

# Molecular and biochemical detection of fengycin- and bacillomycin D-producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat

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**Abstract:** *Bacillus* species are well known for their ability to control plant diseases through various mechanisms, including the production of secondary metabolites. *Bacillus subtilis* DFH08, an antagonist of *Fusarium graminearum*, and other *Bacillus* spp. that are antagonists of common fungal pathogens of canola were screened for peptide synthetase biosynthetic genes of fengycin and bacillomycin D. Specific polymerase chain reaction (PCR) primers identified *B. subtilis* strains DFH08 and 49 for the presence of the *fenD* gene of the fengycin operon. *Bacillus cereus* DFE4, *Bacillus amyloliquefaciens* strains DFE16 and BS6, and *B. subtilis* 49 were identified for the presence of the *bamC* gene of the bacillomycin D synthetase biosynthetic operon. Both fengycin and bacillomycin D were detected in the culture extract of strain Bs49, characterized through MALDI-TOF-MS (matrix-assisted laser desorption ionization – time of flight – mass spectrometry), and their antifungal activities demonstrated against *F. graminearum* and *Sclerotinia sclerotiorum*. This study designed and used specific PCR primers for the detection of potential fengycin- and bacillomycin D-producing bacterial antagonists and confirmed the molecular detection with the biochemical detection of the corresponding antibiotic produced. This is also the first report of a *B. cereus* strain (DFE4) to have bacillomycin D biosynthetic genes. Bacteria that synthesize these lipopeptides could act as natural genetic sources for genetic engineering of the peptide synthetases for production of novel peptides.

**Key words:** *Bacillus* spp., bacillomycin D, fengycin, PCR detection, MALDI-TOF-MS.

**Résumé :** Les espèces appartenant à *Bacillus* sont bien connues pour leur capacité à contrôler des maladies des végétaux par le biais de différents mécanismes, incluant la production de métabolites secondaires. La souche DFH08 de *Bacillus subtilis*, un antagoniste de *Fusarium graminearum*, et d'autres *Bacillus* spp. antagonistes de pathogènes fongiques communs du canola, ont été criblés pour détecter l'expression de gènes de peptide synthétases impliquées dans la biosynthèse de la fengycine et de la bacillomycine D. Des amorces de la réaction de la polymérase en chaîne spécifiques ont permis de détecter la présence du gène *fenD* de l'opéron fengycine chez les souches *Bacillus subtilis* DFH08 et 49. La présence du gène *bamC* de l'opéron bacillomycine D synthétase a été démontrée chez les souches *B. cereus* DFE4, *Bacillus amyloliquefaciens* BS6 et DFE16, ainsi que chez *B. subtilis* 49. La fengycine et la bacillomycine D ont été toutes deux détectées dans les extraits de culture de la souche Bs49 par MALDI-TOF-MS (spectrométrie de masse à désorption-ionisation laser assistée par matrice – analyse en temps de vol, et leur activité anti-fongique a été démontrée contre *F. graminearum* et *Sclerotinia sclerotiorum*. Cette étude a permis de concevoir et d'utiliser des amorces PCR spécifiques pour détecter des bactéries antagonistes potentielles produisant de la fengycine et de la bacillomycine D, et a confirmé par la détection biochimique de l'antibiotique correspondant les résultats de la détection moléculaire. Elle rapporte aussi pour la première fois la présence de gènes biosynthétiques de la bacillomycine D chez une souche de *B. cereus* (DFE4). Ces bactéries qui synthétisent des lipopeptides pourraient constituer des sources génétiques naturelles pour l'ingénierie génétique des peptides synthétases lors de la production de nouveaux peptides.

**Mots-clés :** *Bacillus* spp., bacillomycine D, fengycine, détection par PCR, MALDI-TOF-MS.

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**Table 1.** List of bacterial isolates and their origins used in this study.

Bacterium	Origin
<i>Bacillus subtilis</i> B49	Rhizosphere of tea
<i>B. subtilis</i> ATCC 13952	American Type Culture Collection
<i>B. subtilis</i> DF-HO8	Isolated from wheat head
Strain DFE3	Endophyte from <i>Brassica napus</i> 'Cresor' leaves from bud stage
<i>Bacillus cereus</i> DFE4	Endophyte from <i>B. napus</i> 'Westar' cotyledon leaves
Strain DFE6	Endophyte from <i>B. napus</i> 'Westar' cotyledon leaves
<i>Bacillus pumilus</i> DFE11	Endophyte from <i>B. napus</i> 'Cresor' cotyledon leaves
<i>B. pumilus</i> DFE12	Endophyte from <i>B. napus</i> 'Cresor' cotyledon leaves
<i>B. cereus</i> DFE13	Endophyte from <i>B. napus</i> 'Cresor' cotyledon leaves
Strain DFE15	Endophyte from <i>B. napus</i> 'Westar' leaves from bud stage
<i>Bacillus amyloliquefaciens</i> DFE16	Endophyte from <i>B. napus</i> 'Cresor' leaves from bud stage

## Introduction

*Bacillus* species produce a wide range of secondary metabolites that display 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 nonribosomally (Moyne et al. 2004). The lipopeptide antibiotics such as iturin, surfactin, fengycin, and plispastin fall under this category. The lipopeptides have a hydrophilic peptide portion and a hydrophobic fatty acid portion (Roongsawang et al. 2002). Most of them are cyclic in nature, mediated by either  $\beta$ -hydroxy fatty acid ( $\beta$ -hydroxy type) or  $\beta$ -amino fatty acid ( $\beta$ -amino type). Bacillomycin D, which is a member of the iturin family along with mycosubtilin and iturin A, is made of one  $\beta$ -amino fatty acid and 7  $\alpha$ -amino acids. Fengycins are cyclic lipodecapeptides containing a  $\beta$ -hydroxy fatty acid with a side chain length of 16–19 carbon atoms. Four D-amino acids and ornithine (a nonproteinogenic residue) have been identified in the peptide portion of fengycin (Koumoutsi et al. 2004). The members of the iturin family exhibit strong antifungal and hemolytic activities and limited antibacterial activity (Maget-Dana and Peypoux 1994). Fengycin shows specific antifungal activity against filamentous fungi and inhibits phospholipase A<sub>2</sub> (Nishikori et al. 1986). The non-ribosomal synthesis of these lipopeptides is achieved through the involvement of large, modular, multienzyme templates designated peptide synthetases. Each module of the multimodular peptide synthetase is made up of approximately 1000 amino acids and catalyzes the incorporation of one amino acid in the lipopeptide product (Stachelhaus et al. 1995).

