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Molecular and Biochemical Approaches for Characterization of Antifungal Trait of a Potent Biocontrol Agent *Bacillus subtilis* RP24

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Abstract *Bacillus subtilis* strain RP24, isolated from rhizoplane of field grown pigeon pea, exhibited in vitro antagonism against a wide range of phytopathogenic fungi. An attempt was made to partially purify and characterize the diffusible antifungal metabolite/s produced by the strain RP24 and its negative mutant (NM) in potato dextrose medium. High performance liquid chromatography (HPLC) of partially purified extract of RP24 showed the presence of lipopeptide antibiotic iturin as a major peak that was comparable to that of standard iturin A (5.230 min) from Sigma–Aldrich whereas the corresponding peak was absent in extract of NM. The structure was further confirmed by liquid chromatographic mass spectrometric (LCMS) analysis as iturin A. LCMS analysis also showed the presence of surfactin and fengycin besides iturin A. Amplification of the *lpa*-14 (encodes the 4′-phosphopantetheinyl transferase required for the maturation of template enzyme of iturin A) and *itu*D (encodes a putative malonyl coenzyme A transacylase, whose disruption results in a specific deficiency in iturin A production) genes of iturin operon of strain RP24 was carried out and the sequences obtained were compared with the existing database of NCBI. The sequences of *lpa*-14 and *itu*D gene of RP24 showed 98% and 97% homology with *lpa*-14 and *itu*D genes of *B. subtilis* in the existing database. The results indicated that strain RP24 harbors iturin operon in its genome and a chemical mutation in this operon might have resulted in loss of antifungal activity in the negative mutant.

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Introduction

With the intensification of agricultural production the dependency on agrochemicals as means of crop protection has also increased many folds over the past few decades. But with the growing cost of the chemical pesticides and increasing awareness about their negative effect the farmers are looking for environmental and soil friendly substitutes for these products to fulfill the consumers demand for pesticide-free food while maintaining soil fertility and environmental safety.

The use of microorganisms for biological purposes has become an effective alternative to control plant pathogens. There are many examples of formulations using bacteria or fungal strains with biocontrol applications. Among them, members of genus *Bacillus* are well-known antibiotic producers [9, 12] which have advantage over other biocontrol microorganisms due to their inherent property to form endospores and resistance to extreme conditions. *Bacillus* species strains produce a broad spectrum of bioactive peptides with great potential for biotechnological and biopharmaceutical applications. A known class of such compounds includes the lipopeptides surfactin, fengycin, and iturin compounds which are amphiphilic membrane active peptide antibiotics with potent antimicrobial activities and can be used as biopesticides for plant protection [1, 5]. The peptide portions of these lipopeptides are

produced nonribosomally with templates of the multifunctional peptide synthetases [11, 21, 28]. The surfactin and iturin compounds are cyclic lipoheptapeptides that contain a β-hydroxy fatty acid and a β-amino fatty acid, respectively, as lipophilic component. Fengycin is a lipodecapeptide with a β-hydroxy fatty acid in its side chain. The lipopeptides belonging to the iturin family are potent antifungal agents which can be used as biopesticides for plant protection. McKeen et al. [12] obtained a heat resistant antifungal compound obtained from *B. subtilis* that was identified as a member of iturin group. Besson et al. [4] identified the presence of antifungal antibiotics of the iturin group in about 30 *B. subtilis* strains.

Bacillus subtilis strain RP24 isolated from rhizosphere of pigeonpea has a suppressive effect against several phytopathogens and expected to be used as a potential biocontrol agent and a promising plant growth promoting rhizobacteria [13, 14]. The biocontrol activity of the RP24 can be attributed to diffusible antifungal metabolites [15]. In the present investigation an attempt was made for partial purification and characterization of the antifungal metabolite/s produced by the *B. subtilis* RP24 and amplification of the genes responsible for the antifungal trait. The information generated could be used for commercial exploitation of the strain for biofungicide production.

Materials and Methods

Iturin A

Iturin A standard was procured from Sigma–Aldrich. The solvents used were laboratory grade and distilled prior to use. All the chemicals and reagents were analytical grade. For HPLC acetonitrile used was HPLC grade. Deionised distilled water was obtained from a Milli Q, millipore water purification system.

