Ann Microbiol (2016) 66:407–416
DOI 10.1007/s13213-015-1123-0

ORIGINAL ARTICLE

A novel strain of *Bacillus amyloliquefaciens* displaying broad spectrum antifungal activity and its underlying mechanism

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Received: 5 March 2015 / Accepted: 16 June 2015 / Published online: 3 July 2015

Abstract One isolate among the various bacterial soil isolates screened in our study showed broad spectrum and profound antifungal activity and was characterized by biochemical and molecular approaches. Sequence analysis of the 16S rRNA gene indicated more than 98 % sequence similarity with *Bacillus amyloliquefaciens* ATCC 23350^T and its variant *Bacillus velezensis* BCRC 17467^T. However, the biochemical characteristics of the isolated strain, such as cellular fatty acid composition, differed significantly from those of these two *Bacillus* strains, with the isolated strain containing anteiso-C15:0 (terminally branched saturated fatty acids) as a predominant fatty acid. The band pattern of the pulsed field gel electrophoresis profile of *Avr*II-digested genomic DNA of the isolate also showed significant variation from those of the two closely related *Bacillus* type strains. Based on these differences, the isolate was considered to be a new strain of *B. amyloliquefaciens* and deposited as *Bacillus amyloliquefaciens* strain fiply 3A in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) culture collection (DSM 22646) and in MTCC, IMTECH, Chandigarh (DSM 22646). Purification of the extracellular antifungal compound produced by the isolate by HPLC and its analysis by LC-ESI-MS revealed it to be a bacillomycin D-like cyclic lipopeptide. The antifungal activity of the compound was found to be due to its inhibitory effect on β-1,3-glucan biosynthesis, a major fungal cell-wall component. Thus, we describe here the identification and characterization of a novel strain of *Bacillus amyloliquefaciens* (fiply 3A) which produces antifungal lipopeptide and the deciphering of its mechanism of action.

Keywords *Bacillus* · Lipopeptide · β-1,3-Glucan · *Aspergillus parasiticus* · Pulsed field gel electrophoresis

Introduction

Fungi have a tremendous potential to grow and survive in nature, primarily because they can utilize wide range of organic substrates as their nutrient and energy source, making it difficult to control them (Quiroga et al. 2001). This leads to the ubiquitous presence of fungal organisms and, consequently, a menace to the health of both animals and humans as many fungi cause diseases. In humans, major fungal infections range from superficial skin infections to biofilm formation on open wounds (Ramage et al. 2014). Invasive fungal infections pose major management problems for hematopoietic cell transplant patients (Wingard and Leather 2004). Two major fungal genera, *Candida* and *Aspergillus*, account for most of the fungal infections in such patients (Wingard and Leather 2004). Four major classes of antifungal drugs are currently used to control these infections, namely, the polyenes, nucleoside analogs, azoles and echinocandins (Wingard and Leather 2004). Their mechanism of action includes binding to ergosterol (polyenes), inhibiting nucleic acid biosynthesis (flucytosine, a nucleoside analog) or inhibiting glucan biosynthesis (echinocandins).

Certain fungi can produce mycotoxins in food and feed, such as aflatoxins (*Aspergillus parasiticus*) and ochratoxins (*Aspergillus ochraceus*), making them unsafe for consumption,

Electronic supplementary material The online version of this article (doi:10.1007/s13213-015-1123-0) contains supplementary material, which is available to authorized users.

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while others can also cause diseases, such as rust or smuts, in the crop plants (Ellis et al. 2007). The losses due to fungal contamination and infections have been reported to be more than 25 % of the agricultural crops produced (Kabak 2010). To control such fungal contaminations in food crops, synthetic chemicals, such as thiabendazole and carbendazim, are used either alone or in combination (Leelasuphakul et al. 2008). However, the use of such chemicals has raised public concerns regarding possible toxicological risks and environmental effects, leading to several such fungicides being banned for field applications (Leelasuphakul et al. 2008). Moreover, it has been suggested that overuse of these chemicals could also lead to the development of novel fungicide-resistant strains (Janisiewicz and Korsten 2002). Thus, it is of utmost importance to develop an alternative strategy with the potential to be translated into an effective fungal control measure. Biological control methods which involve the use of either microorganisms or their extracellular metabolites for controlling the pathogenic fungi provide a viable option to be explored (Mercier and Jiménez 2004). Some *Pseudomonas* and *Bacillus* strains have been reported to have such potential.

The major objective of our study was to isolate a microorganism having the potential to produce a broad spectrum antifungal compound which can work against both plant (e.g. *Aspergillus* sp.) and human pathogenic (e.g. *Candida* sp.) fungi. To this end we identified a new isolate using biochemical and molecular approaches. The secondary metabolite produced by this isolate had been purified in an earlier study and characterized as a cyclic lipopeptide using standard chromatographic [high-performance liquid chromatography (HPLC)] and spectroscopic [liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)] approaches (Hajare et al. 2013). We also investigated the mechanism of action of this antifungal metabolite and evaluated its efficacy to control the growth of mycotoxigenic fungus on spiked food grains (e.g. corn).