*Bacillus* spp., especially *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus amyloliquefaciens*, are effective for the control of plant diseases caused by soil-borne, foliar, and post-harvest fungal pathogens (Silo-Suh et al. 1994; Raupach and Kloepper 1998; Shoda 2000; Janisiewicz and Korsten 2002; Chiou and Wu 2003; Tjamos et al. 2004). 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 et al. 2005). Similarly, bacillomycin D exhibits strong antifungal activity towards aflatoxin-producing fungi, such as *Aspergillus flavus* (Moyne et al. 2001). Other lipopeptides such as iturin A

also exhibit strong antifungal activity and potential for biocontrol (Yoshida et al. 2002; Cho et al. 2003). Selection and identification of antibiotic-producing bacteria from natural environments through random isolation and screening procedures is time consuming and laborious (de Souza and Raaijmakers 2003). However, when discovery of strains that produce specific antibiotics is desired, screening candidate strains for particular antibiotic-encoding sequences represents a more expeditious approach.

In this study, bacteria isolated from canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.) that exhibited antifungal activity towards economically important pathogens of canola and wheat were screened using specific polymerase chain reaction (PCR) primers designed to detect the presence of biosynthetic genes of fengycin and bacillomycin D peptide synthetases. *Bacillus subtilis* 49 (Bs49), which was earlier identified as a fengycin and bacillomycin D producer through "whole cell" matrix-assisted laser desorption ionization – time of flight – mass spectroscopy (MALDI-TOF-MS) analysis (Vater et al. 2003) was also included in the PCR screening. This study is unique in that it follows up the PCR detection of the biosynthetic genes of fengycin and bacillomycin D synthetase in strain Bs49 with biochemical extraction, purification, and characterization of the lipopeptides produced. The biochemical analysis serves as a confirmation of the PCR detection, and establishes the prospective use of PCR screening in identifying potential producers of fengycin and bacillomycin D antibiotics.

## Materials and methods

### Bacterial and fungal cultures and culture conditions

The bacterial identifications and the origin of the 11 Gram-positive bacterial isolates used in this study are presented in Table 1. Pure cultures of each bacteria were maintained in Luria–Bertani broth, amended with 20% glycerol (Fisher Scientific, Fair Lawn, New Jersey, USA) and stored at  $-80^{\circ}\text{C}$ . Bacterial strains were streaked from the stock onto Luria–Bertani agar or nutrient agar (Difco Laboratories, Detroit, Michigan) plates and incubated at  $28^{\circ}\text{C}$  for 24 h in an incubator. The fungal cultures of *Fusarium graminearum* and *Sclerotinia sclerotiorum* were maintained in potato dextrose agar (PDA; Difco) plates at room temperature under a continuous source of light. Fungal discs from the edge of the colony were used for the inhibition assays.

### In vitro and in vivo assays for the control of economically important pathogens of canola and wheat

The in vitro assays (plate) and in vivo plant assays (greenhouse) for the inhibition of *Leptosphaeria maculans* (Desm.) Ces (anamorph *Phoma lingam* (Tode: Fr./Desm.)), the causal agent of Blackleg disease of canola for bacterial strains (DFE3, DFE4, DFE6, DFE11, DFE12, DFE13, DFE15, and DFE16), were done as previously described (Ramarathnam and Fernando 2006). Similarly, the in vitro assays (plate) and in vivo assays (greenhouse and field) for the inhibition of *S. sclerotiorum* (Lib.) de Bary, the causal agent of stem rot of canola for bacterial strains (DFE16 and BS6), were done as previously described (Fernando et al. 2007). *Bacillus subtilis* B49 isolated from rhizosphere soil of tea exhibited strong inhibition of radial mycelial growth of *F. graminearum*, *Rhizoctonia solani*, *S. sclerotiorum*, *Fusarium oxysporum* f.sp. *vasinfectum*, and *Botrytis fabae* in in vitro plate assays (data not presented).

The ability of *B. subtilis* DFH08 (DFH08) to inhibit radial mycelial growth of *F. graminearum* Schwabe (Teleomorph = *Gibberella zeae* (Schwein.) Petch), the head blight pathogen of wheat, was assayed on PDA and nutrient agar plates and the percent mycelial inhibition was calculated. In greenhouse conditions (25 °C, 14 h photoperiod/day), potential *Fusarium* head blight antagonistic bacterial strains were individually applied onto the seeds and heads of the highly susceptible cultivar AC-Teal (hard red spring wheat) to investigate the microbial interaction between antagonists and the pathogen in vivo. For seed-coating treatment, germinated seeds were immersed in a bacterial suspension ( $4.5 \times 10^8$  cfu/mL) for 30 min before seeding. When wheat was at 50% flowering, 5 µL of each bacterial suspension was applied to the heads by injecting directly into the floret. The pathogen macroconidia ( $5 \times 10^5$  macroconidia/mL) were inoculated in the same spot either before or after bacterial inoculation. Head inoculation was undertaken as follows: the middle floret of the head was injected with 2 µL of *Fusarium* macroconidia suspension ( $5 \times 10^5$  macroconidia/mL and 0.04% Tween 80). After inoculation, wheat plants were incubated in a mist chamber for 72 h at 22 °C and transferred to a greenhouse bench. There were 6 treatments (10 pots per replicate and 5 plants in each pot): (i) seed coating with bacteria and bacterial application on head 4 h prior to *Fusarium* inoculation (BST-BBI), (ii) seed coating with bacteria and bacterial application on head 4 h post *Fusarium* inoculation (BST-BAI), (iii) seed coating with bacteria and no bacterial application on head (BST) prior to *Fusarium* application, (iv) bacterial application on head 4 h prior to *Fusarium* inoculation on head and no seed coating of bacteria (BBI), (v) bacterial application on head 4 h post *Fusarium* inoculation and no seed coating of bacteria (BAI), and (vi) no seed coating of bacteria and no bacterial application on head prior to *Fusarium* application (CK). The FHB incidence (the number of heads infected) and severity (the number of diseased spikes on each head) were estimated 16 days after inoculation. Analysis of variance (ANOVA) and a mean separation test (Fisher's Least Significant Difference), at  $P = 0.05$  were performed using the analyst procedure of SAS<sup>TM</sup>, Version 8.1 (SAS Institute, Cary, North Carolina).