Microorganisms

The bacterial culture RP24 selected for this study was isolated from rhizoplane of field grown pigeonpea plant [14] and identified as *Bacillus subtilis* (unpublished data). An antifungal negative mutant (NM) of the strain RP24 generated by chemical mutagenesis [15] was also used in the present study. Bacterial cultures were stored frozen in 10% glycerol at −70°C. Working cultures were maintained on tryptone yeast extract (TY; tryptone 5.0 g/l, yeast extract 3.0 g/l, $CaCl_2$ 0.66 g/l) agar slants at 4°C. The phytopathogenic fungal cultures (Table 1) were obtained from Indian Type Culture Collection, Division of Plant Pathology, Indian Agricultural Research Institute, India and maintained (4°C) and multiplied (25 ± 1°C) on potato dextrose agar (PDA) medium.

Table 1 In vitro antagonism of phytopathogenic fungi by *B. subtilis* RP24

Fungus	Inhibition zone (mm)[a]
Macrophomina phaseolina	9.00 ± 0.82
Pythium ultimum	7.50 ± 0.72
P. aphanidermatum	8.25 ± 0.90
Rhizoctonia solani	8.80 ± 0.78
Fusarium solani	7.85 ± 0.83
F. oxysporum	8.25 ± 0.75
F. moniliforme	7.50 ± 0.71
F. udum	8.50 ± 0.83
Alternaria solani	7.25 ± 0.71
A. alternata	8.30 ± 0.84
Aspergillus niger	8.50 ± 0.76

[a] Values are the mean of triplicates

Antifungal Activity of the Whole Culture of *Bacillus subtilis* RP24

Bacterial culture grown in TY broth to a concentration of ca 10^8 cfu/ml was streaked on PDA plate (3 cm away from the center). Simultaneously a 5 mm diameter agar plug containing actively growing fungal pathogen was placed in the center of the plate (dual culture method). All the pathogens (Table 1) were tested in triplicates. The plates were incubated at 28 ± 1°C for 7 days and the inhibitory effect of the bacterial strain on fungal growth was evaluated and expressed in terms of inhibition zone.

Extraction and Partial Purification of Antifungal Metabolite/s

For production of antifungal metabolites the organism was grown aerobically on potato dextrose broth maintained at pH 7.0. The culture was grown at 30 ± 1°C for 48, 96, and 144 h in 750 ml Erlenmeyer flasks containing 250 ml of medium with shaking at 200 rpm in a shaker incubator. After incubation bacterial cells were removed by centrifugation (8,000×*g* for 25 min) and the supernatant was passed through 0.45 µm nitrocellulose membrane filter. The pH of the filtered sterilized supernatant was adjusted to 2.0 by adding 6 N HCl. The acid precipitates were recovered by centrifugation (10,000 rpm for 15 min at 4°C) and were extracted with methanol. The methanol extract (100 µl) was bioassayed against the test pathogen *M. phaseolina* by well diffusion method. Quantification of the crude extract was done by drying the methanol extract under vacuum and weighing the remaining light brown

residue. For further purification the crude fraction was extracted with different non-polar and polar solvents and all the fractions were bioassayed against the test fungus. The active fraction was dissolved in methanol and used for HPLC studies. The NM strain was also subjected to extraction and partial purification in a similar way for a comparative analysis.

High Performance Liquid Chromatographic (HPLC) Analysis

Partially purified extract was analyzed by high performance liquid chromatography (HPLC). A reverse phase HPLC technique was used for quantitative analysis. A Hewlett Packard HPLC instrument (series 1100) equipped with degasser, quaternary pump, photo diode-array detector connected with rheodyne injection system (20 µl loop) and a computer (model Vectra) was used for analysis. The stationary phase consisted of Lichrospher on C-18 packed stainless steel column (250 mm × 4 mm i.d). Chromatogram was recorded in a Windows' NT based HP chemstation programme. Acetonitrile: water (70:30) at 1 ml/min flow rate was used as mobile phase. HPLC analysis was performed at wavelength of 240 nm, which was detected for absorption maxima using photodiode array. Twenty microliters of sample and standard iturin were injected into HPLC under standardized conditions. Each run was repeated thrice and the detector response was measured in terms of peak areas.