Materials and methods

Chemicals and bacterial strains

The chemicals and media used in this study were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), unless otherwise specified. The bacterial type strains, namely *Bacillus amyloliquefaciens* MTCC 1270 (ATCC 23350^T) and *B. subtilis* MTCC 121 (ATCC 6051^T), were procured from the Microbial Type Culture Collection (MTCC), IMTE CH, Chandigarh, India, whereas the type strain of *B. velezensis* BCRC 17467^T was obtained from Colección Española de Cultivos Tip (CECT), Valencia, Spain.

Isolation of bacterial strain

The bacterial strain was isolated from a sample of field soil from the Institute's campus. The soil sample (1 g) was suspended in 0.85 % saline, streaked on nutrient agar (NA) plates and incubated at ambient temperature (26±2 °C) for 24 h. About 45 randomly selected bacterial isolates were screened for antifungal activity as described by Souto et al. (2004). Pure bacterial culture was grown overnight in nutrient broth (NB), and then an aliquot of the broth was streaked in a straight line on one side of a petri dish containing potato dextrose agar (PDA) or a mix of NA:PDA (1:1, v/v). Simultaneously, an agar plug (diameter 9 mm) containing mycelium of *Aspergillus parasiticus* NRRL 3145 grown for 48 h in PDA was placed in the center of the plate. The plate was incubated for 7 days at 28 °C, following which time the inhibitory effect of the bacterial isolate on fungal growth was evaluated in terms of size of the inhibition zone.

Identification and characterization of the new bacterial isolate

The isolate was characterized by conventional microbiological methods for morphological parameters, including shape of the cells, morphology of sporangia and shape and position of spores. Further characterization of the strain was carried out using biochemical and molecular analysis involving sequencing and comparison of conserved genes, cellular fatty acid analysis and pulsed field gel electrophoresis (PFGE).

Conserved genes, such as the 16S rRNA gene, *recA*, *gyrB* and *rpoB*, were amplified by PCR (Master Thermal Cycler, Eppendorf AG, Hamburg, Germany) and sequenced using the primers described in detail in Electronic Supplementary Material (ESM) Table S1. The sequences of the cloned genes were obtained using the ABI PRISM^{TM} 3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and subsequently compared with existing bacterial sequences using NCBI nucleotide BLAST tool and CLUSTAL_W version 1.83 (Kyoto University, Kyoto, Japan). Cellular fatty acids of the isolate were extracted using the MIDI protocol (Van den Velde et al. 2006), and the fatty acid methyl esters obtained were analyzed using a gas chromatograph-MS system equipped with a GC-17A Gas Chromatograph (Shimadzu Corp., Kyoto, Japan) with a DB-5 Capillary Column (J&W Scientific, Folsom, CA, USA): 5 %-phenyl-methylpolysiloxane, length 30 m, internal diameter 0.25 mm; film thickness 0.25 m. The PFGE analysis of bacterial strains was carried out as described by Yavuz et al. (2004).

Purification and characterization of the antifungal secondary metabolite

The isolate was grown in potato dextrose broth (PDB) at 30 °C with shaking (150 rpm) for 6 days, following which time the

cells were removed by centrifugation (12,000 *g*, 10 min, 4 °C). The supernatant containing the antifungal secondary metabolite (ASM) was subjected to ammonium sulfate precipitation at 60 % (w/v) saturation concentration. The precipitate was dissolved in distilled water (DW) and dialyzed through a tubular cellulose membrane (molecular weight cutoff 10 kDa) against DW to remove all traces of ammonium sulfate. The insoluble residue was removed by centrifugation (18,000 *g*, 15 min, 4 °C). Finally, the supernatant was freeze-dried and stored at −20 °C until further use. This fraction, referred to as the partially purified ASM (PPASM), was used for the antifungal assay. To compare the profile of antifungal metabolite production of the isolate to those of closely related species, we grew *Bacillus subtilis* ATCC 6051^T and *B. amyloliquefaciens* ATCC 23350^T in Luria broth for 48 h (Yang et al. 2015) and *B. velezensis* BCRC 17467^T in tryptic soy broth medium for 6 days (Ruiz-Garcia et al. 2005). The antifungal activity of filter-sterilized (0.22 μm) culture supernatants of all of the strains was evaluated against *Candida albicans* MTCC 183 using the disc diffusion assay.

