Molecular and biochemical detection of lipopeptide antibiotics producing *Bacillus* spp., antagonistic to common fungal pathogens of canola (*Brassica napus* L.)

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Abstract

Bacillus cereus strain DFE4, *B. amyloliquefaciens* strains DFE16 and BS6 exhibited *in vitro* and *in vivo* antagonistic activity towards *Leptosphaeria maculans* (Desm.) Ces & De Not, the blackleg pathogen and *Sclerotinia sclerotiorum* (Lib.) de Bary, the stem rot pathogen of canola (*Brassica napus* L.). Role of antifungal lipopeptide antibiotics, produced by these *Bacillus* spp., in plant disease control was investigated. The presence of the biosynthetic genes of the multi-modular peptide synthetases, responsible for the production of the lipopeptides, was detected with newly designed specific-PCR primers. Strains DFE4 and DFE16 tested positive for the presence of the biosynthetic genes of surfactin, iturin A and bacillomycin D, and strain BS6 for iturin A and bacillomycin D. The surfactin specific primers amplified a 441 bp product, while the bacillomycin D and iturin A specific primers amplified an 875 bp and 647 bp product, respectively. The purified PCR products, when sequenced and searched in the nBLAST–NCBI database, showed very high homology to the sequences of their respective biosynthetic genes in reference *B. subtilis* strains from the GenBank. Production of the lipopeptide antibiotics by these isolates was detected through MALDI-TOF-MS, which confirmed the molecular detection of the peptide synthetase biosynthetic genes. This is the first report on the molecular and biochemical detection of antifungal lipopetide antibiotics produced by *Bacillus* spp., isolated from canola and antagonistic to economically important fungal pathogens of canola worldwide. This is also the first report of a *B. cereus* strain to harbor the biosynthetic genes and produce surfactin, iturin A and bacillomycin D.

Key words: Canola, Blackleg, *Bacillus* spp., lipopeptide antibiotics, biosynthetic genes, biocontrol

Introduction

Bacillus species produce a wide range of secondary metabolites, with a broad spectrum of activity, and very diverse structures. These metabolites range from gene-encoded antibiotics to a variety of small antibiotic peptides, which are synthesized non-ribosomally (Moyne et al. 2004). The lipopeptide antibiotics such as iturin, surfactin, fengycin and plispastin fall under this category. Among the lipopeptide antibiotics, fengycin has been shown to exhibit strong antifungal activity in the biocontrol of damping-off of bean seedling caused by *Pythium ultimum* and gray mold disease of apple caused by *Botrytis cinerea* (Ongena 2005). Similarly, bacillomycin D has been reported for exhibiting strong antifungal activity towards aflatoxin producing fungi- such as *Aspergillus flavus* (Moyne et al. 2001). Other lipopeptides such as iturin A have also been shown to exhibit strong antifungal activity and potential for biocontrol (Cho et al. 2003). Specific-PCR primers have been extensively used in the detection of pathogenic bacteria, fungi, viruses, nitrogen-fixing bacteria and other microorganisms from various environmental samples, but for the first time were used for the detection of natural strains of bacteria harboring antibiotic producing genes of phenazine and 2,4-diacetylphloroglucinol by Raaijmakers et al (1997). These primers, unlike the random isolation and screening procedures that are time-consuming and laborious, allow the specific and efficient detection of the antibiotic producing strains from the natural environments.

Bacillus cereus strain DFE4, *B. amyloliquefaciens* strains DFE16 and BS6 exhibited *in vitro* and *in vivo* antagonistic activity towards *Leptosphaeria maculans* (Desm.) Ces & De Not, the blackleg pathogen and *Sclerotinia sclerotiorum* (Lib.) de Bary, the stem rot pathogen of canola (*Brassica napus* L.) (Ramarathnam and Fernando 2006; Fernando et al 2006). The correlation of agar-diffusible antifungal activity to the disease suppression in the plants indicated the possible role of antibiotics in disease suppression. The objectives of this study are: 1) Design specific PCR-primers and screen for the presence of biosynthetic genes of iturin A, bacillomycin D and surfactin; 2) Matrix-Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (MALDI-TOF-MS) based biochemical detection of lipopeptide antibiotics in bacterial cell-surface extracts.