Liquid Chromatographic Mass Spectrometric (LCMS) Analysis

LCMS of the partially purified fraction was done on Water Alliance 2695 HPLC system with auto-sampler coupled with a mass detector with positive and negative mode. The mass spectrometer was operated in positive ionization mode with selected ion recorder (SIR) acquisition. Mobile phase was acetonitrile and 10 mM ammonium acetate (60:40) at a flow rate of 0.3 ml/min. Major peaks were produced by SIR of 10 channels in the TIC.

Amplification of Antifungal Genes

Genomic DNA preparation and electrophoretic analysis of DNA in agarose gel were performed according to standard protocols. Amplification of *itu*D and *lpa*-14 genes was carried out by PCR with specific primers. Primers of *itu*D-f (5′-ATGAACAATCTTGCCTTTTTA-3′) and *itu*D-r (5′-TTATTTTAAAATCCGCAA TT-3′) were used for amplification of 1,203-bp *itu*D fragment corresponding to the *B. subtilis itu*D gene (GenBank Accession No. AB050629), whereas primers of *lpa*-14f (5′-GAAAATTTACGGAGTATATATGGACCGC-3′) and *lpa*-14r (5′-TTATAACAGCTCTTCATACGTTTTCATCTC-3′) were employed for the 675-bp *lpa*-14 gene (GenBank Accession No. D21876). Amplification reaction was accomplished with a DNA thermal cycler (peqlab Primus 96 advanced) using the step-cycle program set for denaturing at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.5 min, for a total of 30 cycles.

Results

The rhizobacterial strain *Bacillus subtilis* RP24 could inhibit all the test pathogens in dual culture test (Table 1) due to the production of diffusible antifungal metabolites. Based on the size of inhibition zones, *M. phaseolina* was selected for further bioassay studies.

Production of extracellular antifungal metabolite/s by the parent strain was studied under shaking conditions in PDB at 30°C. After incubation for 48, 96, and 144 h the antifungal metabolites were extracted by acid precipitation. After 48 h incubation 120 mg/l of the crude extract was produced which increased by more than fourfolds by 96 h (620 mg/l). Further incubation up to 144 h did not show any significant increase in the concentration of the crude extract indicating that 96 h incubation is sufficient for maximum recovery of the metabolite/s. The observation was further supported by size of inhibition zone produced by crude extract of the culture broth incubated for 48, 96, and 144 h. The crude extract was partially purified by partitioning with different non-polar and polar organic solvents (data not shown). The active fraction (205 mg/l) was dissolved in methanol and used for further analysis.

The methanol extracts of the culture broth of the *B. subtilis* RP24 and its NM were analyzed by HPLC as described in the "Materials and Methods" section. In comparison to negative mutant, methanolic extract of the *B. subtilis* showed three extra peaks at retention time 5.236, 6.118, and 7.163 min (Fig. 1). When compared with iturin A standard, the peak at 5.236 min having the same elution profile as commercial iturin A, and was regarded as a positive result for iturin A production. HPLC analysis confirmed the production of iturin A by the parent strain whereas the peak corresponding to iturin A was absent in the methanol extract of culture broth of NM strain. The presence of iturin A was further confirmed by co-injecting the iturin A standard with extracted sample where enhancement in the area of peak at 5.236 min was observed. The results indicated that mutagenesis with EMS might have altered the structure of the gene/s responsible for the production of iturin A in the NM resulting in the loss of antagonistic trait in the strain.