Further purification of the ASM was achieved using RP (reverse phase)-HPLC as described by Kim et al. (2004). The pH of the PPASM was adjusted to 2.0 using 3 N HCl, which resulted in the precipitation of the metabolites. The precipitate was centrifuged, and the pellet obtained was dried and suspended in $CHCl_3$:MeOH (2:1). Insoluble material was removed by centrifugation, and the supernatant obtained was filtered through a 0.22-μm filter. The filtrate was injected into an HPLC column (ODS Hypersil C_{18}; Thermo Fisher Scientific, Waltham, MA, USA): internal diameter 4.6 mm, length 250 mm, particle size 10 μm. The mobile phase consisted of 0.1 % trifluoroacetic acid (TFA) in water (solvent A) and 0.1 % TFA in acetonitrile (solvent B). The sample was analyzed with a linear gradient of solvent B, which increased from 30 to 100 %, at a flow rate of 1.0 ml/min. The elution was monitored at 225 nm. Peaks obtained were collected separately and analyzed for antifungal activity. The peaks showing antifungal activity were characterized by LC-ESI-MS; Thermo Finnigan LCQ Deca Electrospray quadrupole ion trap mass spectrometer; ThermoElectron Co., Hemel Hempstead, Herts, UK. The flow rate of the analyte was maintained at 5 μl/min. Nitrogen was used as the sheath and auxiliary gas. The ion source conditions were: sheath gas flow rate of 20 arbitrary units, capillary temperature was maintained at approximately 200 °C and capillary voltage was kept at 15 V. The ion-spray voltage and tube lens offset were maintained at 4.5 kV and −7 V, respectively. The ion optics was tuned to achieve a maximum ion count.

Microscopic examination of fungal morphology upon treatment with purified ASM

Spores of *A. parasiticus* (approx. 10^3 spores/ml) were inoculated in PDB containing purified ASM (PASM; 2 μg/ ml) and incubated for 24 h at ambient temperature (26±2 °C). Slides were then prepared from treated and control cultures and observed under a light microscope (Axiolab, Carl Zeiss, Jena, Germany) using 40× objective.

Estimation of β-1, 3-glucan from fungal cells

Aspergillus spores (approx. 10^5 spores/ml) were grown for 72 h in PDB either in absence (untreated) or presence of PPASM (100 μg/ml) or PASM (5 μg/ml), the mycelium was then harvested, and the cell walls from both the treated and untreated mycelia were prepared as described by Momany et al. (2004). The freeze-dried cell-wall material (5 mg) was suspended in 1 ml of 50 mM sodium acetate buffer (pH5.2) and then 40 μl of β-1,3 glucanase (Sigma, St. Louis, USA; 10 mg/ml) was added to the suspension and the mixture incubated at 37 °C for 48 h for complete degradation of β-1,3-glucan. The glucose released from this degradation was estimated using a glucose estimation kit (Sigma) according to the manufacturer's guidelines. In this assay glucose is oxidized by glucose oxidase to gluconic acid and hydrogen peroxide, and the latter reacts with *o*-dianisidine in the presence of peroxidase to form a colored product. The colored product further reacts with sulfuric acid to form a more stable pink-colored product; the intensity of the latter is measured at 540 nm and is directly proportional to the amount of glucose in the sample. Glucose released from the cell wall was quantified using a calibration curve.

Antifungal activity of PASM and determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the PASM was determined against 13 fungal cultures obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India, using the broth macrodilution method (Pujol et al. 1996). These fungal strains were maintained on PDA. A spore suspension of each strain was prepared by flooding 15-day-old cultures with sterile 0.01 % Tween 80 water. Viable spore count was determined by serially diluting the spore suspension with sterile 0.01 % Tween 80 water and plating the dilutions on PDA plates. PASM stocks were prepared at tenfold the strength of the required concentration and dispensed in 0.1-ml aliquots in 1.5-ml polystyrene tubes. During the assay, approximately 10^3 spores were inoculated in 0.9-ml PDB containing 0.1 ml of the tenfold PASM and incubated at 26±2 °C. The lowest concentration of PASM which reduced the growth measured in terms of a turbidity by ≥80 % compared to that of control ones was considered to be the MIC (Barchiesi et al. 1994).

Use of PPASM as a biocontrol agent

Partially purified ASM was favored over PASM as a biocontrol agent mainly due to our objective to develop a cost-effective fungicide. Hence, spiked corn kernels (10 g) with *A. parasiticus* spores (approx. 10^3 spores) were treated with PPASM (5 mg/ml). Test samples together with proper controls were incubated at ambient temperature (26±2 °C) for up to 21 days at 90 % relative humidity, following which time the samples were crushed to powder using a mortar and pestle and extracted with a chloroform:water (1:1) mixture. The extract was separated and partially purified using a silica gel column consisting of sodium sulfate for the top and bottom layers and activated silica gel for the middle layer. The chloroform extract was added to the column and washed initially with hexane, followed by ether and finally with methanol:chloroform (3:97) for aflatoxin elution. The purified fractions were dried and processed for quantification of total aflatoxins (Velasco 1975).

