Study of the Antifungal Ability of Bacillus subtilis Strain PY-1 in Vitro and Identification of its Antifungal Substance (Iturin A)

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Abstract A Bacillus strain, denoted as PY-1, was isolated from the vascular bundle of cotton. Biochemical, physiological and 16S rDNA sequence analysis proved that it should belong to Bacillus subtilis. The PY-1 strain showed strong ability against many common plant fungal pathogens in vitro. The antibiotics produced by this strain were stable in neutral and basic conditions, and not sensitive to high temperature. From the culture broth of PY-1 strain, five antifungal compounds were isolated by acidic precipitation, methanol extraction, gel filtration and reverse-phase HPLC. Advanced identification was performed by mass spectrometry and nuclear magnetic resonance spectroscopy. These five antifungal compounds were proved to be the isomers of iturin A: A2, A3, A4, A6 and A7. In fast atom bombardment mass spectrometry/mass spectrometry collision-induced dissociation spectra, fragmentation ions from two prior linear acylium ions were observed, and the prior ion, Tyr-Asn-Gln-Pro-Asn-Ser-βAA-Asn-CO⁺, was first reported.

Key words Bacillus subtilis; antifungal compound; FAB MS/MS CID spectrum; NMR spectroscopy; iturin A

Fusarium wilt causes huge economic losses in a wide variety of crops [1]. The pathogen, Fusarium oxysporum, infects plants through the roots by direct penetration or wounds, colonizes the vascular tissue and causes plant death [2]. Chemical soil fumigation is the main treatment of Fusarium wilt. Broad-spectrum biocides, particularly methyl bromide, can be used to fumigate the soil, but they cause serious environmental damage [3]. Safer and more efficient methods are not available at present.

Recently, scientists have paid attention to biological methods of defense against plant diseases. Control of pathogens by antagonistic microorganisms or their anti-biotic products is now considered a viable disease control technology [4–6]. A Bacillus strain with an effective ability against the Fusarium wilt pathogen was isolated from the vascular tissue of a cotton Fusarium wilt-resistant strain, and named PY-1.

In our study, we identified this strain to be a Bacillus subtilis strain by biochemical, physiological and 16S rDNA sequence analysis. In vitro antagonism experiments showed that B. subtilis PY-1 was effective against not only F. oxysporum, but also many other agricultural fungal pathogens, implying it has great potential as an agent for biological control of many fungal diseases. To explore the antifungal mechanism, we analyzed the culture broth of B. subtilis PY-1. Five compounds with high antifungal activity were separated from the culture filtrate by reverse-phase HPLC (RP-HPLC) and proved to be a series of isoforms of iturin A by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. A new fragmentation of iturin A was also observed in the fast atom bombardment mass spectrometry/mass spectrometry collision-induced dissociation (FAB MS/MS CID) experiment.

Materials and Methods

Microbial strains and culture conditions

B. subtilis PY-1 strain was separated from cotton vascular bundle by the Key Laboratory of Biotechnology and

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Crop Quality Improvement at the Southwest Agricultural University of China (Chongqing, China) and stored in sterilized dry sand at 4 °C. Before experimental use, it had been activated on potato dextrose agar (PDA) plates at 30 °C for 12 h and transferred twice by streaking. The fungal strains were kindly provided by the Life Science College of Sichuan University (Chengdu, China). These fungi were routinely grown on PDA at 28 °C and stored at 4 °C on the same medium.

**Identification of B. subtilis PY-1 by 16S rDNA analysis**

The biochemical and physiological identification of the PY-1 strain was performed using the BD Phoenix 100 Automated Microbiology System (BD Diagnostic Systems, Sparks, USA). The 16S rDNA of *B. subtilis* PY-1 was amplified using forward primer 5’-ATGGATCCGAGA-GTTTGATCCTGGCTCAG-3’ and reverse primer 5’-TATCTGCACTGTGGTGACGGGCGGTGT-3’. The polymerase chain reaction program was 94 °C for 4 min, followed by 94 °C for 1 min, 53 °C for 1 min and 72 °C for 1.8 min for 30 cycles, and a final 10 min extension at 72 °C. The amplified 16S rDNA fragment was ligated into pMD18-T vector, then transformed into Escherichia coli. Sequencing was done by Invitrogen (Shanghai, China). The result was analyzed on the website http://rdp.cme.msu.edu. DNAMAN software (version 5.1; Lynnon Biosoft, Quebec, Canada) was used to analyze the relationship of *B. subtilis* PY-1 with other *Bacillus* strains to construct a phylogenetic tree.

