of binding of specific primers with genomic DNA and amplifying with the fragment size of 875 bp. with the remaining isolates of *Bacillus* spp.

Zwittermycin

The positive reaction of binding of specific primers with the genomic DNA and amplifying to form an amplicon size of 779 bp was noticed with only one isolate TLB2 of *B. tequilensis*.

Bacilysin bac D

The test performed for the detection of bacilysin bac D gene was found to be positive with the four native isolates *viz. B. licheniformis* (TSB3), *B. tequilensis* (TLB2), *B. thuringiensis* (TLBRE2) and *B. cereus* (CLB2D) based on binding of specific primers with the genomic DNA and amplifying to form an amplicon size of 749 bp which is characteristic of bacilysine bac D gene.

Bacilysin bac AB

Among the ten native isolates of *Bacillus* spp. tested for the presence of bacilysin bac AB gene, only five isolates *viz. B. licheniformis* (TSB3), *B. weihenstephanensis* (TSB4), *B. tequilensis* (TLB2), *B. thuringiensis* (TLBRE2) and *B. cereus* (CLB2D) were found to be positive through binding of specific primers with the genomic DNA and

amplifying to form an amplicon size of 815 bp due to the possession of bacilysine bac AB gene.

Fengycin CAE

Six native isolates *viz*. *B. licheniformis* (TSB3), *B. weihenstephanensis* (TSB4), *B. thuringiensis* (TLBRE2) and *B. cereus* (CLB2, CLB2D and TSB4D) were found to be positive in the test performed for the detection of the above gene. In this test the positive reaction was confirmed through binding of specific primers with the genomic DNA and amplifying to form an amplicon size of 820 bp which is characteristics to denote the presence of fengycin CAE gene. In the above test undertaken for the detection of antibiotic biosynthetic genes, the iturin A gene was found to be present in all the ten isolates of *Bacillus* spp. Although all the above ten native isolates of *Bacillus* spp. were found in possession of one or more genes, the isolates of *Bacillus* spp. *viz., B. thuringiensis* (TLBRE2), *B. cereus* (CLB2D) were found to possess higher number of eight antibiotic biosynthetic genes. It was followed by *B. tequilensis* (TLB2) with seven genes, *B. licheniformis* and *B. cereus* (TSB3, CLB2 and TSB4D) with six genes; *B. weihenstephanensis* (CLB3) with five genes; *B. weihenstephanensis* (TSB4) and *B. subtilis* (TLBRE1) with four genes and *B. subtilis* (TSB5) with three genes. The antimicrobial peptides are potent, broad spectrum antibiotics and they are being currently used as novel therapeutic agents. Antimicrobial peptides have been demonstrated to kill pathogens, envelop pathogenic bacteria, virus, fungi and nematodes. The AMPs produced by *Bacillus* spp. have been implicated in the biocontrol of several plant pathogens causing aerial, soil and postharvest diseases and in the promotion of plant growth. In addition to lipopeptides, other peptidic compounds such as bacilysin, a dipeptide described in *B. amyloliquefaciens* FZB42 and subtilin, a lantibiotic described in *B. subtilis* are active in the biocontrol of plant pathogens. The biocontrol ability of several strains of *Bacillus* in the management of plant pathogens has been linked to the presence of the AMP biosynthetic genes *viz.* bmyB, fenD, ituC, srfAA, and srfAB. According to Mora *et al.*, (2011) the simultaneous production of different AMPs were important for the antagonistic activity of *Bacillus* spp.

In addition, the genome analysis of the *B. amyloliquefaciens* FZB 42 revealed the presence of several AMP genes rather than one or two genes responsible for broad spectrum of action against plant pathogens as indicated by Chen *et al.,* (2009). Similar genes have been reported in the commercialized strains of *B. subtilis viz.* GB03, QST713 and MBI 600 which are presently available commercially in the USA market (Arguelles *et al.,* 2009). Besides, srrfAA, bmyB, bacA, and fend genes dominant in plant environment and reinforces the competitive role of surfactin, bacillomycin, fengycin and bacilysin in conferring strain fitness in natural environment (Mora *et al.,* 2011). Hence, an understanding of the role of AMPs in the ecological fitness of the plants is imperative.

Antibiotic production by the bacterial endophytes plays a major role in plant disease suppression. For example the endophytic *Bacillus* spp. produces more than 24 antibacterial (Ono and Kimura 1991 and Silo-Suh *et al.,* 1994), antifungal (Nishikiori *et al.,* 1986 and Maget- Dana and Peypoux, 1994), antinematicidal (Broderick *et al.,* 2003) and antiviral and antimycoplasmal (Vollenbroich *et al.,* 1997) compounds with diverse structures and function belonging to peptides.

Table.1 Identification of antimicrobial peptide genes associated with different native Bacillus spp.