Results and discussion

In the context of identifying an ASM for use in the control of both mycotoxigenic and pathogenic fungi, we examined bacterial isolates from a soil sample for their ability to produce effective and broad spectrum antifungal compounds and chose one such potent strain for further characterization.

Bacterial strain displaying antifungal activity

About 45 randomly selected bacterial isolates were screened for antifungal activity against *Aspergillus parasiticus*. Of these, five isolates (No. 1, 2, 3, 7 and 12) showed this activity, with isolate number 3 displaying the highest antifungal activity in terms of size of the inhibition zone (ESM Table S2). This isolate was selected for further analysis.

Biochemical and molecular characterization indicated the isolate was a *Bacillus* strain

Microscopy of the isolate confirmed it to be Gram-positive, medium-sized (0.6×1.4–3.5 μm), motile rods. Analysis of the spores indicated the presence of centrally located oval-shaped endospores which were produced in swollen sporangia.

The sequence of the 16S rRNA, *gyrB* and *rpoB* genes of the new isolate showed 98.2, 95.5 and 99.0 % similarity, respectively, with *Bacillus amyloliquefaciens* ATCC 23350^T, while the sequence of the *recA* gene of the new isolate showed 97.8 % similarity with *B. amyloliquefaciens* FZB42 strain (Table 1). Phylogenetic reconstructions generated using the 16S rRNA, *gyrB* and *rpoB* genes also revealed that the isolate was more closely placed to *B. amyloliquefaciens* type strain than to the *B. subtilis* type strain (ESM Fig. S1).

Thus, taking into account all of the sequence data (Table 1), it would appear that the new isolate showed more similarity to the *B. amyloliquefaciens* type strain; more specifically, it showed significant similarity with *B. velezensis* which is now considered to be a *B. amyloliquefaciens* species. Thus, for further comparison, the *B. velezensis* BCRC 17467^T strain was selected along with the *B. amyloliquefaciens* strain ATCC 23350^T.

Analysis of the cellular fatty acid composition showed that the new *Bacillus* isolate contained saturated straight-chain, saturated terminally branched and monounsaturated fatty acids in the ratio of 13:83:4, respectively (Table 2). In genus *Bacillus*, terminally branched fatty acids are always more predominant that the other two types of fatty acids taken together (Albert et al. 2005; Wan et al. 2008). Hexadecanoic acid (16:0), 13-methyl tetradecanoic acid (15:0 iso), 15-methyl hexadecanoic acid (17:0 iso), 12-methyl tetradecanoic acid (anteiso-C15:0) and 14-methyl hexadecanoic acid (anteiso-C17:0) were found to be the major fatty acids in the new isolate (Table 2). Saturated straight-chain fatty acid 16:0 accounted for approximately 10 % of all fatty acids in the new isolate, while in two of the *Bacillus* type strains it accounted for much less (<5 %).

The PFGE profile of *Avr*II-digested DNA of the new *Bacillus* isolate was found to be distinct from those of the closely related type strain (*B. amyloliquefaciens* ATCC 23350^T and its variant *B. velezensis* BCRC 17467^T) (Fig. 1). Restriction analysis of the *B. amyloliquefaciens* ATCC 23350^T genome resulted in about 11 distinct fragments which ranged from 291 to 48.5 kbp (lane 2), while that of the new isolate showed about 14 distinct fragments in the range of 436.5 to 48.5 kbp (lane 3) (Fig. 1). Restriction analysis of *B. velezensis* BCRC 17467^T resulted in about 12 distinct bands in the range of 485 to 145.5 kbp (lane 4). The difference in PFGE banding pattern indicated high genetic diversity among three strains. As PFGE analysis spans the entire genome, the technique is considered a relatively conclusive method to determine the similarity or dissimilarity of strains.

Submission of the strain in culture collection

The new strain was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) culture collection (DSM 22646) and in the MTCC, IMTECH, Chandigarh (MTCC 11707) under the name *Bacillus* sp. strain fiply 3A.

New isolate displayed better antifungal activity than the other *Bacillus* strains tested

The new isolate displayed better antifungal activity against *C. albicans* in terms of size of the inhibition zone (10 mm) than *B. velezensis* BCRC 17467^T (inhibition zone 7 mm)

Table 1 Sequence similarity of new isolate with related *Bacillus* type strains

Isolate/strain	Characteristics	Genes sequenced			
		16S rRNA	*gyrB*	*rpoB*	*recA*
New isolate	% identity	100	100	100	100
	Accession no.	EF 392817	GQ850459	GU057406	GQ900665
Bacillus amyloliquefaciens ATCC 23350^T	% identity	98.2	95.5	99	97.8
	Accession no.	X60605	DQ309294	FN597644	CP000560
B. subtilis ATCC 6051^T	% identity	99.3	80.1	91.3	N.A.
	Accession no.	EF423592	DQ309293	CP010052	N.A.
B. velezensis BCRC 17467^T	% identity	99.7	99.4	N.A.	N.A.
	Accession no.	AY603658	DQ903176	N.A.	N.A.