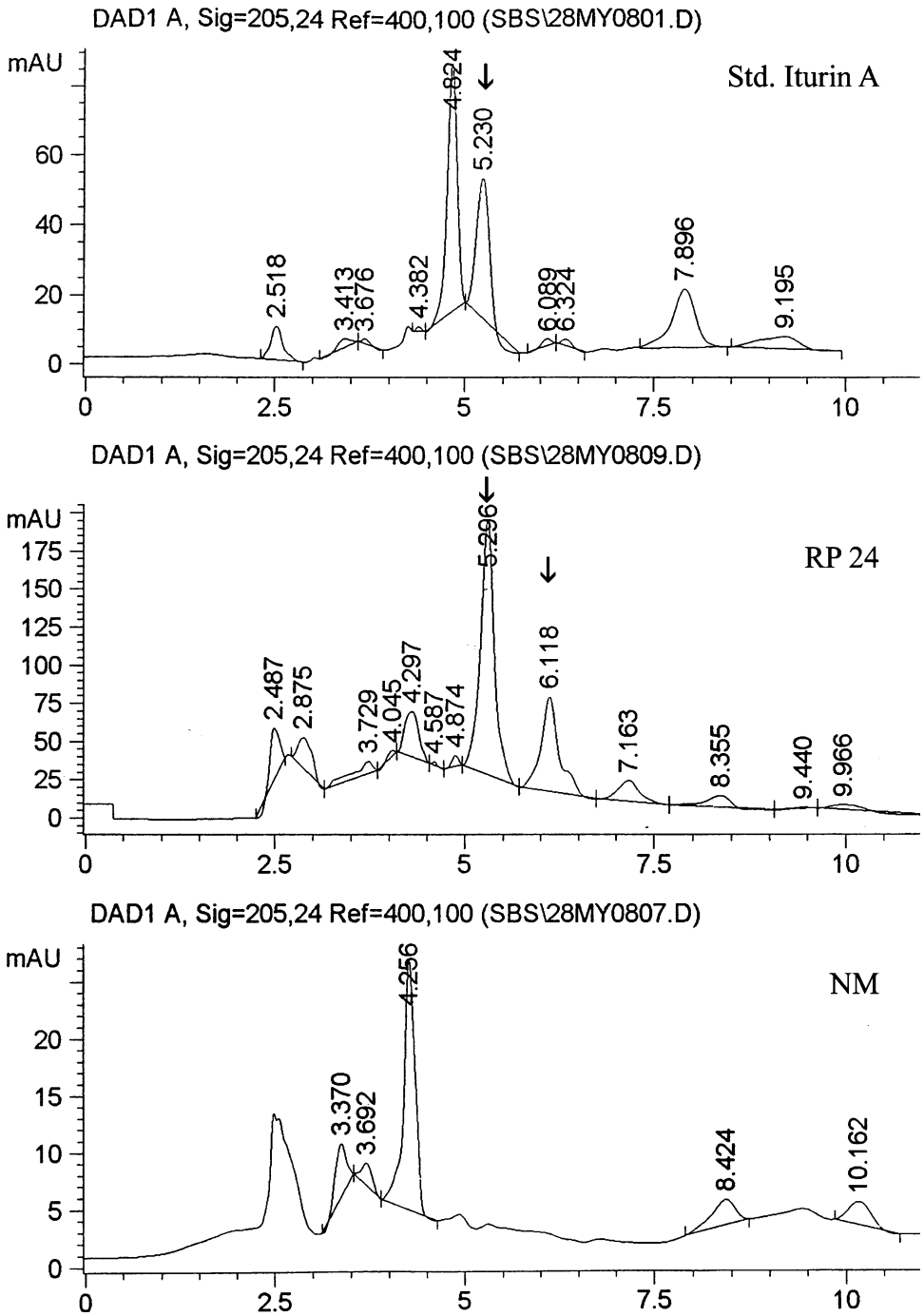

Fig. 1 HPLC analysis of partially purified extracts of RP24 and NM

The partially purified extract of the culture broth of the *B. subtilis* RP24 was also analyzed by LCMS. Although HPLC comparison by standard iturin A indicated the presence of iturin A in the extract but the authenticity of the produced iturin A was further established by LCMS analysis. The other two peaks (6.118 and 7.163 min), which were found to be absent in negative mutant strain were also analyzed by LCMS. Mass spectrum of sample showed three well-resolved groups of peaks at *m*/*z* values between 967 and 1,073, between 1,082 and 1,110, and between 1,483 and 1,549 (Fig. 2). The groups of peaks could be attributed to the isoform ensembles of surfactins, iturins, and fengycins, which represent the well-known biosurfactant families by *B. subtilis* strains. Mass numbers of all the surfactin, iturin, and fengycin peaks obtained by LCMS of partially purified extracts and tentatively identified on the basis of literature information [27] are given in Table 2. Besides comparison with authentic sample of iturin in HPLC, it is the 14 mass unit difference in peaks that suggests the presence of an iturin, as this difference