**In vitro antagonism experiments**

The ability of the PY-1 strain to inhibit the growth of various plant fungal pathogens was tested in Petri dishes containing PDA medium. The mycelial plugs of each fungus were deposited in the center of the plates, and the bacterium was inoculated on the edge, approximately 3 cm from the center. The slow growth fungi were inoculated from the center. The slow growth fungi were inoculated 24−48 h prior to the bacterium. After incubation for 3−5 d at 28 °C, the inhibition of fungal growth was evaluated by the percentage of reduction of mycelium expansion compared to control plates without bacteria [7].

**Production of antifungal compounds**

The PY-1 strain was cultured in 100 ml KMB liquid medium (2% tryptone, 1% glycerin, 1.5% K2HPO4, 1.5% MgSO4·7H2O, pH 7.0) on a constant temperature shaker (30 °C, 200 rpm) for 12 h. The broth was transferred to 900 ml fresh medium and incubated at 30 °C, 200 rpm for 72 h. During incubation, a 1-ml sample was taken every 12 h for antifungal assay. In this study, all antifungal assays were performed by the paper disk assay (40 μl sample per paper disk) against *Aspergillus niger*. The experiment was repeated three times and the data were combined for analysis.

**Effects of pH and temperature on antimicrobial activity**

In the pH stability test, the filter-sterilized crude supernatant was adjusted to pH 1.0−14.0, and maintained for 24 h at 4 °C. The antifungal activity was assayed after the solution had been readjusted to pH 7.0. To test the effect of temperature, the samples of the culture broth were exposed at 60 °C, 80 °C and 100 °C for 30 min and 121 °C for 15 min, then the remaining activity was assayed after the samples were cooled to room temperature.

**Isolation of the antifungal compounds**

After centrifugation at 8000 g for 30 min at 4 °C, the cell-free culture broth was acidized by 3 M acetic acid to pH 3.0. The precipitation was collected by centrifugation at 10,000 g for 25 min at 4 °C and extracted twice with five times volume of methanol. After removal of methanol *in vacuo*, the crude extract was separated by gel filtration and the antifungal fraction was collected. Sephacryl S-100 (Amersham Pharmacia Biotech, Uppsala, Sweden) column (16 mm×800 mm) was used and the mobile phase was 0.05 M NaHCO3 (pH 8.0). A sample of approximately 100 mg was uploaded every time. Further purification was carried out by RP-HPLC with a C18 column (Sephasil Peptide C18, 4.6 mm×250.0 mm, 12 μm; Amersham Pharmacia Biotech) on ÅKTA purifier 10 (Amersham Pharmacia Biotech). Elution was carried out by 32%−55% acetonitrile (Tedia, Fairfield, USA) with a linear gradient in 30 min, maintaining a flow rate of 0.8 ml/min. Active compounds were collected and freeze-dried. During the purification, the paper disk assay against *A. niger* guided the collection of active fraction.

**Structure characterization by mass spectrometry and NMR spectroscopy**

The electrospray ionization (ESI) time of flight (TOF) mass spectrometry carried out by a BioTOF Q Bruker (Bremen, Germany) was chosen initially to measure the mass of the antifungal compounds. The FAB-TOF/TOF CID analysis was performed on a Micromass Q-TOF2 (Waters, Milford, USA) in positive mode with 20 kV argon for bombardment. All samples were dissolved in methanol. CID spectrum analysis followed the nomenclature of Roepstorff and Fohlman [8], which was modified by Biemann [9]. NMR spectra were recorded on a Bruker Ultrashield 600 MHz NMR spectrometer. Purified anti-
fungal compounds were dissolved in dimethylsulphoxide-
$_6$ at 3 mg/ml 3-(trimethylsilyl)-1-propane-sulfonic acid
was used as the standard substance and chemical shifts
were reported in p.p.m. relative to it. 1D $^1$H NMR, $^1$H-$^1$H
correlated spectroscopy (COSY), $^1$H-$^1$H total correlation
spectroscopy (TOCSY), $^1$H-$^1$H nuclear overhauser effect
correlated spectroscopy (NOESY), $^1$H-$^1$C heteronuclear
single quantum correlation (HSQC) and $^1$C distortionless
enhancement by polarization transfer (DEPT) experiments
were performed.