### Design of specific primers for the detection of fengycin and bacillomycin D synthetase biosynthetic genes

We designed 2 primer pairs for the detection of the fengycin and bacillomycin D synthetase biosynthetic cluster using the web software Primer3<sup>®</sup> (Rozen and Skaletsky 2000). A 7.7 kb region (GenBank accession No. AJ011849 (Region: 1–7774) of *B. subtilis* F29-3) of the *fenD* gene proposed to be involved in the biosynthesis of 2 modules of fengycin synthetase (Lin et al. 1999) was used for the design of the FEND1F and FEND1R specific primer pair. The 2 amino acid activating modules, FenD1 and FenD2, activate L-Tyr and L-Thr, the third and fourth amino acids in fengycin, respectively (Lin et al. 2005). Similarly, for designing the bacillomycin D specific primer pair, BACC1F and BACC1R, a 7.8 kb region (GenBank accession No. AY137375.1 (Region: 1–7860) of *B. subtilis* ATTCAU195) involved in the synthesis of bacillomycin D synthetase C was used (Moyné et al. 2004). The details of the 2 specific primer pairs are presented in Table 2. The specificity of the primers were checked with a nucleotide-to-nucleotide BLAST search and also by including unrelated bacterial species in the PCR analysis.

### PCR analysis

Total genomic DNA was isolated from bacterial strains by a cetyltrimethylammoniumbromide-based miniprep protocol (Ausubel et al. 1995). PCR amplifications were performed in a 25 µL reaction mixture containing 20 ng of template DNA; 1× PCR buffer; 1.75 mmol/L (for BACC1F/BACC1R) and 2 mmol/L (for FEND1F/FEND1R) MgCl<sub>2</sub>; 200 µmol/L concentration of each dATP, dCTP, dGTP, and dTTP (Invitrogen, Carlsbad, California); 20 pmol of each primer (Invitrogen); and 0.5 U of Platinum<sup>®</sup> Taq (Invitrogen). PCR amplifications were carried out with a PTC-100<sup>TM</sup> programmable thermal controller (MJ Research, Waltham, Massachusetts). The PCR programs used were as follows: (i) BACC1F/BACC1R—initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min 45 s; and final extension at 72 °C for 6 min; (ii) FEND1F/FEND1R—initial denaturation at 94 °C for 3 min; 45 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 1 min 45 s; and final extension at 72 °C for 6 min. *Bacillus subtilis* ATCC 13952 was used as the positive control for fengycin detection and strain Bs49 was used as the positive control for the bacillomycin D detection. Eight microlitres of each sample was loaded onto a 1% or 1.5% agarose gel containing ethidium bromide and electrophoresed in 1× Tris-acetate ethylenediamine tetraacetic acid (TAE) buffer at 100–180 V for 1–2 h. The gels were visualized with an ultraviolet illuminator and digitally recorded. The bacterial DNA that tested positive with a specific amplification product were re-amplified with the specific primers and the desired bands were purified with a High Pure<sup>TM</sup> PCR product purification kit (Roche Diagnostics, Basel, Switzerland). The amplified products were quantified in agarose gel with the 1 kb ladder and were sequenced at MacrogenUSA (Rockville, Maryland). The sequences obtained were searched for homology with sequenced genes in the

**Table 2.** Characteristics of specific primers developed for the detection of biosynthetic genes of fengycins and bacillomycin D synthetases.

Lipopeptide	Primer name	Primer sequence (5' to 3')	Length (bp)	G+C (%)	Temperature (°C)	Position in GenBank sequence	Product length (bp)	Positive strains
Fengycin	FENDIF	ttggcagcaggagaagtt	20	45	60	3687–3706	964	<i>Bacillus subtilis</i> F29-3
	FENDIR	gctgccgttcgcttttc	20	50	60	4650–4631		
Bacillomycin	BACCIF	gaaggacacggcagagagtc	20	60	60	34274–34293	875	<i>B. subtilis</i> ATTCAU195
	BACCIR	cgtgatgactgtcatgct	20	50	60	35148–35129		

GenBank database through the National Center for Biotechnology Information Blast search for nucleotides.

#### Culture of strain Bs49 and preparation of lipopeptides

*Bacillus subtilis* Bs49 was grown in Landy medium (glucose, 20 g; L-glutamic acid, 5 g; MgSO<sub>4</sub>, 0.5 g; KCl, 0.5 g; KH<sub>2</sub>PO<sub>4</sub>, 1 g; Fe<sub>2</sub>SO<sub>4</sub>·6H<sub>2</sub>O, 15 mg; MnSO<sub>4</sub>·H<sub>2</sub>O, 5.0 mg; and CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.16 mg; 1000 mL distilled water; final pH 7.0) (Landy et al. 1948). A loop of strain Bs49 grown on nutrient agar at 32 °C for 24 h was inoculated in 500 mL of Landy medium in a 2 L Erlenmeyer flask, and incubated at 33 °C and 180 r/min for 30 h. Strain Bs49 cells were removed from the cell culture by centrifugation at 4000g for 10–15 min; pH of the supernatant was adjusted to 2.0 by the addition of 6 mol/L HCl and then gently stirred for 2 h or overnight. The precipitate was collected by centrifugation, resuspended in 1 mol/L NaOH to adjust the pH to 7.0, and extracted twice with methanol. The crude lipopeptide was further purified by gel filtration on a 2 cm × 50 cm Sephadex™ LH-20 column. Finally, the pure lipopeptide was collected and stored at –20 °C.