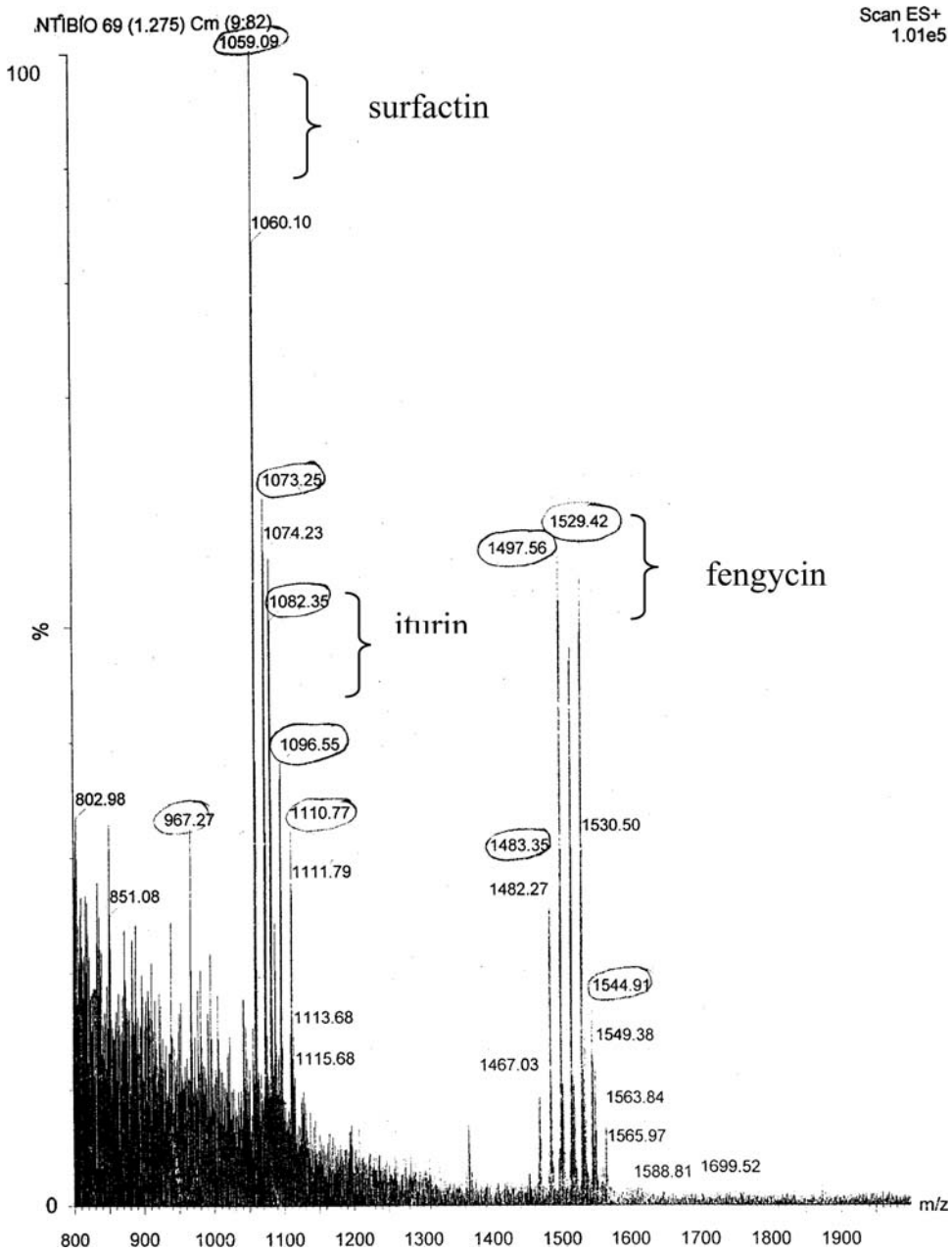

Fig. 2 MS of partially purified extract RP24

is characteristic of the two fatty acids of 14 and 15 C-atom that are called ituric acids and are associated with iturin.