Results

Relationship between B. subtilis PY-1 and other Bacillus
strains

Biochemical and physiological identification showed that
the PY-1 strain should be a B. subtilis. By 16S rDNA
analysis, B. subtilis PY-1 showed approximately 99%
similarity to B. subtilis P45B. A phylogenetic tree showing
the relationship between B. subtilis PY-1 and other Bacillus
strains is shown in Fig. 1.

![Phylogenetic tree of Bacillus subtilis PY-1 and other Bacillus strains based on their 16S rDNA sequences](image)

Table 1  Antagonism developed by Bacillus subtilis strain
PY-1 on potato dextrose agar plates against various plant fungal
pathogens

<table>
<thead>
<tr>
<th>Fungal pathogen</th>
<th>$I_R$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria kikuchiana</td>
<td>29</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>26</td>
</tr>
<tr>
<td>Bipolaris maydis</td>
<td>24</td>
</tr>
<tr>
<td>Exserohilum turcicum</td>
<td>33</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>32</td>
</tr>
<tr>
<td>Gibberella zeae (Schw.)</td>
<td>27</td>
</tr>
<tr>
<td>Penicillium digitatum Sac.</td>
<td>30</td>
</tr>
<tr>
<td>Pestalotia funereal</td>
<td>12</td>
</tr>
<tr>
<td>Pyricularia oryzae</td>
<td>23</td>
</tr>
<tr>
<td>Trichoderma viride</td>
<td>25</td>
</tr>
</tbody>
</table>

Data are expressed as the following equation: $I_R=([A_0-A_1]/A_0)/N$. $I_R$, relative
mycelium growth inhibition per bacterial colony; $A_0$, area of mycelium on control
plates without bacteria; $A_1$, area of mycelium on plates inoculated with B. subtilis
PY-1; $N$, the number of bacterial colonies.

Activity of the culture broth and its stability

As shown in Fig. 2, the culture broth had the strongest
antifungal activity after incubation for 72 h and 10 μl of
the filter-sterilized crude supernatant was sufficient to
clearly inhibit mycelium expansion. The result of a pH
stability test is shown in Fig. 3(A). In a neutral condition,
the antifungal activity of the culture broth was the highest,
and it was also stable, when the pH ranged from 5.0 to 13.0
with only moderate reduction. The antibiotics had weak
solubility and stability in acidic conditions. When the culture
broth was adjusted to pH 3.0, with large precipitation, the

![Relation between antifungal activity of Bacillus subtilis strain PY-1 culture broth and culture time](image)

In vitro inhibition of fungal growth

In this experiment, PY-1 strain showed strong inhibition
ability against all tested fungal plant pathogens (Table 1).
F. oxysporum appeared to be one of the most sensitive
species with 32% relative inhibition of mycelial growth.

![In vitro inhibition of fungal growth](image)
supernatant lost activity. If the precipitation was dissolved immediately in neutral phosphate-buffered saline, more than 80% activity could be recovered. But long-term treatment in acidic conditions significantly weakened activity. Only approximately 30% activity remained after the broth had been at pH 3.0 for 24 h, and the activity was completely destroyed at pH 1.0 for 24 h. However, in alkaline pH, activity was more stable than in acidic pH. A thermal stability test showed that the antifungal activity was not sensitive to high temperature [Fig. 3(B)]. The culture broth even maintained approximately 60% activity after being exposed to 121 °C for 15 min.

**Isolation of the active compounds**

The result of a pH stability test demonstrated that the antifungal compounds were insoluble in acidic aqueous solution, and could be precipitated completely at pH 3.0. Methanol could effectively dissolve the antifungal compounds. The methanol extract of the precipitation at pH 3.0 showed dramatic activity against fungi (approximately 30 times stronger than that of culture broth, assessed by two-fold dilution), which also implied that the antifungal compounds should have higher hydrophobicity. As shown in Fig. 4, using gel filtration and RP-HPLC, five pure antifungal compounds (called Compounds 1–5) were obtained. Compound 1 was the component with the largest

Fig. 3 Effects of pH and temperature on antimicrobial activity of the *Bacillus subtilis* strain PY-1 culture broth

(A) Culture supernatant fluids were incubated at various pH values. Activity was expressed as the percentage of relative residual activity. The antifungal compounds were more stable in basic conditions than in acid conditions. (B) Culture supernatant fluids were exposed to various temperatures for 30 min (or 121 °C for 15 min). Activity was expressed as the percentage of relative residual activity. The antibiotic secreted by strain PY-1 was so stable that 60% activity was maintained at 121 °C for 15 min.