#### Preparative separation of strain Bs49 lipopeptides

After precipitation with HCl, the crude lipopeptide fraction dissolved in methanol was evaporated in a rotary evaporator under a vacuum. The dried material was dissolved in a minimum volume of chloroform–methanol (1:1 v/v), applied to a 5 cm × 150 cm LH-20 column, and fractionated by size exclusion chromatography by using the same solvent for elution. The products were monitored by determining the absorbance at 220 nm.

#### Analytical scale purification of strain Bs49 lipopeptides

Isolated lipopeptides were purified further and fractionated into isoforms by reversed-phase high-performance liquid chromatography (HPLC) on a  $\mu$ RPC SC 2.1/10 column (Amersham Biosciences, Buckinghamshire, England) at room temperature. A 10–50  $\mu$ L portion of the lipopeptide fraction was loaded on a  $\mu$ RPC SC 2.1/10 column and separated by high-resolution reversed-phase HPLC by using a Pharmacia Smart™ microseparation system (Amersham Pharmacia Biotech, New Jersey, USA). The products were eluted with a linear gradient of 20%–100% acetonitrile – 0.1% trifluoroacetic acid (TFA) in 60 min by using a flow rate of 100  $\mu$ L/min. Solvent A was 20% acetonitrile in 0.1% TFA (v/v). Solvent B was acetonitrile containing 0.1% TFA (v/v). The eluted biosurfactants were detected by measuring the absorbance at 220 nm. In the fractions obtained, the compounds were detected by MALDI–TOF–MS.

#### MALDI–TOF–MS analysis of strain Bs49 lipopeptides fractions

Lipopeptides produced by strain Bs49 were characterized through MALDI–TOF–MS, with recording provided by a Bruker™ Reflex instrument (Bruker Daltonics, Billerica, Massachusetts) using a 337 nm nitrogen laser for desorption and ionization. The matrix was  $\alpha$ -cyano-4-hydroxycinnamic acid.

**Table 3.** In vitro and in vivo assays for the inhibition of *Leptosphaeria maculans* and *Sclerotinia sclerotiorum* by bacterial antagonistic agents.

Treatment	Bacteria ID	Pathogen inhibited	% mycelial inhibition	Cotyledon stage	3-4 leaf stage	Sclerotinia stem rot severity			Source
						Greenhouse	2003	2004	
						Field			
DFE3		<i>Leptosphaeria maculans</i>	—	3.0	0.7				Ramarathnam and Fernando 2006
DFE4	<i>Bacillus cereus</i>	<i>L. maculans</i>	69.5	0.6	0.0				Ramarathnam and Fernando 2006
DFE6		<i>L. maculans</i>	53.4	2.4	0.6				Ramarathnam and Fernando 2006
DFE13	<i>B. cereus</i>	<i>L. maculans</i>	58.3	0.2	0.0				Ramarathnam and Fernando 2006
DFE11	<i>Bacillus pumilus</i>	<i>L. maculans</i>	55.6	2.0	0.8				Ramarathnam and Fernando 2006
DFE12	<i>B. pumilus</i>	<i>L. maculans</i>	74.6	2.2	0.6				Ramarathnam and Fernando 2006
DFE15		<i>L. maculans</i>	29.8	2.6	1.5				Ramarathnam and Fernando 2006
DFE16	<i>Bacillus amyloliquefaciens</i>	<i>L. maculans</i>	80.3	1.8	0.0				Ramarathnam and Fernando 2006
Control			0.0	9.0	9.0	0.2	22.5	36.9	Ramarathnam and Fernando 2006
DFE16	<i>B. amyloliquefaciens</i>	<i>Sclerotinia sclerotiorum</i>							Fernando et al. 2007
DFBS6	<i>B. amyloliquefaciens</i>	<i>S. sclerotiorum</i>				0	13.8	35.4	Fernando et al. 2007
Control						4	33.8	44.1	Fernando et al. 2007

\*IP Rating — interaction phenotype (IP) scale of 0-9, where 0 = no darkening around the wounds; 1 = limited blackening around the wound; 3 = dark necrotic lesions; 5 = nonsporulating lesions, sharply limited by dark necrotic margin; 7 = gray-green tissue collapse; and 9 = rapid tissue collapse accompanied by profuse sporulation.

**Table 4.** In vitro plate assays and in vivo green house assays for the inhibition of *Fusarium graminearum* by *Bacillus subtilis* DFH08.

Treatment	% mycelial inhibition	Disease severity	Relative control
PDA assay	60		
BST-BBI		45.4c	49.1a
BST-BAI		58.9bc	34.0abc
BST		72.0b	19.3c
BBI		55.6c	37.7ab
BAI		64.3bc	27.9bc
CK		89.2a	0.0d

**Note:** BST-BBI, seed coating with bacteria and bacterial application on head 4 h prior to *Fusarium* inoculation; BST-BAI, seed coating with bacteria and bacterial application on head 4 h post *Fusarium* inoculation; BST, seed coating with bacteria and no bacterial application on head prior to *Fusarium* application; BBI, bacterial application on head 4 h prior to *Fusarium* inoculation on head and no seed coating of bacteria; BAI, bacterial application on head 4 h post *Fusarium* inoculation and no seed coating of bacteria; CK, no seed coating of bacteria and no bacterial application on head prior to *Fusarium* application. Treatments followed by the same letter were not significantly different from each other ( $P = 0.05$ ).

**Detection of antifungal activity of strain Bs49 lipopeptides against plant pathogenic fungi**

Purified bacillomycin D and fengycin were tested for their antifungal activities by spotting them on sterile filter paper discs placed around the fungal disc located at the centre of PDA plates. Sterile filter paper discs were loaded with 5 µL of the pure lipopeptide suspended in methanol when the radial growth of the fungal mycelium reached 2 cm. The concentration of the lipopeptide suspended in methanol was about 10 mg/mL for fengycin and 20 mg/mL for bacillomycin D. Sterile filter paper discs loaded with methanol were used as the negative control. The results were recorded when the fungal mycelium grew over the control disc.