Amplification of *itu*D (~1,203 bp) (Fig. 3) and *lpa*-14 (~675 bp) (Fig. 4) genes of *B. subtilis* RP24 suggested that the strain harbor the gene cluster required for iturin A biosynthesis. The PCR products were sequenced (Link Biotech, India) and the sequences obtained were subjected to nucleotide BLAST analysis and submitted to NCBI GenBank. Sequence of *itu*D gene of RP24 (GeneBank Accession No. FJ494832) showed 97% homology with *itu*D gene of *B. subtilis* (NCBI database, Accession No. EU263005). The sequence of *lpa*-14 gene of RP24 (Accession No. EU797520) showed 98% homology with *lpa*-14 gene of *B. subtilis* (NCBI database, Accession No. D21876).

Discussion

Bacillus subtilis, the most common representative of the genus, exhibits broad spectrum of action against different plant pathogens due to its ability to produce a great abundance of antibiotics with an amazing varieties of structures [20]. These compounds include predominantly peptides that are resistant to hydrolysis by proteinases and proteases. Their activity is also resistant to high

Table 2 Assignment of all mass peaks produced by *B. subtilis* by LCMS

Mass peaks (*m/z*)	Probable assignment
1044.6	C_{14} surfactin $[M + Na]^+$
1059.09	C_{15} surfactin $[M + Na]^+$
1060.10	C_{14} surfactin $[M + K]^+$
1073.25	C_{16} iturin $[M + H]^+$
1082.35	C_{17} iturin $[M + H]^+$
1095.52	C_{18} iturin $[M + H]^+$
1110.79	C_{19} iturin $[M + H]^+$
1467.03	C_{15} fengycin $[M + Na]^+$
1483.35	C_{16} fengycin $[M + Na]^+$
1497.8	C_{17} fengycin $[M + Na]^+$
1513.9	C_{16} fengycin $[M + Na]^+$
1529.42	C_{16} fengycin $[M + K]^+$

The mass data represent the monoisotopic mass numbers

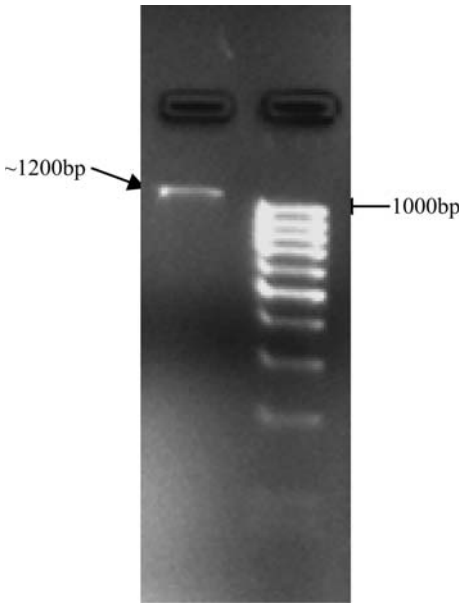

Fig. 3 Amplicon (~1,200 bp) of *itu*D gene of *B. subtilis* RP24

temperature and a wide range of pH [19]. In our study, the *Bacillus subtilis* strain RP24 exhibited in vitro antagonism against a wide range of phytopathogenic fungi due to the production of diffusible antifungal metabolites. An incubation period of 96 h was found to be optimum for the maximum production of antifungal metabolites by the strain RP24 in PD broth at 30°C. The antifungal metabolite/s was thermostable, pH stable, proteinase K resistant, soluble in methanol, ethanol, and acetic acid but insoluble in water indicating toward lipopeptide nature of the metabolite/s [15]. To clarify the involvement of antimicrobials in the biocontrol performance, additional evidence may be offered by mutagenizing the strain to reduce or avoid biocontrol activity. In the present study, an