Fig. 4 Purification of antifungal compounds from the fermentation broth of *Bacillus subtilis* PY-1

(A) Gel filtration chromatogram of crude extract by methanol. Column: Sephacryl S100 column (16 mm×800 mm). Mobile phase: 0.05 M NaHCO₃ (pH 8.0). Flow rate: 1.0 ml/min. (B) Reverse phase HPLC Chromatogram of the antifungal component separated by gel filtration. Five antifungal compounds were isolated. As the numbers shown, these five antifungal compounds were called Compounds 1–5. The ratio of the abundance of Compounds 1–5 was about 4.4:1:1.4:1.5:1 from peak area integral. Column: Sephasil Peptide C18, 4.6 mm×250 mm, 12 μm. Mobile phase: water (A), acetonitrile (B). Flow rate: 0.8 ml/min. Gradient program: 32% (B) in 0–15 min; 32%–55% in 15–45 min.
abundance. The ratio of the abundance of Compounds 1–5 was approximately 4.4:1:4.1:5:1.

Mass spectrometry analysis

ESI-TOF MS analysis showed that these five compounds had three different molecular weights: (M+H)+ ions at m/z 1043 (Compound 1), 1057 (Compounds 2 and 3) and 1071 (Compounds 4 and 5). It was implied that Compounds 1–5 might be five isoforms of iturin A [10,11]. For further identification, their sequences were analyzed by FAB-MS/MS CID spectrometry. The CID spectrum of Compound 1 is shown in Fig. 5. From the spectrum, five compositive α-amino acids could be identified from their special fragments: Asn (m/z 87.06); Pro (m/z 70.07); Ser (m/z 60.04); Gln (m/z 101.07); and Tyr (m/z 136.08).

An immonium ion of the β-amino acid belonging to the iturin family (H₂N+=CH-C₁₁H₂₃) could also be found at m/z 184.21 (198.23 for Compounds 2 and 3; 212.24 for Compounds 4 and 5). For a cyclopeptide, the peptide ring should be opened at a certain peptide bond and first form a linear acylium ion, then other peptide bonds could be broken to form fragment ions. Because of a random ring-opening reaction, it was difficult to sequence the cyclopeptide without the presence of proline residue. Easier breakage on the peptidyl-prolyl (Xaa-Pro) bond led to the formation of the major linear acylium ions [12]. For the presence of proline residue in iturin A molecules, the main linear acylium ion should be Pro-Asn-Ser-βAA-Asn-Tyr-Asn-Gln-CO⁺ (βAA denotes β-amino acid) [13]. The formation of b-type and y-type ions is shown in Fig. 6(A). In the CID spectrum, most of these fragment ions as well as some related a- or c-type ions were found, as listed in Table 2. This result confirmed that the compounds were isoforms of iturin A. However, another series of ions with high abundance were also observed (Table 2). It was implied that the prior ion, Tyr-Asn-Gln-Pro-Asn-Ser-βAA-Asn-CO⁺ [Fig. 6(B)], should be also formed in a high ratio.

Fig. 5 Fast atom bombardment mass spectrometry/mass spectrometry collision-induced dissociation spectrum of Compound 1 produced by Bacillus subtilis PY-1

Five compositive α-amino acids could be identified from their special fragments at the light mass area.

http://www.abbs.info; www.blackwellpublishing.com/abbs
Other major ions included the fragment ions from molecular ions that lost NH$_3$ or H$_2$O groups: m/z 1026.53 (M+H-NH$_3$)$_3$, m/z 1025.55 (M+H-H$_2$O)$_3$, m/z 1009.50 (M+H-2NH$_3$)$_3$, m/z 1008.54 (M+H-H$_2$O-NH$_3$)$_3$, m/z 992.48 (M+H-3NH$_3$)$_3$, m/z 991.52 (M+H-H$_2$O-2NH$_3$)$_3$, m/z 975.47 (M+H-4NH$_3$)$_3$, m/z 974.51 (M+H-H$_2$O-3NH$_3$)$_3$; ions with double positive charges: m/z 522.31 (M+2H)$_2$, m/z 505.29 (M+2H-2NH$_3$)$_2$; and some internal fragment ions: m/z 392.16 (AsnTyrAsn-H$_2$O), m/z 375.15 (AsnTyrAsn-H$_2$O$_2$), m/z 358.16 (AsnTyrAsn-H$_2$O$_3$) 