Materials and methods

PCR screening

Endophytic gram positive bacteria, isolated from canola cotyledons and true leaf, were used for the PCR assay. Total genomic DNA was isolated from bacterial strains by a cetyltrimethylammoniumbromide (CTAB)-based miniprep protocol. Specific-PCR primer pairs for the detection iturin A, bacillomycin D and surfactin synthetase biosynthetic genes, were designed using the web-software Primer3 (Rozen and Staletsky, 2000). The details of the specific primer pairs are presented in

Table 1. PCR reactions were performed using standard reagents. The following thermocycler program was used for the PCR amplification: initial denaturation at 94 °C for 3 min; 36 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s and extension at 72 °C for 1 m 45 s; and final extension at 72 °C for 6 min. The specificity of the primers were checked with a nucleotide to nucleotide BLAST search, and also by including non – related bacterial species in the PCR analysis. The specific PCR products were extracted, purified, quantified in agarose gel with the 1kb-ladder, and sequenced. The sequences obtained were searched for homology, with sequenced genes in the GenBank database, through the NCBI-BLAST search for nucleotides.

Table 1. Characteristics of specific-primers developed for the detection of biosynthetic genes of iturin A, bacillomycin D, and surfactin.

Primer Name	Primer Sequence (5' to 3')	Length (bp)	G+C (%)	Tm (oC)	Position in GenBank Sequence	Product length (bp)	Positive Strain
ITUD1F	gatgcgatctccttggatgt	20	50	60	4041	647	*B. subtilis* strain RB14
ITUD1R	atcgtcatgtgctgcttgag	20	50	60	4687		
BACC1F	gaaggacacggcagagagtc	20	60	60	3382	875	*B. subtilis* strain ATTCAU195
BACC1R	cgctgatgactgttcatgct	20	50	60	4256		
SUR3F	acagtatgggaggcatggtc	20	55	62	313	441	*B. subtilis* strain B3
SUR3R	ttccgccacttttttcagttt	20	40	56	743		

MALDI-TOF-MS analysis of cell-surface extracts

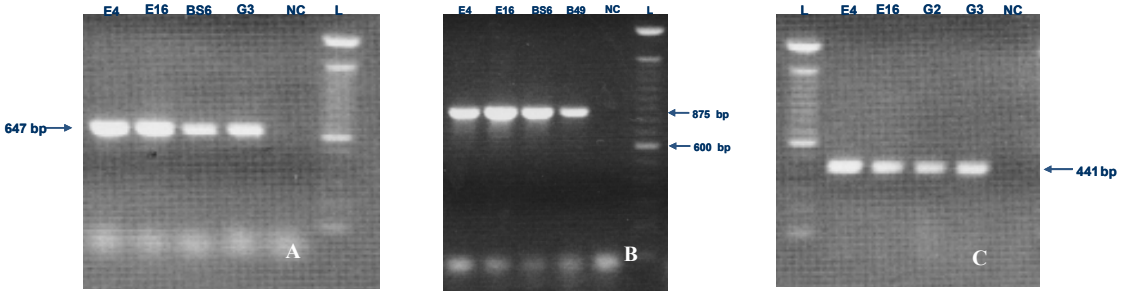
Surface extracts were prepared by suspending 1-2 loops of bacterial cells, grown on Landy agar plates, in 500 µL of 70% acetonitrile with 0.1% trifluoroacetic acid for 1-2 min. The bacterial cells were pelleted by centrifugation and the cell-free surface extract was transferred to a new microcentrifuge tube and stored in 4°C, until further analysis. For the MALDI-TOF-MS analysis, 1 µL of the surface extract was spotted onto the target with an equal volume of matrix solution. Dihydroxy benzoic acid (DHB) was used as the matrix. The sample spots were air dried and subjected to MALDI-TOF-MS analysis. The *m/z* ratio range was 500-4000. The mass spectra were analyzed and *m/z* ratio identified using the *moverz* software.

Results

PCR screening:

Antibiotic	Isolate	Primer	Product size (bp)	GenBank Accession #	Obtained GenBank Match	Score	E value
Bacillomycin	*B. cereus* strain DFE4	BACC1F/1R	875	AY137375	Bacillomycin D operon of *B. subtilis* ATTCAU195	1447	0
Bacillomycin	*B. amyloliquefaciens* strain DFE16	BACC1F/1R	875	AY137375	Bacillomycin D operon of *B. subtilis* ATTCAU195	1463	0
Bacillomycin	*B. amyloliquefaciens* strain BS6	BACC1F/1R	875	AY137375	Bacillomycin D operon of *B. subtilis* ATTCAU195	848	0
Bacillomycin	*B. subtilis* strain 49	BACC1F/1R	875	AY137375	Bacillomycin D operon of *B. subtilis* ATTCAU195	1495	0
Iturin A	*B. cereus* strain DFE4	ITUD1F/1R	647	AB050629	*ituD* - iturin A operon of *B. subtilis* strain RB14	1031	0
Iturin A	*B. amyloliquefaciens* strain DFE16	ITUD1F/1R	647	AB050629	*ituD* - iturin A operon of *B. subtilis* strain RB14	1035	0
Iturin A	*B. amyloliquefaciens* strain BS6	ITUD1F/1R	647	AB050629	*ituD* - iturin A operon of *B. subtilis* strain RB14	789	0
Iturin A	*B. subtilis* strain ATTC13952	ITUD1F/1R	647	AB050629	*ituD* - iturin A operon of *B. subtilis* strain RB14	724	0
Surfactin	*B. cereus* strain DFE4	SUR3F/3R	441	AY040867	*srfDB* - surfactin operon of *B. subtilis* strain B3	678	0
Surfactin	*B. amyloliquefaciens* strain DFE16	SUR3F/3R	441	AY040867	*srfDB* - surfactin operon of *B. subtilis* strain B3	684	0
Surfactin	*B. subtilis* strain ATTC6633	SUR3F/3R	441	AY040867	*srfDB* - surfactin operon of *B. subtilis* strain B3	694	0

Figure 1. PCR - detection of biosynthetic genes of lipopeptide antibiotics from antagonistic endophytic bacteria, using specific-primers. Detection of *ituD* gene of iturin A biosynthetic operon (A) and *bamC* gene of the bacillomycin D operon (B) in *Bacillus cereus* strain DFE4, *B. amyloliquefaciens* strains DFE16 and BS6 and *B. subtilis* strains ATTC13952 (iturinA) and B49 (bacillomycin D). Detection of *srfDB* gene of the surfactin operon (C) in *B. cereus* strain DFE4, *B. amyloliquefaciens* strains DFE16 and BS6 and *B. subtilis* strains ATTC13952 and ATTC6633.