**Results**

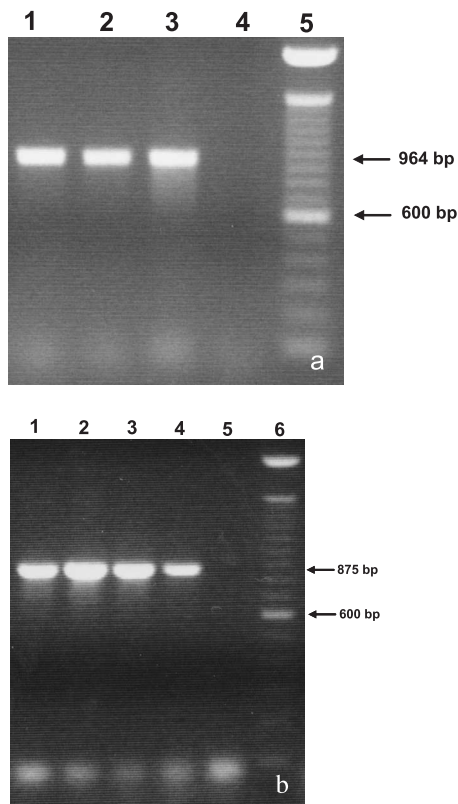
**In vitro and in vivo assays for the control of economically important pathogens of canola and wheat**

The results of the in vitro and in vivo assays for the inhibition of *L. maculans* and *S. sclerotiorum* are presented in Table 3. Almost all of the endophytic bacteria tested against *L. maculans* had >50% inhibition of the radial mycelial growth on plates and suppression of disease to levels of resistant ratings on a highly susceptible cultivar (Ramarathnam and Fernando 2006). Bacterial strains DFE16 and BS6 showed consistent reduction of *S. sclerotiorum* stem rot in canola (both disease incidence and disease severity) compared with the pathogen control in the greenhouse and field assays (Fernando et al. 2007). Strain DFH08 significantly inhibited the radial mycelial growth of *F. graminearum* on PDA plates by 60% compared with the control (Table 4). In plant assays on greenhouse grown wheat at 50% flowering, it was observed that strain DFH08 significantly reduced disease severity in all the 5 treatments when compared with the pathogen control. Seed coating plus application of bacteria on head prior to *Fusa-*

**Table 5.** Nucleotide Blast search results of the specific primers developed for the detection of fengycin and bacillomycin D synthetase genes.

Lipopeptide	Primer	GenBank acc. No.	Position in GenBank sequence	Positive strain	E-value
Fengycin	FEND1F	AJ011849	3687–3706	<i>Bacillus subtilis</i> F29-3	0.02
Fengycin	FEND1R	AJ011850	4650–4631	<i>B. subtilis</i> F29-3	0.02
Bacillomycin D	BACC1F	AY137375	34274–34293	<i>B. subtilis</i> ATTCAU195	0.02
Bacillomycin D	BACC1R	AY137376	35148–35129	<i>B. subtilis</i> ATTCAU195	0.02

**Fig. 1.** Polymerase chain reaction screening using specific primers for antibiotic biosynthetic genes in antagonistic bacteria. (a) Detection of the *fenD* gene of the fengycin synthetase biosynthetic cluster. Lane 1, *Bacillus subtilis* 49; lane 2, *B. subtilis* DFH08; lane 3, *B. subtilis* ATCC 13952; lane 4, negative control; lane 5, 100 bp ladder. (b) Detection of *bamC* gene of the bacillomycin D synthetase biosynthetic operon. Lane 1, *B. cereus* DFE4; lane 2, *Bacillus amyloliquefaciens* DFE16; lane 3, *B. amyloliquefaciens* BS6; lane 4, *B. subtilis* 49; lane 5, negative control; lane 6, 100 bp ladder. Eight microlitres of the PCR product was loaded in a 1% agarose gel, amended with ethidium bromide, run in TAE buffer at 150–180 V for 1 h, and visualized under ultraviolet light.



*rium* inoculation (treatment 1) gave the best disease reduction, even though it was not significantly different from treatment 2 and treatment 4.

#### PCR analysis

The specificity of the specific primers FEND1F/FEND1R and BACC1F/BACC1R were checked with a nucleotide-to-nucleotide BLAST search to see if the primer would hybridize to any other known sequence. The specificity of the primer sequence to its corresponding target sequence in the

GenBank and the E-value are presented in Table 5. Of the 11 bacterial strains tested for the presence of the *fenD* gene, which is involved in the biosynthesis of FenD1 and FenD2 modules of fengycin synthetase, only isolates DFH08, Bs49, and the positive control (*B. subtilis* ATCC 13952) produced the 964 bp product (Fig. 1a). The PCR products of FEND1F/FEND1R were sequenced and analyzed using the National Center for Biotechnology Information nBlast database. The sequences of the PCR products of strains DFH08 and Bs49 showed very high homology to the sequence of the *fenD* gene of *B. subtilis* F29-3 (AJ011849) (Table 6). Of the 11 isolates screened with primer pairs BACC1F/BACC1R, only *B. cereus* DFE4 (DFE4), *B. amyloliquefaciens* DFE16 (DFE16), *B. amyloliquefaciens* BS6 (BS6), and the positive control Bs49 yielded the 875 bp product indicative of the presence of the *bamC* gene of the bacillomycin D biosynthetic cluster (Fig. 1b). The PCR products derived from primers BACC1F/BACC1R were sequenced and analyzed using the National Center for Biotechnology Information nBlast database. The sequences of the PCR products of strains DFE4, DFE16, BS6, and Bs49 showed very high homology to the sequence of bacillomycin D operon of *B. subtilis* ATTCAU195 (AY137375) (Table 6). Other *Bacillus* spp. and Gram positive bacteria included in the analysis did not yield any PCR product, thus establishing the specificity of primer pairs FEND1F/FEND1R and BACC1F/BACC1R.

#### Chromatography and spectroscopy detection and characterization of strain BS49 lipopeptides

The individual lipopeptide fractions eluted from a  $\mu$ RPC SC 2.1/10 column by high-resolution reversed-phase HPLC using a linear solvent gradient were subjected to MALDI-TOF-MS analysis. Bacillomycin D eluted in HPLC fractions 22–26, while fengycin was detected in fractions 27–33 (Fig. 2). Peaks in the range of  $m/z = 1069.5, 1083.7, 1097.6,$  and  $1111.6$  were detected in fractions 22–26, which could be attributed to the potassium adducts of C14–C17 bacillomycin D (Fig. 3a). Chromatographic fractions 27–33 produced peaks in the range of  $m/z = 1449.8–1543.8$ , which corresponded to the protonated forms and adducts of C15–C17 fengycins with alkali metal ions (Fig. 3b).