NMR spectroscopy analysis

The entire spin systems of amino acid residues were identified through $^{1}H$-$^1$H COSY and TOCSY experiments [14]. Amide groups of asparagine and glutamine and the phenolic group of tyrosine could be clarified from $^{1}H$-$^1$H COSY and NOESY spectra. Corresponding with the mass spectrometry result, the NOESY experiment could also be used to induce the amino acid sequence (cyclo-Asn1-Tyr2-Asn3-Gln4-Pro5-Asn6-Ser7-βAA8) [15] (Fig. 7). A strong NOE between Gln4 and Ser7 suggested that Gln4-Pro5-Ser7 should form a β-turn structure. The trans-amide bond of Gln4-Pro5 was indicated by the strong NOE correlation between the α proton of Gln4 and δ protons of Pro5 [12,15]. β-amino acid could be discriminated by $^{1}H$-$^1$H COSY experiment because its aminomethenyl (NH$_2$-CH) group connected with two methylene groups (-CH$_2$-). Their diolefinic tails were identified by 1D $^{1}$H-NMR, $^{13}$C-DEPT and $^{3}$H-$^{13}$C HSQC experiments [10]. A series of conspicuous overlapping methylene signals (δ$_H$ about

Table 2  Fragment ions from two different linear acylium ions found on fast atom bombardment mass spectrometry/mass spectrometry collision-induced dissociation spectrum of Compound 1 produced by Bacillus subtilis PY-1

<table>
<thead>
<tr>
<th>Fragmentsions</th>
<th>b-type ion (m/z)</th>
<th>y-type ion (m/z)</th>
<th>a-type ion (m/z)</th>
<th>c-type ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>from prior linear</td>
<td>b1 212.11 – 481.16 NH$_3$</td>
<td>y1 243.11 226.08 αH$_3$</td>
<td>a1 70.07 c1 –</td>
<td>–</td>
</tr>
<tr>
<td>acylium ion (A)</td>
<td>b3 299.15 – 278.12 CH$_2$</td>
<td>y3 406.19 389.16 αH$_3$</td>
<td>a3 – c3 –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>b4 524.37 – 520.22 CH$_2$</td>
<td>y4 456.36 439.31 αH$_3$</td>
<td>a4 216.36 c4 –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>b5 528.40 621.37 αH$_3$</td>
<td>y5 – –</td>
<td>a5 – c5 –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>b6 801.46 – 784.41 CH$_2$</td>
<td>y6 – –</td>
<td>a6 – c6 –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>b7 915.52 898.48 αH$_3$</td>
<td>y7 946.56 929.53 αH$_3$</td>
<td>a7 – c7 932.55</td>
<td>–</td>
</tr>
<tr>
<td>from prior linear</td>
<td>b1 – –</td>
<td>y1 243.11 226.08 αH$_3$</td>
<td>a1 136.08 c1 –</td>
<td>–</td>
</tr>
<tr>
<td>acylium ion (B)</td>
<td>b2 278.12 261.09 αH$_3$</td>
<td>y2 340.27 323.24 αH$_3$</td>
<td>a2 250.11 c2 295.15</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>b4 503.22 486.19 βH$_3$</td>
<td>y4 541.41 524.37 βH$_3$</td>
<td>a4 – c4 520.25</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>b5 617.37 600.30 αH$_3$</td>
<td>y5 638.40 621.37 αH$_3$</td>
<td>a5 – c5 –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>b6 704.30 –</td>
<td>y6 766.47 749.44 αH$_3$</td>
<td>a6 – c6 –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>b7 929.53 912.50 αH$_3$</td>
<td>y7 880.51 863.48 αH$_3$</td>
<td>a7 – c7 –</td>
<td>–</td>
</tr>
</tbody>
</table>