N.A., Not available

(ESM Fig. S2).The other strains tested, *B. subtilis* ATCC 6051^T and *B. amyloliquefaciens* ATCC 23350^T, did not show any antifungal activity against *C. albicans* MTCC 183 (ESM Fig. S2). In another study, the culture supernatants of both *B. velezensis* BCRC 17467^T and *B. amyloliquefaciens* ATCC 23350^T grown in PDB for 6 days did not show any significant antifungal activity against *C. albicans*. Thus, the findings indicated that the growth media did not have any major impact on the production of an antifungal factor in other *Bacillus* species. Among these bacterial species, the new isolate displayed maximum antifungal activity and hence maximum ASM production.

Based on the results of these analyses, the newly isolated strain showed significant variation from the other related *B. amyloliquefaciens* strains and was considered to be a novel strain of this genus.

Many species of *Bacillus* produce various biologically active ASM which have the potential to inhibit different phytopathogenic and mycotoxigenic fungi (Peypoux et al. 1999). It has been reported that about 4–5 % of the genome of *B. subtilis* (Lee and Kim 2012) and more than 8.5 % of the genome of *B. amyloliquefaciens* (Chen et al. 2007) are engaged in antibiotic biosynthesis, with the potential to produce more than two dozen structurally diverse antimicrobial

Table 2 Cellular fatty acid composition of the new isolate *Bacillus amyloliquefaciens* strain fiply 3A and related *Bacillus* species

Fatty acid	Fatty acid content (%)			
	Bacillus amyloliquefaciens fiply 3A	*Bacillus velezensis* BCRC 17467^T	*Bacillus amyloliquefaciens* ATCC 23350^T	*Bacillus subtilis* ATCC 6051^T
Saturated straight-chain fatty acid				
12:0	0.3	–	–	–
14:0	1.42	3.8	0.7	–
16:0	10.42	18.3	5.40	3.65
18:0	0.40	–	–	–
Saturated terminally branched fatty acid				
13:0 iso	0.36	–	–	–
14:0 iso	0.7	–	1.48	0.87
15:0 iso	29.08	30.4	28.63	24.08
16:0 iso	1.19	1.0	3.12	2.47
17:0 iso	9.26	7.8	13.18	11.53
anteiso- C15:0	35.11	27.6	33.10	38.48
anteiso- C17:0	6.81	3.4	8.65	12.52
Monounsaturated fatty acid				
16:1ω11*c*	2.87	3.5	2.73	1.75
ISO 17:1ω10*c*	1.34	1.3	2.05	2.41

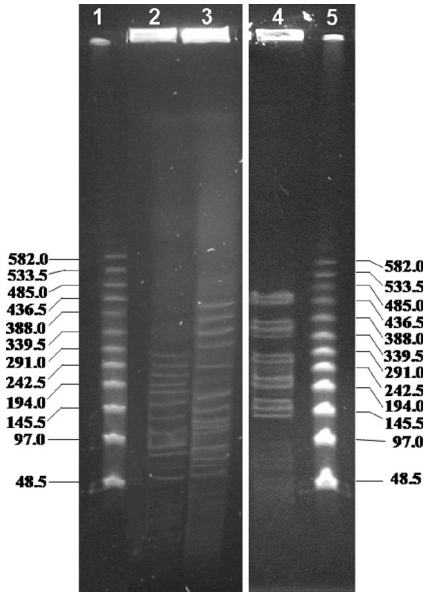

Fig. 1 Pulsed field gel electrophoresis profile of *Avr*II-digested DNA of *Bacillus amyloliquefaciens* strain fiply 3A (*lane 3*) in comparison with those of *B. amyloliquefaciens* ATCC 23350^T (*lane 2*) and *B. velezensis* BCRC 17467^T (*lane 4*). *Lanes 1, 5* Molecular weight marker

compounds. Lee and Kim (2012) isolated *B. amyloliquefaciens* strains from *Meju* (a traditional dish of Korea) that displayed antifungal activity against toxigenic *Aspergillus* and *Penicillium* species.

Characterization of ASM

As the new isolate produced an extracellular antifungal compound during its stationary phase, this compound can be considered to be a secondary metabolite. Among the different media assessed, PDB was found to be the best to support the production of the ASM (data not shown), with the new isolate producing between 300 and 390 mg/l ASM on PDB based on both dry weight and HPLC analyses. The metabolite displayed functional stability over a wide range of pH (pH 2–10) and temperature (up to 121 °C) (data not shown).