S.No.	Antimicrobial peptide genes	*Bacillus* spp.									
		B. s **(TSB5)**	***B. l*** **(TSB3)**	***B. w*** **(TSB4)**	***B. w*** **(CLB3)**	***B. t*** **(TLB2)**	***B. th*** **(TLBRE2)**	***B. s*** **(TLBRE1)**	***B. c*** **(CLB2)**	***B. c*** **(TSB4D)**	***B. c*** **(CLB2D)**
1	Iturin A	+	+	+	+	+	+	+	+	+	+
2	Iturin C	-	-	-	+	-	+	+	+	-	+
3	Iturin D	+	-	-	+	+	+	-	+	+	-
4	Surfactin	-	-	-	+	+	+	+	+	+	+
5	Bacillomycin D	+	+	-	+	+	+	+	-	+	+
6	Fengycin D	-	+	+	-	-	-	-	+	+	+
7	Zwittermycin	-	-	-	-	+	-	-	-	-	-
8	Bacilysin bac D	-	+	-	-	+	+	-	-	-	+
9	Bacilysin bac AB	-	+	+	-	+	+	-	-	+	+
10	Fengycin CEA	-	+	+	-	-	+	-	+	-	+

Note: '+': Positive and '-': Negative reaction

B. s.	*B. subtilis*
B. l.	*B. licheniformis*
B. w.	*B. weihenstephanensis*
B. c.	*B. cereus*
B. t.	*B. tequilensis*
B. th.	*B. thuringiensis*

Montesinos, (2007) reported that this type of short sequence peptides with generally fewer than 50 amino acid residues present in living system acting as first line of defence in plants and animals. Hence it is considered that the detection of antibiotic production by *Bacillus* spp. is a prime need to determine the effectiveness of the bacterial isolates as it serves as an index to decide the ability of an individual organism to control plant diseases.

Therefore in the present work an attempt has been made to detect the production of antibiotic compounds in the above ten isolates of *Bacillus* spp. The present study indicated the presence of iturin A gene in all the ten isolates of *Bacillus* spp. Whereas the presence of other genes *viz.* iturin C gene in five isolates of *B. weihenstephanensis* (CLB3), *B. thuringiensis* (TLBRE2), *B. subtilis* (TLBRE1) and *B. cereus* (CLB2D and CLB2); iturin D gene in six isolates of *B. subtilis* (TSB5), *B. weihenstephanensis* (CLB3), *B. tequilensis* (TLB2), *B. thuringiensis* (TLBRE2) and *B. cereus* (TSB4D and CLB2); surfactin gene in seven isolates of *B. weihenstephanensis* (CLB3), *B. tequilensis* (TLB2); *B. thuringiensis* (TLBRE2); *B. subtilis* (TLBRE1), *B. cereus* (TSB4D,CLB2D and CLB2) and bacillomycin D gene in eight isolates of *B. licheniformis* (TSB3), *B. weihenstephanensis* (CLB3), *B. tequilensis* (TLB2), *B. thuringiensis* (TLBRE2), *B. subtilis* (TLBRE1 and TSB5), *B. cereus* (CLB2D and TSB4D); fengycin D gene in five isolates of *B. licheniformis* (TSB3), *B. weihenstephanensis* (TSB4), *B. cereus* (CLB2, CLB2D and TSB4D); zwittermycin gene in one isolate of *B. tequilensis* (TLB2); bacilysin bac D gene in four isolates of *B. licheniformis* (TSB3), *B. tequilensis* (TLB2), *B. thuringiensis* (TLBRE2) and *B. cereus* (CLB2D); bacilysin bac AB gene found only in four isolates *viz. B. licheniformis* (TSB3), *B. weihenstephanensis* (TSB4), *B. tequilensis* (TLB2), *B. thuringiensis* (TLBRE2) and *B. cereus* (CLB2D and CLB2D); fengycin CAE gene in five isolates of *B. licheniformis* (TSB3), *B. weihenstephanensis* (TSB4), *B. thuringiensis* (TLBRE2), *B. cerus* (CLB2, CLB2D) is noticed with the tested ten isolates of *Bacillus* spp. in the present study. The available literature quoting the presence of different antibiotic biosynthetic genes *viz.* iturin A, iturin C, iturin D (Montesinos, 2007 and Assie *et al.,* 2002); surfactin, fengycin (Mora *et al.,* 2011) in *B. amyloliquifaciens*, bacilysin D and AB in *B. subtilis* (Montesinos 2007), bacilomycine D (Ramanatham *et al.,* 2007) in *B. subtilis* and *B. amyloliquifaciens* is in conformity with the findings of the present study. Ongena *et al.,* (2005) reported cyclic lipopeptides of the surfactin, iturin and fengycin families from *Bacillus,* impart successful biocontrol activity by direct suppression of phytopathogens and reinforcing of the potentiality of host plants through stimulating induced systemic resistance phenomenon.

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