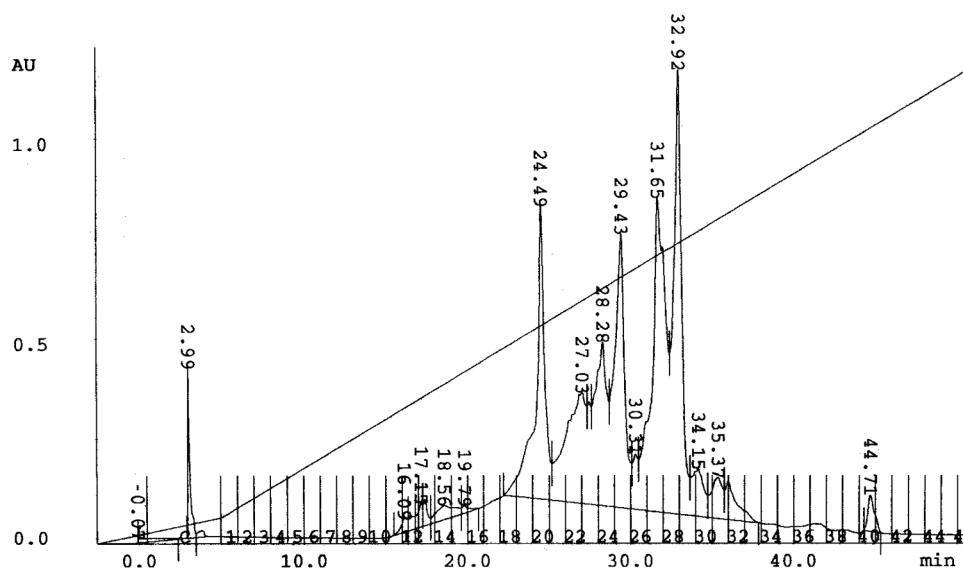
#### Detection of antifungal activity of strain Bs49 lipopeptides against plant pathogenic fungi

The lipopeptides fengycin and bacillomycin D isolated from Bs49 culture and loaded onto sterile filter paper discs exhibited a significant inhibitory effect on the radial mycelial growth of *F. graminearum* and *S. sclerotiorum* on PDA compared with the control discs. Both fengycin and bacillomycin D exhibited higher agar-diffusible antifungal activity

**Table 6.** Blast results of the sequenced products obtained from polymerase chain reaction amplification using gene-specific primers for biosynthetic genes of fengycin and bacillomycin D synthetase.

Isolate	Primer	Product size (bp)	GenBank acc. No.	Obtained GenBank match	S-score	E-value
<i>Bacillus subtilis</i> DFH08	FEND1F/ FEND1R	964	AJ011849	<i>fenD</i> gene of <i>Bacillus subtilis</i> F29-3	1562	0
<i>B. subtilis</i> 49	FEND1F/ FEND1R	964	AJ011849	<i>fenD</i> gene of <i>B. subtilis</i> F29-3	1578	0
<i>B. subtilis</i> ATCC 13952	FEND1F/ FEND1R	964	AJ011849	<i>fenD</i> gene of <i>B. subtilis</i> F29-3	1594	0
<i>Bacillus cereus</i> DFE4	BACC1F/ BACC1R	875	AY137375	Bacillomycin D operon of <i>B. subtilis</i> ATTCAU195	1447	0
<i>Bacillus amyloliquefaciens</i> DFE16	BACC1F/ BACC1R	875	AY137375	Bacillomycin D operon of <i>B. subtilis</i> ATTCAU195	1463	0
<i>B. amyloliquefaciens</i> BS6	BACC1F/ BACC1R	875	AY137375	Bacillomycin D operon of <i>B. subtilis</i> ATTCAU195	848	0
<i>B. subtilis</i> 49	BACC1F/ BACC1R	875	AY137375	Bacillomycin D operon of <i>B. subtilis</i> ATTCAU195	1495	0

**Note:** S-score is a measure of the similarity of the query to the sequence shown. E-value is a measure of the reliability of the S-score.

**Fig. 2.** Chromatogram of purified lipopeptides from culture extract of *Bacillus subtilis* 49. The lipopeptides were purified by reversed phase-HPLC and the eluted fractions were detected at an absorbance of 220 nm. Bacillomycin D eluted in fractions 22–26, while fengycin was detected in fractions 27–33.

towards *S. sclerotiorum* (Fig. 4a) compared with *F. graminearum* (Fig. 4b). Fengycin exhibited higher antifungal activity than bacillomycin D, even though the purified lipopeptide suspension of fengycin was half the concentration of the bacillomycin D concentration.