In an earlier study the ASM had been characterized as a cyclic lipopeptide bacillomycin D, having three structural variants of the molecular weight (1031, 1045 and 1059 Da; Hajare et al. 2013). The lipopeptide preparation obtained by ammonium sulfate precipitation followed by acid precipitation, solvent extraction and RP-HPLC (ESM Fig. S3) was found to be pure, as evaluated by LC-ESI-MS, with no contaminating peaks were detected. This purified cyclic lipopeptide was used in analyses aimed at gaining an understanding of its mechanism of antifungal activity and determining its MIC against 13 different fungal strains, including *C. albicans*, *Aspergillus* sp. and *Penicillium* species.

Possible mechanism contributing to the antifungal activity of the PASM

Microscopic examination of the fungus revealed that treatment with the PASM caused abnormal mycelial growth, as evidenced by increases in mycelial apex offshoots, distortion and tumescence (Fig. 2b). These abnormalities indicated a possible defect in fungal cell-wall synthesis, whereas no such abnormalities were was seen inf untreated mycelium (Fig. 2a).

PASM treatment resulted in impairment of β-1,3-glucan synthesis in the fungal cell wall

The fungal cell wall is an important cell organelle which not only maintains cell shape and integrity but also provides the mechanical strength to withstand changes in the osmotic pressure imposed by the environment. Hence, disruption of cell-wall structure has a profound effect on the growth and morphology of a fungal cell, often rendering it susceptible to lysis and death. Consequently, an antifungal agent which damages the cell wall is of immense significance. The fungal cell wall is primarily composed of chitin, glucans, mannans and glycoproteins. Chitin accounts for approximately 1–2 % of the yeast cell wall by dry weight and approximately 10–20 % of the cell wall of filamentous fungi. Glucan is the major structural polysaccharide of the fungal cell wall, constituting approximately 50–60 % by dry weight. One of the major constituents of glucan is β-1,3-glucan (approx. 65–90 %), and this polymer serves as the main structural constituent to which other cell-wall components are covalently attached. Hence, β-1,3-glucan synthesis is a very important process for proper fungal cell-wall formation and the normal development of fungi (Bowman and Free 2006). Therefore, we studied the effect of PASM on β-1,3-glucan synthesis in the fungal cell wall. Interestingly, β-1,3-glucan biosynthesis was found to be inhibited by the PASM treatment (Table 3). The amount of this polymer in the cell-wall component of the fungus was measured in terms of released glucose upon treatment with purified β-1,3 glucanase which eventually indicated the proportion of β-1,3-glucan in the fungal cell wall. As seen in Table 3, PPASM treatment resulted in about 78 % reduction in the glucan production in the cell wall compared to the untreated control and a more than 99 % reduction in cell-wall glucan content (Table 3).

Our findings confirmed that the PASM acted on the fungal cells mostly by inhibiting β-1,3-glucan synthesis, resulting in abnormal mycelial growth. Similar results were reported by Kurtz et al. (1994) and Huang et al. (1990) in *Aspergillus fumigatus* and *C. albicans*, respectively, when these fungi were treated with a cyclic lipopeptide, pneumocandin B_0. Gong et al. (2014) recently studied the effect of bacillomycin D from *B. subtilis* fmbj on *Aspergillus flavus* hyphae and spores and reported that both hyphal

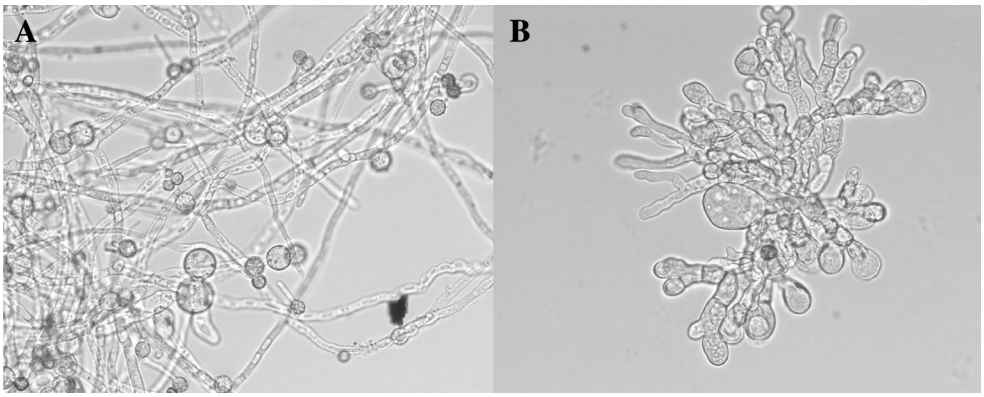

Fig. 2 Morphology of control and purified antifungal secondary metabolite-treated *Aspergillus parasiticus* NRRL 3145 hyphae under light microscope. **a** Control mycelium, not treated with metabolite (magnification 40× 10), **s** test mycelium after being treated with the purified lipopeptide (2 μg/ml) for 24 h (magnification 40×10)