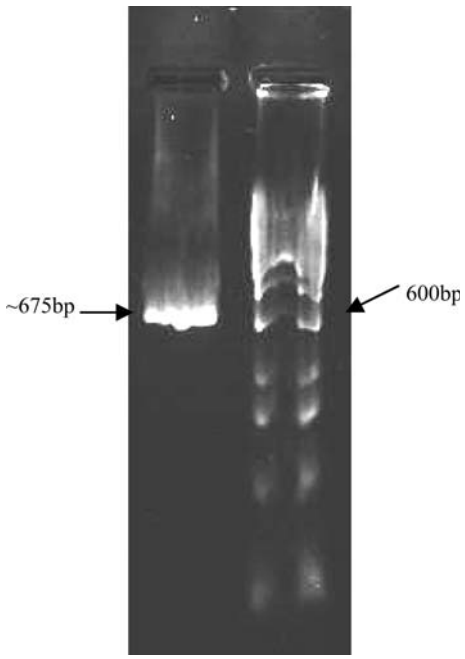

Fig. 4 Amplicon (~675 bp) of *lpa*-14 gene of *B. subtilis* RP24

antifungal negative mutant of *B. subtilis* RP24 created by chemical mutagenesis [15] could not exhibit biocontrol properties in vitro. The chromatographic analysis using HPLC and LCMS showed the occurrence of three different lipopeptide antibiotics, iturin A (as major) fraction and fengycin and surfactin (as minor fractions) in the partially purified extract of strain RP24 whereas none of these compounds was detected in the corresponding extract of negative mutant. Thus the production of three different lipopeptide antibiotics could be related with the biocontrol efficiency of RP24. The simultaneous excretion of different lipopeptides is often observed in *Bacillus* spp. [18, 19]. *Bacillus subtilis* GA1 is a producer of a wide variety of lipopeptides, iturin A, surfactin, and fengycin with various lengths of the fatty acid chains from C14 to C18 [24]. Coproduction of iturin A, fengycin, and surfactin by *B. subtilis* strains UMAF6614 and UMAF6639 was found responsible for the biocontrol of cucurbit powdery mildew *Podosphaera fusca* [17]. Mixture of surfactin and iturin produced by *B. subtilis* RB14 and *B. amyloliquefaciens* BNM 122 increased the antifungal activity since the former compound is able to form mixed micelles with iturin and thereby improves its activity [23]. Furthermore, surfactin seem to help the organisms in biofilm formation thus contributing to the protective activity by preventing the growth of other microorganisms as shown in *Arabidopsis* against *Pseudomonas syringae* [2]. Increasing the diversity of antibiotics excreted by the organism to the soil might result in an increase of the range of action on different phytopathogens. The target site for lipopeptide antibiotics is the fungal cytoplasmic membrane. Iturin antibiotics

increase the membrane permeability of the target microorganism due to the formation of ion channels on the cell membranes thereby increasing the permeability to K^+ that is associated with fungicidal activity. Modification of membrane permeability and lipid composition of *S. cerevisiae* cells by iturin A has been reported [3, 10, 29].

In the present study, an attempt was also made to amplify the *lpa*-14 and *itu*D genes of iturin operon. In *Bacillus* Iturin operon is 38–40 kb in size and consists of four open reading frames *itu*A, *itu*B, *Itu*C, and *itu*D [25]. The *ituD* encodes a putative malonyl coenzyme A transacylase, whose disruption results in a specific deficiency in iturin A production [8, 25]. The *itu*A (*lpa*-14) encodes the 4′-phosphopantetheinyl transferase required for maturation of template enzyme of iturin A [7]. Both these genes play leading role in the production of iturin A. The PCR method using specific primers could be applied to detect the presence of *itu*D and *lpa*-14 genes [6]. The *lpa*-14 and *idu*D genes of RP24 showed 98% and 97% homology with the corresponding genes of iturin operon of *Bacillus subtilis* in the existing database, respectively. *Bacillus subtilis* 168 Marburg could not produce lipopeptide surfactin, fengycin, and iturin due to a frameshift mutation in *sfp* gene coding for 4′-phosphopantetheinyl transferase which is responsible for conversion of nascent antibiotic synthetases to their active holoforms [16]. Interestingly introduction of a native *sfp* gene into *B. subtilis* 168 provoked surfactin and fengycin production [26]. In the present study, the NM could not produce any of the antibiotics, as detected in parent strain. This could be due to simultaneous mutations in the operons of three antibiotics or due to the mutation in some common gene/s that might be involved in the production of all three antibiotics in *B. subtilis* RP24.

Iturin and fengycin are lipopeptide antibiotics with a broad antifungal spectrum. Surfactin are strong biosurfactants and have some synergistic effects on the antifungal activity of iturin A. They have wide application in industries and medicine [22]. In our present observation, an efficient iturin A producing *B. subtilis* RP24 strain along with fengycin and surfactin provide a broad antifungal spectrum which can be further exploited as a biocontrol agent and for the commercial production of antifungal compounds.

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