† no corresponding ion observed; † the loss of NH$_3$ group; † the loss of H$_2$O group.
1.27 p.p.m.; δc 30.5–30.7 p.p.m.) in every compound. The NMR spectra implied that there might be a long carbon chain. The NMR spectra of Compound 1 were similar to those of Compound 5: there was one aliphatic methyl signal at δH = 0.88 p.p.m. (3H, t, J = 6.0 Hz), δc = 15.55 p.p.m., and in the HSQC spectrum, the connected methylene (δc = 1.27 p.p.m.; δH = 23.68 p.p.m.) could be observed. The NMR spectra of Compounds 3 and Compound 4 were similar: the signal at δH = 0.87 p.p.m. (6H, d, J = 6.7 Hz), δc = 24.14 p.p.m. indicated a pair of symmetric methyl groups, and at δH = 1.52 p.p.m. (1H, m, J = 6.7 Hz), δc = 28.97 p.p.m., a methenyl group was connected with them, confirmed by HSQC and DEPT experiments. The spectra of Compound 2 were unique: there were two different methyl signals (6H) overlapping at 0.8–0.9 p.p.m. by HSQC and DEPT spectra, one (δH = 0.85 p.p.m., δc = 12.82 p.p.m.) connected with a methylene group (δH = 1.31 p.p.m., 1.12 p.p.m.; δc = 12.95 p.p.m.), and the other (δH = 0.84 p.p.m., δc = 20.63 p.p.m.) connected a methenyl (δH = 1.31 p.p.m., 1H, m; δc = 35.33 p.p.m.). Considering the molecular weight, we deduced the structures of these five β-amino acids (Fig. 8). Compounds 1–5 were iturin A5, A3, A4, A6 and A7 [16].

![Diagram](image)

**Fig. 8** Structures of the five β-amino acids deduced by 1H nuclear magnetic resonance, 13C distortionless enhancement by polarization transfer and 1H-13C heteronuclear single quantum correlation

A–E represent the structure of β-amino acid of Compound 1–5 (iturin A5, A3, A4, A6 and A7, respectively) produced by *Bacillus subtilis* strain PY-1, respectively.

### Discussion

Antagonism is ubiquitous in nature among different species. For a long time, people have been interested in rationally making use of it in the areas of agricultural defense or therapy of diseases. Plant fungal diseases are difficult to control and can cause huge damage to economic crops. Environmental pollution, caused by abusing chemical biocides, is another serious problem. Using antibiotic production bacteria to control plant fungal diseases is a popular topic and has been studied extensively [17]. Compared with chemical biocides, many antibiotics produced by antagonistic strains have the advantage of being easily decomposed, leaving no harmful residues [18]. According to the pathogenesis mechanism, we know that, as well as soil fumigation, inhibiting *F. oxysporum* from invading the vascular tissue of plants might be another valid method to control Fusarium wilt. *B. subtilis* PY-1 was isolated from the vascular tissue of a cotton Fusarium wilt resistant strain, and showed strong inhibitory ability against many plant fungal pathogens in vitro, especially *F. oxysporum* and *Exserohilum turcicum*, which may be why this cotton strain can resist Fusarium wilt.

Analysis of the *B. subtilis* PY-1 broth showed that the main antifungal compounds were five isomers of iturin A. Iturins are a group of similar cyclic lipopeptides with high antifungal activity, which can modify the membrane permeability and lipid composition, and inhibit the mycelium growth and sporulation of fungi [19]. The structure of iturins is characterized by an amphiphilic peptide ring, which is composed of seven chiral amino acids including an invariable D-Tyr2 with the constant sequence LDDLDDL, and a rare β-amino acid with a long hydrophobic diolefin tail [20]. There are some isomeric compounds in iturins, such as iturin A6 (βAA:C16) and mycosubtilin (βAA:C14):C48H74N12O14 (MW 1070 D) [21, 22]; iturin A5 (βAA:C14) and mixirin A [23]:C48H74N12O14 (MW 1042 D). It is not enough to identify these compounds just by their molecular weight, and sequence analysis is necessary. FAB-TOF/TOF CID MS is a valid method. Because of the presence of proline residue, the main linear acylium ion would be formed by the ring-opening reaction at the peptidyl-prolyl bond. As for iturin A, major fragment ions were derived from the prior ion: Pro-Asn-Ser-βAA-Asn-Tyr-Asn-Gln-CO’. However, in our research, by CID spectra data analysis, two series of ions from different prior linear acylium ions were observed, and the second prior ion, Tyr-Asn-Gln-Pro-Asn-Ser-βAA-Asn-CO’, was first reported in FAB-MS/MS CID spectrometry experiment of iturin A. CID spectrum can be used as a “fingerprint” to identify iturin A, especially in a mixture [13,24]. This finding will be very useful to analyze the spectrum and discriminate some special fragment ions, so as to identify iturin A quickly and correctly. NMR spectroscopy is necessary to determine the structure of the β-amino acid residue and gives us more information about...
the space conformation of the molecule. But NMR spectroscopy needs an amount of pure sample and is inappropriate for fast identification.

With the high production of iturin A and its endogenic character, *B. subtilis* PY-1 is a promising agent in the biocontrol of fungal diseases in agriculture. The application in farmland and the biologic security of *B. subtilis* PY-1 needs advanced testing.

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