growth and spore germination rate were reduced with increasing concentration of bacillomycin D. These authors concluded that due to its amphipathic nature, bacillomycin D entered the spores and the hyphae where it caused pores to be formed in the membrane, resulting in the leakage of cell contents. However, they did not report any inhibition of β-1,3-glucan synthesis in the cell wall of the spores or mycelium. In a more recent study, Romano et al. (2013) identified a new surfactin-like lipopeptide from *B. amyloliquefaciens* strain BO5A that shows antifungal activity against pathogenic *Fusarium oxysporum* and *Aspergillus niger*. Kumar et al. (2014) reported the biocontrol of *A. flavus* on peanut kernels by the antifungal metabolite diketopiperazine produced by *Bacillus cereus*. In an earlier study, Hassan et al. (2010) isolated three *Bacillus* strains, namely *B. subtilis* NH-100, *B. subtilis* NH-160 and *Bacillus* sp. NH-217, which produced surfactin with an antagonistic activity against *Colletotrichum falcatum*. A novel strain of *Bacillus velezensis*, BCRC 17467^T, was identified by Ruiz-Garcia et al. (2005) as producing biosurfactant; this strain was later found to be a variant of *B. amyloliquefaciens* (Wang et al. 2008). Other authors have also reported similar findings (Chen et al. 2010; Thasana et al. 2010; Basurto-Cadena et al. 2012; Cui et al. 2012; Martinez-Absalon et al. 2012).

To the best of our knowledge, there has been no earlier report in which extracellular lipopeptide (bacillomycin D) from a *Bacillus* species has been shown to inhibit β-1,3-glucan synthesis.

Table 3 Effect of treatment with partially purified antifungal secondary metabolite (PPASM) and purified ASM (PASM) on β-1,3-glucan level in the cell wall of *Aspergillus parasiticus*

Treatment of *A. parasiticus*	Glucose concentration (μg/mg mycelium)
Control (untreated)	102±2.6 a
PPASM	23±1.9 b
PASM	1±0.1 c

Data are presented as the mean±standard deviation (SD) of 3 different sets of experiments performed in triplicate. Values followed by different lowercase letters are significantly different at the 0.05 level of significance ($P<0.05$) as analyzed by one-way analysis of variance (ANOVA)

The MIC of PASM against different fungal strains

The MIC values of the PASM ranged from 4 (*Penicillium oxalicum* strain TMPS3 and *Aspergillus niger* NCIM 501) to 40 μg/ml (*Alternaria solani* NCIM 887 and *Fusarium moniliforme* NCIM 1099) (Table 4). For the opportunistic human pathogen *Candida albicans* MTCC 183, the MIC value was found to be 10 μg/ml. Intermediate susceptibility to PASM was observed for *Aspergillus parasiticus* NRRL 3145, *Penicillium expansum* NCIM 939 and *P. pinophyllum* NCIM 759, with the MIC value being 20 μg/ml for these three strains. *Aspergillus flavus* NCIM 535, *A. oryzae* NCIM 635, *Fusarium oxysporum* NCIM 1008 and *Penicillium chrysogenum* NCIM 708 were found to show some degree of resistance to the antifungal properties of the PASM and required higher concentrations of the PASM (MIC 30 μg/ ml) compared to the other strains (Table 4). As the PASM was found to inhibit various plant pathogenic and toxigenic fungi as well as human pathogenic fungi, it can be considered as a compound with broad spectrum antifungal activity.

Different fungi showed variable MIC values, possibly due to differences in their cell-wall components, which seems to be the major target of this lipopeptide. Carrillo et al. (2003) reported that the presence of cholesterol in the phospholipid bilayer can attenuate the membrane destabilizing effects of the lipopeptide surfactin. During the course of our study, we found that β-1,3-glucan is one of the targets of PASM. The extent of the sensitivity of fungal strains to this molecule was found to be positively correlated with the β-1,3-glucan content of the cell wall of the fungal strain. Fungal strains containing more β-1,3-glucan in their cell wall (e.g. *C. albicans*—up to 39 %) have bee reported to be more susceptibility to PASM than those containing a relatively lower amount of this polymer (Free 2013).