MALDI-TOF-MS analysis of cell-surfac

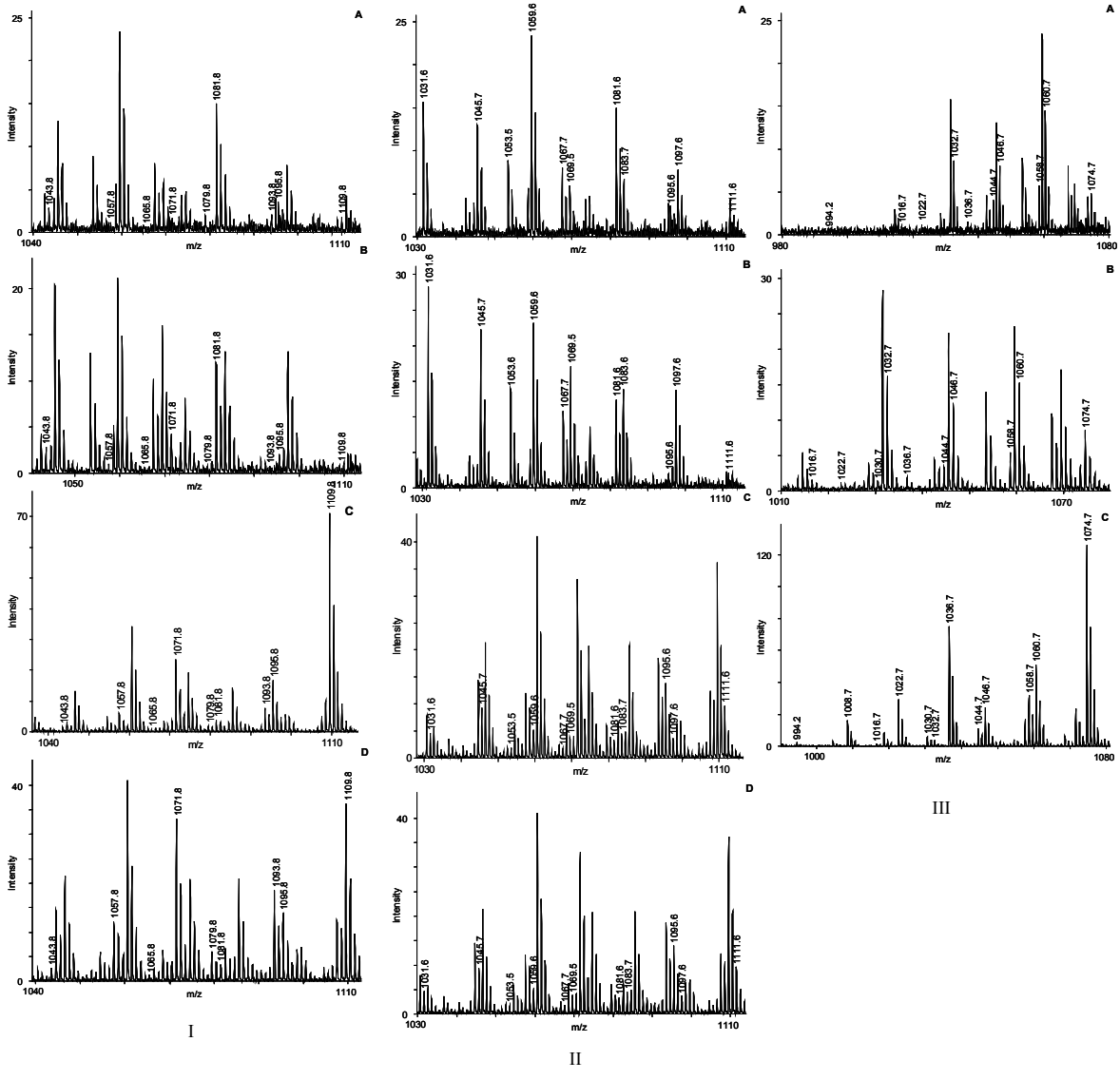


Figure 2. MALDI-TOF-MS mass spectra of bacterial cell-surface extracts analyzed for the presence of lipopeptide antibiotics, iturin A (I); bacillomycin D (II); and surfactin (III). Peaks at the range of *m/z* = 1081.8, 1095.8, and 1109.8, which correspond to the potassium adducts of C14-C16-iturin A, were identified in the mass spectra of *Bacillus cereus* strain DFE4 (IA), *B. amyloliquefaciens* strains DFE16 (IB) and BS6 (IC) and the positive control *B. subtilis* strain ATCC13952 (ID). Peaks in the range of *m/z* = 1069.5, 1083.7, 1097.6, and 1111.6 were detected in the surface extracts of DFE4 (IIA), DFE16 (IIB), BS6 (IIC) and the positive control *B. subtilis* strain B49 (IID), which can be attributed to the potassium adducts of C14-C17 bacillomycin D. The mass spectra of strains DFE4 (IIIA) and DFE16 (IIIB) showed a group of mass peaks at *m/z* = 1032.7, 1046.7, 1060.7, and 1074.7, which correspond to the potassium adducts of C13-C15-surfactins, which were also detected in the mass spectrum of the positive control strain ATCC 13952 (IIIC).

Discussion

To our knowledge, this study for the first time designed specific- PCR primers for the detection of biosynthetic genes of multi-modular enzymes, the peptide synthetases, involved in the synthesis of the antifungal antibiotics iturin A, bacillomycin D, and surfactin. The detection of the lipopetide antibiotics in the cell-surface extracts, by MALDI-TOF-MS analysis, confirms and validates the PCR-detection. This is also the first report of a *B. cereus* strain not only to harbor biosynthetic genes of iturin A, bacillomycin D and surfactin, but also to produce them. The strong agar-diffusible antifungal activity in plates, which correlates to disease suppression in plants, as observed for strains DFE4 (Ramarathnam and Fernando 2006), DFE16 (Fernando et al 2006; Ramarathnam and Fernando 2006) and BS6 (Fernando et al 2006) could be linked to their ability to produce multiple antibiotics. Also, this opens up avenues to investigate if a synergistic activity of these antibiotics is involved in disease suppression or if it is a single antibiotic effect. It is very important to understand the role of different antibiotics in disease control for which mutational studies and isolation of the antibiotics from the site of infection is required. It was earlier observed that strain BS6 not only inhibited germination of ascospores of *S. sclerotiorum* on petals of canola, but also induced systemic resistance for disease suppression when applied away from the pathogen (Zhang et al 2004). From an environmental

point of view, understanding the different mechanisms, such as direct antagonism or induced systemic resistance, employed by these potential biocontrol bacteria would give us more options to consider, and to integrate them in to a disease management program involving use of less hazardous synthetic fungicides.

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