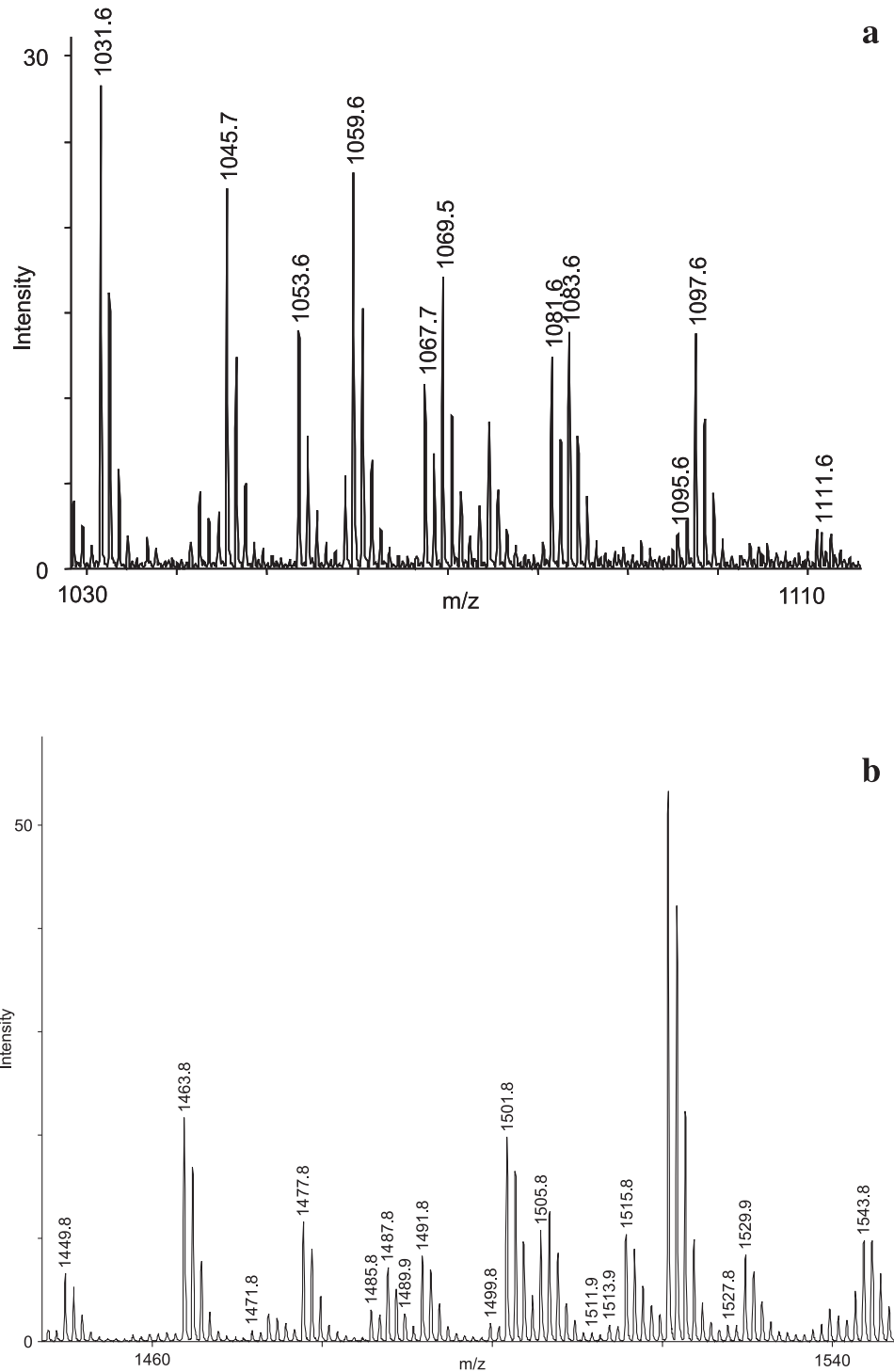
## Discussion

To our knowledge this is the first study involving identification of disease-suppressive bacterial antagonists isolated from canola and wheat, which could be potential producers of antifungal lipopeptides such as fengycins and bacillomycin D. This study employed specific PCR primers for the detection of biosynthetic genes of multimodular enzymes, the peptide synthetases, involved in the synthesis of the antifungal lipopeptides fengycin and bacillomycin D. The PCR detection was backed up with the biochemical detection of the

compounds, which established the presence of the biosynthetic genes corresponding to the production of the compounds. To our knowledge, this is also the first report of the presence of bacillomycin D biosynthetic genes in a *B. cereus* strain.

In our earlier studies, we identified bacterial antagonists involved in vitro and in vivo suppression of *L. maculans* (Ramarathnam and Fernando 2006) and *S. sclerotiorum* (Fernando et al. 2007); these organisms were also included in this study for the PCR analysis. In the present work, we have identified a bacterial antagonist isolated from a wheat head, *B. subtilis* DFH08, which shows significant suppression of *F. graminearum* in vitro and in vivo. The competence of a biocontrol agent and synchronization of its activity in time and space with the pathogen are key factors that determine the efficiency of the agent (Folman et al. 2003). Hence, we considered testing strain DFH08 in vivo

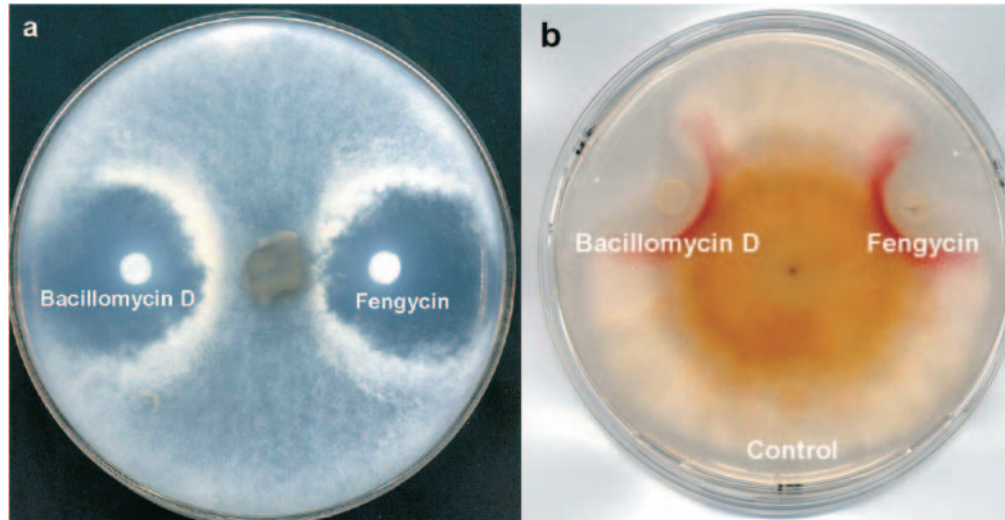
**Fig. 3.** Mass spectroscopy (MALDI-TOF-MS) spectra of molecular mass of strain Bs49 lipopeptides. (a) Spectra of bacillomycin D produced by *Bacillus subtilis* 49. Peaks in the range of  $m/z = 1069.5, 1083.7, 1097.6,$  and  $1111.6$  were detected in fractions 22–26, which could be attributed to the potassium adducts of C14–C17 bacillomycin D. (b) Spectra of fengycin produced by *B. subtilis* 49. Peaks in the range of  $m/z = 1449.8$ – $1543.8$  corresponded to the protonated forms and adducts of C15–C17 fengycins with alkali metal ions.