Table 4 Sensitivity of various fungal strains to PASM treatment

Fungal strain	Pathological significance	MIC of the PASM (μg/ml)
1. *Aspergillus niger* NCIM 501	Human pathogenic	4±1
2. *Penicillium oxalicum* strain TMPS3	Non-pathogenic	4±1
3. *Botrytis allii* NCIM 1041	Plant pathogenic	5±1
4. *Candida albicans* MTCC 183	Human pathogenic	10±1
5. *Aspergillus parasiticus* NRRL 3145	Mycotoxigenic	20±2
6. *Penicillium expansum* NCIM 939	Plant pathogenic	20±1
7. *Penicillium pinophyllum* NCIM 759	Non-pathogenic	20±2
8. *Aspergillus flavus* NCIM 535	Mycotoxigenic	30±2
9. *Aspergillus oryzae* NCIM 635	Non-pathogenic	30±2
10. *Fusarium oxysporum* NCIM 1008	Plant pathogenic	30±2
11. *Penicillium chrysogenum* NCIM 708	Human pathogenic	30±2
12. *Alternaria solani* NCIM 887	Plant pathogenic	40±3
13. *Fusarium moniliforme* NCIM 1099	Plant pathogenic	40±2

Values are presented as the mean ± SE

PPASM as a biocontrol agent

As the PASM was shown to possess broad antifungal activity, we then focused on evaluating its efficacy as a biocontrol agent. We addressed this question in a preliminary study by first applying PPASM onto corn kernels spiked with approximately 10^3 spores of *A. parasiticus* and then after a prolonged incubation measuring the aflatoxin accumulation. Table 5 shows the inhibition of aflatoxin production on the corn kernels following PPASM treatment. Aflatoxin biosynthesis was reduced by about 84 % during 7 days of incubation at ambient temperature (26±2 °C). Although continued incubation up to 21 days resulted in some recurrence of toxin biosynthesis in the PPASM-treated kernels (Table 5), the increase was considerably less in the treated samples than in the untreated ones. In a separate experiment in which a higher concentration of PPASM (20 mg/10 g) was used and the samples were incubated at ambient temperature (26±2 °C) for 1 month, a complete inhibition of *Aspergillus* growth and aflatoxin production was achieved (ESM Fig. S4). Gong et al. (2014) performed similar study with purified bacillomycin D and reported a concentration-dependent inhibition of *A. flavus* growth on corn kernels for up to 8 days.

Other potential applications of the PASM

In another study from this laboratory, the biosynthesis of this molecule was found to be well correlated with the extent of sporulation, indicating a possible correlation between its biosynthesis and cellular stress. However, further studies are needed to validate these unpublished data. Interestingly, this molecule also exhibited preferential toxicity to cancer cells, and the mechanism of cell death was found be the induction of programmed cell death in these cells (Hajare et al. 2013). The study involved evaluating the effect of purified lipopeptide on three cancer cell lines, namely, A549 (human alveolar adenocarcinoma), A498 (human renal carcinoma) and HCT-15 (colon adenocarcinoma), as well as one normal cell line, L-132 (pulmonary epithelial cells), and one primary cell type, i.e. mouse splenocytes. Purified lipopeptide in the concentration range of 15–120 μg/ml (i.e. concentrations used in antifungal assays) displayed induced cytotoxicity preferentially to the cancer cell lines in a concentration-dependent manner. The normal cell line did not show any significant toxicity, while the splenocytes were unaffected, even at concentrations of 200 μg/ml (Hajare et al. 2013). These results open an avenue to explore the use of this lipopeptide as a possible

Table 5 Inhibition of aflatoxin production by *Aspergillus parasiticus* on corn samples treated with PPASM

Days of incubation (*n*)	Aflatoxin concentration (μg/g corn)		% Inhibition
	Untreated	PPASM treated	
7	36.0±2.1 a	5.6±3.7 b	84.4
14	87.5±3.2 c	17.0±6.4 d	80.6
21	167.5±7.4 e	34.5±8.3 f	79.4

Data are presented as the mean± SD of 3 different experiments with 3 replicates. Means followed by different lowercase letters across rows are significantly different at the 0.05 level of significance ($P<0.05$) as analyzed by two-way ANOVA

chemotherapeutic agent. However, in some studies bacillomycin-D-like lipopeptides have been reported to show hemolytic activity (Tabbene et al. 2011), with one of the isoforms showing 50 % hemolytic activity at 22.14 μM. This is an important issue which directly pertains to the potential application of any lipopeptide in the clinical setting, and further studies are required on these lipopeptides to evaluate their safety for therapeutic applications.

Conclusion

In our search for a better source of antifungal compound we identified and characterized a new soil bacterial isolate as a novel strain of *Bacillus amyloliquefaciens*. This new strain was deposited in two culture collections (DSMZ, Germany and MTCC, IMTECH, Chandigarh, India).

The antifungal compound identified, which had been characterized as a cyclic lipopeptide bacillomycin D in an earlier study, displayed broad spectrum antifungal activity against aflatoxigenic and human pathogenic fungi. The antifungal activity of the compound was found to be due to the inhibition of β-1,3-glucan synthesis in the fungal cell wall. This compound showed quite promising results in terms of controlling fungal growth and aflatoxin production in a pilot scale study carried out on spiked corn kernels. As the compound displayed higher stability at broad range of temperature and pH, its wider potential for field applicability looks promising.

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