at the anthesis stage, which is the most crucial stage of head blight infection. It is the infection at anthesis that enables the fusarium fungus to gain access into the head and cause head blight, producing mycotoxins such as deoxynivalenol. If the head can be protected during this narrow window of infection then much of the disease can be controlled. In our

study, significant disease control was achieved when the bacteria was applied prior to the inoculation of *F. graminearum* macroconidia (Table 4). The time of application of the bacterial antagonist at the site of infection (on the head) seems to be more crucial than the bacterial seed coating treatment. In this case, no systemic resistance effect

**Fig. 4.** Inhibition of radial mycelial growth of *Sclerotinia sclerotiorum* (a) and *Fusarium graminearum* (b) by purified fengycin and bacillomycin D. Five microlitres of the purified lipopeptide was loaded onto sterile filter paper discs. The plates were scored when the mycelium grew over the control discs.



was observed with the bacterial seed coating, and it seemed to be more of an antifungal antibiotic effect mediated by strain DFH08 when applied at the site of infection.

Agar-diffusible antifungal activity and inhibition of radial mycelial growth is an indication of the potential of a bacterial antagonist to produce antibiotics (Burkhead et al. 1995). But such in vitro assays and several other screening procedures are time consuming and are not useful in assessing whether a given strain produces a particular antibiotic. Advances in molecular biology have led to the cloning and partial or complete sequencing of antibiotic biosynthetic and regulatory genes, which has facilitated the development of specific primers and probes that can be used for the PCR-based detection of specific antibiotic-producing bacteria (de Souza and Raaijmakers 2003). This enables processing of environmental bacterial samples in a much quicker and easier manner for the detection of biosynthetic genes involved in antibiotic synthesis. Also, unlike the laborious screening procedures, PCR screening enables precise identification of a particular antibiotic produced by the bacterium since these primers are targeted toward specific antibiotic biosynthetic genes. The primer pairs developed in this study target the biosynthetic genes of the multimodular fengycin and bacillomycin D synthetases. With the help of these primer pairs, strains DFH08 and Bs49 have been identified as potential fengycin producers, and strains DFE4, DFE16, BS6, and Bs49 have been identified as potential bacillomycin D producers. The specificity of the primers is clearly established when the positive data from the PCR was correlated with the purification and characterization of fengycin and bacillomycin D in the purified lipopeptide fraction derived from culture extracts of strain Bs49. This correlation may also exist for the other PCR positive strains to actually synthesize these antifungal lipopeptides. Indeed, this is expected, based on the high antifungal activity and disease suppressive qualities of strains DFE4 (Ramarathnam and Fernando 2006), DFE16 (Ramarathnam and Fernando 2006; Fernando et al. 2007), and BS6 (Fernando et al. 2007). To our knowledge, this is the first identification of a *B. cereus*

strain (DFE4) positive for the presence of bacillomycin D genes. Strain DFE4 contains biosynthetic genes encoding the aminopolyol antibiotic zwittermicin A (Ramarathnam and Fernando 2006), thus making it a potential multiple antibiotic producer and likely a strong candidate as a bio-control agent. Strains DFE4, DFE16, and BS6, isolated from canola phytosphere, with their high disease suppressive and antifungal activities, add further support to the findings of Berg et al. (2002) and Johansson and Wright (2003), who isolated a high number of disease suppressive, antagonistic bacteria from the *Brassicaceae*. Other than the presence of the bacillomycin D synthetase gene, strains DFE4, DFE16, and BS6 also test positive for the presence of the *ituD* gene of the iturin A operon involved in the biosynthesis of the lipopeptide iturin A (data not presented). This potential to produce multiple antibiotics could explain the ability of these bacteria to suppress multiple canola diseases. 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, and whether it is a means to compete with other microorganisms. The synergistic action of multiple antibiotics enables the members of the sessile actinomycetes to compete with other microorganisms for food and space (Challis and Hopwood 2003). *Bacillus subtilis* M4 produces multiple lipopeptides such as surfactins, fengycins, and iturins, but only fengycin was recovered from disease suppressed apple tissue in a study where the bacterium was challenge inoculated with the pathogen (Ongena et al. 2005). Hence, it is very important to understand the role of different antibiotics in disease control for which mutational studies are required. Single or multiple antibiotic production and activity could be the possible explanation for the antifungal nature of strains DFE4 and DFE16. Both strains exhibit disease suppression when applied at the site of pathogen infection, but no systemic resistance effect in disease reduction when applied away from the pathogen (data not presented). Similar results were obtained in this study for strain DFH08, a potential fengycin producer, where reduction in disease severity of *Fusarium*

head blight was seen when the bacterium was challenge inoculated with the pathogen on the wheat head, and no disease reduction or systemic resistance effect observed when applied as a seed treatment. These findings could be further strengthened by the isolation of the antibiotics from the site of infection.

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 integrate them into a disease management program involving minimal use of hazardous synthetic fungicides. Also, because many of these metabolites exhibit various biologically active ingredients, they are useful targets for biotechnological and biopharmaceutical interest (Vater et al. 2003). The antifungal activities of fengycin (Ongena et al. 2005) and bacillomycin D (Moyné et al. 2001), antiviral and antimicrobial activity of surfactins (Peypoux et al. 1999), and strong antifungal, hemolytic, and limited antibacterial activities of the iturins (Maget-Dana and Peypoux 1994) are few good examples. The natural pools of these lipopeptides can be extended in a directed manner by biocombinatoric efforts involving recombinant peptide synthetases, which synthesize these lipopeptides nonribosomally (Vater et al. 2003). Recombinant peptide synthetases can be obtained by swapping their module and domain, and also by site-specific mutagenesis of the amino acid residues involved in the intermediate steps of peptide synthesis. The *Bacillus* species, which are natural producers of these lipopeptides, could act as a rich genetic source for the construction of large biocombinatorial libraries of genetically engineered peptide synthetases for novel peptide design.

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